LETTERS

Methanotrophy below pH 1 by a new Verrucomicrobia species

Arjan Pol[1], Klaas Heijmans[1], Harry R. Harhangi[1], Dario Tedesco[2], Mike S. M. Jetten[1] & Huub J. M. Op den Camp[1]

Mud volcanoes, mudpots and fumaroles are remarkable geological features characterized by the emission of gas, water and/or semi-liquid mud matrices[1] with significant methane fluxes to the atmosphere (10^{-1} to 10^{3} t y^{-1})[2–4]. Environmental conditions in these areas vary from ambient temperature and neutral pH to high temperatures and low pH. Although there are strong indications for biological methane consumption in mud volcanoes[4,5], no methanotrophic bacteria are known that would thrive in the hostile conditions of fumaroles (temperatures up to 70 °C and pH down to 1.8)[2]. The first step in aerobic methane oxidation is performed by a soluble or membrane-bound methane mono-oxygenase. Here we report that *pmoA* (encoding the β-subunit of membrane-bound methane mono-oxygenase) clone libraries, made by using DNA extracted from the Solfatara volcano mudpot and surrounding bare soil near the fumaroles, showed clusters of novel and distant *pmoA* genes. After methanotrophic enrichment at 50 °C and pH 2.0 the most distant cluster, sharing less than 50% identity with any other described *pmoA* gene, was represented in the culture. Finally we isolated an acidiphilic methanotrophic bacterium *Acidimethylosilex fumarolicum* SolV belonging to the Planctomycetes/Verrucomicrobia/Chlamydiae superphylum[6], 'outside' the subphyla of the Alpha- and Gammaproteobacteria containing the established methanotrophs. This bacterium grows under oxygen limitation on methane as the sole source of energy, down to pH 0.8—far below the pH optimum of any previously described methanotroph. *A. fumarolicum* SolV has three different *pmoA* genes, with two that are very similar to sequences retrieved from the mudpot. Highly homologous environmental 16S rRNA gene sequences from Yellowstone Park show that this new type of methanotrophic bacteria may be a common inhabitant of extreme environments. This is the first time that a representative of the widely distributed Verrucomicrobia phylum, of which most members remain uncultivated[6], is coupled to a geochemically relevant reaction.

Significant amounts of geological methane, produced within the Earth's crust, are currently released naturally into the atmosphere[3,7,8]. The preliminary global estimate of these methane emissions indicates that there are probably more than enough sources to provide the amount of methane required to account for the suspected missing source of global methane[8]. Recent findings from the Haakon Mosby and Carpatian mud volcanoes showed that these systems may also act as sinks for this geological methane[4,5,9]. At these sites with moderate environmental conditions (2–25 °C and a neutral pH), 16S rRNA genes of both aerobic and anaerobic methane-oxidizing microorganisms were present. In contrast, fumaroles such as those located in the Solfatara at Pozzuoli near Naples (southern Italy), which also emit significant amounts of methane (73 tonnes of CH_4 per km^2 per year)[2], are characterized by soils with a low pH (down to 1.0) and elevated temperatures (up to 70 °C). The H_2S-rich sulphurous fumes at these sites are microbially oxidized into sulphuric acid, creating an extremely acidic environment. The very acidic soil of the Solfatara was shown to support significant methane consumption[2], but so far it is unknown which microbes could be responsible for this consumption. Obligately aerobic methanotrophs are assumed to be a unique group of bacteria, belonging to either the Alpha or Gamma subclass of the Proteobacteria, which use methane as the sole source of energy and carbon[10]. So far, all aerobic methanotrophs have been shown to contain a membrane-bound particulate methane mono-oxygenase (pMMO), except for *Methylocella* sp. that was reported to have only the soluble, cytoplasmic form of MMO (sMMO)[11]. The *pmoA* gene (encoding the 24 kDa β-subunit of this membrane bound MMO[12]) is generally used as a phylogenetic marker for methanotrophic bacteria. Methanotrophs are widespread in nature and are mostly neutrophilic and mesophilic. However, on the basis of molecular surveys, in the last decade isolation and characterization of more extremophilic proteobacterial methanotrophs was initiated[13]. Thus far, the lowest pH values still supporting methanotrophic activity were reported for bacteria isolated from peat bogs[11,14,15]. These bacteria belong to genera of the Alpha subclass of the Proteobacteria (*Methylocella*, *Methylocapsa* and *Methylocystis*), and showed growth between pH 4.2 and 7.5 with a maximum methane-oxidizing activity around pH 5.0.

