Trace methane oxidation studied in several Euryarchaeota under diverse conditions

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Summary We used ^{13}C-labeled methane to document the extent of trace methane oxidation by *Archaeoglobus fulgidus*, *Archaeoglobus lithotrophicus*, *Archaeoglobus profundus*, *Methanobacterium thermoautotrophicum*, *Methanosarcina barkeri* and *Methanosarcina acetivorans*. The results indicate trace methane oxidation during growth varied among different species and among methanogen cultures grown on different substrates. The extent of trace methane oxidation by *Mb. thermoautotrophicum* ($0.05 \pm 0.04\%$, ± 2 standard deviations of the methane produced during growth) was less than that by *M. barkeri* ($0.15 \pm 0.04\%$), grown under similar conditions with H_2 and CO_2. *Methanosarcina acetivorans* oxidized more methane during growth on trimethylamine ($0.36 \pm 0.05\%$) than during growth on methanol ($0.07 \pm 0.03\%$). This may indicate that, in *M. acetivorans*, either a methyltransferase related to growth on trimethylamine plays a role in methane oxidation, or that methanol is an intermediate of methane oxidation. Addition of possible electron acceptors (O_2, NO_3^-, SO_4^{2-}, SO_3^{2-}) or H_2 to the headspace did not substantially enhance or diminish methane oxidation in *M. acetivorans* cultures. Separate growth experiments with FAD and NAD^+ showed that inclusion of these electron carriers also did not enhance methane oxidation. Our results suggest trace methane oxidized during methanogenesis cannot be coupled to the reduction of these electron acceptors in pure cultures, and that the mechanism by which methane is oxidized in methanogens is independent of H_2 concentration. In contrast to the methanogens, species of the sulfate-reducing genus *Archaeoglobus* did not significantly oxidize methane during growth (oxidizing $0.003 \pm 0.01\%$ of the methane provided to *A. fulgidus*, $0.002 \pm 0.009\%$ to *A. lithotrophicus* and $0.003 \pm 0.02\%$ to *A. profundus*). Lack of observable methane oxidation in the three *Archaeoglobus* species examined may indicate that methyl-coenzyme M reductase, which is not present in this genus, is required for the anaerobic oxidation of methane, consistent with the "reverse methanogenesis" hypothesis.

Keywords: anaerobic methane oxidation, Archaeoglobus, methanogen, reverse methanogenesis, stable isotope label.

Introduction

The anaerobic oxidation of methane (AOM) is a significant methane sink in marine sediments, oxidizing up to 90% of the biological methane produced in these environments (Hinrichs et al. 2000). Organisms responsible for AOM (ANME-1 and ANME-2) have not been isolated, but genetic and lipid analyses indicate the methane-consuming organisms are archaeal and closely related to cultured methanogenic genera, specifically *Methanosarcina* and *Methanococcoides* (Hinrichs et al. 1999, 2000, Boetius et al. 2000, Orphan et al. 2001*a*). Lipid analyses and fluorescence in situ hybridization (FISH) suggest that certain bacteria form tight consortia with the archaeal methanotrophs (ANME-2), coupling methane oxidation to sulfate-reduction and rendering the overall reaction thermodynamically favorable (Boetius et al. 2000, Orphan et al. 2001*b*). Nauhaus et al. (2002), investigating methane seep sediments, showed a 1:1 reduction of sulfate to sulfide with the oxidation of methane to CO_2 when methane served as the only available electron donor. The bacterial component of these natural consortia is most closely related to isolated sulfate-reducers from the Desulfosarcinales (Orphan et al. 2001*a*), and stable isotope evidence suggests their biomass is, at least in part, composed of carbon from oxidized methane (Hinrichs et al. 2000, Pancost et al. 2000, Orphan et al. 2001*b*, Michaelis et al. 2002). Some archaeal methane-oxidizing organisms (ANME-1) may perform AOM and associated sulfate reduction unaided by bacteria (Michaelis et al. 2002, Orphan et al. 2002).

