



Methanotrophic activity and diversity of methanotrophs in volcanic geothermal soils at Pantelleria (Italy)

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Abstract. Volcanic and geothermal systems emit endogenous gases by widespread degassing from soils, including CH₄, a greenhouse gas twenty-five times as potent as CO₂. Recently, it has been demonstrated that volcanic or geothermal soils are not only a source of methane, but are also sites of methanotrophic activity. Methanotrophs are able to consume 10–40 Tg of CH₄ 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 (Italy), Favara Grande, whose total methane emission was previously estimated at about 2.5 Mg a⁻¹ (t a⁻¹). Laboratory incubation experiments with three top-soil samples from Favara Grande indicated methane consumption values of up to 59.2 nmol g⁻¹ soil d.w. h⁻¹. One of the three sites, FAV2, where the highest oxidation rate was detected, was further analysed on a vertical soil profile, the maximum methane consumption was measured in the top-soil layer, and values greater than 6.23 nmol g⁻¹ h⁻¹ 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⁻¹ h⁻¹) was recorded. The soil total DNA extracted from the three samples was probed by Polymerase Chain Reaction (PCR) using standard proteobacterial primers and newly designed verrucomicrobial primers, targeting the unique methane monooxygenase gene *pmoA*; the presence of methanotrophs was detected at sites FAV2 and FAV3, but not at FAV1, where harsher chemical–physical conditions and negligible methane ox-

idation were detected. The *pmoA* gene libraries from the most active site (FAV2) pointed to a high diversity of gammaproteobacterial methanotrophs, distantly related to *Methylocaldum*-*Metylococcus* genera, and the presence of the newly discovered acido-thermophilic Verrucomicrobia methanotrophs. 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 of 3.5 to 8 and temperatures of 18–45 °C, and consume 160 nmol of CH₄ h⁻¹ mL⁻¹ of culture. Soils from Favara Grande showed the largest diversity of methanotrophic bacteria detected until now in a geothermal soil. While methanotrophic Verrucomicrobia are reported as dominating 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 large 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 CH₄, produced within

the earth's crust, are currently released naturally into the atmosphere (Etiopie et al., 2008). Volcanic/geothermal systems emit endogenous gases, including CH₄, by widespread degassing from soils. Indirect estimations based on CO₂ or H₂O outputs and CO₂/CH₄ or H₂O/CH₄ ratios of the main gas manifestations gave a total CH₄ emission from European geothermal/volcanic systems in the range of 4–16 Gg a⁻¹ (4000–16 000 t a⁻¹) (Etiopie et al., 2007). Methanotrophy is a metabolic process by which bacteria obtain energy via the oxidation of CH₄ to CO₂ (Murrell and Jetten, 2009). Methanotrophs are a subset of methylotrophic bacteria that use methane as the sole C source (Hanson and Hanson, 1996). They are abundant at the anoxic–oxic interfaces of methanogenic environments such as wetlands, peatlands (Kip et al., 2012), aquatic sediments (Rahalkar et al., 2009), landfills (Ait-Benichou et al., 2009) and, as recently discovered, also in geothermal areas, which have long been 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 CH₄ a⁻¹ and to trap more than 50 % of the methane degassing through the soils (IPCC, 2001; Reeburgh, 2003). The effectiveness of biological oxidation processes 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 favour methanotrophic activity consuming the atmospheric CH₄ efficiently (Hanson and Hanson, 1996; Op den Camp et al., 2009). In such a situation, methanotrophic activity is sustained by a CH₄ flux coming from the atmosphere above the soil, but this activity can also be sustained by CH₄ fluxes coming from below. Such a flux can be of biological origin (CH₄ 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 CH₄ flux often exceeds the biologic oxidation capacity, and soils become a source of endogenous CH₄ for 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, starting in recent years (Etiopie and Klusman, 2002; Castaldi and Tedesco, 2005), accounted for a new, previously neglected, source of atmospheric CH₄. Castaldi and Tedesco (2005) hypothesised 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 with the Verrucomicrobia phylum. 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 the methanotrophic Verrucomicrobia that are affiliated with the Methylococcaceae fam-

