Ammonia Oxidation and Nitrite Reduction in the Verrucomicrobial Methanotroph *Methylacidiphilum fumariolicum* SolV

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The Solfatara volcano near Naples (Italy), the origin of the recently discovered verrucomicrobial methanotroph *Methylacidiphilum fumariolicum* SolV was shown to contain ammonium (NH\textsubscript{4}\textsuperscript{+}) at concentrations ranging from 1 to 28 mM. Ammonia (NH\textsubscript{3}) can be converted to toxic hydroxylamine (NH\textsubscript{2}OH) by the particulate methane monooxygenase (pMMO), the first enzyme of the methane (CH\textsubscript{4}) oxidation pathway. Methanotrophs rapidly detoxify the intermediate NH\textsubscript{2}OH. Here, we show that strain SolV performs ammonium oxidation to nitrite at a rate of 48.2 nmol NO\textsubscript{2}\textsuperscript{-}.h\textsuperscript{-1}.mg DW\textsuperscript{-1} under O\textsubscript{2} limitation in a continuous culture grown on hydrogen (H\textsubscript{2}) as an electron donor. In addition, strain SolV carries out nitrite reduction at a rate of 74.4 nmol NO\textsubscript{2}\textsuperscript{-}.h\textsuperscript{-1}.mg DW\textsuperscript{-1} under anoxic condition at pH 5–6. This range of pH was selected to minimize the chemical conversion of nitrite (NO\textsubscript{2}\textsuperscript{-}) potentially occurring at more acidic pH values. Furthermore, at pH 6, we showed that the affinity constants (K\textsubscript{s}) of the cells for NH\textsubscript{3} vary from 5 to 270 µM in the batch incubations with 0.5–8% (v/v) CH\textsubscript{4}, respectively. Detailed kinetic analysis showed competitive substrate inhibition between CH\textsubscript{4} and NH\textsubscript{3}. Using transcriptome analysis, we showed up-regulation of the gene encoding hydroxylamine dehydrogenase (haoA) cells grown on H\textsubscript{2}/NH\textsubscript{4}\textsuperscript{+} compared to the cells grown on CH\textsubscript{4}/NO\textsubscript{3} which do not have to cope with reactive N-compounds. The denitrifying genes *nirk* and *norC* showed high expression in H\textsubscript{2}/NH\textsubscript{4}\textsuperscript{+} and CH\textsubscript{4}/NO\textsubscript{3} grown cells compared to cells growing at µ\textsubscript{max} (with no limitation) while the *norB* gene showed downregulation in CH\textsubscript{4}/NO\textsubscript{3} grown cells. These cells showed a strong upregulation of the genes in nitrate/nitrite assimilation. Our results demonstrate that strain SolV can perform ammonium oxidation producing nitrite. At high concentrations of ammonium this may results in toxic effects. However, at low oxygen concentrations strain SolV is able to reduce nitrite to N\textsubscript{2}O to cope with this toxicity.

Keywords: Methylacidiphilum, methanotroph, ammonia, methane, nitrite, reactive N compounds

INTRODUCTION

Methane (CH\textsubscript{4}) is a powerful greenhouse gas, which is released in to the atmosphere both from natural and anthropogenic sources (Conrad, 2009). Understanding sources and sinks of CH\textsubscript{4} is important for future models of climate change on our planet. Methane oxidizing microorganisms are one of the most important biological sinks of CH\textsubscript{4} (Murrell and Jetten, 2009).
Aerobic methanotrophic bacteria belong to a physiological group of bacteria recognized as methylotrophs. The proteobacterial methanotrophs are distinctive in their ability to exploit CH\textsubscript{4} as the only carbon and energy source (Hanson and Hanson, 1996). Recently, three independent research groups discovered extreme acidophilic methanotrophic *Verrucomicrobia* in geothermal regions (Dunfield et al., 2007; Pol et al., 2007; Islam et al., 2008). Prior to this finding, obligate aerobic methanotrophs were speculated to be exclusively represented in the *Alpha* and *Gamma* subclasses of the *Proteobacteria*. Analysis of the 16S ribosomal RNA and *pmoA* genes demonstrated that the new *Verrucomicrobia* species do not form a monophyletic group with this subclasses (Heyer et al., 2005), and the new genus name *Methylacidiphilum* was suggested (Op den Camp et al., 2009). Furthermore, it has been shown that growth of the new acidophilic methanotrophic bacterium *Methylacidiphilum furarilicium* SolV is strictly dependent on the presence of lanthanides acting as a cofactor of the methanol dehydrogenase (Kelijsens et al., 2014; Pol et al., 2014). Recently, new species of mesophilic acidophilic verrucomicrobial methanotrophs were isolated and characterized from a volcanic region in Italy and the new genus *Methylacidimicrobium* was proposed (Sharp et al., 2014; van Teeseling et al., 2014). This finding expands the diversity of verrucomicrobial methanotrophs and demonstrates that they could be present in more ecosystems than formerly supposed (Chistoserdova et al., 2009). The new verrucomicrobial strains from both genera were shown to be autotrophs that use CH\textsubscript{4} as the sole energy source and fix CO\textsubscript{2} using the Calvin-Benson-Bassham Cycle (Khadem et al., 2011; Sharp et al., 2012, 2013, 2014; van Teeseling et al., 2014), and strain SolV was shown to be able to fix N\textsubscript{2} (Khadem et al., 2010).

