Expanding the Verrucomicrobial Methanotrophic World: Description of Three Novel Species of *Methylacidimicrobium* gen. nov.

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Methanotrophic *Verrucomicrobia* have been found in geothermal environments characterized by high temperatures and low pH values. However, it has recently been hypothesized that methanotrophic *Verrucomicrobia* could be present under a broader range of environmental conditions. Here we describe the isolation and characterization of three new species of mesophilic acidophilic verrucomicrobial methanotrophs from a volcanic soil in Italy. The three new species showed 97% to 98% 16S rRNA gene identity to each other but were related only distantly (89% to 90% on the 16S rRNA level) to the thermophilic genus *Methylacidiphilum*. We propose the new genus *Methylacidimicrobium*, including the novel species *Methylacidimicrobium fagopyrum*, *Methylacidimicrobium tartarophylax*, and *Methylacidimicrobium cyclopophantes*. These mesophilic *Methylacidimicrobium* spp. were more acid tolerant than their thermophilic relatives; the most tolerant species, *M. tartarophylax*, still grew at pH 0.5. The variation in growth temperature optima (35 to 44°C) and maximum growth rates (μmax; 0.013 to 0.040 h^{-1}) suggested that all species were adapted to a specific niche within the geothermal environment. All three species grew autotrophically using the Calvin cycle. The cells of all species contained glycogen particles and electron-dense particles in their cytoplasm as visualized by electron microscopy. In addition, the cells of one of the species (*M. fagopyrum*) contained intracytoplasmic membrane stacks. The discovery of these three new species and their growth characteristics expands the known diversity of verrucomicrobial methanotrophs and shows that they are present in many more ecosystems than previously assumed.

Methane oxidation is a microbial process which limits the amount of methane released to the atmosphere (1). Microbial methane oxidation can occur via different routes, including either anaerobically (2, 3, 4) or aerobically (5, 6, 7). The diversity of the biochemistry of methane oxidation is also reflected in the diversity of organisms performing these processes. For a long time, *Alpha-* and *Gammaproteobacteria* were considered to be the only organisms to perform aerobic methane oxidation. However, in 2007, methane-oxidizing *Verrucomicrobia* were discovered (genus *Methylacidiphilum* [8, 9, 10]); also, intra-aerobic bacteria belonging to the NC10 candidate phylum (3, 11) were recently described to oxidize methane using internally produced oxygen. Like most other aerobic methane oxidizers, the *Verrucomicrobia* use particulate methane monooxygenase (pMMO) to catalyze the first step of the methane oxidation. Unlike most proteobacterial methanotrophs (7), but like *Methylomirabilis oxyfera* (belonging to the NC10 phylum) (12), *M. fumariolicum* SolV (13) and *M. infernorum* V4 (14) grow as autotrophs, using only carbon dioxide as the carbon source via the Calvin cycle.

All verrucomicrobial methanotrophs known to date have been enriched from geothermal environments (7, 15, 16), and 16S rRNA gene surveys indicated their presence to be mainly limited to such environments (16). The three thermophilic strains belonging to the genus *Methylacidiphilum* share low pH optima (2 to 3.5) and high temperature optima (55 to 60°C) (7). In addition, 16S rRNA gene sequences from different geothermal sites showed identities of only 95% to 99% to the 16S rRNA gene sequence of *M. fumariolicum* SolV (8, 14, 16). This indicated that a larger diversity in verrucomicrobial methanotrophs might exist in geothermal environments. Geothermal environments are often characterized not only by extreme conditions but also by local and temporal fluctuations in pH, temperature, and methane concentrations. The flux and composition of geothermal gasses, includ-ing methane and hydrogen sulfide, differ considerably over time (17). The hot gasses escape directly as fumaroles or seep through soil layers, via routes that may change continuously. Hydrogen sulfide is oxidized by microbes into sulfuric acid, leading to very low pH values. It is likely that the pH is also influenced by rainfall.

During the isolation of the first (thermophilic) verrucomicrobial methanotrophs (8, 9), mesophilic isolates were also encountered. A mesophilic verrucomicrobial enrichment culture was obtained during the isolation of *M. infernorum* V4 (9). Recently, strain LP2A was isolated in pure culture from this mesophilic enrichment culture (16). Strain LP2A has a growth optimum temperature of 30°C, and its 16S rRNA gene sequence identity to the *Methylacidiphilum* genus is only 89.6%. In our search for methane-oxidizing species that are more acid tolerant and mesophilic, we started enrichment cultures from volcanic soil. Here we describe the isolation, physiology, phylogeny, and morphology of three new highly acid-tolerant, methanotrophic mesophilic verrucomicrobial strains.

MATERIALS AND METHODS

Enrichment and isolation. Soil samples were taken from various spots at the Solfatara crater, which is at the center of the Campi Flegrei caldera, near Naples (Italy). Spots were chosen at least 20 m away from the hot area which surrounds the central mud pot. Their temperatures as judged manually were well below 50°C, and pH values ranged between 1 and 1.5. pH values were measured at the spots with indicator strips and confirmed by slurry measurements (about 0.3 ml g^{-1} soil). The rather dry top layer of about 0.5 to 1 cm in thickness was first removed, and samples of the humid layer just below were sampled into 50-ml sterile plastic tubes. The tubes were closed, and within 10 h, 15 ml of samples was mixed with 15 ml of store-bought mineral water. The mixtures were shaken by hand for 2 min in order to extract microorganisms from the soil. After the mixtures were allowed to settle for a few minutes, 1 ml from the liquid phase was transferred to a dilution series at 1 ml each time into 19 ml of medium in 60-ml bottles. The growth medium (8) was supplemented with 20% filtered (using a 0.22-μm-pore-size filter) liquid from the central mud pot and adjusted to pH 2.5 (using H$_2$SO$_4$). Bottles had a gas phase of 5% carbon dioxide and 10% methane in air and were incubated at 29°C with slow shaking (100 rpm) for 4 months. Bottles that showed growth were transferred three times and subsequently serially diluted (up to 10^{-8}) to extinction. Serial dilutions were incubated for growth or immediately transferred onto floating membrane filters and incubated at 30°C as described previously (8). Single colonies that appeared on the membranes were transferred to liquid medium. The composition of the enrichments was investigated by light microscopy and fluorescence *in situ* hybridization (FISH; EubIII). The purity of the strains was investigated via 16S rRNA gene sequence analysis.

Growth. Temperature and pH optima for the different strains were determined on the basis of growth rates in medium as described before (18). The growth rate was determined by following the optical density at 600 nm (OD$_{600}$) of batch cultures under conditions of excess (>2%) methane and fast (350-rpm) shaking.

