

# Methanotrophic activity and diversity of methanotrophs 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<sub>4</sub>, a greenhouse gas twenty-five times as potent as CO<sub>2</sub>. Recently, it has been demonstrated that volcanic/geothermal soils are not only a source of methane, but also sites of methanotrophic activity. Methanotrophs are able to consume 10-40 Tg of CH<sub>4</sub> a<sup>-1</sup> 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 Mg a<sup>-1</sup> (t a<sup>-1</sup>). Laboratory incubation experiments with three top-soil samples from Favara Grande indicated methane consumption values up to 59.2 nmol g<sup>-1</sup> soil d.w. h<sup>-1</sup>. 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 and values >6.23 nmol g<sup>-1</sup> h<sup>-1</sup> were still detected up to a depth of 13 cm. The highest consumption rate was measured at 37°C, but a still detectable consumption at 80°C (>1.25 nmol g<sup>-1</sup> h<sup>-1</sup>) was recorded. The soil total DNAs extracted from the three 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 re-

lated 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 a pH range from 3.5 to 8, temperatures of 18 – 45 °C and consume 160 nmol of CH<sub>4</sub> h<sup>-1</sup> ml<sup>-1</sup> of culture. Soils 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<sub>4</sub>, produced within the Earth's crust, are currently released naturally into the atmosphere (Etiopie et al., 2008). Volcanic/geothermal systems emit endogenous gases, including CH<sub>4</sub>, by widespread degassing from soils. Indirect estimations based on CO<sub>2</sub> or H<sub>2</sub>O outputs and CO<sub>2</sub>/CH<sub>4</sub> or H<sub>2</sub>O/CH<sub>4</sub> ratios of the main

gas manifestations gave a total CH<sub>4</sub> emission from European geothermal/volcanic systems in the range of 4-16 Gg a<sup>-1</sup> (4,000 - 16,000 ta<sup>-1</sup>) (Etiopie et al., 2007). Methanotrophy is a metabolic process by which bacteria obtain energy via the oxidation of CH<sub>4</sub> to CO<sub>2</sub> (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<sub>4</sub> a<sup>-1</sup> 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 depends 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<sub>4</sub> (Hanson and Hanson, 1996; Op den Camp et al., 2009). In such situation methanotrophic activity is sustained by a CH<sub>4</sub> flux coming from the atmosphere above the soil but this activity can also be sustained by CH<sub>4</sub> fluxes coming from below. Such flux can be of biological origin (CH<sub>4</sub> 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<sub>4</sub> flux often exceeds the biologic oxidation capacity, and soils become a source of endogenous CH<sub>4</sub> towards the atmosphere (Cardellini et al., 2003; Castaldi and Tedesco, 2005; D'Alessandro et al., 2009, 2011; Etiopie and Klusman, 2010). Methane flux measurements in volcanic/geothermal areas, started in recent years (Etiopie and Klusman, 2002; Castaldi and Tedesco, 2005), accounted for a new, previously neglected, source of atmospheric CH<sub>4</sub>. 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). Before the discovery of methanotrophic Verrucomicrobia, that are affiliated to the family Methylococcaceae, known methanotrophic bacteria were taxonomically affiliated to the phylum Proteobacteria in the classes Gammaproteobacteria and Alphaproteobacteria. 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 (RuBisCO), 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). The RuBisCO pathway is used by Verrucomicrobia *Methylococcoides burtonii* to fix CO<sub>2</sub> using CH<sub>4</sub> as energy source (Khadem et al., 2011).

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 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 Mg a<sup>-1</sup> (t a<sup>-1</sup>). The same authors suggested the presence of methanotrophic activity within the soils of this area. The main reason was because concurrent CO<sub>2</sub> and CH<sub>4</sub> flux measurements showed nearly always a CO<sub>2</sub>/CH<sub>4</sub> 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<sub>4</sub> 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-dependent and culture-independent approaches.