Methanotrophic and nitrifying microorganisms share many similarities. They grow obligately on the specific substrates, CH\textsubscript{4} for methanotrophs and NH\textsubscript{3} for nitrifiers. These molecules are structurally comparable and both are highly reduced. Many of these types of microorganisms have intracellular membrane structures where the membrane bound ammonia monoxygenase (AMO) or CH\textsubscript{4} monooxygenase (pMMO) are localized. In the first step of aerobic CH\textsubscript{4} or NH\textsubscript{3} oxidation, the monoxygenase enzymes introduce a single oxygen atom from O\textsubscript{2} into CH\textsubscript{4} or NH\textsubscript{3}, producing methanol from CH\textsubscript{4} and hydroxylamine from NH\textsubscript{3} (Stein et al., 2012). Both microorganisms are able to co-oxidize a range of other substrates and are inhibited by similar compounds (Bédard and Knowles, 1989; Stein et al., 2012). Nitrifiers are able to oxidize CH\textsubscript{4}, and methanotrophs are capable of nitrification. It has been shown that in nutrient limited situations, methanotrophs do participate in soil nitrification, mainly in the production of N\textsubscript{2}O. Nitrification by aerobic methanotrophs relies on CH\textsubscript{4}, because they cannot grow on NH\textsubscript{3} (Stein et al., 2012). Recent studies of CH\textsubscript{4} oxidation and N\textsubscript{2}O production in soils using stable isotopes and particular inhibitors offered more evidence for a role of methanotrophic bacteria in nitrification (Mandernack et al., 2000; Lee et al., 2009; Acton and Baggs, 2011; Im et al., 2011).

NH\textsubscript{3}\textsuperscript{+} is a nitrogen source for methanotrophic bacteria but was also shown to inhibit CH\textsubscript{4} oxidation in the model organism *Methylosinus sporium*, especially due to accumulation of NO\textsubscript{3}\textsuperscript{-} (He et al., 2017). The pMMO enzyme catalyzing the first step of CH\textsubscript{4} oxidation in methanotrophs, also oxidizes NH\textsubscript{3} (NH\textsubscript{3}\textsuperscript{+}) to hydroxylamine (NH\textsubscript{2}OH; Hanson and Hanson, 1996; Nyerges and Stein, 2009; Stein and Klotz, 2011; Stein et al., 2012). Ammonia-oxidizers can convey electrons from hydroxylamine oxidation to the quinone pool to conserve energy and support cellular growth (Klotz and Stein, 2008), but methanotrophs lack this system and cannot conserve energy from this oxidation. Since the intermediate NH\textsubscript{2}OH is highly toxic, methanotrophs use mechanisms to quickly detoxify it. In the natural environment strain SolV cells are faced with 1–28 mM NH\textsubscript{2}OH concentrations (Khadem et al., 2010) meaning that the cells have to balance assimilation and tolerance in response to reactive-N molecules. Detoxification can be achieved by conversion of NH\textsubscript{2}OH back to NH\textsubscript{4}\textsuperscript{+} or to NO\textsubscript{3} using a hydroxylamine dehydrogenase enzyme. Nitrite, which is also toxic, can be further converted to nitrous oxide (N\textsubscript{2}O) via toxic nitric oxide (NO) by denitrification enzymes under anoxic conditions (Campbell et al., 2011). Recently, Kits et al. (2015) reported the reduction of nitrate coupled with aerobic methane oxidation under extreme oxygen limited conditions in which N\textsubscript{2}O production was directly supported by CH\textsubscript{4} oxidation in *Methylocomas denitrificans* strain FJG\textsuperscript{1}.

In the genome of strain SolV, genes encoding enzymes responsible for NO\textsubscript{3} reduction (*nirK*) and NO reduction (*norB* encoding the catalytic subunit, *norC* encoding the electron-accepting subunit), were identified but the gene encoding N\textsubscript{2}O reductase was absent. A *haoAB* gene cluster encoding hydroxylamine dehydrogenase was also identified, suggesting the ability of nitrification and handling of reactive N-compounds (Khadem et al., 2012c; Anvar et al., 2014). Previously a pH of 2–3 has been used for physiological studies of strain SolV (Khadem et al., 2010, 2011, 2012a,b,c). However, since strain SolV has a rather broad pH range for growth (Pol et al., 2007) and can be easily adapted to grow at higher pH values, we used the pH range of 5–6 in the present study. This minimized the chemical conversion of NO\textsubscript{3} at acidic pH (Matthew et al., 2005; Ryabenko et al., 2009).

Recently, using growth experiments (batch and continuous cultures) together with transcriptome and kinetics analyses, *M. furarilicium* SolV was shown to be able to grow as a real “Knallgas” bacterium on hydrogen/carbon dioxide, without addition of CH\textsubscript{4} (Mohammadi et al., 2017). Cells grown on H\textsubscript{2} still express active pMMO similar to the CH\textsubscript{4} culture (Mohammadi et al., 2017). Since we hypothesized that the NH\textsubscript{4}\textsuperscript{+} oxidation is limited by the presence of CH\textsubscript{4}, we tested NH\textsubscript{4}\textsuperscript{+} oxidation to NO\textsubscript{3} using a continuous culture grown on hydrogen in the absence of CH\textsubscript{4} (Mohammadi et al., 2017). Furthermore, we examined the affinity of cells for NH\textsubscript{4}\textsuperscript{+} using batch cultures with different concentrations of CH\textsubscript{4} in a range of 0.5–8% (v/v). The aim of this study was first to investigate whether strain SolV can perform NH\textsubscript{4}\textsuperscript{+} oxidation, and secondly, how it could detoxify the reactive N-compounds resulting from this oxidation using physiological experiments and transcriptome analysis.
MATERIALS AND METHODS

