More Than a Methanotroph: A Broader Substrate Spectrum for Methylacidiphilum fumariolicum SolV

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Volcanic areas emit a number of gases including methane and other short chain alkanes, that may serve as energy source for the prevailing microorganisms. The verrucomicrobial methanotroph Methylacidiphilum fumariolicum SolV was isolated from a volcanic mud pot, and is able to grow under thermoacidophilic conditions on different gaseous substrates. Its genome contains three operons encoding a particulate methane monooxygenase (pMMO), the enzyme that converts methane to methanol. The expression of two of these pmo operons is subjected to oxygen-dependent regulation, whereas the expression of the third copy (pmoCAB3) has, so far, never been reported. In this study we investigated the ability of strain SolV to utilize short-chain alkanes and monitored the expression of the pmo operons under different conditions. In batch cultures and in carbon-limited continuous cultures, strain SolV was able to oxidize and grow on C₁–C₃ compounds. Oxidation of ethane did occur simultaneously with methane, while propane consumption only started once methane and ethane became limited. Butane oxidation was not observed. Transcriptome data showed that pmoCAB1 and pmoCAB3 were induced in the absence of methane and the expression of pmoCAB3 increased upon propane addition. Together the results of our study unprecedently show that a pMMO-containing methanotroph is able to co-metabolize other gaseous hydrocarbons, beside methane. Moreover, it expands the substrate spectrum of verrucomicrobial methanotrophs, supporting their high metabolic flexibility and adaptation to the harsh and dynamic conditions in volcanic ecosystems.

Keywords: Methylacidiphilum fumariolicum, higher alkanes, ethane, propane, butane, thermoacidophilic, methanotroph

INTRODUCTION

Methane (CH₄) is a powerful greenhouse gas, which is released to the atmosphere from both natural and anthropogenic sources. About 70–80% of CH₄ is generated biologically and a large part of it is removed in the stratosphere and troposphere through reactions with chlorine and OH radicals (Le Mer and Roger, 2001). In addition, microbial methane oxidation is an important terrestrial methane sink (Conrad, 2020). Bacteria can convert CH₄ to methanol aerobically using the enzyme methane monooxygenase (MMO; Conrad, 2009). Under anaerobic conditions, mostly methanotrophic archaea remove methane via reverse methanogenesis (Welte et al., 2016).
After the original discovery by Soehngen (1906), it was believed for a long time that aerobic methanotrophy was restricted to the phylum Proteobacteria, specifically in the subphyla α- and γ-Proteobacteria (Op den Camp et al., 2009). During the past decade, it was discovered that two bacterial phyla contained new methanotrophic representatives: the intra-aerobic NC10 (Raghoebarsing et al., 2006; Ettwig et al., 2010; Hu et al., 2014) and the Verrucomicrobia (Dunfield et al., 2007; Pol et al., 2007; Islam et al., 2008).

The phylum Verrucomicrobia includes highly acidophilic and mesophilic Methylacidimicrobium species (optimum pH 1–3; temperature 30–44°C) (Sharp et al., 2014; van Teeseling et al., 2014) and thermophilic but less acidophilic strains of the genus Methylacidiphilum (optimum pH 2–2.7; temperature 50–55°C) (Dunfield et al., 2007; Pol et al., 2007; Islam et al., 2008; Erikstad et al., 2019). Methylacidiphilum fumarolicum SolV is the most studied verrucomicrobial methanotroph to date and was initially discovered in the Solfatara volcano near Naples (Italy) (Pol et al., 2007). Strain SolV grows optimally at pH 2.7 and 55°C and it fixes CO₂ via the Calvin cycle and N₂ gas through a nitrogenase enzyme (Khadem et al., 2010, 2011). Methane can be used as energy source, but strain SolV is also able to use hydrogen gas as substrate, even at sub-atmospheric concentrations (Mohammadi et al., 2017; Schmitz et al., 2020). Beside methane, the Solfatara volcano in Naples (and many other volcanic areas) emits a mixture of gas that also includes ethane (C₂H₆, 805–1218 ppbv), propane (C₃H₈, 68–178 ppbv), and butane (C₄H₁₀, 8–18 ppbv) (Capaccioni and Mangani, 2001). These gases are particularly important because they could serve as additional substrate for microorganisms. Further, they are present, together with methane, in natural gas, which is commonly used in households and industries.

