Diversity of Methane-Oxidizing Bacteria in Soils from “Hot Lands of Medolla” (Italy) Featured by Anomalous High-Temperatures and Biogenic CO₂ Emission

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(Received May 3, 2016—Accepted July 13, 2016—Published online September 17, 2016)

“Terre Calde di Medolla” (TCM) (literally, “Hot Lands of Medolla”) refers to a farming area in Italy with anomalously high temperatures and diffuse emissions of biogenic CO₂, which has been linked to CH₄ oxidation processes from a depth of 0.7 m to the surface. We herein assessed the composition of the total bacterial community and diversity of methane-oxidizing bacteria (MOB) in soil samples collected at a depth at which the peak temperature was detected (0.6 m). Cultivation-independent methods were used, such as: i) a clone library analysis of the 16S rRNA gene and pmoA (coding for the α-subunit of the particulate methane monoxygenase) gene, and ii) Terminal Restriction Fragment Length Polymorphism (T-RFLP) fingerprinting. The 16S rRNA gene analysis assessed the predominance of Actinobacteria, Acidobacteria, Proteobacteria, and Bacillus in TCM samples collected at a depth of 0.6 m along with the presence of methanotrophs (Methylocaldum and Methylobacter) and methylotrophs (Methylbacillus). The phylogenetic analysis of pmoA sequences showed the presence of MOB affiliated with Methylocaldum, Methylobacter, Methylococcus, and Methylocaldum in addition to as yet uncultivated and uncharacterized methanotrophs. Jaccard’s analysis of T-RFLP profiles at different ground depths revealed a similar MOB composition in soil samples at depths of 0.6 m and 0.7 m, while this similarity was weaker between these samples and those taken at a depth of 2.5 m, in which the genus Methylocaldum was absent. These results correlate the anomalously high temperatures of the farming area of “Terre Calde di Medolla” with the presence of microbial methane-oxidizing bacteria.

Key words: methanotrophs, methane-oxidizing bacteria, pmoA gene, Terre Calde di Medolla (“Hot Lands of Medolla”), soil high temperature

Methane (CH₄) is the most abundant organic gas in the atmosphere; it represents the second most important greenhouse gas after carbon dioxide (CO₂) (11, 35) and is strictly involved in carbon cycle processes. Therefore, processes with the ability to consume atmospheric CH₄ may play important roles in preventing climate change (21, 28). The origin of CH₄ in the atmosphere is related to different anthropogenic (e.g., rice paddies, livestock farms, biomass burning, oil and gas mining, and waste disposal) and natural (e.g., wetlands, oceans, freshwaters, and seepage from deep reservoirs in both sedimentary and volcanic environments) sources (6, 37). The total amount of atmospheric CH₄ is 520 Tg year⁻¹, 90% of which is oxidized by photochemical reactions in the troposphere while 10% is removed by microbiological activities (15).

Methanotrophs (also named Methane-Oxidizing Bacteria, MOB) are responsible for most of the biological processes of CH₄ oxidation. MOB are a unique group of Gram-negative aerobic bacteria that metabolize CH₄ as the only source of carbon and energy (15). They are ubiquitous microorganisms in nature that are able to adapt to different environmental conditions (10). Methane consumption by MOB occurs through an oxidation reaction led by the methane monoxygenase enzyme (MMO), which catalyzes the conversion of methane in methanol (26). This is the first step in the metabolic pathway of methanotrophs, which ends with the production of CO₂ (24). Methanotrophs have been classified into two distinct taxonomic groups based on phenotypic and genotypic properties. Type I methanotrophs belong to the γ-subdivision of Proteobacteria, whereas type II methanotrophs belong to the α-subdivision of Proteobacteria (15). Two types of MMO systems have been identified: a soluble cytoplasmic complex (sMMO) and membrane-bound particulate system (pMMO) (18). pMMO genes are universal in MOB, with the possible exception of Methylocella palustris (11). In contrast, sMMO genes are restricted to type II methanotrophs with the exception of certain representatives of two type I genera (27).

The application of molecular biological tools has greatly facilitated the study of methanotroph communities in natural environments. The pmoA gene, which encodes the α-subunit of pMMO, has been widely used as a phylogenetic marker for the identification of methanotrophs through cultivation-independent approaches. The pmoA sequence has provided information on the diversity of these organisms in different environments (19, 25).