Microorganism and Medium Composition

*M. fumariolicum* strain SolV used in this study was initially isolated from the volcanic region Campi Flegrei, near Naples, Italy (Pol et al., 2007). In this study the medium to obtain an OD_{600} of 1.0 was composed of 0.2 mM MgCl₂, 6H₂O; 0.2 mM CaCl₂, 2H₂O; 1 mM Na₂SO₄; 2 mM K₂SO₄; 2 mM (NH₄)₂SO₄ (or 5 mM KNO₃) and 1 mM NaH₂PO₄, H₂O. A trace element solution containing 1 µM NiCl₂, CoCl₂, MoO₄Na₂, ZnSO₄ and CeCl₃; 5 µM MnCl₂ and FeSO₄; 10 µM CuSO₄ and 40–50 µM nitrilotriacetic acid (NTA). The pH of medium was adjusted to 2.7 using 1 M HCl. To avoid precipitation, CaCl₂, 2H₂O and the rest of medium were autoclaved separately and mixed after cooling. This medium composition was used in batch and continuous cultures, unless otherwise stated.

Chemostat Cultivation

The continuous culture with CH₄ as an electron donor and nitrate (NO₃⁻) as N-source (CH₄/NO₃⁻), liquid volume 500 ml, was operated at 55°C with stirring at 900 rpm with a stirrer bar. The chemostat was supplied with medium at a flow rate of 14.5 ml h⁻¹ (D = 0.026 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 500 ml level sensor in the reactor. A supply of 10% CH₄ (v/v), 8% O₂ (v/v), and 68% CO₂ (v/v) took place by mass flow controllers through a sterile filter and was sparged into the medium just above the stirrer bar (total gas flow rate ≈ 20 ml min⁻¹). The initial pH was 3.4 and was regulated with 1 M carbonate connected to the vessel by a peristaltic pump. The pH was gradually increased to 6 and after obtaining a steady state, all experiments were performed at this pH. In the continuous culture with H₂ as an electron donor and NH₄⁺ as N-source (H₂/NH₄⁺), liquid volume was 1.2 L and this culture was operated at 55°C with stirring at 1,000 rpm. The chemostat was supplied with medium at a flow rate of 29.9 ml h⁻¹ (D = 0.023 h⁻¹). A gas supply of 12% H₂ (v/v), 10% air (v/v), and 5% CO₂ (v/v) was provided by mass flow controllers through a sterile filter and sparged into the medium (total gas flow rate ≈ 16.5 ml min⁻¹). The initial pH was 2.9 and the pH was regulated by 1 M NaOH. A pH range from 3 to 5.5 was investigated in the steady state. In the continuous culture with CH₄ as an electron donor and NH₄⁺ as N-source (CH₄/NH₄⁺), the liquid volume was 0.3 L and the culture was operated at 55°C with stirring at 700 rpm at pH 2.7. The chemostat was supplied with medium at a flow rate of 0.35 ml h⁻¹ (D = 0.0012 h⁻¹). A gas supply of 0.16% CH₄ (v/v), 0.6% O₂ (v/v), and 5% CO₂ (v/v) was directed by mass flow controllers through a sterile filter and sparged into the medium (total gas flow rate ≈ 10 ml min⁻¹). An O₂ sensor in the liquid was coupled to a Biocontroller (Applikon) regulating the O₂ mass controller in each reactor.

Batch Cultivation

In order to obtain cells growing at maximum growth rate (μ_max), cells were grown without any limitation in 250-ml serum bottles containing 40 ml medium (4 mM NH₄⁺; pH 2.7), and sealed with red butyl rubber stoppers. The headspace contained air with (v/v) 10% CH₄, 5% CO₂ at 55°C with shaking at 250 rpm. Incubations were performed in duplicate.

Gas Analysis

Nitric oxide and nitrous oxide (NO and N₂O) were analyzed on an Agilent series 6890 gas chromatograph (Agilent, USA) equipped with a Porapak Q and a Molecular sieve column, coupled to a thermal conductivity detector and a mass spectrometer (MS; Agilent 5975 Cinert MSD; Agilent, USA) as described before (Ettwig et al., 2008). For all gas analyses, 100 µl gas samples were injected into the gas chromatograph. Furthermore, nitric oxide production was monitored directly from the gas outlet of the reactors using a nitric oxide analyzer (NOA 280i, GE) with a suction rate of 11.6 ml min⁻¹.

Dry-Weight Determination and Elemental Analysis

To determine the dry weight, samples of 8–10 ml from the culture suspension were filtered through pre-weighed 0.45 µm filters and dried to constant weight in a vacuum oven at 70°C (n = 3). In order to determine the total content of carbon and nitrogen, 10 ml of the culture suspension (duplicate) was centrifuged at 4,500 g for 30 min and the clear supernatant was used for the analysis. The nitrogen and carbon content in the supernatant was compared with the corresponding values in the whole cell suspension. The total carbon and nitrogen contents were measured using TOC-L and TNM-1 analyzers (Shimadzu).