The inner part of the Solfatara, characterized by a central mudpool (fangaia) surrounded by bare, acid soil (pH 1–2), was sampled and DNA was extracted to start a molecular survey of *pmoA* genes. Here we report the presence of *pmoA* genes in an environmental clone library constructed using this DNA as a PCR template for the widely applied *pmoA* primer set A189/A682 (ref. 16), which also may amplify the gene of ammonium mono-oxygenase β subunit (*amoA*). We were only able to amplify *pmoA* genes using non-restrictive conditions (annealing temperature lowered from 56 °C to 48 °C; no false-positive clones obtained), pointing to the presence of *pmoA* genes with low similarity to known sequences. This is supported by the phylogenetic analyses of the *pmoA* sequences, which show that the Solfatara *pmoA* sequences group into two clusters: one represents a completely new, deep branch within the *pmoA*/*amoA* phylogenetic tree, sharing very low homology to known sequences (Fig. 1); the other cluster groups with the Gammaproteobacterial methanotrophs.

Intrigued by the new *pmoA* sequences, we used mud and mixed-soil samples from this site to start enrichment cultures at 50 °C and pH 2 with methane as the sole source of energy and carbon. After 3 weeks, methane consumption was observed in both soil and mud incubations. Non-restrictive PCR amplification of *pmoA* sequences, with DNA from the enrichment as a template, resulted in five clones (from two different enrichments) with sequences almost identical to the distant group within the environmental clones (Fig. 1). Repeated

[1]Department of Microbiology, IWWR, Radboud University Nijmegen, Toernooiveld 1, NL-6525 ED Nijmegen, The Netherlands. [2]Dipartimento di Scienze Ambientali, Seconda Università di Napoli, Via Vivaldi 43, 81100 Caserta, Italy.

serial transfers of the mud culture into fresh medium (see Methods) and finally diluting the culture onto floating polycarbonate filters[17] resulted in a pure culture, named strain SolV. Tiny whitish colonies appeared on the filters after 1 week and microscopic observation revealed only one rod-shaped morphotype. When exponentially growing cells of SolV were tested, the sMMO activity test (conversion of naphthalene to naphthol) was negative, but pMMO activity (particulate MMO; using propylene) could easily be measured (50 nmol per min per mg of protein). Genomic DNA from SolV was extracted and subjected to pyrosequencing[18]. From these data, we could identify many genes of C1 metabolism (Table 1), indicating that strain SolV may use a new combination of the serine, tetrahydrofolate and ribulose-1,5 bisphoshosphate pathways for carbon assimilation. The diagnostic genes of the ribulose-monophosphate pathway seem to be absent (Table 1). Conversion of formaldehyde seems to be mediated by a tetrahydrofolate-dependent pathway or directly by formaldehyde dehydrogenase (activity 110 nmol per min per mg of protein). The methanol dehydrogenase activity was 60 nmol per min per mg of protein and the *mxaF* gene showed 50% identity to *mxaF* of *Methylococcus capsulatus*. None of the subunits of sMMO was found. However, two complete *pmoCAB* operons and one *pmoCAB* cluster with a partial *pmoC* were identified. Several (two to nine) mismatches with *pmoA* primers A189/A682 were found (Supplementary Fig. 1), explaining the low recovery in PCR amplification from environmental DNA. However, all signature amino acids of PmoA were present, whereas the signature amino acids of AmoA were absent[19]. Of all 42 highly conserved amino acids in all bacterial PmoA/AmoA proteins[19], 6 to 8 were not shared by one or more of the *pmoA* genes from strain SolV (Supplementary Fig. 2). Phylogenetic analysis of the *pmoA* genes showed that *pmoA1* and *pmoA2* are highly similar to the environmental sequences from the Solfatara and the enrichments (Fig. 1, and see above). The *pmoA3* gene represents another completely new, deep branch. Together these three new *pmoA* sequences indicate that methanotrophic bacteria are phylogenetically much more diverse than currently assumed. Recent genomic data have shown that two either identical or distantly related *pmoA* genes can be present in one Alpha- or Gamma-proteobacterial methanotroph[20–22]. Expression of *pmoA1* and *pmoA2* messenger RNA was confirmed by RT–PCR on mRNA extracted from methane-grown SolV cells using specific primers (see Methods). The stacked membrane structures characteristic for methanotrophs expressing pMMO were not observed in SolV by transmission electron microscopy (Supplementary Fig. 3). Instead, circular bodies of about 50–70 nm were observed after fixation with glutaraldehyde or cryofixation. These bodies may be reminiscent of the vesicles observed in the acidiphilic methanotroph *Methylocella palustris*[23].