The pH range at which growth occurs was tested by gradually changing the pH when cultures were transferred (steps of 1 pH unit between pH 2 and pH 6; below pH 1, steps were only 0.1 pH unit). For pH 3 and below, the medium pH was adjusted by addition of sulfuric acid, which also acted as a buffer. For pH 3 and above, 50 mM MES (morpholineethanesulfonic acid) was included as a buffer (adjusted by addition of NaOH), which did not influence the growth rates. After growth, only pH values above 3 were different from the starting value but never differed by more than 0.1 pH unit. The ability of the strains to grow on methanol was tested by adding 10 mM methanol to the growth media in the absence of methane.

The ability of the strains to grow autotrophically was tested by incubation with labeled methane. ^{13}C-labeled CH$_4$ experiments were done in batch cultures (in duplicate for each of the three strains) as described previously (13), using 150-ml serum bottles containing 10 ml of culture medium and 40% of CO$_2$ in air. ^{13}C-labeled CH$_4$ (Sigma-Aldrich) (99 atom% ^{13}C; 3 to 5.5 ml) was added. Initial and final gas concentrations and amounts as well as mass ratios for CO$_2$ were verified by gas chromatography-mass spectrometry (GC-MS) analysis at the start and after growth (when at least 90% of the added CH$_4$ had been consumed) in order to calculate the recovery of ^{13}C from CH$_4$ in CO$_2$. At the end of the experiment, the ^{13}C/^{12}C ratio of the biomass was determined by isotope ratio mass spectrometry (IRMS) as described before (13).

Phylogenetic and genomic analysis. The draft genomes of strains 3B and 4AC were assembled from Illumina sequencing runs using CLCBio software with standard settings. This resulted in 604 and 314 contigs for strains 3B and 4AC, respectively. The contigs were submitted to the RAST server for annotation (19). The draft genome for strain 3C was assembled and annotated as part of a JGI Bioproject (PRJNA199166). The draft genomes of the three strains and the published genome of *M. fumariolicum* SolV (8, 20) were used to obtain scores corresponding to average nucleotide identity using BLAST [ANI(b)] and JSpecies with standard settings (21). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (22). The 16S rRNA gene sequences used for the analysis were full length. The 16S rRNA gene similarity percentage analysis was performed via the Jukes-Cantor model (23).

Morphological investigation by EM using different sample preparation techniques. High-pressure freezing, freeze-substitution, Epon embedding, and sectioning were performed as described previously (24). For high-pressure freezing, cells were taken at one time point. Freeze-substitution was performed in acetone containing 2% osmium tetroxide, 0.2% uranyl acetate, and 1% H$_2$O. Respectively, 66, 180, 88, and 173 images containing 1 to 50 typical cells were obtained by transmission electron microscopy (TEM) for *M. fumariolicum* SolV and strains 3B, 3C, and 4AC.

For freeze-etching (FE), cryo-scanning electron microscopy (cryo-SEM), and negative staining, cells were taken from batch cultures at two time points. Freeze-etching on the three mesophilic strains of verrucomicrobial methanotrophs and on *M. fumariolicum* SolV was performed as described previously (25). Respectively, 73, 60, 79, and 66 typical images containing 1 to 4 freeze-etched cells per image were obtained using a CM12 transmission electron microscope (TEM) (FEI, Eindhoven, the Netherlands) for *M. fumariolicum* SolV and strains 3B, 3C, and 4AC.

Negative staining was performed by applying a cell suspension (concentrated by centrifugation for 4 min at 12,000 × *g*) on a (Formvar) carbon-coated copper grid. The grid was then immediately washed in two drops of MilliQ and subsequently incubated for 15 to 20 s on 2% uranyl acetate in MilliQ. The cells were then investigated by the use of a 1010 TEM (Jeol, Tokyo, Japan). Per strain, 24 electron-dense (ED) particles were examined, accounting for 27 cells for strains 3B and 4AC, 20 cells for strain 3C, and 24 cells for *M. fumariolicum* SolV.

CryoSEM was performed on plunge-frozen cells from the four strains as described previously (25) using a 6301F EDS FESEM instrument (Jeol, Tokyo, Japan). A total of 100 typical nondividing cells of each strain were analyzed. The length and width at the broadest part of the cell were measured for each cell.

A polysaccharide stain was performed to investigate if the electron-light particles identified in the three mesophilic strains were polysaccharide inclusions, as described for *M. fumariolicum* SolV (26). The stain and matching negative-control procedures were performed as described previously (24) on ultrathin sections of high-pressure-frozen cells (freeze-substituted in acetone containing 2% osmium tetroxide, 0.2% uranyl acetate, and 1% H$_2$O) of the mesophilic strains and visualized using a TEM 1010 instrument (Jeol, Tokyo, Japan). In this method, electron-dense silver albumin aggregates indicate the presence of polysaccharide molecules.

Energy-dispersive X-ray (EDX) analysis was performed on all four strains to investigate the contents of the electron-dense particles. For this purpose, cells were high-pressure frozen, freeze-substituted in 2% osmium tetroxide, and sectioned as described previously (24). The ultrathin sections (ca. 60 nm thick) were visualized (without poststaining) in STEM mode in a JEM 2100 instrument (Jeol, Tokyo, Japan). Qualitative maps were made in which an enrichment of certain elements in specific locations of the cell could be investigated. Maps were acquired after 600 s of measurement time using the Bruker Quantax 200 esprit 1.9.4 software package. For each strain, 10 cells were analyzed in this fashion.

Deposition of cultures. Preservation of the strains in any other state than as living cultures proved difficult. We are currently testing methods to generate viable frozen stocks. In the meantime, live cultures are available from us by request.

Nucleotide sequence accession numbers. The genome data of strain 3C have been deposited under BioProject PRJNA165235 and those for strains 3B and 4AC under BioProject PRJNA255456. The *pmoA* and 16S rRNA sequences can be found in GenBank under the following numbers: KM210549 (3C_pmoA1), KM210550 (3B_pmoA1), KM210551 (3B_pmoA2), KM210552 (4AC_pmoA1), KM210553 (3C_16S), KM210554 (4AC_16S), and KM210555 (3B_16S).