The oxidation of C₂–C₄ compounds is mainly observed in a group of bacteria that includes the genera Corynebacterium, Nocardia, Mycobacterium, Rhodococcus, Pseudomonas and the sulfate-reducing bacteria Desulfosarcina/Desulfococcus and Desulfotomaculum (Takahashi, 1980; Ashraf et al., 1994; Hamamura et al., 1999; Kinnaman et al., 2007; Kniemeyer et al., 2001). Recently, oxidation of alkanes under anoxic conditions was reported in archaea and catalyzed by the enzyme ethyl/methyl-coenzyme M reductase (MCR; Laso-Pérez et al., 2016; Borrel et al., 2019; Chen et al., 2019; Seitz et al., 2019; Wang et al., 2019).

In the past, the aerobic oxidation of methane and short-chain alkanes was considered to be carried out by separate groups of microorganisms (Crombie and Murrell, 2014). However, early studies already obtained indications that methanotrophs might be able to oxidize ethane, propane and butane (Leadbetter and Foster, 1960; Hazeu and de Bruyn, 1980; Shennan, 2006). In 2010, Stable Isotope Probing (SIP) experiments linked the oxidation of ethane to the family Methylococcaceae and the oxidation of propane to unclassified y-Proteobacteria (Redmond et al., 2010). Methylcella silvestris (α-Proteobacteria) was the first strain that showed simultaneous growth on methane and propane. This strain contained both a soluble methane monooxygenase (sMMO) and a soluble propane monooxygenase (PrMMO) (Crombie and Murrell, 2014). Genes encoding proteins of the methylmalonyl-CoA pathway of propionate oxidation were induced during growth on propane.

The enzymes involved in aerobic hydrocarbon oxidation are usually soluble di-iron monooxygenases complexes consisting of multiple associated proteins (Shennan, 2006). sMMO also has this structure and exhibits a larger substrate range than the copper-containing particulate methane monooxygenase (pMMO; Burrows et al., 1984). However, the butane monooxygenases of Nocardiodes CF8 and Mycobacterium probably contain copper (Hamamura and Arp, 2000; Coleman et al., 2012).

The mechanism of hydrocarbon oxidation starts with the conversion of the alkane into an alcohol. More specifically, ethane is oxidized to ethanol, acetaldehyde and acetate; propane can be oxidized at the terminal or subterminal carbon atom, leading to the formation of 1-propanol, propionaldehyde, and propanoic acid in case of terminal oxidation and to 2-propanol and acetone in case of sub-terminal oxidation. Butane, instead, is oxidized to 1-butanol, butyraldehyde, and butyric acid (Shennan, 2006).

The genome of strain SolV does not encode sMMO, nor propane or butane monooxygenases, but it shows the presence of three operons for the membrane-bound pMMO. pmocAB1 and pmocAB2 operons are located in close proximity in the genome and their PmoA subunits share 84% amino acid identity. The pmocAB3 operon, instead, is distantly located and its PmoA3 subunit only shares 41% amino acid identity to PmoA1 and PmoA2. Experimental data have demonstrated that the expression of pmocAB1 and pmocAB2 is regulated by oxygen concentrations (Khadem et al., 2012), whereas pmocAB3 expression was so far not detected under any growth condition tested. One hypothesis proposes that pmocAB3 is of ancestral origin and its function could differ from methane oxidation (Fuerst, 2014). Therefore, the aim of this study was to test the ability of strain SolV to grow on short-chain alkanes and to investigate the expression of the three pmoc operons.

Here we report that M. fumarolicum SolV can grow on ethane and propane, but not on butane. When methanol is supplied to a SolV culture with no oxygen limitation, expression of pmocAB1 and pmocAB3 could be detected. Furthermore, pmocAB3 expression increased upon propane addition.

MATERIALS AND METHODS

Microorganism and Medium Composition

Methylacidiphilum fumarolicum strain SolV used in this study was initially isolated from the volcanic region Campi Flegrei, near Naples, Italy (Pol et al., 2007). The medium was composed of 0.2 mM MgCl₂, 6H₂O; 0.2 mM CaCl₂, 2H₂O; 1 mM Na₂SO₄; 2 mM K₂SO₄; 4 mM (NH₄)₂SO₄; 1 mM NaH₂PO₄, H₂O. A trace element solution was added resulting in the following end concentrations: 1 μM NiCl₂, CoCl₂, MoO₃Na₂, ZnSO₄ and CeCl₃; 5 μM MnCl₂ and FeSO₄; 10 μM CuSO₄ and 40–50 μM nitritotriacetic acid (NTA). The pH of medium was adjusted to 2.7 using 1 M H₂SO₄. To avoid precipitation, CaCl₂, 2H₂O
and the rest of the medium were autoclaved separately and mixed after cooling.

