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RESEARCH ARTICLE

The effect of methane and methanol on the terrestrial ammonia-oxidizing archaeon '*Candidatus* Nitrosocosmicus franklandus C13'

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Abstract

The ammonia monooxygenase (AMO) is a key enzyme in ammonia-oxidizing archaea, which are abundant and ubiquitous in soil environments. The AMO belongs to the copper-containing membrane monooxygenase (CuMMO) enzyme superfamily, which also contains particulate methane monooxygenase (pMMO). Enzymes in the CuMMO superfamily are promiscuous, which results in co-oxidation of alternative substrates. The phylogenetic and structural similarity between the pMMO and the archaeal AMO is well-established, but there is surprisingly little information on the influence of methane and methanol on the archaeal AMO and terrestrial nitrification. The aim of this study was to examine the effects of methane and methanol on the soil ammonia-oxidizing archaeon '*Candidatus* Nitrosocosmicus franklandus C13'. We demonstrate that both methane and methanol are competitive inhibitors of the archaeal AMO. The inhibition constants (K_i) for methane and methanol were 2.2 and 20 μM, respectively, concentrations which are environmentally relevant and orders of magnitude lower than those previously reported for ammonia-oxidizing bacteria. Furthermore, we demonstrate that a specific suite of proteins is upregulated and downregulated in '*Ca.* Nitrosocosmicus franklandus C13' in the presence of methane or methanol, which provides a foundation for future studies into metabolism of one-carbon (C1) compounds in ammonia-oxidizing archaea.

INTRODUCTION

Ammonia-oxidizing archaea (AOA) are among the most numerous living organisms on Earth (Karner et al., 2001; Leininger et al., 2006) and play an important role in the global biogeochemical cycling of nitrogen. AOA are ubiquitous in soils, including acidic, neutral and alkaline soils, in both natural and agricultural soil habitats. The key enzyme responsible for initiating their energy metabolism is the ammonia monooxygenase (AMO), a member of the copper-containing membrane monooxygenase (CuMMO) protein superfamily. The CuMMO superfamily also contains particulate methane monooxygenase (pMMO), which is expressed by most methanotrophs and catalyses the oxidation of methane. Furthermore, enzymes belonging to the CuMMO superfamily are known for their ability to oxidize a broad range of substrates. Studies on the inhibition of AOA by alkynes (Taylor et al., 2013; Taylor et al., 2015; Wright et al., 2020) have suggested that the substrate range of the archaeal AMO may include short-chain-length hydrocarbons (≤C_5), including the one-carbon (C1) compounds methane and methanol. Despite the well-established phylogenetic and structural similarity between the pMMO and the archaeal AMO, the effect

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of the C1 compounds methane and methanol on AOA, and nitrification in the terrestrial environment, remains surprisingly underexplored.

Both methane and methanol are biologically important C1 compounds which are actively cycled in soil ecosystems. An estimated 550 Tg of the greenhouse gas methane are emitted annually into the Earth's atmosphere (Saunois et al., 2020). Approximately 150 Tg of methanol is produced annually in the biosphere, with roughly 85% contributed by terrestrial plants and their decay (Galbally & Kirstine, 2002). Aerobic soils act as important sinks for both methane and methanol. Approximately 30 Tg of atmospheric methane is oxidized by soil microorganisms annually (Boeckx & Van Cleemput, 1996), but much more than this, produced by methanogens in anoxic regions, is oxidized before it ever reaches the surface (Reeburgh, 2007). The majority of methanol generated in soil also does not enter the atmosphere due to oxidation and assimilation by soil microorganisms (Kolb, 2009). A previous study suggested that AOA competed with methanotrophs for ammonia and oxygen in volcanic grassland soil (Daebeler et al., 2014). It seems plausible that if methane and methanol interact with the archaeal AMO, then they would also influence nitrogen cycling by AOA in soil ecosystems.

There are three known groups of ammonia-oxidizing microorganisms, AOA, AOB (canonical ammonia-oxidizing bacteria) and comammox (*Nitrospira*, complete ammonia oxidisers). Ammonia oxidation is the first step in nitrification, which results in large-scale environmental impacts in soils, including emission of the greenhouse gas nitrous oxide (N_2O) and groundwater pollution through nitrate leaching (Canfield et al., 2010; Prosser & Nicol, 2012). Both the AMO and pMMO initiate energy metabolism by the hydroxylation of their respective substrates. The pMMO oxidizes methane to methanol, which is analogous to the oxidation of ammonia to hydroxylamine catalysed by the AMO. The AMO is the only enzyme of the ammonia oxidation pathway which is shared by all aerobic ammonia-oxidizing microorganisms, and the downstream enzymology to produce nitrite from hydroxylamine differs considerably between them (Caranto & Lancaster, 2017; Martens-Habbena et al., 2015; Vajrala et al., 2013). In methanotrophs, methanol is further oxidized to formaldehyde, formate and CO_2. The oxidation of ammonia or methane requires reductant and does not yield net energy. Instead, the downstream reactions in the pathways (hydroxylamine oxidation in ammonia oxidisers, methanol and formaldehyde oxidation in methanotrophs) generates energy and a source of reductant to sustain AMO/pMMO activity (Khadka et al., 2018).

Whilst the pMMO and AMO demonstrate clear specificity for one substrate (i.e., their normal physiological substrate, methane or ammonium, respectively), enzymes of the CuMMO superfamily are promiscuous and can co-oxidize a broad range of alternative substrates. For example, pMMO from methanotrophic bacteria can oxidize ammonia, short-chain-length hydrocarbons and halogenated hydrocarbons, although none of these reactions support growth (Hutton & Zobell, 1953; Lontoh et al., 1999; Nyerges & Stein, 2009). Similarly, the AMO from AOB can oxidize numerous hydrocarbons including methane and methanol, which is the product of methane oxidation (Hyman & Wood, 1983; Voysey & Wood, 1987). It is interesting to consider the degree of crossover and synergy between carbon and nitrogen cycling as a result of the promiscuous nature of the pMMO and AMO. The soil AOB *Nitrosomonas europaea* can produce considerable quantities of methanol when incubated with methane in the presence of a source of reductant (ammonia and/or hydroxylamine; Hyman & Wood, 1983; Taher & Chandran, 2013). Isotope tracer experiments also found that ^{13}C-formaldehyde and ^{13}C-formate were detectable in cell suspensions of *N. europaea* following incubations with ^{13}C-methane or ^{13}C-methanol (Voysey & Wood, 1987). Furthermore, both *N. europaea* and the marine AOB *Nitrosococcus oceani* produced ^{14}C–CO_2 and incorporated ^{14}C into the cell biomass from ^{14}C-methane or ^{14}C-methanol (Jones & Morita, 1983; Ward, 1987, 1990). The pathway by which methane-derived carbon is incorporated into the biomass of ammonia oxidisers is not known. AOB can assimilate CO_2 produced from methane oxidation through the Calvin cycle, but the dynamics of CO_2 and cell biomass production in *N. oceani* (Ward, 1987) suggested that the intermediates (formaldehyde and formate) might also be assimilated. In addition, formaldehyde can react with hydroxylamine to produce formaldoxime, which is a substrate for the hydroxylamine dehydrogenase (Voysey & Wood, 1987). Formation of formaldoxime may represent another sink for formaldehyde in AOB.