Growth of strain SolV occurred between pH 0.8 and 5.8 (Fig. 2). The temperature optimum is 55 °C, with only minor growth observed below 40 °C and above 65 °C. The maximum-specific-growth rate on methane was 0.07 h^{-1} (doubling time 10 h). Carbon dioxide and the

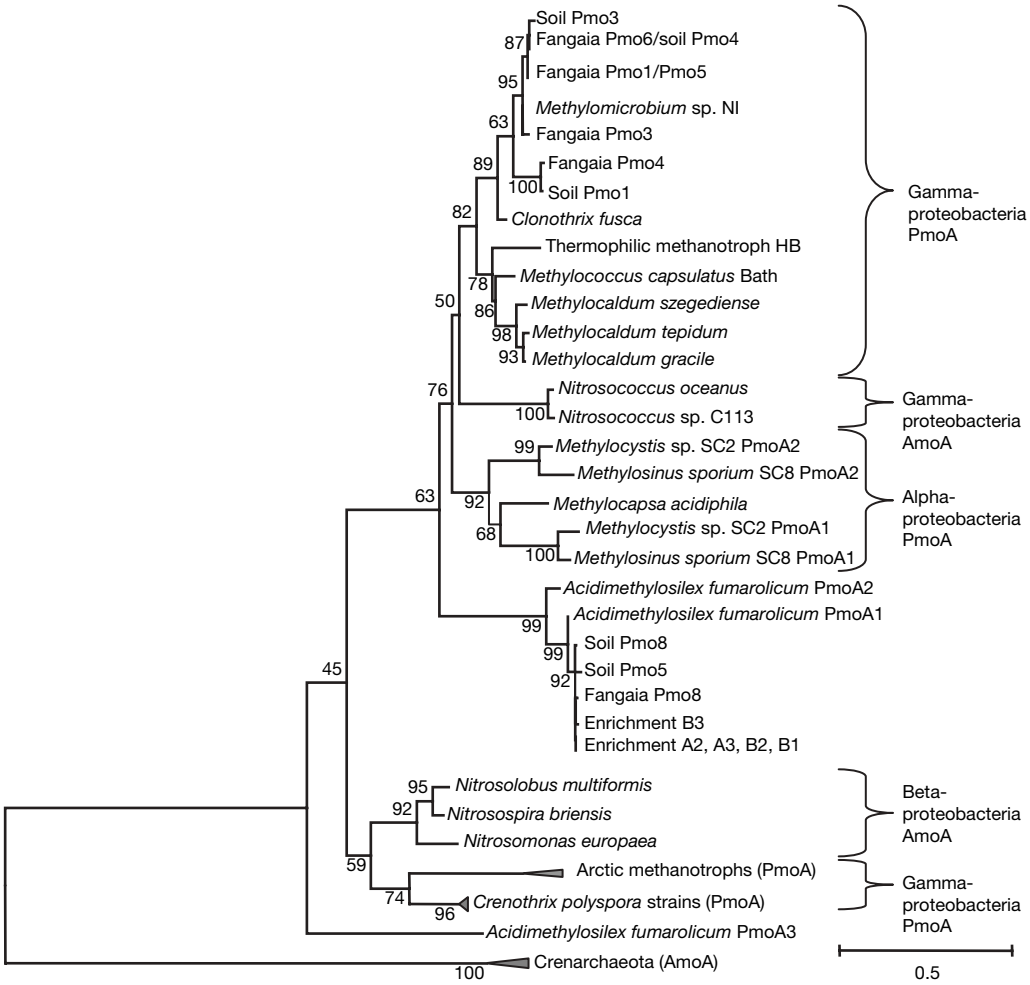

Figure 1 | Phylogenetic relationship among deduced PmoA and AmoA proteins. The neighbour-joining tree calculated with the PAM Dayhoff matrix is shown with bootstraps values of 500 replicates at the branches. The bar represents a 50% estimated-sequence divergence. Application of different methods of compiling trees revealed congruent tree topologies. The fangaia Pmo and soil Pmo prefix refer to environmental clones from DNA extracted from the central mudpot and bare, acid soil, respectively. Enrichment refers to clones obtained from DNA extracted from two different enrichments (A and B).

Table 1 | Genes of C1 metabolism of *Acidimethylosilex fumarolicum* SolV

Enzyme	Enzyme Commission (EC) number	Gene	BLASTP search against *Methylococcus capsulatus*			
			Identity (%)	Similarity (%)	Expected (*E*)-value	GenBank
Methane mono-oxygenase	1.14.13.25	*pmoA1*	53	71	4.6×10^{-80}	mca1797
		pmoA2	57	74	5.5×10^{-81}	mca1797
		pmoA3	41	62	1.2×10^{-51}	mca1797
		pmoB1	39	57	6.8×10^{-75}	mca2853
		pmoB2	40	58	1.6×10^{-76}	mca2853
		pmoB3	38	56	8.7×10^{-75}	mca2853
		*pmoC1**				
		pmoC2	58	72	5.9×10^{-77}	mca0295
		pmoC3	43	60	8.0×10^{-50}	mca0295
