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Methylacidiphilum kamchatkense gen. nov., sp. nov., an extremely acidophilic and moderately thermophilic methanotroph belonging to the phylum *Verrucomicrobiota*

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Abstract

The thermo-acidophilic aerobic methanotrophic *Verrucomicrobia* bacterium, designated strain Kam1^T was isolated from an acidic geothermal mud spring in Kamchatka, Russia. Kam1^T is Gram-stain-negative, with non-motile cells and non-spore-forming rods, and a diameter of 0.45–0.65 µm and length of 0.8–1.0 µm. Its growth is optimal at the temperature of 55 °C (range, 37–60 °C) and pH of 2.5 (range, pH 1–6), and its maximal growth rate is ~0.11 h^{-1} (doubling time ~6.3 h). Its cell wall contains peptidoglycan with *meso*-diaminopimelic acid. In addition to growing on methane and methanol, strain Kam1^T grows on acetone and 2-propanol. Phylogenetically, it forms a distinct group together with other *Methylacidiphilum* strains and with the candidate genus Methylacidimicrobium as a sister group. These findings support the classification of the strain Kam1^T as a representative of a novel species and genus of the phylum *Verrucomicrobiota*. For this strain, we propose the name *Methylacidiphilum kamchatkense* sp. nov. as the type species within *Methylacidiphilum* gen. nov. Strain Kam1^T (JCM 30608^T=KCTC 4682^T) is the type strain.

The phylum *Verrucomicrobiota* Hedlund 2021 [1, 2] currently contains three classes with validly published names, namely *Optituae* [3], *Terrimicrobiia* [4] and *Verrucomicrobiae* [1], and four validated orders, namely *Opitutales* [3], *Puniceicoccales* [3], *Terrimicrobiales* [4] and *Verrucomicrobiales* [5]. In addition, a number of *incertae sedis* taxa not formally assigned to any class have been described (https://lpsn.dsmz.de/; accessed 18 June 2023) [6] including the candidate order Methylacidiphilales [7]. *Candidatus* Methylacidiphilales Op den Camp *et al.* 2009 encompasses thermoacidophilic methanotrophs thriving at extremely acidic conditions (growth at pH as low as 0.8) and elevated temperatures, which have been recovered from geothermal environments worldwide and given the genus name *Candidatus* Methylacidiphilum [7–11]. The discovery of these micro-organisms has broadened our understanding of the taxonomic diversity and evolution of methanotrophs, as all previously described methane-oxidizing bacteria belonged to the phylum *Pseudomonadota* [7]. They are phylogenetically similar and share a common cellular morphology (short rods), lack intracellular membrane systems, and form extensive intracellular glycogen storage vesicles under various growth conditions [7, 12, 13]. Carbon is assimilated through the Calvin–Benson–Bassham cycle following the oxidation of methane to carbon dioxide in a process that has been termed 'autotrophic methanotrophy' [14], in contrast to assimilation from formaldehyde through either the serine or ribulose monophosphate pathways in methanotrophic *Pseudomonadota* [15]. These micro-organisms also share the presence of three phylogenetically distinct *pmoCAB* gene clusters which encode three different particulate monooxygenase enzymes (pMMO; EC 1.14.18.3) [7, 16]. No genes encoding the soluble methane monooxygenase (sMMO; EC 1.14.13.25) have been found, but complete sets of genes for the dinitrogen fixation pathway and the tricarboxylic acid cycle are present. The *pmoCAB1* and *pmoCAB2* clusters are organized in tandem, whereas *pmoCAB3* are located elsewhere in the genome. The *pmoCAB1-pmoCAB2* tandem cluster and *pmoCAB3* are each linked to a single *pmoD* gene, which has been speculated to have a role as a copper chaperone [17–19]. The *pmoCAB1-pmoCAB2 clusters are* differentially regulated, with *pmoCAB2* being highly expressed under high oxygen partial pressure, i.e. in atmospheric air and *pmoCAB1* being highly expressed under low oxygen conditions [12, 16, 20].

