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DOI
10.1007/s00203-025-04630-7

Publication date
2026

Document Version
Final published version

Published in
Archives of Microbiology

Citation (APA)
Schmitz, R. A., Peeters, S. H., van Alen, T. A., Mets, A., Hogendoorn, C., Nuijten, G. H. L., Iosif, C. A., Pol, A., Schouten, S., Jetten, M. S. M., & Op den Camp, H. J. M. (2026). Response and adaptation of verrucomicrobial methanotrophs to heat and acidity. *Archives of Microbiology*, *208*(1), Article 66. https://doi.org/10.1007/s00203-025-04630-7

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Archives of Microbiology (2026) 208:66
https://doi.org/10.1007/s00203-025-04630-7

ORIGINAL PAPER

Response and adaptation of verrucomicrobial methanotrophs to heat and acidity

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Received: 25 August 2025 / Revised: 24 November 2025 / Accepted: 24 November 2025
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Abstract
Acidophilic microorganisms thrive in environments where the external pH is orders of magnitude lower than their intracellular pH. Verrucomicrobial methanotrophs of the family *Methylacidiphilaceae*, including *Methylacidiphilum* and *Methylacidimicrobium*, inhabit extremely acidic geothermal environments and can grow at a pH < 1.0 and temperatures up to 65 °C. We analyzed and compared their membrane fatty acid compositions at pH 3.0 across strains with different temperature optima. Thermophilic *Methylacidiphilum* strains almost exclusively contain saturated fatty acids, while the mesophilic *Methylacidimicrobium* strains we studied incorporate 16–47% unsaturated fatty acids. Notably, the thermophile *Methylacidiphilum fumariolicum* SolV increases unsaturated fatty acid content in response to a 10 °C temperature decrease but not to a decrease in pH from 3.0 to 1.7. Genomic analysis revealed a conserved fatty acid biosynthesis pathway. Despite constitutive expression of predicted pH homeostasis genes, SolV did not upregulate them upon changing the pH from 3.0 to 1.7. However, genes involved in methane oxidation were strongly upregulated, suggesting a potential metabolic adaptation to extreme acidity.

Keywords Acidophiles · Verrucomicrobial methanotrophs · Membrane fatty acids · pH homeostasis · Thermophiles · Methane oxidation

Introduction

Acidophilic microorganisms thrive in environments that are either naturally acidic (e.g., acidic geothermal environments and sulfide-rich caves) or acidic due to anthropogenic activity (e.g., acid mine drainage systems) (Schoen and Rye 1970; Baker and Banfield 2003; Jones et al. 2012). Moderate acidophiles have a pH optimum between 3.0 and 5.0, whereas extreme acidophiles grow optimally below pH 3.0 (Johnson 2007). Prokaryotic acidophiles are found in a range of archaeal and bacterial phyla (Johnson and Quatrini 2020). Methanotrophs of the bacterial phylum *Verrucomicrobiota* (order *Methylacidiphilales*, class *Methylacidiphilae*) are found in acidic geothermal environments and grow optimally at a pH of 1.0 to 3.0 (Schmitz et al. 2021a). These harsh environments are located across the globe and are characterized by geothermal features such as mud pools, fumaroles and hot springs from which acidic fluids and gases such as methane (CH_4), hydrogen gas (H_2), carbon dioxide (CO_2) and hydrogen sulfide (H_2S) are expelled (Castaldi and Tedesco 2005; Picone et al. 2020; Schmitz et al. 2021a; Benson et al. 2011). Such habitats can become extremely acidic if the buffering capacity of the environment is exceeded through microbial oxidation of H_2S to sulfuric acid (H_2SO_4) (Johnson and Quatrini 2020). Interestingly, acidophiles have developed various mechanisms

Communicated by Kristina Beblo-Vranesevic.

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Published online: 11 December 2025

to maintain their intracellular proton concentration several orders of magnitude lower than their surroundings, which is critical to survive in extremely acidic environments (Baker-Austin and Dopson 2007). Distinctive structural and functional characteristics of acidophiles include cell membranes highly impermeable to protons, membrane channels with reduced pore size, reversed membrane potential, pumping out excess protons and cytoplasmic buffering.

Prior to the discovery of verrucomicrobial methanotrophs from acidic geothermal soils, aerobic methanotrophs were thought to be restricted to the Alpha- and Gammaproteobacteria, of which no extreme acidophiles (growth at pH < 3.0) have been isolated (Schmitz et al. 2021a; Whittenbury et al. 1970; Hanson and Hanson 1996). Hereafter, a member of the N10 phylum (*Candidatus* Methylomirabilis oxyfera) was shown to perform aerobic methanotrophy in anoxic environments through an intra-aerobic pathway (Ettwig et al. 2010). Recently, a newly isolated *Mycobacterium* strain from the phylum *Actinomycetota* was found to oxidize methane at a pH as low as 0.75, expanding the known phylogenetic diversity of aerobic methanotrophs (Kambara et al. 2025).

Currently, it is well established that members of the verrucomicrobial genus *Methylacidiphilum*, which exhibit optimal growth at 50–60 °C and grow up to 65 °C, inhabit acidic geothermal environments across the globe (Dunfield et al. 2007; Pol et al. 2007; Islam et al. 2008; Erikstad et al. 2019; Awala et al. 2021). More recently, verrucomicrobial methanotrophs were isolated from comparable, but significantly cooler environments (Sharp et al. 2014; van Teeseling et al. 2014). These mesophilic strains, belonging to the genus *Methylacidimicrobium*, exhibit optimal growth at 33–44 °C and grow up to 49 °C, with some able to grow at a pH as low as 0.5 (van Teeseling et al. 2014). An exception within this mesophilic genus is *Methylacidimicrobium thermophilum* AP8, which grows optimally at 50 °C and up to 55 °C (Picone et al. 2021b). Overall, the primary distinction between *Methylacidiphilum* and *Methylacidimicrobium* appears to be their thermal preference, with the former generally favoring higher temperature.

Interestingly, although all known verrucomicrobial methanotrophs have been isolated from methane-rich acidic geothermal environments, pyrosequencing of samples derived from sulfide-rich concrete sewage pipes has revealed the presence of *Methylacidimicrobium* strains in this non-geothermal habitat (Pagaling et al. 2014). Indeed, recently the verrucomicrobial methanotroph *Methylacidiphilum fumariolicum* SolV was shown to degrade methanethiol (CH_3SH) and hydrogen sulfide (H_2S) (Schmitz et al. 2022, 2023). The presence of verrucomicrobial methanotrophs in these man-made environments indicates that they are not limited to acidic geothermal habitats but can also thrive in other acidic niches.

pH homeostasis is essential for all living organisms since a lowered intracellular pH could result in protein instability, reduced enzyme activity, and structural changes of both DNA and RNA (Madshus 1988; Slonczewski et al. 2009). Hence, acidophiles need to maintain a proton gradient of several orders of magnitude between the interior and the external environment (Slonczewski et al. 2009; Moll and Schäfer 1988). Indeed, the verrucomicrobial methanotroph *Methylacidiphilum* sp. RTK17.1 was shown to maintain an intracellular pH of 6.5 when growing in medium with an external pH of 1.5 (Carere et al. 2021). The most convenient adaptation to acid is to possess a membrane with low proton permeability (Driessen et al. 1996). Maintaining such a membrane is especially challenging in thermophiles since with increasing temperature, the membrane becomes increasingly permeable to protons (Driessen et al. 1996). Through homeoviscous adaptation to fluctuations in the environment, microbial membranes can retain the required fluidity for integrity, nutrient permeability and membrane protein functioning (Sinensky 1974). Accordingly, saturated fatty acids decrease membrane fluidity whereas unsaturated fatty acids and branched-chain fatty acids increase membrane fluidity (Denich et al. 2003; Zhang and Rock 2008). The fatty acid chains of thermophiles are generally longer, more saturated and include more branched-chain *iso*-fatty acids, whereas acidophiles possess various adaptations such as a relatively high percentage of branched-chain fatty acids and cyclopropane fatty acids (Siliakus et al. 2017). Previously published fatty acid compositions of eleven thermophilic *Methylacidiphilum* strains grown at 55 °C and pH 3.0−3.5 revealed an exceptionally large proportion (on average 99%) of saturated fatty acids (Erikstad et al. 2019; Op den Camp et al. 2009; Awala et al. 2023). The high share of saturated fatty acids in *Methylacidiphilum* strains could be an adaptation to establish optimal membrane fluidity even under the combination of a high temperature and an extremely low pH (Konings et al. 2002; Sohlenkamp 2017). Besides having a membrane with low proton permeability, acidophiles seem to share several mechanisms with neutrophilic microorganisms to cope with an excess of protons intracellularly, such as active proton efflux and cytoplasmic buffering (Baker-Austin and Dopson 2007; Foster 2004). While neutrophiles use these mechanisms to swiftly respond to acid stress, acidophiles may use them to live and grow perpetually in acidic environments (Foster 2004; Kanjee and Houry 2013; Guan and Liu 2020).

In this study, we analyzed 11 genomes of different verrucomicrobial methanotrophs to predict how they synthesize membrane fatty acids. Membrane fatty acid profiles for several thermophilic *Methylacidiphilum* strains have been

published, whereas the profile of only one mesophilic *Methylacidimicrobium* strain was recently published (Op den Camp et al. 2009; Erikstad et al. 2019; Awala et al. 2023). To fill this knowledge gap, we analyzed the fatty acid compositions of three mesophilic verrucomicrobial methanotrophs and compared them with those found in the thermophilic genus *Methylacidiphilum*. In addition, we grew the thermophilic strain *M. fumariolicum* SolV at different temperatures and pH values in continuous cultures (chemostats) to assess how temperature and pH affect the fatty acid composition and how pH affects gene expression.

Materials and methods

Cultivation of *Methylacidiphilum* and *Methylacidimicrobium* strains

Methylacidiphilum fumariolicum SolV was grown as methane-limited continuous culture as described before (Schmitz et al. 2020), except that the cells were grown at a pH value of 1.7 and 3.0, at a temperature of 45 and 55 °C and that a 400 mL bioreactor was used. For the temperature decrease experiment (from 55 to 45 °C) and the pH decrease experiment (from pH 3.0 to 1.7), two different chemostats were used, inoculated by *M. fumariolicum* SolV cells of different batch incubations. The growth medium was composed of 0.2 mM $MgCl_2 \cdot H_2O$, 0.2 mM $CaCl_2 \cdot H_2O$, 1 mM Na_2SO_4, 2 mM K_2SO_4, 2 mM $(NH_4)_2SO_4$, and 50 µM $NaH_2PO_4 \cdot H_2O$. The final trace element concentration was 1 µM $NiCl_2 \cdot 6H_2O$, $CoCl_2 \cdot 6H_2O$, $NaMoO_4 \cdot 2H_2O$, and $ZnSO_4 \cdot 7H_2O$, 5 µM $MnCl_2 \cdot 4H_2O$ and $FeSO_4 \cdot 7H_2O$, 10 µM $CuSO_4 \cdot 5H_2O$, and 20 nM $CeCl_3 \cdot 6H_2O$. To assess the effect of a temperature and pH decrease, the temperature and pH were decreased abruptly, without the use of a gradient. RNA and fatty acids were extracted and isolated after new steady states were reached after at least three generations. *M. tartarophylax* 4AC was grown in a methane-limited chemostat at pH 3.0 and 38 °C as described by Mohammadi et al. (2019). *M. cyclopophantes* 3B was grown as batch culture at pH 3.0 and 44 °C and *M. fagopyrum* 3 C as batch culture at pH 3.0 and 35 °C as described by van Teeseling et al. (2014). These temperatures were chosen as they are the temperature optima of the different strains (van Teeseling et al. 2014). To isolate fatty acids, 10 mL bacterial cell culture was centrifuged at 5000 × *g* at 4 °C for 5 min. The supernatant was decanted, and the pellet was resuspended in the remaining liquid by gently pipetting up and down. The cell suspension was transferred to a glass bowl, which was immediately frozen in liquid nitrogen. Finally, the frozen pellet was freeze-dried overnight.