Methane and methanol were both reported to be competitive inhibitors of ammonia oxidation in *N. europaea* (Suzuki et al., 1976), although *N. europaea* has a poor affinity for methane with an estimated half-inhibition constant (K_i) of 2 mM (Hyman & Wood, 1983). For comparison, apparent half-saturation constants ($K_{m\,(app)}$) for methanotrophs ranges between 0.8 and 45 μM in whole cells (Holmes et al., 1999). The marine AOB *N. oceani* is more sensitive to methane inhibition compared to *N. europaea* and had a lower K_i (6.6 μM), although the significance of this value was uncertain due to the fact that methane inhibition in this strain does not follow Michaelis–Menten kinetics (Ward, 1987).

While nothing is known about the potential inhibitory effects or oxidation kinetics of methane or methanol in AOA, it is interesting to note that ammonia substrate affinity of some marine and acidophilic AOA, for example, the representatives in *Nitrosopumilales* and '*Ca.* Nitrosotaleales' is orders of magnitude greater than that of AOB (Jung et al., 2022; Martens-Habbena

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et al., 2009). The response of AOA to methane and methanol is an important outstanding research question. Inhibition of AOA by methane and methanol would potentially have important consequences for our understanding of the function of CuMMO proteins and the global biogeochemical nitrogen cycle. Members of the archaeal genus *Nitrosocosmicus* are widespread and abundant in soil environments. Therefore, in this study, we used the terrestrial ammonia-oxidizing archaeon '*Candidatus* Nitrosocosmicus franklandus C13' as a model organism. The aim of this study was to characterize the inhibition of archaeal ammonia oxidation by methane and methanol using whole cell kinetics, and to investigate the key cellular responses of '*Ca*. Nitrosocosmicus franklandus C13' to additions of methane and methanol using proteomics.

EXPERIMENTAL PROCEDURES

Culture maintenance

Pure cultures of '*Ca*. Nitrosocosmicus franklandus C13' were maintained static at 37°C (Lehtovirta-Morley et al., 2016) in freshwater medium (FWM) at pH 7.3. FWM contained 1 g NaCl, 0.4 g $MgCl_2 \cdot 6H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.2 g KH_2PO_4, 0.5 g KCl per litre of ddH_2O. Medium was supplemented with 5 mM NH_4Cl, 2 mM $NaHCO_3$, 10 mM HEPES, 1 ml/L modified non-chelated trace element mixture (Könneke et al., 2005), 7.5 μM FeNaEDTA (Tourna et al., 2011), 1 ml/L vitamin solution (Lehtovirta-Morley et al., 2011) and 50 mg/L streptomycin.

Culture purity was regularly verified by microscopy, plating onto R2A agar plates and by PCR assays targeting the 16S rRNA gene using bacteria-specific primers 27f and 1492r (Lane, 1991; Nicol et al., 2008), and the identity of the archaeal strain was verified by PCR and Sanger sequencing of the archaeal 16S gene amplified using the primer set A 109f and 1492r (Nicol et al., 2008). All glassware was soaked with 10% (vol/vol) nitric acid overnight, thoroughly rinsed with ddH_2O and autoclaved before use.

Growth and activity of cultures was monitored by nitrite accumulation. Nitrite concentration was determined colorimetrically using the Griess test as previously described (Lehtovirta-Morley et al., 2014). Briefly, 20 μl of sulphanilamide (5 g/L in 2.4 M HCl) was added into 100 μl sample or standard, followed by addition of 20 μl *N*-(1-naphthyl)ethylenediamine (3 g/L in 0.12 M HCl) in a 96-well plate format. Absorbance was recorded at 540 nm using a VersaMax platereader (Molecular Devices).

Cell counts were performed using fluorescent microscopy. One millilitres of culture was fixed in 5% (vol/vol) formaldehyde and stained with 30 μl of 200 μg/ml DAPI (4,6-diamidino-2-phenylindole) in the dark for 5 min. Stained cells were filtered onto a 0.22 μm pore-size black polycarbonate filter (Whatman [Cytiva]), mounted on a microscopy slide with Citifluor antifadent solution (Citifluor) and visualized by microscopy. Cell counts were performed in 10 fields of view for each sample. The volume of the cell suspension and areas of the filter and the fields of view were known, therefore it was possible to calculate the cell count per ml.

Methane and methanol inhibition kinetics

Cells from the '*Ca*. Nitrosocosmicus franklandus C13' culture (1.6 L) were harvested at mid-exponential phase (approximately 600 μM NO_2^- accumulated) by filtration onto 0.22 μm pore-size membrane filter (PES; Merck Millipore), washed and resuspended in 200 ml of FWM salts (i.e., FWM without added NH_4Cl and other media components as described above) buffered with 10 mM HEPES buffer (pH 7). Cells were placed in the 37°C incubator for ~1 h until endogenous respiration ceased. Four millilitres of FWM salts with HEPES were placed in 24 ml glass vials, closed with grey butyl rubber stoppers (twice autoclaved in ultrapure water in order to remove contaminants) and crimp sealed. Vials were preincubated at 37°C for 30 min with methane [2.5%–10% (vol/vol)] or methanol (0.1–2 mM). NH_4Cl was added to final concentrations of 0.01–6 mM. Reactions were initiated by adding 1 ml of concentrated '*Ca*. Nitrosocosmicus franklandus C13' cells to the vials (corresponding to a final concentration of $\sim 5 \times 10^6$ cells/ml) and the assays were carried out in a shaking water bath at 37°C. Nitrite concentration was measured at 15-min intervals over 1 h. Assays were performed in triplicate and repeated >10 times with similar results. The aqueous concentration of methane was calculated using a Henry's law constant (1.4×10^{-3} M/atm; Sander, 2015).