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Abbreviations: ANI, average nucleotide identity; dDDH, digital DNA-DNA hybridization; DPM, 2,6-diaminopimelic acid; GBDP, genome blast distance phylogeny; MAG, metagenome-assembled genome; pMMO, particulate methane monooxygenase; sMMO, soluble methane monooxygenase; TYGS, type strain genome server.
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They can also use methanol as their energy and carbon source [21], which is oxidized by the lanthanide-dependent XoxF methanol dehydrogenase (EC 1.1.2.10) [22].

Recently, the *pmoCAB3* cluster was shown to be strongly expressed during oxidation of the C3 substrates propan-2-ol, acetone and hydroxyacetone, and was suggested to encode an acetone monooxygenase with substrate specificity for short-chain ketones [23]. Likewise, *Methylacidiphilum* members have recently been demonstrated to be able to oxidize and grow on ethane and propane [24]. While the sequences of *pmoCAB1-2* indicate evolution from a common verrucomicrobial ancestor, *pmoCAB3* appears to have been obtained horizontally from a distantly related bacterium [8, 16]. Furthermore, growth as 'knallgas' bacteria with hydrogen and carbon dioxide as the sole energy and carbon sources, respectively, has also been demonstrated for *Candidatus* Methylacidiphilum fumariolicum SolV [25, 26], as well as the capability of *Candidatus* Methylacidiphilum infernorum strain RTK17.1 to grow on formate [27]. All verrucomicrobial methanotrophs contain genes encoding methanthiol oxidase (MTO; EC 1.8.3.4) and sulphide:quinone oxidoreductase (SQR; EC 1.8.5.4), indicating a capability to oxidize sulphur compounds, which are common compounds emitted in geothermal systems [21]. Oxidation of methanethiol and sulfide has been demonstrated for strain SolV [28]. Verrucomicrobial methanotrophs seem thus to be much more metabolically versatile than their classical proteobacterial counterparts.

To date, three candidate species have been tentatively assigned to the genus *Methylacidiphilum*, namely *Methylacidiphilum kamchatkense* Kam1^T, *Candidatus* M. fumariolicum SolV and *Candidatus* M. infernorum V4 and RTK17.1, isolated from extremely acidic geothermal environments in Uzon Caldera, Kamchatka, Russia [10], a mudpot in the Solfatara volcano in Italy [11] and steaming soil at Tikitere, New Zealand [8], respectively. In addition, two tentative novel species, termed *Candidatus* Methylacidiphilum sp. Yel and Phi, were recovered from the Norris Geyser Basin in Yellowstone National Park, USA, and the Makiling Mud Spring in the Philippines, respectively [9]. Two novel strains, termed IT5 and IT6, which were recently recovered from the Pisciarelli Hot Spring in Pozzuoli, Italy and used to demonstrate the growth on C3 substrates mentioned above, were phylogenetically affiliated with *Candidatus* M. infernorum V4 and *Candidatus* M. fumariolicum strain Phi, respectively [23]. Growth of *Methylacidiphilum* strains has been recorded from pH 0.8 to 6.0 and from 37 to 65 °C, with optima at pH 2–3 and 55–60 °C [7, 21].

Mesophilic representatives of the related candidate genus Methylacidimicrobium with a lower growth optimum (35–50 °C) were isolated in 2014 from geothermal environments in Italy [29] and New Zealand [30]. These organisms possess only one *pmoCAB* cluster and phylogenetically belong to a well-separated taxonomic cluster [29]. A thermophilic member of the same cluster, *Candidatus* Methylacidimicrobium thermophilum, with a growth optimum at 50 °C and possessing two *pmoCAB* gene clusters was also recently described [31]. Metagenomic analyses of DNA extracted from acidic volcanic soil in Italy have indicated that methanotrophic *Verrucomicrobia* are even more diverse than previously thought, exemplified by the metagenome-assembled genome of *Candidatus* Methylacidithermus pantelleriae strain PQ17, reported to form an intermediate phylogenetic lineage between *Methylacidiphilum* and *Candidatus* Methylacidimicrobium [32].