## 2 Material and methods

### 2.1 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<sup>2</sup> 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 manifestations 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 mmol mol<sup>-1</sup>) followed by CO<sub>2</sub> (about 23 mmol mol<sup>-1</sup>). Among the minor components the fumarolic gases of Favara Grande display relatively high contents of H<sub>2</sub> and CH<sub>4</sub> (about 1.30 and 0.80 mmol mol<sup>-1</sup>, respectively) and low contents of H<sub>2</sub>S (<0.02 mmol mol<sup>-1</sup>). This leads, after condensation of water vapor, to high CH<sub>4</sub> concentrations in the soils (up to 44 mmol mol<sup>-1</sup>) and high CH<sub>4</sub> fluxes from the soil (up to 221 mmol m<sup>-2</sup> day<sup>-1</sup>) in the area of Favara Grande (D'Alessandro et al., 2009).

## 2.2 Soil sampling and chemical-physical characterization

Soil samples used in this study were collected at Favara Grande during two field campaigns in 2011 in an area that has previously ascertained to be the site of intense geothermal degassing (D'Alessandro et al., 2009). 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 0-13 cm (FAV2A to FAV2E) (Fig.1, Table 1). Ground temperature measurements (at 2-50 cm of depth) were taken using thermal probes and a digital thermometer. All the soil samples used for geochemical and microbiological analyses were taken using a sterile hand shovel and stored in sterile plastic bags. Soil sub-samples for molecular analyses were stored at -20°C until analysis. Soil sub-samples for geochemical analysis were air-dried overnight, sieved at 2 mm and homogenised. Organic matter in soils was measured by loss-on-ignition analysis with heating stages of 105 °C for 4 h (for % of H<sub>2</sub>O 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.

## 2.3 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. The samples were collected sequentially from the shallowest to the deepest level. To avoid atmospheric contamination the suction through the syringe is made very slowly (> 60 sec for 20 ml). Two aliquots of about 20 ml of soil gas were extracted. The first was discarded and the second 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<sub>4</sub>, CO<sub>2</sub>, N<sub>2</sub>, O<sub>2</sub> and H<sub>2</sub> 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 1,000 µl loop. The introduction system of the GC (total volume about 2 ml) was evacuated with a vacuum pump before the introduction of the sample and was provided with a pressure sensor to correct small (positive or negative) deviations from the introduction pressure (atmospheric) of the calibration standards. Calibration was made with certified gas mixtures. Analytical precision ( $\pm 1\sigma$ ) was always better than  $\pm 3\%$ . The detection limits were about 0.1 µmol mol<sup>-1</sup> for CH<sub>4</sub>, 2 µmol mol<sup>-1</sup> for H<sub>2</sub>, 10 µmol mol<sup>-1</sup> for CO<sub>2</sub> and 200 µmol mol<sup>-1</sup> for O<sub>2</sub> and N<sub>2</sub>.

## 2.4 Methanotrophic activity

Methane oxidation potential of the soils was analyzed by transferring about 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<sub>4</sub> to reach about 1 mmol mol<sup>-1</sup>. Bottles were incubated at controlled room temperature (23-25°C) and the CH<sub>4</sub> concentration was measured on the same bottles at the beginning of the experiment and at about 24h intervals for 5 days. To better monitor the methane consumption in samples that after 24h consumed more than 30% of the initial CH<sub>4</sub> the experiments were repeated measuring the concentrations at 2h intervals. Samples collected in autumn from the FAV2 vertical profile were also incubated at 5°C, 37°C, 50°C and 80°C under the same conditions. Finally, the variation of the soil CH<sub>4</sub> oxidation potential was analysed on sample FAV2A with different starting CH<sub>4</sub> concentrations at room temperature (from about 0.1 to 85 mmol mol<sup>-1</sup>). Methane concentration inside the vials was measured using CG as above withdrawing about 2 ml gas for each analysis. All incubation experiments were in duplicate and the results expressed as nmol CH<sub>4</sub> per g of soil dry weight per h (nmol g<sup>-1</sup> h<sup>-1</sup>). To report methane oxidation potential to the dry weight of the soil, subsamples of the air-dried soil were oven-dried at 105 °C. Taking into account all the instrumental errors, we consider that only values above 0.6 nmol g<sup>-1</sup> h<sup>-1</sup> indicate significant oxidation activity.