The reaction velocity of the ammonia oxidation by AMO was calculated from nitrite accumulation during four or five time points. Methane and methanol inhibition kinetics were analysed by plotting the velocity of AMO activity (in the presence or absence of various inhibitor concentrations) against total ammonium ($NH_3 + NH_4^+$) concentration. The apparent half-saturation constant and maximum velocity ($K_{m(app)}$ and $V_{max(app)}$) were estimated by nonlinear regression using the Hyper32 kinetics software. K_i was calculated from the apparent K_m using formula:

$$K_i = \frac{K_m(1+[CH_4])}{K_m'},$$

where K_m' equals the apparent K_m in the presence of methane or methanol (Ward, 1987), and K_m refers to the K_m value with no added methane or methanol (uninhibited control).

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Proteome analysis

Triplicate samples were prepared for the proteomic analyses as follows: sterile FWM medium was prepared with 1 mM NH_4Cl, and $NaHCO_3$ at a final concentration of 400 μM instead of 2 mM in the standard medium. One litre of medium was placed into a 2 L Duran bottle fitted with an additional septum port, inoculated with exponentially growing '*Ca*. Nitrosocosmicus franklandus C13', and closed with an gas-tight screw cap. Methane [10% (vol/vol)] or methanol (500 μM) were added through the septum. Cultures were incubated shaking at 37°C and 70 rpm. Growth was monitored using the Griess assay. Once the nitrite had accumulated to ~700 μM, a further 1 mM NH_4Cl was added to maintain exponential growth. When the nitrite concentration in the culture reached 1.2 mM, the cells were harvested by filtration as described above and washed with 200 ml of FWM salts buffered with 10 mM HEPES (pH 7.5). To obtain sufficient biomass, 3 L of '*Ca*. Nitrosocosmicus franklandus C13' culture was harvested for each sample for proteomic analysis. After concentrating the cells initially by filtration, the cells were then centrifuged and the cell pellet was stored at −20°C for protein extraction.

Cell pellets were resuspended in buffer containing 50 mM Tris–HCl (pH 8, containing 0.2 M NaCl), complete protease inhibitor cocktail [SIGMAFAST (Catalogue Number: S8830); Sigma Aldrich] and 10 mM dithiothreitol, and broken using a French press [~20,000 psi (137 MPa), 3 cycles]. Lysate was then mixed with 2% SDS, boiled at 100°C for 10 min and spun down (10,000 × *g* for 10 min). Protein in the soluble supernatant was then quantified using the Pierce BCA Protein assay kit and precipitated using the chloroform methanol method. Briefly, 250 μg of protein was diluted to final volume of 100 μl, mixed with 400 μl of methanol and vortexed. Then 100 μl of chloroform was added and vortexed, followed by 300 μl of H_2O resulting in precipitation. The protein flake was collected using centrifugation (14,000 × *g*, 1 min). After discarding the top layer, 400 μl of methanol was added and the mixture was vortexed and centrifuged (20,000 × *g*, 5 min). The aqueous layer was removed, and the protein pellet was air-dried.

Proteins samples were prepared for tandem mass tagging using a TMTpro 16plex kit (Lot VK309613, Thermo Fisher Scientific) according to the manufacturer's instructions with modifications as previously described (Crombie, 2022). Briefly, peptides were analysed by nanoLC-MS/MS on an Orbitrap Eclipse Tribrid mass spectrometer coupled to an UltiMate 3000 RSLCnano LC system (Thermo Fisher Scientific). Peptides were identified using real-time search (RTS) against the protein database '*Ca*. Nitrosocosmicus franklandus C13' (Nicol et al., 2019) (3188 entries) with default corrections. The acquired raw data were processed, quantified and statistically analysed in Proteome Discoverer 2.4.1.15 (Thermo Fisher Scientific) using the incorporated search engine Sequest HT and the Mascot search engine (Matrix Science; Mascot version 2.8.0). To identify statistically significant differences between upregulated and downregulated proteins, *p* values were calculated using a background-based *t*-test and adjusted *p* value calculation performed by Benjamini–Hochberg method. For analysing upregulation and downregulation of proteins, we considered the change in expression significant when the adjusted $p < 0.05$, and the expression fold-change >2. Clusters of orthologous groups were assigned using the eggNOG-mapper v2 software (Cantalapiedra et al., 2021; Huerta-Cepas et al., 2019). Protein annotations were manually curated in MaGe (Vallenet et al., 2009)

Phylogenetic analyses

Amino acid sequences were retrieved from NCBI and aligned using BioEdit (Hall, 1999). Phylogenetic analyses of the conserved positions were performed in MEGA 11.0 package (Tamura et al., 2021) using Maximum Likelihood analysis based on a Jones–Taylor–Thornton matrix-based model with 100 bootstrap replicates. Nodes with bootstrap support of <50% were multifurcated on the phylogenetic trees.

RESULTS AND DISCUSSION

Inhibition of the archaeal AMO by methane and methanol

To test if methane inhibited ammonia oxidation by '*Ca*. Nitrosocosmicus franklandus C13', whole-cell activity assays were performed in the presence of 1 mM NH_4Cl with methane headspace concentrations ranging from 10% to 50% (vol/vol) (Figure 1A). Ammonia oxidation was inhibited at all methane concentrations tested, and inhibition was greater at higher methane concentrations (Figure 1A). To determine whether inhibition of the archaeal ammonia oxidation was specific to the AMO enzyme, activity assays were performed using 200 μM hydroxylamine as a substrate. Methane had no effect on hydroxylamine oxidation by '*Ca*. Nitrosocosmicus franklandus C13' at even the highest methane concentration tested [50% (vol/vol)], indicating that the observed inhibition was specific to the AMO, and not due to inhibition of downstream reactions in the archaeal ammonia oxidation pathway (Figure 1A,B).

To investigate the threshold and mode of inhibition by methane in '*Ca*. Nitrosocosmicus franklandus C13', ammonia oxidation kinetics were determined using whole-cell activity assays with methane headspace