“Terre Calde di Medolla” (TCM) (literally, “Hot Lands of Medolla”) is an ancient toponym that dates back to 1893 (6, 33) and describes a heating surface phenomenon affecting agricultural soils located in the area of Medolla, a small town in the province of Modena (Italy) (Fig. 1A). This phenomenon has a patchy distribution, with a sub-circular shaped area (a few meters in diameter) in which temperatures up to ≅50°C were measured (Fig. 1C and B). The anomalously warm areas are easily recognizable during winter because snow melts within them and during summer when soil heating leads to the death of corn plants (6). Previous studies conducted in 2012 reported a clear correlation between soil heating and gas
seepage (CH₄ and CO₂) (29). As shown in Fig. 2B, the maximum temperature (42.2°C) is reached at a depth of 0.6 m, where horizons with high permeability are present (loamy sandy layer and sandy layer). The CO₂/CH₄ flux ratio (ϕCO₂/ϕCH₄) increases from this level to the surface by three orders of magnitude, from values <1 to >1,000 (Fig. 2D). The chemical composition of soil gases together with the gradual increase of ¹³C in CO₂ and decrease in CH₄ at shallower depths (from 0.7 m to 0.25 m, Fig. 2A) indicate that the marked increase in the CO₂/CH₄ flux ratio is due to the biological oxidation of rising CH₄, suggesting the occurrence of biological methane oxidation processes in the most aerated layers. Conceptual modeling and numerical simulations have indicated that the exothermic nature of the CH₄ →CO₂ conversion (800 KJ mol⁻¹ of CH₄) represents a heat source with the ability to produce the soil thermal anomalies observed in TCM soil (29). This previous finding and thermodynamic prediction prompted us to verify the hypothesized correlation between methanotrophs and soil hot temperatures by examining microbial methanotrophic diversity in TMC soil samples using a cultivation-independent approach. Three soil samples were collected at different ground depths (0.6, 0.7, and 2.5 m). A clone library analysis of the 16S rRNA gene and pmoA gene was performed on 0.6-TCM soil in order to characterize the total bacterial community and define the MOB fraction, respectively. The methanotrophic community composition identified in 0.6-TCM soil was compared to those detected in 0.7- and 2.5-TCM soil by Terminal Restriction Fragment Length Polymorphism (T-RFLP) fingerprinting of the pmoA gene. The results presented here strongly support earlier geophysical models implicating the presence of microbial methane-oxidizing activity in the farming area of “Terre Calde di Medolla” in the anomalous heating of soil.

Materials and Methods

Sample collection

During a field campaign conducted in July 2013, a 2.5-m-deep piezometer was drilled within an area selected on the basis of the presence of an anomalously high surface soil temperature (36.4°C) and significant CO₂ fluxes (up to 103.4 g m⁻² d⁻¹) (Fig. 2B and D). During drilling, three different soil samples were collected within the sediment corer at depths of 0.6, 0.7, and 2.5 m (hereafter referred to as 0.6-TCM, 0.7-TCM, and 2.5-TCM) and stored within small polystyrene tubes kept at -20°C for later use. During drilling, CO₂
and CH₄ fluxes were also measured at 0.1-m intervals to a depth of 0.80 m and then at intervals of 0.5 m thereafter.

**Extraction of DNA**

Genomic DNA was extracted from 0.35 g of 0.6-TCM, 0.7-TCM, and 2.5-TCM soils in duplicate using the Power Soil DNA isolation kit (MOBIO Laboratories, Solana Beach, CA, USA) according to the manufacturer’s instructions with some modifications. Briefly, 0.35 g of each soil sample was incubated in IRS solution supplied with 5 mg mL⁻¹ proteinase K and 8 mg mL⁻¹ lysozyme at 37°C for 30 min. After the addition of 0.1% of SDS, cells were further disrupted by bead beating for three cycles of 1 min each. DNA was eluted in a final volume of 50 μL and stored at −20°C in milli-Q water.

**Construction of gene clone libraries and RFLP screening**

Isolated genomic DNA was used for PCR amplification of the 16S rRNA gene and/or functional genes (pmoA and mmoX) using the primers listed in Table 1. Bacterial DNA (1 μL of 10× diluted genomic DNA) was added to a 50-μL (final volume) mixture containing 1.25% (v/v) DMSO and 1.25 U Taq polymerase (Thermo Scientific). The following conditions were used to amplify the 16S rRNA gene: at 94°C for 4 min for initial denaturation, 30 cycles (at 94°C for 40 s and 55°C for 40 s for alignment and at 72°C for 40 s for elongation), and at 72°C for 15 min for the final extension step. The pmoA gene was amplified using the following PCR program: at 94°C for 4 min, 30 cycles (at 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min), and a final elongation at 72°C for 20 min. The PCR reactions and cycling conditions used for the amplification of mmoX were as described for pmoA, except for an annealing temperature of 60°C.