## 2.5 Extraction of soil DNA

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 1X TAE agarose gel (1%) electrophoresis and spectrophotometric analysis using Nanodrop (NanoDrop ND-1000, Celbio SpA).

## 2.6 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 (Holmes et al., 1995), targeting the  $\beta$ -subunit of the proteobacterial *pmoA* gene. PCRs were carried out in a final volume of 50  $\mu$ l, containing 100 ng of total DNA, 200 nM of each oligonucleotide primer, 0.20 mM dNTPs, and 1 u 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 sec, annealing at 56 °C for 45 sec and extension at 72 °C for 45 sec 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 PCR II 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 (M13F/M13R, supplied by the TOPO-TA® cloning kit). Positive clones were sequenced using the universal T7 primer. The sequences of the *pmoA* clones were deposited in Genbank under accession numbers KJ207214-19. Two novel couples of primers, 298f/599r (5'-CAGTGGATGAAYAGGTAYTGGAA-3'/5'-ACCATGCGDGTAYTCAGG-3') and 156f/743r (5'-TGGATWGATTGGAAAGATCG-3'/5'-TTCTTTACCCAACGRITTTCT-3'), targeting Verrucomicrobial *pmoA1/A2* and *pmoA3*, respectively, were designed and positively validated on *Methylophilum 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 sec followed by 5 cycles consisting of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec and extension at 68°C for 30 sec; the following 35 cycles consisted of a denaturation at 94°C for 30 sec, annealing at 52°C for 10 sec and extension at 68°C for 30 sec. A final extension at 68°C was carried out for 5 min. Amplicons were purified and cloned into PCR II TOPO-TA® (Invitrogen, USA) as described above. Clones

containing an insert of the correct size were sequenced as described above.

## 2.7 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 13 cm (samples FAV2A to FAV2E) were placed in 125-ml sealed serum bottles in atmosphere supplemented with methane (25%) and incubated either at 37°C or 65°C for 2 weeks. After incubation, 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. 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. 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<sub>4</sub> atmosphere at different temperatures. Genomic DNA was extracted from 10 ml of M3-CH<sub>4</sub> 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 16S rRNA gene with universal primers fD1/rD1 (Weisburg et al., 1991) 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 *Methylocystis* sp. 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 to an (OD<sub>600</sub>) of 0.25, corresponding to 2.6·10<sup>7</sup> CFU ml<sup>-1</sup>) and subsequently inoculated in three 160-ml serum bottles (2 ml each) containing 20 ml of M3 mineral medium and ~95 mmol mol<sup>-1</sup> methane; growth was monitored as turbidity using a spectrophotometer. Methane concentration was periodically measured in the cultures and in the uninoculated control bottles incubated under the same conditions.

### 3 Results

#### 3.1 Soil gas composition

Soil gases collected in June 2011 at the sites FAV1, FAV2 and FAV3 (Fig.1, Table 1) display a composition that is the result of the mixing process between a hydrothermal component rich in H<sub>2</sub>, CH<sub>4</sub> and CO<sub>2</sub> and an atmospheric component rich in O<sub>2</sub> and N<sub>2</sub> (Table S1). 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<sub>2</sub> concentrations would still be enough to sustain aerobic methanotrophic activity (Kumaresan et al., 2011).

#### 3.2 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 and Table S1). FAV1 has the highest temperature (62°C at 2 cm of depth) and lowest pH (3.4); FAV2 is similar to FAV3 and both show significantly milder conditions than FAV1. Organic matter was in a range of 1.8 to 6.2 % 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<sub>4</sub> consumption values in a range from 0.31 (FAV1) to 59.2 nmol g<sup>-1</sup> h<sup>-1</sup> (FAV2), (Fig.2). Since FAV2 was the most active site, its methane oxidation was further investigated on a vertical profile up to a depth of 13 cm (Fig.2). Temperature, in FAV2, increases with depth from 33°C to 83 °C while pH decreases from 6.6 to 5.9. The maximum methane oxidation rate in the FAV2 vertical profile was measured in the shallowest soil layers (0-2 cm), but significant values (6.23 nmol g<sup>-1</sup> h<sup>-1</sup>) were still detected at 13 cm depth (Fig.2). When samples from the vertical profile were incubated at different temperatures, the CH<sub>4</sub> 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<sub>4</sub> concentration in the headspace: methane oxidation values of 592 nmol g<sup>-1</sup> h<sup>-1</sup> are measured with an initial CH<sub>4</sub> concentration of 85 mmol mol<sup>-1</sup> at room temperature and decrease down to 8.17 nmol g<sup>-1</sup> h<sup>-1</sup> with a starting concentration of 0.15 mmol mol<sup>-1</sup> (data not shown).