Fatty acid extraction and analysis

Freeze-dried bacterial biomass was extracted with a modified Bligh and Dyer extraction. The samples were ultrasonically extracted for 10 min with a solvent mixture containing methanol, dichloromethane (DCM) and phosphate buffer (2:1:0.8, vol/vol/vol). After centrifugation, the solvent was collected, combined and the residues were re-extracted twice. A biphasic separation was achieved by adding additional DCM and phosphate buffer to a ratio of methanol, DCM and phosphate buffer of 1:1:0.9 (vol/vol/vol). The aqueous layer was washed two more times with DCM and the combined organic layers were dried over a Na_2SO_4 column followed by drying under N_2. The extracts were subsequently hydrolyzed with 1 N 5% HCl in methanol by reflux for 3 h. The hydrolysate was adjusted to pH 4 with 2 N KOH-methanol (1:1, vol/vol) and, after addition of water to a final water-methanol ratio of 1:1, extracted three times with DCM. The DCM fractions were collected and dried over Na_2SO_4. The obtained extract was methylated with diazomethane and silylated with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA). The derivatized extract was analyzed by GC and GC-mass spectrometry (MS). GC-MS was performed using a Triple Quad 7000 C GC-MS (Agilent Technologies, Santa Clara, CA, USA) in full scan mode. A CP-Sil5 CB column (25 m x 0.32 mm with a film of 0.12 µm, Agilent Technologies) was used for the chromatography with He as carrier gas with a constant flow of 2 mL · min^{-1}. The samples (1 µL) were injected onto the column at 70 °C, after which the temperature was increased at 20 °C · min^{-1} to 130 °C, raised further by 4 °C · min^{-1} to 320 °C, at which it was held for 20 min. The fatty acids analyzed in this study of strains *M. tartarophylax* 4AC, *M. cyclopophantes* 3B, *M. fagopyrum* 3 C and *M. fumariolicum* SolV were compared with fatty acids of *M. kamchatkense* Kam1, *M. infernorum* V4 and *M. fumariolicum* SolV as reported by Op den Camp et al. (2009), with *Methylacidiphilum* strains IT6 and IT5 and *Methylacidimicrobium* strain B4 as reported by Awala et al. (2023), and with *Methylacidiphilum* strains Fur, Rib, Fdl, Yel, Ice and Phi as reported by Erikstad et al. (2019).

Genetic analyses

To investigate how membrane fatty acids could be synthesized in verrucomicrobial methanotrophs, the following amino acid sequences of enzymes of *Escherichia coli* strains were retrieved from GenBank (Clark et al. 2016): acetyl-CoA carboxylase subunits AccA (CAD6021894.1), AccB (CAD6001842.1), AccC (CAD6001830.1), AccD (CAD6008109.1); acyl carrier proteins AcpP (CAD6016753.1), AcpS (CAD6006565.1); malonyl CoA-acyl carrier protein transacylase FabD (CAD6010950.1);

beta-ketoacyl-ACP synthase FabH (CAD6016771.1); acetolactate synthase IlvB (CAD5998393.1); acetolactate synthase regulatory subunit IlvH (CAD6022235.1); ketol-acid reductoisomerase IlvC (CAD6023092.1); dihydroxy-acid dehydratase IlvD (CAD6021386.1); branched-chain amino acid aminotransferase IlvE (CAD6023096.1); 2-isopropylmalate synthase LeuA (CAD6022244.1); 3-isopropylmalate dehydrogenase LeuB (CAD6022247.1); 3-isopropylmalate dehydratase subunits LeuC (CAD6022250.1), LeuD (CAD6022253.1); pyruvate dehydrogenase subunits PdhA (WP_214096071.1), PdhB, (WP_151040350.1), PdhC (ADX52374.1); phospholipase D Pld (WP_216348498.1); 3-ketoacyl-ACP reductase FabG (EGT67884.1); 3-hydroxyacyl-ACP dehydratase FabZ (CAD6021915.1); enoyl-ACP reductase FabI (CAD6015223.1); beta-ketoacyl-ACP synthase II FabF (CAD6016748.1), HTH-type transcriptional repressor FabR (CCP46023.1); fatty acid metabolism transcriptional regulator FadR (CAA30881.1), 3-ketoacyl-CoA thiolase FadA (AAA23751.1); fatty acid oxidation complex subunit alpha FadB (AAA23750.1); long-chain-fatty-acid-CoA ligase FadD (CAA50321.1); acyl-coenzyme A dehydrogenase FadE (AAB08643.1). In addition, the amino acid sequence of the HTH-type transcriptional activator FasR of *Mycobacterium tuberculosis* was used (CCP46023.1). Subsequently, these sequences were blasted against the following TaxIds in NCBI (Edgar 2010): *Methylacidiphilales* (taxid: 717963), *Methylacidiphilum* (taxid: 511745), *M. fumariolicum* SolV (taxid: 1156937), *M. kamchatkense* Kam1 (taxid: 431057), *Methylacidiphilum* sp. RTK17.1 (taxid: 1776078), *Methylacidiphilum* sp. Yel (taxid: 1847730), *Methylacidiphilum* sp. Phi (taxid: 1847729), *M. infernorum* V4 (taxid: 481448), *M. tartarophylax* 4AC (taxid: 1041768), *M. cyclopophantes* 3B (taxid: 1041766), *M. fagopyrum* 3 C (taxid: 1041767), *Methylacidimicrobium* sp. LP2A (taxid: 478741), *M. thermophilum* A8 (taxid: LR797830) and *Methylacidithermus pantelleriae* PQ17 (taxid: GCA_905250085). BLOSUM62 was used as matrix to compare sequences with an e-value of 10^{-6}.

Core gene cluster analysis

Protein-coding genes from assemblies of verrucomicrobial methanotrophs were clustered using usearch (Edgar 2010), with a 0.5 cutoff. These clusters were visualized using the pheatmap package (Kolde 2015) in the R environment (version 4.5.1, R Core Team, 2025).

RNA isolation, RNA-seq and data analysis

M. fumariolicum SolV cells (OD_{600}~2.0) were grown in a chemostat at pH 3.0 and subsequently at pH 1.7. Per condition, three technical replicates consisting of 10 mL cells were collected from the chemostat to create triplicates for transcriptomics. Cells were immediately pelleted for 3 min at 15,000 × *g*, snap-frozen in liquid nitrogen and stored at −80 °C. Total RNA was isolated using the RiboPure™ RNA Purification Kit for bacteria (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Ribosomal RNA was removed from the total RNA samples to enrich for mRNA using the MICROBExpress Bacterial mRNA Enrichment Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. The Qubit high sensitivity RNA and the Agilent RNA 6000 Nano kits and protocols were used for the quantitative and qualitative analysis of the extracted total RNA and enriched mRNA. The latter was used for library preparation by using the TruSeq Stranded mRNA Reference Guide (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. For quantitative and qualitative assessment of the synthesized cDNA, the Qubit double-stranded DNA High Sensitivity and the Agilent High Sensitivity DNA kits and protocols were used.

Transcriptome reads were checked for quality using FastQC (Andrews 2010) and subsequently trimmed 10 base pairs at the 5' end and 5 base pairs at the 3' end of each read. Reads were mapped against the strain SolV Mfumv_2 genome (accession number LM997411) using Bowtie2 (Langmead and Salzberg 2012). The remainder of the analysis and the production of images were done in the R environment (version 4.5.1, R Core Team, 2025). The mapped read counts per gene were determined using Rsubread (Liao et al. 2019) and fold change and dispersion were estimated using DEseq2 (Love et al. 2014). Before performing any statistics, a principal component analysis on the top 1000 genes by variance of each sample was performed to check whether samples within the same condition were both similar to each other and dissimilar to any other sample. For differential expression, a Wald test was employed by DEseq2 to calculate adjusted p-values. Differences in counts were considered to be significant if the base mean was higher than 4, the $\log_2$ fold change was higher than [0.58], and the adjusted p-value was ≤ 0.05. For easy comparisons between samples, we calculated TPM (Transcripts Per Million) values.

Results and discussion

Verrucomicrobial methanotrophs possess a conserved fatty acid biosynthesis pathway

Based on genomic analyses and comparisons with fatty acid biosynthesis pathways in diverse microorganisms, the fatty acid biosynthesis pathway in verrucomicrobial methanotrophs could be predicted (Supplementary File S1).

Although several differences are found between the genera *Methylacidiphilum* and *Methylacidimicrobium*, their fatty acid biosynthesis pathways show strong similarity to the well-described pathway of *Escherichia coli* (Zhang and Rock 2008). The fatty acid biosynthesis in verrucomicrobial methanotrophs is thought to be initiated by the conversion of acetyl-CoA to malonyl-CoA by the acetyl-CoA carboxylase (Cronan and Waldrop 2002). The enzyme involved is encoded by the genes *accABCD*, which are not organized in an operon (Supplementary File S1). Subsequently, the malonyl-group of malonyl-CoA could be transferred to the acyl carrier protein (ACP) by the enzyme FabD to form malonyl-ACP (Ruch and Vagelos 1973). ACP is typically activated by the ACP synthase and subsequently all intermediates in the fatty acid biosynthesis pathway are attached to this carrier protein (Marcella et al. 2017). The enzyme FabH could condense malonyl-ACP with an acyl-CoA molecule, leading to the formation of a β-ketoacyl-ACP molecule (Jackowski and Rock 1987). FabH has been shown to utilize acyl-CoA primers of different lengths, which results in the synthesis of fatty acids of different acyl chain lengths (Khandekar et al. 2001; Qiu et al. 2005). After the synthesis of β-ketoacyl-ACP, the elongation phase of the fatty acid biosynthesis pathway is initiated, in which verrucomicrobial methanotrophs are predicted to use well-studied enzymes. FabG is known to oxidize an NADPH molecule and reduce the product of FabH to β-hydroxy-acyl-ACP (Lai and Cronan 2004). Subsequently, this product is hydrolyzed to form *trans*-2-enoyl-ACP. In verrucomicrobial methanotrophs, a predicted bifunctional enzyme LpxC/FabZ is encoded that might catalyze this reaction. The final step in the elongation cycle to produce acyl-ACP is presumably catalyzed by the enoyl-ACP reductase FabI in verrucomicrobial methanotrophs. In other microorganisms, the isoforms FabV, FabK and FabL with a similar function have been found as well (Rana et al. 2020). In order to add two additional C-atoms to the acyl-chain, the cycle could rerun, condensing another malonyl-group of malonyl-CoA with acyl-ACP. This condensation reaction is presumably catalyzed by FabF in verrucomicrobial methanotrophs (Wang and Cronan 2004; Supplementary File S1).

Saturated fatty acids to which a methyl group is attached to the acyl chain increase membrane fluidity compared to non-methylated saturated fatty acids (Kaneda 1991). In turn, a methyl group attached in *anteiso*-fashion increases membrane fluidity compared to *iso*-fatty acids (Zhang and Rock 2008). Indeed, in *Methylacidiphilum* strains various *anteiso*- and *iso*-fatty acids were previously found (Erikstad et al. 2019; Op den Camp et al. 2009), which could be an adaptation to both a low pH and high temperature (Siliakus et al. 2017; Santiago et al. 2012). FabH, the enzyme that initiates the elongation phase, utilizes an acyl-CoA molecule as primer to produce straight-chain fatty acids. Alternatively, FabH can utilize branched-chain acyl-CoA primers to produce *anteiso*- and *iso*-fatty acids (Qiu et al. 2005). These primers are formed through modification of branched-chain amino acids and are subsequently modified through the same elongation cycle as for the synthesis of straight-chain fatty acids (Kaneda 1991; Choi et al. 2000; Li et al. 2005; Singh et al. 2009; Yu et al. 2016). Verrucomicrobial methanotrophs possess the gene *ilvE* encoding a branched-chain aminotransferase (Santiago et al. 2012). Depending on the type of *anteiso*- and *iso*-fatty acid to be synthesized, this enzyme can convert valine to 3-methyl-oxobutyric acid, leucine to 4-methyl-oxopentanoic acid and isoleucine to 3-methyl-oxopentanoic acid, respectively (Qiu et al. 2005). Subsequently, the branched-chain α-keto acid dehydrogenase (BKD complex) can convert 3-methyl-oxobutyric acid to isobutyryl-CoA, 4-methyl-oxopentanoic acid to isovaleryl-CoA and 3-methyl-oxopentanoic acid to 2-methyl-butyryl-CoA, respectively. These molecules are the precursors that could be used by FabH to produce branched-chain fatty acids and subsequently enter the elongation cycle (Qiu et al. 2005). The even-numbered *iso*-C14:0 and *iso*-C16:0 fatty acids are formed with isobutyryl-CoA as primer, whereas odd-numbered *iso*-C15:0 and *iso*-C17:0 fatty acids are formed by using isovaleryl-CoA. In addition, odd-numbered fatty acids *anteiso*-C15:0 and *anteiso*-C17:0 originate from the primer 2-methyl-butyryl-CoA.

