Archaea catalyze iron-dependent anaerobic oxidation of methane

Katharina F. Ettwig,1,2 Baoli Zhu,1,3 Daan Speth,4,5 Jan T. Keltjens,6 Mike S. M. Jetten7, and Boran Kartal8,2,5

*Department of Microbiology, Institute for Water and Wetland Research, Radboud University, Heyendaalseweg 135, 6525 AJ Nijmegen, The Netherlands

Edited by Donald E. Canfield, Institute of Biology and Nordic Center for Earth Evolution, University of Southern Denmark, Odense M, Denmark, and approved September 16, 2016 (received for review June 13, 2016)

An aerobic oxidation of methane (AOM) is crucial for controlling the emission of this potent greenhouse gas to the atmosphere. Nitrite,- nitrate-, and sulfate-dependent methane oxidation is well-documented, but AOM coupled to the reduction of oxidized metals has so far been demonstrated only in environmental samples. Here, using a freshwater enrichment culture, we show that archaea of the order Methanosarcinales, related to “Candidatus Methanoperedens nitroreducens,” couple the reduction of environmentally relevant forms of Fe(III) and Mn(IV) to the oxidation of methane. We obtained an enrichment culture of these archaea under anaerobic, nitrate-reducing conditions with a continuous supply of methane. Via batch incubations using [3H]methane, we demonstrated that soluble ferric iron (Fe(III), as Fe-citrate) and nanoparticulate forms of Fe(III) and Mn(IV) supported methane-oxidizing activity. CO2 and ferric iron (Fe(III)) were produced in stoichiometric amounts. Our study connects the previous finding of iron-dependent AOM to microorganisms detected in numerous habitats worldwide. Consequently, it enables a better understanding of the interaction between the biogeochemical cycles of iron and methane.

Methane is of interest to scientists and society for many reasons: It is one of the most important fossil fuels, a significant greenhouse gas, as well as an abundant natural hydrocarbon and substrate for microorganisms. However, due to its high activation energy, it can only be used by a few specialized groups of archaea and bacteria. Although aerobic methane-oxidizing bacteria have been known for 110 years (1), it was only at the turn of the last century that organisms performing anaerobic oxidation of methane (AOM) were first identified (2). These organisms couple AOM to sulfate reduction and belong to distinct clades within the methanogens (phylum Euryarchaeota), microorganisms that normally produce methane under anoxic conditions. These anaerobic methanotrophic (ANME) archaea have vast importance in regulating Earth’s climate, as they prevent the escape of the large quantities of methane contained in the oceans’ sediments and gas hydrates (2). Because CO2 has a 12 times weaker radiative forcing per molecule than methane (3), converting methane-carbon to CO2, assimilating it into biomass, or precipitating it as carbonates altogether reduce global warming effects considerably (2).

Until a decade ago, sulfate, a major constituent of seawater, was the only known electron acceptor for anaerobic oxidation of methane (2, 4). However, a variety of other environmentally important oxidized compounds—most notably Fe(III), Mn(IV), nitrate (NO3−) (5), and nitrite (NO2−) (6)—are thermodynamically more favorable electron acceptors than sulfate. The lack of known methanotrophs capable of coupling AOM to metal oxide reduction in particular represents a fundamental gap in our understanding of the dynamics of AOM in the environment.

For nitrogenous electron acceptors, two microbial players and processes have by now been identified: Methylophilus arsenitoxigenus, a bacterium of the NC10 phylum, is capable of producing its own oxygen from nitrite, and uses this oxygen to activate methane, analogous to aerobic methanotrophs (6). In addition, archaea of a new group within Methanosarcinales [termed AOM-associated archaea (AAA) (2), sometimes also referred to as ANME-2d], originally coenriched together with M. oxyfera (7), were recently found to couple the oxidation of methane to the reduction of nitrite to nitrate (5). In reaching these insights, laboratory enrichment cultures of the responsible microorganisms were of crucial importance.