The enzymatic mechanisms mediating AOM in marine sediments remain unknown. One possible mechanism, proposed by Hoehler et al. (1994), is reverse methanogenesis. In this paper, we refer to "reverse methanogenesis" as the reversal of a methanogen's typical metabolism such that methane production is replaced by net methane oxidation. Hallam et al. (2004) recently found nearly all genes associated with methanogensis in environmental ANME-1 sequences. It is hypothesized that reverse methanogenesis begins with methane activation by either methyl-coenzyme M reductase (MCR) or methyl transfer-

ase (MT) as described by Hoehler and Alperin (1996). Methyl-coenzyme M reductase catalyzes the final reductive step in all known biogenic methane formation and the operon (*mcrA*) encoding this enzyme is observed in all known methanogen species. Consistent with the reverse methanogenesis hypothesis is the recent discovery of *mcrA* or a close variant in both ANME-1 and ANME-2 environmental samples (Hallam et al. 2003, 2004, Krüger et al. 2003*b*). In the event that ANME-2 methanotrophy is initiated by reverse MCR activity, analysis of closely related, culturable species with active MCR may provide a suitable model system for studying archaeal methane oxidation until the indigenous archaeal methanotrophs can be cultured.

We use the term "trace methane oxidation" (TMO) to refer to the conversion of labeled methane to CO_2 in methanogen cultures during typical methanogenic growth (Zehnder and Brock 1979, Harder 1997). The extent to which reverse methanogenesis and TMO are related is undetermined. Yet, if the two processes are mechanistically similar, providing an organism capable of TMO with favorable environmental conditions could potentially initiate reverse methanogenesis.

Although never experimentally demonstrated, Hohler and Alperin (1996) used a thermodynamic approach to argue that under the proper conditions, principally low H_2 pressures, reverse methanogenesis is favored. Also, exposure to typical methanogenesis inhibitors (such as bromoethane or methyl fluoride) halts AOM activity within marine consortia (Krüger et al. 2003*a*), suggesting a possible link to methanogenesis pathways. The reverse methanogenesis hypothesis invokes consortia associations to help maintain thermodynamic favorability by consuming the methanotroph's metabolic end product, keeping its concentration below a critical thermodynamic threshold. One possible end product is H_2, which could be produced by an archaeal methanotroph and consumed by sulfate-reducing bacteria. Valentine et al. (2000) monitored H_2 production rates in stressed methanogen cultures subjected to high methane concentrations and low methanogenic substrate levels. No link between methane abundance and H_2 production was observed, suggesting reverse methanogenesis is either unrelated to H_2 production or it is not a metabolism adoptable by the methanogens tested. Acetate has been proposed as an alternative AOM intermediate (Valentine and Reeburgh 2000). Yet, Nauhuas et al. (2002) tested multiple potential intermediates (including acetate, formate, hydrogen and methanol) and demonstrated these possible intermediates did not greatly enhance sulfate reduction rates in methane seep sediment samples, suggesting that acetate is not an intermediate for in situ AOM.

In order to further understanding of TMO and evaluate the reverse methanogenesis hypothesis, we examined TMO in pure cultures of three *Archaeoglobus* species and three methanogen species. Our experiments were aimed at determining (1) if archaeal species more closely related to AOM methanotrophs exhibited a stronger propensity for methane oxidation expressed through enhanced TMO, (2) whether the inclusion of different potential electron acceptors make conditions more amenable to methane oxidation, increasing the level of TMO

and (3) if archaeal sulfate-reducers, which share genetic similarity to the ANME group but lack the potential enzymatic methane activators (namely MCR) required for reverse methanogensis, exhibit TMO.

For each experiment, a set of cultures was grown under headspaces containing differing proportions of ^{13}C-labeled methane. Following growth, isotopic analysis was used to quantify the amount of ^{13}C label converted from methane to CO_2, indicating the extent of TMO in the culture.

Materials and methods

Microorganisms

Archaeoglobus fulgidus VC16, *Archaeoglobus profundus* AV18 and *Methanobacterium thermoautotrophicum* ΔH were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). *Methanosarcina barkeri* MS was obtained from the Oregon Collection of Methanogens (Portland, OR). *Archaeoglobus lithotrophicus* TF2 was a kind gift from Karl Stetter (emeritus, Universität Regensburg, Germany). *Methanosarcina acetivorans* 2CA was provided by J.G.F.