		mmoX	Not present in SolV			
Methanol dehydrogenase	1.1.99.8	*mxaF*	50	64	2.4×10^{-169}	mca0299
		mxaJ	36	55	1.7×10^{-36}	mca0300
		mxaG	34	51	2.0×10^{-8}	mca0781
Formaldehyde dehydrogenase	1.2.99.3	*adhP*	41	58	8.5×10^{-68}	mca0775
Formaldehyde-activating enzyme	4.3.-.-	*fae*	Not present in SolV			
Formate dehydrogenase		*fdhA*	50	67	3.1×10^{-28}	mca1393
		fdhB	62	78	6.7×10^{-172}	mca1392
		fdhC	68	81	0	mca1391
		fdhD	48	67	3.0×10^{-12}	mca1389
Serine–glyoxylate aminotransferase	2.6.1.45	*agxt/spt*	31	50	6.1×10^{-40}	mca1406
Hydroxypyruvate dehydrogenase	1.1.1.29	*hprA*	32	52	5.0×10^{-23}	mca1407
Formate–tetrahydrofolate ligase	6.3.4.3	*fhs*	53	70	1.5×10^{-165}	mca2219
Serinehydroxymethyl transferase	2.1.2.1	*glyA*	57	75	2.6×10^{-135}	mca1660
5-formyltetrahydrofolate cycloligase	6.3.3.2	*mthfs*	29	45	5.7×10^{-11}	mca2773
Methylenetetrahydrofolate dehydrogenase/ methenyltetrahydrofolate cyclohydrolase	1.5.1.5/3.5.4.9	*folD*	Not present in *M. capsulatus* †			
Hexulose-6-phosphate synthase	4.1.2.-	*hspA*	Not present in SolV			
Hexulose-6-phosphate isomerase	5.-.-.-	*sgbU*	Not present in SolV ‡			
Ribulose bisphosphate carboxylase	4.1.1.39	*cbbL*	60	75	4.9×10^{-165}	mca2743
		cbbS	41	62	4.5×10^{-18}	mca2744
Phosphoribulokinase	2.7.1.19	*cbbP*	64	80	6.6×10^{-108}	mca3051
Phosphoglycerate kinase	2.7.2.3	*cbbK*	40	61	1.5×10^{-76}	mca2021
Glyceraldehyde-3-phosphate dehydrogenase	1.2.1.13	*cbbG*	49	64	1.4×10^{-72}	mca2598

