LETTERS

# Methane oxidation by an extremely acidophilic bacterium of the phylum Verrucomicrobia

Peter F. Dunfield[1]†, Anton Yuryev[2], Pavel Senin[3,4], Angela V. Smirnova[1], Matthew B. Stott[1], Shaobin Hou[3,4], Binh Ly[3,4], Jimmy H. Saw[3], Zhemin Zhou[5], Yan Ren[5], Jianmei Wang[5], Bruce W. Mountain[1], Michelle A. Crowe[1], Tina M. Weatherby[6], Paul L. E. Bodelier[7], Werner Liesack[8], Lu Feng[5], Lei Wang[5] & Maqsudul Alam[3,4]

**Aerobic methanotrophic bacteria consume methane as it diffuses away from methanogenic zones of soil and sediment[1]. They act as a biofilter to reduce methane emissions to the atmosphere, and they are therefore targets in strategies to combat global climate change. No cultured methanotroph grows optimally below pH 5, but some environments with active methane cycles are very acidic[2,3]. Here we describe an extremely acidophilic methanotroph that grows optimally at pH 2.0–2.5. Unlike the known methanotrophs, it does not belong to the phylum Proteobacteria but rather to the Verrucomicrobia, a widespread and diverse bacterial phylum that primarily comprises uncultivated species with unknown genotypes. Analysis of its draft genome detected genes encoding particulate methane monooxygenase that were homologous to genes found in methanotrophic proteobacteria. However, known genetic modules for methanol and formaldehyde oxidation were incomplete or missing, suggesting that the bacterium uses some novel methylotrophic pathways. Phylogenetic analysis of its three *pmoA* genes (encoding a subunit of particulate methane monooxygenase) placed them into a distinct cluster from proteobacterial homologues. This indicates an ancient divergence of Verrucomicrobia and Proteobacteria methanotrophs rather than a recent horizontal gene transfer of methanotrophic ability. The findings show that methanotrophy in the Bacteria is more taxonomically, ecologically and genetically diverse than previously thought, and that previous studies have failed to assess the full diversity of methanotrophs in acidic environments.**

Methane is the second most important greenhouse gas, estimated to contribute 18% of the total atmospheric radiative forcing[4]. It is produced in anoxic environments primarily through the microbial degradation of organic matter[1], but abiogenic methane emitted from seeps and geothermal areas is also a major contribution to the atmospheric budget, estimated at 45–75 Tg annually[2,5]. Hell's Gate (Tikitere), New Zealand, is a geothermal area rich in abiogenic methane[6]. We studied methane oxidation in an area of woody vegetation that had recently died as a result of the onset of steam emission from below. There was a temperature gradient in the soil, which consisted of an organic horizon (0–2 cm, pH 3.1, 31 °C), an A horizon (2–5 cm, pH 3.8, 42 °C), a B horizon (5–10 cm, pH 4.3, 53 °C), black ash (10–15 cm, pH 4.5, 63 °C) and pumice mixed with ash (below 15 cm, pH 4.7, 71 °C at 20 cm). Soil gas at a depth of 60 cm contained about 1% (v/v) methane. This did not decline linearly towards the soil surface. Instead it approached a minimum at 10–20 cm below the surface, indicating that a subsurface sink consumed the methane as it diffused upwards (Fig. 1). Bacterial 16S rRNA genes amplified by polymerase chain reaction (PCR) from the soil layer at 10–15 cm depth were dominated (33 of 35 cloned genes) by a single sequence that showed low identity (less than 83%) to any cultivated bacterium and grouped phylogenetically within the phylum Verrucomicrobia (Supplementary Fig. 1). A bacterium possessing this 16S ribosomal RNA gene sequence (isolate V4) was subsequently isolated at 60 °C in air supplemented with 25% (v/v) methane as the sole energy source. Isolate V4 was able to grow and oxidize methane down to at least pH 1.5 (Fig. 2). The optimum was pH 2.0–2.5 (Fig. 3). The growth rate was near optimal between pH 1.5 and pH 3.0, but weak growth was observed as low as pH 1.0 and as high as pH 6.0.

Two properties of this bacterium are unique in comparison with all known methanotrophs: its phylogenetic placement in the phylum Verrucomicrobia and its extremely acidophilic phenotype.

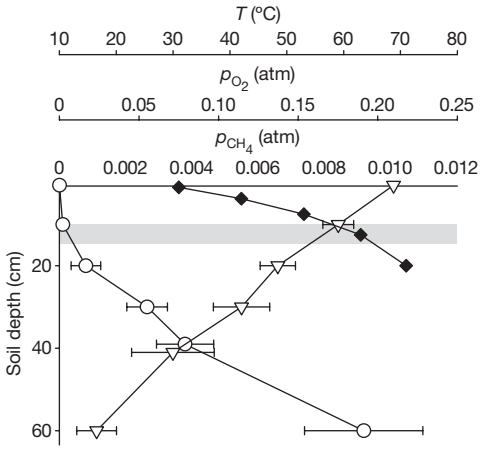


**Figure 1 | Vertical profiles of methane partial pressures (open circles), $O_2$ partial pressures (open triangles) and temperature (filled diamonds) in a geothermal soil.** Partial pressures are shown as means ± s.e.m. ($n = 5$). Methane declined from 0.01 atm at 60 cm depth to below the detection limit (less than 100 p.p.m.) at 10–20 cm depth, suggesting that there was a subsurface methane sink. The linear decline in $O_2$ indicates that there was a deeper sink for this gas and that the diffusion rate was relatively constant in different soil layers. The grey area represents the soil sample (10–15 cm depth) from which bacterial 16S rRNA genes were amplified by PCR.