Media

Strict anaerobic technique was employed for cell culturing. Media for *A. fulgidus* and *A. profundus* were prepared as recommended by DSMZ (media 399 and 519, respectively). *Methanobacterium thermoautotrophicum* was cultured on medium containing: 3.0 g Na_2SO_4, 0.2 g KH_2PO_4, 0.3 g NH_4Cl, 0.3 g KCl, 0.2 g $CaCl_2 \cdot 2H_2O$, 0.3 g $MgCl_2 \cdot 6H_2O$, 1.0 ml 10× Wolfe solution (Wolin et al. 1963), 0.5 mg resazurin, 3.0 ml NaOH solution (10%) and 0.5 g $Na_2S \cdot 9H_2O$. All reagents except Na_2S were mixed in solution and degassed by bubbling with N_2 for 20 min followed by Na_2S addition. The medium (20 ml per bottle) was dispensed into 120-ml culture bottles in an anaerobic chamber followed by three flushes with a mixture of H_2 and CO_2 (80:20 (v/v)) and subsequent filling of the bottles to 0.3 MPa pressure with the gas mixture and sterilization in an autoclave. *Archaeoglobus lithotrophicus* was cultured in the same medium, but with the addition of 20 g NaCl l^{-1}. *Methanosarcina barkeri* was cultured in medium containing (per liter): 0.29 g $K_2HPO_4 \cdot 3H_2O$, 0.23 g KH_2PO_4, 0.23 g $(NH_4)_2SO_4$, 0.45 g NaCl, 0.06 g $CaCl_2 \cdot 2H_2O$, 0.09 g $MgSO_4 \cdot 7H_2O$, 1.0 ml $(NH_4)_2Ni(SO_4)_2$ solution (0.2%), 1.0 ml $FeSO_4 \cdot 7H_2O$ solution (0.2%), 0.5 mg resazurin, 1.0 ml 10× Wolfe mineral solution (Wolin et al. 1963), 6.0 ml vitamin solution, 4.0 ml NaOH solution (10%) and prepared as above with the addition of 0.5 g l^{-1} $Na_2S \cdot 9H_2O$. Medium for *M. acetivorans* contained (per liter final solution): Mix A: 23.4 g NaCl, 3.8 g $NaHCO_3$, 1.0 g KCl, 1.0 ml 10× Wolfe mineral solution, 2.0 ml vitamin solution and 0.5 mg resazurin; Mix B: 11.0 g $MgCl_2 \cdot 6H_2O$, 0.30 g $CaCl_2 \cdot 2H_2O$ and 0.68 g KH_2PO_4; Mix C: 1.0 g NH_4Cl, 0.50 g cysteine hydrochloride and appropriate substrate; and Mix D: 0.50 g $Na_2S \cdot 9H_2O$. Mixes A and B in 500 ml water each were degassed under N_2 then combined in an anaerobic chamber followed by addition of Mix C and finally the addition of Mix D. Substrates were

trimethylamine (9.56 g l^{-1}) or methanol (5.0 ml l^{-1}). Once dispensed (30 ml per culture bottle), individual bottles were flushed three times, then filled to 0.2 MPa pressure with a mixture of N_2 and CO_2 (80:20 (v/v)) and autoclaved. For the experiment involving *M. acetivorans* and H_2, the culture bottles were pressurized to 0.2 MPa with a mixture of H_2 and CO_2 (80:20 (v/v)). For experiments involving *M. acetivorans* and oxygen, neither Na_2S nor resazurin were included in the growth media and sterile oxygen was injected into the bottles after autoclaving.

Cultivation

Two sets of four culture bottles were used for each species investigated. A total of 40 ml (at 0.1 MPa $\approx$1.8 mmol) of methane was added to each culture bottle with increasing proportions of labeled methane (99% $^{13}CH_4$, 1% $^{12}CH_4$) added to each bottle in the series (Lot 00-04, Cambridge Isotope Labs, Cambridge, MA). For each tested species, one set of bottles was inoculated for growth and the other was kept uninoculated as a negative control. All species, except *M. barkeri*, were incubated for 1 week to ensure maximum growth and substrate consumption. Because of its slower growth, *M. barkeri* was incubated for 2 months. The amount of methane produced in each culture was calculated from established stoichiometric relationships for each substrate and final methane production. The cultures were grown to completion, ensuring complete substrate usage. Measurement of methane concentration in parallel experiments, by gas chromatography with a thermal conductivity detector, confirmed the accuracy of calculated methane productivity. Cell counts following growth were performed by phase-contrast microscopy with a counting chamber for all species but *M. barkeri* where cell clumping prevented accurate counting.