Methylacidiphilum and *Methylacidimicrobium* strains possess dissimilar fatty acid profiles

The large percentage of saturated fatty acids of thermophilic *Methylacidiphilum* strains could be an adaptation to the extreme environment in which these methanotrophs live. To assess the effect of temperature on fatty acid composition, three mesophilic *Methylacidimicrobium* strains (strain 3B, 3 C and 4AC) were grown at their optimal temperatures (35–44 °C) and pH 3.0. Their fatty acid profiles were analyzed and compared to those of eleven thermophilic *Methylacidiphilum* strains grown at their respective optima of 55 °C and pH 3.0 to 3.5 (Op den Camp et al. 2009; Erikstad et al. 2019; Awala et al. 2023) and the recently isolated *Methylacidimicrobium* sp. B4 (Awala et al. 2023), with growth optima of 37–42 °C and pH 1.5–3.0. The most common fatty acids detected in the four mesophilic *Methylacidimicrobium* strains (with an average percentage > 10%) are, in sequential order, stearic acid (C18:0), *anteiso*-pentadecanoic acid (aC15:0), oleic acid (C18:1 (Δ9), palmitic acid (C16:0), and 2-methyltridecanoic acid (iC14:0) (Table 1; Supplementary File S2). Strain 3 C and strain 4AC possess similar fatty acid profiles whereas strain 3B and strain B4 produce various other fatty acids in high

Table 1 Percentages of identified fatty acids of four mesophilic verrucomicrobial methanotrophs and one thermophilic (strain SolV) verrucomicrobial methanotroph

Fatty acid	strain 3B	strain 3 C	strain 4AC	strain B4	strain SolV
C13:1 (at 12–13)	–	–	–	0.7	–
C14:0	–	0.2	1.9	7.8	3.4
iso-C14:0	–	1.6	6.4	26.2	9.4
C15:0	–	–	–	–	1.7
anteiso-C15:0	2.9	25.9	22.9	34.0	17.6
iso-C15:0	–	0.2	0.7	0.9	–
C16:0	10.1	13.8	17.9	10.6	17.5
iso-C16:0	1.1	2.7	2.3	1.7	5.4
C16:0 (3-OH)	–	–	–	4.1	0.1
C17:0	3.9	0.5	0.3	–	0.5
anteiso-C17:0	–	4.9	1.2	–	1.4
iso-C17:0	–	0.9	0.7	–	0.3
C18:0	31.4	29.7	28.9	4.4	41.8
iso-C18:0	–	–	–	–	0.9
C18:0 (3-OH)	3.6	0.4	0.6	–	0.1
C18:1 (unknown position)	–	–	0.4	–	–
C18:1 (Δ9)	26.3	16.4	14.8	9.6	–
C18:1 (Δ11)	8.4	1.3	1.0	–	–
C18:1 (6-OH+8-OH)	5.9	0.7	–	–	–
C19:1 (unknown position)	–	0.8	–	–	–
C19:1 (Δ9, Δ10 & Δ13)	6.3	–	–	–	–
References	This study	This study	This study	Awala et al. (2023)	This study

Strain 3B: *M. cyclopophantes* 3B (grown at 44 °C and pH 3.0 in batch); strain 3C: *M. fagopyrum* 3 C (grown at 35 °C and pH 3.0 in batch); strain 4AC: *M. tartarophylax* 4AC (grown at 38 °C and pH 3.0 in a chemostat); strain B4: *Methylacidimicrobium* strain B4 (unspecified, presumably at it growth optima of 37–42 °C and pH 1.5–3.0 in batch; Awala et al. 2023); strain SolV: *M. fumariolicum* SolV (grown at 55 °C and pH 3.0 in a chemostat)

amounts. Unexpectedly, the *Methylacidimicrobium* strain grown at the highest temperature (44 °C; strain 3B) contains the highest amount of unsaturated fatty acids in its membrane (Supplementary File S2). The strain seems to balance the high proportion of unsaturated fatty acids with a low percentage branched-chain fatty acids, as its membrane consists of only 1% *iso*-fatty acids (the other *Methylacidimicrobium* strains 5 to 29%) and only 3% *anteiso*-fatty acids (the other *Methylacidimicrobium* strains 24 to 35%). In strain B4, low amounts of the fatty acid C18:0 were detected, whereas large amounts of *iso*-C14:0 are present (Supplementary File S2). In general, large differences in membrane fatty acids profiles of *Methylacidimicrobium* strains were detected, indicating multiple membrane fatty acid compositions enable living at low pH.

Analysis of the fatty acids of *M. fumariolicum* SolV retrieved in this study shows small differences to the fatty acids retrieved from this bacterium several years ago, but in general the profiles are similar (Op den Camp et al. 2009). In this study the fatty acids were released by acid hydrolysis while previously base hydrolysis was used, which may induce differences in fatty acid composition (Lambert and Moss 1983). Furthermore, minor differences may be caused by the fact that in the current study, *M. fumariolicum* SolV was grown in a chemostat instead of in batch cultures. Nevertheless, differences in fatty acids profiles of strain SolV grown in batch and chemostat are small. In addition, *Methylacidimicrobium* strain 3 C and 4AC also have similar fatty acids profiles, although they were grown in batch and chemostat, respectively.

Whereas the thermophilic *Methylacidiphilum* strains almost exclusively possess saturated fatty acids, the membranes of the mesophilic *Methylacidimicrobium* strains consist on average for 23% of unsaturated fatty acids (Fig. 1a; Supplementary File S2). This difference between genera

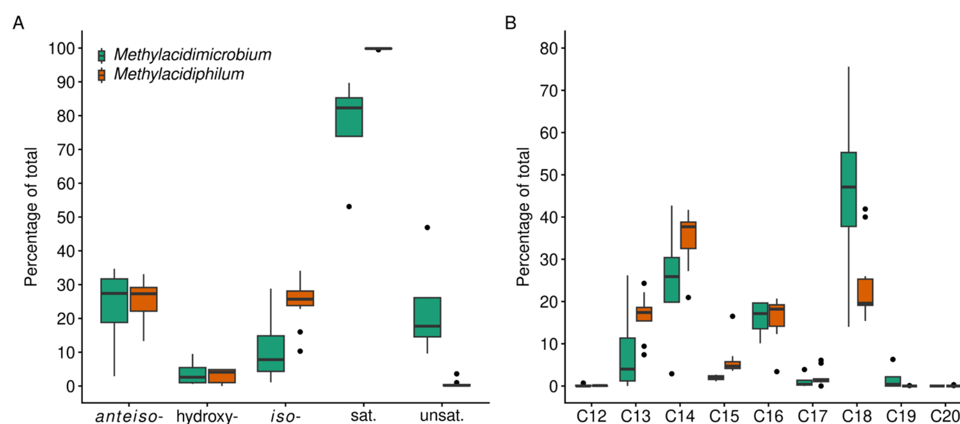

Fig. 1 **a** Boxplots of different types of fatty acids and **b** of different chain lengths detected in four mesophilic *Methylacidimicrobium* strains (*M. tartarophylax* 4AC, *M. cyclopophantes* 3B *M. fagopyrum* 3 C, *Methylacidimicrobium* sp. B4) and eleven *Methylacidiphilum* strains (*M. fumariolicum* SolV, *M. kamchatkense* Kam1, *M. infernorum* V4, *Methylacidiphilum strains* IT6, IT5, Ice, Yel, Phi, Fdl, Rib and Fur). *anteiso*: *anteiso* fatty acids; hydroxy: hydroxyl fatty acids; *iso*: *iso*-fatty acids; sat: saturated fatty acids; unsat: unsaturated fatty acids. The C-number indicates the length of the acyl chain of the fatty acids, without taking methyl-groups into account

is not statistically significant due to substantial variation in membrane composition between species within the genus *Methylacidimicrobium* (Welch's two-sample t-test, $p = 0.11$). The minimal proportion of unsaturated fatty acids in thermophilic *Methylacidiphilum* strains is consistent with the general trend that microbes growing at higher temperatures contain lower levels of unsaturated fatty acids in comparison with those thriving at lower temperatures, to maintain proper membrane fluidity (Suutari and Laakso 1994). In general, the fatty acid profiles between the four analyzed *Methylacidimicrobium* strains show more variation than the profiles between the eleven analyzed *Methylacidiphilum* strains (Fig. 1; Supplementary File S2). Another general phenomenon in thermophiles is the longer acyl chain of fatty acids compared to microorganisms growing at lower temperatures (Siliakus et al. 2017). The longer the acyl chain the more interaction occurs between neighboring acyl chains of fatty acids and hence results in an increased rigidity (Russell 1989). Surprisingly, however, the thermophilic *Methylacidiphilum* strains possess shorter acyl chain lengths compared to their mesophilic counterparts (Fig. 1b). In *Methylacidiphilum* strains, C14 fatty acids are most common, whereas C18 fatty acids are most common in *Methylacidimicrobium* strains. In the former genus, only about a quarter of all fatty acids is C18 or longer, whereas strains of the latter genus consist for almost half of C18 fatty acids or longer.

M. fumariolicum SolV synthesizes unsaturated fatty acids at lower temperature

In the natural environment, verrucomicrobial methanotrophs could encounter rapid fluctuations in temperature and would therefore need to modify their fatty acid composition accordingly to maintain the desired membrane fluidity. To investigate the effect of temperature on fatty acids, *M. fumariolicum* SolV was grown in a chemostat at 55 °C and subsequently abruptly at 45 °C, after which the fatty acids were analyzed when the culture reached steady state. *M. fumariolicum* SolV reacts to a decrease in growth temperature of 10 °C by synthesizing the unsaturated fatty acid C18:1, which is not detected when grown at 55 °C (Supplementary Fig. 1). This monounsaturated fatty acid increases the membrane fluidity, which is a common strategy in response to a decrease in temperature (Siliakus et al. 2017). Indeed, the mesophilic *Methylacidimicrobium* strains that were grown at temperatures of 35–44 °C possess large proportions of monounsaturated C18 fatty acids (Table 1). However, the membrane fatty acid composition of *M. fumariolicum* SolV grown at pH 3.0 and 45 °C is still distinct from the fatty acid composition of *M. cyclopophantes* 3B grown at pH 3.0 and 44 °C. The former strain was grown in a continuous chemostat, while the latter was grown in batch at maximum specific growth rate (μ_{max}), which may influence the fatty acid composition of the membrane. In general, closely related microorganisms cultivated under the same extreme conditions can have various ways to achieve the necessary membrane fluidity and integrity, which is observed in a variety of acidophiles (Siliakus et al. 2017).

Based solely on genomic comparisons, it cannot be concluded how unsaturated fatty acids are produced by verrucomicrobial methanotrophs *de novo*. Alpha- and Gammaproteobacteria possess a gene encoding FabA, catalyzing the same dehydration reaction in the elongation cycle as FabZ, which these Proteobacteria also possess (Zhang and Rock 2008). Unlike FabZ, FabA is also able to convert *trans*-2-decenoyl-ACP (i.e., a *trans*-2-enoyl-ACP molecule with 10 carbons) to *cis*-3-enoyl-ACP, creating the precursor for the synthesis of *cis*-unsaturated fatty acids (Heath and Rock 1996; Dodge et al. 2019). FabA therefore represents a branch point between the elongation of saturated fatty acids and the synthesis of unsaturated fatty acids. In *Methylacidimicrobium* strains, but not in *Methylacidiphilum* strains, a gene is found that is classified by InterPro as part of the family "Beta-hydroxydecanoyl thiol ester dehydrase FabA/FabZ" (IPR013114). Accordingly, the *Methylacidimicrobium* strains might use this enzyme for the synthesis of unsaturated fatty acids, which are present only in very low quantity in *Methylacidiphilum* strains (Erikstad et al. 2019; Op den Camp et al. 2009). However, to elongate unsaturated fatty acids produced via FabA, the enzyme FabB is required as well, which is absent in all verrucomicrobial methanotrophs (Heath and Rock 1996; Campbell and Cronan 2001).