[1]GNS Science, Extremophile Research Group, Private Bag 2000, Taupo, New Zealand. [2]Ariadne Genomics, Inc., 9430 Key West Avenue no. 113, Rockville, Maryland 20850, USA. [3]Department of Microbiology, University of Hawaii, Snyder Hall no. 207, 2538 The Mall, Honolulu, Hawaii 96822, USA. [4]Advanced Studies in Genomics, Proteomics and Bioinformatics, College of Natural Sciences, Keller Hall 319, 2565 McCarthy Mall, Honolulu, Hawaii 96822, USA. [5]TEDA School of Biological Sciences and Biotechnology, Nankai University, 23 HongDa Street, Tianjin 300457, China. [6]Biological Electron Microscopy Facility, Pacific Biosciences Research Center, University of Hawaii at Manoa, Snyder Hall no. 118, Honolulu, Hawaii 96822, USA. [7]Netherlands Institute of Ecology (NIOO-KNAW), Centre for Limnology, Department of Microbial Wetland Ecology, Rijksstraatweg 6, 3631 AC, Nieuwersluis, The Netherlands. [8]Max Planck Institute for Terrestrial Microbiology, Karl-von-Frisch-Strasse, 35043 Marburg, Germany. †Present address: Department of Biological Sciences, University of Calgary, 2500 University Drive NW, Calgary, Alberta, T2N 1N4, Canada.

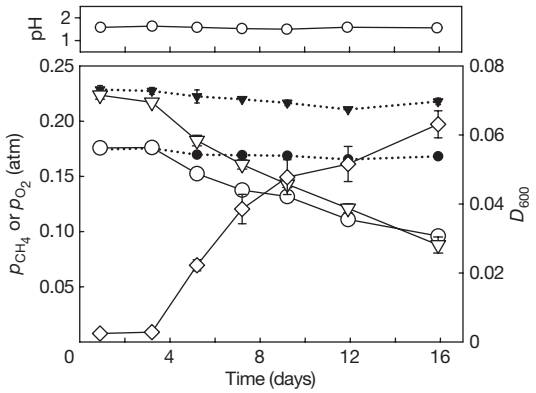


**Figure 2 | $D_{600}$ (open diamonds), methane consumption (open circles), and $O_2$ consumption (open triangles) of isolate V4 growing in liquid medium at pH 1.5 and 50 °C.** Data are shown as means ± s.e.m. for triplicate culture vials. Where error bars are not visible they are smaller than the symbols. The upper panel shows that the pH was constant throughout the experiment. The dotted lines represent partial pressures of methane (solid circles) and $O_2$ (solid triangles) in uninoculated blanks (means ± s.e.m. for duplicate vials); the slight decline is due to removal during sampling.

Decades of previous study have identified only 13 genera of aerobic methanotrophs. All belong to the phylum Proteobacteria, in the classes Alphaproteobacteria and Gammaproteobacteria. They include two moderate acidophiles, *Methylocella* and *Methylocapsa*, which have pH optima of 5.0–5.5 and a lower limit of 4.0–4.5 (refs 3, 7). Isolate V4 is therefore by far the most acidophilic methanotroph yet found. Cells were non-motile rods with dimensions 0.3–0.5 μm × 1–4 μm. A tubular membrane inside an invagination of the inner membrane was observed in a small proportion of cells (Fig. 4). Intracytoplasmic membranes, usually occurring in parallel stacks, are common in methanotrophs and are suspected to house the membrane-bound particulate methane monooxygenase (pMMO) enzyme. The membrane structure in isolate V4 could serve the same function; however, its rare occurrence indicates that it may be only a stage in a morphological life cycle or that it may occupy a small volume of the cell.

Aerobic methanotrophic bacteria use monooxygenase enzymes to convert methane to methanol, which is then oxidized to formaldehyde, formate and $CO_2$. Two forms of methane monooxygenase are known, a soluble form (sMMO) present in a few species, and a membrane-bound form (pMMO) present in all known genera except *Methylocella*[8]. Microbial ecologists have taken advantage of the nearly universal occurrence of pMMO to design detection assays specific to methanotrophs. Degenerate oligonucleotide primer sets have been employed to amplify *pmoA* genes (encoding a subunit of pMMO) from various environments by means of PCR. On the basis of *pmoA* sequence phylogeny, individual species can then be identified[9,10]. We were unable to amplify a *pmoA* gene product from a DNA extract of isolate V4, nor directly from a soil DNA extract, using standard primer sets and PCR protocols[10]. To obtain a better understanding of methane metabolism by this bacterium, we therefore sequenced and assembled a draft genome using an eightfold-coverage whole-shotgun approach[11].