Even though rock weathering supplies ample amounts of iron, manganese, and other metal oxides suitable as electron acceptors to freshwater bodies (8), and eventually oceans, evidence for the occurrence of iron- and manganese-dependent methane oxidation in nature is scarce. By geochemical profiling or isotope tracer studies, the coupling of methane oxidation to metal reduction has been demonstrated or suggested in lake sediments (9–11), lake water (12), a contaminated aquifer (13), a mud volcano (14), and marine sediments (15–18). The possible role of a cryptic sulfur cycle supplying sulfate via Fe(III)-mediated reoxidation of sulfides (19, 20) could often not be excluded in these studies, and none of them identified the microorganisms responsible for the process nor succeeded in obtaining an enrichment

Significance

Carbon and nitrogen cycles have been altered dramatically by human activities. Methane-producing (methanogenic) and methane-consuming (methanotrophic) microorganisms control the emission of methane, one of the most potent greenhouse gases, to the atmosphere. Earlier studies identified methanotrophic microorganisms that use methane as an electron donor and oxygen, sulfate, nitrite, and nitrate as electron acceptors. Previous research showed that methanotrophy coupled to the reduction of oxidized metals could be important in the environment. In the current paper, we identified archaea of the order Methanosarcinales, related to “Candidatus Methanoperedens nitroreducens,” which couple the reduction of environmentally relevant particulate forms of iron and manganese to methane oxidation, filling one of the remaining lacunas in anaerobic methane oxidation.

Author contributions: K.F.E., B.Z., M.S.M.J., and B.K. designed research; B.Z. and D.S. performed research; D.S. carried out sequencing and genome assembly; K.F.E., B.Z., J.T.K., M.S.M.J., and B.K. analyzed data; and K.F.E. and B.K. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The sequence reported in this paper has been deposited in the DDBJ/EMBL/GenBank database (metagenome accession no. LKCM01000000).

1K.F.E. and B.Z. contributed equally to this work.

2To whom correspondence may be addressed. Email: k.ettwig@science.ru.nl or bkartal@mpi-bremen.de.

3Present address: Institute of Groundwater Ecology, Helmholtz Zentrum München, 85764 Neuhberg, Germany.

4Present address: Division of Geological and Planetary Sciences, California Institute of Technology, Pasadena, CA 91125.

5Present address: Microbial Physiology Group, Max Planck Institute for Marine Microbiology, 28359 Bremen, Germany.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1609534113/-/DCSupplemental.
CH$_4$ + NO$_3^-$ + 2H$^+$ → CO$_2$ + NH$_4^+$ + 2H$_2$O. \[2\]

This observation adds a new aspect to the current understanding of AAA physiology (5): In the absence of ammonium-oxidizing bacteria, ammonium production in our enrichment culture could be measured. Despite M. oxyfera-like bacteria reducing nitrite to N$_2$, a transient accumulation of nitrite was also often observed, although it was consumed when nitrate became limiting (Fig. 2B). Nitrate reduction in the enrichment culture was largely (>95%) dependent on methane. All genes necessary for nitrate reduction to nitrite and nitrite reduction to ammonium are present in the genome of AAA (5, 24). Together, these results suggest that AAA most likely couples methane oxidation to dissimilatory nitrate reduction to ammonium via nitrite, a trait rarely observed in archaea (25).

We incubated this enrichment culture with AAA as the only archaea with a range of oxidized metal compounds. In the absence of an electron acceptor in batch incubations, methane oxidation did not occur, but activity began immediately after addition of ferric citrate (Fig. 3A). Recently, using a sample that
CH$_4$ + 8Fe$^{3+}$ + 2H$_2$O → CO$_2$ + 8Fe$^{2+}$ + 8H$^+$

($\Delta G^\circ = -454$ kJ mol$^{-1}$ CH$_4$).