PCR amplification products were confirmed by electrophoresis with a 1% (w/v) agarose gel, purified with the Qiagen PCR purification kit (Qiagen, Hilden, Germany). PCR products were ligated into the pCRII vector supplied with the TOPO TA cloning kit (Invitrogen, San Diego, CA, USA), according to the manufacturer’s instructions, and cloned into Escherichia coli DH5α for clone library construction.

A Restriction Fragment Length Polymorphism (RFLP) analysis was performed on at least 100 clones from each library. Individual colonies containing inserts of the appropriate size were suspended in 20 μL of TE pH 8 and boiled for 5 min. Cell debris was removed by centrifugation and 1-μL portions of the supernatant were used as a template for PCR amplifications to re-amplify the gene inserts that were further used in restriction digestion with tetrameric restriction enzymes. The genes were digested with 5 U of each enzyme at 37°C for approximately 200 ng of fluorescently labeled PCR amplification products was labeled at the 5ʹ end with hexachlorofluorescein dye (Hex). After purification with Qiagen spin columns (Qiagen), approximately 200 ng of fluorescently labeled PCR amplification products were digested with 10 U of the restriction enzymes (Roche) MspI and HaeIII. Digestion was performed in a total volume of 20 μL at 37°C for 3 h. The digests obtained were sent to BMR Padova for automated DNA sequencing.

Regarding sequence identification, plasmids (for the 16S rRNA gene) or PCR products (for pmoA) were purified from one representative clone of each group with a Qiagen plasmid purification kit or QiAquick PCR Purification kit (Qiagen). Sequencing was performed by the BMR Genomics Service (Padova, Italy) using T7 and T3 primers (Table 1). Sequences were checked for chimeras using the CHECK_CHIMERA program at the Ribosomal Database Project (RDP) (https://rdp.cme.msu.edu/).

### Sequence analysis

An analysis of the 16S rRNA gene and pmoA gene sequences obtained from the libraries was performed using the Ribosomal Database Project (8) as well as BLASTn (1) hits against GenBank to generate the best hits. A phylogenetic tree of the derived pmoA gene nucleotide sequences was created using Geneious Tree Builder with the Juke-Cantor genetic distance model and using the neighbor-joining method. One thousand parametric bootstrap replications were simultaneously computed to statistically consolidate the branching topology of the inferred tree.

### T-RFLP analysis

A T-RFLP analysis was performed for each sample in triplicate as described previously (7). The same PCR primers and conditions as those described above were used, although the forward primer A189f was labeled at the 5ʹ end with hexachlorofluorescein dye (Hex).

After purification with Qiagen spin columns (Qiagen), approximately 200 ng of fluorescently labeled PCR amplification products were digested with 10 U of the restriction enzymes (Roche) MspI and HaeIII. Digestion was performed in a total volume of 20 μL at 37°C for 3 h. The digests obtained were sent to BMR Padova for automated DNA sequencing.

### T-RF profile and statistical analyses

The lengths of the labeled fragments were assessed by comparison with an internal standard (ROX-labeled GS500) using Peak Scanner version 1.0 software (Applied Biosystems). A tolerance limit of +/− 2 bp was used for peak assignment on the sizing accuracy of T-RFs ranging from 35/50 to 500 bp. In order to avoid the detection of primers and uncertainties of size determination, terminal fragments smaller than 50 bp were typically excluded from the analysis. In the digestion of HaeIII only, T-RFs ranging from 35 to 50 bp were taken in the analysis because the HaeIII restriction sites included in this size range were expected in pmoA amplics. A 1% threshold was used to define the baseline. A peak height threshold of 50 fluorescence units was used in the initial analysis of the electropherogram. In order to compare the T-RFLP data obtained from the different soil samples being analyzed, T-RFLP profiles were normalized as previously described by Stralis-Pave et al. (36).

### Table 1: Primers used in the present study for PCR amplification and sequencing.

| Target gene or scope | Primer set | Sequence (5ʹ to 3ʹ) | Fragment length | Reference |
|----------------------|------------|---------------------|-----------------|-----------|
| 16S rRNA gene        | 27f 1492r  | AGAGTTTGATCCTGAGCTCAG  |
|                      |            | TACGGYTACCTGTTCAGACTT  | 1465 bp         | (12)      |
| pmoA                 | A189f A605r| GGNGACTGGGGACCTCTGG    |
|                      |            | GAAAGCNGAGAAGAAGGGC    | 525 bp          | (18)      |
| mmoX                 | A189f mb661r| GGNGACTGGGGACCTCTGG   |
|                      |            | CCGGCGCAAGCATCYYTACC   | 510 bp          | (9)       |
| mmoX                 | mmoXA      | ACCAAAGGARCAATCAAG     |
|                      |            | CGATCCAGATDCCRCCCA     | 790 bp          | (2)       |
| mmoX                 | mmoXD      | ATCCGCAAARGATAYGCGCG   |
|                      |            | ACCANGGCTCGACTTGAA     | 719 bp          | (22)      |

**Sequencing**

| T7 | TAATACGACTCACTATAGGG | variable | Invitrogen |
| T3 | ATTAACCCTCACTAAAGGGA | variable | Invitrogen |
T-RF peaks were regarded as binary characters and analyzed using NTSYS software as described previously (13) to calculate the distance between soil samples analyzed in terms of T-RFs detected.