Genes of C1 metabolism were identified in an assembly of the genome after pyrosequencing. The assembly was produced from 88.9 Mb of sequence information and resulted in a 35-fold coverage, based on an estimated genome size of 2.5 Mb. Translated protein sequences, based on genes identified, were used for a BLAST search in the *Methylococcus capsulatus* genome (http://pedant.gsf.de/). *partial gene (48 amino acids); †best NCBI BLAST hit with *folD* from *Prosthecochloris aestuarii* (identity 51%; similarity 71%; *E*-value 1.0×10^{-73}); ‡best NCBI BLAST hit with *gutQ* (sugar phosphate isomerase family) from *Burkholderia phytofirmans*, (identity 45%; similarity 66%; *E*-value 2.0×10^{-78}).

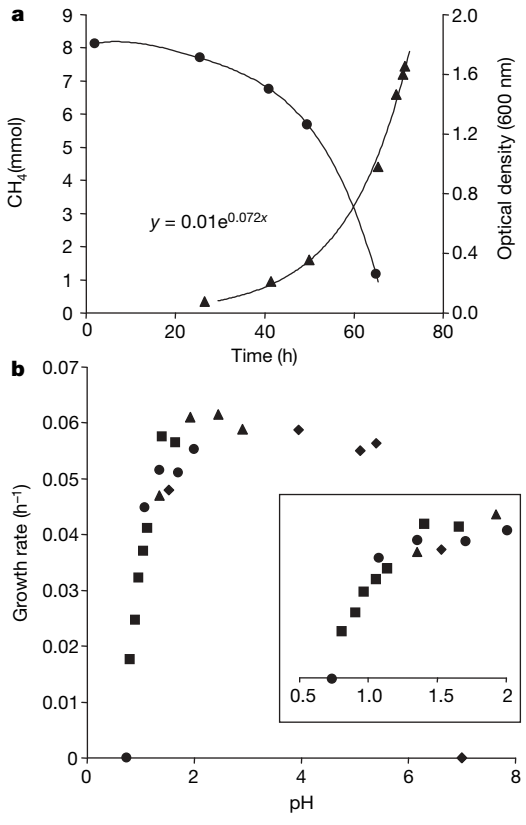

Figure 2 | Growth characteristics of strain SolV. a, Typical growth curve showing decrease of methane (circles) and increase of optical density (triangles) at pH 2 and 55 °C. The equation is the best exponential fit through the data points. **b**, Growth rate in relation to pH. The insert shows an enlargement of the data below pH 2. Different symbols indicate experiments performed on different days.

inorganic fraction of mud water stimulated growth. Methane was converted to carbon dioxide according to a stoichiometry that is typical for methanotrophs: $CH_4 + 1.6\,O_2 \rightarrow 0.65\,CO_2 + 1.55\,H_2O + 0.35\,CH_20$ (biomass) with a yield of 6.4 g of dry weight per mol of methane.