Strain Kam1^T was initially reported to have a doubling time of 38 h [10]. This was, however, before lanthanides were found to be necessary for the functioning of Xox-F type methanol dehydrogenase in these methanotrophs, thus being necessary for optimal growth conditions [22]. To determine the growth rate and pH dependency of Kam1^T under optimal conditions, it was grown in a mineral medium (P14) supplemented with 550 nM Ce(III) at 55 °C with shaking at 150 rpm, as described previously [22]. Triplicate batch cultures were prepared with pH adjusted with sulphuric acid to values ranging from pH 0.5 to 6.5 at increments of 0.5 pH units. A culture with pH 0.8 was also included. The head space contained atmospheric air supplemented with CH_4 and CO_2 to initial concentrations of 20 and 2% CO_2, respectively. Bacterial growth was measured as OD_{600} at 12 h intervals. The greatest growth rate was observed at pH 2.5 (μ=0.11 h^{-1}; g=6.3 h). Growth was observed from pH 1.0 to 6.0, whereas no growth was observed at pH ≤0.8 and >6.0. The cell wall peptidoglycan of strain Kam1^T contained *meso*-diaminopimelic acid, as determined at the DSMZ-German Collection of Microorganisms and Cell Cultures Gmbh by thin-layer chromatography of purified peptidoglycan and GC-MS chromatographic identification of the Dpm derivative *N*-heptafluorobutyryl Dpm isobutylester fragment-ions in whole-cell hydrolysates, as described previously [33, 34]. The peptidoglycan most likely belongs to type A1γ.

M. kamchatkense Kam1^T and the *Candidatus* Methylacidiphilum species strains share main phenotypic characteristics as outlined above, most likely including the ability to oxidize C3 compounds like propan-2-ol, acetone and hydroxyacetone as all sequenced genomes possess the *pmoCAB3* gene cluster. Growth with propan-2-ol and acetone as the sole carbon and energy source has been confirmed for strain Kam1^T (Birkeland, unpublished). Ability to grow on hydrogen and CO_2 seems to be variable within this group and has only been shown for strain SolV. Growth on formate has so far only been demonstrated for *Candidatus* M. infernorum [12]. Likewise, capability to grow on alternative hydrocarbons, like ethane and propane has only been shown for *Candidatus* M. fumariolicum strain SolV [24]. Trials to grow strain Kam1^T on hydrogen, formate, ethane or propane were unsuccessful. Comparison of the 16S rRNA gene sequences, shows that the *Methylacidiphilum* strains share from 97.2 (strain IT5 versus Yel) to 99.6% (Kam1^T versus SolV) pairwise sequence identity (Fig. 1) and are thus above the generally accepted bacterial species demarcation value of 97%. A phylogenetic tree encompassing all the methanotrophic *Verrucomicrobia* candidate taxa including two metagenome-assembled genomes (MAGs) was reconstructed (Fig. 2). The tree firmly placed *Methylacidiphilum* and *Candidatus* Methylacidimicrobium as sister groups with *Candidatus* Methylacidithermus pantelleriae PQ17 (an MAG)

