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Methanotrophic activity and bacterial diversity in volcanic-geothermal soils at Pantelleria island (Italy)

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Abstract

Volcanic and geothermal systems emit endogenous gases by widespread degassing from soils, including CH_4, a greenhouse gas twenty-five times as potent as CO_2. Recently, it has been demonstrated that volcanic/geothermal soils are source of methane, but also sites of methanotrophic activity. Methanotrophs are able to consume 10–40 Tg of $CH_4\,a^{-1}$ and to trap more than 50 % of the methane degassing through the soils. We report on methane microbial oxidation in the geothermally most active site of Pantelleria island (Italy), Favara Grande, whose total methane emission was previously estimated in about 2.5 $t\,a^{-1}$. Laboratory incubation experiments with three top-soil samples from Favara Grande indicated methane consumption values up to 950 $ng\,g^{-1}$ dry soil h^{-1}. One of the three sites, FAV2, where the highest oxidation rate was detected, was further analysed on a vertical soil profile and the maximum methane consumption was measured in the top-soil layer but values > 100 $ng\,g^{-1}\,h^{-1}$ were maintained up to a depth of 15 cm. The highest consumption rate was measured at 37 °C, but a still recognizable consumption at 80 °C (> 20 $ng\,g^{-1}\,h^{-1}$) was recorded. In order to estimate the bacterial diversity, total soil DNA was extracted from Favara Grande and analysed using a Temporal Temperature Gradient gel Electrophoresis (TTGE) analysis of the amplified bacterial 16S rRNA gene. The three soil samples were probed by PCR using standard proteobacterial primers and newly designed verrucomicrobial primers targeting the unique methane monooxygenase gene *pmoA*; the presence of methanotrophs was detected in sites FAV2 and FAV3, but not in FAV1, where harsher chemical-physical conditions and negligible methane oxidation were detected. The *pmoA* gene libraries from the most active site FAV2 pointed out a high diversity of gammaproteobacterial methanotrophs distantly related to *Methylococcus*/*Methylothermus* genera and the presence of the newly discovered acido-thermophilic methanotrophs Verrucomicrobia. Alphaproteobacteria of the genus *Methylocystis* were isolated from enrichment cultures, under a methane containing atmosphere at 37 °C. The isolates grow at pH 3.5–8 and temperatures of 18–45 °C, and show a methane oxidation rate of ~ 450 $\mu mol\,mol^{-1}\,h^{-1}$. Soils

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from Favara Grande showed the largest diversity of methanotrophic bacteria until now detected in a geothermal soil. While methanotrophic Verrucomicrobia are reported to dominate highly acidic geothermal sites, our results suggest that slightly acidic soils, in high enthalpy geothermal systems, host a more diverse group of both culturable and uncultivated methanotrophs.

1 Introduction

Methane plays an important role in the Earth's atmospheric chemistry and radiative balance, being the second most important greenhouse gas after carbon dioxide. It is released into the atmosphere by a wide number of sources, both natural and anthropogenic, with the latter being twice as large as the former (IPCC, 2001). It has recently been established that significant amounts of geologic CH_4, produced within the Earth's crust, are currently released naturally into the atmosphere (Etiope et al., 2008). Volcanic/geothermal systems emit endogenous gases, including CH_4, by widespread degassing from soils. Indirect estimations based on CO_2 or H_2O outputs and CO_2/CH_4 or H_2O/CH_4 ratios of the main gas manifestations gave a total CH_4 emission from European geothermal/volcanic systems in the range of 4–16 $kt\,a^{-1}$ (Etiope et al., 2007). Methanotrophy is a metabolic process by which bacteria obtain energy via the oxidation of CH_4 to CO_2 (Murrell and Jetten, 2009). Methanotrophs are a subset of methylotrophic bacteria that use methane as the sole carbon source (Hanson and Hanson, 1996). They are abundant at the anoxic/oxic interfaces of methanogenic environments such as wetlands, peat lands (Kip et al., 2012) aquatic sediments (Rahalkar et al., 2009), landfills (Ait-Benichou et al., 2009) and, as recently discovered, also in geothermal areas, that have been long considered incompatible with methanotrophic activity (Op den Camp et al., 2009). Methanotrophy in soils is one of the main sinks of atmospheric methane; methanotrophs are able to consume 10 to 40 Tg of $CH_4\,a^{-1}$ and to trap more than 50 % of the methane degassing through the soils (IPCC, 2001; Reeburgh, 2003). The effectiveness of biological oxidation process within the soil de-

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pends not only on the type and quantity of methanotrophic microorganisms but also on the characteristics of the soils. Dry soils with high permeability and circumneutral pH favor methanotrophic activity consuming efficiently the atmospheric CH_4 (Hanson and Hanson, 1996; Op den Camp et al., 2009). In such situation methanotrophic activity is sustained by a CH_4 flux coming from the atmosphere above the soil but this activity can also be sustained by CH_4 fluxes coming from below. Such flux can be of biological origin (CH_4 production in deeper anoxic layers) or of more deeper geogenic origin in areas rich in hydrocarbon reservoirs or in geothermal/volcanic areas. In these cases the CH_4 flux often exceeds the biologic oxidation capacity, and soils become a source of endogenous CH_4 towards the atmosphere (Cardellini et al., 2003; Castaldi and Tedesco, 2005; D'Alessandro et al., 2009, 2011; Etiope and Klusman , 2010). Methane flux measurements in volcanic/geothermal areas, started in recent years (Etiope and Klusman , 2002; Castaldi and Tedesco, 2005), accounted for a new, previously neglected, source of atmospheric CH_4. Castaldi and Tedesco (2005) hypothesized for the first time the presence of methanotrophic microorganisms in such areas. Actually, soon after, a new group of obligately methanotrophic bacteria was isolated from different geothermal/volcanic sites and affiliated to the phylum Verrucomicrobia. These new isolates thrive at very low pH (down to 0.8) and high temperatures (up to 60 °C optimal temperature) and may consume 10–90 % of the methane before its emission from soils (Pol et al., 2007; Islam et al., 2008; Dunfield et al., 2007). Methanotrophic bacteria are taxonomically affiliated to the phylum Proteobacteria in the classes Gammaproteobacteria and Alphaproteobacteria and to the phylum Verrucomicrobia in the family Methylacidiphilaceae. Among proteobacterial methanotrophs, type I methane-oxidizing bacteria use the ribulose monophosphate pathway for formaldehyde fixation while type II use the serine pathway. Type X are similar to type I methanotrophs, but they also have low levels of enzymes of the serine pathway Ribulose 1,5-bisphosphate carboxylase, an enzyme present in the Calvin–Benson cycle (Hanson and Hanson, 1996). Type I and type II are sometimes used as synonyms for Gamma- and Alphaproteobacteria, respectively (Op den Camp et al., 2009) and type X methanotrophs have been included, together with type I, in the family Methylococcaceae (Gammaproteobacteria, Wise et al., 1999). Methanotrophic communities in natural areas can be investigated and characterized using functional genes such as, *pmoA* and *mmoX* (McDonald et al., 2008) encoding subunits of the two forms of the methane monooxygenase enzyme (the particulate pMMO and the soluble methane sMMO, respectively), which catalyzes the first step in the methane oxidation pathway and can only be found in methanotrophs, Hanson and Hanson, 1996). Italy is a geodynamically active region with several active volcanic/geothermal areas including Pantelleria island. Previously, D'Alessandro et al. (2009) estimated a total methane output at Pantelleria island close to $10\,t\,a^{-1}$. The same authors suggested the presence of methanotrophic activity within the soils of this area. The main reason was because concurrent CO_2 and CH_4 flux measurements showed nearly always a CO_2/CH_4 ratio lower than that measured in the fumarolic manifestations of the area which are representative of the gas composition coming up from the geothermal system of the island. Such pattern points to a loss of CH_4 during the travel of the gases within the soil towards the earth's surface. The aim of this work was to estimate the methane oxidation potential of the geothermal soils of Pantelleria through laboratory soil incubation experiments and to detect and characterize the methane oxidizing bacteria that thrive in these soils using cultural and culture-independent approaches.