RESULTS AND DISCUSSION

Enrichment and isolation. Samples from the bare soil area of the Solfatara were taken at spots at least 20 m away from hot fumaroles or the central mud pot. The temperatures of these soils range from 25 to 40°C ([27](#)). Soil samples were extracted with water, serially diluted in medium, and incubated with methane for over 4 months. Four of eight soil sample enrichments showed methane consumption and growth at up to a 10^4 dilution. Lack of growth might be due to the sudden change in acidity that occurred when soils (pH 1) were extracted with water and diluted into medium (pH 2.5). Such pH shocks were found later to prevent the onset of growth of isolates from the enrichments. The positive enrichments contained almost exclusively verrucomicrobia, as judged by FISH microscopy using verrucomicrobium-specific probe EubIII ([28](#)) (data not shown). The positive 10^1 and 10^4 dilutions were chosen for further cultivation and isolation. After three transfers into new liquid medium, serial dilutions of the final cultures were transferred to floating membrane filters. After 4 weeks at 30°C, the highest dilutions (10^7) showed isolated colonies that were either small, yellowish, and shiny or larger and off-white. Different colony types were picked and purified by streaking on floating filters. Finally, this resulted in the isolation of strain 4AC (from the 10^1 dilution) and strains 3C and 3B (both from the 10^4 dilution). We observed that strain 4AC was sensitive to oxygen and showed a growth rate at an oxygen concentration of 5% that was 2-fold higher than that seen at the ambient oxygen concentration. Especially when shaken at an ambient oxygen concentration, cultures of strain 4AC appeared to have a long lag phase or even failed to grow. This may have prohibited the appearance of strain 4AC in higher serial dilutions. Strains 4AC and 3B consisted of rod-shaped bacteria as investigated with light microscopy. Cells of strain 3C were bigger and morphologically less homogenous than those of strains 4AC and 3B. Both long and short rod-shaped cells were observed as well as cells with a shape reminiscent of buckweed seeds, some of which were connected to each other at distances of up to a few micrometers. For all three isolates, only one rRNA operon was obtained upon genome sequencing (see below) and the isolates were considered to be a pure culture.

Genome properties. For further characterization, the draft genomes of the new strains were assembled and annotated (see Materials and Methods). The GC content was 60.9% for both strain 3C and strain 4AC and 63.8% for strain 3B. The draft genome sizes (and numbers of identified protein-encoding genes) were 2.77 Mb (2,945 open reading frames [ORFs]), 2.75 Mb (2,804 ORFs), and 2.44 Mb (2,511 ORFs) for strains 3C, 3B, and 4AC, respectively. In general, the metabolic machinery was similar to that seen with thermophilic strains SolV ([20](#)) and V4 ([29](#)) and mesophilic strain LP2A ([16](#)). The annotated genomes were used to compile a table with the key predicted methylotrophy genes (see Table S1 in the supplemental material). The three new isolates possessed only one complete *pmoCAB* operon and thus differ from the thermophilic strains, which have three complete operons ([20](#), [29](#)). The soluble methane monooxygenase (sMMO)-encoding genes were absent in all strains. Other features of the draft genomes are included in the descriptions below.

Growth conditions. Acid tolerance of the isolated strains was tested by gradually changing the acid concentration in batch cultures (with an OD of between 0.1 and 0.4). For strains 3C and 3B, growth was optimal between pH 1.5 and 3. Strain 4AC was the

TABLE 1 Conditions for optimal growth of the three mesophilic verrucomicrobial methanotroph strains as tested in batch cultures[a]

Strain	Optimum temp (°C) (maximum temp)	μ_{max} (h^{-1}) (doubling time [h])	Optimum pH range	Lowest pH (% of μ_{max})
3B	44 (49)	0.042 (16)	1.5–3	0.6 (30)
4AC	38 (43)	0.035 (20)	1–3	0.5 (40)
3C	35 (39)	0.013 (53)	1.5–3	0.6 (35)

[a] Because of the oxygen sensitivity of strain 4AC, this organism was grown at 5% O_2 compared to 17% O_2 for the other two strains. μ_{max} = ln2/doubling time.

most acid tolerant; its optimum growth range extended down to pH 1, and it exhibited the lowest pH allowing growth, namely, pH 0.5. This was lower than for the thermophilic verrucomicrobial methanotrophs (pH 0.8) ([7](#)). This makes strain 4AC the most acidophilic methanotroph isolated thus far. Above pH 3, the growth rate of all strains dropped gradually. The maximum pH allowing growth was between pH 5 and 6.

The temperature and pH optima and ranges for these three mesophilic strains ([Table 1](#)) reflected the conditions in the acid soils (pH ranging from 1.5 to below 1) and indicated that they occupy different niches in their natural environment. The fact that these strains were enriched from the same soils indicates that local conditions are variable. This is evident for ecosystems such as that of the Solfatara, where heterogeneity in soil conditions occurs because of the irregular rainfall and the various routes the volcanic gasses take through the soil.

Like the thermophilic verrucomicrobial methanotrophs ([7](#)), the three mesophilic strains were able to grow on methanol. All strains were strictly dependent on the addition of mud pot water to the growth medium. This water could be replaced by cerium (III), which is one of the lanthanides that can serve as a metal cofactor in methanol dehydrogenase of *M. fumariolicum* SolV ([30](#)). All mesophilic strains contain two different *xoxF* genes encoding methanol dehydrogenases (see Table S1 in the supplemental material). All genes possess the typical lanthanide (III) coordination-specific aspartate located two amino acids downstream of the catalytic aspartate ([31](#)).

Most proteobacterial methanotrophs use methane both as an energy source and as a carbon source. Carbon is assimilated at the formaldehyde oxidation level via the ribulosemonophosphate (RuMP) pathway or serine pathway ([32](#)). In contrast, *M. fumariolicum* SolV ([13](#)) and *M. infernorum* V4 ([14](#)) and employ carbon dioxide as a carbon source using the Calvin cycle and therefore have an autotrophic lifestyle. Since all of the three new strains contain the genes involved in the Calvin cycle (including the two subunits of the key enzyme RuBisCO) (see Table S1 in the supplemental material), they were predicted to grow as autotrophs. The *cbbL* genes encoding the RuBisCO large subunit of the three strains were previously shown to form a phylogenetically distinct cluster closely related to *M. fumariolicum* SolV and *M. infernorum* V4 ([13](#)). The ability to grow autotrophically was tested by culturing the strains on ^{13}C-labeled methane (2% to 3% of the headspace) added to batch cultures that contained a high percentage (40%) of unlabeled $^{12}CO_2$ in the gas phase to serve as a trap for the produced $^{13}CO_2$. After growth, 94% to 102% of the ^{13}C label from methane was recovered as $^{13}CO_2$. The finding that only 4% to 5% of the biomass produced was labeled with ^{13}C, matching the average ^{13}C percentage of the CO_2 in the bottles during growth (which increased from 1.2% to 7% to 10%), confirmed that CO_2 was the
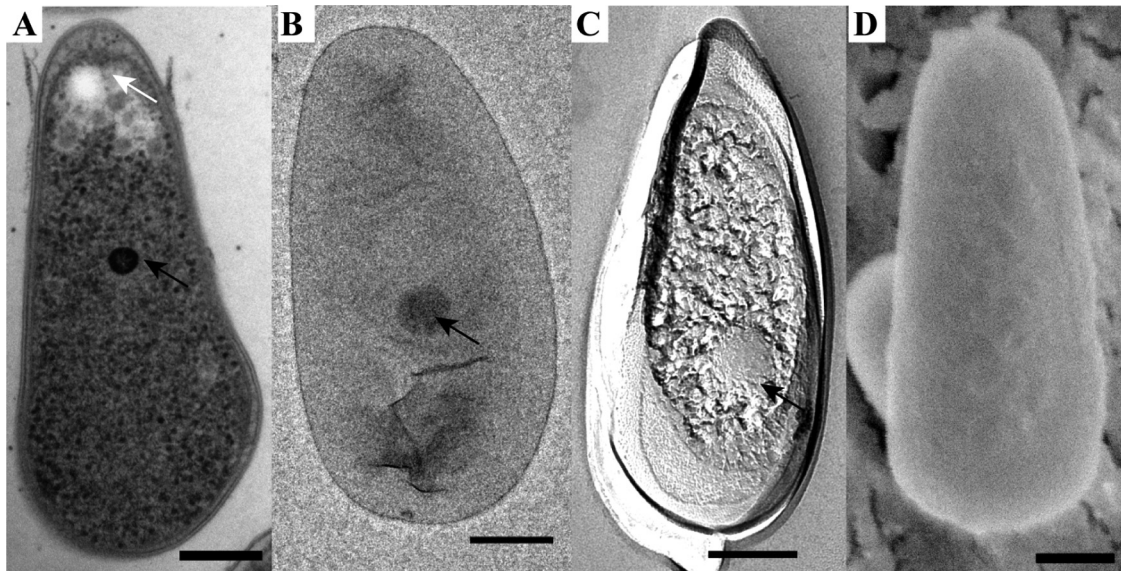
FIG 1 Morphology of the mesophilic verrucomicrobial methanotroph strain 3B as visualized by the use of thin sections of high-pressure-frozen and freeze-substituted cells (A), negative staining (B), FE (C), and cryoSEM (D). The cells are rod shaped with one broader cell pole and contain electron-dense (black arrows) and electron-light (white arrow) particles. Scale bars, 200 nm.