In nature, iron reduction is often limited by the bioavailability of iron oxides, and soluble or complexed Fe$^{3+}$ is scarce (19, 23). Therefore, we also incubated the enrichment culture with more environmentally relevant forms of oxidized metals: nanoparticulate ferrhydrite (Fe$^{3+}$) and birnessite (Mn$^{4+}$). These were suitable as electron acceptors for the oxidation of methane to CO$_2$ by the enrichment culture, albeit at lower rates (Fig. 3C): 6 and 21 nmol CO$_2$ mg$^{-1}$ protein$^{-1}$ h$^{-1}$, compared with 80 and 180 nmol CO$_2$ mg$^{-1}$ protein$^{-1}$ h$^{-1}$, with soluble Fe$^{3+}$ (ferric citrate) and nitrate as electron acceptors, respectively.

In a setup with destructive sampling, where total CO$_2$ was measured and 2036), closely related to the ones of 43% identity, respectively), and the other by an enrich-
ment culture directly coupled methane oxidation to Fe$^{3+}$ reduction in batch incubations and its late ferrihydrite (Fe$^{3+}$). In controls without added sulfate (1 mM) (Fig. 3A), the enrichment culture did not produce any Fe$^{3+}$ (above the background of ~2 μM remaining from 3.75 μM FeSO$_4$ supplied as a micronutrient), the end product of Fe$^{3+}$ reduction in incubations with methane. Finally, an enrichment culture containing M. oxyfera as the only methanotroph (26) did not oxidize methane coupled to the reduction of Fe$^{3+}$. Taken together, these findings strongly indicated that the AAA in the enrichment culture directly coupled methane oxidation to Fe$^{3+}$ and Mn$^{4+}$ reduction.

AAA and an M. oxyfera-like microorganism dominated our enrichment culture (Fig. 1). The analyses of the 16S rRNA gene reads of the metagenome (DDBJ/EMBL/GenBank accession no. LKCM01000000) revealed that other archaea or known iron-reducing microorganisms [Geo bacter or Shewan ella (27)] were absent from the enrichment culture; this was expected, as the culture had never been exposed to iron exceeding the aforementioned 3.75 μM FeSO$_4$ in the medium. Furthermore, the immediate onset of Fe$^{3+}$ reduction in batch incubations and its linear course excluded the growth of an iron-reducing subpopulation interacting with a methanotroph being responsible for AOM in our culture.

Rather, the analysis of the partially reconstructed AAA genome (24) supported that the AAA alone were able to couple methane oxidation to the reduction of different electron acceptors. All genes of the reverse methanogenesis pathway (28, 29) were present in the enriched strain of AAA, in complete agreement with its closest relative, M. nitroreducens (81 and 83% identity, respectively), and the other by an nzarB-related production; data points represent the average of four incubations of 77 ± 3.6 μg protein each. (C) Stimulation of 13CO$_2$ production from 13CH$_4$ by the addition of nanoparticulate suspensions of ferrhydrite (Fe$^{3+}$) and birnessite (Mn$^{4+}$). Protein content in these experiments was 77.0 ± 3.6 μg per vial. Error bars indicate SD.
operon (MPEBLZ_01127 and 1129) absent from *M. nitroreducens* and similar to that in nitrite-oxidizing bacteria (68/78% similarity to *Nitrolancea hollandica* and 68/69% to *Nitrobacter winogradskyi*). The further reduction of nitrite to N₂ gas could be accomplished by the *M. oxyfera*-like microorganism as previously demonstrated (6) or, alternatively, ammonium could be formed by a NrfAH-like protein complex (MPEBLZ_01114 and 1115) transcribed by the AAA (24). As the likely genetic basis for Fe⁺⁺ reduction, we identified a wealth of genes encoding heme c-containing proteins (c-type cytochromes), which are generally recognized to function as electron shuttles from the cell to soluble or solid electron acceptors such as iron minerals (27, 30). The AAA in our enrichment culture encode numerous (41 species) multiheme c-type cytochromes (Table S1). This number is much higher than for methanogens, of which only *Methanosarcinales* encodes few multiheme cytochromes, but similar to *M. nitroreducens* (38 species) (5), the sulfate-dependent ANME-1 (11 species) (28), ANME-2a (16 species) (29, 31), as well as the iron-reducing archaea *Ferroglobus placidus* (22 species) and *Geoglobus* (15 species) (32).