**Chemostat Cultivation With Methanol/Ethane**

To test the consumption of ethane, a continuous culture with the standard medium containing 50 mM methanol (CH₃OH; added through a 0.2 μm sterile filter to the medium) was used. The bioreactor was operated at 55°C with stirring at 700 rpm using a stirrer bar. The chemostat (liquid volume of 300 ml) was supplied with the medium at a flow rate of 3.9 ml h⁻¹ (D = 0.013 h⁻¹), using a peristaltic pump. The cell-containing medium was removed automatically from the chemostat by a peristaltic pump when the liquid level reached the sensor in the reactor. A supply of 10% O₂ (v/v) and 5% CO₂ (v/v) in argon (total gas flow = 10.6 ml min⁻¹) was directed to the reactor by mass flow controllers through a sterile filter and sparged into the medium just above the stirrer bar. The initial pH was 2.7 and it was regulated with 0.2 M NaOH connected to the vessel by a peristaltic pump. The pH at steady state was kept at about 2.2. At steady state, while cells were grown under methanol limitation, a supply of ethane was introduced to the reactor. An O₂ sensor (Applikon, Delft, Netherlands) in the liquid was coupled to a Biocontroller (Applikon) to monitor the dO₂ values during growth.

**Chemostat Cultivation With Natural Gas and Methanol/Propane**

Chemostat cultivations with methanol and natural gas were performed in a bioreactor (500 ml MiniBio Reactor, Applikon Biotechnology, Delft, Netherlands) with a working volume of 350 ml liquid medium. The system was run at 55°C and 1500 rpm stirring speed. pH, dO₂, and medium level were monitored by Applikon MyControl Reactor sensors. The natural gas reactor was operated with a dilution rate of 0.024 h⁻¹ (8.4 ml h⁻¹ fresh medium was added) and a constant gas inflow consisting of air, N₂, and natural gas. The natural gas mix used in this experiment consisted of 78.53% methane, 3.34% ethane, 0.46% propane, and 0.09% butane. The remaining 17.58% consisted of N₂, O₂, CO₂, and trace amounts of higher alkanes. In the Netherlands, 1.8 ppm of the sulfur compound tetrahydrothiophene (THT) is added for safety reasons. During the oxygen limiting condition, 0.60 ml/min of natural gas was flowing through the bioreactor, with 2.20 ml/min air and 7.19 ml/min N₂. Under methane limiting condition, 0.60 ml/min of natural gas was supplied to the bioreactor, with 4.40 ml/min air and 4.99 ml/min N₂. Under both conditions, the total gas flow was 9.99 ml/min. In the experiments where propane was supplied, the reactor was operated with a dilution rate of 0.012 h⁻¹ (medium contained 50 mM methanol) and a constant gas inflow consisting of air and CO₂. Propane was added at a rate of 0.1 ml/min, resulting in a total gas flow of 1.6 ml/min. Biomass was measured as optical density at 600 nm (OD₆₀₀) using 1 ml cuvettes with the spectrophotometer Spectronic 200 (Thermo Fisher Scientific, Waltham, MA, United States).

**Dry Weight Determination**

To determine biomass dry-weight concentration, 10 ml of the culture suspension (triplicate) were filtered through pre-weighed 0.45 μm filters and dried to stable weight in a vacuum oven at 70°C.

**Batch Cultivation**

The batch growth experiments were performed using 120 and 250 ml serum bottles containing 10 and 20 ml medium, respectively, with a headspace containing air, CO₂ (10%) and CH₄ (2%), C₂H₆ (4%), or C₃H₈ (4%). All incubations were performed at 55°C at 350 rpm.

**Gas Analysis**

Alkane concentrations were measured injecting 100 μl of sample with a Hamilton glass syringe in a HP 5890 gas chromatograph (Agilent, United States) equipped with a Porapak Q column (1.8 m, ID 2 mm) and a flame ionization detector.