Nucleotide sequence accession numbers

The pmoA gene sequences identified in this study have been deposited in the DDBJ/EMBL/GenBank databases under accession numbers from KX035110 to KX035129.

Results

Microbial community characterization of soils sampled at a depth of 60 cm by clone library screening

16S rRNA gene sequences

A total of 100 randomly selected non-chimeric rDNA clones containing inserts from the 16S rRNA gene clone library were subjected to RFLP analyses (with the tetrameric restriction enzymes HaeIII, AluI, and Rsal) and placed into groups based on their RFLP patterns. One representative clone for each group was sequenced. A total of 22 bacterial phylotypes were found to be affiliated with 7 distinct phyla (Table 2). One representative clone for each group was sequenced. A total of 22 bacterial phylotypes were found to be affiliated with 7 distinct phyla (Table 2). 16S rRNA gene sequences identified in this study have been deposited in the DDBJ/EMBL/GenBank databases under accession numbers from KX035110 to KX035129.

Table 2. Total eubacterial community composition in 0.6-TCM soil on the basis of a 16S rRNA gene clone library analysis.

| Phylum             | Class           | Order         | Family           | Genus            | Clone library | Best RDP hit | % ID |
|--------------------|-----------------|---------------|------------------|------------------|---------------|--------------|------|
| Acidobacteria      | Acidobacteria   | unspecified   | unspecified      | Gp3              | 3%            | JQ712939     | 96   |
|                    |                 |               |                  | Gp4              | 2%            | AY094624     | 99   |
|                    |                 |               |                  | Gp6              | 6%            | HQ597776     | 99   |
|                    |                 |               |                  | Gp10             | 6%            | JQ408035     | 98   |
| Actinobacteria     | Actinobacteria  | Acidimicrobales | Acidimicrobinea | Aciditerrimonas | 7%            | LN573416     | 99   |
|                    |                 | Actinomycteales | Micrococcaceae   | Phycicoccus      | 8%            | HJ323449     | 99   |
|                    |                 |               | Streptosporangiaceae | Arthrobacter | 5%            | KF9193442    | 99   |
|                    |                 |               |                  | Microbispera    | 1%            | KF886293     | 96   |
|                    |                 | Gaiellae      | Gaiellaceae      | Gaiella         | 2%            | KC554071     | 99   |
| Armimatamonadetes  | Chthonomonadetes | Chitonomonadacea | Chitonomonadacea | Chitonomonas  | 2%            | GJ454980     | 95   |
|                    | Bacteroidetes   | Sphingobacteria | Sphingobacteriales | Sphingobacter | 5%            | GQ487995     | 99   |
| Chloroflexi        | Thermomicrobia  | Sphaerobacteriales | Sphaerobacteraceae | Sphaerobacter | 3%            | KC432559     | 95   |
| Fircmutes          | Bacilli         | Bacillales    | Bacillaceae      | Bacillus        | 16%           | EU221338     | 99   |
| Alphaproteobacteria | Rhodospirillae | Rhodospirillae | Rhodospirillae | Dongia          | 3%            | FJ478641     | 97   |
|                    | Sphingomonadales | Sphingomonadaceae | Sphingomonadaceae | Skermanella | 1%            | KF010774     | 99   |
|                    |                 |               |                  | Sphingomonas     | 6%            | JN182709     | 99   |
| Betaproteobacteria | Burkholderiales | Acaligenaceae | Comamonadaceae | Derria           | 1%            | GQ009540     | 99   |
|                    | Methylophilaceae | Methylophilaceae | Methylophilaceae | Methylobacillum | 2%            | FJ447673     | 99   |
| Gammaproteobacteria | Methylcooccales | Methylcooccales | Methylcooccales | Methyllobacter   | 7%            | AY921679     | 98   |
|                    |                 |               |                  | Methylobacillum  | 3%            | HM362553     | 94   |

a The amount of clones representing each bacterial phylotype within the clone library out of 100 screened clones.

b The best hit resulting from a comparison of each 16S rRNA gene partial sequence with sequences in the small-subunit rRNA database of the Ribosomal Database Project (RDP).

c The % of nucleotide identity revealed by a BLAST analysis of the RDP best hit.