This genomic blueprint suggests that AAA, and possibly also the cytochrome-rich ANME-1 (28) and ANME-2a (29, 31) archaea, known for sulfate-dependent methane oxidation, could be versatile methanotrophs that are able to switch between different electron acceptors, depending on environmental conditions (17). It is also conceivable that ANME-2a and ANME-2c archaea, which were shown to couple the reduction of solubilized iron species (iron citrate) to methane oxidation recently (22), could also be capable of converting particulate Fe⁺⁺ or Mn⁺⁺. Compared with *M. oxyfera*, AAA probably extends into more reduced environments, as these microorganisms can also use oxidized metals. However, AAA can form stable cocultures with microorganisms related to the nitrite-dependent methanotroph *M. oxyfera*, as previously reported (7). While AAA supply nitrite, which is often a limiting electron acceptor in nature, to *M. oxyfera*-like bacteria, or as in the culture described by ref. 5, to anaerobic ammonium-oxidizing (ammonox) bacteria, the bacterial partner in these cocultures keeps the concentration of the potentially toxic nitrite low. A bioreactor containing all three microorganisms could be applied in wastewater treatment to simultaneously convert ammonium, methanol, and oxidized nitrogen species to harmless N₂ and CO₂.

Molecular surveys of 16S rRNA or *mcrA* genes in the environment have often retrieved AAA-affiliated sequences in ecosystems poor in nitrite but rich in oxidized metals (14, 33) (Fig. S1), so far without an ecophysiological explanation. Our results show that the AAA directly coupled the reduction of these environmentally relevant, particulate metal species to methane oxidation and could play an as-yet largely overlooked role in decreasing methane emissions from anoxic environments to the atmosphere. Given the 8:1 stoichiometry of Fe⁺⁺ reduced to methane oxidized [3], their contribution to iron cycling, thereby impacting also the geochemistry of phosphorus and sulfur, may be even more important than their impact on the carbon cycle (11, 16, 18, 20). The enrichment of at least one of the microbial players not only now enables a targeted investigation of iron-dependent methane oxidation in physiological and molecular detail, but may also shed light on the long-standing discussion about Fe⁺⁺-producing processes on early Earth, when AAA-related organisms may have thrived under the methane-rich atmosphere in the ferruginous Archean oceans (12, 18, 30).

**Methods**

**Enrichment of the Coculture of AAA and the *M. oxyfera*-like Bacteria.** The enrichment of the biomass used here was described in brief previously (24). Biomass from the settler of an *M. oxyfera*-dominated reactor (26) containing about 10% (of the total microbial community) AAA was used to inoculate a continuous culture reactor (2-L working volume) to further enrich AAA. The reactor was run at room temperature with a pH of 7.5 (±0.15) and stirred gently with a magnetic bar (100 rpm). Initially, the reactor was coupled to the effluent of an *M. oxyfera* reactor, receiving once per day the methane- saturated, nonacidified (95:5, vol:vol) effluent containing up to 50 mM *M. oxyfera*-like bacteria and 68/69% to *Nitrobacter winogradskyi*. After about 1 year of incubation, the enrichment culture was uncoupled from the *M. oxyfera* reactor. Methane was provided by constant sparging with CH₄·CO₂ (95:5, vol:vol; ca. 10 mL·min⁻¹). Nitrate (0.5 to 1.5 mM final concentration) as the sole electron acceptor was injected daily into the reactor for over 2 years. Supernatant (ca. 0.8 L) was removed and replenished with fresh anoxic mineral medium (as previously described by ref. 34, omitting nitrate and nitrite) after settling of biomass every 2 weeks.

**Nitrate Reduction and Nitrite and Ammonium Production by the Enrichment Culture.** Conversion of nitrogenous compounds was measured in the continuous culture while sparging the reactor with CH₄·CO₂ (95:5, vol:vol; ca. 10 mL·min⁻¹). After nitrate depletion (to <10 μM), fresh nitrate was added to a final concentration of ~1 mM on 3 consecutive days. Liquid samples (1.5 mL) were taken every 3 to 4 h from the reactor and centrifuged at 14,000 × g, and supernatants were stored at 4 °C before nitrate, nitrite, and ammonium analysis.