**Respiration Experiments**

Respiration rates were determined polarographically in a respiration cell with an oxygen microsensor (RC350, Strathkelvin, Motherwell, United Kingdom) using 3 ml of whole cell suspensions of strain SolV. Methane, propane, or oxygen saturated media were injected into the respiration chamber to obtain the desired dissolved gas concentrations. The O₂ signal was monitored and recorded using SensorTrac Basic software (Unisense, Aarhus, Denmark). The temperature and stirring rate in the respiration chamber was adjusted to 55°C and 1000 rpm, respectively. Rates were expressed as nmol O₂ min⁻¹ mg DW⁻¹ and when necessary corrected for endogenous respiration.

**RNA Extraction and Transcriptome Analysis**

A volume of 5 ml cell suspension was harvested from a continuous culture at steady state. After centrifugation (10,000 × g, 5 min) the pellet was used for RNA isolation using the RiboPure™-Bacteria kit (ThermoFisher, Waltham, MA, United States) according to manufacturer’s instructions. mRNA was purified with the MegaClear kit (Ambion) and MICROBexpress™ kit (Thermo Fisher Scientific, Waltham, MA, United States) according to manufacturer’s protocol. The efficiency of rRNA and small RNAs removal was analyzed using the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, United States). The mRNA extracted from cells grown with natural gas and with ethane was then converted to cDNA with the Ion Total RNA-Seq™ Kit v2 (Thermo Fisher Scientific, Waltham, MA, United States) following manufacturer’s instructions. cDNA was amplified and purified to prepare barcoded libraries. Ion Sphere™ Particles (ISPs) were used to create template positive ISPs by the Ion OneTouch™ 2 instrument, which were enriched in the Ion OneTouch™ ES instrument. This was performed with the Ion PGM™ Template OT2 200 Kit (Ion Torrent, Life technologies). Sequencing of these templates was conducted on an Ion 318™ Chip v2 using the
Ion PGM™ sequencing 200 Kit v2. The mRNA extracted from cells grown on propane and methanol was used to construct libraries using the TrueSeq Stranded mRNA Library Prep protocol (Illumina) according to the manufacturer’s instructions. Libraries were normalized, pooled and sequenced using the Illumina MiSeq sequencing machine. For sequencing, the 150 bp single-read sequencing chemistry was performed using the MiSeq Reagent Kit v3 (Illumina, San Diego, CA, United States) according the manufacturer’s protocol. The RNA-Seq Analysis tool of the CLC Genomic Work bench software (version 10.1.1, CLC-Bio, Aarhus, Denmark) was used for sequence analysis. As a template for the transcriptome analysis, the complete genome sequence of strain SolV, available at the Microscope annotation platform (Vallenet et al., 2009), was used. Expression values were expressed as RPKM (Mortazavi et al., 2008). Statistical analyses were performed using the DESeq2 package in R Studio (Love et al., 2014). Transcriptomics data were deposited in NCBI Bioproject database with accession number PRJEB39356.

RESULTS

Oxidation of Short-Chain Alkanes by *M. fumariolicum* SolV

The ability of strain SolV to oxidize short-chain alkanes was tested as follows: cells were incubated in bottles containing natural gas (in v/v: 75% CH₄, 3% C₂H₆, and 0.5% C₃H₈), and this resulted in exponential growth with a doubling time of 10.2 h and a growth rate of 0.068 h⁻¹. After 20 h, a strong drop in the methane concentrations was observed and within two days the values were below the detection limit (5 ppm). Ethane seemed to be consumed simultaneously. Propane consumption only started once ethane and methane became limiting. At the end of the experiment, concentrations of both ethane and propane were below detection levels. Remarkably, the sulfur compound THT seemed to be degraded as well as concluded from effluent gas analysis on a gas chromatograph equipped with a sulfur-specific flame-photometric detector (data not shown).

In a follow up experiment, growth of strain SolV was tested in serum bottles with separate methane/alkane mixtures. Results for methane/butane (2 and 4%, v/v), methane/propane (2 and 4%, v/v) and methane/ethane (2 and 4% v/v) are shown in Figures 2A,C,E. Consumption of butane in the presence and/or absence of methane was not observed (Figures 2A,B). Propane was consumed from the start but the rate increased after methane became limiting and, although the exponential growth phase was very short, we could calculate a growth rate of 0.007 h⁻¹ (about 10% of $\mu_{max}$ on CH₄), equivalent to a doubling time of 102 h (Figures 2C,D).