Mutation studies in *E. coli* and *Pseudomonas aeruginosa* strains revealed FabA to be essential for the synthesis of unsaturated fatty acids (Wang and Cronan 2004). Interestingly, however, in the Gram-positive bacterium *Enterococcus faecalis*, two FabZ homologs and two FabF homologs are found (Wang and Cronan 2004). Complementation studies revealed that one of the FabZ and FabF homologs of *E. faecalis* can take over the function of FabA and FabB in *E. coli*, respectively. Thus, FabA and FabB are not necessarily required for *de novo* synthesis of unsaturated fatty acids in all bacteria. In this light it is interesting to note that only *Methylacidimicrobium* strains possess two dissimilar homologues of FabZ and FabF, whereas the *Methylacidiphilum* strains only possess one of each. Nevertheless, Wang and Cronan (2004) noted that amino acid sequence comparisons do not suffice in functional predictions, since one FabZ homolog of *E. faecalis* functions as a FabA enzyme but is much more similar in amino acid sequence identity to *E. coli*-FabZ than to *E. coli*-FabA.

The fact that *Methylacidimicrobium* strains possess large amounts of unsaturated fatty acids whereas in

Methylacidiphilum strains they were barely detected, combined with the additional genes encoding FabZ and FabF homologs in *Methylacidimicrobium* strains that are absent in *Methylacidiphilum* strains, could point to a specific regulation for unsaturated fatty acid synthesis. Nevertheless, mutagenesis studies and isolation of these enzymes are needed for verification. Apart from the *de novo* synthesis of unsaturated fatty acids, microorganisms possess mechanisms to desaturate straight-chain fatty acids by introducing a *cis*-bond in response to environmental changes such as a decrease in temperature (Mansilla et al. 2004; Aquilar and de Mendoza 2006). Interestingly, both *Methylacidiphilum* and *Methylacidimicrobium* strains code for desaturases to catalyze this reaction, but of different types (Supplementary File S1). A temperature decrease from 55 °C to 45 °C in *M. fumariolicum* SolV led to a decrease in C18:0 and a corresponding increase in C18:1 membrane fatty acids, suggesting that a desaturase may have catalyzed this conversion as a mechanism to respond to a sudden temperature decrease (Supplementary Figure S1). Genes encoding regulatory proteins involved in fatty acid biosynthesis (i.e., FabR and FadR of *E. coli* and FasR of *Mycobacterium tuberculosis*) and beta-oxidation of fatty acids (encoded by *fad* genes of *E. coli*) were not detected in the genomes of the verrucomicrobial methanotrophs analyzed in this study.

Little is known about changes in fatty acid composition as a response to acid stress in acidophiles. Interestingly, the membrane fatty acid content of *M. fumariolicum* SolV does not change upon a decrease from pH 3.0 to 1.7 (Supplementary Figure S2), indicating other mechanisms may be used by verrucomicrobial methanotrophs for pH homeostasis that are not related to the membrane fatty acid composition. Notably, even the membrane fatty acid composition of the same strain grown at different times under the same conditions can differ. Strain SolV grown in the first chemostat (at 55 °C and pH 3.0 before the decrease in temperature) contains *iso*-C14:0 and C14:0 fatty acids, which are absent in the strain grown in the second chemostat (grown under the same conditions, before the decrease in pH). In contrast, the cells of the second chemostat contain larger amounts of *iso*-C16:0, *anteiso*-C17:0 and *iso*-C18:0 fatty acids. Hence, related microbes and even the same strain grown at different times but under the same conditions can have distinct membrane fatty acid compositions, indicating multiple ways exist to obtain the required membrane fluidity under harsh conditions such as low pH and higher temperature. The observation that the membrane fatty acid composition of strain SolV does not change upon a decrease in pH is distinct from what is observed in some other acidophiles. The acidophilic sulfur-oxidizer *Acidithiobacillus ferrooxidans* responds to a decrease in pH by an increase in the ratio of saturated/unsaturated fatty acids (Mykytczuk et al. 2010). In addition, microorganisms were shown to convert monounsaturated fatty acids to cyclopropane fatty acids in response to a decrease in pH, catalyzed by a cyclopropane fatty acid synthase (Zhang and Rock 2008; Sohlenkamp 2017). However, the gene encoding this enzyme is absent in verrucomicrobial methanotrophs. Acidophiles seem to synthesize various combinations of fatty acids to grow in an environment with low pH, such as *iso*- and *anteiso*-branched chain fatty acids and β-hydroxy fatty acids (Siliakus et al. 2017). Nevertheless, acidophiles can have very distinct fatty acid compositions and therefore multiple mechanisms to thrive at low pH seem to exist.

Gram-negative bacteria such as verrucomicrobial methanotrophs possess a cytoplasmic bilayer composed of various phospholipid fatty acids and an outer membrane composed of phospholipids in the inner leaflet and polysaccharides in the outer leaflet (Speth et al. 2012; Konovalova et al. 2017; Vergalli et al. 2020). Cells of *E. coli* that suddenly encountered a lower pH in the environment were observed to have a similarly low pH in the periplasm, suggesting that the outer membrane is relatively permeable to protons compared to the cytoplasmic membrane (Wilks and Slonczewski 2007). The outer membranes of acidophiles could either be less permeable to protons or they could possess periplasmic proteins that are adapted to a low pH. Interestingly, the purified periplasmic lanthanide-dependent methanol dehydrogenase XoxF of *M. fumariolicum* SolV has an optimum activity at neutral pH, which could imply a relatively proton-impermeable outer membrane (Keltjens et al. 2014). In addition to this, verrucomicrobial methanotrophs do not possess genes encoding the periplasmic chaperones HdeA and HdeB found in many Gram-negative neutrophiles to prevent protein aggregation at low pH (Tapley et al. 2009; Hong et al. 2012). The fatty acid analyses of different thermophilic verrucomicrobial methanotrophs revealed fatty acid profiles with large amounts of saturated fatty acids, which represents a combination of fatty acids in the cytoplasmic membrane and the outer membrane (Erikstad et al. 2019).

Putative pH homeostasis mechanisms encoded in the genomes

To investigate whether verrucomicrobial methanotrophs possess genes that could be involved in pH homeostasis, a two-pronged approach was used. Firstly, the genomes of five *Methylacidiphilum* strains, five *Methylacidimicrobium* strains and the metagenome-derived genome of *Methylacidithermus pantelleriae* PQ17, representing a third genus of verrucomicrobial methanotrophs, were investigated for shared gene clusters. 407 core gene clusters were found, of which 403 could be assigned a pfam domain using the

gathering threshold (Fig. 2). These gene clusters were used to search for genes involved in pH homeostasis. In general, large numbers of gene clusters are shared between verrucomicrobial methanotrophs, although clear differences between genera were found (Fig. 2). Secondly, genes known or predicted to be involved in pH homeostasis in acidophiles and genes encoding proteins known or predicted to respond to a sudden decrease in pH in neutrophilic microorganisms were BLASTed against the genomes of the verrucomicrobial methanotrophs.

The most convenient mechanism for pH homeostasis is preventing protons from entering the cell, which can be achieved by synthesizing a membrane with relatively low proton permeability (Konings et al. 2002). Still, protons enter the cell via the ATP synthase at the expense of the proton motive force. This force is made up of a membrane potential (ΔΨ) and a pH gradient (ΔpH), creating an energized membrane. In acidophiles, ΔpH is very large due to the acidic environment and the circumneutral interior pH. Under acid stress, *E. coli* was shown to invert the membrane potential to limit proton influx, resulting in a net positive charge close to the inside of the cytoplasmic membrane compared to the outside of the membrane (Baker-Austin and Dopson 2007; Matin et al. 1982; Zychlinsky and Matin 1983; Richard and Foster 2004). Experiments with acidophilic archaea of the genus *Sulfolobus* suggest that this inverted electrical gradient is obtained by the influx of potassium (K^+) ions via dedicated transporters, diminishing the entry of protons over the membrane (Buetti-Dinh et al. 2016). Indeed, *Methylacidiphilum* sp. RTK17.1 was shown to maintain a reversed electrical gradient at a range of pH values (Carere et al. 2021). In verrucomicrobial methanotrophs, the operon *kdpABC* is found (Supplementary File S3). This operon is present in a variety of microorganisms and encodes a high-affinity potassium-transporting ATPase involved in potassium uptake (Epstein 2003; Pedersen et al. 2019; Neira et al. 2022). When *E. coli* is deprived of potassium, the sensor kinase KdpD and response regulator KdpE activate the expression of the potassium-translocating ATPase, which are also found in verrucomicrobial methanotrophs (Altendorf et al. 1994; Heermann and Jung 2010). However, in the genome of *Methylacidithermus pantelleriae* PQ17 (CheckM completeness 98.6%) this potassium-transporting system could not be detected. Since this genome was derived from a metagenome and no isolate is available, it is unknown at what pH range this bacterium lives and therefore whether an inverted electrical gradient is necessary (Picone et al. 2021a). Nonetheless, *Methylacidithermus pantelleriae* PQ17 seems to code for a dedicated potassium transport channel, as do the other verrucomicrobial methanotrophs. In addition, several verrucomicrobial methanotrophs code for a protein annotated as low-affinity kup-type potassium transporter (Supplementary File S3).

Nutrients and solutes enter the cell via primary or secondary transporters. Through the latter, acidophiles can make use of the influx of protons along the large pH gradient. Protons that have entered the cell either through transporters, leakage, or through the ATP synthase, also need to be removed from the cell to prevent acidification (Baker-Austin and Dopson 2007). Verrucomicrobial methanotrophs possess two types of proton-translocating ATPases used to conserve energy through respiration (Schmitz et al. 2021a; Kruse et al. 2019). Since these protein complexes can work in both directions, they might be used to extrude protons (Slonczewski et al. 2009; Hou et al. 2008). In addition, Kruse et al. (2019) detected two possible sodium/proton antiporters KefB, and a sodium/proton-translocating pyrophosphatase HppA that could all be involved in proton extrusion and

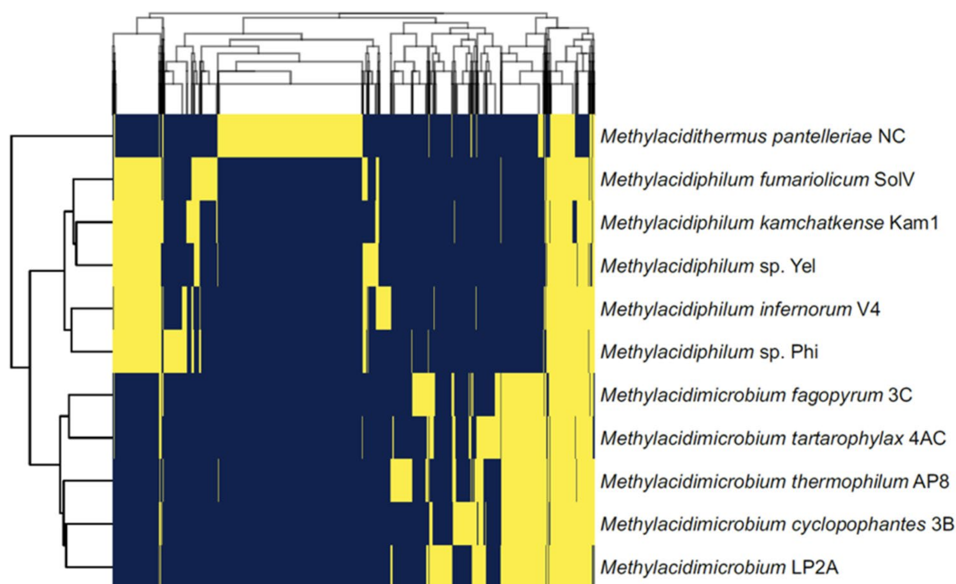

Fig. 2 Absence and presence of gene clusters in verrucomicrobial methanotrophs. The core genome consists of 407 gene clusters, out of a total of 7326 detected gene clusters. Genes are assigned to gene clusters using usearch, with a 0.5 cutoff (in yellow > 0.5, in blue < 0.5) (colour figure online)

are also found in other verrucomicrobial methanotrophs, except for *Methylacidithermus pantelleriae* PQ17 (Supplementary File S3). Genes encoding enzymes with the same proton-pumping mechanisms were found in the genomes of proteobacterial methanotrophs that live in acidic forest soil at a pH of 3 to 4 (Nguyen et al. 2018).