2 Geological setting

The island of Pantelleria is a strato-volcano located in the Strait of Sicily, about 100 km SW of Sicily and 70 km NE of Tunisia, on the axis of the Sicily Channel Rift Zone (Fig. 1). Pantelleria island has a surface of 83 km^2 and it is entirely covered by volcanic products from both effusive and explosive activity, with dominant peralkaline rhyolites ("pantellerites") and trachytes and minor alkali basalts (Civetta et al., 1984). The most recent volcanic activity of the island was an underwater eruption in 1891 4 km NNW off its coast. Although at present in quiescent status, the widespread thermal manifes-

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tations on Pantelleria attest to a sustained heat flow (Parello et al., 2000). Many hot springs and thermal wells occur in the NW and SW part of the island. Persistent fumaroles are concentrated on the young eruptive centres and/or along active faults. In the central part of the island, within the younger caldera, many fumaroles with temperatures between 40 °C and 100 °C are recognizable. Previous surveys identified many areas characterized by intense gas flux from the soil (Chiodini et al., 2005). The most important fumarolic manifestations of the island can be detected at le Favare, south of Montagna Grande (Fig. 1), an area located at the intersection of a regional tectonic lineament with many volcano-tectonic structures. It comprises the main fumarolic field of Favara Grande with strong steam emission and many fumarolic manifestations all with temperatures close to boiling water. Fumarolic emissions have typical hydrothermal composition (Chiodini et al., 2005; Fiebig et al., 2013) with water vapor as the main component (about 970 000 $\mu mol\,mol^{-1}$) followed by CO_2 (about 23 000 $\mu mol\,mol^{-1}$). Among the minor components the fumarolic gases of Favara Grande display relatively high contents of H_2 and CH_4 (about 1300 and 800 $\mu mol\,mol^{-1}$, respectively) and low contents of H_2S (< 20 $\mu mol\,mol^{-1}$). This leads, after condensation of water vapor, to high CH_4 concentrations in the soils (up to 44 000 $\mu mol\,mol^{-1}$) and high CH_4 fluxes from the soil (up to 3550 $mg\,m^{-2}\,day^{-1}$) in the area of Favara Grande (D'Alessandro et al., 2009).

3 Material and methods

3.1 Soil sampling and chemical-physical characterization

Soil samples used in this study were collected at Favara Grande during two field campaigns in 2011. Top-soil samples (0–3 cm) were collected in June 2011 from three sites (FAV1, FAV2, FAV3) and a further sampling was carried out in November 2011 at site FAV2 on a vertical profile of 0–15 cm (FAV2A to FAV2E) (Fig. 1, Table 1). All the samples used for geochemical and microbiological analyses were taken using a ster-

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ile hand shovel and stored in sterile plastic bags. Soil sub-samples for microbiological analysis were air-dried, sieved at 2 mm and stored at −20 °C. Organic matter in soils was measured by loss-on-ignition analysis with heating stages of 105 °C for 4 h (for % of H_2O by mass), 400 °C for 16 h (for % organic matter by mass) (Heiri et al., 2001); soil pH was determined using a pH meter in a mixture of 1/2.5 of soil and distilled deionised water. Ground temperature measurements were taken at 10 cm depth using thermal probes and a digital thermometer.

3.2 Gas sampling and characterization

Soils gas samples from the three sites were taken through a special sampling device with three 2 mm ID tubes tapping soil gases at 13, 25 and 50 cm depth, using a gas-tight plastic syringe. About 20 mL of soil gas was injected through a three-way valve and a needle into a 12 mL pre-evacuated sampling vial (Exetainer®, Labco Ltd.). The overpressured vials were sent to the laboratory for CH_4, CO_2, N_2, O_2 and H_2 analysis by using a Perkin Elmer Clarus 500 GC equipped with Carboxen 1000 columns and two detectors (HWD and FID) and argon as carrier gas. The gas samples were injected through an automated injection valve with a 1000 µL loop. Calibration was made with certified gas mixtures. Analytical precision (±1σ) was always better than ±3 %. The detection limits were about 0.1 µmol mol^{-1} for CH_4, 2 µmol mol^{-1} for H_2, 10 µmol mol^{-1} for CO_2 and 200 µmol mol^{-1} for O_2 and N_2.