	Kam1	SolV	Yel	Phi	V4	IT5	LP2A	3B	3C	4AC	AP8	PQ17
	1	2	3	4	5	6	7	8	9	10	11	12
Methylacidiphilum kamchatkense Kam1^T (EF127896)	1	100.00	6	21	23	33	33	157	160	162	174	166	187
Ca. Methylacidiphilum fumariolicum SolV (EF591088)	2	99.61	100.00	21	22	36	36	155	161	163	173	169	190
Ca. Methylacidiphilum sp. Yel (LXQB00000000)	3	98.63	98.63	100.00	35	41	43	171	172	174	167	176	192
Ca. Methylacidiphilum sp. Phi (LXQC00000000)	4	98.50	98.56	97.72	100.00	28	26	149	161	156	165	166	182
Ca. Methylacidiphilum infernorum V4 (CP000975.1)	5	97.84	97.65	97.33	98.17	100.00	16	160	157	158	170	153	170
Ca. Methylacidiphilum sp IT5 (CP065956)	6	97.84	97.65	97.20	98.30	98.95	100.00	156	153	153	171	161	176
Ca. Methylacidimicrobium sp. LP2A (AM900834)	7	89.79	89.92	88.93	90.31	89.59	89.85	100.00	35	35	60	68	187
Ca. Methylacidimicrobium cyclopophantes 3B (NR_126315)	8	89.58	89.53	88.85	89.53	89.75	90.01	97.72	100.00	36	65	57	186
Ca. Methylacidimicrobium fagopyrum 3C (NR_126313)	9	89.45	89.39	88.72	89.85	89.68	90.01	97.72	97.65	100.00	61	67	179
Ca. Methylacidimicrobium tartarophylax 4AC (NR_126314)	10	88.78	88.85	89.21	89.37	89.00	88.96	96.13	95.80	96.06	100.00	94	198
Ca. Methylacidimicrobium thermophilum AP8 (LR797830)	11	89.19	89.00	88.58	89.20	89.96	89.48	95.58	96.28	95.62	93.92	100.00	164
Ca. Methylacidithermus pantelleriae PQ17 (CAJNOB00000000)	12	87.88	87.70	87.60	88.22	88.90	88.56	87.90	87.91	88.37	87.26	89.26	100.00

Fig. 1. Pairwise comparison of the 16S rRNA gene sequences of *Methylacidiphilum kamchatkense* Kam1^T and other methanotrophic *Verrucomicrobia* strains. The upper right triangle indicates the number of nucleotide mismatches and the lower left triangle the pairwise sequence identities (%). The matrix was made using the CLC Genomics Workbench, version 20.0.4. Sequence accession numbers are provided in brackets.

between the two. However, based on whole genome comparisons, the ANI values between Kam1^T and its most highly related strains, SolV, Rib, Fur, Fdl and Yel are in the range 92.49–92.85% and thus well below the genome-based species cut-off value of 95%. This is in accordance with the Genome Taxonomy Database (https://gtdb.ecogenomic.org/; accessed 18 June 2023) [35] which classifies these organisms into the three previously suggested species, *Candidatus* M. fumariolicum, *Candidatus* M. infernorum and *M. kamchatkense*. A whole proteome-based analysis using the TYGS server (https://tygs.dsmz.de; accessed 18 June 2023) [36] confirms a separate species cluster for *M. kamchatkense* with a 100% pseudo-bootstrap support value (Fig. 3). A maximum digital DNA–DNA hybridization (dDDH) value of 50.4% confirms classification of strain Kam1^T as representing a separate genomic species.

The candidate genus, *Candidatus* Methylacidimicrobium differs from *Methylacidiphilum* in several aspects, e.g. possessing only one or two *pmoCAB* gene clusters, lower temperature and pH optima, and a significantly higher G+C content (Table 1). The phylogenetic and whole-proteome analyses (Figs 2 and 3) support placement of these organisms in a separate genus. *Candidatus* Methylacidithermus pantelleriae represents an additional candidate genus as it forms a separate lineage between the other two and differs also in possessing only a single *pmoCAB* cluster and lack of nitrogen fixation genes (Fig. 2; Table 1).