ily, known methanotrophic bacteria were taxonomically affiliated with the Proteobacteria phylum in the Gammaproteobacteria and Alphaproteobacteria classes. Among proteobacterial methanotrophs, type I methane-oxidising bacteria use the ribulose monophosphate pathway for formaldehyde fixation, while type II methane-oxidising bacteria use the serine pathway. Type X methanotrophs are similar to type I methanotrophs, but they also have low levels of enzymes of the Ribulose 1,5-bisphosphate carboxylase (RuBisCO) serine pathway, 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 Alpha-proteobacteria, respectively (Op den Camp et al., 2009), and type X methanotrophs have been included, together with type I, in the Methylococcaceae family (Gammaproteobacteria) (Wise et al., 1999). The RuBisCO pathway is used by Verrucomicrobia *Methylococcoides burtonii* to fix CO₂ using CH₄ as an energy source (Khadem et al., 2011).

Methanotrophic communities in natural areas can be investigated and characterised 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 catalyses 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. Previously, D'Alessandro et al. (2009) estimated a total methane output at Pantelleria of close to 10 Mg a⁻¹ (t a⁻¹). The same authors suggested the presence of methanotrophic activity within the soils of this area. The main reason was that concurrent CO₂ and CH₄ flux measurements nearly always showed a CO₂/CH₄ 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 a pattern points to a loss of CH₄ during the travelling 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 characterise the methane-oxidising bacteria that thrive in these soils using culture-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 has a surface area of 83 km², and it is entirely covered by volcanic products from both effusive and explosive

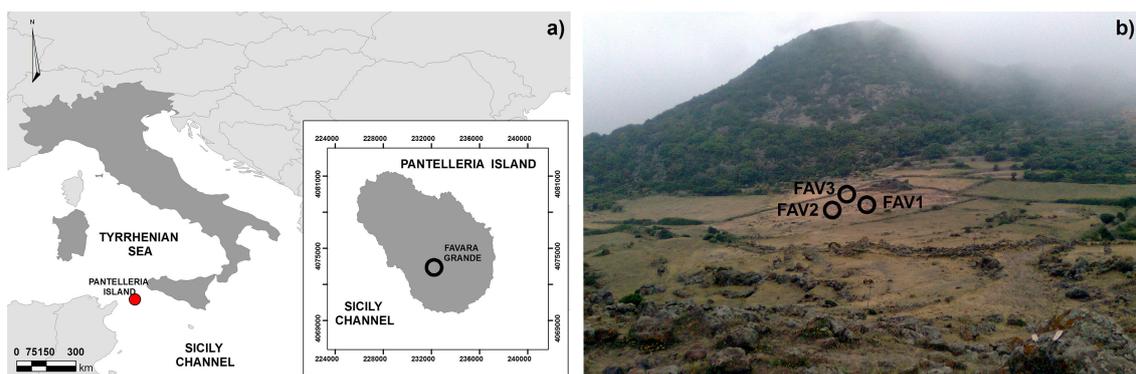


Figure 1. (a) Pantelleria (Italy) and (b) the three main sampling points at the geothermal field of Favara Grande.

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 of its coast. Although at present in quiescent status, the widespread thermal manifestations on Pantelleria attest to a sustained heat flow (Parrello et al., 2000). Many hot springs and thermal wells occur in the northwestern and southwestern parts 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 recognisable. Previous surveys identified many areas characterised by an 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 a strong steam emission and many fumarolic manifestations, all with temperatures close to boiling water. Fumarolic emissions have typical hydrothermal compositions (Chiodini et al., 2005; Fiebig et al., 2013) with water vapour as the main component (about 970 mmol mol⁻¹) followed by CO₂ (about 23 mmol mol⁻¹). Among the minor components, the fumarolic gases of Favara Grande display relatively high contents of H₂ and CH₄ (about 1.30 and 0.80 mmol mol⁻¹, respectively) and low contents of H₂S (< 0.02 mmol mol⁻¹). This leads, after condensation of water vapour, to high CH₄ concentrations in the soils (up to 44 mmol mol⁻¹) and high CH₄ fluxes from the soil (up to 221 mmol m⁻² day⁻¹) in the area of Favara Grande (D'Alessandro et al., 2009).