3.3 Methanotrophic activity

Methane oxidation potential of the soils was analyzed by transferring 15 g of each air-dried soil sample in a 160 mL glass serum bottle, that was capped with a rubber stopper and sealed with aluminium crimps, after wetting with 1 mL sterile distilled water. After sealing the bottle, the atmosphere was enriched in CH_4 to reach about 1000–2000 µmol mol^{-1}. Bottles were incubated at controlled room temperature (23–25 °C) and the CH_4 concentration was measured at the beginning of the experiment and at

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about 24 h intervals for 5 days. To better monitor the methane consumption in samples that after 24 h consumed more than 30 % of the initial CH_4 the experiments were repeated measuring the concentrations at 2 h intervals. Samples collected in autumn from the FAV2 vertical profile were also incubated at 5, 37, 50 and 80 °C under the same conditions. Finally, the variation of the soil CH_4 oxidation potential was analysed on sample FAV2A with different starting CH_4 concentrations at room temperature (from about 100 to 85 000 $\mu mol\,mol^{-1}$). Methane concentration inside the vials was measured using CG as above. All incubation experiments were in duplicate and the results expressed as ng CH_4 per g of soil dry weight per h (ng $g^{-1}\,h^{-1}$). Taking into account all the instrumental errors, we consider that only values above 10 $ng\,g^{-1}\,h^{-1}$ indicate significant oxidation activity.

3.4 Extraction of soil DNA and PCR-TTGE

The extraction of total DNA from soil samples was performed using the FastDNA® Spin Kit for Soil (MP Biomedicals, Solon, OH, USA), from 0.5 g of dried soil, following the manufacturer's protocol/instructions. The DNA quality and concentration was assessed on 1XTAE agarose gel (1 %) electrophoresis and spectrophotometric analysis using Nanodrop (NanoDrop ND-1000, Celbio SpA). For Temporal Thermal Gradient gel Electrophoresis (TTGE) the hypervariable V3 region of the 16S rRNA gene, about 200 bp long, was PCR amplified using the primer pair 341F-GC/534R (Table 3) and soil DNA as template. The PCR reaction mixture (50 µL) contained about 100 ng of soil DNA, 1X PCR buffer, 0.20 mM dNTPs, 500 nM of each primer and 1 µL of Phire Hot Start II DNA Polymerase (Thermo Scientific, USA). PCR was carried out in a Biometra Thermocycler using the following thermal cycling: initial denaturation at 98 °C for 30 s, followed by 35 cycles of 10 s at 98 °C, 10 s at 66 °C, 10 s at 72 °C and final extension at 72 °C for 1 min. PCR amplification products were visualized after electrophoresis in a 1.5 % agarose gel stained with ethidium bromide, under UV light. For TTGE analysis, 10 µL of each PCR mix were loaded in a 8 % (w/v) acrylamide gel (acrylamide : bis-acrylamide

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29 : 1) containing 7 M urea and 10 % formamide in 1.5X TAE buffer (60 mM Tris-Acetate, 1.5 mM Na2 EDTA; pH 8). The gels were run in a DCode (Bio-Rad, Richmond, CA, USA) apparatus, at 70 V for 17 h, with a temperature ramping rate of 0.4 °C h^{-1} with a starting temperature of 57 °C. Gels were stained with SYBR Gold (Invitrogen, USA) in 1X TAE for 45 min and visualized under a UV light using the ChemiDoc apparatus (Bio-Rad). Richness and diversity were determined by using the executable PAST version 2.17c (Hammer et al., 2001).

3.5 Detection of methane oxidation genes and construction of a *pmoA* gene library

The gene encoding the key methane oxidation enzyme pMMO was detected by amplification of total soil DNA using the primers A189f and A682r (Table 3), targeting the β-subunit of the proteobacterial *pmoA* gene. PCRs were carried out in a final volume of 50 µL, containing 100 ng of total DNA, 200 nM of each oligonucleotide primer, 0.20 mM dNTPs, and 1 unit of recombinant Taq polymerase, (Invitrogen, USA). PCR program consisted of an initial denaturation step at 95 °C for 4 min, followed by 28 cycles consisting of a denaturation step at 95 °C for 45 s, annealing at 56 °C for 45 s and 45 s of extension at 72 °C and a final extension at 72 °C for 5 min. For the *pmoA* clone library, amplicons were purified using QIAquick spin columns (Qiagen, Germany) and cloned into PCRII TOPO TA (Invitrogen, USA) according to the manufacturer's instructions. The ligation mixture was used to transform One Shot TOP10 chemically competent cells. Plasmids were extracted by using GenElute Plasmid Miniprep Kit (Sigma-Aldrich, USA) and screened for the correct-size insert by PCR amplification using vector specific primers (Table 3). Positive clones were sequenced using the universal T7 primer. The sequences of the *pmoA* clones were deposited in Genbank under accessions numbers KJ207214-19. Two novel couples of primers, 298f/599r and 156f/743r (Table 3), targeting Verrucomicrobial *pmoA1/A2* and *pmoA3*, respectively, were designed and positively validated on *Methylacidiphilum fumarolicum* strain SolV. To detect Verrucomicrobial *pmoA* gene, PCR was carried out as described above using the OneTaq®

DNA Polymerase (New England Biolab, MA, USA) with an initial denaturation at 94 °C for 60 s followed by 5 cycles consisting of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s and extension at 68 °C for 30 s; the following 35 cycles consisted of a denaturation at 94 °C for 30 s, annealing at 52 °C for 10 s and extension at 68 °C for 30 s. A final extension at 68 °C was carried out for 5 min. Amplicons were purified and cloned into PCRII TOPO-TA® (Invitrogen, USA) as described above. Clones containing an insert of the correct size were sequenced using the vector specific primer M13 (Table 3).