Analysis of the draft genome (2.1 megabases) allowed the identification of genetic systems involved in methanotrophic growth. Three complete *pmoCAB* operons encoding pMMO were found, each with the same gene arrangement found in Proteobacteria[12,13] and each with a putative upstream $\sigma^{70}$ promoter sequence. Membrane topologies predicted from derived amino acid sequences were identical to the corresponding proteins in Proteobacteria[13], although the maximum sequence identities were only 34–65% (Supplementary Table 1). The *pmoA* gene is a conserved and largely reliable phylogenetic marker[9,10], so a comparative phylogenetic analysis was made of the three derived PmoA sequences of isolate V4. These grouped into a single evolutionary lineage distinct from PmoA and AmoA sequences of other methane-oxidizing and ammonia-oxidizing microorganisms (Fig. 5), indicating an ancient divergence of Verrucomicrobia and Proteobacteria methanotrophs rather than a recent horizontal transfer of *pmo*.

Three divergent *pmo* operons have never before been detected in a methanotroph. *Methylococcus capsulatus* and some other species possess two nearly identical copies of *pmoCAB*[12,13], whereas two divergent operons have been found in a *Methylocystis* species[14]. Two of the derived PmoA sequences in isolate V4 were 90% identical (Supplementary Table 2), but copy 3 was less than 50% identical with the other two, suggesting that different selection pressures have acted on it. However, it is unlikely that any of the three operons encode an ammonia monooxygenase. The bacterium did not grow on ammonium, and an analysis of the three derived PmoA sequences for putative 'signature' amino acids found a higher proportion of methanotroph-specific than nitrifier-specific signatures in each[15] (Supplementary Table 2). However, Hell's Gate is rich in inorganic nitrogen[6], so the three putative pMMO enzymes may be differentially sensitive to competitive inhibition by ammonia. Alternatively, they may have different kinetic properties or pH optima.

The genome was also searched for homologues of other methylotrophy genes, especially those identified in the fully sequenced methanotroph *Methylococcus capsulatus*[12] (Supplementary Table 1). Besides pMMO, gene clusters encoding formate dehydrogenase and methylamine dehydrogenase were present, although several genes encoding accessory proteins for assembly and stabilization of the latter enzyme[16] were not identified, and we were unable to

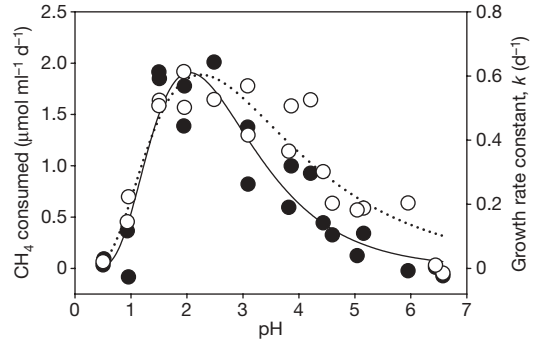


**Figure 3 | Growth rate constant based on $D_{600}$ (open circles) and average methane consumption rates (filled circles) of isolate V4 at a range of pH values.** Each point represents the mean of two culture vials, measured over 7–14 days of incubation. The lines were estimated as log-normal iterative best fits to the data.

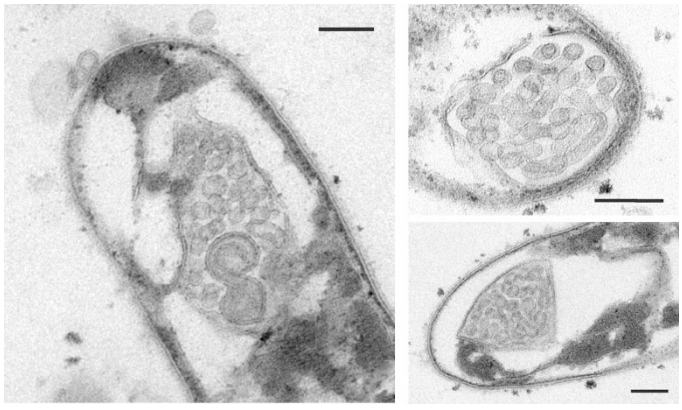

**Figure 4 | Transmission electron micrographs of internal membrane structures observed in some cells of Verrucomicrobia isolate V4.** A sac containing tubular membrane structures is attached to the inner cell membrane. Scale bar, 100 nm.