Methylacidiphilum sp. RTK17.1 was shown to maintain an intracellular pH of 6.5 at an extracellular pH of 1.5 to 3.0 (Carere et al. 2021). When its electron transport chain was uncoupled with the use of the chemicals valinomycin and nigericin, the internal pH only decreased to 6.1, which may be the result of the buffering capacity of the cytoplasm and the strong membrane barrier (Carere et al. 2021). In neutrophilic microorganisms, many proteins involved in intracellular proton consumption are upregulated under acid stress (Slonczewski et al. 2009). Verrucomicrobial methanotrophs possess two genes encoding enzymes typically involved in intracellular proton consumption (Supplementary File S3). The glutamate decarboxylase (GadB) is a cytoplasmic enzyme found in many microorganisms to cope with acute acid stress (Lin et al. 1995; Lund et al. 2014). The enzyme catalyzes the decarboxylation of glutamate to γ-aminobutyric acid (GABA), which involves the take-up of a proton and the formation of one CO_2 molecule (Castanie-Cornet et al. 1999). Via the glutamate/GABA antiporter (GadC), GABA is subsequently removed from the cell by exchanging it for glutamate (Castanie-Cornet et al. 1999; Cotter et al. 2001). In addition, all verrucomicrobial strains code for the biosynthetic arginine decarboxylase (SpeA), which catalyzes the decarboxylation of arginine to agmatine, consuming a proton and producing one CO_2 molecule (Richard and Foster 2004) (Supplementary File S3). The decarboxylated product can subsequently be removed from the cell by an antiporter (Slonczewski et al. 2009). Based on genome analyses only, it is not possible to decipher which genes encode the dedicated antiporters in verrucomicrobial methanotrophs. In *E. coli*, GadB and GadC are inactive above pH 6.5, and both have pH optima of 4 to 5.5 (Ma et al. 2012; Pennacchietti et al. 2018). If the homologs in verrucomicrobial methanotrophs have similar pH optima, it is questionable whether this system is employed since even at very low external pH, the internal pH was shown not to decrease below pH 6 (Carere et al. 2021).

As opposed to neutrophilic microorganisms, acidophiles are constantly surrounded by an (extremely) acidic environment. If the intracellular proton concentration becomes too high, acidophiles could use cytoplasmic buffering and proton efflux pumps to restore the internal pH and the membrane potential (Mangold et al. 2013). Still, at a too-low intracellular pH, proteins and DNA could become damaged due to a high concentration of protons (Baker-Austin and Dopson 2007). In verrucomicrobial methanotrophs, a large variety of chaperones are present that were shown to be produced in response to acid in neutrophiles, such as GroEL, GroES, DnaK, and DnaJ (Supplementary File S3) (Carere et al. 2021; Frees et al. 2003; Len et al. 2004; Zanotti and Cendron 2010; Mols et al. 2010). These proteins are used by a wide range of organisms and assist in protein folding during times of stress, while damaged proteins are degraded by the enzyme Clp (Wawrzynow et al. 1996; Lemos and Burne 2002). In addition, RecA and UvrABCD were shown to be involved in acid-induced DNA repair (Hanna et al. 2001; Cappa et al. 2005; Jin et al. 2011; Das et al. 2015). Still, all these proteins are used in response to various stressors and therefore their presence does not necessarily indicate an adaptation to a low pH.

M. fumariolicum SolV upregulates methane respiration genes in response to a low pH

Interestingly, none of the genes proposed to be involved in pH homeostasis in *M. fumariolicum* SolV are significantly upregulated when the bacterium is grown at pH 1.7 compared to growth at pH 3.0 (Supplementary Table 1). The absence of gene regulation when grown at these different pH values could have several explanations. It is possible that a shift in external pH from 3.0 to 1.7 (i.e., a 20-fold increase in proton concentration) is insufficient to modulate expression of the putative pH homeostasis genes. Indeed, the internal pH of *Methylacidiphilum* sp. RTK17.1 remains 6.5 when in an external pH of 1.5 to 3.0 and only fell below 6.5 at an external pH of 1 or lower (Carere et al. 2021). Feasibly, regulation of the genes proposed to be involved in pH homeostasis only occurs at even lower external pH values than those used here. Alternatively, it could be that the genes listed in Supplementary Table S1 are not essential to *M. fumariolicum* SolV in terms of pH homeostasis. Although several genes were shown to be upregulated in response to a decrease in pH in acidophiles and neutrophiles, many proteins encoded by these genes could serve multiple purposes in the cell, which may explain their constitutive expression.

Remarkably, whereas genes predicted to be involved in pH homeostasis were not upregulated or downregulated at different pH values, several genes involved in respiration were heavily upregulated at pH 1.7 compared to pH 3.0 (Table 2; Supplementary Table S1; Supplementary File S4). The *pmoBAC2*, one of three *pmo* operons in *M. fumariolicum* SolV encoding a particulate methane monooxygenase was upregulated 18- to 77-fold. This operon was shown to be highly upregulated under maximum growth conditions in batch cultures (Khadem et al. 2012). In addition, an aa_3-type cytochrome *c* oxidase (Mfumv2_0388−92), an orphan subunit I of an aa_3-type cytochrome *c* oxidase (Mfumv2_2181) and two genes involved in heme *a*

Table 2 Regulation of *Methylacidiphilum fumariolicum* SolV genes of cells grown at pH 1.7 compared to cells grown at pH 3.0

ORF	Annotation	Regulation factor
Mfumv2_0275	Conserved exported protein of unknown function	1.6
Mfumv2_0388	Cytochrome *c* oxidase polypeptide III	2.2
Mfumv2_0389	Predicted small integral membrane protein	1.9
Mfumv2_0390	Conserved protein of unknown function	2.0
Mfumv2_0391	Conserved protein of unknown function	1.7
Mfumv2_0392	Cytochrome *c* oxidase (CyoA)	1.8
Mfumv2_0393	Conserved protein of unknown function	1.6
Mfumv2_0526	Sulfate adenylyltransferase subunit 2 (CysD)	1.8
Mfumv2_0527	Phosphoadenosine phosphosulfate reductase (CysH)	1.9
Mfumv2_0644	Transposase	2.1
Mfumv2_0815	Sulfite reductase [NADPH] hemoprotein beta-component (CysI)	1.7
Mfumv2_1257	Conserved protein of unknown function	1.6
Mfumv2_1449	Hemoglobin-like protein	1.8
Mfumv2_1462	Pyrroloquinoline-quinone synthase (PqqC)	1.7
Mfumv2_1480	Protein of unknown function	2.1
Mfumv2_1562	Putative HupH hydrogenase expression protein	4.2
Mfumv2_1563	Putative Ni/Fe-hydrogenase B-type cytochrome subunit (HupZ)	5.1
Mfumv2_1564	Hydrogenase 1 large subunit (HyaB)	5.3
Mfumv2_1565	Hydrogenase 1 small subunit (HyaA)	5.0
Mfumv2_1616	Ribonuclease HIII	1.6
Mfumv2_1631	Ribonuclease HIII (RnhC)	1.6
Mfumv2_1714	Heme A synthase, cytochrome oxidase biogenesis protein (CtaA)	2.5
Mfumv2_1715	Protoheme IX farnesyltransferase (CtaB)	2.4
Mfumv2_1758	Multicopper oxidase family protein (CueO)	1.6
Mfumv2_1759	Conserved protein of unknown function	1.5
Mfumv2_1794	Methane monooxygenase subunit alpha (PmoB2)	18.0
Mfumv2_1795	Methane monooxygenase subunit beta (PmoA2)	29.3
Mfumv2_1796	Methane monooxygenase subunit gamma (PmoC2)	76.8
Mfumv2_1798	Conserved protein of unknown function	2.2
Mfumv2_1799	DNA-binding response regulator, NarL family REC-HTH domains	2.2
Mfumv2_1800	ABC transport system, permease component YbhR	1.7
Mfumv2_1819	Conserved protein of unknown function	1.6
Mfumv2_1832	Globin domain	2.2
Mfumv2_2181	Alternative cytochrome *c* oxidase subunit 1 (CoxN)	7.1
Mfumv2_2188	Conserved protein of unknown function	2.9
Mfumv2_2215	Conserved membrane protein of unknown function	1.6
Mfumv2_2381	Opacity protein or related surface antigen	1.8
Mfumv2_0170	Outer membrane receptor protein, mostly Fe transport (CirA)	−26.7
Mfumv2_0171	Conserved protein of unknown function	−5.3
Mfumv2_0553	Iron-sulfur cluster insertion protein (ErpA)	−3.3
Mfumv2_0556	Pyridoxal phosphate-dependent enzyme	−1.8
Mfumv2_1062	Protein of unknown function	−1.5
Mfumv2_1470	Glutaredoxin-related protein	−1.7
Mfumv2_2500	Fe-S cluster scaffold complex subunit (SufC)	−1.7

Listed genes have a basemean higher than 4, an upregulation (positive values) or downregulation (negative values) factor higher or lower than 1.5 and an adjusted p-value ≤ 0.05. Numbers are averages of three technical replicates

synthesis (Mfumv2_1714-15) were upregulated (Svensson et al. 1993). *E. coli* was shown to upregulate proton-pumping respiratory complexes in response to acid stress (Slonczewski et al. 2009; Krulwich et al. 2011). Accordingly, the upregulation of genes involved in the aerobic oxidation of methane could be a response to an elevated ΔpH (i.e., an increase in the pH gradient component of the proton motive force due to a larger difference in proton concentration between the cytoplasm and the environment) and as such as a mechanism to extrude excess protons (Siliakus et al. 2017; Carere et al. 2021). Alternatively, greater energetic investments are needed for cellular maintenance at low pH (Mangold et al. 2013). To further investigate these possibilities, detailed physiological studies on methane respiration at different pH values are desirable. Remarkably, the group 1 d [NiFe] hydrogenase (Mfumv2_1562-66), known to oxidize H_2 and feed electrons to the electron transport chain, is upregulated 5-fold in the absence of H_2, suggesting it may

work bidirectionally (Søndergaard et al. 2016; Mohammadi et al. 2017). In addition, three genes (Mfumv2_0526-7 and Mfumv2_0815) involved in the proton-consuming assimilatory conversion of sulfate (SO_4^{2-}) to sulfide (H_2S) were upregulated.

Several genes were downregulated at pH 1.7 compared to pH 3.0, including a 27- and 5-fold downregulation of the outer membrane receptor protein (CirA) (Mfumv2_0170) and the adjacent gene with unknown function (Mfumv2_0171), respectively (Table 2). In *E. coli*, CirA was shown to be involved in the uptake of iron-chelating molecules (siderophores) (Hantke 1990). At lower pH, the solubility and bioavailability of metals generally increase, which may lead to downregulation of receptor protein expression to balance metal uptake. Interestingly, the outer membrane receptor protein CirA of *M. fumariolicum* SolV shares sequence similarity, albeit at 28% identity, with LutH, a protein thought to play a role in periplasmic lanthanide uptake (Groom et al. 2019; Roszczenko-Jasińska et al. 2020; Gorniak et al. 2023; Liu et al. 2025). Verrucomicrobial methanotrophs, including *M. fumariolicum* SolV, rely on lanthanides as essential cofactors for the activity of the methanol dehydrogenase XoxF (Pol et al. 2014; Schmitz et al. 2021b). While considerable progress has been made in identifying lanthanide-binding and -uptake systems in proteobacterial methylotrophs, the molecular mechanisms underlying lanthanide acquisition in verrucomicrobial methanotrophs remain poorly understood. Further experimental investigation is required to identify and characterize novel proteins involved in lanthanide binding and uptake in these extremophiles.