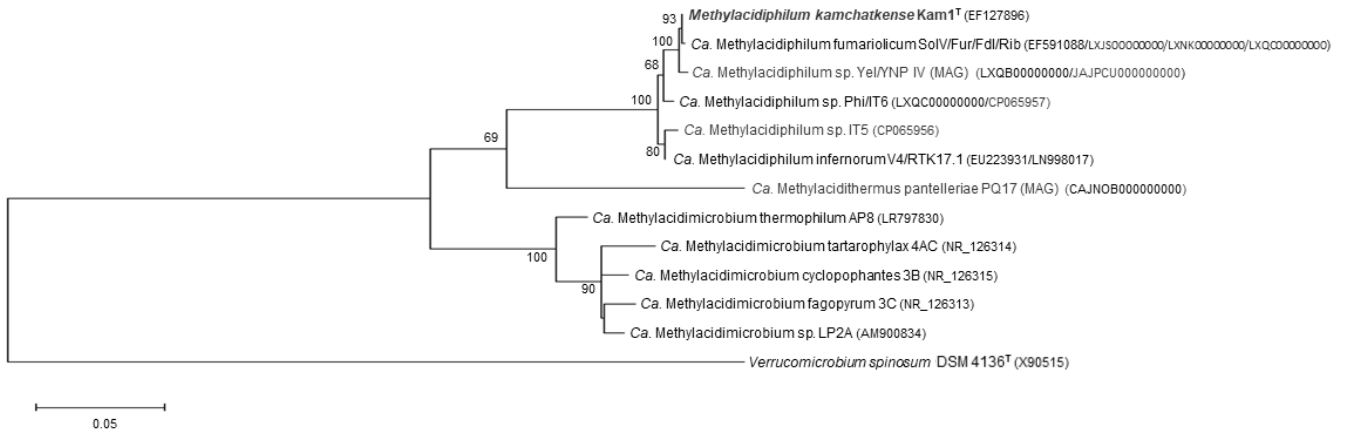

Fig. 2. Phylogenetic tree of methanotrophic *Verrucomicrobia* inferred from 16S rRNA gene sequences using the maximum-likelihood method based on the Tamura–Nei model [37]. The 16S rRNA gene sequence of *Verrucomicrobium spinosum* DSM 4136^T was used as outgroup. The tree with the highest log likelihood (−4648.84) is shown. The percentage of trees in which the associated taxa clustered together using 500 bootstrap replications is shown next to the branches [38]. Initial trees for the heuristic search were obtained automatically by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (five categories (+*G*, parameter=0.3744)). The analysis involved 13 nucleotide sequences. All positions with less than 95% site coverage were eliminated. There were a total of 1491 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [39]. Accession numbers are provided in brackets. Sequences from metagenome-assembled genomes are indicated by 'MAG'.