The same batch experiments were also performed with methane/ethane (2 and 4%, v/v) at starting $OD_{600}$ 0.07 (Figure 2E). Both gases were consumed simultaneously. Once methane was completely consumed (250 h), $OD_{600}$ reached 0.8. At this time, about 65% of ethane was still present and growth continued till $OD_{600}$ 1.0 and ethane became limited (Figure 2E). At this point, 5 ml ethane, 10 ml O₂, 4 mM NH₄⁺ and extra trace elements (enough for $OD_{600}$ 5) were added. Growth continued to $OD_{600}$ 1.3.

To further investigate the consumption of short-chain alkanes by SolV, cells were cultivated in a continuous system where oxygen and nutrient concentrations can be easily monitored and controlled. The chemostat was first kept under oxygen-limited conditions, using natural gas as energy source. The culture reached a steady state at $OD_{600}$ 0.96 ± 0.09 and primarily consumed methane and ethane (Table 1). After 25 days, the air inflow was doubled, so that the amount of natural gas became limiting. The $OD_{600}$ increased from 0.96 ± 0.09 to 1.7 ± 0.1 and the dissolved oxygen concentration from 0 to 4%. In this situation, methane consumption doubled and ethane consumption was 4.4 times higher. In addition, significant propane consumption could be detected. Butane consumption was not observed (Table 1). Ethane oxidation happened simultaneously with methane, but propane oxidation...
Oxidation of butane, propane and ethane by strain SolV. 

(A) Consumption of methane and butane in batch cultures. (B) Butane concentration over time in batch cultures containing SolV cells. In panels (A,B) data points represent the average over three biological replicates. All error bars indicating standard deviation (SD) are smaller than the symbols. (C) Batch growth of strain SolV in serum bottles with methane/propane. The first arrow indicates an addition of 4 mM NH$_4^+$ and 10 ml O$_2$. The second arrow indicates an addition of extra trace element solution to avoid any limitation. (D) Growth of strain SolV with only propane using an inoculum from the incubation depicted in panel (C). (E) Growth of strain SolV with methane and ethane mixture, followed by a growth only using ethane. The arrow indicates an addition of 5 ml ethane, 10 ml O$_2$, 4 mM NH$_4^+$ and extra trace elements. In panels (C–E), data points represent the average over two biological replicates.
only started once methane became limiting, confirming the results of the batch experiments.

The capability of multiple substrates consumption by co-metabolism provided further opportunities to establish a correlation between physiological activity and gene expression in strain SolV. The genome of strain SolV shows the presence of three pmo operons. To investigate their expression, a transcriptome analysis was performed under both the oxygen and natural gas limited conditions described above. Under oxygen limited conditions, pmoCAB2 had the highest expression levels, whereas pmoCAB1 had the highest expression under natural gas limitation (Supplementary Figure 1). These data confirmed the oxygen-dependent regulation of these operons observed before in strain SolV (Khadem et al., 2012). The third operon, pmoCAB3, did not show high levels of expression in both conditions.

Natural gas only contains minor amounts of ethane and propane. To investigate the consumptions of these alkanes in more detail, higher concentrations were used in a chemostat with methanol as the additional substrate. Methanol, which is as effective as methane for growth, was used instead of methane to induce its expression. The transcriptome data did not reveal any upregulation of genes that could point to the pathway used for further oxidation of ethane after conversion to acetate.

Similarly, to test the growth of strain SolV in a continuous culture grown with propane, another bioreactor using methanol as electron donor was started (D = 0.012 h\(^{-1}\)). Under methanol limitation conditions, biomass reached a steady state at OD\(_{600}\) 0.84 ± 0.05. Propane was then added at a rate of 4.5 \(\mu\)mol min\(^{-1}\). The propane addition resulted in a new steady state at OD\(_{600}\) 1.13 ± 0.04, which equals an increase of 34% in biomass. Out of the supplied propane, only 0.1% was removed and the consumption of propane was measured at a rate of 0.46 ± 0.14 nmol min\(^{-1}\) mg DW\(^{-1}\).

Since the alkane consumption was so low, we checked the propane oxidation rate in respiration experiments using SolV cells from a continuous culture grown on methane (oxygen limited; D = 0.017 h\(^{-1}\)) (Khadem et al., 2012) and we calculated a respiration rate on propane of 2.4 nmol O\(_2\) min\(^{-1}\) mg DW\(^{-1}\). This value corresponds to 0.48 nmol propane min\(^{-1}\) mg DW\(^{-1}\), which is in agreement with what calculated in the reactor (0.46 nmol propane min\(^{-1}\) mg DW\(^{-1}\)).