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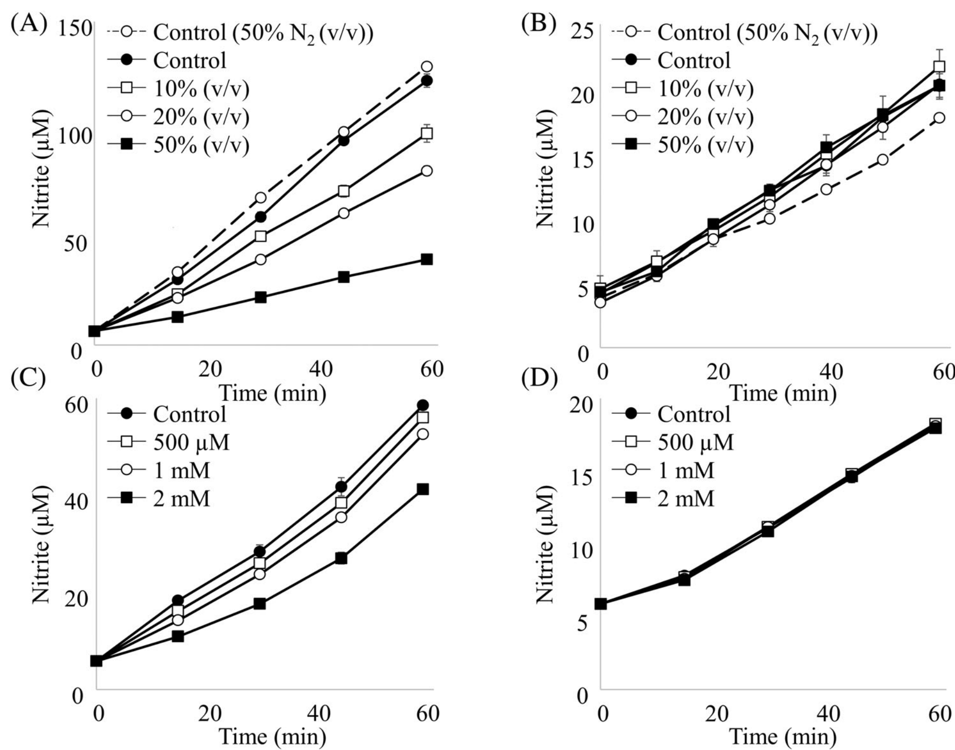

FIGURE 1 The effect of methane (A, B) and methanol (C, D) on nitrite accumulation in short-term activity assays with '*Ca*. N. franklandus C13' using ammonia (1 mM) (A, C) or hydroxylamine (200 μM) (B, D) as a substrate. For panels A and B: Closed circles: control (no amendments), open squares: 10% (vol/vol) methane, open circles: 20% (vol/vol) methane, closed squares: 50% (vol/vol) methane, open circles with dashed line: control with 50% (vol/vol) N_2. For panels C and D: Closed circles: control, open squares: 500 μM methanol, open circles: 1 mM methanol, closed squares: 2 mM methanol.

TABLE 1 Kinetic parameters of NH_3-dependent NO_2^- production by "*Ca*. Nitrosocosmicus franklandus C13" in the presence of methane.

Methane (%) (vol/vol)	$K_{m(app)}(NH_3 + NH_4^+)$ (μM)	$V_{max(app)}$ (nmol mg prot^{-1} min^{-1})	$K_{m(app)}NH_3$ (μM)
0	12.2 (2.4)	101.3 (2.4)	0.13
2.5	169.7 (13.1)	108.0 (1.8)	1.80
5	483.0 (58.8)	107.2 (3.9)	5.12
10	771.9 (107.8)	112.4 (4.7)	8.18

Note: SE is in parentheses ($n = 3$).

concentrations ranging from 2.5% to 10% (vol/vol). Increasing the NH_4^+ concentration alleviated the inhibitory effect of methane on ammonia oxidation, and when the cells were incubated with 6 mM NH_4^+ there was very little inhibition even at 10% methane. Accordingly, the $K_{m(app)}(NH_3 + NH_4^+)$ increased from 12.2 ± 2.4 μM for the uninhibited control to 771.9 ± 107.8 μM in the presence of 10% (vol/vol) methane, while the V_{max} was not significantly affected (Table 1). This indicated that methane is a competitive inhibitor for ammonia oxidation in the AOA '*Ca*. Nitrosocosmicus franklandus C13' (Figure 2). This is consistent with previous findings that small substrates, which includes ammonia, acetylene and now methane, compete for the same binding site on the AMO from '*Ca*. Nitrosocosmicus franklandus C13' whilst larger substrates are non-competitive and presumably bind at a different site (Taylor et al., 2015; Wright et al., 2020). The $K_{i(app)}$ of methane was 2.2 ± 0.19 μM (mean ± SE) corresponding to 0.15% (vol/vol) methane in the headspace, which was below the lowest concentration tested.

Methanol is the reaction product of methane oxidation in both methanotrophs and in AOB. Methanol is structurally similar to methane, and interestingly, it is also a competitive inhibitor and a substrate for the bacterial AMO (Voysey & Wood, 1987). In addition, methanol is also an alternative substrate for sMMO in the methanotroph *Methylocella silvestris* (Crombie, 2022). We therefore hypothesised that methanol may also be an inhibitor for the archaeal AMO in a manner analogous to methane. Whole-cell kinetics were determined in '*Ca*. Nitrosocosmicus franklandus C13' using methanol concentrations ranging from 0.1 to 2 mM. As with methane, $K_{m(app)}(NH_3 + NH_4^+)$ increased in the presence of methanol, ranging from 12.3 ± 1.0 μM for the uninhibited control to 1.65 ± 0.33 mM in the presence

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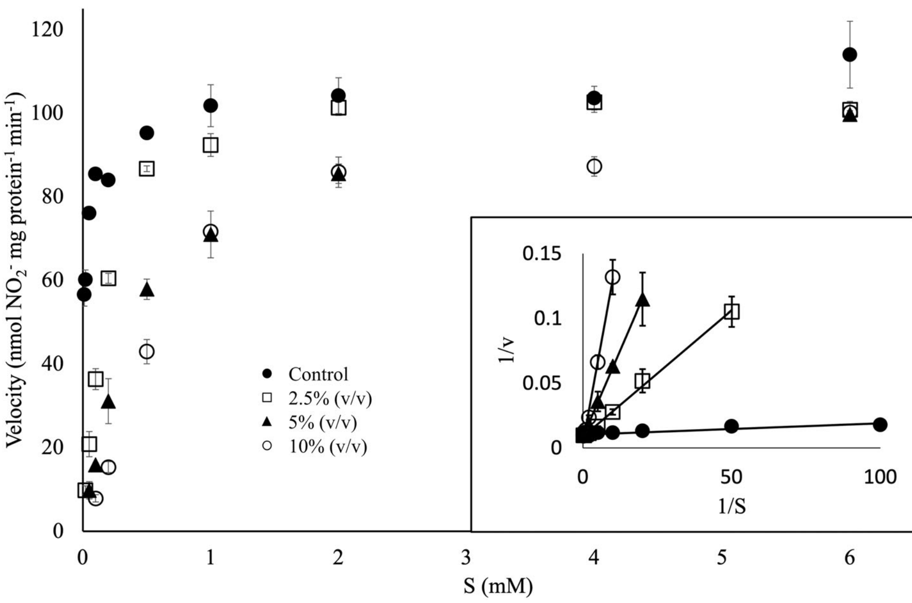

FIGURE 2 The effect of methane on the whole-cell ammonia oxidation kinetics in '*Ca.* N. franklandus C13' shown on the Michaelis–Menten and Lineweaver–Burk (inset) plots. Treatments are as follows: Closed circles: Control, open squares: 2.5% (vol/vol) CH_4 , closed triangles: 5% (vol/vol) CH_4 , open circles: 10% (vol/vol) CH_4. Error bars represent standard error ($n = 3$).