2.2 Soil sampling and chemical–physical characterisation

Soil samples used in this study were collected at Favara Grande during two field campaigns in 2011 in an area that was 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 and 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 in 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 H₂O by mass) and 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 characterisation

Soil 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 in 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 done very slowly (> 60 s 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 CH₄, CO₂, N₂, O₂ and H₂ analysis by using a Perkin Elmer Clarus 500 GC equipped with Carboxen 1000 columns, two detectors (HWD and FID), and argon as the carrier gas. The gas samples were injected through an automated injection valve with a 1000 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

Table 1. Chemical–physical characteristics of the soil sampled at the Favara Grande geothermal site (Pantelleria, Italy), and detection of the functional gene for methane monooxygenase *pmoA*. Thermal probe precision ± 0.01 °C; pH meter precision ± 0.02 ; OM and H₂O precision ± 0.01 %.

Soil sample	Depth cm	T^a °C	pH	OM %	H ₂ O %	Methane monooxygenase gene detection ^b	
						Proteobacterial <i>pmoA</i>	Verrucomicrobial <i>pmoA</i>
FAV1	0–3	62	3.4	3.7	12.9	– ^c	–
FAV2	0–3	60	5.8	3.1	2.8	+ ^d	+
FAV3	0–3	50	5.2	2.9	3.3	+	–
FAV2A ^e	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	+	+

^a: Table S1 shows temperatures measured up to 50 cm in depth.

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

^c: Absence of an amplicon of the expected size.

^d: Presence of an amplicon of the expected size.

^e: Soil samples FAV2A to FAV2E were sampled at the FAV2 site at different depths (0–13 cm) in November.

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 $0.1 \mu\text{mol mol}^{-1}$ for CH₄, $2 \mu\text{mol mol}^{-1}$ for H₂, $10 \mu\text{mol mol}^{-1}$ for CO₂ and $200 \mu\text{mol mol}^{-1}$ for O₂ and N₂.

2.4 Methanotrophic activity

The methane oxidation potential of the soils was analysed 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 CH₄ to reach about 1 mmol mol^{-1} . Bottles were incubated at controlled room temperature (23–25 °C), and the CH₄ concentration was measured on the same bottles at the beginning of the experiment and at about 24 h intervals for 5 days. To monitor the methane consumption better in samples that, after 24 h, consumed more than 30 % of the initial CH₄, the experiments were repeated by 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 in the soil CH₄ oxidation potential was analysed on sample FAV2A with different starting CH₄ concentrations at room temperature (from about 0.1 to 85 mmol mol^{-1}). 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 CH₄ per g of soil dry weight per h ($\text{nmol g}^{-1} \text{ h}^{-1}$). 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 $0.6 \text{ nmol g}^{-1} \text{ h}^{-1}$ 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 were assessed with 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 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 one unit of recombinant Taq polymerase, (Invitrogen, USA). The 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, an extension at 72 °C for 45 s, 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'-ACCATGCGDTGTAYTCAGG-3') and 156f/743r (5'-TGGATWGATTGGAAAGATCG-3'/5'-TTCTTTACCCAACGRTTCT-3'), targeting Verrucomicrobial *pmoA1/A2* and *pmoA3*, respectively, were designed and positively validated on *Methylococcus thermophilus* strain SolV. To detect the 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 an 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 an 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 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 an 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 a methane-enriched atmosphere and incubated as described above for 2 weeks. As soon as colonies appeared, they were transferred to a 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 bottles, in an atmosphere enriched in methane (25 %) added every week, and transferred to a 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 CH₄ atmosphere at different temperatures. Genomic DNA was extracted from 10 mL of M3–CH₄ 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 the *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 *Methylococcus* 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 OD₆₀₀ of 0.25, corresponding to 2.6×10^7 CFU mL⁻¹, and subsequently inoculated in three 160 mL serum bottles (2 mL each) containing 20 mL of M3 mineral medium and ~ 95 mmol mol⁻¹ methane; growth was monitored as turbidity using a spectrophotometer (OD₆₀₀). 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 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 H₂, CH₄ and CO₂ and an atmospheric component rich in O₂ and N₂ (Table S1). The hydrothermal component coming from below is always enriched at the deeper sampling points, while the atmospheric component diffusing from above is enriched at the shallower soil levels. Although, at least at FAV1 and FAV2, at 50 cm in depth, the gas composition is very close to that of the fumarolic gases, O₂ 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 in 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 in depth) and the 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 decreasing in the shallowest layers (Table 1). Laboratory incubation experiments with soil samples from 0 to 3 cm detected CH₄ consumption values in a range from 0.31 (FAV1) to 59.2 nmol g⁻¹ h⁻¹ (FAV2) (Fig. 2). Since FAV2 was the most active site, its methane oxidation was investigated further 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 (November measurements), 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⁻¹ h⁻¹) were still detected at