#### 3.3 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  $\beta$ -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 13 cm depth (Table 1). 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.

#### 3.4 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 twenty-six 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% nt identity) and to the reference strain *Methylococcus capsulatus* bath (82% nt identity), (Fig.3). The closest sequences had been recently detected in a methanotrophic community of tropical alkaline landfill upland soils (Chang et al., 2010). Two of the verrucomicrobial *pmoA* clones, obtained with the couple of primers targeting the *pmoA3* gene (see above) were sequenced and showed 99% identity with *Methylacidiphilum fumarolicum* strain SolV (Fig.3).

#### 3.5 Isolation of methanotrophic bacteria from the geothermal site FAV2

In order to isolate methanotrophic bacteria from the geothermally active site, soil enrichment cultures were set in methane-enriched atmosphere. 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 proteobacterial *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 an uncultured *Methylocystis* (Fig.3). Amplification of verrucomicrobial *pmoA* genes gave negative results since the first M3 enrichment step. The enrichment cultures were subcultured under the same conditions, and after streaking on

M3 agar-slants, in sealed serum bottles with a CH<sub>4</sub>-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 was 3.5 to 8, and they grew up to 45°C but were 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 OBPP). The growth curve of *Methylocystis parvus* strain Pan1, indicates a correlation between methane consumption and turbidity, and a methane oxidation in the order of 160 nmol per hour h<sup>-1</sup> ml<sup>-1</sup> of culture (Fig.4). 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% 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. appeared 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 <sup>13</sup>C-DNA during a Stable Isotope Probing (SIP) experiment, aiming to characterize the active methylotroph populations in forest soil microcosms (Radajewski et al., 2002).

#### 4 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 Mg a<sup>-1</sup> (t a<sup>-1</sup>) of CH<sub>4</sub> (D'Alessandro et al., 2009). In many sampling points of Favara Grande CO<sub>2</sub>/CH<sub>4</sub> 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 allow-

ing 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<sub>2</sub>/CH<sub>4</sub> 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<sub>2</sub> 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<sub>2</sub> and N<sub>2</sub>) 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<sub>2</sub> to sustain the detected microbial CH<sub>4</sub> oxidation. The conditions detected at Favara Grande appear favorable for methanotrophs, that were actually detected by culture-independent methods in sites FAV2 and FAV3. These sites have high temperatures (up to 60°C at 2 cm of depth), and are only slightly acidic; incubation experiments point out the highest methanotrophic activity in the shallowest soil layers at FAV2 and FAV3 with rapid decrease with depth. The oxidation potential in the deepest layer (10-13 cm) is probably too low (6.23 nmol g<sup>-1</sup> h<sup>-1</sup>) to significantly affect the CO<sub>2</sub>/CH<sub>4</sub> ratio in the deepest sampling point FAV2E. Chemical-physical analysis and total bacterial diversity analyzed by TTGE (Supplement, Fig.S1) suggest that sites FAV2 and FAV3 have very similar environmental conditions and microbial diversity. In both sites proteobacterial methane monoxygenase 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, low pH, low oxygen availability and chemical conditions at FAV1, that prevent survival even of the most thermophilic Verrucomicrobia. 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 were milder (pH 2 and temperature of 55°C) than those detected *in situ*. The extreme physical conditions, however, do not prevent bacterial life, as the TTGE (Supplement, Fig.S1) 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) methan-

otrophs but could coexist with a more complex archaeal community (Kan et al., 2011).