Nitrite, Ammonium, and Hydroxylamine Analysis

To determine nitrite (NO₂⁻) concentrations, 50 µl of sample, and 450 µl of MilliQ water were added to a cuvette. Then, 500 µl of reagent A [1% (w/v) sulfanilic acid in 1M HCl; kept in the dark] and 500 µl of reagent B [0.1% (w/v) naphthylethylene diaminedihydrochloride (NED) in water; kept at 4°C in the dark] were added to the same cuvette and mixed well. After incubation for 10 min at room temperature, the absorbance at 540 nm was measured and the values were compared with a calibration curve using known concentrations of nitrite in a range of 0–0.5 mM. If necessary, the sensitivity of this assay could be increased 10-fold using 500 µl samples without addition of water. NH₄⁺ concentrations were measured using the ortho phthalaldehyde (OPA) method (Taylor et al., 1974). In order to determine hydroxylamine concentrations, 200 µl reagent A (50 mM potassium phosphate buffer pH 7), 160 µl demineralized water, 200 µl sample, 40 µl reagent B [12% (w/v) trichloroacetic acid in water, kept in the dark], 200 µl reagent C (1% w/v 8-hydroxyquinoline (quinolinol) in 100% ethanol, kept in the dark) and 200 µl reagent D (1 M Na₂CO₃) were mixed and incubated at 100°C for 1 min. The absorption was measured at 705 nm and the values were compared to a calibration curve using hydroxylamine concentrations 0.02–0.1 mM.
Activity Assays
To determine the affinity constant of pMMO for NH₃ of each sample, a volume of 5 ml of cells from the CH₄/NO₂⁻ continuous culture were washed and resuspended in the same medium at pH 6 (The pH of the medium was adjusted to 6 using MES buffer at a final concentration of 25 mM), transferred to a 60-ml serum bottle and capped. After a pre-incubation for 30 min, CH₄ was added to each bottle at final concentrations of 0.5, 1, 2, 3, 4, and 8% (v/v). To each incubation, with a certain concentration of CH₄, NH₄⁺ was added in a range of 0.5–16 mM. The initial production of NO₂⁻ was measured, and the values were normalized to the total protein content of the cells. Incubations were performed at 55°C and shaking at 380 rpm. Each condition was performed in duplicate and values did not deviate more than 5%.

RNA Isolation and Transcriptome Analysis
The complete genome sequence of strain SolV (Anvar et al., 2014), which is also available at the MicroScope annotation platform (https://www.genoscope.cns.fr/agc/microscope/home/), was used as the template for the transcriptome analysis (RNA-seq). A 4-ml volume of cells (OD₆₀₀ = 1) was sampled from the continuous cultures (H₂ and CH₄ grown cells under O₂ limitation) and from a batch culture (cells at μ_max grown on CH₄ without limitation) and harvested by centrifugation. The pellet was further used for mRNA isolation using the RiboPure™-Bacteria Kit according to the manufacturer’s protocol (ThermoFisher, Waltham, USA). Briefly, cells were disrupted by cold Zirconia beads and after centrifugation, 0.2 volumes of chloroform was added to the supernatant for initial RNA purification. Next, 0.5 volumes of 100% ethanol was added to the aqueous phase obtained after chloroform addition and the whole sample was transferred to a filter cartridge. After washing, the RNA was eluted from the filter cartridge. Afterwards, using MICROBExpress™ kit (ThermoFisher, Waltham, USA) the ribosomal RNAs were removed from the total RNA. The rRNA removal efficiency was checked using the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, USA). Next, Ion Total RNA-Seq Kit v2 (ThermoFisher, Waltham, USA) was used to construct the cDNA libraries from rRNA-depleted total RNA. Briefly, the rRNA-depleted total RNA was fragmented using RNase III and then, reverse transcription was performed on the fragmented RNAs. The obtained cDNAs were amplified and further purified to prepare barcoded libraries. To prepare the template for the Ion Personal Genome Machine® (PGM™) System, a volume of 15 µl from two sample libraries with a concentration of 14 pM were mixed. This mixture of two libraries was used to prepare the template-positive Ion Sphere™ particles (ISPs) using the Ion OneTouch™ 2 instrument. Afterwards, the template-positive ISPs were enriched using the Ion OneTouch™ ES instrument. Both template preparation and enrichment were performed using the Ion PGM™ Template OT2 200 Kit (Ion Torrent, Life technologies). Enriched templates were sequenced on an Ion 318™ Chip v2 using the Ion PGM™ sequencing 200 Kit v2. Expression analysis was performed with the RNA-seq Analysis tool from the CLC Genomic Work bench software (version 7.0.4, CLC-Bio, Aarhus, Denmark). The sequencing reads were first mapped to the ribosomal RNA operon and all tRNA and ncRNA genes, and mapped reads were discarded. The remaining reads were mapped to the CDS sequences extracted from the genome sequence of strain SolV (Anvar et al., 2014). Expression values are given as RPKM (Reads per Kilo base of exon model per Million mapped reads; Mortazavi et al., 2008). The total number of reads obtained and mapped on the coding sequences of the genome for each sample together with the calculated expression levels (RPKM) is provided in the Supplementary Material (Table S1).

RESULTS
Physiological Tests Regarding Ammonium Oxidation to Nitrite and Nitrite Reduction to N₂O
To study the effect and conversion of nitrogenous compounds, three different continuous cultures were used which are referred to as CH₄/NH₄⁺, H₂/NH₄⁺, and CH₄/NO₂⁻. In the second and third cultures, oxygen was limiting. Using a NOx analyzer and GC-MS, we demonstrated that in the CH₄/NH₄⁺ culture with low actual CH₄ concentrations in the liquid (0.3 µM) and with NH₄⁺ (4 mM), NO₂⁻ was not detected, and N₂O production rate was only 0.015 nmol N₂O.h⁻¹.mg DW⁻¹ (Table 1) which was 12,000-fold less than the CH₄ conversion rate (180 nmol.h⁻¹.mg DW⁻¹). To increase NO₂⁻ concentrations and study potential toxic effects of this compound, we used the H₂/NH₄⁺ continuous culture applying different conditions. Initially, the production of NO₂⁻, NO, and N₂O were measured under steady state conditions at a pH range of 3–5.5 under O₂ limitation (Figure 1). We showed that the NO₂⁻, NO and N₂O concentrations were elevated by increasing the pH from 3 to 5.5 in the presence of 4 mM NH₄⁺. Changing pH from 3 to 5.5 introduces more NH₃ in the medium. The NH₃ concentration in a range of 12 nM to 5 µM was calculated using the Henderson–Hasselbalch equation (Hütter, 1992), considering the temperature of 55°C at pH 3 to 5.5, respectively. At pH 5.5, we measured a NO₂⁻ concentration TABLE 1 | Overview of NH₄⁺ oxidation and NO₂⁻ reduction rates calculated in each continuous culture at two different pH values.