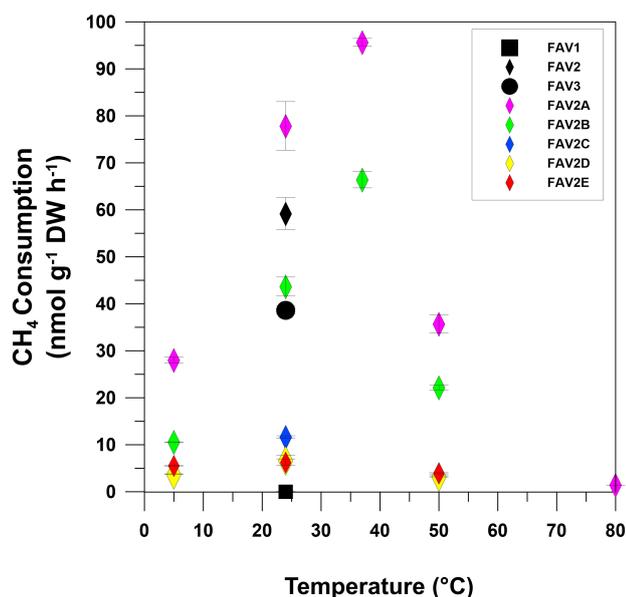


Figure 2. Methane consumption of soils sampled at sites FAV1, FAV2 and FAV3 (after incubation at controlled room temperature), and at different depths of FAV2 (soils incubated at different temperatures).

13 cm in depth (Fig. 2). When samples from the vertical profile were incubated at different temperatures, the CH₄ 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 CH₄ concentration in the headspace: methane oxidation values of 592 nmol g⁻¹ h⁻¹ are measured with an initial CH₄ concentration of 85 mmol mol⁻¹ at room temperature, and decrease to 8.17 nmol g⁻¹ h⁻¹, with a starting concentration of 0.15 mmol mol⁻¹ (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 and 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 all samples from the FAV2 vertical profile up to 13 cm in 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 primer couple 298f/599r (targeting *pmoA1/A2*) and the 156f/743r couple (targeting *pmoA3*) yielded the expected PCR products of about 300 and 600 bp, respectively (data not

shown) (Table 1). Accordingly, soil samples from the 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 the 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 an 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 FAV2 geothermal site

In order to isolate methanotrophic bacteria from the geothermally active site, soil enrichment cultures were set in a 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 the 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 the FAV2E soil sample was sequenced, and its sequence was close (96 % identity) 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 sub-cultured under the same conditions, and after streaking on M3 agar slants in sealed serum bottles with a CH₄-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 layer soil samples (2–10 cm) and characterised further. 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 with the Alphaproteobacteria species *Methylocystis parvus* (99 % identity with *Methylocystis parvus* strain OBBP). The growth curve of