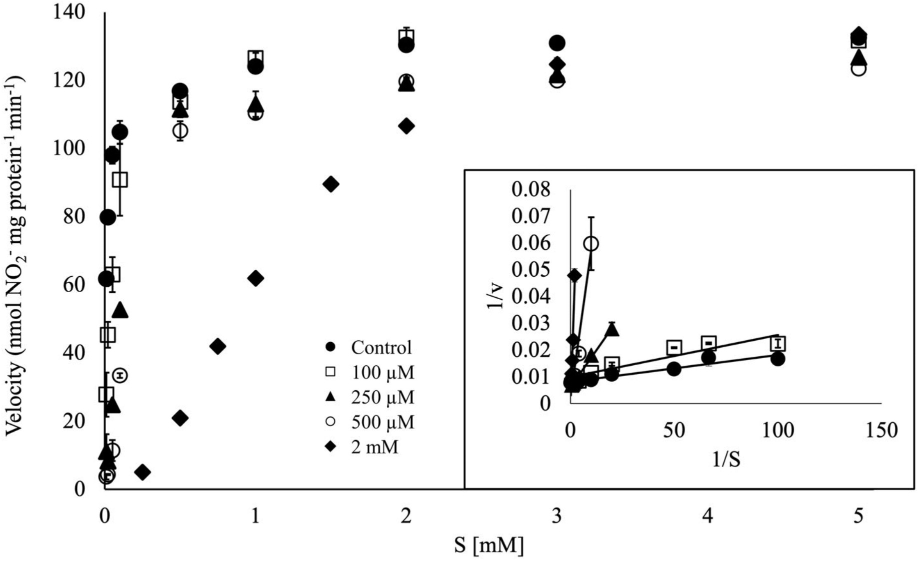

FIGURE 3 The effect of methanol on the whole-cell ammonia oxidation kinetics in '*Ca.* N. franklandus C13' shown on the Michaelis–Menten and Lineweaver–Burk (inset) plots. Treatments are as follows: Closed circles: Control, open squares: 100 μM methanol, closed triangles: 250 μM methanol, open circles: 500 μM methanol, closed diamonds: 2 mM methanol. Error bars represent standard error ($n = 3$).

of 2 mM methanol. The V_{max} remained similar between the different methanol treatments (126 ± 1.6 to 163.7 ± 13.9 nmol mg prot^{-1} min^{-1}) (Figure 3; Table 2). This suggested that methanol, like methane, is also a competitive inhibitor of ammonia oxidation in '*Ca.* Nitrosocosmicus franklandus'. As with methane, methanol had no effect on hydroxylamine oxidation by '*Ca.* Nitrosocosmicus franklandus C13' (Figure 1C,D). The $K_{i(app)}$ for methanol was higher than the $K_{i(app)}$ for methane, 20 ± 2.4 μM (mean ± SE).

The K_i value for methane in '*Ca.* Nitrosocosmicus franklandus C13' is remarkably low compared to the terrestrial AOB *N. europaea* and certainly within a range of concentrations found in some soils, for instance, in wetland sediments and landfill cover soils which are associated with high methanogenic activity (Hyman & Wood, 1983; Jones & Nedwell, 1993; Laanbroek, 2010). At 2.2 μM, the $K_i(CH_4)$ of '*Ca.* Nitrosocosmicus franklandus' is in a comparable range to that of the γ-proteobacterial AOB *N. oceani* (6.6 μM)

TABLE 2 Kinetic parameters of NH_3-dependent NO_2^- production by "*Ca.* Nitrosocosmicus franklandus C13" in the presence of methanol.

Methanol (μM)	$K_{m(app)}(NH_3 + NH_4^+)$ (μM)	$V_{max(app)}$ (nmol mg prot^{-1} min^{-1})	$K_{m(app)}NH_3$ (μM)
0	12.3 (1.0)	126.0 (1.6)	0.13
250	154.0 (14.3)	131.0 (2.3)	1.63
500	243.9 (29.8)	134.1 (3.4)	2.58
2000	1651.0 (333.3)	163.7 (13.9)	17.50

Note: SE is in parentheses ($n = 3$).

(Ward, 1987). However, comparisons between AOA and AOB are complicated by the fact that the methane inhibition in *N. oceani* does not follow Michaelis–Menten kinetics (Hyman & Wood, 1983; Ward, 1987). In contrast to *N. oceani*, we found that methane appeared to be a simple competitive inhibitor in '*Ca.* Nitrosocosmicus franklandus C13' and our data are consistent with Michaelis–Menten kinetics. These experiments were performed in a whole-cell system, and evalution using purified enzymes would be useful for gaining further insights into the inhibition kinetics. The $K_i(CH_4)$ value in '*Ca.* Nitrosocosmicus franklandus' is in a similar range with the $K_m(CH_4)$ of oligotrophic atmospheric methane oxidisers, (Knief et al., 2005; Tveit et al., 2019) and also lower than, or comparable to, some of the previously reported values of the $K_m(CH_4)$ of methanotrophs in general, which range from 0.8 to 66 μM (Joergensen & Degn, 1983; O'Neill & Wilkinson, 1977). '*Ca.* Nitrosocosmicus franklandus C13' had a $K_i(CH_3OH)$ of 20 μM, which is also low considering that the $K_m(CH_3OH)$ values in extant obligate and facultative methylotrophs typically range from 20 to 70 μM for MxaF methanol dehydrogenases (MDH) (Khmelenina et al., 2019) and from 0.8 to 55 μM for XoxF MDH (Picone & Op den Camp, 2019). Furthermore, this value is similar to the K_m calculated for the purified MDH from the methylotrophic denitrifying bacteria *Hyphomicrobium denitrificans*, which ranges from 0.3 to 10.5 μM (Nojiri et al., 2006). Methanol concentrations in the environment are extremely variable, however, higher concentrations can be expected in the proximity to plants as most of the methanol released is associated with degradation of plant polymers (Kolb, 2009). The oxidation of hydroxylamine, the product of NH_3 oxidation, provides the only known source of internal reducing equivalents to sustain AMO activity. Consequently, methane and methanol inhibition, or oxidation, will slow the rate of ammonia turnover by the AMO by reducing the supply of reductant. It has also been shown that formaldehyde, the product of methanol oxidation, can react with hydroxylamine to form formaldoxime which inhibits hydroxylamine oxidation by *N. europaea* (Voysey & Wood, 1987). Taken together, these results suggests that AOA can be inhibited by, and interact with, methane and methanol at environmentally relevant concentrations which typically support the growth of methanotrophs. Therefore, both C1 compounds could markedly influence nitrifying activity in the environment.