The 16S rRNA gene sequences of two methanotrophs belonged to the genera *Methylobacter* and *Methylocaldum*, both of the *Gammaproteobacteria* class, while one methylotroph belonged to the genus *Methylobacillum* of the *Betaproteobacteria* class.

pmoA sequences

A molecular analysis of the pmoA gene was used to characterize the methane-oxidizing microbial community, thereby identifying the most representative strains involved in CH$_4$ consumption.

The genes coding for the α-subunit of both types of methane monoxygenases (particulate and soluble MMO) were targeted in the present study. The pmoA gene encoding the α subunit of the pMMO was successfully amplified with the primer sets, A189f/mb661r and A189f/A682r, while no PCR product was obtained using the primers for the α subunit of the soluble isoform of the methane monoxygenase (mmoxA/mmoxXD and mmoxX206f/mmoxX866r, Table 1) (data not shown). Corresponding to each primer set (amplification lengths of 510 bp and 525 bp with A189f/mb661r and for A189f/A682r, respectively), two clone libraries were constructed. A total of 200 clones were screened from the two clone libraries through the RFLP analysis using HaeIII and MspI restriction enzymes. Similar to 16S rRNA gene screening, the representative clone of each RFLP group was detected and sequenced in both strands. The pmoA gene sequences were aligned, analyzed through a BLAST search and grouped (group A–H), if possible, using a threshold of 90% sequence identity (Table S1). Sequences that did not show any affiliation

The 16S rRNA gene sequences of two methanotrophs belonged to the genera *Methylobacter* and *Methylocaldum*, both of the *Gammaproteobacteria* class, while one methylotroph belonged to the genus *Methylobacillum* of the *Betaproteobacteria* class.
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with pmoA sequences were excluded from further analyses.

A distance-based neighbor-joining tree was constructed with the pmoA sequences obtained from the two libraries and their closely related reference sequences obtained from the GenBank database (Fig. 3, Table 3). The phylogenetic analysis of the pmoA sequences revealed the presence of MOB in soil related to the genera Methylocystis (group A), Methylococcus (groups F, D, and E), Methylocaldum (group G), and Methyloomonas (group H) (Fig. 3), indicating wide biodiversity in terms of bacteria associated with CH₄ consumption. Moreover, two groups of clones (groups B and C in Fig. 3 and Table 3) clustered with the pmoA clones of uncultured or uncharacterized methanotrophs without a defined taxonomic affiliation (uncultured pmoA groups I and II, Fig. 3). In the clone library with A189f/A682r, one clone corresponded to a partial amoA gene that clustered in the phylogenetic analysis with the amoA gene of Nitrospira sp. (Fig. 3, Table 3). The clone groups C and D, belonging to uncultured methanotrophs and the genus Methylococcus, respectively, were the most dominant in both libraries; however, the percentage of clones belonging to each clone group was different (Table 3). In the A189f/mb661r library, 49% and 18% of clones belonged to groups C and D, respectively, while in the A189f/A682r library, 57% and 23% of clones belonged to groups D and C, respectively. The clones of group B represented 10% of all the clones characterized in the A189f/A682r library, while the clones of group A as well as those of groups F, G, and H were detected in the A189f/mb661r library only. The latter represented 1%, 17%, and 2%, respectively, of all clones characterized in the A189f/mb661r library. Notably, group G was the third most represented phylotype in the library and was closely related to the genus Methylocaldum. The different results obtained from the screening of the two libraries may be related to the different abilities of the two primer sets (A189f/A682r and A189f/mb661r) to assess methanotroph diversity in soil (4). In the present study, the A189f/mb661r primer set retrieved the largest diversity of methanotroph pmoA sequences. Therefore, this primer set was used in T-RFLP analyses. As shown in Table 3 and Fig. 4 (0.6-m HaeIII and 0.6-m MspI), the pmoA-based T-RFLP profiles obtained from 0.6-TCM soil correlated with the results of clone library screening. Additionally, the minor T-RFs that were not affiliated with any pmoA sequence from the libraries may represent new methanotrophic species. Among these, the T-RFs not corresponding to any expected digestion fragments with a relative area greater than 1% were 65, 78, 93, 144, and 210 bp in the HaeIII digestion, and 63 and 75 bp in the MspI digestion (0.6-m HaeIII and 0.6-m MspI in Fig. 4).