Acetate, malate, succinate, formate, formaldehyde and yeast extract (all at $1\,g\,l^{-1}$) completely inhibited growth of SolV on methane at pH 2. The bacterium apparently is very sensitive towards uncoupling by small organic acids at low pH values, because at pH 5 formate (pK_a 3.75) did not inhibit growth. No growth took place above 100 mM NaCl or in media containing glucose. In addition to methane, hydrogen gas was also oxidized. Strain SolV grew well on methanol, but the added methanol completely repressed methane consumption. After methanol was depleted, methane consumption and growth started only after 4 h. Ethane inhibited growth although it was converted simultaneously with methane as a competitive substrate at virtually the same rate. Acetylene (0.1% v/v) instantaneously caused a complete inhibition of methane consumption, an observation that supports pMMO being the primary methane-oxidizing system. SolV could use both ammonium and nitrate as a nitrogen source. No growth occurred on ammonium without methane. Nitrogen fixation and anoxic nitrate-dependent methanol oxidation was not observed.

SolV has a typical K_s value for methane, namely 6 μM. However, the affinity for oxygen was exceptionally high (K_s 0.7 μM), reflecting

the need to compete for oxygen in its natural habitat, where microbial oxygen consumption and a constant flux of oxygen-depleted fumarolic gases, containing mainly carbon dioxide, will cause oxygen concentrations to be very low.

Fluorescence *in situ* hybridization (FISH) analysis of the isolate using the probe EUBIII, which is designed to mainly cover the Verrucomicrobiales[24,25], showed a strong hybridization signal (Supplementary Fig. 4). No signal was obtained with EUBI, EUBII or the alpha (ALF968), beta (BET42a) or gamma (GAM42a) proteobacterial probes[25,26]. The Verrucomicrobia-like identity was confirmed by the sequence of its 16S rRNA gene obtained from pyrosequencing (see above). A specific probe was designed on the basis of this sequence (SolV830, see Methods) and used together with probe EUBIII to confirm the purity of the SolV culture. All cells from an exponentially growing culture showed double hybridization (Supplementary Fig. 4). Phylogenetic analysis of the 16S rRNA sequence of SolV indicated that the isolate represents the first member of a new subdivision within the Verrucomicrobia phylum (Fig. 3 and Supplementary Fig. 5). Pairwise distance analysis revealed $<81\%$ identity with members of other subdivisions[6,27].

Strain SolV is the first reported extreme acidiphilic methanotrophic bacterium and is phylogenetically placed outside the subphyla of the Alpha- and Gammaproteobacteria containing the established methanotrophs, and we propose to name it: '*Acidimethylosilex fumarolicum*', gen. nov. sp. nov. (Supplementary Information).

So far the Verrucomicrobia phylum contains only a few cultivated strains that are anaerobic or aerobic heterotrophs, growing on sugars in more or less complex media. However environmental clone libraries show that there is a large biodiversity of Verrucomicrobia and they are encountered in many ecosystems (soils, peat bogs, acid rock drainage and landfill leachate) often in relatively high numbers, but with an unknown physiology[6]. It is interesting to speculate that the widely distributed Verrucomicrobia phylum, from which most members remain uncultivated[6], may be coupled to a geochemically relevant reaction. BLAST searches with the strain SolV 16S rRNA gene sequence showed very high identity (98–99%) to six environmental clones (Fig. 3) that were obtained during a geochemical study on microbial communities in acidic hot springs (Rainbow and Joseph's Coat) in Yellowstone National Park (unpublished; NCBI accession numbers: AY882698, AY882699, AY882710, AY882819, AY882820 and AY882834). This shows that bacteria similar to