Changes in the proteome of '*Ca.* Nitrosocosmicus franklandus C13' induced by methane and methanol

The fact that methane and methanol did not affect hydroxylamine oxidation in '*Ca.* Nitrosocosmicus franklandus C13' (Figure 1) strongly suggested that the inhibition was AMO-specific in terms of the ammonia oxidation pathway. However, we hypothesised that methane and methanol may influence other metabolic pathways of AOA, including energy metabolism and stress responses. We further hypothesised that methane and methanol may be further metabolized and serve as additional sources of carbon for assimilation, as previously demonstrated in AOB (Jones & Morita, 1983; Ward, 1987). To test these hypotheses, the proteomes of '*Ca.* Nitrosocosmicus franklandus C13' cultures grown in the presence of methane [10% (vol/vol)] or methanol (0.5 mM), were examined and compared to control cultures grown without methane and methanol. All cultures were grown in bicarbonate-limited (400 μM) medium to increase the possibility that carbon derived from methane and methanol might be assimilated. Bicarbonate limitation resulted in a slightly lower growth rate of 0.40 d^{-1} compared to the typical cultures from bicarbonate-replete conditions (~0.47 d^{-1}) (Klein et al., 2022). Addition of 10% (vol/vol) methane resulted in a further decline of the growth rate to 0.24 d^{-1}. The presence of methanol (0.5 mM) was inhibitory and resulted in an atypical growth curve, with a growth rate which declined over time.

Late-exponential cultures of '*Ca.* Nitrosocosmicus franklandus C13' were harvested and proteomic analysis of soluble and membrane bound proteins was performed. A total of 1868 polypeptides were detected, accounting for 68.7% of all predicted proteins. This proteome coverage is comparable to previous proteomics studies of AOA, where the expression of 48% of 3123 predicted protein-coding genes was detected in *Nitrososphaera viennensis* (Kerou et al., 2016) and 70% of 1445 predicted protein-coding genes in *Nitrosopelagicus brevis* (Santoro et al., 2015).

Methane and methanol had a significant effect on the proteome of '*Ca.* Nitrosocosmicus franklandus

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C13' and the differential expression of proteins was visualized using volcano plots (Figures S1 and S2) and a heatmap (Figure S3). In the presence of methane, a total of 24 proteins were upregulated and 33 downregulated compared to the control. A total of 43 proteins were upregulated and 52 downregulated in the presence of methanol compared to the control (Table S1). The effect of methane on the proteome of '*Ca*. Nitrosocosmicus franklandus C13' was different compared to methanol. Methanol is the presumed product of methane oxidation by AMO, as is the case with pMMO. Therefore, a larger overlap between the effects of methane and methanol on the proteome might be expected, if both C1 compounds were metabolized via the same pathways.

Classification of the proteome of '*Ca.* Nitrosocosmicus franklandus C13' into functional categories

To gain a broad overview of the cellular metabolism in different treatments, proteins were assigned to clusters of orthologous proteins (COGs) and COG assignments compared. The assignment of proteins into COGs was possible for 54% of the upregulated and downregulated proteins (Figure 4). The proteome of '*Ca*. Nitrosocosmicus franklandus C13' control cultures consisted of proteins related to metabolism (53%), information storage and processing related proteins (23%), cellular processes and signalling related proteins (12%) and poorly characterized proteins (12%) (Figure 4).

In the presence of 10% (vol/vol) methane in the headspace, most upregulated proteins were related to energy production and conversion (alcohol dehydrogenase, 4Fe–4S dicluster domains, nitroreductase), amino acid transport and metabolism (urease) and carbohydrate transport and metabolism (aldose sugar dehydrogenase). Downregulated proteins were related to cellular processes and signalling, energy production and conversion (multicopper oxidases) and secondary metabolites biosynthesis (methyl transferases).

In the presence of 0.5 mM methanol, there was upregulation of proteins related to energy production and conversion (4Fe–4S dicluster domains, aconitate hydratase, alcohol dehydrogenase) and downregulation