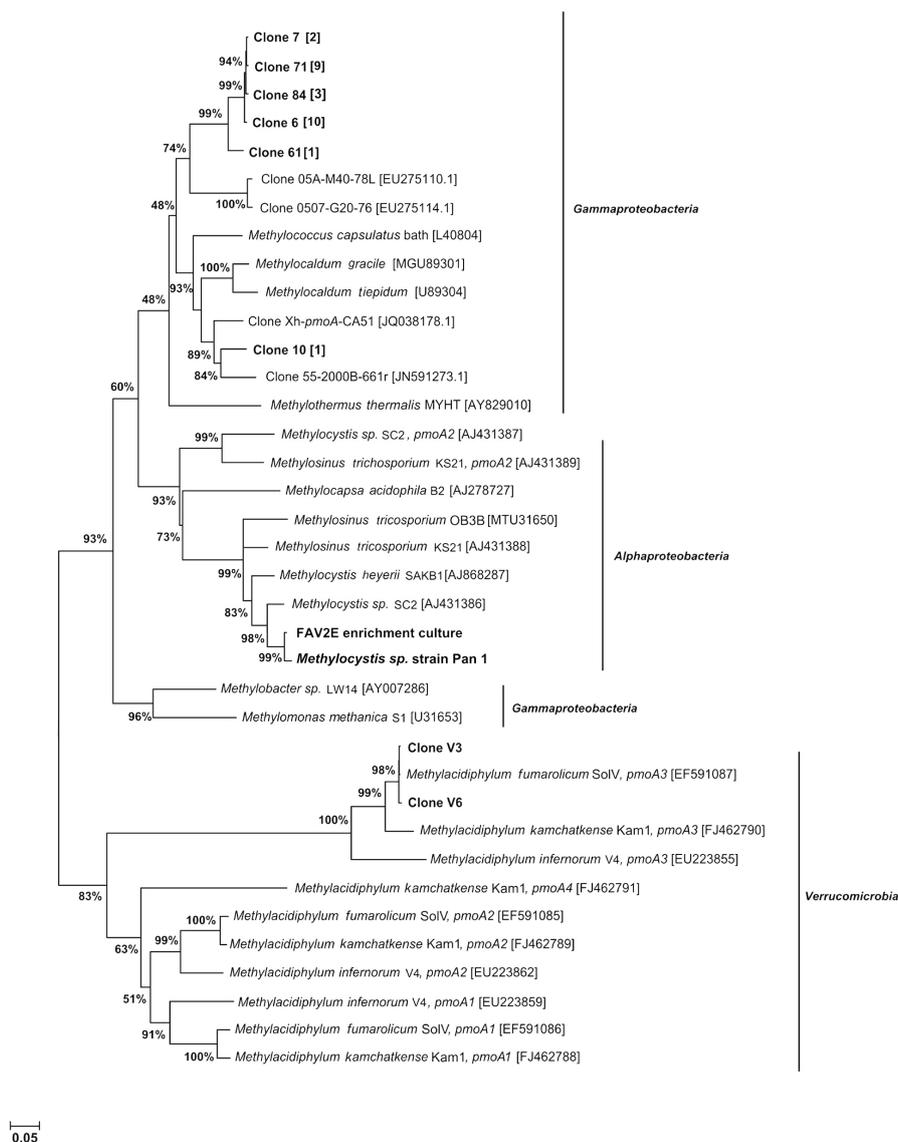


Figure 3. Phylogenetic tree constructed based on partial sequences (529 nt) of *pmoA* genes, showing the relative position of the genes and isolates from the FAV2 geothermal site where high methane oxidation rates were detected. The tree was constructed in the Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (Tamura et al., 2007), and a maximum composite likelihood (MCL) model was used (Schmidt et al., 2002). A neighbour-joining distance correction method was applied. Node support values are indicated for the primary nodes. The scale bar represents a 0.05 change per position. FAV2E enrichment culture derives from amplification of the final stage of the enrichment; all the others are from the FAV2 soil clone library. Pan1 was 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.