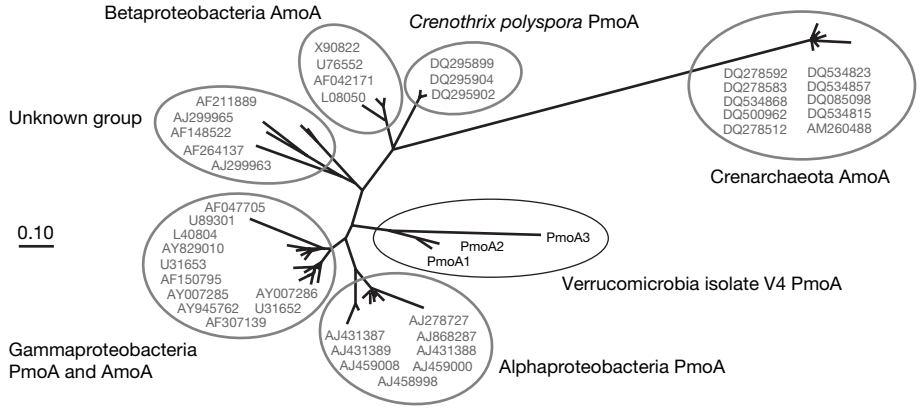


**Figure 5 | Phylogenetic tree constructed from derived PmoA and AmoA sequences (subunits of particulate methane monooxygenase or ammonia monooxygenase), showing the relative positions of the three sequences from Verrucomicrobia isolate V4.** Distinct groups corresponding to broad microbial taxa can be delineated, with the exception of *Crenothrix polyspora*, which groups apart from other Gammaproteobacteria. The tree was constructed on the basis of 165 amino-acid positions by using TREE-PUZZLE[29], a quartet maximum-likelihood method. The support value from 10,000 puzzling steps for the branch to the Verrucomicrobia was 86%. The scale bar represents 0.1 change per amino-acid position.

cultivate the bacterium on methylamine. Other similarities to *Methylococcus* included genes encoding nitrite reductase, hydroxylamine oxidoreductase and nitric oxide reductase. These enzymes may remove toxic byproducts of competitive ammonia oxidation by pMMO.

However, there was no genomic evidence for some key enzymes of methylotrophy, including methanol dehydrogenase and tetrahydromethanopterin enzymes for formaldehyde oxidation. Methanol is the immediate product of pMMO, so some form of methanol dehydrogenase should be present. However, no homologues were identified for the *mxaI* gene encoding the small subunit of this enzyme, nor for a variety of other essential genes[16]. Genomes of several methylotrophic and non-methylotrophic bacteria contain truncated *mxa*-like gene clusters similar to that in isolate V4, but it is doubtful whether these express a methanol dehydrogenase[17,18]. To our knowledge, isolate V4 is also the first fully sequenced methylotroph to lack the tetrahydromethanopterin pathway for formaldehyde oxidation. Enzymes of this pathway are taxonomically widespread, occurring in Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria[12,16–18], as well as in Archaea and Planctomycetes[19]. A potential alternative for formaldehyde oxidation is by means of tetrahydrofolate enzymes[12]. Several are present in isolate V4 (Supplementary Table 1), but a complete pathway cannot be verified at this stage. Another alternative would be a glutathione-dependent or glutathione-independent formaldehyde dehydrogenase. Although we found no homologues to formaldehyde dehydrogenases from *Methylococcus capsulatus* or *Methylobacterium extorquens*, there were homologues to alcohol dehydrogenases that could conceivably oxidize formaldehyde.

Methanotrophs fix most of their carbon heterotrophically through serine or ribulose monophosphate pathways. No homologues were found for key enzymes (hexulose-6-phosphate synthase and hexulose-6-phosphate isomerase) of the ribulose monophosphate pathway[20,21]. However, genes encoding some serine cycle enzymes were identified, indicating that a variant of this cycle may function (Supplementary Table 1). In addition, all genes necessary for a complete Calvin–Benson cycle were present. Carbon dioxide fixation contributes only a minor proportion of the total carbon assimilation of proteobacterial methanotrophs[20], but the genomic data imply an increased role in isolate V4. Experiments confirmed that the maximum growth rate (on methanol) was strongly dependent on $CO_2$. It decreased by two orders of magnitude when no $CO_2$ was added to culture vials, from 0.91 $d^{-1}$ (s.e.m. 0.061) under 2–10% (v/v) $CO_2$ to less than 0.01 $d^{-1}$ under ambient $CO_2$. A complete tricarboxylic acid cycle was also predicted, but we were unable to cultivate the bacterium on multicarbon substrates. Robust growth occurred only on the $C_1$ compounds methane and methanol. Although most methanotrophs are obligately methylotrophic, the failure of isolate V4 to grow on short-chain organic acids such as acetate is curious, because these should be protonated under acidic growth conditions and diffuse freely across the cell membrane. Other factors must restrict growth on these substrates.

Some bacterial phyla, particularly the Verrucomicrobia and Acidobacteria, are widespread and abundant in nature but are represented by only a few isolates in culture collections[22]. Determining the metabolic lifestyles of these bacteria (for example, the recent discovery of photosynthesis in the Acidobacteria[23]) is a major task facing microbial ecologists[22]. Stable-isotope-probing experiments using $^{13}CH_4$ to label bacterial DNA have identified Verrucomicrobia as potential members of methane-based food webs in an acidic soil[24] and an alkaline sediment[25], so methanotrophic members of this phylum may be ecologically diverse. We have recently obtained a methanotrophic mixed culture at 25 °C from an acidic mud (pH 3), which is dominated by a Verrucomicrobia showing less than 90% 16S rRNA gene sequence identity to isolate V4 (culture LP2A in Supplementary Fig. 1). Although the inoculum source was also a geothermal area, this finding does suggest that verrucomicrobial methanotrophs are a taxonomically broad group with varying environmental tolerances. Environmental surveys of methanotrophs based on *pmoA* recovery have failed to detect these methanotrophs in the past. Standard PCR primers[9,10] show 3–13 mismatches with *pmoA* target regions in isolate V4, so this failure may be due to methodological limitations. Isolate V4 also possesses none of the membrane lipids considered unique to methanotrophs: 16:1ω8c,16:1ω5t, or 18:1ω8c (ref. 26) (Supplementary Table 3), so studies targeting signature phospholipids have overlooked this group as well. The physiological and genomic analysis of our isolate, for which we propose the name '*Methylokorus infernorum*', will facilitate the design of more complete surveys of methanotrophs in different environments.