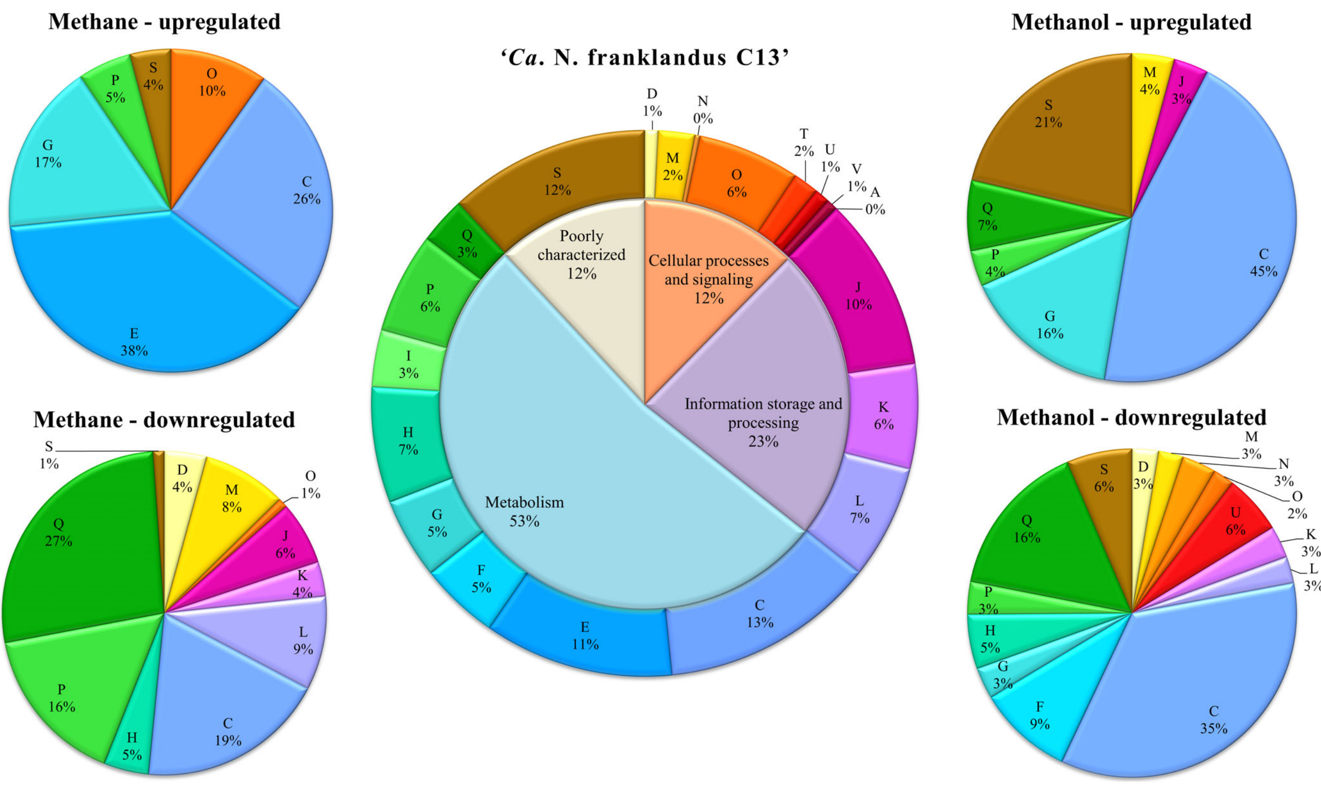

FIGURE 4 The assignment of proteins of '*Ca*. N. franklandus C13' into the COG categories. Baseline proteome in typical control cultivation conditions (middle), proteins upregulated and downregulated in the presence of methane and methanol (left panel and right panel, respectively) are shown. (D: Cell cycle control, cell division, chromosome partitioning; M: Cell wall/membrane/envelope biogenesis; N: Cell motility; O: Post-translational modification, protein turnover, chaperones; T: Signal transduction; U: Intracellular trafficking, secretion, vesicular transport; V: Defence mechanisms; J: Translation, ribosomal structure and biogenesis; K: Transcription; L: Replication, recombination and repair; C: Energy production and conversion; E: Amino acid transport and metabolism; F: Nucleotide transport and metabolism; G: Carbohydrate transport and metabolism; H: Coenzyme transport and metabolism; I: Lipid transport and metabolism; P: Inorganic ion transport and metabolism; Q: Secondary metabolites biosynthesis, transport and catabolism; S: Function unknown).

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of secondary metabolites biosynthesis (methyltransferases), energy production (multicopper oxidases, antibiotic biosynthesis monooxygenase, copper binding proteins, cytochrome *b* subunit) and cellular processes and signalling.

Energy metabolism and respiration

Since methane and methanol inhibited ammonia oxidation and growth of '*Ca*. Nitrosocosmicus franklandus C13', we expected to observe changes in the expression of proteins involved in energy metabolism. Interestingly, methane and methanol did not significantly affect the expression of the subunits of AMO in the proteome. Whilst there was a consistent pattern in the expression of all subunits, including the recently identified subunits AmoX, AmoY and AmoZ (Hodgskiss et al., 2022), the differences between cultures grown with and without C1 compounds were not statistically significant (Figure S4). Contrary to our expectations, there was a significant upregulation of the urease enzyme subunits UreA, UreB and UreC in response to methane. Urease is an enzyme catalysing the hydrolysis of urea to ammonia and CO_2. Although '*Ca*. Nitrosocosmicus franklandus C13' can grow on urea (Lehtovirta-Morley et al., 2016), no urea was added at any point of these experiments. While the regulation of the archaeal urease is not well understood, the upregulation of urease and its accessory proteins could be a response to energy limitation (Reyes et al., 2020). A previous study found that the *ureC* subunit of the AOA '*Ca*. Nitrosocosmicus agrestis' was transcribed in cultures grown on ammonia as the sole nitrogen and energy source, although transcription was upregulated in the presence of urea (Liu et al., 2021).

Multiple proteins involved in electron transport and energy generation were affected by the presence of methane and methanol. NADH dehydrogenase subunit I (NFRAN_2088) was highly upregulated in both treatments (Table S1). Ferredoxin (NFRAN_2188) was fourfold upregulated in the presence of methane and eightfold upregulated in the presence of methanol. These proteins contain the 4Fe–4S dicluster domain and are involved in electron transport and energy conservation in the form of proton motive force and the electrochemical membrane potential (Buckel & Thauer, 2013). Several multicopper oxidases (NFRAN_2029, NFRAN_2030, NFRAN_2792, NFRAN_2798) putatively involved in the electron transport pathway were downregulated in the presence of both methane and methanol (Table S1) (Kerou et al. 2016; Walker et al. 2010). It has been previously shown that upon Cu-limitation, these multicopper oxidases were upregulated in the transcriptome of *N. viennensis*, and although they are conserved in AOA, their role has not been experimentally validated (Reyes et al., 2020). In addition, in the presence of methanol, amicyanin (NFRAN_0912) and cytochrome *b*6 (NFRAN_0910), putatively involved in electron transport, were downregulated (Table S1).

Stress responses induced by methane and methanol in '*Ca*. Nitrosocosmicus franklandus C13'

In addition to energy acquisition, we hypothesised that additional stress may be caused by further factors, such as methanol being a solvent and membrane permeable. Proteins putatively involved in cell wall modifications (NFRAN_0106, NFRAN_0021) were downregulated by both methane and methanol. In addition, undecaprenyl-phosphate mannosyltransferase (NFRAN_1957) was downregulated in the presence of methane. These changes may be related to remodelling of the cell envelope in response to stress from energy limitation. There appeared to be an increase in the cell size in the presence of methanol compared to other treatments. However, this observation was made in a small subset of samples and would require a more systematic study in the future to understand the mechanisms responsible for it. Putative DNA protection during starvation protein (NFRAN_2658) was fourfold upregulated by methane and sixfold upregulated by methanol. This cytoplasmic protein was among the most highly upregulated proteins and likely protects DNA during starvation. The homologues of this protein belong to the ferritin superfamily, suggesting that it may bind iron (Grant et al., 1998). Universal stress protein (NFRAN_2131) was also upregulated in the presence of methanol.

Can proteomics provide insights into further metabolism of C1 compounds in '*Ca*. Nitrosocosmicus franklandus C13'?

Although the oxidation of methane and methanol by AOA was not tested in this study, the fact that methane and methanol compete for the same active site as ammonia in the archaeal AMO raises an interesting possibility that they may act as substrates as well as inhibitors. Thus, we examined the proteomics dataset for upregulation of any genes putatively involved in C1 oxidation pathways.