Sample preparation

Following culture growth, two distillations were performed to collect and purify CO_2 from each sample for isotope analysis. The first distillation was done with an apparatus (Figure 1) we developed for collection of CO_2 from high pressure cultures with up to 0.4 MPa of mixed gases. After growth, a sample bottle was connected to the evacuated distilling apparatus through a gas-tight needle. A liquid nitrogen trap froze CO_2 from the culture bottles into a separate collection tube, and gaseous methane was pumped from the sample. A secondary liquid nitrogen trap ensured quantitative CO_2 collection. During growth, the *Archaeoglobus* spp. produced H_2S, which interfered with preliminary CO_2 isotope analysis. For these samples, about 100 mg of silver foil was included in the sample collection vial at room temperature over 24–48 h prior to analysis on the mass spectrometer. The foil removed all H_2S artifacts from the isotopic analysis. A second distillation cryogenically trapped and removed water (2-propanol and dry ice) and condensed CO_2 (liquid nitrogen) to assure residual non-condensable gasses were removed. Isotope analysis to determine the $^{13}C/^{12}C$ ratio for the CO_2 in each culture bottle was performed with a Finnigan MAT 252 dual inlet mass spectrometer (Thermo Finnigan, Bremen, Germany).

Data analysis

Isotope data were plotted as the ^{13}C fractional abundance of the initial methane ($F_{CH4} = {}^{13}C/({}^{13}C + {}^{12}C)$) in each culture bottle versus the measured fractional abundance of CO_2 (F_{CO2}) in each bottle after incubation (Figure 2). Least-squares calculations provide the slope and intercept for the resulting relationships and a positive slope indicates methane oxidation. We next consider mass balance for methane and CO_2 produced by *M. acetivorans* cultures during growth on either trimethylamine or methanol. The methane in a culture bottle after growth represents that contributed during growth and methane lost by oxidation:

$$F_T n_T = F_i n_i + F_b n_b - F_{ox} n_{ox} \tag{1}$$

where F_T, F_i, F_b and F_{ox} represent the fractional abundance of carbon isotopes in total, initial, biogenic and oxidized methane, respectively. Likewise, n_T, n_i, n_b and n_{ox} represent the moles of total, initial, biogenic and oxidized methane, respectively. We assume that any fractionation in the methane oxidation process is insignificant in our strongly labeled system, such that $F_{ox} = F_T$; we can therefore simplify Equation 1 and solve for F_T:
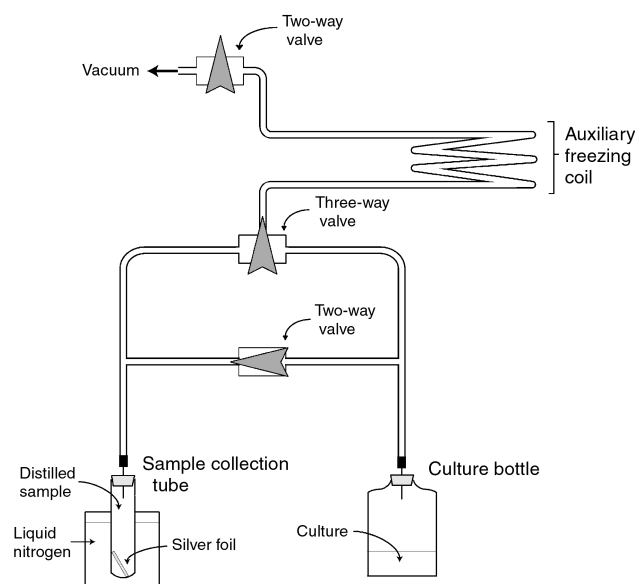
$$F_T = \frac{F_i n_i + F_b n_b}{n_i + n_b} \tag{2}$$

Figure 1. The distilling apparatus used to isolate CO_2 cryogenically from pressurized culture bottles after growth. Sample was introduced to the system through a needle. Liquid nitrogen collected CO_2 in a separate sample tube, allowing for subsequent removal of non-condensable gases by a vacuum. The auxiliary freezing coil was also cooled by liquid nitrogen and served as a secondary trap for assuring no CO_2 loss. Heating the auxiliary freezing coil after removing non-condensable gases allowed any trapped CO_2 to migrate to the sample tube.

We assume oxidized CH_4 is converted to CO_2, such that:

$$F_{T_{CO2}} n_{T_{CO2}} = F_{i_{CO2}} n_{i_{CO2}} + F_{b_{CO2}} n_{b_{CO2}} + F_{T_{CH4}} n_{ox} \qquad (3)$$

where $F_{T_{CO2}}$, $F_{i_{CO2}}$ and $F_{b_{CO2}}$ represent fractional abundance of carbon isotopes in total, initial and biogenic CO_2, respectively. Similarly, $n_{T_{CO2}}$, $n_{i_{CO2}}$ and $n_{b_{CO2}}$ represent moles of total, initial and biogenic CO_2, respectively. In a culture bottle containing unlabeled methane, we can ignore the methane oxidation input into the CO_2 mass balance because the small n_{ox} does not significantly affect $F_{T_{CO2}}$ or $n_{T_{CO2}}$. In cases with unlabeled methane we have:

$$F_{T_{CO2}*} n_{T_{CO2}*} = F_{i_{CO2}*} n_{i_{CO2}*} + F_{b_{CO2}*} n_{b_{CO2}*} \qquad (4)$$

where terms are defined as in Equation 3, and an asterisk refers to bottles cultured with unlabeled methane. We prepared incubation bottles such that $F_{i_{CO2}} = F_{i_{CO2}*}$ and $n_{i_{CO2}} = n_{i_{CO2}*}$. Therefore, unlabeled incubations account for CO_2 produced during cell growth:

$$F_{T_{CO2}} n_{T_{CO2}} = F_{T_{CO2}*} n_{T_{CO2}*} + F_T n_{ox} \qquad (5)$$

Combining Equations 2 and 5 and solving for n_{ox} gives:

$$n_{ox} = (F_{T_{CO2}} n_{T_{CO2}} - F_{T_{CO2}*} n_{T_{CO2}*}) \frac{F_i n_i + F_b n_b}{n_i + n_b} \qquad (6)$$

where $F_{T_{CO2}}$ and $F_{T_{CO2}*}$ were measured in multiple sample cultures with varied F_i (Figure 2). The value of $F_{T_{CO2}}$ was interpolated from the linear regression of the data at the median F_i, whereas $F_{T_{CO2}*}$ was interpolated from the same line at the point with no labeled methane. We controlled the amount of labeled methane added to each bottle, with n_i fixed at 1.8 mmol. We estimated the biogenic methane composition to be $\delta = -50\permil$, and therefore $F_b = 0.0106$. Because of the strong label, small variations in F_b become inconsequential to the results. The value of n_b was estimated based on the stoichiometry of methanogenesis for a given substrate and confirmed in parallel experiments by gas chromatography with a thermal conductivity detector. The value of $n_{T_{CO2}*}$ was estimated by summing $n_{i_{CO2}*}$ and $n_{b_{CO2}*}$; CO_2 input from CH_4 oxidation was small in comparison with other CO_2 sources.

We solved for n_{ox} using an iterative method. First, $n_{T_{CO2}}$ was estimated as equal to $n_{T_{CO2}*}$. We then solved for n_{ox} by adjusting $n_{T_{CO2}}$ to account for CO_2 derived from CH_4 oxidation. The n_{ox} values converged within two to three iterations. We calculated n_{ox} for both the growth experiments and control bottles (media with labeled CH_4 but no cells) and the amount of CH_4 oxidized in each experiment is reported as the difference between these two values. Error estimates are based on the standard error of the slope used to calculated $F_{T_{CO2}}$ and $F_{T_{CO2}*}$. Final reported error was estimated by repeating the above calculations using an experimental slope plus twice the slope standard error (i.e., 2 SD) and a control slope minus twice the slope standard error. The same system of calculations was used for determining CH_4 oxidation in the other methanogen and sul-
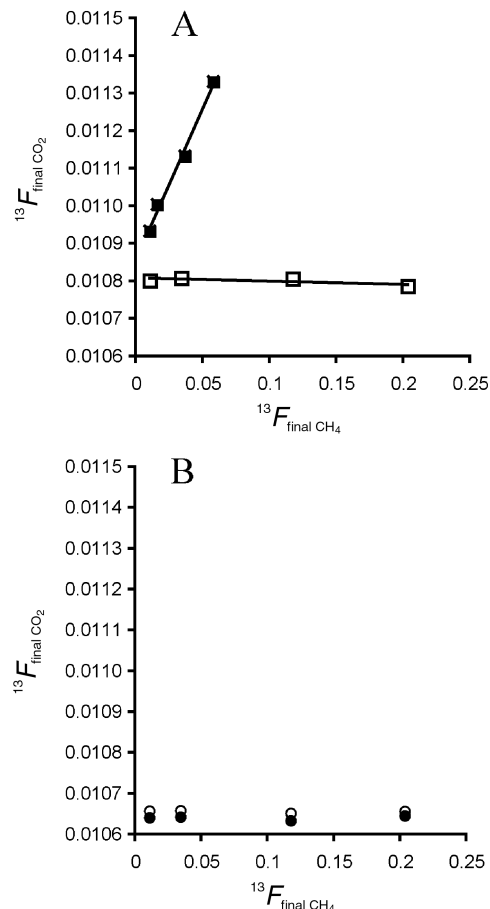