actual carbon source. This showed that these strains do not use intermediates of methane oxidation for their carbon supply as in the case of the serine or RuMP pathway (which would lead to, respectively, 50% or 100% ^{13}C from methane incorporated into the biomass [5, 32]). The key genes of the serine and RuMP pathways were indeed absent from the draft genomes (see Table S1). With the finding that the three mesophilic strains are autotrophic, five of five studied methanotrophic *Verrucomicrobia* have been proven to be autotrophs. Furthermore, the genome of strain LP2A indicates that this organism too is an autotroph (16). This suggests that all methanotrophic *Verrucomicrobia* have an autotrophic lifestyle. For the methanotrophic proteobacteria, thus far only *Methylococcus capsulatus* was shown to possess Calvin cycle genes (33), although physiological evidence for an active Calvin cycle is lacking.

Description of morphology. The morphology of the three mesophilic strains was studied in detail using multiple electron microscopy (EM) techniques. For comparison, the thermophilic verrucomicrobial methanotroph *M. fumariolicum* SolV was included in this study. The shape and dimensions of the cells were investigated using cryoSEM. This method showed the whole cells (in contrast to sections) in a near-native state (in contrast to negative staining, where the cells can collapse during the procedure). The cells of all four studied microorganisms were rod shaped, with one of the cell poles being broader than the other pole (Fig. 1D, 2D, 3D, and 4D). Using freeze-etching (FE), replicas of whole or fractured cells show the overall morphology and especially the appearance of the cell surface. FE showed the same morphology of the cells as cryoSEM (Fig. 1 to 4). No S-layers were observed; instead, the cell surface was smooth, as demonstrated by both FE and cryoSEM. Cells of strain 3C exhibited a wider variety in shapes than those of the other strains, as was also observed by light microscopy. In cryoSEM, cells of strain 3C were occasionally attached to each other via a pilus-like structure. The dimensions of all strains were similar (approximately 1.3 µm by 0.6 µm) (see

Table S2 in the supplemental material). The width (measured at the broadest pole) was clearly greater for strain 3C, as was also obvious from the length/width (L/W) ratios (see Table S2). This leads to the resemblance of these cells to buckwheat seeds.

Thin sections of high-pressure-frozen and freeze-substituted cells were studied to get an insight into the ultrastructure of the cells. Both *M. fumariolicum* SolV and the three mesophilic strains feature Gram-negative cell envelopes, as two membranes can be visualized in thin sections (Fig. 1 to 4). There have been reports that multiple verrucomicrobial species (*Verrucomicrobium spinosum*, *Prosthecobacter dejongeii*, *Chthoniobacter flavus*, and *Pedosphaera parvula* strain Ellin514) have a *Planctomycetes-Verrucomicrobia-Chlamydia* (PVC)-specific cell plan in which the outermost membrane is in fact a cytoplasmic membrane (34). This is not apparent from our ultrastructural study of the four studied verrucomicrobial methanotrophs. In addition to the Gram-negative cell envelope, all four strains feature a typical-looking cytoplasm containing ribosomes. No condensed DNA has been observed in the thin sections of the four strains.

In strain 3C, intracytoplasmic membrane (ICM) stacks orthogonal to the membrane were observed in many sections (Fig. 3A), after FE (Fig. 3C), and during cryoSEM (data not shown). Although ICMs have been observed in the verrucomicrobial methanotroph *Methylacidiphilum infernorum* in rare occurrences (9), strain 3C is the first verrucomicrobial methanotroph described where membrane stacks are present in most cells. In the proteobacterial methanotrophs, a complex ICM system consisting of membrane stacks is a widespread characteristic and the membranes are thought to harbor the pMMO enzyme. The appearance and orientation of ICMs of type I methanotrophs differ from the appearance and orientation of ICMs of type II methanotrophs (5). The membranes occur as layers lining the periphery of the cell in type II methanotrophs; in type I methanotrophs, the membranes form stacks that are aligned more or less orthogonally to the cytoplasmic membrane. The membrane stacks of strain 3C
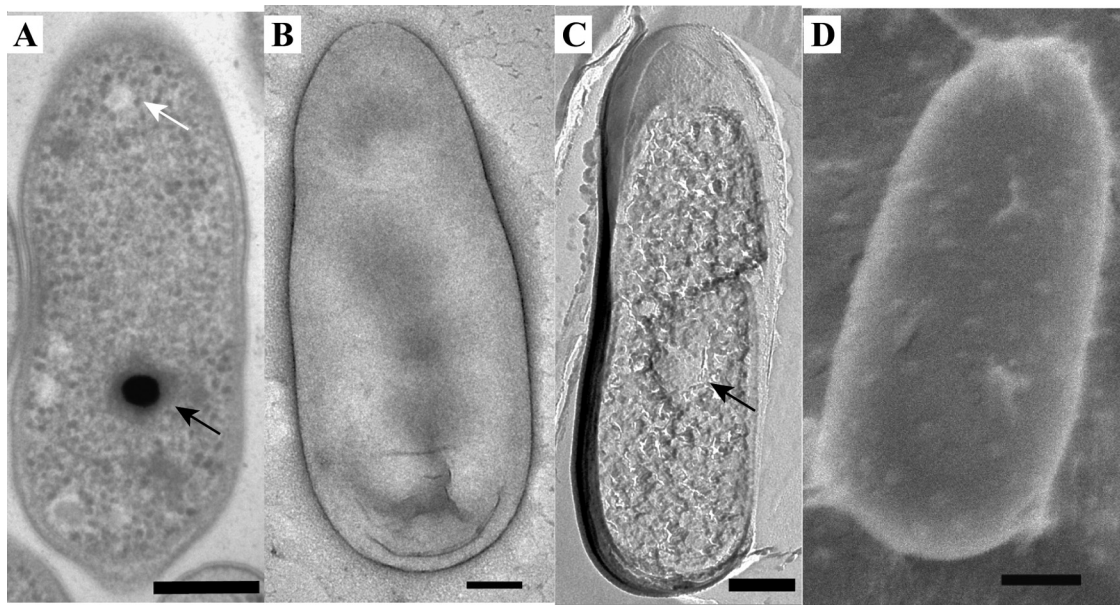
FIG 2 Morphology of the mesophilic verrucomicrobial methanotroph strain 4AC as visualized by the use of thin sections of high-pressure-frozen and freeze-substituted cells (A), negative staining (B), FE (C), and cryoSEM (D). The cells are rod shaped with one broader cell pole and contain electron-dense (black arrows) and electron-light (white arrow) particles. Scale bars, 200 nm.