Methylocystis parvus strain Pan1 indicates a correlation between methane consumption and turbidity and a methane oxidation on the order of $160 \text{ nmol per hour h}^{-1} \text{ mL}^{-1}$ 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 % identity) and to *Brevibacillus agri* (99 % identity), respectively. The isolate obtained from the enrichment culture of FAV2A was assigned to the *Acidobac-*

terium genus (95 % identity) (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 recently been reported as capable of growth on methane as the sole C source under thermophilic conditions, although methane is not its preferred substrate (Laursen et al., 2007). Sequences

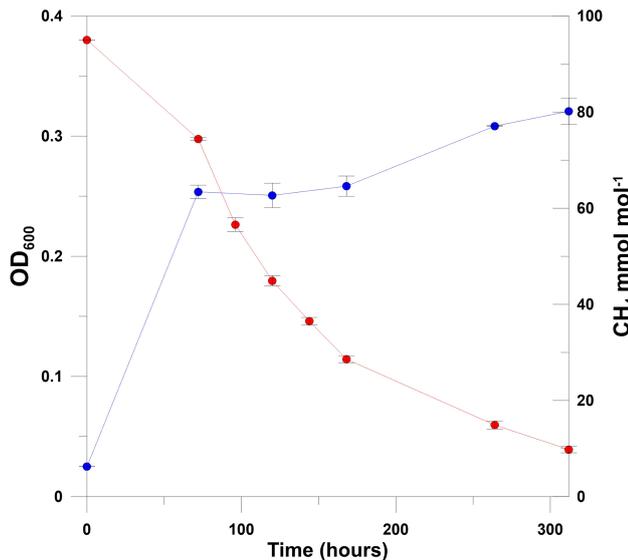


Figure 4. Growth (blue line) and corresponding variation of the methane in the headspace of the serum bottles as a result of methane consumption (red line) of the Pan1 *Methylocystis* sp. strain. The averages of the optical density measures (OD₆₀₀) ± standard error are from three replicate 160 mL serum bottles incubated at 37 °C at pH 5.8. No decrease in headspace methane was observed in uninoculated controls (data not shown).

related to the Acidobacteria phylum have been detected from the ¹³C DNA during a stable isotope probing (SIP) experiment aiming to characterise the active methylotroph populations in forest soil microcosms (Radajewski et al., 2002).

4 Discussion

Pantelleria 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 framework, Favara Grande represents the main exhalative area of Pantelleria, emitting about 2.5 Mg a⁻¹ (t a⁻¹) of CH₄ (D'Alessandro et al., 2009). At many sampling points of Favara Grande, CO₂/CH₄ ratios in the soil gases were higher than in the hydrothermal end members, revealing 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 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 CO₂/CH₄ ratios in the soil gases up to a 13 cm depth were similar to those of the fumarolic emissions, and then they 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 the fact 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 at 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 O₂ for the aerobic methanotrophy is reached only in the shallowest soil layers. Measurements of the soil gases indicate a very high variation in concentrations of the atmospheric gas content (O₂ and N₂) with depth. Air gas contributions at sites FAV2 and FAV3, where higher methane consumptions were measured, form more than 70 % of the total gas content, creating a very favourable environment for methanotrophic bacteria and allowing atmospheric O₂ to sustain the detected microbial CH₄ oxidation.