**T-RFLP analysis to compare MOB at different ground depths (soils sampled at 0.6, 0.7, and 2.5 m)**

A T-RFLP analysis of the amplified pmoA gene was used to elucidate differences and similarities between sediments sampled at different ground depths (0.6, 0.7, and 2.5 m) in TCM. On the basis of the presence or absence of terminal restriction fragments (T-RFs) in the profiles shown in Fig. 4, an analysis of Jaccard’s distance (JD) was performed in order to compare the MOB communities present in soil collected at different depths (Table S1). A similarity index of 1.0 indi-

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**Fig. 3.** Phylogenetic tree of partial pmoA and amoA nucleotide sequences retrieved from an analysis of A189f/A682r and A189f/mb661r clone libraries from 0.6-TCM soil. pmoA clones are grouped into eight groups (A–H) based on at least 90% nucleotide sequence identity. GenBank accession numbers are shown for sequences of cultured methanotrophs and clones from other studies. The bar indicates 10% sequence divergence. Bootstrap values are given and based on 500 data resampling. Boxes marked by dashed lines show the phylogenetic affiliations of the pmoA clones from 0.6-TCM soil.
cated that communities have strong similarities. Jaccard’s index ($S_j$) between 0.6- and 0.7-TCM soil was 0.905, demonstrating stability in the microbial composition of the two soil samples. A lower degree of similarity in the MOB community composition was revealed between 0.6-TCM/0.7-TCM soil and 2.5-TCM soil (JD of 0.6–0.7) (Table S1).

Table 3. Analysis of representative pmoA clones obtained from the amplification of a 0.6-TCM soil extract with two primer sets (A189f/mb661r and A189f/A682r).

| Group | Clone | Library with A189f/mb661r | Library with A189f/A682r | Best Blast hit | % ID | T-RFLP expected | T-RFLP observed |
|-------|-------|---------------------------|---------------------------|----------------|------|----------------|----------------|
| A     | MD-51 | 1% —                      | —                         | Uncultured methanotrophic bacterium clone 16 (pmoA) | 100% | M244, H45      | M242, H45      |
| A     | MD-92 | 1% —                      | —                         | Uncultured bacterium clone ZW200 (pmoA)       | 98%  | M279, H225     | M279, H226     |
| B     | MD-53b| 6% —                      | —                         | Uncultured bacterium clone CH_118 (pmoA)       | 93%  | M208, H225     | M208, H226     |
| B     | MD-58b| 4% —                      | —                         | Uncultured bacterium clone CH_118 (pmoA)       | 93%  | M208, H225     | M208, H226     |
| C     | MD-2  | 6% —                      | —                         | Uncultured bacterium clone JX148 (pmoA)        | 94%  | M36, (H19)     | M35, (H19), H82 |
| C     | MD-5  | 32% —                     | —                         | Uncultured bacterium clone JX148 (pmoA)        | 94%  | M33, (H19)     | M33, (H19)     |
| C     | MD-32 | 10% —                     | —                         | Uncultured bacterium clone JX148 (pmoA)        | 94%  | M36, (H19)     | M37, (H19), H82 |
| C     | MD-8b | 15% —                     | —                         | Uncultured bacterium clone JX148 (pmoA)        | 94%  | M208, H225     | M208, H226     |
| C     | MD-10b| 8% —                      | —                         | Uncultured bacterium clone JX148 (pmoA)        | 95%  | M208, H225     | M208, H226     |
| D     | MD-1  | 17% —                     | —                         | Uncultured methanotroph pmoA-61 (pmoA)         | 91%  | M36, (H19)     | M35, (H19), H82 |
| D     | MD-9  | 1% —                      | —                         | Uncultured bacterium RB18 (pmoA)               | 90%  | M33, (H19)     | M33, (H19)     |
| D     | MD-103b| 57%                      | —                         | Uncultured methanotroph pmoA-61 (pmoA)         | 91%  | M36, (H19)     | M37, (H19), H82 |
| E     | MD-14 | 10% —                     | —                         | Uncultured Methylococcus sp. clone Xh_pmoA_CA51 (pmoA) | 93%  | M33, (H19)     | M35, (H19), H82 |
| E     | MD-57b| 5% —                      | —                         | Uncultured Methylococcus sp. clone Xh_pmoA_CA51 (pmoA) | 93%  | M33, (H19)     | M35, (H19), H82 |
| E     | MD-5b | 3% —                      | —                         | Uncultured Methylococcus sp. clone Xh_pmoA_CA51 (pmoA) | 93%  | M33, (H19)     | M35, (H19), H82 |
| F     | MD-47 | 1% —                      | —                         | Uncultured methanotrophic bacterium clone 37 (pmoA) | 94%  | M36, (H19)     | M35, (H19), H82 |
| G     | MD-3  | 17% —                     | —                         | Uncultured bacterium JX16 (pmoA)               | 99%  | M36, (H19)     | M35, (H19), H82 |
| H     | MD-11 | 2% —                      | —                         | Uncultured Methylococcus sp. clone 13RH2Omb16 (pmoA) | 99%  | M36, (H19)     | M35, (H19), H82 |
| H     | MD-41 | 1% —                      | —                         | Uncultured bacterium clone AOB-A0-21 (amoA)    | 97%  | M36, (H19)     | M35, (H19), H82 |

* The % of clones representing each clone group within the 100 clones screened.