resemble type I ICMs. It remains to be established if the pMMO enzymes are also localized in these membranes of strain 3C. Furthermore, the location of pMMO in the other verrucomicrobial methanotrophs that seem to lack internal membranes needs to be investigated.

In all strains, both electron-dense (ED) and electron-light (EL) particles were observed in the thin sections (Fig. 1A, 2A, 3A, and 4A) and after FE. The EL particles were smaller and more numerous than the ED particles. The ED particles were typically located near the center of the cell, and the EL particles seemed to localize preferentially to the cell poles. EL particles were observed in the majority of cells, and they resembled the glycogen storage particles in *M. fumariolicum* SolV (26). A polysaccharide stain verified that the EL particles contained glycogen (Fig. 5). Accordingly, the genomes of all studied verrucomicrobial methanotrophs harbor the

key genes for glycogen synthesis and glycogen degradation (see Table S1 in the supplemental material).

The amount and the size of the ED particles were studied via negative staining. The amount of ED bodies was on average close to one per cell (see Table S3 in the supplemental material). Two ED bodies per cell were observed only in some cells of strain 3C and in many dividing cells. The average diameters of the ED particles were similar for all strains and were slightly lower than 200 nm (see Table S3).

To investigate the composition of the cells and of the ED particles in particular, EDX was performed on thin sections of cryosectioned, freeze-substituted cells. Qualitative maps showed enrichments of specific elements throughout the cell and made clear that multiple elements were enriched in the electron-dense particles. For *M. fumariolicum* SolV (Fig. 6) and strains 3C and 3B, the
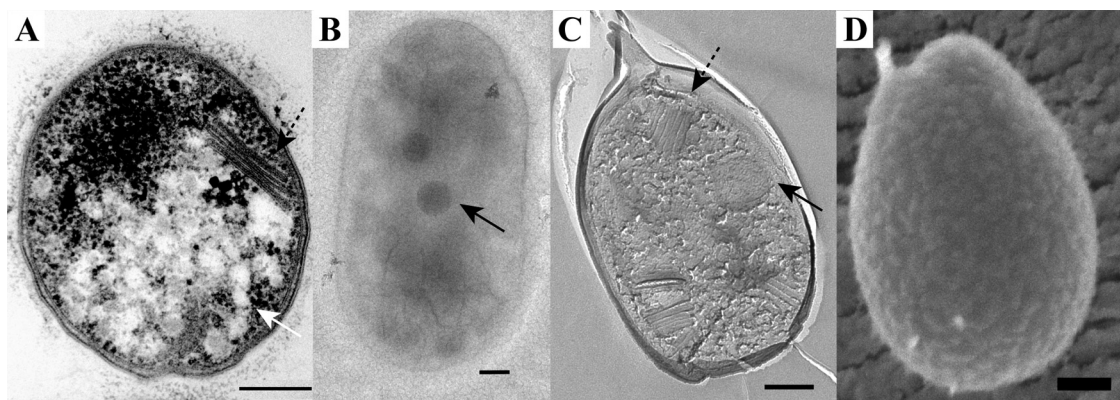

FIG 3 Morphology of the mesophilic verrucomicrobial methanotroph strain 3C as visualized by the use of thin sections of high-pressure-frozen and freeze-substituted cells (A), negative staining (B), FE (C), and cryoSEM (D). The cells are rod shaped with one broader cell pole and contain membrane stacks (dashed arrow) and electron-dense (black arrows) and electron-light (white arrow) particles. Scale bars, 200 nm.
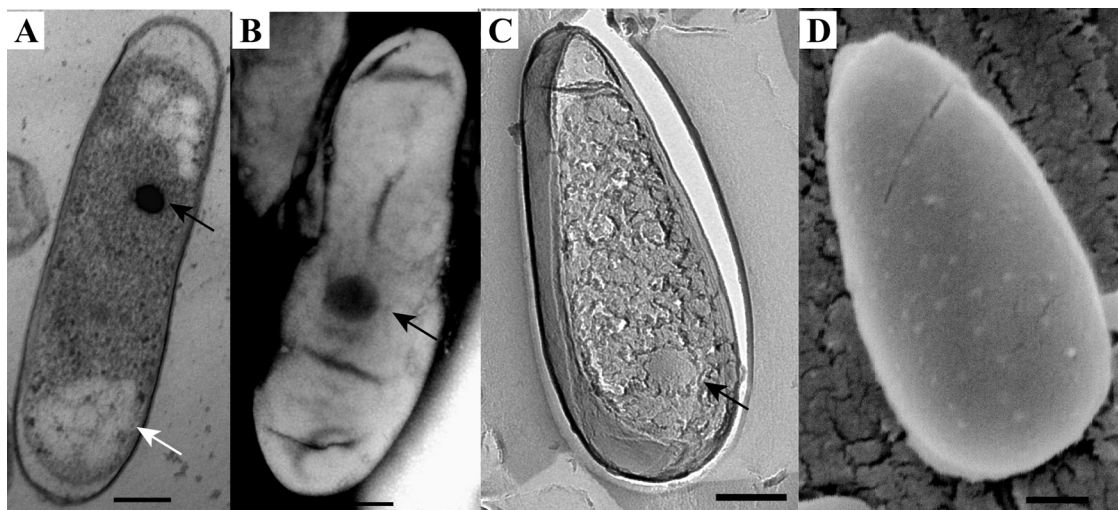
FIG 4 Morphology of the thermophilic verrucomicrobial methanotroph *M. fumariolicum* SolV as visualized by the use of thin sections of high-pressure-frozen and freeze-substituted cells (A), negative staining (B), FE (C), and cryoSEM (D). The cells are rod shaped with one broader cell pole and contain electron-dense (black arrows) and electron-light (white arrow) particles. Scale bars, 200 nm.
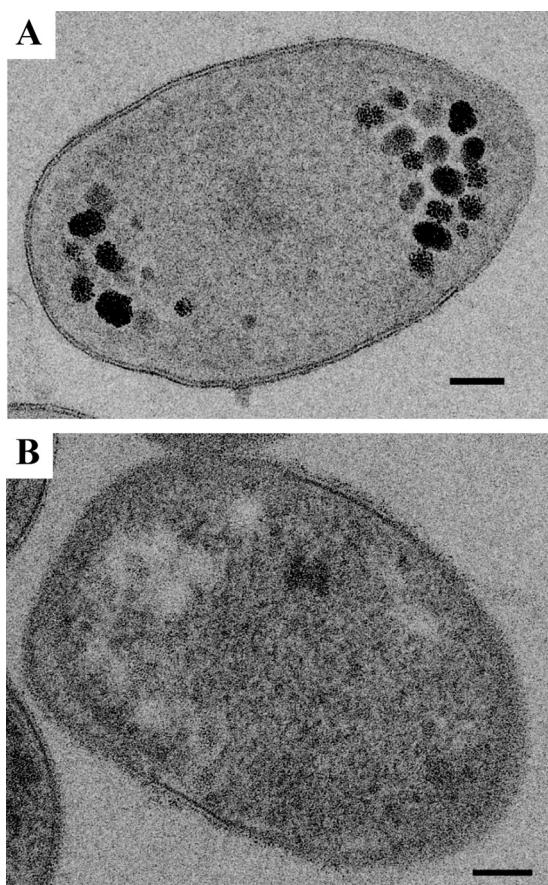