For the methanol/propane culture, a transcriptome analysis was performed, but this time we analyzed both cells growing on methanol/propane and on methanol only. As shown in Figure 4, pmoCAB1 had the highest expression in both conditions, followed by pmoCAB3. The pmoCAB2 operon was not expressed. An upregulation of pmoCAB3 and downregulation of pmoCAB1 in presence of propane seemed to happen. However, the difference in the expression of pmoCAB1 was not statistically significant (p > 0.05), whereas expression of pmoCAB3 was significantly higher (p = 0.00). These data shows that pmoCAB3 expression is linked to the absence of methane or presence of methanol, but its expression levels increase when propane is supplied. The transcriptome data did not reveal upregulation of genes that could point to the pathway used for further oxidation of propane after conversion to propanoate.

### Oxidation of Ethane and Propane in Strain SolV

A continuous culture using methanol as an electron donor (medium flow rate = 3.9 ml h\(^{-1}\); D = 0.013 h\(^{-1}\)) was established. At the steady state, when methanol was the limiting growth factor, the optical density was stable (OD\(_{600}\) = 1.03 ± 0.02) and the consumption of methanol occurred at a rate of 25.9 nmol min\(^{-1}\) mg DW\(^{-1}\). After reaching a steady state, ethane was supplied to the cells. The amount of ethane provided to the bioreactor was slowly increased from 0.36 to 7.2 \(\mu\)mol min\(^{-1}\). Average consumption of the supplied ethane was 50% and this resulted in an increase of OD\(_{600}\) from 1.01 to 1.71 (70% increase). The highest ethane consumption rate measured was 15.6 nmol min\(^{-1}\) mg DW\(^{-1}\), which is about 60% of the methanol consumption rate (25.9 nmol min\(^{-1}\) mg DW\(^{-1}\)), supporting the increase in optical density.

Further, cells of *M. fumariolicum* SolV grown with methanol and ethane were harvested and RNA was extracted to perform transcriptome analysis. Analysis of the expression of the pmo operons showed that pmoCAB1 had the highest expression values while expression of pmoCAB2 was hardly detectable (Figure 3). Surprisingly, pmoCAB3 expression was noticed for the first time, suggesting that either the presence of methanol or ethane could induce its expression. The transcriptome data did not reveal any upregulation of genes that could point to the pathway used for further oxidation of ethane after conversion to acetate.

### DISCUSSION

In this study, we show that *M. fumariolicum* SolV is able to co-metabolize ethane and propane with methane or methanol, but not butane. To our knowledge, the ability to use other gaseous hydrocarbons for growth is unprecedented in a pMMO-encoding methanotroph (with no sMMO present). Most methanotrophs are highly selective, to the point that they only grow on methane and its one-carbon derivatives such as methanol, and cannot grow on complex, multi-carbon substrates like sugars or organic acids. However, the facultative methanotroph *Methylcella silvestris* that only contains sMMO (and PrMMO) is able to grow on acetate, ethanol, pyruvate, succinate, malate and propane in addition to methane and methanol (Dedysh et al., 2005; Chen et al.,

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**TABLE 1** | Consumption of methane, ethane, propane, and butane by *M. fumariolicum* SolV during O\(_2\) and natural gas limitation.

|          | O\(_2\) limitation\(^a\) | Natural gas limitation\(^b\) |
|----------|--------------------------|-----------------------------|
| Methane  | 35.3 ± 1.4%              | 73.2% (73.0–73.3)           |
| Ethane   | 15.5 ± 1.3%              | 67.9% (67.6–68.1)           |
| Propane  | 0 ± 0%                   | 10.2% (10.2–10.2)           |
| Butane   | ND\(^c\)                 | ND\(^c\)                    |

\(^a\)Values are the average ± standard deviation (n = 8).

\(^b\)Values are the average of two measurements with separate values between brackets.