## METHODS SUMMARY

**Isolation.** Soil crumbs were spread on plates of mineral salts medium at pH 4.5–5.5 and incubated at 60 °C in sealed jars containing 25% (v/v) $CH_4$ and 8% (v/v) $CO_2$ in air. Colony growth was restreaked onto new medium until pure. Purity was ensured by observation of colony and cell morphology and by genome sequencing of 43,008 reactions, which detected no contamination.

**Characterization of isolate V4.** Transmission electron microscopy[27] and phospholipid fatty acid analyses[26] were performed as described previously. For growth curve experiments, medium was adjusted to various pH values with $H_2SO_4$ and NaOH, and divided into aliquots in serum vials closed gas-tight with septa. $CO_2$ (2–5% v/v) and $CH_4$ (8–15%) were added. The pH, $CH_4$ and attenuance at 600 nm ($D_{600}$) were monitored at 2-day intervals. Methane was measured on a gas chromatograph equipped with a thermal conductivity detector (Varian Star 3600, 5 m × 0.75 mm Molecular Sieve 5A column, oven 35 °C, detector

270 °C). To test organotrophic growth, medium was supplemented with various sugars, organic acids, alcohols, methylamines or complex media at 0.05% (w/v). To test the effect of $CO_2$, cells were grown at pH 3.5 on methanol (2.5 $ml l^{-1}$), and 0, 2 or 10% (v/v) $CO_2$ was added to the headspace of vials.

**Soil analyses.** Soil gas was extracted into gas-tight syringes through a stainless steel tube. DNA was extracted with the PowerSoil Kit (Mo Bio Laboratories, Inc.). PCR amplification of 16S rRNA and *pmoA* genes, cloning with the TOPO TA Cloning Kit (Invitrogen) and DNA sequencing were performed as described previously[10,11].

**Genome analyses.** The genome was sequenced, assembled and mapped as described previously[11]. Open reading frames were mapped into different metabolic pathways by using BLAST against the entire collection of bacterial protein sequences in the RefSeq database (National Centre for Biotechnology Information) and separately against the genome of *Methylococcus capsulatus* str. Bath. Orthologous pairs were calculated from BLAST results from the full-length sequence similarity, and best orthologues were estimated with a reciprocal best-hit method[28]. The functional analysis was completed by manual annotation.