FIG 5 A polysaccharide stain on thin sections of high-pressure-frozen and freeze-substituted cells of the mesophilic verrucomicrobial methanotroph strain 4AC indicates that polysaccharides are present in the electron-light particles. Electron-light particles, appearing as white spots in the negative control (B), show clear staining in panel A (black silver aggregates) because of the polysaccharide content. Similar results were obtained for strains 3B and 3C (data not shown).

ED particles were enriched in phosphorus and oxygen (see Table S3 in the supplemental material). This suggests that (poly)phosphate is present in the ED particles. Genes encoding polyphosphate kinase and exopolyphosphatase were present in both mesophilic and thermophilic strains (see Table S1). Polyphosphate-encompassing vesicles have been found in other organisms, where they are known as acidocalcisomes (35). Because polyphosphate has a negative charge, positive counterions are often found in the vicinity of these molecules. In the case of *M. fumariolicum* SolV and strain 3C, magnesium ions seemed to have this role. In strain 4AC, a minority of the ED particles showed a phosphorus signal. In addition, these particles showed sulfur enrichment. The role for sulfur, however, remains puzzling, since no genes for sulfur storage/utilization have been detected thus far in the draft genome (data not shown).

Phylogenetic and genomic analysis. Phylogenetic analysis of the 16S rRNA gene of the three mesophilic strains showed them to be affiliated with the previously described (16) mesophilic LP2A strain (Table 2). The cluster of mesophiles separates clearly from the thermophilic cluster, with which they share only just below 90% identity (Fig. 7). The *pmoA*-based phylogeny agreed well with the 16S rRNA-based phylogeny, with strain LP2A as the closest cultivated neighbor (see Table S1 in the supplemental material). The *pmoA* genes of new isolates showed about 92% identity to those of strain LP2A at the amino acid level and about 70% to 72% amino acid identity to the *pmoA1* and *pmoA2* genes of the thermophilic strains. The same clustering was obtained using the *mxaF* (*xoxF*) (31) gene. It was previously asserted that verrucomicrobial 16S rRNA genes putatively belonging to methanotrophs cluster in three distinct groups (16). Analysis of the 16S rRNA genes that also included the environmental operational taxonomic units (OTUs) described previously (16) showed that the newly described mesophilic isolates belong to the same group as LP2A (data not shown).

To investigate if the three new strains represent three strains of the same species or multiple species, analyses based on the 16S rRNA gene and the average nucleotide identity using BLAST
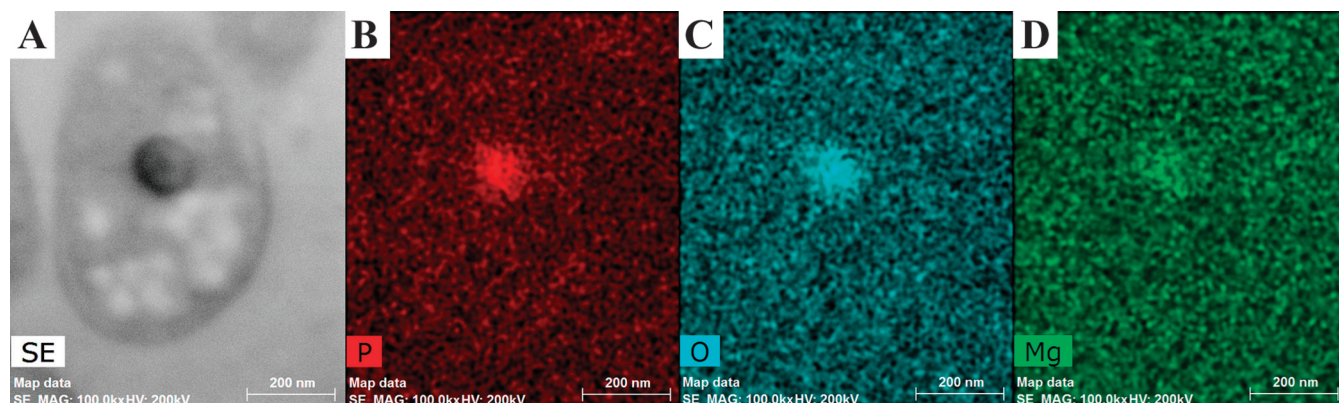
FIG 6 Thin section of *M. fumariolicum* SolV analyzed by EDX shows the ED particle (dark particle in the sectioned cell in STEM mode [A]) to be enriched in phosphorus (B), oxygen (C), and magnesium (D). SE, secondary electron detector; MAG: 100.0kx, magnification 100,000 times; HV, high voltage.