575 Enrichment cultures with methane as sole C and energy 630  
source and culture-independent techniques, based on func-  
tional gene probes, were used to describe the diversity of  
methanotrophs at the most active site FAV2. Matching the  
580 results obtained from the *pmoA* gene library and the isola-  
tion by enrichment cultures on the soil profile, FAV2 site 635  
at Favara Grande recorded the highest diversity of methan-  
otrophs ever described before in a geothermal soil (Op den  
Camp et al., 2009; Kizilova et al., 2013). In the same soil, in  
585 fact, we could isolate and cultivate in pure culture type II Al-  
phaproteobacterial methanotrophs of the genus *Methylocys-* 640  
*tis* and, contemporarily, we detected, by amplification of the  
functional methane monooxygenase gene *pmoA*, as yet un-  
cultivated methanotrophic Gammaproteobacteria related to  
*Methylococcus capsulatus* and *Methylocaldum spp.*

590 As standard primers are not adequate for detecting *pmoA* 645  
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 Verrucomi-  
595 crobial methane monooxygenase genes of *Methylacidiphilum* 650  
*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  
600 presence of both phyla of methanotrophs, Proteobacteria and  
Verrucomicrobia, is recorded and their coexistence is demon-  
strated. Interestingly, the results obtained from the *pmoA*  
gene libraries do not overlap with those from enrichment cul-  
tures. Gammaproteobacterial and verrucomicrobial methane  
605 monooxygenase were only detected in the clone library from 660  
soil DNA, while only Alphaproteobacteria type II methan-  
otrophs could be isolated after enrichment in a highly con-  
centrated methane atmosphere at 37°C. This would indicate  
a preponderance of the most thermo-tolerant Gammapro-  
610 teobacterial methanotrophs close to the genera *Methylococ-* 665  
*cus* and *Methylocaldum* (Hanson and Hanson, 1996; Trot-  
senko et al., 2002) in the geothermal FAV2 soil.

These as yet uncultivated Type I methanotrophs are prob-  
ably responsible for methanotrophy in the shallowest soil  
615 layer, but also in the hottest deeper layers. Type I methan- 670  
otrophs are reported to be dominant in environments that  
allow the most rapid growth while type II methanotrophs,  
are more abundant in environments with fluctuating nutri-  
ent availability (Hanson and Hanson, 1996). Type I *pmoA*  
620 sequences, however, could be preferentially amplified over 675  
those from type II methanotrophs due to variations in the  
guanine and cytosine content of their DNA (Murrell and Jet-  
ten, 2009). Type II methanotrophs in fact could not be de-  
tected by molecular methods but resulted as the dominant  
625 cultivable methanotrophs in the top 10 cm soil layers. Un- 680  
der laboratory conditions in the presence of high methane  
concentrations at 37°C, type II methanotrophs, that tend to

survive better, took precedence over the other methanotrophs  
both Gammaproteobacteria and Verrucomicrobia.

The conditions used in this study for enrichment culture  
setting, actually, were those described for the isolation of  
methanotrophic Verrucomicrobia by Islam and colleagues  
(2008); following their procedure, the medium pH was ad-  
justed to 6 (instead of 3.5), in order to restore the FAV2  
511 soil condition, but we were unable to isolate any verrucomi-  
crobial member, although they were detected by molecular  
methods in the same soil samples. Cultivation of Verrucomi-  
crobia methanotrophs under laboratory conditions is hard to  
obtain and seems to be limited by still unknown factors. New  
knowledge on the positive role of rare earth elements (REE)  
in Verrucomicrobia methanotrophs growth and cultivation  
was recently provided by Pol et al. (2014). They suggested  
that a good strategy, to cultivate Verrucomicrobia methan-  
otrophs, could be the addition of REE in the culture media.  
In fact, recently it was demonstrated that lanthanides are es-  
sential cofactors of homodimeric methanol dehydrogenase by  
providing superior catalytic properties to pyrroloquinoline  
quinone (PQQ)-dependent methanol dehydrogenase, which  
is a key enzyme for both methanotrophs and methylotrophs  
(Pol et al., 2014).