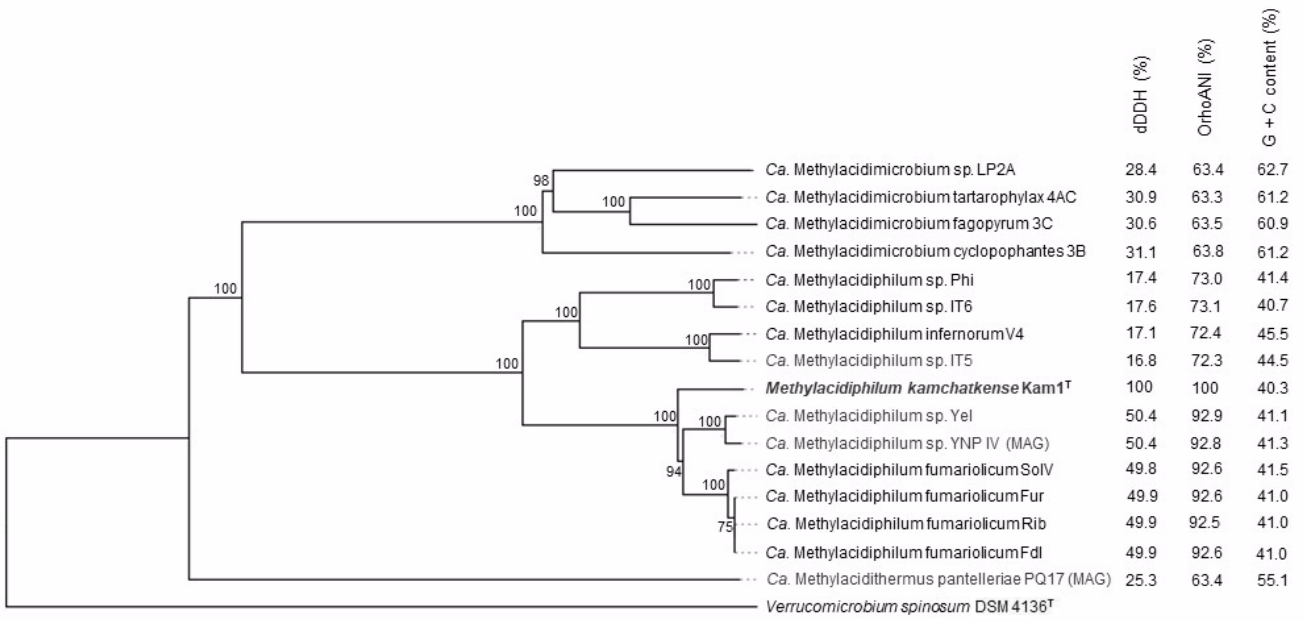

Fig. 3. Whole-proteome-based tree of methanotrophic *Verrucomicrobia* inferred with FastME 2.1.6.1 [40] from Genome Blast Distance Phylogeny (GBDP) distances calculated from genome sequences using the TYGS server (https://tygs.dsmz.de) [36]. Branch lengths are scaled via GBDP distance formula d5. Branch values are GBDP pseudo-bootstrap support values>60% from 100 replications, with an average branch support of 93.4%. The tree was midpoint-rooted [41]. Pairwise dDDH (d4) and ANI values are indicated as compared with those of strain Kam1^T and the genomic G+C content is shown on the right. *Verrucomicrobium spinosum* DSM4136^T was used as outgroup. The genome sequence accession numbers are as follows: *Ca*. Methylacidimicrobium sp. LP2A, JAFS00000000.1; *Ca*. M. tartarophylax 4AC, GCF_902143375.2; *Ca*. M fagopyrum 3C, GCF_000379365.1; *Ca*. M. cyclopophantes 3B, NZ_CABFUZ000000000.2; *Ca*. Methylacidiphilum sp. Phi, GCA_004421255.1; *Ca*.Methylacidiphilum sp. IT6, GCA_017310505.1; *Ca*. M. infernorum V4, CP000975.1; *Ca*. Methylacidiphilum sp. IT5, GCA_017310525.1, *M. kamchatkense* Kam1^T, CP037899.1, *Ca*. Methylacidiphilum sp. Yel; GCA_004421185.1, *Ca*. Methylacidiphilum sp. YNP IV, GCA_021323575.1; *Ca*. M. fumariolicum SolV, GCA_000953475.1; *Ca*. M. fumariolicum Fur, LXJS00000000; *Ca*. M. fumariolicum Rib, LXNK00000000; *Ca*. M. fumariolicum Fdl, LXNL00000000; *Ca*. M. pantelleriae PQ17, GCF_905250085.1; *V. spinosum* DSM4136^T, GCA_000172155.1.

Table 1. Differential characteristics of *Methylacidiphilum* and related candidate genera of methanotrophic *Verrucomicrobia*

+, Positive; –, negative; ND, no data.