[ANI(b)] (with draft genomes) were performed. *M. fumariolicum* SolV was included in both analyses, and LP2A was included in the 16S rRNA gene sequence analysis. The three mesophilic strains (3B, 3C, and 4AC) share 89.1% to 98.1% 16S rRNA gene sequence identity (Table 2). They would therefore be characterized as three different species by the use of 16S rRNA gene analysis with the strict species cutoffs for similarity of 98.5% (36) or 98.7 to 99.1% (37, 38) (Table 2). Using the same species cutoffs, mesophilic strain LP2A should be classified as a fourth species, since it shares 89.6% to 98.1% 16S rRNA gene sequence identity with the three mesophiles. However, multiple studies have shown that 16S rRNA gene analysis is often not fit for resolution on the species level and that the ANI technique is better suited to such analyses (36, 39, 40). Therefore, we have also analyzed the ANI(b) values for the three mesophilic strains and *M. fumariolicum* SolV. The three strains share ANI(b) values between 77.3% and 87.8% (Table 3) and are therefore proposed to belong to three different species using the standard species identity cutoff value of 95% (41). Based on the ANI(b) and 16S rRNA gene similarity values (using a strict species cutoff), it should be concluded that each strain represents a distinct species.

A minimal 95% identity of the 16S rRNA gene sequences is widely accepted to affiliate two strains to the same genus (42, 43). Because of the 16S rRNA gene sequence identity of 89.1% to 89.7% (Table 2) between the four mesophilic species and *M. fumariolicum* SolV, it should be concluded that the four species belong to a different genus than *M. fumariolicum* SolV. The four mesophilic species have 16S rRNA gene sequence identities between 97.3% and 98.1% and therefore belong to the same genus.

TABLE 2 Distance analysis (complete deletion) of 16S rRNA genes showing the similarities between the three mesophilic verrucomicrobial strains and the previously described mesophilic LP2A and thermophilic *M. fumariolicum* SolV strains

Strain no.	Strain name	% similarity with strain:				
		1	2	3	4	5
1	*Methylacidiphilum fumariolicum* SolV					
2	*Methylacidimicrobium fagopyrum* 3C	89.1				
3	*Methylacidimicrobium tartarophylax* 4AC	89.6	97.3			
4	*Methylacidimicrobium cyclopophantes* 3B	89.7	97.6	97.1		
5	LP2A	89.6	98.1	97.3	98.1	

For this genus, we propose the name *Methylacidimicrobium*. The proposed names for the three species described in this article are as follows: *Methylacidimicrobium tartarophylax* 4AC, *Methylacidimicrobium fagopyrum* 3C, and *Methylacidimicrobium cyclopophantes* 3B. We do not propose a species name for strain LP2A but propose referring to this strain as *Methylacidimicrobium* strain LP2A.

Here we described three new species of a new genus of methanotrophic *Verrucomicrobia* isolated from a geothermal environment. The three species featured in this study indicate that a wide variety of methanotrophic verrucomicrobial species exist and that they are quite well adapted to different niches present in geothermal environments with respect to both temperature and acidity. The previously described LP2A strain (16), which, according to our analysis, also belongs to the genus *Methylacidimicrobium*, has a lower growth temperature range than the three *Methylacidimicrobium* species described here. The *Methylacidiphilum* species described before have a higher growth temperature range than all *Methylacidimicrobium* species. Although the optimum pH range for growth is essentially the same for all three *Methylacidimicrobium* species (1.5 to 3), these new mesophilic strains are more acid tolerant, with strain 4AC even growing at pH 0.5. The pH range for growth of *Methylacidimicrobium* strain LP2A is similar but slightly higher (1 to 5.2) (16), and the same is true for the *Methylacidiphilum* species (0.8 to 6) (7). It would be very interesting to study whether verrucomicrobial methanotrophs exist that live at higher pHs or if they are outcompeted by proteobacterial methanotrophs in these environments. It is therefore important to isolate and characterize additional verrucomicrobial methanotrophs.

So far, all studies in acidic, volcanic environments have exclusively yielded verrucomicrobial enrichments. No proteobacteria have been isolated from these environments to date (see, e.g., reference 16). This suggests that *Verrucomicrobia* dominate these ecosystems, although additional (especially metagenomic) surveys would be needed to verify this hypothesis.

It appears that many methanotrophic species are adapted to slightly different conditions, which would indicate that even when circumstances change, methanotrophy occurs in the ecosystem. The diversification of methanotrophs in the ecosystem may thus lead to a more stable ecosystem. As has been shown before in other ecosystems with a high number of microbial species per functional
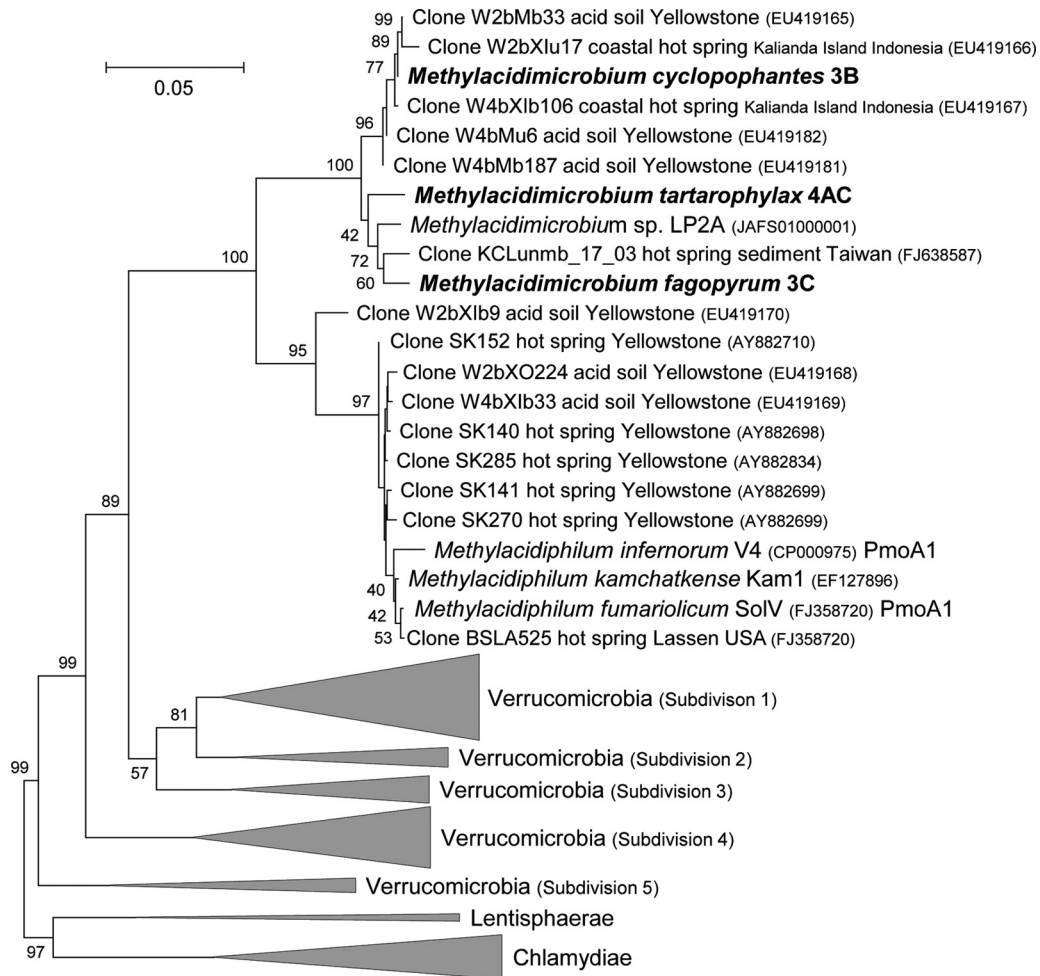
FIG 7 16S rRNA gene-based phylogenetic tree of methanotrophic and other *Verrucomicrobia* showing that the methanotrophic mesophilic strains cluster separately from the methanotrophic thermophilic species. The evolutionary history was inferred using the neighbor-joining method. The optimal tree with the branch length sum of 4.46361805 is shown. The percentage of replicate trees (values above 40%) in which the associated taxa clustered in the bootstrap test (10,000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths shown in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site. The analysis involved 128 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1,647 positions in the final data set. Evolutionary analyses were conducted in MEGA5.