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

**Received 13 August; accepted 24 October 2007.**
**Published online 14 November 2007.**

1. Conrad, R. Soil microorganisms as controllers of atmospheric trace gases ($H_2$, CO, $CH_4$, OCS, $N_2O$, and NO). *Microbiol. Rev.* **60,** 609–640 (1996).
2. Castaldi, S. & Tedesco, D. Methane production and consumption in an active volcanic environment of southern Italy. *Chemosphere* **58,** 131–139 (2005).
3. Dedysh, S. N. *et al.* Isolation of acidophilic methane-oxidizing bacteria from northern peat wetlands. *Science* **282,** 281–284 (1998).
4. Intergovernmental Panel on Climate Change. *Climate Change 2007: The Physical Science Basis. Working Group I Contribution to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change* (eds Solomon S. *et al.*) (Cambridge Univ. Press, Cambridge, 2007).
5. Kvenvolden, K. A. & Rogers, B. W. Gaia's breath—global methane exhalations. *Mar. Petrol. Geol.* **22,** 579–590 (2005).
6. Giggenbach, W. F., Sheppard, D. S., Robinson, B. W., Stewart, M. K. & Lyon, G. L. Geochemical structure and position of the Waiotapu geothermal field, New Zealand. *Geothermics* **23,** 599–644 (1994).
7. Dedysh, S. N. Methanotrophic bacteria of acidic *Sphagnum* peat bogs. *Microbiology (Russia)* **71,** 638–650 (2002).
8. Theisen, A. R. *et al.* Regulation of methane oxidation in the facultative methanotroph *Methylocella silvestris* BL2. *Mol. Microbiol.* **58,** 682–692 (2005).
9. Dumont, M. G. & Murrell, J. C. Community-level analysis: key genes of aerobic methane oxidation. *Methods Enzymol.* **397,** 413–427 (2005).
10. Knief, C., Lipski, A. & Dunfield, P. F. Diversity and activity of methanotrophic bacteria in different upland soils. *Appl. Environ. Microbiol.* **69,** 6703–6714 (2003).
11. Hou, S. *et al.* Genome sequence of the deep-sea γ-proteobacterium *Idiomarina loihiensis* reveals amino acid fermentation as a source of carbon and energy. *Proc. Natl Acad. Sci. USA* **101,** 18036–18041 (2004).
12. Ward, N. *et al.* Genomic insights into methanotrophy: the complete genome sequence of *Methylococcus capsulatus* (Bath). *PLoS Biol.* **2,** E303 (2004).
13. Gilbert, B. *et al.* Molecular analysis of the *pmo* (particulate methane monooxygenase) operons from two type II methanotrophs. *Appl. Environ. Microbiol.* **66,** 966–975 (2000).
14. Ricke, P., Erkel, C., Kube, M., Reinhardt, R. & Liesack, W. Comparative analysis of the conventional and novel *pmo* (particulate methane monooxygenase) operons from *Methylocystis* strain SC2. *Appl. Environ. Microbiol.* **70,** 3055–3063 (2004).
15. Holmes, A. J. *et al.* Characterization of methanotrophic bacterial populations in soils showing atmospheric methane uptake. *Appl. Environ. Microbiol.* **65,** 3312–3318 (1999).
16. Chistoserdova, L., Chen, S. W., Lapidus, A. & Lidstrom, M. L. Methylotrophy in *Methylobacterium extorquens* AM1 from a genomic point of view. *J. Bacteriol.* **185,** 2980–2987 (2003).
17. Chistoserdova, L. *et al.* Genome of *Methylobacillus flagellatus,* molecular basis for obligate methylotrophy, and polyphyletic origin of methylotrophy. *J. Bacteriol.* **189,** 4020–4027 (2007).
18. Kane, S. R. *et al.* Whole-genome analysis of the methyl tert-butyl ether-degrading beta-proteobacterium *Methylibium petroleiphilum* PM1. *J. Bacteriol.* **189,** 1931–1945 (2007).
19. Chistoserdova, L. *et al.* The enigmatic *Planctomycetes* may hold a key to the origins of methanogenesis and methylotrophy. *Mol. Biol. Evol.* **21,** 1234–1241 (2004).
20. Trotsenko, Y. Metabolic features of methane- and methanol-utilizing bacteria. *Acta Biotechnol.* **3,** 269–277 (2004).
21. Kelly, D. P., Anthony, C. & Murrell, J. C. Insights from the complete genome sequence of the obligate methanotroph, *Methylococcus capsulatus. Trends Microbiol.* **13,** 195–198 (2005).
22. Janssen, P. H. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Appl. Environ. Microbiol.* **72,** 1719–1728 (2006).
23. Bryant, D. A. *et al. Candidatus Chloracidobacterium thermophilum*: an aerobic phototrophic *Acidobacterium. Science* **317,** 523–526 (2007).
24. Morris, S. A., Radajewski, S., Willison, T. W. & Murrell, J. C. Identification of the functionally active methanotroph population in a peat soil microcosm by stable-isotope probing. *Appl. Environ. Microbiol.* **68,** 1446–1453 (2002).
25. Lin, J. L. *et al.* Molecular diversity of methanotrophs in Transbaikal soda lake sediments and identification of potentially active populations by stable isotope probing. *Environ. Microbiol.* **6,** 1049–1060 (2004).
26. Dedysh, S. N. *et al. Methylocystis heyerii* sp. nov., a novel type II methanotrophic bacterium possessing the 'signature' fatty acid of type I methanotrophs. *Int. J. Syst. Evol. Microbiol.* **57,** 472–479 (2007).
27. Weatherby, T. M. & Lenz, P. H. Mechanoreceptors in calanoid copepods: Designed for high sensitivity. *Arthropod Struct. Dev.* **29,** 275–288 (2000).
28. Ispolatov, I., Yuryev, A., Mazo, I. & Maslov, S. Binding properties and evolution of homodimers in protein–protein interaction networks. *Nucleic Acids Res.* **33,** 3629–3635 (2005).
29. Schmidt, H. A., Strimmer, K., Vingron, M. & von Haeseler, A. TREE-PUZZLE: maximum likelihood phylogenetic analysis using quartets and parallel computing. *Bioinformatics* **18,** 502–504 (2002).

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

**Acknowledgements** We thank S. Dedysh, J. C. Murrell and J. Euzéby for comments; A. Malahoff for supporting this research programme; and the Tikitere Trust for permission to sample at Hell's Gate. This work was supported in part by the Wairakei Environmental Mitigation Charitable Trust (P.D.). The genome analysis was funded by the US Department of Defense (M.A.).

**Author Contributions** P.F.D., B.W.M., M.A.C. and M.B.S. performed field sampling, methane measurement and molecular 16S rRNA analyses. P.F.D. and M.B.S. isolated and characterized the culture. S.H., B.L., J.H.S., Z.Z., Y.R., J.W., L.F., M.B.S., L.W., W.L. and M.A. conducted genome sequencing. P.F.D., P.S., A.Y., A.V.S., J.S., P.S. and M.A. conducted genome analyses. T.M.W. and M.B.S. performed electron microscopy. P.B. undertook phospholipid fatty-acid analysis.