3.6 Isolation of methanotrophic bacteria

In order to enrich soil microbial populations for methanotrophs, 15-g aliquots of FAV2 soil from the vertical profile 0 to 15 cm (samples FAV2A to FAV2E) were placed in 125 mL sealed serum bottles in atmosphere supplemented with methane (25 %) and incubated either at 37 or 65 °C for 2 weeks. Two grams of enriched soil crumbles were transferred to 125 mL serum bottles containing 20 mL of low salt mineral medium M3 (Islam et al., 2008) adjusted to pH 6 under the same conditions. After incubation, aliquots of M3 enrichment cultures were inoculated on M3 agar-slants in 125 mL sealed serum bottles under methane enriched atmosphere and incubated as described above for 2 weeks. As soon as colonies appeared, they were transferred to fresh medium to obtain pure cultures that were checked for methane consumption by GC analysis, as described above. Growth on alternative C sources was assessed by streaking each isolate on M3 agar plates containing methanol (0.5 %), glucose (1 %), fructose (1 %) and ethanol (1 %), respectively, and in the absence of any C source and incubating at 37 °C. The isolates were also incubated in M3 agar in a CH_4 atmosphere at different temperatures. Each isolate was routinely grown in M3-agar slants in 120 mL serum bottle, in atmosphere enriched in methane (25 %) added every week and transferred to fresh medium every three weeks. Genomic DNA was extracted from 10 mL of M3-CH_4 broth culture of each isolate grown in the conditions described above following the method described by Sambrook et al. (1989) and used as template for the amplification of the

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16S rRNA gene with universal primers (Table 3) and *pmoA* gene as described above. The 16S rRNA and *pmoA* gene sequences of the isolates were deposited in Genbank under accession numbers KJ207210-14 and KJ207220, respectively. A growth curve of strain Pan1 was obtained by pre-inoculating a single colony in a 125 mL serum bottle containing 10 mL of M3 mineral medium and 25 % methane. The pre-culture was incubated for 10 days at 37 °C and subsequently split in five 200 mL serum bottles (2 mL each) containing 30 mL of M3 mineral medium and 25 % methane; growth was monitored as turbidity using a spectrophotometer at a wavelength of 600 nm (OD_{600}). Methane concentration was measured every 12 h in the cultures and in the uninoculated control bottles incubated under the same conditions.

4 Results

4.1 Soil gas composition

Soil gases collected in June 2011 at the sites FAV1, FAV2 and FAV3 display a composition that is the result of the mixing process between a hydrothermal component rich in H_2, CH_4 and CO_2 and an atmospheric component rich in O_2 and N_2 (Table 2). The hydrothermal component coming from below is always enriched in the deeper sampling points, while the atmospheric component diffusing from above is enriched in the shallower soil levels. Although, at least at FAV1 and FAV2, at 50 cm depth the gas composition is very close to that of the fumarolic gases, O_2 concentrations would still be enough to sustain aerobic methanotrophic activity (Kumaresan et al., 2011).

4.2 Methanotrophic activity in the geothermal area

Soils sampled from the three sites at the most active fumarolic area of Favara Grande show significant differences in chemical-physical parameters (Table 1). FAV1 has the highest temperature (82.7 °C) and lowest pH (3.41); FAV2 is similar to FAV3 and both show significantly milder conditions than FAV1. Organic matter was in a range of 1 to

6 % by mass with the maximum value measured in the shallowest layer and decreased in the deeper layers. Water content was higher in the deeper layers and decreased in the shallowest layers (Table 1). Laboratory incubation experiments with soil samples from the 0–3 cm detected CH_4 consumption values in a range from 5 (FAV1) to 950 ng g^{-1} h^{-1} (FAV2) (Table 1). Since FAV2 was the most active site, its methane oxidation was further investigated on a vertical profile up to a depth of 15 cm (Table 1). Temperature, in FAV2, increases with depth from 33 to 83 °C while pH decreases from 6.62 to 5.88. The maximum methane oxidation rate (1200 ng g^{-1} h^{-1}) in the FAV2 vertical profile was measured (at controlled room temperature) in the shallowest soil layers (0–2 cm), but significant values (100 ng g^{-1} h^{-1}) were still detected at 15 cm depth. When samples from the vertical profile were incubated at different temperatures, the CH_4 consumption increased with temperature from 5 °C, to a maximum at 37 °C and then decreased to a minimum, but still detectable, methane consumption value at 80 °C (Fig. 2). The methane oxidation potential of FAV2 soil strongly depends on the initial CH_4 concentration in the headspace: methane oxidation values of 9500 ng g^{-1} h^{-1} are measured with an initial CH_4 concentration of 85 000 µmol mol^{-1} at room temperature and decrease down to 131 ng g^{-1} h^{-1} with a starting concentration of 148 µmol mol^{-1} (data not shown).

4.3 Bacterial diversity at the geothermal site

Total bacterial diversity of sites FAV1, FAV2 and FAV3 was analysed by Temporal Thermal Gradient gel Electrophoresis (TTGE) of PCR-amplified bacterial 16S rRNA gene fragments from total soil DNA (Fig. 3); TTGE band profiles indicate the presence of several putative bacterial phylotypes in Pantelleria geothermal soils. FAV2 and FAV3 samples share most TTGE bands, which probably reflect their similar chemical physical conditions (Table 1). The Chao1 richness estimator was 153 for FAV1, 231 for FAV2 and 253 for FAV3. The bacterial diversity Shannon's index (H'), was 2.8 in FAV1, 3.05

and 3.1 in FAV2 and FAV3, respectively; these indices are similar to those found in other geothermal areas (Yim et al., 2006).

4.4 Detection of methane oxidation genes

The presence of methanotrophs was verified by detecting the unique methane oxidation gene in the total soil DNA extracted from the three sites FAV1, FAV2, FAV3 and also in all the samples from the FAV2 vertical profile; PCR was carried out using the couple of primers targeting the *pmoA* gene, encoding the β-subunit of the proteobacterial methane monooxygenase. A unique band of the expected size (580 bp, data not shown) was obtained from FAV2, FAV3 and in all samples from the FAV2 vertical profile up to 15 cm depth (Table 2). Conversely, no PCR product was obtained from FAV1 (Table 1). The two newly designed couples of primers targeting the three verrucomicrobial methane monooxygenase genes, produced positive results only for FAV2 soil where the couple of primers 298f/599r (targeting *pmoA1/A2*) and the couple 156f/743r (targeting *pmoA3*) yielded the expected PCR products of about 300 and 600 bp, respectively (data not shown) (Table 1). Accordingly, soil samples from FAV2 profile showed the presence of verrucomicrobial methane monooxygenase genes with the exception of FAV2D. No amplification products were obtained with the verrucomicrobial *pmoA* primers from FAV1 and FAV3.