- Expected T-RFLP peaks outside of the valid T-RF range are between brackets (on the basis of the DNA fragment length standard [Rox 500]).

- Second cut.

Fig. 4. Comparison of representative T-RFLP patterns of pmoA products amplified from DNA extracted from different ground depths of TCM (0.6-, 0.7- and 2.5-TCM soil). The lengths of significant T-RFs (signal intensity greater than the threshold of 1%) are reported. See Table 3 for the assignment of T-RFs to methanotrophic species/clones revealed from clone library screening. Putative peaks representing partial digestion are depicted by asterisks.
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50 MJ (kgCH4 )–1 (23). The specific heat released by methanotrophy to be in the order of 0.6-TCM soil (29). Indirect support for this hypothesis is provided by an early study on permafrost, which quantified the unusual ground temperatures that reach up to 50°C at a depth of 0.6 m, which exceed local average values. This phenomenon is associated with diffuse CH4 and biogenic CO2 seepage (6), and conceptual modeling linked to numerical simulations have suggested that the exothermic nature of the CH4→CO2 conversion (800 KJ mol–1 of CH4) represents a heat source with the ability to produce the thermal anomalies observed in TCM soil (29). Indirect support for this hypothesis is provided by an early study on permafrost, which quantified the specific heat released by methanotrophy to be in the order of

The area known as TCM in the Po river valley (Italy) has been attracting interest since 1893 (6, 29, 33) due to its unusual ground temperatures that reach up to 50°C at a depth of 0.6 m, which exceed local average values. This phenomenon is associated with diffuse CH4 and biogenic CO2 seepage (6), and conceptual modeling linked to numerical simulations have suggested that the exothermic nature of the CH4→CO2 conversion (800 KJ mol–1 of CH4) represents a heat source with the ability to produce the thermal anomalies observed in TCM soil (29). Indirect support for this hypothesis is provided by an early study on permafrost, which quantified the specific heat released by methanotrophy to be in the order of

Fig. 5. Visual representation of the vertical variability of sedimentological features (from the surface downwards to a depth of 2.5 m) associated with the corresponding profiles of temperature, the CO2/CH4 flux ratio, and T-RFLP patterns (HaeIII-based digestion) representing methanotrophic diversity in soil collected at depths of 0.6, 0.7, and 2.5 m. The T-RF corresponding to the genus Methylocaldum is highlighted in samples from depths of 0.6 and 0.7 m.

Discussion

The 16S rRNA gene clone library analysis of 0.6-TCM soil (this work) indicates the predominance of bacteria belonging to the phyla Proteobacteria (with representatives of the three classes Alpha-, Beta-, and Gammaproteobacteria), Actinobacteria, and Acidobacteria (17). Among Proteobacteria, two methanotrophic genera belonging to Gammaproteobacteria, while one methylothetic genus in the library belonged to Betaproteobacteria. A total of 12% of the clones of the 16S rRNA gene library, constructed with the universal 27f/1492r primer set, are representative of methanotrophs and/or methylotrophs, suggesting the occurrence of methane-oxidizing activities in high-temperature soil, i.e. 0.6-TCM soil.

An examination of the pmoA clone libraries of 0.6-TCM soil revealed the large biodiversity of methanotrophs including various MOB affiliated to Methylomonas, Methylomethylocystis, and Methylocaldum. In the A189f/mb661r pmoA library, most clones (±50%) were found within group C, which represents uncultivated methanotrophic bacteria related to methanotrophs isolated from upland grassland soil consuming atmospheric CH4 and a saline alkaline environment (21, 34). In the A189f/mb661r library of 0.6-TCM soil, most clones (75%) were found within group D, which shared a maximum of 90% nucleotide similarity with the uncultivated methanotrophic bacteria identified in paddy fields cultivated with rice over a long period of time (16). The relative abundance of the two groups in the two clone libraries may be associated with the different specificities of the two primer sets used to amplify the molecular marker gene pmoA. In particular, the primer pair (A189f/mb661r) was able to detect a larger set of pmoA sequences and did not amplify any pmoA from the 0.6-TCM extract. This is in line with previous findings showing the superior ability of A189f/mb661r over A189f/A688r to detect a larger part of methanotrophic bacteria and its higher specificity to amplify pmoA (4, 9, 31). Therefore, this primer set was used in the T-RFLP analysis of pmoA in 0.6-TCM soil. T-RFs corresponding to all the groups detected in the clone libraries were found in T-RFLP fingerprinting. Although the genus Methyllobacter was detected in the 16S rRNA gene clone library, but not in the pmoA clone library, MspI and HaeIII T-RFs (505 and 350 bp, respectively) were found in T-RFLPs, which may correspond to Methyllobacter pmoA digestion fragments on the basis of the MspI T-RFLP analysis reported by Horz et al. (20) and also the in silico HaeIII digestion of representatives of this gene present in the database (e.g. the pmoA gene with GenBank ID EU124862 and JQ038155).