Pantelleria soils naturally contain REE (Gagliano, 2014),  
but have higher pH values with respect to the soils of Sol-  
fatara where the Verrucomicrobia (Pol et al., 2007, 2014)  
were isolated. Soil acidity strongly increases REE mobility  
and consequently their availability to soil microbes (Grawun-  
der and Merten, 2012). Due to the relatively high pH of the  
soil collected at FAV2 and, consequently, of the M3 medium  
used for enrichment cultures, REE could have been limiting  
for the growth of Verrucomicrobia methanotrophs in the M3  
culture medium.

High CH<sub>4</sub> concentration and a temperature of 37°C fa-  
vored the growth of *Methylocystis* from the first top soil lay-  
ers and of the facultative *Methylobacterium* in the deepest  
layer. *Methylocystis* is one of the most ubiquitous genera be-  
ing capable to oxidize methane both in high and low amounts  
(Kip et al., 2012) and under acidic 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). These *Methylocystis* isolates could contribute  
to methane oxidation in the shallowest soil layers up to 7 cm,  
characterized by T<46°C and almost neutral pH.

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 uncul-  
tured (and perhaps unculturable) methanotrophs, detected by  
culture-independent methods, that are distantly related to the  
thermophilic genera *Methylocaldum* and *Methylococcus*.  
Moreover, most *pmoA* sequences show very low identity with  
other methanotrophs, indicating that Pantelleria geothermal  
soils host new species of thermophilic methanotrophs that

are adapted to these specific site conditions. 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<sub>4</sub> fluxes and differences in environmental conditions shape the complex methanotroph community structure at this geothermal area.

This study on Pantelleria soils suggests that a physiologically and taxonomically diverse group of methanotrophs are responsible for CH<sub>4</sub> consumption at FAV2 on a layer of 0 - 13 cm and, presumably, at FAV3 site; at the same time our results assess that temperatures above 80°C hinder methane oxidation and probably survival of methanotrophs. 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*. Thermal probe precision  $\pm 0.01^\circ\text{C}$ ; pH-meter precision  $\pm 0.02$ ; OM and  $\text{H}_2\text{O}$  precision  $\pm 0.01\%$ .

Soil sample	Depth cm	T <sup>a</sup> °C	pH	OM %	H <sub>2</sub> O %	Methane monooxygenase gene detection <sup>b</sup>	
						Proteobacterial <i>pmoA</i>	Verrucomicrobial <i>pmoA</i>
FAV1	0 - 3	62	3.4	3.7	12.9	- <sup>c</sup>	-
FAV2	0 - 3	60	5.8	3.1	2.8	+ <sup>d</sup>	+
FAV3	0 - 3	50	5.2	2.9	3.3	+	-
FAV2A <sup>e</sup>	0 - 2	33	6.6	2.3	2.5	+	+
FAV2B	2 - 4	37	6.7	6.2	2.7	+	+
FAV2C	4 - 7	46	6.6	1.8	3.1	+	+
FAV2D	7 - 10	74	6.0	1.9	4.3	+	-
FAV2E	10 - 13	83	5.9	1.9	6.0	+	+

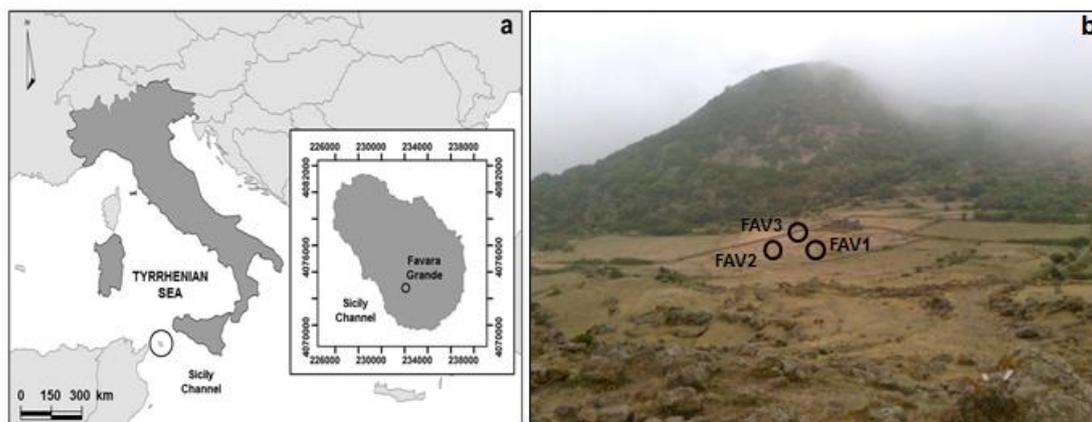
<sup>a</sup>: Table S1 shows temperatures measured up to 50 cm of depth.