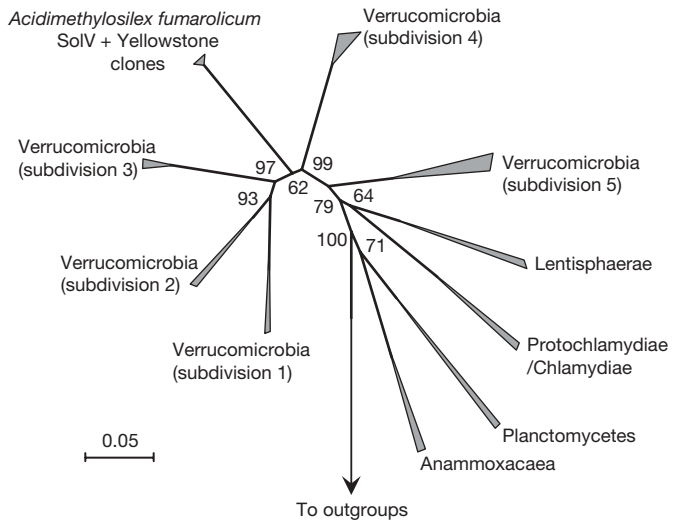

Figure 3 | Phylogenetic relationship between the 16S rRNA gene sequence of strain SolV and representatives of the Planctomycetes/Verrucomicrobia/Chlamydiae superphylum. The tree was calculated using the neighbour-joining algorithm with Kimura 2-parameter correction. Bootstrap values of 500 replicates are shown at the nodes. The scale bar represents 0.05 nucleotide changes per position.

A. fumarolicum may be common inhabitants of these extreme environments. The new *pmoA* and 16S rRNA gene sequences may help to identify the Planctomycetes/Verrucomicrobia/Chlamydiae superphylum methanotrophs from less extreme habitats and to show how they are globally distributed.

METHODS SUMMARY

Enrichments were started with mud and mixed soil samples from the Solfatara and incubated at 50 °C and pH 2.0 with methane as the sole source of energy and carbon. When methane consumption was observed, serial transfers into fresh medium were started. Finally a pure culture was obtained using the floating-filter technique[17]. Purity was checked by FISH and plating on medium enriched with yeast extract, without methane in the head space. DNA from environmental samples and genomic DNA from strain SolV was isolated as described[28]. The *pmoA* and 16S rRNA genes were amplified by hot start using primers A189 and A682 (ref. 16), and 616F (5′-AGA GTT TGA TYM TGG CTC AG-3′) and 630R (5′-CAK AAA GGA GGT GAT CC-3′)[28], respectively. Pyrosequencing on genomic DNA was done as described[18]. FISH microscopy was performed as described[29] using the following nucleotide probes: EUBI, EUBII, EUBIII and SolV830 (5'-GGT CGA TTC CGC CAA CGC-3′). The latter probe was designed with the ARB program[30]. Expression of *pmoA* mRNA was analysed by RT–PCR. The affinity for methane was estimated using cells from a batch culture ($OD_{600} = 0.24$). The apparent affinity constant for oxygen was estimated by measuring the methane respiration of a stirred culture in a 1 ml glass chamber equipped with a micro-oxygen sensor (Unisense A/S). Enzyme activities mentioned in Table 1 were measured according to ref. 23.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions A.P. and D.T. performed the sampling; A.P. did the enrichment and isolation; K.H. and A.P. carried out the physiological experiments; K.H. and H.R.H. were responsible for the molecular analysis; A.P. and H.J.M.O.d.C. performed phylogenetic analyses, alignments and probe design. The research was conceived by A.P., M.S.M.J. and H.J.M.O.d.C. and was based on observations made by D.T. A.P., M.S.M.J., D.T. and H.J.M.O.d.C. contributed to interpreting the data and writing the paper.

Author Information The nucleotide sequence data have been deposited in GenBank under accession numbers EF591085 (pmo_1), EF591086 (pmo_2), EF591087 (pmo_3) and EF591088 (16S rRNA). Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to M.S.M.J. (m.jetten@science.ru.nl) or H.J.M.O.d.C. (h.opdencamp@science.ru.nl).

METHODS

After 3 weeks, methane consumption was observed and repeated serial transfers of the mud and mixed-soil culture into fresh medium (see below) were started. Finally the culture was serially diluted and aseptically filtered through 25-mm polycarbonate filters (0.2 μm, Nucleopore), which were placed floating on medium in Petri dishes and incubated in closed jars under a methane atmosphere (see below)[17]. Tiny whitish colonies appeared on the filters after 1 week and microscopic observation revealed only one rod-shaped morphotype. Purity was checked by FISH and plating on medium enriched with yeast extract, without methane in the head space. No growth was observed on this medium.