**Author Information** Gene sequences referenced in this paper are deposited DDBJ/EMBL/GenBank under accession numbers AM900833–AM900834 and EU223838–EU223931. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to P.D. (p.dunfield@gns.cri.nz), M.A. (alam@hawaii.edu) or L.W. (wanglei@nankai.edu.cn).

## METHODS

**Isolation.** The mineral salts medium V41 contained 0.1 g $l^{-1}$ $NH_4Cl$, 0.015 g $l^{-1}$ $KH_2PO_4$, 0.01 g $l^{-1}$ $Na_2HPO_4 \cdot 12H_2O$, 10 mg $l^{-1}$ yeast extract (added to the medium as a 0.2-μm-filter-sterilized solution after autoclaving), 3 ml $l^{-1}$ FeEDTA solution (see below), 3 ml $l^{-1}$ trace elements 1 solution and 1 ml $l^{-1}$ trace elements 2 solution. The medium was adjusted to pH 5.5 and solidified by adding 22 g $l^{-1}$ agar. The FeEDTA solution contained 1.54 g $l^{-1}$ $FeSO_4 \cdot 7H_2O$ and 2.06 g $l^{-1}$ $Na_2EDTA$. Trace elements solution 1 contained 0.44 g $l^{-1}$ $ZnSO_4 \cdot 7H_2O$, 0.20 g $l^{-1}$ $CuSO_4 \cdot 5H_2O$, 0.19 g $l^{-1}$ $MnCl \cdot 4H_2O$, 0.06 g $l^{-1}$ $Na_2MoO_4 \cdot 2H_2O$, 0.10 g $l^{-1}$ $H_3BO_3$ and 0.08 g $l^{-1}$ $CoCl_2 \cdot 6H_2O$. Trace elements solution 2 contained 1.5 g $l^{-1}$ nitrilotriacetic acid, 0.2 g $l^{-1}$ $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.2 g $l^{-1}$ $Na_2SeO_3$, 0.1 g $l^{-1}$ $CoCl_2 \cdot 6H_2O$, 0.1 g $l^{-1}$ $MnSO_4 \cdot 2H_2O$, 0.1 g $l^{-1}$ $Na_2MoO_4 \cdot 2H_2O$, 0.1 g $l^{-1}$ $Na_2WO_4 \cdot 2H_2O$, 0.1 g $l^{-1}$ $ZnSO_4 \cdot 7H_2O$, 0.04 g $l^{-1}$ $AlCl_3 \cdot 6H_2O$, 0.025 g $l^{-1}$ $NiCl_2 \cdot 6H_2O$, 0.01 g $l^{-1}$ $H_3BO_3$ and 0.01 g $l^{-1}$ $CuSO_4 \cdot 5H_2O$ at pH 7.

Soil crumbs were spread on plates and these were placed in sealed jars containing 25% (v/v) $CH_4$ and 8% (v/v) $CO_2$ in air. Jars contained open vials of water to hydrate the air. Plates were incubated at 60 °C and viewed at 2-week intervals for at least 12 weeks. Colonies that formed around soil crumbs were restreaked onto new medium. Optimization of the medium during this process indicated that pH 5.5 was too high for optimal growth, and the following altered medium composition (V42) was made: 0.4 g $l^{-1}$ $NH_4Cl$, 0.05 g $l^{-1}$ $KH_2PO_4$, 0.02 g $l^{-1}$ $MgSO_4 \cdot 7H_2O$ and 0.01 g $l^{-1}$ $CaCl_2 \cdot 6H_2O$, plus yeast extract and trace elements as above, adjusted to pH 4.5 and solidified with 15 g $l^{-1}$ Phytagel plus 1 g $l^{-1}$ $MgCl_2 \cdot 6H_2O$. V42 medium was occasionally supplemented with 25 mg $l^{-1}$ of a filter-sterilized vitamin mixture containing (per 100 mg): 0.8 mg folic acid, 8 mg vitamin $B_1$, 4 mg vitamin $B_2$, 1 mg niacin, 10 mg niacinamide, 15 mg pantothenate, 15 mg pyridoxine, 5 mg cobalamin, 5 mg biotin, 15 mg choline, 15 mg inositol and 7 mg *p*-aminobenzoic acid. The vitamin mixture resulted in no obvious stimulation of growth.

Colonies of the isolate V4 were light brown to copper in colour. Purity was ensured as described previously for methanotrophs[3], by observation of colony growth on plates, by phase-contrast and transmission electron microscopy, by lack of growth on various heterotrophic substrates (see below), and by extraction of DNA, amplification and cloning of the 16S rRNA gene, and restriction-fragment-length polymorphism and sequencing analysis of 20 clones. All sequences were identical. Genome sequencing of 43,008 reactions detected no contaminating sequences (see below).