<sup>b</sup>: Gene detection was performed by PCR with primers A189F/A62R (Holmes et al., 1995), 298f/599r and 156f/743r (this study, see text).

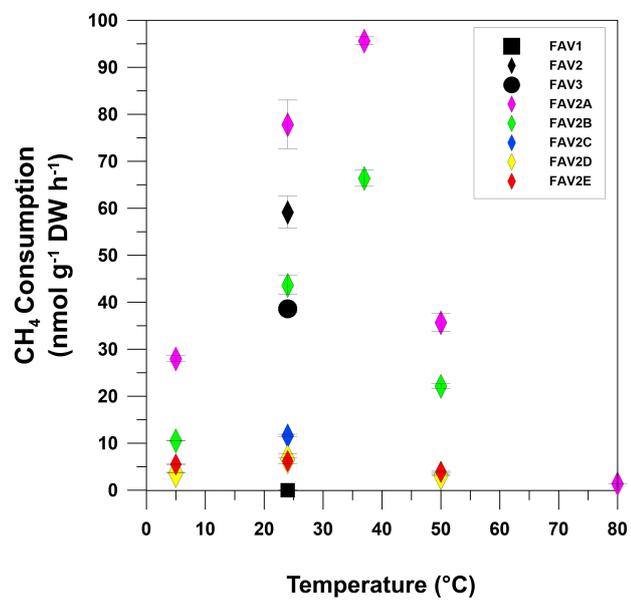
<sup>c</sup>: Absence of amplicon of the expected size.

<sup>d</sup>: Presence of amplicon of the expected size.