Conclusion

In conclusion, in this study we show the fatty acid compositions of three strains of mesophilic *Methylacidimicrobium* strains 4AC, 3B, and 3 C for the first time. In general, diverse membrane fatty acid compositions were observed for verrucomicrobial methanotrophs, suggesting that multiple adaptive strategies exist to modulate membrane properties for survival under low pH and high-temperature conditions. The membranes of mesophilic *Methylacidimicrobium* strains have almost a quarter (10 to 47%) of their membranes made up of non-saturated fatty acids, whereas thermophilic *Methylacidiphilum* strains have almost none. The level of unsaturation could be explained by mechanisms to preserve appropriate membrane fluidity at different growth temperatures. Indeed, the thermophile *M. fumariolicum* SolV was shown to synthesize monounsaturated C18 fatty acids in response to a decrease in temperature of 10 °C. Still, the membrane fatty acid composition of *M. fumariolicum* SolV after decrease in temperature to 45 °C and *M. cyclopophantes* 3B grown at almost identical pH and temperature are not in the least identical. Hence, even for closely related strains, multiple ways exist to thrive at elevated temperatures at extremely low pH. The fatty acid biosynthesis pathway in verrucomicrobial methanotrophs is conserved, although *Methylacidimicrobium* strains may possess a more versatile pathway to synthesize unsaturated fatty acids. Many genes used by neutrophiles for pH homeostasis were detected in verrucomicrobial methanotrophs and expressed in *M. fumariolicum* SolV. However, a decrease in pH from 3.0 to 1.7 did not result in regulation of these genes nor in a change in membrane fatty acid composition. *M. fumariolicum* SolV primarily responded to a decrease in pH by the upregulation of genes involved in methane respiration pathway, presumably to pump out excess protons. This study highlights the importance of validated predictions through comparative genomics with experimental studies.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00203-025-04630-7.

Author contributions RAS, AP and HJMOdC designed the projects and experiments. SHP, CAI and TAvA performed molecular work including transcriptome sequencing and analyses. CH and GHLN performed cultivation of the microorganisms and physiological experiments supervised by RAS and AP. AM and SS performed lipid extraction and analysis. RAS, MSMJ and HOdC wrote the manuscript. All authors contributed to revision of the manuscript, and read and approved the submitted version.

Funding RAS was supported by the Netherlands Organisation for Scientific Research (NWO) grant VI.Veni.242.027. RAS, CH, and HJMOdC were supported by the European Research Council (ERC advanced grant project VOLCANO 669371). SHP was supported by the Netherlands Organisation for Scientific Research (NWO) grant ALWOP.308. GHLN and MSMJ were supported by the European Research Council (ERC advanced grant project Eco_MoM 339880).

Data availability Data is provided within the manuscript or supplementary information files.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Aguilar PS, de Mendoza D (2006) Control of fatty acid desaturation: a mechanism conserved from bacteria to humans. Mol Microbiol 62:1507–1514. https://doi.org/10.1111/j.1365-2958.2006.05484.x

Altendorf K, Voelkner P, Puppe W (1994) The sensor kinase KdpD and the response regulator KdpE control expression of the kdpFABC operon in Escherichia coli. Res Microbiol 145:374–381. https://doi.org/10.1016/0923-2508(94)90084-1

Andrews S (2010) FastQC: a quality control tool for high throughput sequence data. http://www.bioinformatics.babraham.ac.uk/projects/fastqc

Awala SI, Gwak JH, Kim YM, Kim SJ, Strazzulli A, Dunfield PF, Yoon H, Kim G-J, Rhee S-K (2021) Verrucomicrobial methanotrophs grow on diverse C3 compounds and use a homolog of particulate methane monooxygenase to oxidize acetone. ISME J 15:3636–3647. https://doi.org/10.1038/s41396-021-01037-2

Awala SI, Gwak JH, Kim Y, Seo C, Strazzulli A, Kim SG, Rhee SK (2023) *Methylacidiphilum caldifontis* gen. nov., sp. nov., a thermoacidophilic methane-oxidizing bacterium from an acidic geothermal environment, and descriptions of the family *Methylacidiphilaceae* fam. nov. and order *Methylacidiphilales* ord. nov. Int J Syst Evol Microbiol 73:006085. https://doi.org/10.1099/ijsem.0.006085

Baker BJ, Banfield JF (2003) Microbial communities in acid mine drainage. FEMS Microbiol Ecol 44:139–152. https://doi.org/10.1016/S0168-6496(03)00028-X

Baker-Austin C, Dopson M (2007) Life in acid: pH homeostasis in acidophiles. Trends Microbiol 15:165–171. https://doi.org/10.1016/j.tim.2007.02.005

Benson CA, Bizzoco RW, Lipson DA, Kelley ST (2011) Microbial diversity in nonsulfur, sulfur and iron geothermal steam vents. FEMS Microbiol Ecol 76:74–88. https://doi.org/10.1111/j.1574-6941.2011.01047.x

Buetti-Dinh A, Dethlefsen O, Friedman R, Dopson M (2016) Transcriptomic analysis reveals how a lack of potassium ions increases *Sulfolobus acidocaldarius* sensitivity to pH changes. Microbiology 162:1422–1434. https://doi.org/10.1099/mic.0.000314

Campbell JW, Cronan JE Jr (2001) Bacterial fatty acid biosynthesis: targets for antibacterial drug discovery. Annu Rev Microbiol 55:305–332. https://doi.org/10.1016/S1359-6446(01)01774-3

Cappa F, Cattivelli D, Cocconcelli PS (2005) The UvrA gene is involved in oxidative and acid stress responses in *Lactobacillus helveticus* CNBL1156. Res Microbiol 156:1039–1047. https://doi.org/10.1016/j.resmic.2005.06.003

Carere CR, Hards K, Wigley K, Carman L, Houghton KM, Cook GM, Stott MB (2021) Growth on formic acid is dependent on intracellular pH homeostasis for the thermoacidophilic methanotroph *Methylacidiphilum* sp. RTK17.1. Front Microbiol 12:651744. https://doi.org/10.3389/fmicb.2021.651744

Castaldi S, Tedesco D (2005) Methane production and consumption in an active volcanic environment of Southern Italy. Chemosphere 58:131–139. https://doi.org/10.1016/j.chemosphere.2004.08.023

Castanie-Cornet MP, Penfound TA, Smith D, Elliott JF, Foster JW (1999) Control of acid resistance in *Escherichia coli*. J Bacteriol 181:3525–3535. https://doi.org/10.1128/jb.181.11.3525-3535.1999

Choi KH, Heath RJ, Rock CO (2000) Beta-ketoacyl-acyl carrier protein synthase III (FabH) is a determining factor in branched-chain fatty acid biosynthesis. J Bacteriol 182:365–370. https://doi.org/10.1128/jb.182.2.365-370.2000

Clark K, Karsch-Mizrachi I, Lipman DJ, Ostell J, Sayers EW (2016) GenBank. Nucleic Acids Res 44:D67–D72. https://doi.org/10.1093/nar/gkv1276

Cotter PD, Gahan CG, Hill C (2001) A glutamate decarboxylase system protects *Listeria monocytogenes* in gastric fluid. Mol Microbiol 40:465–475. https://doi.org/10.1046/j.1365-2958.2001.02398.x

Cronan JE Jr, Waldrop GL (2002) Multi-subunit acetyl-CoA carboxylases. Prog Lipid Res 41:407–435. https://doi.org/10.1016/S0163-7827(02)00007-3

Das S, Ganeriwal S, Mangwani N, Patel B (2015) Survival and expression of DNA repair genes in marine bacteria *Pseudomonas pseudoalcaligenes* NP103 and *P. aeruginosa* N6P6 in response to environmental stressors. Microbiology 84:644–653. https://doi.org/10.1134/S0026261715050057

Denich T, Beaudette L, Lee H, Trevors J (2003) Effect of selected environmental and physico-chemical factors on bacterial cytoplasmic membranes. J Microbiol Methods 52:149–182. https://doi.org/10.1016/S0167-7012(02)00155-0

Dodge GJ, Patel A, Jaremko KL, McCammon JA, Smith JL, Burkart MD (2019) Structural and dynamical rationale for fatty acid unsaturation in *Escherichia coli*. Proc Natl Acad Sci USA 116:6775–6783. https://doi.org/10.1073/pnas.1818686116

Driessen AJM, van de Vossenberg JLCM, Konings WN (1996) Membrane composition and ion-permeability in extremophiles. FEMS Microbiol Rev 18:139–148. https://doi.org/10.1016/0168-6445(96)00007-1

Dunfield PF, Yuryev A, Senin P, Smirnova AV, Stott MB, Hou S, Ly B, Saw JH, Zhou Z, Ren Y, Wang J, Mountain BW, Crowe MA, Weatherby TM, Bodelier PLE, Liesack W, Feng L, Wang L, Alam M (2007) Methane oxidation by an extremely acidophilic bacterium of the phylum verrucomicrobia. Nature 450:879–882. https://doi.org/10.1038/nature06411

Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26:2460–2461. https://doi.org/10.1093/bioinformatics/btq461

Epstein W (2003) The roles and regulation of potassium in bacteria. Prog Nucleic Acid Res Mol Biol 75:293–320. https://doi.org/10.1016/S0079-6603(03)75008-9

Erikstad H-A, Ceballos RM, Smestad N, Birkeland N-K (2019) Global biogeographic distribution patterns of thermoacidophilic verrucomicrobia methanotrophs suggest allopatric evolution. Front Microbiol 10:1129. https://doi.org/10.3389/fmicb.2019.01129

Ettwig KF, Butler MK, Le Paslier D, Pelletier E, Mangenot S, Kuypers MM, Schreiber F, Dutilh BE, Zedelius J, de Beer D, Gloerich J, Wessels HJ, van Alen T, Luesken F, Wu ML, van de Pas-Schoonen KT, Op den Camp HJM, Janssen-Megens EM, Francoijs KJ, Stunnenberg H, Weissenbach J, Jetten MSM , Strous M (2010) Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. Nature 464:543–548. https://doi.org/10.1038/nature08883

Foster JW (2004) *Escherichia coli* acid resistance: tales of an amateur acidophile. Nat Rev Microbiol 2:898–907. https://doi.org/10.1038/nrmicro1021

Frees D, Vogensen FK, Ingmer H (2003) Identification of proteins induced at low pH in *Lactococcus lactis*. Int J Food Microbiol 87:293–300. https://doi.org/10.1016/S0168-1605(03)00104-1

Gorniak L, Bechwar J, Westermann M, Steiniger F, Wegner C-E (2023) Different lanthanide elements induce strong gene expression changes in a lanthanide-accumulating Methylotroph. Microbiol Spectr 11:e0086723. https://doi.org/10.1128/spectrum.00867-23

Groom JD, Ford SM, Pesesky MW, Lidstrom ME (2019) A mutagenic screen identifies a TonB-dependent receptor required for the lanthanide metal switch in the type I methanotroph "*Methylotuvimicrobium buryatense*" 5GB1C. J Bacteriol 201:e00120-19. https://doi.org/10.1128/jb.00120-19

Guan N, Liu L (2020) Microbial response to acid stress: mechanisms and applications. Appl Microbiol Biotechnol 104:51–65. https://doi.org/10.1007/s00253-019-10226-1

Hanna MN, Ferguson RJ, Li YH, Cvitkovitch DG (2001) *uvrA* is an acid-inducible gene involved in the adaptive response to low pH in *Streptococcus mutans*. J Bacteriol 183:5964–5973. https://doi.org/10.1128/jb.183.20.5964-5973.2001

Hanson RS, Hanson TE (1996) Methanotrophic bacteria. Microbiol Rev 60:439–471. https://doi.org/10.1128/mr.60.2.439-471.1996

Hantke K (1990) Dihydroxybenzoylserine–a siderophore for E. coli. FEMS Microbiol Lett 55:5–8. https://doi.org/10.1016/0378-1097(90)90158-M

Heath RJ, Rock CO (1996) Roles of the FabA and FabZ beta-hydroxyacyl-acyl carrier protein dehydratases in *Escherichia coli* fatty acid biosynthesis. J Biol Chem 271:27795–27801. https://doi.org/10.1074/jbc.271.44.27795

Heermann R, Jung K (2010) The complexity of the "simple" two-component system KdpD/KdpE in *Escherichia coli*. FEMS Microbiol Lett 304:97–106. https://doi.org/10.1371/journal.ppat.1003201