group, the amount of biomass and the density were more stable (44). High numbers of species that are adapted to slightly different conditions are also expected to stabilize ecosystem process rates in response to changes in environments (45).

The recent findings are just the beginning of understanding the diversity in verrucomicrobial methanotrophs. Much more re-search is needed to verify that an even broader methanotrophic diversity can exist, as is suggested from a study of operational taxonomic units (OTUs) that were retrieved from multiple geo-thermal sites (16). For the organisms that these OTUs repre-sented, it has to be investigated whether they indeed perform methane oxidation and to find out which circumstances they need for their growth. Additional studies investigating the ecosystems are necessary to understand the interplay between the different genera and species of verrucomicrobial methanotrophs and the role they play in different environments.

Description of *Methylacidimicrobium* gen. nov. *Methylacidi-microbium* (Me.thyl.a.ci.di.mi.cro′bi.um. N.L. n. *methyl*, the methyl group; N.L. n. *acidum*, acid [from L. adj. *acidus*, sour]; N.L. n. *microbium*, microbe; N.L. n. *Methylacidimicrobium*, methyl-using microbe living in an acid environment).

Gram-negative, rod-shaped bacteria with a broadening at one of the two cell poles. Cells occur as single cells or as small groups;

TABLE 3 ANIb analysis results show the similarities between the draft genomes of the three mesophilic verrucomicrobial strains and that of the thermophilic *M. fumariolicum* SolV strain

Strain no.	Strain name	% similarity with strain:			
		1	2	3	4
1	*Methylacidiphilum fumariolicum* SolV		63.7	63.2	63.7
2	*Methylacidimicrobium fagopyrum* 3C	63.8		87.8	77.3
3	*Methylacidimicrobium tartarophylax* 4AC	62.9	87.6		77.6
4	*Methylacidimicrobium cyclopophantes* 3B	63.4	77.6	77.7	

do not form rosettes. Reproduce by binary fission. Nonmotile. Produce intracellular glycogen granules and additional intracellular (electron-dense) particles. Except for the type species *M. fagopyrum* 3C, no ICM were observed. sMMO is not present in the genomes. Representatives are extremely acidophilic and mesophilic. No growth occurs without lanthanides in the medium. Growth is also possible on methanol. Carbon is fixed via the Calvin cycle. The G-C content is 60.9% to 63.8%. Belongs to the *Verrucomicrobia*; the closest described methanotrophic bacterial genus is the verrucomicrobial thermo- and acidophilic genus *Methylacidiphilum* (7). Contains the type species *Methylacidimicrobium fagopyrum* 3C and *Methylacidimicrobium tartarophylax* 4AC, *Methylacidimicrobium cyclopophantes* 3B, and *Methylacidimicrobium* strain LP2A (16) as additional species. Habitat is acidic soil of elevated temperature, particularly in volcanic mud pots or near fumaroles.

Description of *Methylacidimicrobium fagopyrum* sp. nov. *Methylacidimicrobium fagopyrum* (fa.go′py.rum. N.L. neuter n. *fagopyrum*, buckwheat; referring to the shape of the cell).

Description as for the genus plus the following traits. Cells are ca. 1.2 μm long and 0.7 μm wide. An ICM system was observed (Fig. 3) that consists of membrane stacks orthogonal to the cell wall. One or two particles (0.16 ± 0.08 μm in diameter) containing phosphorus, oxygen, magnesium, and nitrogen are present in most cells. The optimum temperature for growth is 35°C; no growth occurs above 39°C. Growth occurs at or above pH 0.6, with an optimum range of 1.5 to 3.0. The type strain is strain 3C^T, which was isolated from soil of the Solfatara, at Pozzuoli, near Naples, Italy.

Description of *Methylacidimicrobium tartarophylax* sp. nov. *Methylacidimicrobium tartarophylax* (tar.ta.ro′phy.lax. L. masc. n. *Tartarus*, underworld; Gr. masc. n. *phylax*, guardian; N.L. adj. *tartarophylax*, guardian of the underworld; referring to the enrichment location which in Roman times was believed to be in the vicinity of an entrance to the underworld).

Description as for the genus plus the following traits. Cells are ca. 1.4 μm long and 0.9 μm wide. No ICM system was observed (Fig. 2). One particle (0.15 ± 0.03 μm in diameter) containing sulfur, oxygen, and in some cases phosphorus is present in most cells. The optimum temperature for growth is 38°C; no growth occurs above 43°C. Growth occurs at and above pH 0.5, with pH 1 to 3 as optimum. Growth is inhibited by oxygen (growth at 5% oxygen is about two times faster than at ambient oxygen concentration). The type strain is strain 4AC^T, which was isolated from soil of the Solfatara, at Pozzuoli, near Naples, Italy.

Description of *Methylacidimicrobium cyclopophantes* sp. nov. *Methylacidimicrobium cyclopophantes* (cy.clo.po.phan′tes. L. masc. *cyclops*, cyclops; Gr. adj. suffix -*phantes*, resembling; N.L. n. adj. *cyclopophantes*, resembling a Cyclops; referring to the single large electron-dense particle which is present in each cell).

Description as for the genus plus the following traits. Cells are ca. 1.2 μm long and 0.6 μm wide. No ICM system was observed (Fig. 1). One particle (0.12 ± 0.04 μm in diameter) containing phosphorus and oxygen is present in most cells. The optimum temperature for growth is 44°C; no growth occurs above 49°C. Growth occurs at or above pH 0.6, with an optimum range of 1.5 to 3.0. The type strain is strain 3B^T, which was isolated from soil of the Solfatara, at Pozzuoli, near Naples, Italy.

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