<table>
<thead>
<tr>
<th>Continuous cultures</th>
<th>CH₄/NH₄⁺</th>
<th>H₂/NH₄⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 3</td>
<td>pH 5.5</td>
</tr>
<tr>
<td>NH₄⁺ (NH₄⁺) oxidation</td>
<td>4 (0.02)</td>
<td>4 (5)</td>
</tr>
<tr>
<td>NH₄⁺ oxidation a</td>
<td>BOLb</td>
<td>NDc</td>
</tr>
<tr>
<td>NO₂⁻ reduction b</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NO₂⁻ reduction c</td>
<td>0.015</td>
<td>ND</td>
</tr>
</tbody>
</table>

aNH₄⁺ and NH₃ concentrations are in mM and µM, respectively.
bND, not determined.
cAll values are the average of two replicates of the same continuous culture with <5% difference between duplicates.

dEqual to or below detection limit.
at steady state of about 420 µM in the reactor (Figure 2) resulting from a production rate of ≈ 48 nmol NO$_3^-$·h$^{-1}$·mg DW$^{-1}$, while nitrite production at pH 3 was very limited. Based on the clear effect of increasing pH on the production of NO$^-_2$, one could speculate that the real substrate for pMMO to produce NO$^-_2$ is NH$_3$ (not NH$_4^+$). Furthermore, the NO$^-_2$ reduction activities (NO and N$_2$O production) were measured at 0.81 nmol NO$^-_2$·h$^{-1}$·mg DW$^{-1}$ (1.7% of NH$_4^+$ oxidation rate) which is 53-fold higher than that in the CH$_4$/NH$_4^+$ culture (Table 1). A rapid NO$^-_2$ consumption (=83 nmol NO$^-_2$·h$^{-1}$·mg DW$^{-1}$) was observed when O$_2$ supply was switched off completely (Figure 2), and the NO$^-_2$ reduction rate (as NO and N$_2$O) increased about 100-fold (74.4 nmol NO$^-_2$·h$^{-1}$·mg DW$^{-1}$). A rapid initial increase of NO suggests that conversion to N$_2$O is the rate limiting step. The decrease of N$_2$O levels was due to the continuous dilution of the gas present in the reactor headspace (total gas flow rate in the outlet ≈ 15 ml·min$^{-1}$). Concentrations of 1–5 µM NH$_3$·OH were measured in data points before and after switching off O$_2$ supply.

We further tested the effect of different concentrations of NH$_4^+$ (4–20 mM) on the NO$^-_2$, NO and N$_2$O production at pH 4 under oxygen limitation in the H$_2$/NH$_4^+$ continuous culture (Figure 3). We showed that the concentrations of NO$^-_2$, NO, and N$_2$O slightly increased once the NH$_4^+$ concentration was gradually elevated. This observation indicates that at pH 4, even a 4-fold increase in the NH$_4^+$ concentration did not result in a high production of NO$^-_2$ similar to what we observed at pH 5.5 supporting our assumption that pH plays an important role regarding the availability of NH$_3$ molecules. Furthermore, we showed that the cells in the CH$_4$/NO$^-_3$ continuous culture were able to perform NO$^-_2$ reduction at a rate of 120 nmol NO$^-_2$·h$^{-1}$·mg DW$^{-1}$ by converting the added NO$^-_2$ (50 µM) to NO and further to N$_2$O in the absence of oxygen (Figure 4). Table 1 shows an overview of rates of ammonium oxidation to nitrite and nitrite reduction to NO/N$_2$O in the different continuous culture.

**Kinetics of Ammonia Oxidation**

The affinity constants (K$_M$) for NH$_4^+$ and NH$_3$ were determined using SolV cells from the CH$_4$/NO$^-_3$ continuous culture. From the initial production rates of nitrite the best fitting curves to Michaelis–Menten kinetics were predicted (Figure S1). Since part of the NH$_4^+$ is present as NH$_3$ at pH 6 (1 M NH$_4^+$ is about 3 mM NH$_3$ at pH 6), the Michaelis–Menten curves were also produced based on the NH$_3$ concentrations (Figure S1). Therefore, we
calculated apparent affinity constants ($K_a$) for both $\text{NH}_4^+$ and $\text{NH}_3$ in strain SolV (Table 2).