Figure 2. The ^{13}C fractional abundance (^{13}F) of a culture bottle's CO_2 after incubation versus ^{13}F of final CH_4 tracks methane oxidation to CO_2. (A) The ^{13}F CO_2 in culture bottle headspace following incubation versus ^{13}F of the final methane in each bottle for *Methanosarcina acetivorans* cultures (■) grown with trimethylamine and for identical, but not inoculated, control bottles (□). The difference in slopes between the cultured (8.0×10^{-3}) and control samples (-8.6×10^{-5}) determined the amount of methane oxidation in the experiment. (B) Results from *Archaeoglobus profundus* (●) and control samples (○) showing no direct relationship between the extent of final methane enrichment and ^{13}F of final CO_2, indicating no methane oxidation by the cultures.

fate reducer cultures, adjusted to account for stoichiometric input (or uptake) of biogenic methane and CO_2 (n_b, $n_{b_{CO2}}$). Methane oxidation is reported as both the percent of total post-growth methane oxidized in each bottle and, for methanogens, as the percent of methane produced during growth that was oxidized to CO_2.

Results and discussion

Archaeoglobus is an archaeal sulfate-reducing genus with multiple culturable representatives (Stetter et al. 1987, 1993). There are several reasons we chose to look at *Archaeoglobus* for studying TMO. First, recent environmental genomic sequencing of uncultured AOM methanotrophs revealed a gen-

eral core similarity between the ANME-1 and the Archaeo-globales (Hallam et al. 2004). Second, sulfate reduction by *Archaeoglobus* could provide an electron sink for methane oxidation. Third, *Archaeoglobus* species possess much of the enzymatic machinery associated with methanogenesis (Klenk et al. 1998). *Archaeoglobus fulgidus*, for example, contains enzymes involved with the reduction of CO_2 to a methyl group in methanogenesis, but operates these enzymes in the reverse direction, oxidizing methyl groups to CO_2. Yet, *Archaeoglobus* species lack MCR (Stetter et al. 1987, Klenk et al. 1998), used in all known methanogenic pathways and thought likely to be required for reverse methanogenesis. Although the role of MCR in methanotrophy is undetermined, its absence from *Archaeoglobus* species would prohibit the initiation steps required for reverse methanogenesis unless an alternative enzymatic pathway for methane activation is present. We examined methane oxidation in three *Archaeoglobus* species, each utilizing different growth substrates for sulfate reduction: *A. fulgidus* utilizes lactate; *A. profundus* utilizes acetate and H_2; and *A. thermolithotrophicus* grows chemolithotrophically on media containing H_2 and CO_2. In each case, the amount of methane oxidized was not detected within the experimental error (Figure 3, Table 1). Lack of observable TMO in these species suggests MCR is required for TMO, which is consistent with the reverse methanogenesis hypothesis for AOM.

The methanogens, *Mb. thermoautotrophicum*, *M. barkeri* and *M. acetivorans*, each displayed small amounts of methane oxidation (Figure 3, Table 1), consistent with previous reports based on similar experimental work (Zehnder and Brock 1979, 1980, Harder 1997). We further explored the reaction in *M. acetivorans*, which has a close rRNA phylogenetic relationship with the ANME-2 methanotroph lineage (Orphan et al. 2001*a*) and also has a large and complex genome (~5.8 Mb) with high metabolic diversity (Galagan et al. 2002). Such a close relationship to indigenous AOM species coupled with its large genome makes *M. acetivorans* a reasonable candidate for harboring genes required for reverse methanogenesis and may make it a valuable model organism for studying archaeal methane oxidation. The TMO we observed suggests a pathway for methane oxidation is present in *M. acetivorans*.

We tested whether O_2, NO_3^-, SO_4^{2-} and SO_3^{2-} could be used as electron acceptors in methane oxidation by *M. acetivorans*. Enriching the culture medium with these electron acceptors could stimulate methane oxidation if electrons can be transferred to them effectively. Sulfite is a metabolic intermediate in bacterial sulfate reduction and, therefore, we hypothesize that it could be transferred between the archaeal and bacterial members of in vivo AOM consortia. In our model, sulfite transfer within the AOM consortia from the sulfate reducer to the archaeal ANME-2 would provide oxidizing potential for the methanotrophic reactions. However, none of the electron acceptors tested with *M. acetivorans* substantially enhanced the amount of methane oxidized in the cultures (Figure 3, Table 1), indicating that these electron acceptors, including sulfite, are not utilized by this methanogen to oxidize methane, even in trace amounts.