\(^c\)Butane oxidation under oxygen and methane limitation could not be determined accurately because butane concentration in natural gas were close to the detection limit of the gas chromatograph.
It is expected that ethane and propane are first converted by pMMO to ethanol and propanol, respectively. The XoxF-type PQQ-dependent methanol dehydrogenase of strain SolV was shown to convert these alcohol to their aldehyde (acetaldehyde, propanaldehyde) and acid (acetate, propanoate) forms (Pol et al., 2014). The transcriptome data did not reveal upregulation of genes that could point to the further oxidation of these metabolites. Acetate could be shuttled into the central metabolism by the acetyl-CoA synthetase present in the genome of strain SolV (Mfumv2_2288). The enzymology of propane oxidation after the first two steps is poorly understood. Crombie and Murrell (2014) found an induction of the methylmalonyl-CoA pathway enzymes during growth on propane. However, the genome of strain SolV does not encode for these enzymes. However, acetyl-CoA synthetase (EC 6.2.1.1) is also known to convert propanoate into propanoyl-CoA. Metabolomic studies could shed light on the pathways involved.

The oxidation of ethane and propane in *M. fumaroliicum* SolV proceeded at different rates. In particular, ethane seemed to be preferred over propane since (i) it could be oxidized simultaneously with methane, (ii) the oxidation rate was faster, and (iii) it led to a higher increase in biomass (70% vs 34%).
This probably depends on the ability of pMMO of binding and converting molecules with different number of carbon atoms. Moreover, differences in the ethane oxidation rate could be noticed in different conditions. In particular, a 10-fold increase in the ethane consumption rate was calculated in the continuous culture compared to the batch incubations. This discrepancy could be due to CO₂ or NH₄⁺ limitations in the batch experiments. The solubility of the different alkanes at 55°C do not differ much. The values calculated from mole fractions taken from Wilhelm et al. (1977) were 0.92 mM for methane, 1.04 mM for ethane, 0.79 mM for propane, and 0.56 mM for butane.

Contrary to ethane and propane, butane consumption was not observed in strain SolV. Butane oxidation, together with propane and ethane, was detected in Methylosinus trichosporium OB3b. This bacterium encodes both sMMO and pMMO (Burrows et al., 1984), but its pMMO is different than the ones encoded by strain SolV. PmoA from M. trichosporium OB3b only shares 53% amino acid identity to strain SolV’s PmoA1, 51% to PmoA2 and 40% to PmoA3.

The sMMO-containing methanotroph Methylococcus capsulatus Bath also showed the ability of oxidizing C₁–C₈ compounds (Colby et al., 1977). The oxidation of these alkanes in M. capsulatus and M. trichosporium, however, was not linked to growth. Additionally, butane oxidation has been documented in the genera Nocardioides, Mycobacterium, Giesbergeria, Ramlilbacter, Arthrobacter, Brevibacterium (McLee et al., 1972; Hamamura et al., 1999; Hamamura and Arp, 2000; Deng et al., 2018) and in the β-proteobacterium Thauera butanovora (Arp, 1999). The butane monooxygenase of T. butanovora (sBMO) presents high identity (38–65%) to sMMO as it contains three subunits α, β and γ encoded by bmoX, bmoY and bmoZ genes and a non-haem carboxylate-bridged diiron site (Sluis et al., 2002). The sBMO has a much lower affinity for methane (1.1 mM) compared to sMMO (3–13 µM) (Cooley et al., 2009). The butane degrading strain CF8, instead, seems to possess a pBMO similar to pMMO with subunit identities of 34–47% (Hamamura et al., 1999; Kinnaman et al., 2007; Sayavedra-Soto et al., 2011). A copper containing monooxygenase in Mycobacterium able to oxidize C₂–C₄ alkanes was also described (Coleman et al., 2012).

In conclusion, this study demonstrates that, beside methane and hydrogen, verrucomicrobial methanotrophs are also able to co-metabolize higher alkanes. This result is particularly important in view of the ecological role of these bacteria in the environment. Methanotrophic Verrucomicrobia appear to be not only extremely resistant to thermoacidic geothermal volcanoes, but also remarkably flexible in terms of substrate utilization. Their metabolic flexibility regarding carbon compounds could
be partly provided by the differential expression of the pmoCAB copies in relation to the substrate available.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI BioProject, accession no: PRJEB39356.

AUTHOR CONTRIBUTIONS

NP, SM, AP, MJ, and HO designed the projects and experiments. NP and AW performed the natural gas experiments. NP, SM, and AP performed the ethane/propane experiments. NP and TA sequenced and analyzed the transcriptomes. NP, AP, and HO carried out the data analysis. NP, SM, and HO wrote the manuscript. All authors contributed to revision of the manuscript, and read and approved the submitted version.

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The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.604485/full#supplementary-material
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Contact of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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