It was also previously postulated that the AOA '*Ca*. Nitrosocosmicus hydrocola' may have the genetic potential to metabolize methane, due to the presence of putative formaldehyde dehydrogenases (WP_148687581.1, WP_196777382.1) and formate dehydrogenase (WP_148685767.1), although this has not been tested experimentally (Sauder et al., 2017). '*Ca*. Nitrosocosmicus franklandus C13' lacks any recognizable homologues of the C1 oxidation enzymes proposed in '*Ca*. Nitrosocosmicus hydrocola'.

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Therefore, if methane and methanol are oxidized to CO_2 by '*Ca*. Nitrosocosmicus franklandus C13', these reactions would need to be catalysed by different enzymes.

In methanotrophs and methylotrophs, methanol is converted to CO_2 via formaldehyde and formate. Methanol oxidation to formaldehyde in these microorganisms is usually carried out by PQQ-dependent MDH (Mxa-MDH andXox-MDH). PQQ-dependent dehydrogenases of the quinoprotein family can also oxidize a range of sugars, alcohols and aldehydes (Anthony & Ghosh, 1998; Southall et al., 2006; Wu et al., 2015). '*Ca*. Nitrosocosmicus franklandus C13' genome contains 12 coding sequences with homology to PQQ-dependent dehydrogenases, all of them with a slightly higher identity to sugar dehydrogenases than MDH, although similarity to any previously characterized dehydrogenases was low. Of the PQQ-dependent dehydrogenases, NFRAN_2094 was significantly upregulated (approximately four-fold) in the treatments with methane and methanol (Figure S5). Homologues of PQQ-dependent dehydrogenases are found in the AOA genera *Nitrosocosmicus*, *Nitrososphaera* and *Nitrosopolaris*, but are not universally conserved in all AOA. PQQ-dependent aldose sugar dehydrogenases are also found in, for example, the AOA '*Ca*. Nitrosocosmicus arcticus', and might have the ability to contribute reducing equivalents to the respiratory chain by oxidizing sugars to lactones (Alves et al., 2019). The upregulation of PQQ-dependent dehydrogenases may be related to modifications of the cell envelope of '*Ca*. Nitrosocosmicus franklandus C13'. It is difficult, however, to draw conclusions on the upregulation of NFRAN_2094 in '*Ca*. Nitrosocosmicus franklandus C13' due to the lack of closely related, functionally characterized proteins. In addition, it is worth noting that the physiological substrates for some of the PQQ-dependent dehydrogenases are not confirmed even in, for example, *Escherichia coli* or *Pyrobaculum aerophilum*, and although these enzymes can oxidize a range of sugars, there may be other, as-yet unknown substrates they accept (Sakuraba et al., 2010; Southall et al., 2006). It is interesting to note that, although low identity (27%, *e* value $= 1 \times 1^{-10}$) to NFRAN_2094, the homologue in *Hyphomicrobium zavarzinii* is an experimentally confirmed PQQ-dependent formaldehyde dehydrogenase (Jérôme et al., 2007). In contrast, PqqC-like protein (NFRAN_0383) and pyrroloquinoline quinone biosynthesis protein (PqqE) (NFRAN_2125) were significantly downregulated in the presence of methanol.

As well as PQQ-dependent dehydrogenases, the proteomes were searched for other dehydrogenases which may use alcohols, aldehydes or formate as substrates. F_{420}-dependent oxidoreductase (NFRAN_1153) was among the highly upregulated proteins in the presence of both methane and methanol. F_{420}-dependent oxidoreductases oxidize a wide range of substrates, including alcohols, alkanes, alkanesulfonates and glucose-6-phosphate (Greening et al., 2016). The sequence identity of NFRAN_1153 to characterized members of this protein superfamily is low and it is difficult to speculate on its role in growth in the presence of methane and methanol. Aldehyde reductase (NFRAN_2275) was two-fold upregulated in '*Ca*. Nitrosocosmicus franklandus C13' incubated with methanol. This enzyme could be involved in the oxidation of formaldehyde, but also in the oxidation of methanol itself, as it is a presumed alcohol dehydrogenase. It is phylogenetically related to several alcohol dehydrogenases, which have been shown experimentally to oxidize medium-chain length aldehydes and alcohols (Figure S6).

CONCLUDING REMARKS

Although the functional similarity and shared evolutionary history of the ammonia and methane monooxygenases has long been recognized (Holmes et al., 1995), the effect of methane and methanol on the archaeal AMO has remained surprisingly enigmatic. In this study, we provide compelling evidence that both methane and methanol are specific, competitive inhibitors of the archaeal AMO in the model soil archaeon '*Ca*. Nitrosocosmicus franklandus C13'. Furthermore, we demonstrate that the AOA '*Ca*. Nitrosocosmicus franklandus C13' is several orders of magnitude more sensitive to inhibition by methane and methanol than the soil AOB *N. europaea*. This finding has important consequences for soil environments. More studies are needed to determine inhibition thresholds of other β-proteobacterial AOB and AOA. Methane and methanol commonly occur in soil environments and future studies are required to determine whether one-carbon compounds influence and indeed inhibit terrestrial nitrification, particularly in ecosystems where nitrification is driven by AOA.

We also demonstrate that methane and methanol specifically interact with AMO, and the presence of methane or methanol results in changes in the expression of stress response, energy metabolism and dehydrogenase enzymes of a key genus of AOA. There are open questions as to whether AOA can oxidize one-carbon compounds and assimilate carbon derived from them. Although we observed a significant shift in the AOA proteome in response to methane and methanol, we did not directly test for oxidation of methane or methanol by AOA in this study. It is noteworthy, however, that in a previous study, where soil microcosms were incubated with ^{13}C-methanol, incorporation of ^{13}C carbon by AOA was observed (Macey et al., 2020). This indicates that at least some AOA in soil were able to assimilate carbon derived from methanol in soil in

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situ, either directly or indirectly through cross-feeding. Future studies are needed to address the potential roles of methane and methanol as substrates for oxidative metabolism and carbon assimilation by AOA.

AUTHOR CONTRIBUTIONS

Barbora Oudova-Rivera: Formal analysis (equal); investigation (lead); methodology (equal); writing – original draft (lead); writing – review and editing (equal). **Chloe L Wright:** Formal analysis (equal); investigation (equal); methodology (equal); writing – review and editing (equal). **Andrew Crombie:** Supervision (equal); writing – review and editing (equal). **Colin Murrell:** Supervision (equal); writing – review and editing (equal). **Laura Lehtovirta-Morley:** Funding acquisition (lead); supervision (lead); writing – original draft (supporting); writing – review and editing (lead).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the supplementary material of this article.

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