**Transmission electron microscopy.** Cells were fixed in 4% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in a graded ethanol series, and embedded in epoxy resin as described previously[27]. Sections 80–90 nm thick were cut with a diamond knife on a Reichert Ultracut E ultramicrotome, collected on 200-mesh copper grids and stained with uranyl acetate and lead citrate. Sections were examined with a Zeiss/LEO 912 energy-filtering transmission electron microscope (EFTEM) operating at 100 kV, and images were collected with a Proscan 1k × 1k slow-scan frame-transfer charge-coupled device camera operated by Esivision software from Soft Imaging System. Images were saved as eight-bit uncompressed TIFF files and imported into Photoshop; histogram stretching was performed with the 'levels' setting, as permitted by the recommendations on digital manipulation set by the Microscope Society of America.

**Growth experiments.** For growth curve experiments, liquid medium V42 was adjusted to various pH values with $H_2SO_4$ and NaOH, and divided into serum vials closed gas-tight with rubber or Viton septa. $CO_2$ and $CH_4$ were added with syringes. Incubations were performed at 50 °C. pH, $CH_4$ and $D_{600}$ were monitored at 2-day intervals by the extraction of 2–3-ml culture samples with sterile syringes. Gas samples were removed by syringe for the measurement of methane on a gas chromatograph equipped with a thermal conductivity detector. The experiment shown in Fig. 2 used 500 ml of medium in 1-litre serum vials, adjusted to an initial pH of 1.5. As there was a possibility of diffusion limitation of gaseous $O_2$ and $CH_4$ into the liquid phase when using large volumes as in the above experiment, growth rates over a range of pH values (Fig. 3) were tested using only 30-ml amounts of medium in 100-ml vials. Duplicate vials were adjusted to pH values of 0.5–7.0. One replicate was incubated statically; the other was shaken at 150 r.p.m. on a rotary shaker as a further control on potential diffusion limitation. Shaking resulted in only a small increase (less than 30%) in growth rate, so data from two replicates were combined into a single mean value. The growth rate constant ($k^{-1}$) was estimated from $D_{600}$ values measured at 2-day intervals during the exponential growth phase (generally 1–7 days for rapid growth, 1–13 days for slower growth). Average methane consumption over this period was estimated by linear regression. The pH values given in Fig. 3 are average values measured over the incubation, in which the maximum change was observed between pH 4 and 5 (an average decrease of 0.5 pH unit per vial).

To test heterotrophic growth, liquid medium was supplemented with 0.05% (w/v) acetate, casamino acids, oxalic acid, citrate, malic acid, formate, benzoate, glucose, sucrose, ethanol, methanol, methylamine, trimethylamine, tryptophan, tryptic soy broth, propanol, nutrient broth, elemental sulphur, ascorbic acid, glycerol, xylose, arabinose, galactose, pectin, alginic acid or carboxymethylcellulose, or $H_2$ (10% v/v). When organic acids were added, the pH of the medium was readjusted to a control value (3.5 or 5.5 in different assays). All vials were supplemented with 5% (v/v) $CO_2$. To test the effect of $CO_2$ on growth, cells were grown at pH 3.5 on methanol (2.5 ml $l^{-1}$), and 0, 2% or 10% (v/v) $CO_2$ was added to the headspace of vials.

The proposed name *Methylokorus infernorum* is derived from the Greek *methu* (wine) and *hulê* (wood), the Maori *korus* (a spiral) and the Latin *infernorum* (of infernal regions, because the strain was isolated from a location known as Hell's Gate).

**Soil analyses.** Gas samples were taken from soil by inserting to various depths a stainless steel tube with an outside diameter of 1/8 inch fitted with a Luer-Lok cap, and drawing out soil gas into syringes closed with valves. The gas volume in the tube was cleared before taking each sample. Extraction of DNA was performed with the PowerSoil Kit. Molecular PCR amplification of 16S rRNA and *pmoA* genes, cloning using the TOPO TA Cloning Kit and DNA sequencing were performed as described previously[10,11].

**Genome analyses.** Genome sequencing was performed as described previously[11]. In brief, highly purified genomic DNA was extracted and used for 3-kilobase shotgun library construction. A total of 56 plates (384 plasmid preparations) were sequenced with forward and reverse primers (43,008 total sequencing reactions). Rough draft assembly was performed with the Arachne assembler. The sequence reads were trimmed and vector regions removed with the program Lucy. A custom-written Perl program was used to generate XML files needed to construct ancillary information needed for Arachne. Open reading frames were identified with Glimmer, and BLAST comparisons were made against the entire collection of bacterial protein sequences in the RefSeq database (National Centre for Biotechnology Information), and also separately against individual genomes of *Methylococcus capsulatus* str. Bath and *Nitrosococcus oceani* ATCC 19707, and against methylotrophy genes in *Methylobacterium extorquens*[16]. The orthologous pairs were calculated from BLAST results by first calculating the full-length sequence similarity followed by calculation of best orthologues using a reciprocal best-hit method[28]. The subsequent pathway analysis and protein functional annotation was performed by importing the genome into the bacterial database of Pathway Studio (Ariadne Genomics Inc.). This allowed the automatic transfer of protein annotation and pathway assignment from the annotated orthologues in other bacteria to V4 proteins. The functional analysis was completed with manual annotation and targeted BLAST searches. Membrane topologies were predicted for *pmoCAB* genes by using TMHMM (CBS), TMPred (EMBnet) and TopPred (Pasteur Institute).