Hong W, Wu YE, Fu X, Chang Z (2012) Chaperone-dependent mechanisms for acid resistance in enteric bacteria. Trends Microbiol 20:328–335. https://doi.org/10.1016/j.tim.2012.03.001

Hou S, Makarova KS, Saw JHW, Senin P, Ly BV, Zhou Z, Ren Y, Wang J, Galperin MY, Omelchenko MV, Wolf YI, Yutin N, Koonin EV, Stott MB, Mountain BW, Crowe MA, Smirnova AV, Dunfield PF, Feng L, Wang L, Alam M (2008) Complete genome sequence of the extremely acidophilic methanotroph isolate V4, *Methylacidiphilum infernorum*, a representative of the bacterial phylum Verrucomicrobia. Biol Direct 3:26. https://doi.org/10.1186/1745-6150-3-26

Islam T, Jensen S, Reigstad LJ, Larsen O, Birkeland NK (2008) Methane oxidation at 55 degrees C and pH 2 by a thermoacidophilic bacterium belonging to the verrucomicrobia phylum. Proc Natl Acad Sci USA 105:300–304. https://doi.org/10.1073/pnas.0704162105

Jackowski S, Rock CO (1987) Acetoacetyl-acyl carrier protein synthase, a potential regulator of fatty acid biosynthesis in bacteria. J Biol Chem 262:7927–7931. https://doi.org/10.1016/S0021-9258(18)47657-0

Jin J, Liu S, Zhao L, Ge K, Mao X, Ren F (2011) Changes in ffh, uvrA, GroES and DnaK mRNA abundance as a function of acid-adaptation and growth phase in *bifidobacterium longum* BBMN68 isolated from healthy centenarians. Curr Microbiol 62:612–617. https://doi.org/10.1007/s00284-010-9751-x

Johnson DB (2007) Physiology and ecology of acidophilic microorganisms. In: Gerday C, Glansdorf N (eds) Physiology and biochemistry of extremophiles. American Society of Microbiology, Washington, pp 257–270

Johnson DB, Quatrini R (2020) Acidophile microbiology in space and time. Curr Issues Mol Biol 39:63–76. https://doi.org/10.21775/cimb.039.063

Jones DS, Albrecht HL, Dawson KS, Schaperdoth I, Freeman KH, Pi Y, Pearson A, Macalady JL (2012) Community genomic analysis of an extremely acidophilic sulfur-oxidizing biofilm. ISME J 6:158–170. https://doi.org/10.1038/ismej.2011.75

Kambara H, Kawamoto T, Matsushita S, Kindaichi T, Ozaki N, Aoi Y, Takaki Y, Imachi H, Nobu MK, Ogawara M, Ohashi A (2025) First isolation of a methanotrophic *Mycobacterium* reveals ammonia- and pH-tolerant methane oxidation. Appl Environ Microbiol. https://doi.org/10.1128/aem.00796-25

Kaneda T (1991) Iso- and anteiso-fatty acids in bacteria: biosynthesis, function, and taxonomic significance. Microbiol Rev 55:288–302. https://doi.org/10.1128/mr.55.2.288-302.1991

Kanjee U, Houry WA (2013) Mechanisms of acid resistance in *Escherichia coli*. Annu Rev Microbiol 67:65–81. https://doi.org/10.1146/annurev-micro-092412-155708

Keltjens JT, Pol A, Reimann J, Op den Camp HJM (2014) PQQ-dependent methanol dehydrogenases: rare-earth elements make a difference. Appl Microbiol Biotechnol 98:6163–6183. https://doi.org/10.1007/s00253-014-5766-8

Khadem AF, Pol A, Wieczorek AS, Jetten MSM, Op den Camp HJM (2012) Metabolic regulation of "*Ca. Methylacidiphilum fumariolicum*" SolV cells grown under different nitrogen and oxygen limitations. Front Microbiol 3:1–15. https://doi.org/10.3389/fmicb.2012.00266

Khandekar SS, Gentry DR, Van Aller GS, Warren P, Xiang H, Silverman C, Doyle ML, Chambers PA, Konstantinidis AK, Brandt M, Daines RA, Lonsdale JT (2001) Identification, substrate specificity, and inhibition of the *Streptococcus pneumoniae* beta-ketoacyl-acyl carrier protein synthase III (FabH). J Biol Chem 276:30024–30030. https://doi.org/10.1074/jbc.M101769200

Kolde R (2015) pheatmap: pretty heatmaps [Software]. https://cran.r-project.org/web/packages/pheatmap/index.html

Konings WN, Albers SV, Koning S, Driessen AJ (2002) The cell membrane plays a crucial role in survival of bacteria and archaea in extreme environments. Antonie Van Leeuwenhoek 81:6–72. https://doi.org/10.1023/A:1020573408652

Konovalova A, Kahne DE, Silhavy TJ (2017) Outer membrane biogenesis. Annu Rev Microbiol 71:539–556. https://doi.org/10.1146/annurev-micro-090816-093754

Krulwich TA, Sachs G, Padan E (2011) Molecular aspects of bacterial pH sensing and homeostasis. Nat Rev Microbiol 9:330–343. https://doi.org/10.1038/nrmicro2549

Kruse T, Ratnadevi CM, Erikstad HA, Birkeland NK (2019) Complete genome sequence analysis of the thermoacidophilic verrucomicrobial methanotroph "*Candidatus* Methylacidiphilum kamchatkense" strain Kam1 and comparison with its closest relatives. BMC Genomics 20:642. https://doi.org/10.1186/s12864-019-5995-4

Lai CY, Cronan JE (2004) Isolation and characterization of beta-ketoacyl-acyl carrier protein reductase (fabG) mutants of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium. J Bacteriol 186:1869–1878. https://doi.org/10.1128/jb.186.6.1869-1878.2004

Lambert MA, Moss CW (1983) Comparison of the effects of acid and base hydrolyses on hydroxy and cyclopropane fatty acids in bacteria. J Clin Microbiol 18:1370–1377. https://doi.org/10.1128/jcm.18.6.1370-1377.1983

Langmead B, Salzberg SL (2012) Fast gapped-read alignment with bowtie 2. Nat Methods 9:357. https://doi.org/10.1038/nmeth.1923

Lemos JA, Burne RA (2002) Regulation and physiological significance of ClpC and ClpP in *Streptococcus mutans*. J Bacteriol 184:6357–6366. https://doi.org/10.1128/jb.184.22.6357-6366.2002

Len A, Harty D, Jacques N (2004) Stress-response proteins are upregulated in *Streptococcus mutans* during acid tolerance. Microbiology 150:1339–1351. https://doi.org/10.1099/mic.0.27008-0

Li Y, Florova G, Reynolds KA (2005) Alteration of the fatty acid profile of *Streptomyces coelicolor* by replacement of the initiation enzyme 3-ketoacyl acyl carrier protein synthase III (FabH). J Bacteriol 187:3795–3799. https://doi.org/10.1128/jb.187.11.3795-3799.2005

Liao Y, Smyth GK, Shi W (2019) The R package Rsubread is easier, faster, cheaper and better for alignment and quantification of RNA sequencing reads. Nucleic Acids Res 47:e47. https://doi.org/10.1093/nar/gkz114

Lin J, Lee IS, Frey J, Slonczewski JL, Foster JW (1995) Comparative analysis of extreme acid survival in <Emphasis Type="Italic">Salmonella typhimurium</Emphasis>, <Emphasis Type="Italic">Shigella flexneri</Emphasis>, and <Emphasis Type="Italic">Escherichia coli</Emphasis>. J Bacteriol 177:4097–4104. https://doi.org/10.1128/jb.177.14.4097-4104.1995

Liu C, Yin X, Op den Camp HJM, Erb TJ (2025) Perspective: roles of rare Earth elements in bacteria. Green Carbon. https://doi.org/10.1016/j.greenca.2025.02.007

Love MI, Huber W, Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 15:550. https://doi.org/10.1186/s13059-014-0550-8

Lund P, Tramonti A, De Biase D (2014) Coping with low pH: molecular strategies in neutralophilic bacteria. FEMS Microbiol Rev 38:1091–1125. https://doi.org/10.1111/1574-6976.12076

Ma D, Lu P, Yan C, Fan C, Yin P, Wang J, Shi Y (2012) Structure and mechanism of a glutamate-GABA antiporter. Nature 483:632–636. https://doi.org/10.1038/nature10917

Madshus IH (1988) Regulation of intracellular pH in eukaryotic cells. Biochem J 250:1–8. https://doi.org/10.1042/bj2500001

Mangold S, Rao Jonna V, Dopson M (2013) Response of *Acidithiobacillus caldus* toward suboptimal pH conditions. Extremophiles 17:689–696. https://doi.org/10.1007/s00792-013-0553-5

Mansilla MC, Cybulski LE, Albanesi D, de Mendoza D (2004) Control of membrane lipid fluidity by molecular thermosensors. J Bacteriol 186:6681–6688. https://doi.org/10.1128/jb.186.20.6681-6688.2004

Marcella AM, Culbertson SJ, Shogren-Knaak MA, Barb AW (2017) Structure, high affinity, and negative cooperativity of the *Escherichia coli* holo-(acyl carrier protein):holo-(acyl carrier protein) synthase complex. J Mol Biol 429:3763–3775. https://doi.org/10.1016/j.jmb.2017.10.015

Matin A, Wilson B, Zychlinsky E, Matin M (1982) Proton motive force and the physiological basis of delta pH maintenance in *Thiobacillus acidophilus*. J Bacteriol 150:582–591. https://doi.org/10.1128/jb.150.2.582-591.1982

Mohammadi SS, Pol A, van Alen T, Jetten MSM, Op den Camp HJM (2017) *Methylacidiphilum fumariolicum* SolV, a thermoacidophilic 'Knallgas' methanotroph with both an oxygen-sensitive and -insensitive hydrogenase. ISME J 11:945–958. https://doi.org/10.1038/ismej.2016.171

Mohammadi SS, Schmitz RA, Pol A, Berben T, Jetten MSM, Op den Camp HJM (2019) The acidophilic methanotroph *Methylacidimicrobium tartarophylax* 4AC grows as autotroph on H_2 under microoxic conditions. Front Microbiol 10:2352. https://doi.org/10.3389/fmicb.2019.02352

Moll R, Schäfer G (1988) Chemiosmotic H<Superscript>+</Superscript>cycling across the plasma membrane of the thermoacidophilic archaebacterium Sulfolobus acidocaldarius. FEBS Lett 232:359–363. https://doi.org/10.1016/0014-5793(88)80769-5

Mols M, van Kranenburg R, Tempelaars MH, van Schaik W, Moezelaar R, Abee T (2010) Comparative analysis of transcriptional and physiological responses of *Bacillus cereus* to organic and inorganic acid shocks. Int J Food Microbiol 137:13–21. https://doi.org/10.1016/j.ijfoodmicro.2009.09.027

Mykytczuk N, Trevors J, Ferroni G, Leduc L (2010) Cytoplasmic membrane fluidity and fatty acid composition of *Acidithiobacillus ferrooxidans* in response to pH stress. Extremophiles 14:427–441. https://doi.org/10.1007/s00792-010-0319-2

Neira G, Vergara E, Holmes DS (2022) Genome-guided prediction of acid resistance mechanisms in acidophilic methanotrophs of phylogenetically deep-rooted verrucomicrobia isolated from geothermal environments. Front Microbiol 13:900531. https://doi.org/10.3389/fmicb.2022.900531

Nguyen NL, Yu WJ, Gwak JH, Kim SJ, Park SJ, Herbold CW, Kim JG, Jung MY, Rhee SK (2018) Genomic insights into the acid adaptation of novel methanotrophs enriched from acidic forest soils. Front Microbiol 9:1982. https://doi.org/10.3389/fmicb.2018.01982

Op den Camp HJM, Islam T, Stott MB, Harhangi HR, Hynes A, Schouten S, Jetten MSM, Birkeland NK, Pol A, Dunfield PF (2009) Environmental, genomic and taxonomic perspectives on methanotrophic verrucomicrobia. Environ Microbiol Rep 1:293–306. https://doi.org/10.1111/j.1758-2229.2009.00022.x