Characteristic	*Methylacidiphilum*	*Ca.* Methylacidimicrobium*	*Ca.* Methylacidithermus† (MAG)
Cell morphology	Rods (0.3–0.65×0.8–4 µm)	Rods (0.6–0.9×1.2–1.4 µm)	ND
pmoCAB clusters†	2–3	1–2	1
Optimal temperature (maximum) for growth (°C)	55–60 (65)	35–50 (55)	ND
Optimal pH for growth (lowest)	2–3 (0.8)	1–3 (0.5)	ND
Maximal growth rate	~0.11 h^{-1} (Kam1)	~0.05 h^{-1} (strain AP8)	ND
nifHDK genes	+	+	–
Genome size (Mb)	2.2–2.5	2.3–2.8	2.5
G+C content (mol%)	40.3–45.5	60.9–64.3	55.2

*Data compiled from [7–11, 19, 21, 32] except maximal growth rate of strain Kam1^T (this work).
†Data from [29].

Based on the phylogenetic, whole-proteome and phenotypic comparisons we consider Kam1^T to represent a distinctive and novel genus and a novel species within the phylum *Verrucomicrobia*.

DESCRIPTION OF *METHYLACIDIPHILUM* GEN. NOV.

Methylacidiphilum (Me.thyl.a.ci.di'phi.lum. N.L. neut. n. *methyl*-, the methyl group; L. masc. adj. *acidus*, sour; Gr. neut. adj. *philon*, someone beloved, someone dearly loved; N.L. neut. adj. *philum*, dearly loved; N.L. neut. n. *Methylacidiphilum*, a methyl-using acid-lover.).

The cells are rod-shaped, 0.4–0.65 µm wide and 0.8–4.0 µm long, Gram-stain-negative. No motility or resting stages have been observed. Grows at 37–65 °C, with optimal growth at 55–60 °C, and at pH pH 0.8–6.0, with optimal growth at pH 2–3. Grows preferentially with methane or methanol with O_2 as electron acceptor. Aerobic to microaerophilic. The genome encodes only the lanthanide-dependent XoxF type methanol dehydrogenase. Some strains grow chemolithoautotrophically with H_2, O_2 and CO_2, as well as heterotrophically with formate, propan-2-ol, acetone or hydroxyacetone. No growth on sugars or in complex organic media has been observed. Oxidizes methane using particulate methane monooxygenase (pMMO). Does not encode sMMO. Two or three complete *pmoCAB* operons encoding pMMO are present in the genome. Intracellular pMMO-embedded membrane systems typical for *Pseudomonadata* methanotrophs are absent. The genome encodes a complete Calvin–Benson–Bassham cycle for CO_2 fixation. Genes for fixation of dinitrogen are present. Complete tricarboxylic acid cycle present. The major cellular fatty acids are: 18:0 (14–39%), a15:0 (13–31%), i14:0 (7–22%), 16:0 (0.3–14%) and 14:0 (7–13%). The G+C content ranges from 40.3 to 45.5 mol%. Phylogenetically belongs to phylum *Verrucomicrobiota*. Inhabits acidic geothermal habitats.

The type species is *Methylacidiphilum kamchatkense*.

DESCRIPTION OF *METHYLACIDIPHILUM KAMCHATKENSE* SP. NOV.

Methylacidiphilum kamchatkense (kam.chat.ken'se. N.L. neut. adj. *kamchatkense*, pertaining to Kamchatka, from where the organism was isolated).

As for the genus description but grows at 37–60°C and at pH 1.0–6.0, with optima at 55 °C and pH 2.5. Can grow with isopropan-2-ol or acetone in addition to methane and methanol. Fixes dinitrogen. Contains peptidoglycan with meso-diaminopimelic acid. Major cellular fatty acids are: 14:0 (7.0%), i14:0 (22.4%), a15:0 (31.9%), 16:0 (9.9%) and 18:0 (13.7%). The type strain is Kam1^T (=JCM 30608^T=KCTC 4682^T), isolated from an acidic mud spring in Uzon Caldera, Kamchatka, Russia. The DNA G+C content is 40.3 mol%. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and the complete genome of strain Kam1^T are EF127896.1 and CP037899.1, respectively.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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