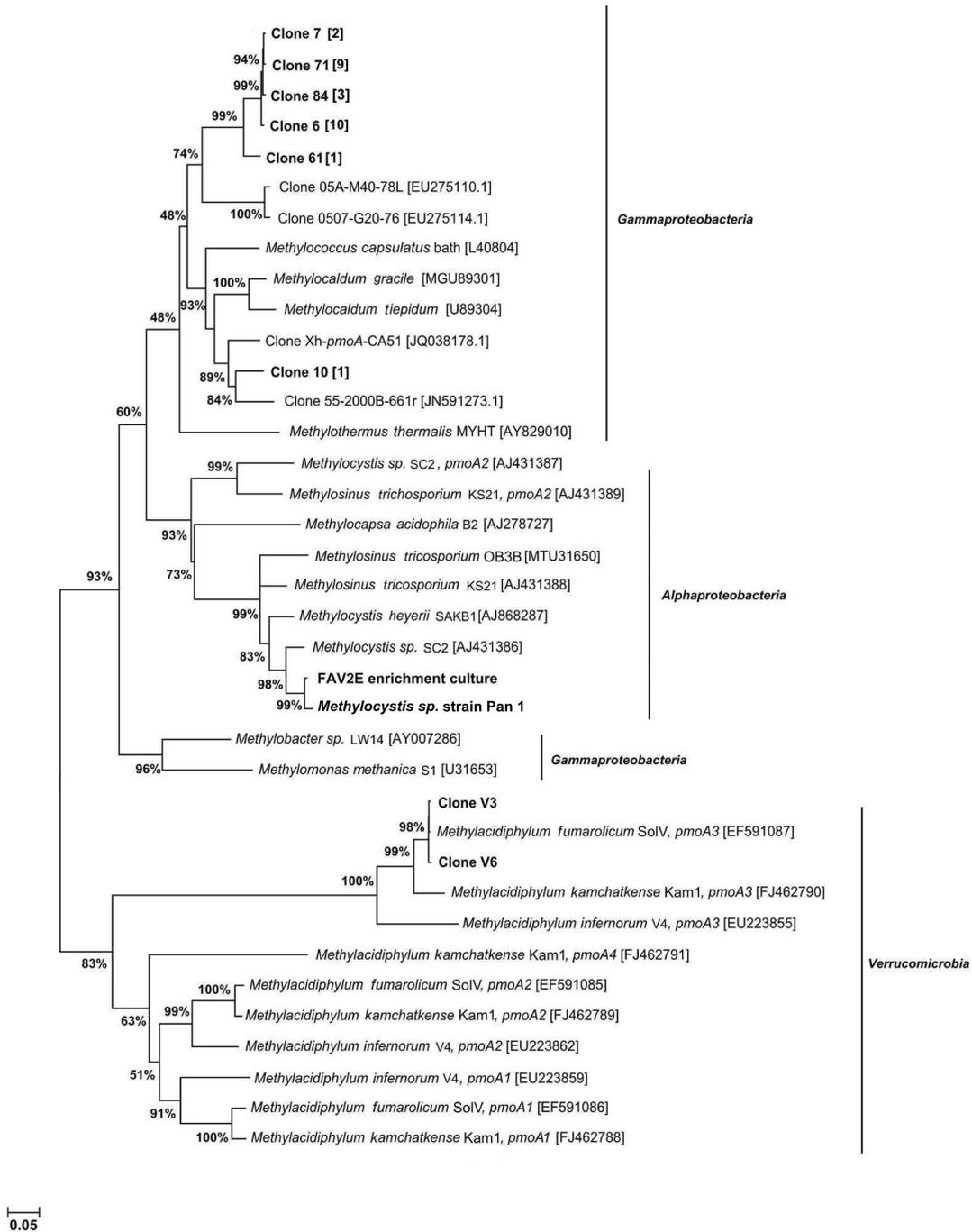
<sup>e</sup>: Soil samples FAV2A to FAV2E were sampled at FAV2 site at different depths (0 – 13 cm), in November.



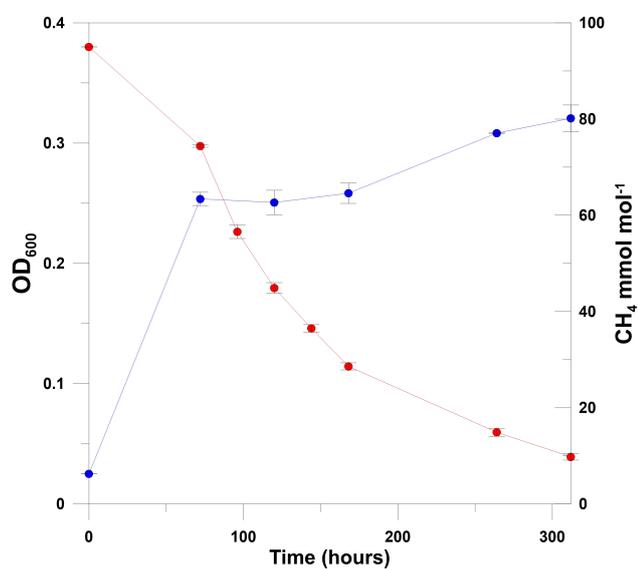
**Fig. 1.** a) Pantelleria island (Italy) and b) the three main sampling points at the geothermal field of Favara Grande.



**Fig. 2.** Methane consumption of soils sampled at sites FAV1, FAV2, FAV3 (after incubation at controlled room temperature), and at different depths of FAV2 (soils incubated at different temperatures).



**Fig. 3.** 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 is isolated from the enrichment cultures together with Pan2 and Pan3 (not shown); the total number of clones with identical sequences is indicated in square brackets.



**Fig. 4.** Growth (blu 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 ( $OD_{600} \pm$  standard error) are from three replicate 160-ml serum bottles incubated at 37°C at pH 5.8. No decrease in headspace methane was observed in uninoculated controls (data not shown).