Pagaling E, Yang K, Yan T (2014) Pyrosequencing reveals correlations between extremely acidophilic bacterial communities with hydrogen sulphide concentrations, pH and inert polymer coatings at concrete sewer crown surfaces. J Appl Microbiol 117:50–64. https://doi.org/10.1111/jam.12491

Pedersen BP, Stokes DL, Apell HJ (2019) The KdpFABC complex - K^+ transport against all odds. Mol Membr Biol 35:21–38. https://doi.org/10.1080/09687688.2019.1638977

Pennacchietti E, D'Alonzo C, Freddi L, Occhialini A, De Biase D (2018) The glutaminase-dependent acid resistance system: qualitative and quantitative assays and analysis of its distribution in enteric bacteria. Front Microbiol 9:2869. https://doi.org/10.3389/fmicb.2018.02869

Picone N, Hogendoorn C, Cremers G, Poghosyan L, Pol A, van Alen TA, Gagliano AL, D'Alessandro W, Quatrini P, Jetten MSM, Op den Camp HJM, Berben T (2020) Geothermal gases shape the microbial community of the volcanic soil of Pantelleria, Italy. mSystems 5:e00517–e00520. https://doi.org/10.1128/msystems.00517-20

Picone N, Blom P, Hogendoorn C, Frank J, van Alen T, Pol A, Gagliano AL, Jetten MSM, D'Alessandro W, Quatrini P, Op den Camp HJM (2021a) Metagenome assembled genome of a novel verrucomicrobial methanotroph from Pantelleria Island. Front Microbiol 12:666929. https://doi.org/10.3389/fmicb.2021.666929

Picone N, Blom P, Wallenius AJ, Hogendoorn C, Mesman R, Cremers G, Gagliano AL, D'Alessandro W, Quatrini P, Jetten MSM, Pol A, Op den Camp HJM (2021b) *Methylacidimicrobium thermophilum* AP8, a novel methane- and hydrogen-oxidizing bacterium isolated from volcanic soil on Pantelleria Island, Italy. Front Microbiol 12:637762. https://doi.org/10.3389/fmicb.2021.637762

Pol A, Heijmans K, Harhangi HR, Tedesco D, Jetten MSM, Op den Camp HJM (2007) Methanotrophy below pH 1 by a new verrucomicrobia species. Nature 450:874–878. https://doi.org/10.1038/nature06222

Pol A, Barends TR, Dietl A, Khadem AF, Eygensteyn J, Jetten MS, Op den Camp HJM (2014) Rare earth metals are essential for methanotrophic life in volcanic mudpots. Environ Microbiol 16:255–264. https://doi.org/10.1111/1462-2920.12249

Qiu X, Choudhry AE, Janson CA, Grooms M, Daines RA, Lonsdale JT, Khandekar SS (2005) Crystal structure and substrate specificity of the beta-ketoacyl-acyl carrier protein synthase III (FabH) from *Staphylococcus aureus*. Protein Sci 14:2087–2094. https://doi.org/10.1110/ps.051501605

Rana P, Ghouse SM, Akunuri R, Madhavi YV, Chopra S, Nanduri S (2020) FabI (enoyl acyl carrier protein reductase) - a potential broad spectrum therapeutic target and its inhibitors. Eur J Med Chem 208:112757. https://doi.org/10.1016/j.ejmech.2020.112757

Richard H, Foster JW (2004) *Escherichia coli* glutamate- and arginine-dependent acid resistance systems increase internal pH and reverse transmembrane potential. J Bacteriol 186:6032–6041. https://doi.org/10.1128/jb.186.18.6032-6041.2004

Roszczenko-Jasińska P, Vu HN, Subuyuj GA, Crisostomo RV, Cai J, Lien NF, Clippard EJ, Ayala EM, Ngo RT, Yarza F, Wingett JP, Raghuraman C, Hoeber CA, Martinez-Gomez NC, Skovran E (2020) Gene products and processes contributing to lanthanide homeostasis and methanol metabolism in *Methylorubrum extorquens* AM1. Sci Rep 10:12663. https://doi.org/10.1038/s41598-020-69401-4

Ruch FE, Vagelos PR (1973) Characterization of a malonyl-enzyme intermediate and identification of the malonyl binding site in malonyl coenzyme A-acyl carrier protein transacylase of *Escherichia coli*. J Biol Chem 248:8095–8106. https://doi.org/10.1016/S0021-9258(19)43198-0

Russell NJ (1989) Function of lipids: structural roles and membrane functions. In: Ratledge C, Wilkinson SG (eds) Microbial lipids. Academic, London, pp 279–365

Santiago B, MacGilvray M, Faustoferri RC, Quivey RG Jr (2012) The branched-chain amino acid aminotransferase encoded by IlvE is involved in acid tolerance in *Streptococcus mutans*. J Bacteriol 194:2010–2019. https://doi.org/10.1128/jb.06737-11
Schmitz RA, Pol A, Mohammadi SS, Hogendoorn C, van Gelder AH, Jetten MSM, Daumann LJ, Op den Camp HJM (2020) The thermoacidophilic methanotroph *Methylacidiphilum fumariolicum* SolV oxidizes subatmospheric $H_2$with a high-affinity, membrane-associated [NiFe] hydrogenase. ISME J 14:1223–1232. https://doi.org/10.1038/s41396-020-0609-3
Schmitz RA, Peeters SH, Versantvoort W, Picone N, Pol A, Jetten MSM, Op den Camp HJM (2021a) Verrucomicrobial methanotrophs: ecophysiology of metabolically versatile acidophiles. FEMS Microbiol Rev 45:fuab007. https://doi.org/10.1093/femsre/fuab007
Schmitz RA, Picone N, Singer H, Dietl A, Seifert KA, Pol A, Jetten MSM, Barends TRM, Daumann LJ, Op den Camp HJM (2021b) Neodymium as metal cofactor for biological methanol oxidation: structure and kinetics of an XoxF1-type methanol dehydrogenase. mBio 12:e0170821. https://doi.org/10.1128/mbio.01708-21
Schmitz RA, Mohammadi SS, Berben T, Jetten MSM, Pol A, Op den Camp HJM (2022) Methanethiol consumption and hydrogen sulfide production by the thermoacidophilic methanotroph *Methylacidiphilum fumariolicum* SolV. Front Microbiol 13:857442. https://doi.org/10.3389/fmicb.2022.857442
Schmitz RA, Peeters SH, Mohammadi SS, Berben T, van Erven T, Iosif CA, van Alen T, Versantvoort W, Jetten MSM, Op den Camp HJM, Pol A (2023) Simultaneous sulfide and methane oxidation by an extremophile. Nat Commun 14(1):2974. https://doi.org/10.1038/s41467-023-38699-9
Schoen R, Rye RO (1970) Sulfur isotope distribution in solfataras, Yellowstone National Park. Science 170:1082–1084. https://doi.org/10.1126/science.170.3962.1082
Sharp CE, Smirnova AV, Graham JM, Stott MB, Khadka R, Moore TR, Grasby SE, Strack M, Dunfield PF (2014) Distribution and diversity of verrucomicrobia methanotrophs in geothermal and acidic environments. Environ Microbiol 16:1867–1878. https://doi.org/10.1111/1462-2920.12454
Siliakus MF, van der Oost J, Kengen SWM (2017) Adaptations of archaeal and bacterial membranes to variations in temperature, pH and pressure. Extremophiles 21:651–667. https://doi.org/10.1007/s00792-017-0939-x
Sinensky M (1974) Homeoviscous adaptation–a homeostatic process that regulates the viscosity of membrane lipids in *Escherichia coli*. Proc Natl Acad Sci USA 71:522–525. https://doi.org/10.1073/pnas.71.2.522
Singh AK, Zhang YM, Zhu K, Subramanian C, Li Z, Jayaswal RK, Gatto C, Rock CO, Wilkinson BJ (2009) FabH selectivity for anteiso branched-chain fatty acid precursors in low-temperature adaptation in *Listeria monocytogenes*. FEMS Microbiol Lett 301:188–192. https://doi.org/10.1111/j.1574-6968.2009.01814.x
Slonczewski JL, Fujisawa M, Dopson M, Krulwich TA (2009) Cytoplasmic pH measurement and homeostasis in bacteria and archaea. Adv Microb Physiol 55:1–79. https://doi.org/10.1016/S0065-2911(09)05501-5
Sohlenkamp C (2017) Membrane homeostasis in bacteria upon pH challenge. In: Geiger O (ed) Biogenesis of fatty acids, lipids and membranes. Springer, New York, pp 1–13
Søndergaard D, Pedersen CNS, Greening C (2016) HydDB: a web tool for hydrogenase classification and analysis. Sci Rep 6:34212. https://doi.org/10.1038/srep34212
Speth DR, van Teeseling MC, Jetten MSM (2012) Genomic analysis indicates the presence of an asymmetric bilayer outer membrane in planctomycetes and verrucomicrobia. Front Microbiol 3:304. https://doi.org/10.3389/fmicb.2012.00304
Suutari M, Laakso S (1994) Microbial fatty acids and thermal adaptation. Crit Rev Microbiol 20:285–328. https://doi.org/10.3109/10408419409113560
Svensson B, Lübben M, Hederstedt L (1993) *Bacillus subtilis* CtaA and CtaB function in haem A biosynthesis. Mol Microbiol 10:193–201. https://doi.org/10.1111/j.1365-2958.1993.tb00915.x
Tapley TL, Körner JL, Barge MT, Hupfeld J, Schauerte JA, Gafni A, Jakob U, Bardwell JCA (2009) Structural plasticity of an acid activated chaperone allows promiscuous substrate binding. Proc Natl Acad Sci USA 106:5557–5562. https://doi.org/10.1073/pnas.0811811106
van Teeseling MC, Pol A, Harhangi HR, van der Zwart S, Jetten MSM, Op den Camp HJM, van Niftrik L (2014) Expanding the verrucomicrobial methanotrophic world: description of three novel species of *Methylacidimicrobium* gen. nov. Appl Environ Microbiol 80:6782–6791. https://doi.org/10.1128/AEM.01838-14
Vergalli J, Bodrenko IV, Masi M, Moynié L, Acosta-Gutiérrez S, Naismith JH, Davin-Regli A, Ceccarelli M, van den Berg B, Winterhalter M, Pagès JM (2020) Porins and small-molecule translocation across the outer membrane of gram-negative bacteria. Nat Rev Microbiol 18:164–176. https://doi.org/10.1038/s41579-019-0294-2
Wang H, Cronan JE (2004) Functional replacement of the FabA and FabB proteins of *Escherichia coli* fatty acid synthesis by *Enterococcus faecalis* FabZ and FabF homologues. J Biol Chem 279:34489–34495. https://doi.org/10.1074/jbc.M403874200
Wawrzynow A, Banecki B, Zylicz M (1996) The Clp ATPases define a novel class of molecular chaperones. Mol Microbiol 21:895–899. https://doi.org/10.1046/j.1365-2958.1996.421404.x
Whittenbury R, Phillips KC, Wilkinson JF (1970) Enrichment, isolation and some properties of methane-utilizing bacteria. J Gen Microbiol 61:205–218. https://doi.org/10.1099/00221287-61-2-205
Wilks JC, Slonczewski JL (2007) pH of the cytoplasm and periplasm of *Escherichia coli*: rapid measurement by green fluorescent protein fluorimetry. J Bacteriol 189:5601–5607. https://doi.org/10.1128/jb.00615-07
Yu YH, Hu Z, Dong HJ, Ma JC, Wang HH (2016) *Xanthomonas campestris* FabH is required for branched-chain fatty acid and DSF-family quorum sensing signal biosynthesis. Sci Rep 6:32811. https://doi.org/10.1038/srep32811
Zanotti G, Cendron L (2010) Functional and structural aspects of *Helicobacter pylori* acidic stress response factors. IUBMB Life 62:715–723. https://doi.org/10.1002/iub.382
Zhang YM, Rock CO (2008) Membrane lipid homeostasis in bacteria. Nat Rev Microbiol 6:222–233. https://doi.org/10.1038/nrmicro1839
Zychlinsky E, Matin A (1983) Cytoplasmic pH homeostasis in an acidophilic bacterium, *Thiobacillus acidophilus*. J Bacteriol 156:1352–1355. https://doi.org/10.1128/jb.156.3.1352-1355.1983

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