To identify the type of inhibition, the Michaelis–Menten curves were transformed to Lineweaver-Burk plots. Figure S2 shows a set of double reciprocal plots, obtained with different $\text{NH}_4^+$ concentrations in the presence of $\text{CH}_4$ at a range of 2, 4, and 8% (v/v). Increasing the $\text{CH}_4$ concentration resulted in a group of lines with a common intercept on the $1/V_0$ axis but with different slopes. The intercept is $1/V_{\text{max}}$ and $V_{\text{max}}$ is constant regardless of increasing $\text{CH}_4$ concentration ($V_{\text{max}} = 1.61 \pm 0.05 \mu\text{mol h}^{-1}\text{mg protein}^{-1}$). The constant intercept of all lines suggests a competitive inhibition between $\text{CH}_4$ and $\text{NH}_3$. The $\text{NO}_2^-$ production rate in the absence of $\text{CH}_4$ was about 3- to 4-fold lower compared to the rate in the presence of 0.5% (v/v) $\text{CH}_4$ suggesting that traces of $\text{CH}_4$ are essential for the pMMO activation. Table 2 shows an overview of affinity constants calculated for $\text{NH}_3$ ($\text{NH}_4^+$) obtained in the incubations with different $\text{CH}_4$ concentrations. Affinity constants for $\text{NH}_3$ were calculated based on the Henderson–Hasselbalch equation considering a temperature of 55°C (Hütter, 1992). These results showed that increasing $\text{CH}_4$ concentration limits the affinity of pMMO for $\text{NH}_3$ significantly, which correlates with the observed competitive inhibition between $\text{CH}_4$ and $\text{NH}_3$.

Whole Genome Transcriptome Analysis of Strain SolV

Expression levels of housekeeping genes and genes involved in metabolism of nitrogenous compounds were determined for $\text{H}_2$- and $\text{CH}_4$-grown cells (both under $\text{O}_2$ limited conditions). These values were compared to the expression values in cells growing at $\mu_{\text{max}}$ on $\text{CH}_4$ (without limitation). To compare baseline expression levels, we selected a group of 384 housekeeping genes (in total 437.9 kbp) involved in energy generation, ribosome assembly, carbon fixation (CBB cycle), C1 metabolism (except for $\text{pmo}$), amino acid synthesis, cell wall synthesis, translation, transcription, DNA replication, and tRNA synthesis (Khadem et al., 2012a,b). All ratios of expression levels of the housekeeping genes under these conditions were between 0.5 and 2 (Table S1). The robustness of the transcriptome data were tested using the method of Chaudhuri et al. (2011). In this method, the logarithmic value of RPKM + 1 of each condition (in duplicates) was calculated and the values were plotted against each other. This resulted in correlation coefficients of 0.80, 0.82, and 0.87 (Figure S3), showing the high robustness of the transcriptome data.

The transcriptome data showed that genes encoding the enzymes involved in $\text{NH}_4^+$ assimilation in strain SolV including glutamine synthase ($\text{GlnA}$)/glutamate synthase ($\text{GltB}$) and the alanine and glutamate dehydrogenases ($\text{Ald}$, $\text{Gdh}$) were equally expressed under all conditions (Table 3). Among these genes, only $\text{glnA}$ was about 2.5-fold less expressed in the continuous cultures compared to the cells grown at $\mu_{\text{max}}$ (Table 3). We also found that the $\text{car}AB$ operons (encoding the glutamine hydrolyzing carbamoyl-phosphate synthase) were constitutively expressed. The conversion of glutamine and carbon dioxide into glutamate and carbamoyl phosphate is performed by this enzyme (Khadem et al., 2012a). Similarly, the $\text{argDHFG}$ operons (encoding enzymes from the urea cycle) were expressed under all conditions (Table 3).

### Table 2 | Kinetics of $\text{NH}_4^+$ oxidation with variable $\text{CH}_4$ supply at pH 6.

<table>
<thead>
<tr>
<th>$\text{CH}_4$</th>
<th>Affinity constant$^c$ ($K_{s(app)}$)</th>
<th>$V_{\text{max}}^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\text{NH}_4^+$ (mM)</td>
<td>$\text{NH}_3$ ($\mu\text{M}$)</td>
</tr>
<tr>
<td>0.5$^a(0.005)^b$</td>
<td>1.25</td>
<td>4.9</td>
</tr>
<tr>
<td>1 (0.01)</td>
<td>1.50</td>
<td>5.8</td>
</tr>
<tr>
<td>2 (0.02)</td>
<td>6</td>
<td>23.3</td>
</tr>
<tr>
<td>3 (0.03)</td>
<td>9</td>
<td>35.0</td>
</tr>
<tr>
<td>4 (0.04)</td>
<td>30</td>
<td>116.7</td>
</tr>
<tr>
<td>8 (0.08)</td>
<td>70</td>
<td>272.3</td>
</tr>
</tbody>
</table>

$^a$CH$_4$ concentrations in % (v/v).
$^b$CH$_4$ concentrations in the liquid in mM.
$^c$Affinity constants were calculated based on two independent experiments.
$^d$V$_{\text{max}}$ values are in $\mu$mol NO$_2^-$ h$^{-1}$mg protein$^{-1}$.
continuous culture compared to the H$_2$/NH$_4^+$ and $\mu_{max}$ cultures, likely due to the absence of NH$_4^+$ in this condition. The haoA gene showed comparable high expression levels in the H$_2$/NH$_4^+$ continuous and batch $\mu_{max}$ culture (Table 3).