The conditions detected at Favara Grande thus appear favourable for methanotrophs, which were actually detected by culture-independent methods at sites FAV2 and FAV3. These sites have high temperatures (up to 60 °C at 2 cm in 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 decreases with depth. The oxidation potential in the deepest layer (10–13 cm) is probably too low (6.23 nmol g⁻¹ h⁻¹) to affect the CO₂/CH₄ ratio significantly at the deepest sampling point (FAV2E). Chemical–physical analysis and total bacterial diversity analysed by TTGE (Supplement, Fig. S1) suggest that sites FAV2 and FAV3 have very similar environmental conditions and microbial diversity. At both sites, proteobacterial methane monooxygenase genes were detected, although verrucomicrobial *pmoA* was only detected at FAV2. Negligible methane oxidation at 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 of even 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 of up to 70 °C (Pol et al., 2007), although the isolation conditions were milder (pH 2 and a temperature of 55 °C) than those detected in situ. The extreme physical-chemical conditions, however, do not prevent bacterial life at FAV1, as the TTGE (Fig. S1) analysis of the 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 the 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, the FAV2 site at Favara Grande recorded the highest diversity of methanotrophs ever described 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.

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 monooxygenase genes of *Methylacidiphilum fumarolicum* SolV, isolated for the first time at Solfatara di Pozzuoli in Italy (Pol et al., 2007). This is an extraordinarily high diversity of methanotrophs as 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 co-existence demonstrated. Interestingly, the results obtained from the *pmoA* gene libraries do not overlap with those from enrichment cultures. Gammaproteobacterial and verrucomicrobial 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 *Methylococcus* and *Methylocaldum* genera (Hanson and Hanson, 1996; Trotsenko et al., 2002) in the geothermal FAV2 soil.

These as yet uncultivated Type I methanotrophs are probably responsible for methanotrophy in the shallowest soil layer, but also in the hottest deeper layers. Type I methanotrophs are reported as being dominant in environments that allow the most rapid growth, while type II methanotrophs are more abundant in environments with fluctuating nutrient availability (Hanson and Hanson, 1996). Type I *pmoA* sequences, however, 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 II methanotrophs in fact could not be detected by molecular methods, but resulted as the dominant cultivable methanotrophs in the top 10 cm soil layers. Under laboratory conditions in the presence of high methane concentrations at 37 °C, type II methanotrophs, which tend to survive more, took precedence over the other methanotrophs (both Gammaproteobacteria and Verrucomicrobia).

The conditions used in this study for the 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 soil condition, but we were unable to isolate any verrucomicrobial member, although they were detected by molecular methods in the same soil samples. Cultivation of Verrucomicrobia methanotrophs under laboratory conditions is hard to obtain, and seems to be limited by still unknown factors. New knowledge of the positive role of rare earth elements (REE) in Verrucomicrobia methanotroph growth and cultivation was recently provided by Pol et al. (2014). They suggested that a good strategy for cultivating Verrucomicrobia methanotrophs could be the addition of REE in the culture media. In fact, recently it was demonstrated that lanthanides are essential 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 Solfatara, where the Verrucomicrobia (Pol et al., 2007; Pol et al., 2014) were isolated. Soil acidity strongly increases REE mobility and consequently their availability to soil microbes (Grawunder 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.

A high CH₄ concentration and a temperature of 37 °C favoured growth of *Methylocystis* from the first top soil layers and the facultative *Methylobacterium* in the deepest layer. *Methylocystis* is one of the most ubiquitous genera, being capable of oxidising 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 wider 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, characterised 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 uncultured (and perhaps unculturable) methanotrophs, as those detected by culture-independent methods that are distantly related to the thermophilic genera *Methylocaldum* and *Methylococcus*.

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 with their ability to survive, grow and oxidise 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 Gamma-proteobacteria was unexpected, and suggests that high CH₄ fluxes and differences in environmental conditions shape the complex methanotroph community structure in this geothermal area.

This study on Pantelleria soils suggests that a physiologically and taxonomically diverse group of methanotrophs is responsible for CH₄ consumption at FAV2 on a layer of 0–13 cm and, presumably, at the FAV3 site; at the same time, our results assess that temperatures above 80 °C hinder methane oxidation and probably the survival of methanotrophs. While methanotrophic Verrucomicrobia are reported as dominating 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 a better understanding of the ecology of methanotrophy at geothermal sites and its impact in atmospheric chemistry.

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