Interestingly, most methanogen species are extremely oxygen sensitive, being killed by even trace amounts of oxygen. However, two superoxide dismutases, a superoxide reductase and a catalase, were uncovered in the *M. acetivorans* genome, suggesting the potential for oxygen resistance (Galagan et al. 2002). The presence of a cytochrome *d* oxidase may even provide a link between oxygen and energy conservation by the organism (Galagan et al. 2002). We cultured *M. acetivorans* under a microaerophilic headspace (0.7% O_2). Diffusional constraints likely lowered the percent of oxygen in the actual liquid growth medium, but *M. acetivorans* still demonstrated oxygen tolerance under microaerophilic conditions. The presence of oxygen had little effect on cell yields, methane production or trace methane oxidation in these cultures (Figure 3, Table 1), indicating that the oxygen reduction, which is likely occurring, cannot be coupled to methane oxidation. Research is now underway to more closely examine the responses of *M. acetivorans* to oxygen.

We also tested the effect of including H_2 in the culture headspace. *Methanosarcina acetivorans* is capable of growth and methanogenesis using a large array of substrates but does not use H_2. Unlike the other methanogens tested, *M. aceti-*
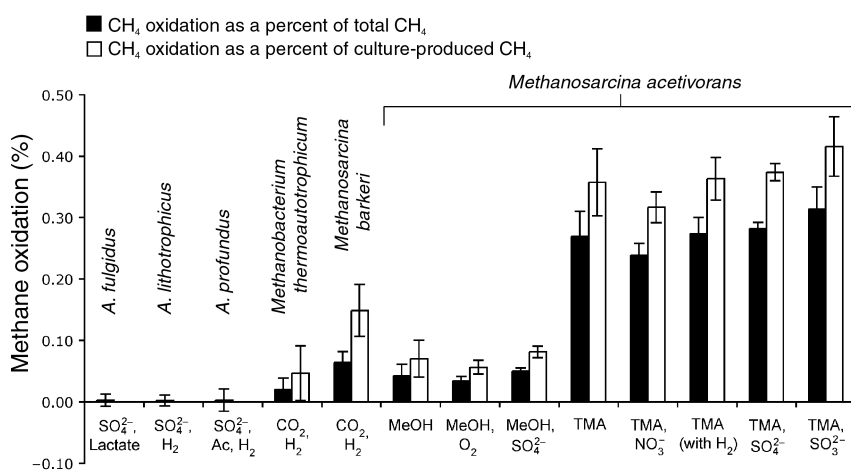

Figure 3. Methane oxidation (± 2 SD) standardized to either the total amount of methane in the culture after growth (black bars) or the methane produced by the culture during growth (white bars) observed in experiments with varying species and substrates. Added concentrations: lactate, 13.4 mM; H_2 (for all but *M. acetivorans* experiment), 0.24 MPa; H_2 (*M. acetivorans* experiment), 0.16 MPa; Ac (acetate), 12.2 mM; SO_4^{2-} (for *A. fulgidus*),14.0 mM; SO_4^{2-} (for *A. profundus*), 33.0 mM; SO_4^{2-} (for *A. thermolithotrophicus*), 21.1 mM; MeOH (methanol), 125 mM; O_2, 0.7 kPa; TMA (trimethylamine), 100 mM; NO_3^-, 6.59 mM; SO_4^{2-} (for *M. acetivorans* experiments), 21.1 mM; and SO_3^{2-}, 2 mM.

Table 1. Growth conditions and methane oxidation per culture experiment. Each culture was initially provided 1.79E–3 mol of methane. In addition, based on methanogenesis, stoichiometry and measurements performed in parallel experiments, the cultures produced 1.3E–3, 2.8E–3 and 5.5E–3 mol of methane for the cultures grown on H_2/CO_2, methanol and trimethylamine (TMA), respectively.