It is important to note that the T-RFLP analysis of the pmoA gene demonstrated that the vertical distribution of MOB (at depths of 0.6, 0.7, and 2.5 m) is relatively stable in
TMC soil, independently of differences in oxygen availability, as has been reported in studies on sediments from Lake Constance (CH) (30, 32). In TCM soil, CO₂ production linked to CH₄ consumption was detected at a depth of 0.7 m to the surface, indicating that methanotrophic activity occurs at shallow depths at which O₂ is present, whereas MOB activity is absent at a depth of 2.5 m at which CH₄ is present and O₂ is absent. The main difference observed with depth was the presence of MOB belonging to group G (Fig. 3, 4 and 5). T-RFLP comparisons highlighted the presence of T-RFs (in the HaeIII- andMspI-based profiles) corresponding to Methylocaldum-affiliated bacteria in soil collected at depths of 0.6 and 0.7 m. These T-RFs were below the detectable limit in 2.5-TMC soil (Fig. 4 and 5). In 0.6-TCM soil, the presence of the genus Methylocaldum was detected by analyzing both the gene markers, the pmoA and 16S rRNA genes. In particular, the pmoA sequence of the clone group G was phylogenetically correlated with Methylocaldum gracile (nt identity 99%). Bacteria belonging to the genus Methylocaldum are widely distributed in nature (3). Their habitats are thermal springs, activated sludge, arable soils, silage waste, and manure (14, 38). Methylocaldum gracile grows at 20°C and Methylocaldum tepidum at 30°C, with both growing optimally at 42°C and at a maximal temperature of 47°C (5). The presence of detectable Methylocaldum-related T-RFs at ground depths of 0.6 and 0.7 m may be related to an optimal growth temperature (42°C) present in this soil layer along with advantageous oxygen concentrations and other undefined environmental conditions occurring at depths of 0.7 and 0.6 m, but not at 2.5 m. Additional T-RFs, not related to any clones in the pmoA library, distinguished 0.6-TCM soil from 2.5-TCM soil; however, the contribution of these putative MOB species has not yet been elucidated. The optimal conditions for the CH₄ conversion are present at depths of approximately about 0.7–0.6 m, at which a significant increase in temperature was detected and bacteria affiliated with the genus Methylocaldum were identified. Chemical and isotopic data together with the presence of MOB appear to indicate that this process may even occur at depths >0.6 m. However, at deeper levels, the lack of free oxygen does not appear to allow efficient and complete CH₄→CO₂ conversion. This is also spatially associated to the absence of significant warming phenomena and not detectability of Methylocaldum-affiliated bacteria.

In conclusion, the results of the present study support and emphasize previous findings by Capaccioni et al. (6) and Nespoli et al. (29) in which strong biological methane-oxidizing activity in TMC soil was proposed to be related to the anomalous temperatures detected at a depth of 0.6 m. Accordingly, the spatial associations between the anomalously high temperatures in TCM soil, the consumption of CH₄ and O₂, the production of biogenic CO₂ at shallow levels, and the detection of MOB all agree with the occurrence of microbial CH₄→CO₂ conversion and soil heating. Although the phenomenon described here occurs in a local farming area in Italy, the importance of our work lies in the relationship we found between methanotrophy and soil heating, a topic that deserves public interest for its implications in climate warming and methane release.

Acknowledgements

The work was supported by “Vasco e GC Rossi—Microbial Biofilm” grant (2012–2015) and by the University of Bologna (RFO grant). The authors thank Dr Stefano Cremonini for his practical support in sample collection from the area of Terre Calde di Medolla, Modena Italy. This paper is in memory of the deceased Prof. Bruno Capaccioni, a dedicated and determined scientist, a clever and passionate man. We feel privileged to have worked with him.

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