4.5 Diversity of methanotrophs at FAV2 site

In order to investigate the diversity of proteobacterial methanotrophs at the most active site FAV2, a *pmoA* gene library was constructed using the PCR product obtained from sample FAV2 (Table 1). The sequencing of sixteen randomly chosen clone inserts from the *pmoA* TOPO-TA library revealed abundance of Gammaproteobacterial methane monooxygenase genes distantly related to those of uncultured methanotrophic bacteria (82–90 % identity) and to the reference strain *Methylococcus capsulatus* bath (82 % nt identity), (Fig. 4). The closest sequences were detected in a methanotrophic community of tropical alkaline landfill upland soils (Chang et al., 2010). Two verrucomicrobial *pmoA* clones obtained with the couple of primers targeting the *pmoA3* gene (Table 3) were also sequenced and showed 99 % nt identity with *Methylacidiphilum fumarolicum* strain SolV (Fig. 4).

4.6 Isolation of methanotrophic bacteria from the geothermal site FAV2

In order to isolate methanotrophic bacteria from the geothermally active site, soil enrichment cultures in methane-enriched atmosphere were set. After one week of growth, the cultures incubated at 37 °C showed a visible increase in turbidity while no growth was observed at 65 °C. Amplification of *pmoA* gene from the enrichment cultures at 37 °C always gave positive results during the incubation period (data not shown). The amplification product of the last enrichment stage from FAV2E soil sample was sequenced, and its sequence was close (96 % id.) to that of uncultured *Methylocystis* (Fig. 4). The enrichment cultures were sub-cultured under the same conditions, and after streaking on M3 agar-slants, in sealed serum bottles with a CH_4-enriched atmosphere, a few single colonies, apparently very similar to each other, were detected after 4–5 days. Three isolates were obtained from the three central layers soil samples (2–10 cm) and further characterized. The isolates were stably able to grow on methane as the sole C sources, could grow on methanol and were unable to grow on glucose, fructose and ethanol. Their pH range of growth is 3.5 to 8, and they grow up to 45 °C but are unable to grow at 65 °C. The 16S rRNA gene sequence revealed that the three FAV2 isolates are all affiliated to the Alphaproteobacteria species *Methylocystis parvus* (99 % identity with *Methylocystis parvus* strain OBBP). The growth curve of *Methylocystis parvus* strain Pan1, indicates a correlation between methane consumption and turbidity OD_{600}. Methane concentration decreases with increasing turbidity, and the average oxidation rate is of 450 $\mu mol\, mol^{-1}\, h^{-1}$ (Fig. 5). Three other isolates, obtained from the methane enrichment cultures, were identified by 16S rRNA gene (partial) sequencing. Two isolates from the enrichment culture of FAV2E were assigned to the facultative methanotroph *Methylobacterium sp.* (95 % id.) and to *Brevibacillus agri* (99 %

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id.), respectively. The isolate obtained from the enrichment culture of FAV2A was assigned to the genus *Acidobacterium* (95 % id.) (data not shown). The cultures of *Brevibacillus agri* and *Acidobacterium sp*. appear pure based on cell morphology, however they might be consortia of tightly syntrophic bacteria. These genera have already been detected in methane-rich environments in association with methanotrophs (Dedysh, 2009). *Brevibacillus agri* was already cultured from thermal features and has been recently reported capable of growth on methane as the sole carbon source under thermophilic conditions, although methane is not its preferred substrate (Laursen et al., 2007). Sequences related to the phylum Acidobacteria have been detected from the ^{13}C-DNA during a Stable Isotope Probing (SIP) experiment, aiming to characterize the active methylotroph populations in forest soil microcosms (Radajewski et al., 2002).

5 Discussion

Pantelleria island represents a high enthalpy geothermal system, with petrological, structural and hydrothermal conditions that allow very high diffusive fluxes of geothermal gases enriched in methane (D'Alessandro et al., 2009; Parello et al., 2000). In this frame, Favara Grande represents the main exhalative area of Pantelleria island, emitting about 2.5 $t\,a^{-1}$ of CH_4 (D'Alessandro et al., 2009). In many sampling points of Favara Grande CO_2/CH_4 ratios in the soil gases were higher than in the hydrothermal end-member revealing a probable methanotrophic activity (D'Alessandro et al., 2009; Parello et al., 2000). Most of these sites corresponded to areas with low fluxes of hydrothermal gases towards the atmosphere and low concentrations of the same gases within the soil, probably allowing a more efficient microbial oxidation. Instead, in the areas where the fumarolic gas fluxes were high, such as the presently studied sites FAV1, FAV2 and FAV3, the CO_2/CH_4 ratio in the soil gases up to a 13 cm depth was similar to that of the fumarolic emissions, and then it decreased in the shallowest soil layers. This indicates that the methanotrophic activity within the soil profile is strongly influenced by the hydrothermal upflow efficiency, which in turn affects soil environmental conditions.

Many studies have highlighted that aerobic methanotrophs increase their efficiency in very aerated soils with high methane fluxes from the underground (Kip et al., 2012). A sustained hydrothermal gas upflow, as in the sites FAV1, FAV2 and FAV3, saturates soils in fumarolic gases such as methane, and the air dilution is hampered. Under these conditions, the required amount of O_2 for the aerobic methanotrophy is reached only in the shallowest soil layers. Measurements of the soil gases indicate a very high variation in concentration in the atmospheric gas content (O_2 and N_2) with depth. Air gases contribution in site FAV2 and FAV3, where higher methane consumptions were measured, is more than 70 % of the total gas content, creating a very favorable environment for methanotrophic bacteria and allowing atmospheric O_2 to sustain the detected microbial CH_4 oxidation. The thermo-acidic geothermal soils, where methanotrophic Verrucomicrobia were isolated for the first time, showed high methane fluxes (Castaldi and Tedesco, 2005), low pH (up to 1) and high temperatures up to 70 °C (Pol et al., 2007), although the isolation conditions for the bacteria were milder (pH 2 and temperature of 50 °C) than those detected in situ. The conditions detected at Favara Grande appear favorable for methanotrophs, that were detected by culture-independent methods in sites FAV2 and FAV3. These sites have high temperatures (up to 60 °C at 2 cm of depth), but are not acid (pH close to 5.8); incubation experiments point out the highest methanotrophic activity in the shallowest soil layers at FAV2 and FAV3 (reaching values up to 1249 $ng\,g^{-1}\,h^{-1}$ at FAV2) with rapid decrease with depth. The oxidation potential in the deepest layer (10–15 cm) is probably too low (100 $ng\,g^{-1}\,h^{-1}$) to significantly affect the CO_2/CH_4 ratio in the deepest sampling point FAV2E. Chemical-physical analysis and total bacterial diversity analyzed by TTGE suggest that sites FAV2 and FAV3 have very similar environmental conditions and microbial diversity. In both sites proteobacterial methane monooxygenase genes were detected, although verrucomicrobial *pmoA* was only detected in FAV2. Negligible methane oxidation in site FAV1 seems in accordance with the negative results obtained by molecular probing of *pmoA* genes and is probably due to high temperatures and low oxygen availability that prevent survival even of the most thermophilic Verrucomicrobia. The extreme physical chemical conditions,