The transcriptome data showed different expression levels of two of the three different pmo operons in strain SolV (Table 4). We found that the pmoCAB2 operon including the mfu$m2_{1796}$, mfu$m2_{1795}$ and mfu$m2_{1794}$ subunits was significantly expressed (RPKM values 14,899–37,218) in the cells growing at $\mu_{max}$ with no limitation and the pmoCAB1 operon showed very low expression. In contrast, cells in the continuous cultures on H$_2$/NH$_4^+$ and CH$_4$/NO$_3^-$ under O$_2$ limitation showed a significantly different expression pattern of the pmoCAB operons. We found that the pmoCAB1 operon including mfu$m2_{1796}$, mfu$m2_{1795}$ and mfu$m2_{1794}$ subunits was very highly expressed under these conditions (RPKM values 5,003–47,785), whereas the expression levels of the pmoCAB2 operon was found to be 2- to 19-fold lower in comparison to the cells growing at $\mu_{max}$. The pmoCAB3 operon including the mfu$m2_{1606}$, mfu$m2_{1605}$ and mfu$m2_{1604}$ subunits showed low expressed under all conditions although expression in H$_2$/NH$_4^+$ grown cells seems to be slightly up-regulated. The conversion of methanol to formaldehyde is the second step in CH$_4$ oxidation pathway. Interestingly, it has been shown
that strain SolV contains a XoxF-type methanol dehydrogenase (MDH) that can convert methanol directly to formate (Pol et al., 2014). We found that the xoxFGJ operon encoding the methanol dehydrogenase and pqqABCDEF operon encoding the proteins involved in biosynthesis of the methanol dehydrogenase cofactor pyrroloquinoline quinone were expressed more or less similar under all conditions tested. The last step of the CH$_4$ oxidation pathway is conversion of formate to CO$_2$ catalyzed by NAD-dependent formate dehydrogenase and a membrane-bound formate dehydrogenase. The genes encoding these enzymes were expressed under all conditions, although the expression levels of these enzymes (except for fdsD and fdsB) in continuous cultures under O$_2$ limitation was 2- to 2.5-fold lower compared to cells grown at $\mu_{\text{max}}$ (Table 4).
DISCUSSION

In the present study, the physiological data of the H$_2$/NH$_4^+$ continuous culture showed that strain SolV is able to oxidize NH$_4^+$ to NO$_3^-$ at a rate of 48.2 nmol NO$_3^-$ h$^{-1}$.mg DW$^{-1}$ at pH 5.5. At pH 3, with less NH$_3$ available this rate was about 400-fold lower (Table 1). We also detected a very limited NH$_3^+$ oxidation rate in the cells of the CH$_4$/NH$_4^+$ chemostat in comparison to the H$_2$/NH$_4^+$ cells. These observations indicate that the higher NH$_3^+$ oxidation activity occurs when CH$_4$ is replaced by H$_2$ as the electron donor. Nitrification was previously reported in methanotrophs. CH$_4$-dependent nitrification was detected in a humisol that was enriched with CH$_4$ (Megraw and Knowles, 1987). It has been shown that methanotrophs are efficient nitrifiers and produce NH$_3$OH as a product of NH$_3$ monooxygenation (Bédard and Knowles, 1989; Nyerges and Stein, 2009).

We observed a similar pattern in the batch experiments using cells from the CH$_4$/NO$_3^-$ continuous culture. In these batch tests, we found higher NO$_3^-$ production rates when the CH$_4$ concentration was limited, although traces of CH$_4$ seemed to be essential for activation of pMMO. In these batch tests, the calculated apparent affinity constants [K$_{s(app)}$] for NH$_4^+$ were approximately between 1.25 and 70 mM. At increasing pH values the equilibrium shifts toward higher NH$_3$ concentrations and the calculated K$_s$ values for NH$_3$ in the same tests were 4–273 µM. Comparable values have been reported in literature (Table 5). Our data showed that increasing the pH from 3 to 5.5 significantly affects the rates of NH$_3^+$ oxidation to NO$_3^-$. This reflects the fact that the pMMO of strain SolV might use NH$_3$ as a substrate (and not NH$_4^+$). This assumption could explain why at low pH, when NH$_3^+$ is present, we observed very limited nitrification. In a study from O’Neill and Wilkinson (1977), they also showed that by increasing pH the rate of NH$_3^+$ oxidation by M. trichosporium OB3B increased, and they also suggested the active species to be NH$_3$.

In the present study, we showed that strain SolV performs NO$_3^-$ reduction to N$_2$O using cells from CH$_4$/NH$_4^+$ and H$_2$/NH$_4^+$ continuous cultures (Table 1). Under anoxic condition, higher NO$_3^-$ reduction rates were observed in cells from the CH$_4$/NO$_3^-$ and H$_2$/NH$_4^+$ cultures (Table 1). The reduction of NO$_3^-$ to N$_2$O may provide a way to remove potentially toxic NO$_3^-$. The lower NO$_3^-$ reduction rate in H$_2$/NH$_4^+$ compared to the CH$_4$/NO$_3^-$ continuous cultures in the absence of oxygen could be explained by the fact that cells in the H$_2$ reactor were confronted with NH$_3$OH and NO$_3^-$ over a relatively long term. Cells might suffer under these conditions and show a decrease in NO$_3^-$ reduction rate. Many methanotrophs possess partial denitrification pathways and they are able to reduce NO$_3^-$ to N$_2$O via NO (Nyerges et al., 2010; Campbell et al., 2011). Recently, two methanotrophic strains were cultured together (Methylomonas album ATCC 33003 and Methylocystis sp. strain ATCC 49242), one with high tolerance to NH$_4^+$ and one with high tolerance to NO$_3^-$, and the nitrite-tolerant strain was shown to be more competitive and produced more N$_2$O compared to the other strain (Nyerges et al., 2010). The highest N$_2$O production rate was reported at about 0.4 nmol.h$^{-1}$ per 10$^6$ cells in M. album ATCC 33003 (Nyerges et al., 2010). Campbell et al. (2011) reported a headspace production of 26.3 µM N$_2$O after 48 h (≈0.24 ppb.h$^{-1}$ per 10$^6$ cells) in Methylococcus capsulatus Bath. Recently, Kits et al. (2015) reported the reduction of nitrate coupled to aerobic CH$_4$ oxidation under extreme oxygen limited conditions in which N$_2$O production (0.414 µmol.h$^{-1}$.L$^{-1}$) was directly supported by CH$_4$ oxidation in M. denitrificans strain FJG1T. The latter N$_2$O production rate is about 60-fold lower compared to our results obtained under anoxic condition in the absence of CH$_4$.