Culture conditions. The culture medium was based on the Fangaia mineral concentrations and composed of 0.4 mM $MgCl_2$, 2 mM $CaHPO_4$, 1 mM Na_2SO_4, 2 mM K_2SO_4, 2 mM $(NH_4)_2SO_4$, 3% autoclaved liquid from the Fangaia mud pool at Pozzuoli; 1 ml l^{-1} trace elements (in mg l^{-1}) $ZnSO_4 \cdot 7H_2O$ (4.4), $MnCl_2 \cdot 4H_20$ (1.0), $FeSO_4 \cdot 7H_2O$ (1.0), $(NH_4)_6MO_7O_{24} \cdot 4H_2O$ (0.22), $CuSO_4 \cdot 5H_2O$ (0.32), $CoCl_2 \cdot 6H_2O$ (0.32). The pH was adjusted with H_2SO_4 or NaOH. Bacteria were grown in 120 ml serum bottles with 10 ml of medium and 2–5% CO_2 and 2–5% CH_4 in the headspace. Bottles were incubated at 50–55 °C on a rotary shaker at 250 r.p.m. To determine the reaction stoichiometry, gas samples were taken from triplicate cultures with a gaslock syringe and methane, carbon dioxide, oxygen and hydrogen were analysed on a HP 6890 gas chromatograph with a Porapak Q column and thermal conductivity detection. Yield on methane was determined by harvesting cells in the late exponential phase. Cells were centrifuged and washed with 1 mM HCl and dried under vacuum at 70 °C until constant weight.

***pmoA* and 16S rRNA gene sequence analysis.** DNA from environmental samples and genomic DNA from strain SolV was isolated as described[28] without the use of lytic enzymes. For *pmoA* PCR under non-restrictive conditions the annealing temperature was lowered from 56 °C to 48 °C. The products were purified from an agarose gel with the QIAEX II gel extraction kit (Qiagen) and cloned using the TOPO TA cloning kit (Invitrogen). Plasmids were purified with FlexiPrep kit (Amersham Biosciences) and sequenced with M13R and M13F primers, which flank the cloning site of the vector. Pyrosequencing on genomic DNA was done as described[18].

Real-time RT–PCR analysis. Samples (50 ml at $OD_{600} = 0.85$) from methane-grown chemostat cultures were rapidly cooled and RNA was isolated using the Omega RNA extraction kit (Omega Bio-Tec) according to the manufacturer's protocol. Transcription products of *pmoA* were detected using SolV-specific primers (REVp1032 5′-GCAAARCTTCTCATYAGTWCC-5′; FORp1034 5′-GTGGATGAATCGGTATTGG-3′). Reverse transcription was performed with primer REVp1032 and RevertAid M_MulV (Fermentas) Quantitative PCR was done using the iQ custom SYBR Green supermix kit (Bio-Rad), according to the manufacturer's instructions. The PCR program on the Biorad MyiQ was 3 min 95 °C and 40 cycles 30 s at 95 °C, 30 s at 54 °C, 30 s at 72 °C.

FISH microscopy. On the basis of the obtained 16S rRNA gene (see above) a new oligonucleotide probe (SolV830, 5′-GGT CGA TTC CGC CAA CGC-3′) was designed using the probe-design software of the ARB program[30]. Optimum formamide concentration for this probe was 20%.

Kinetics and enzyme activities. The affinity for methane was estimated by measuring the consumption rate in a series of incubations of 10 ml samples, taken from a batch culture ($OD_{600} = 0.24$) in 100 ml bottles at 50 °C. Various amounts of methane were added and bottles were shaken vigorously at 500 r.p.m. Virtually linear rates were measured during one hour. Rates were proportional to the cell density in the range used, indicating that there was no mass-transfer limitation for methane. Five ml of the culture were preincubated at 50 °C in a closed 100 ml bottle with 30 ml of methane to ensure excess methane compared to oxygen. The oxygen-consumption rates were calculated from the decrease in oxygen concentration over time.