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however, do not prevent bacterial life, as the TTGE analysis of bacterial 16S rRNA amplified gene describes a low complexity bacterial community that thrives in FAV1 soil. This community probably does not include (known) methanotrophs but could coexist with a more complex archaeal community (Kan et al., 2011). Enrichment cultures with methane as sole C and energy source and culture-independent techniques, based on functional gene probes, were used to describe the diversity of methanotrophs at the most active site FAV2. Matching the results obtained from the *pmoA* gene library and the isolation by enrichment cultures on the soil profile, FAV2 site at Favara Grande recorded the highest diversity of methanotrophs ever described before in a geothermal soil (Op den Camp et al., 2009; Kizilova et al., 2013). In the same soil, in fact, we could isolate and cultivate in pure culture type II Alphaproteobacterial methanotrophs of the genus *Methylocystis* and, contemporarily, we detected, by amplification of the functional methane monooxygenase gene *pmoA*, as yet uncultivated methanotrophic Gammaproteobacteria related to *Methylococcus capsulatus* and *Methylocaldum spp*. Moreover, most *pmoA* sequences show very low identity with other methanotrophs, indicating that Pantelleria geothermal soils host new species of methanotrophs that are adapted to these specific site conditions. As standard primers are not adequate for detecting *pmoA* genes in Verrucomicrobia, two new couples of primers were specifically designed on the consensus sequences of known Verrucomicrobia *pmoA* genes. Using the newly designed primers we were able to detect the presence of Verrucomicrobial methane monooxigenase genes of *Methylacidiphilum fumarolicum* SolV, isolated for the first time at Solfatara di Pozzuoli in Italy (Pol et al., 2007). This is an extraordinary high diversity of methanotrophs that could ever be expected in a geothermal soil and this is the first report in which the presence of both phyla of methanotrophs, Proteobacteria and Verrucomicrobia, is recorded and their coexistence is demonstrated. Different groups of methanotrophs are generally associated to their ability to survive, grow and oxidize methane in different environments. While the presence of Verrucomicrobia in a geothermal soil was predictable due to their thermophilic and acidophilic character, the presence of both Alpha- and Gammaproteobacteria was unexpected and suggests that high CH_4 fluxes and differences in environmental conditions shape the complex methanotroph community structure at this geothermal area. Interestingly, the results obtained from the *pmoA* gene library do not overlap with those from enrichment cultures. Gammaproteobacterial methane monooxygenase were only detected in the clone library from soil DNA, while only Alphaproteobacteria type II methanotrophs could be isolated after enrichment in a highly concentrated methane atmosphere at 37 °C. This would indicate a preponderance of the most thermo-tolerant Gammaproteobacterial methanotrophs close to the genera *Methylococcus* and *Methylocaldum* (Hanson and Hanson, 1996; Trotsenko et al., 2002) in the geothermal soil that probably account for methanotrophy at high temperatures. Under laboratory conditions, type II methanotrophs take over in the presence of high methane concentrations at 37 °C. However it has also been observed that type I methanotroph *pmoA* sequences could be preferentially amplified over those from type II methanotrophs due to variations in the guanine and cytosine content of their DNA (Murrell and Jetten, 2009). Type I methanotrophs are reported to be dominant in environments that allow the most rapid growth while type II methanotrophs, that tend to survive better, are more abundant in environments with fluctuatng nutrient availability (Hanson and Hanson, 1996). The conditions used in this study for enrichment culture setting were those described for the isolation of methanotrophic Verrucomicrobia by Islam and colleagues (2008) but we were unable to isolate any verrucomicrobial member, although they were detected by molecular methods. In particular, high CH_4 concentration and a temperature of 37 °C favored the growth of *Methylocystis* from the first top soil layers and of the facultative *Methylobacterium* in the deepest layer. *Methylocystis* is one of the most ubiquitous genera being capable to oxidize methane both in high and low amounts (Kip et al., 2012) and under acidophilic pH (Op den Camp et al., 2009). Our *Methylocystis* isolates Pan1, Pan2 and Pan3 show a larger pH range (from 3.5 to 8.0) and a higher temperature limit (> 40 °C) than those described for this genus (Kizilova et al., 2013). No isolates, instead, could be obtained from enrichments at 65 °C even though methanotrophic activity was detected in soils up to 80 °C. It can be argued that methanotrophy at higher temperatures could be sustained by the as yet uncultured (and perhaps unculturable) methanotrophs, detected by culture-independent methods, that are distantly related to the thermophilic genera *Methylocaldum* and *Methylococcus*. Analyses on Pantelleria soils suggest that a physiologically and taxonomically diverse group of methanotrophs are responsible for CH_4 consumption at FAV2 on a layer of 0–15 cm and presumably at FAV3 site; at the same time our results assess that temperatures above 80 °C hinder methane oxidation and probably methanotrophs survival. While methanotrophic Verrucomicrobia are reported to dominate highly acidic geothermal sites, our results indicate that slightly acidic soils, in high enthalpy geothermal systems, host a more diverse group of both culturable and uncultivated methanotrophs. This report contributes to better understand the ecology of methanotrophy in geothermal sites and its impact in atmospheric chemistry.

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Table 1. Chemical–physical characteristics of the soil sampled at the geothermal site Favara Grande (Pantelleria, Italy), methane consumption and detection of the functional gene for methane monooxygenase *pmoA*.