In this study, the transcriptome data showed that the pmoCAB1 and pmoCAB2 operons were tightly regulated by oxygen as observed previously (Khadem et al., 2012a). Recently, the down-regulation of pmoCAB gene was detected in response to 30 mM NH$_4^+$ concentration in the medium compared to 10 mM NO$_3^-$ in Methylocystis sp. strain SC2 (Dam et al., 2014). It has been shown that CH$_4$ oxidation in Methylocystis sp. strain SC2 cells supplied with 30 mM NH$_4^+$ was inhibited at CH$_4$ concentrations <400 ppm (v/v); Dam et al., 2014). Our results in all cases showed no expression of the pmoCAB3 operon, suggesting other growth conditions could be examined to elucidate the regulation and role of this pmo operon. Recently, the concurrent growth of the methanotroph Methylomicrobium silvestris was described on CH$_4$ and propane (Crombie and Murrell, 2014). Two soluble di-iron center monooxygenase gene clusters (sMMO).

### Table 5 | Comparison of apparent K$_s$ values for NH$_4^+$

<table>
<thead>
<tr>
<th>Organism</th>
<th>K$_s$ (NH$_4^+$) mM</th>
<th>CH$_4$ % (v/v)</th>
<th>pH</th>
<th>Calculated K$_s$ (NH$_3$) µM</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. fumaricicum</td>
<td>1.25–70</td>
<td>0.5–8</td>
<td>6</td>
<td>4–273</td>
<td>This study*</td>
</tr>
<tr>
<td>Mm. album</td>
<td>2 and 3.9</td>
<td>0.5 and 5</td>
<td>–</td>
<td>–</td>
<td>Nyerges and Stein, 2009</td>
</tr>
<tr>
<td>Methylocystis sp.</td>
<td>0.5 and 1.1</td>
<td>0.5 and 5</td>
<td>–</td>
<td>–</td>
<td>Nyerges and Stein, 2009</td>
</tr>
<tr>
<td>Ms. trichosporium</td>
<td>4.1</td>
<td>–</td>
<td>6.5</td>
<td>–</td>
<td>O’Neill and Wilkinson, 1977</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>–</td>
<td>7.5</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Mb. capsulatus</td>
<td>8*</td>
<td>–</td>
<td>7</td>
<td>–</td>
<td>Dalton, 1977</td>
</tr>
</tbody>
</table>

*See also Table 1.

*At NH$_4^+$ concentrations between 20 and 200 mM, –, not reported.
were identified with different expression during bacterial growth on these alkanes, although both gene sets were essential for efficient propane utilization (Crombie and Murrell, 2014).

In our study, the \( haoAB \) genes encoding hydroxylamine dehydrogenase (HAO) and an associated protein were constitutively expressed in cells grown in the \( \text{H}_2/\text{NH}_3^+ \) continuous and batch cultures (Table 3). In \( M. \text{capsulatus} \) Bath the \( haoAB \) genes were shown to respond to addition of 5 \( \text{mM} \) of \( \text{NH}_4^+ \) (Poret-Peterson et al., 2008). The currently accepted model for oxidation of \( \text{NH}_3 \) to \( \text{NO}_2^- \) proceeds via the intermediate \( \text{NH}_2\text{OH} \) which in a follow up reaction catalyzed by HAO is oxidized to \( \text{NO}_3^- \). Recently, evidence was provided that HAO oxidizes \( \text{NH}_2\text{OH} \) by only three electrons to \( \text{NO}_2^- \) under both aerobic and anaerobic conditions using purified \( \text{Nitrosomonas europaea} \) HAO (Caranto and Lancaster, 2017). This also implies the need for an enzyme converting \( \text{NO} \) to \( \text{NO}_2^- \). For future research we aim at purifying the HAO from strain SolV to test its properties.

The assimilatory nitrite and nitrate reductase genes were found 9- to 45-fold up-regulated in the \( \text{CH}_4/\text{NO}_3^- \) continuous culture compared to the cells at \( \mu_{\text{max}} \). These observations are similar to the down-regulation of assimilatory nitrite and nitrate reductase genes in \( \text{Methylolyticus} \) sp. strain SC2 under 30 \( \text{mM} \) \( \text{NH}_4^+ \) compared to 10 \( \text{mM} \) nitrate or \( \text{NH}_4^+ \) (Dam et al., 2014). It has been proposed that methanotrophs with denitrifying capacity might surpass other methanotrophs in ecosystems with high concentrations of nitrogen, because they have the ability to deal with reactive N-compounds (Nyerges et al., 2010). The \( \text{NO}_2^- \) reducing capacity of strain SolV helps this microorganism to balance assimilation and tolerance in response to reactive N molecules in the extreme conditions of its habitat. Our experiments show that strain SolV is well adapted to cope with the fluctuating conditions (presence of \( \text{H}_2 \), differences in \( \text{NH}_4^+ \) and \( \text{O}_2 \) concentrations and \( \phi \)) that may occur in its natural environment.

**AUTHOR CONTRIBUTIONS**

SM, AP, MJ, and HO designed the project and experiments. Experimental work was performed by SM, TvA, and AP. SM and AP maintained the chemostat cultures. SM, TvA, AP, MJ, and HO performed data analysis and data interpretation. SM and HO wrote the manuscript with input from AP, TvA, and MJ. HO and MJ supervised the research.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01901/full#supplementary-material


Conflict of Interest Statement: 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.