Species	Growth substrate	Media addition	Incubation temperature (°C)	Cell count (ml^{-1})	Experimental slope	Control slope	Methane oxidized (mol)
A. fulgidus	Lactate	–	85	2E+8	3.1E–5	1.7E–5	4.7E–8 (± 1.7E–7)
A. lithotrophicus	H_2, CO_2	–	85	7E+7	9.7E–5	8.1E–5	3.8E–7 (± 1.6E–7)
A. profundus	Acetate, H_2	–	85	1E+8	7.8E–6	–1.3E–5	5.2E–8 (± 2.3E–7)
Mb. thermoautotrophicum	H_2, CO_2	–	65	5E+7	6.1E–4	2.8E–5	6.3E–7 (± 5.9E–7)
M. barkeri	H_2, CO_2	–	33.5	n/a [1]	1.8E–3	6.0E–6	2.0E–6 (± 5.7E–7)
M. acetivorans	Methanol	–	33.5	5E+8	1.0E–3	4.7E–5	2.0E–6 (± 8.5E–7)
M. acetivorans	Methanol	O_2	33.5	5E+8	7.9E–4	1.2E–5	1.6E–6 (± 3.2E–7)
M. acetivorans	Methanol	SO_4^{2-}	33.5	4E+8	1.1E–3	4.9E–6	2.3E–6 (± 2.6E–7)
M. acetivorans	TMA	–	33.5	1E+9	8.0E–3	–8.6E–5	2.0E–5 (± 3.0E–6)
M. acetivorans	TMA	NO_3^-	33.5	9E+8	7.2E–3	9.2E–5	1.7E–5 (± 1.4E–6)
M. acetivorans	TMA	H_2	33.5	2E+9	8.3E–3	7.0E–5	2.0E–5 (± 1.9E–6)
M. acetivorans	TMA	SO_4^{2-}	33.5	5E+8	8.5E–3	3.4E–5	2.0E–5 (± 7.6E–7)
M. acetivorans	TMA	SO_3^{2-}	33.5	1E+9	9.4E–3	–2.5E–5	2.3E–5 (± 2.7E–6)

[1] Clumping of *M. barkeri* during growth prevented accurate cell counting for these cultures.

vorans lacks two essential hydrogenases required for methanogenic growth using hydrogen but does have other hydrogenases frequently associated with methanogenesis (Galagan et al. 2002). Hydrogen transfer between AOM consortia members may be essential for maintaining the low hydrogen concentrations required by thermodynamic constraints (Hoehler et al. 1994). In this experiment, however, the presence of hydrogen did not have a deleterious effect on the amount of methane oxidized (Figure 3, Table 1).

Nauhaus et al. (2002) suggested that AOM depends on the transfer of electron carriers between consortia members. In this model, ANME-2 would donate electrons from methane to an oxidized electron carrier that is then passed to the bacterial sulfate-reducer. We used a simplified protocol to determine whether adding either NAD$^+$ or FAD, the oxidized forms of these carriers, to *M. acetivorans* cultures increased methane oxidation during growth. We incubated cultures with trimethylamine (TMA) as a growth substrate and also provided labeled methane (initial F_{CH4} estimated at 0.117) in media containing 0.1 mM NAD$^+$ or FAD. If these common electron carriers can be used by *M. acetivorans* during TMO we would have observed enhanced methane oxidation. Yet, when compared to the results shown in Table 1, neither NAD$^+$ nor FAD elevated methane oxidation in *M. acetivorans* above typical background values, suggesting the two electron carriers cannot be coupled to methane oxidation.

We also examined *M. acetivorans* for trace methane oxidation when grown on two different substrates, methanol and TMA. More methane was oxidized when TMA, versus methanol, was the substrate for methanogensis. The reason for enhanced methane oxidation in cultures grown on TMA is at present unknown. One potential explanation involves enzymatic differences in the TMA and methanol pathways. Methylamine-based methanogenesis requires three distinct methyltransferase I isozymes specific for mono-, di- and trimethylamines, and only one of two isozymes of methyltransferase II. Methanogenesis from methanol uses only one methyltransferase I and one methyltransferase II, both specific for methanol (Ding et al. 2002). Enhanced TMO in cultures grown with TMA may result from enhanced expression of a methyltransferase involved in TMA metabolism or enhanced expression of a closely related homologue of such a methyltransferase. Alternatively, it is possible that methanol may be an end product of TMO. Nauhaus et al. (2002) explored sulfate reduction in marine AOM samples. They found that when methane was removed and replaced by methanol, sulfate reduction continued at approximately half the original rate. Microbial responses to methanol can be sensitive to concentration (for example, methanol becomes toxic at higher concentrations) and adjusting the methanol concentration used by Nauhuas et al. (2002) may optimize the reactions they observed, increasing sulfate reduction. The ability of consortia of sulfate-reducing bacteria to use methanol as a substrate at all suggests that methanol is an intermediate of AOM. The sulfate-reducing bacteria would be crucial for removing excess methanol, preventing its build up from making AOM energetically unfavorable. If a similar mechanism of methane oxidation occurred in our experiments, TMO might be hindered in cultures grown with methanol because high methanol concentrations could limit methane oxidation.

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