Soil sample	Depth cm	T °C	pH	OM	H_2O %	CH_4 consumption ng g^{-1} h^{-1}	Methane monooxygenase gene detection[a]	
							Proteobacterial *pmoA*	Verrucomicrobial *pmoA*
FAV1	0–3	62	3.4	3.7	12.9	5	–[b]	–
FAV2	0–3	60	5.8	3.1	2.8	950	+[c]	+
FAV3	0–3	50	5.2	2.9	3.3	620	+	–
FAV2A[d]	0–2	33	6.6	2.5	2.3	1249	+	+
FAV2B	2–4	37	6.7	2.7	6.2	701	+	+
FAV2C	4–7	46	6.6	3.1	1.8	186	+	+
FAV2D	7–10	74	6.0	4.3	1.9	107	+	–
FAV2E	10–13	83	5.9	6.0	1.9	100	+	+

[a] Gene detection was performed by PCR with primers described in Table 3.
[b] Absence of amplicon of the expected size.
[c] Presence of an amplicon of the expected size.
[d] Soils FAV2A to FAV2E were sampled at FAV2 site at different depths (0–15 cm).

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Table 2. Chemical composition of soil gases in the Favara Grande area.

Sample	Depth cm	T °C	O_2	N_2 µmol mol^{-1}	CH_4	CO_2
FAV1	13	82.7	143 800	545 200	9900	284 200
	25	103.7	41 000	97 300	38 700	732 700
	50	102.2	24 500	46 200	34 600	759 500
FAV2	13	75	159 200	606 800	8500	216 000
	25	85.9	36 700	69 700	36 000	832 500
	50	111.6	32 500	62 700	38 900	808 400
FAV3	13	52.8	181 800	715 700	1854	77 300
	25	68.5	162 300	633 200	6211	187 000
	50	88.2	106 300	386 800	18 800	482 600

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Table 3. Primer couples used for PCR amplifications.

Primer	Sequence	Target gene	Reference or source
341F 534R	5'-CCTACGGGAGGCAGCAG-3' 5'-ATTACCGCGGCTGCTGG-3'	16S rRNA	Muyzer et al. (1993)
fD1 rD1	5'-AGAGTTTGATCCTGGCTCAG-3' 5'-AAGGAGGTGATCCAGCC-3'	16S rRNA	Weisburg et al. (1991)
A189F A682R	5'-GGNGACTGGGACTTCTGG-3' 5'-GAASGCNGAGAAGAASGC-3'	*pmoA* (Proteobacteria)	Holmes et al. (1995)
M13 F M13 R	5'-GTAAAACGACGGCAG-3' 5'-CAGGAAACAGCTATGAC-3'	TOPO-TA vector	Supplied by TOPO TA cloninig kit
298f 599r	5'-CAGTGGATGAAYAGGTAYTGGAA-3' 5'-ACCATGCGDTGTAYTCAGG-3'	*pmoA1-A2* (Verrucomicrobia)	This study
156f 743r	5'-TGGATWGATTGGAAAGATCG-3' 5'-TTCTTTACCCAACGRTTTCT-3'	*pmoA3* (Verrucomicrobia)	This study

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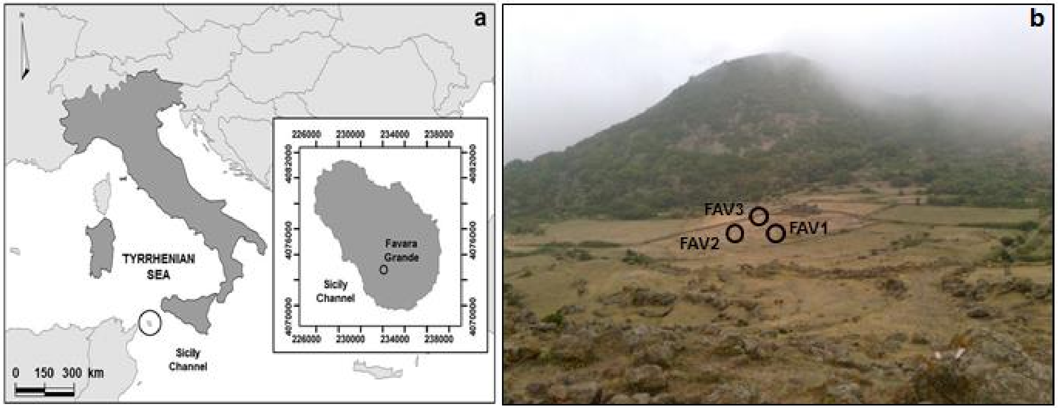

Fig. 1. (a) Pantelleria island (Italy) and **(b)** the three main sampling points at the geothermal field of Favara Grande.

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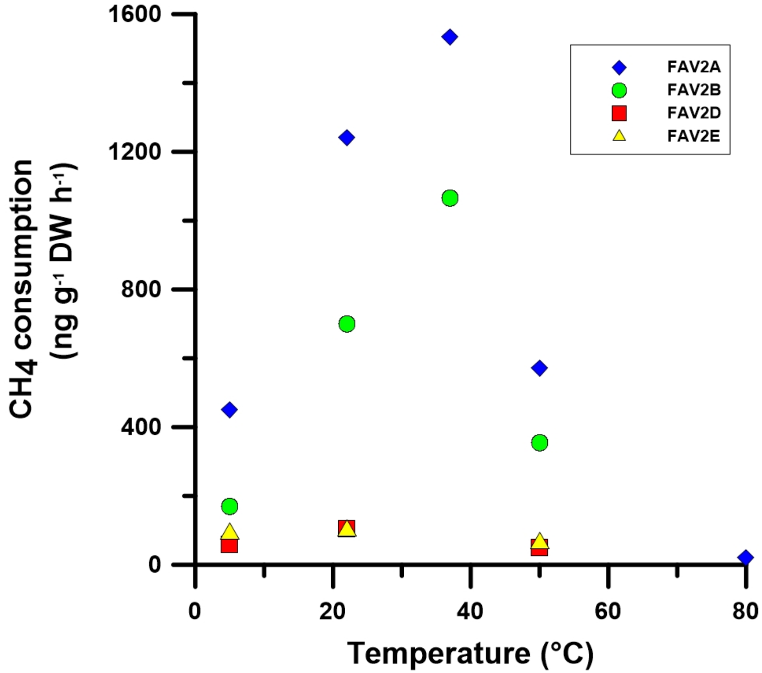

Fig. 2. Methane consumption of soils sampled at different depths of FAV2 and measured at different temperatures.

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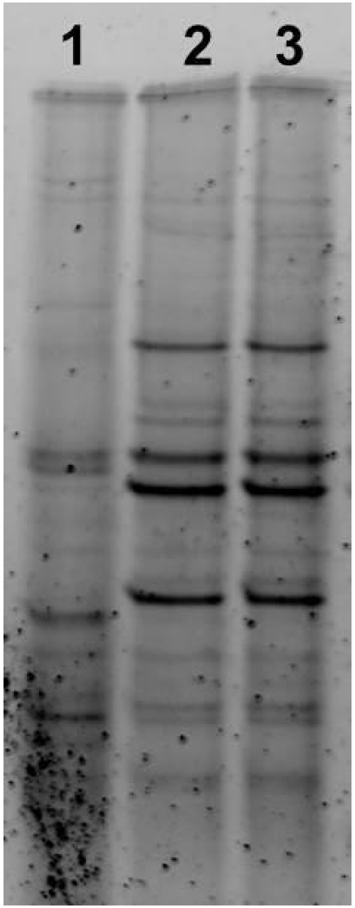

Fig. 3. Bacterial diversity at the geothermal site Favara Grande; Temporal Thermal Gradient gel Electrophoresis (TTGE) profiles of PCR-amplified 16S gene fragments derived from soil DNA extracted from sites (1) FAV1, (2) FAV2, (3) FAV3.

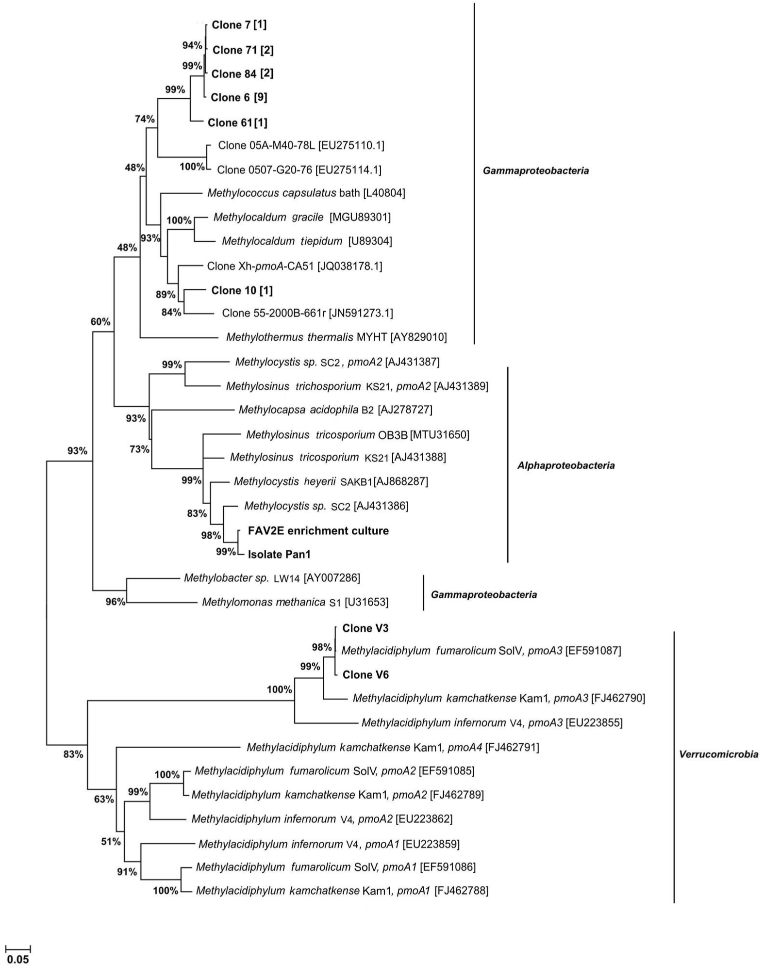

Fig. 4. Caption on next page.

Fig. 4. Phylogenetic tree constructed based on partial sequences (529 nt) of *pmoA* genes, showing the relative position of the genes and isolates from the geothermal site FAV2 where high methane oxidation rates were detected. The tree was constructed in the Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (Tamura et al., 2007), a model Maximum Composite Likelihood (MCL) was used (Schmidt et al., 2002). A neighbor-joining distance correction method was applied. Node support values are indicated for the primary nodes. The scale bar represents 0.05 change per position. FAV2E enrichment culture derives from amplification of the final stage of the enrichment, all the other are from the FAV2 soil clone library. Pan1 was isolated from the enrichment cultures together with Pan2 and Pan3 (not shown); the number of clones with identical sequences is indicated in square brackets.

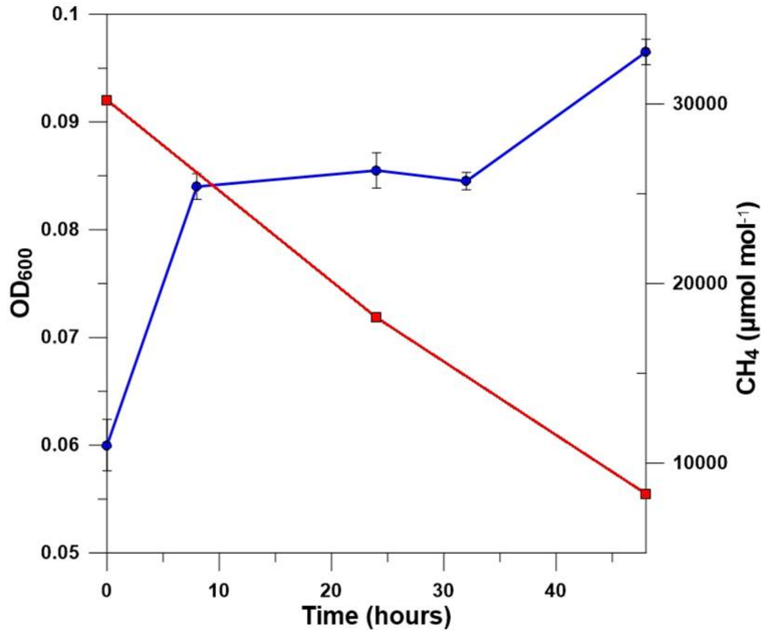

Fig. 5. Growth (blue line) and corresponding variation of the methane in the headspace of the serum bottles as result of methane consumption (red line) of *Methylocystis sp*. strain Pan1. Average of the optical density measures (OD_{600}) ± standard error are from five replicate 200 mL serum bottles incubated at 37 °C for 3 days before starting measurements. The pH was 5.8. No decrease in headspace methane was observed in uninoculated controls (data not shown).

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