**Activity Tests with Different Electron Acceptors.** To test the effect of various electron acceptors, the enrichment culture was left under continuous CH₄·CO₂ (95:5, vol:vol) sparging but without nitrate supply until residual nitrate was depleted. Twenty milliliters of culture liquid (protein content ca. 1.0 mg) was taken and anaerobically transferred to a sealed, evacuated sterile bottle (50 mL). After removal of unlabeled methane by sparging with Ar·CO₂ (90:10, vol:vol), 10 mL of CH₄·CO₂ (99.99% ¹²C, Sigma-Aldrich) was injected into the 1-bar Ar·CO₂ headspace. Ferric citrate (pH 6.8) and sodium sulfate were added from anoxic stock solutions to final concentrations of 2 and 1 mM, respectively. Particulate iron and manganese were added as ferrihydrite or birensite nanoparticles from anoxic suspensions. The incubation of nitrite-depleted *M. oxyfera* biomass (34) with ferric citrate was prepared in the same manner as described above. To control for the effect of electron acceptor addition, additional incubations were performed accordingly, but either without any additions or with sodium citrate only (2 mM final concentration). All serum bottles were incubated at room temperature and shaken gently at 50 rpm. Intervals between headspace sampling for concentration and isotopic composition of CH₄ and CO₂ were chosen according to the observed activity.

**Stoichiometry Determination in Activity Tests with Destructive Sampling.** To be able to precisely determine the stoichiometry of methane oxidation coupled to nitrate or Fe⁺⁺ reduction and minimize the biomass needed, in the experiment, the experimental setup was miniaturized and incubations were performed in 3-mL Exetainer vials (Labco). Before distribution of biomass over the vials, residual methane was stripped from nitrate-depleted enrichment culture by stirring and concomitantly flushing with He·CO₂ (90:10, vol:vol) in a previously evacuated and sealed serum bottle. The biomass was then transferred to an anaerobic vial with an anaerobic atmosphere (O₂ in the anaerobic chamber was removed by passing of the gas over a Pd catalyst resulting in a residual O₂ concentration of <0.2 ppm) and amended with an electron acceptor (nitrate or ferric citrate). The biomass was then equally distributed to aliquots of 1.5 mL in Exetainers, resulting in a protein content of 77.0 ± 3.6 μg per vial. Exetainers were sealed and 1 mL of CH₄·CO₂ (80:20, vol:vol) was injected into the headspace to provide an electron donor, to buffer pH, and create overpressure. The vials were incubated at room temperature in an overpressure shaker inside the anaerobic cabinet. For incubations with ferric citrate, three vials were harvested every 3 h and acidified with 0.5 mL anaerobic HCl solution (final concentration 0.5 mM) and kept in the anaerobic tent for at least 0.5 h before GC/MS measurement to allow CO₂ to equilibrate with the headspace. After gas measurements, biomass from each vial was transferred to a 1.5-mL tube and centrifuged, and the supernatant was stored at 4 °C until Fe⁺⁺ determination. For incubations with nitrate, four vials were harvested every 2 h, two of which were acidified with 0.5 mL anaerobic HCl solution. CO₂ production in all four vials’ headspace was measured. The biomass from the nonacidified vials was transferred to a 1.5-mL cup and centrifuged, and supernatant was stored at 4 °C until nitrate determination by HPLC.

**Analytical Procedures and Synthesis of Reagents.** Quantity and isotopic composition of CH₄, CO₂, and N₂ in the headspace were measured by GC/MS (GC 6890 coupled to MSD 5975C; Agilent) as previously described (34). Total amounts indicated in the incubations take into account the dissolved
fraction of the respective gases. Nitrate and nitrite were measured after a reduction step to NO with a Sievers Nitric Oxide Analyzer (NOA 280; Analytic 2000, Boulder, CO). The nitrite was both reduced with a saturated solution of vanadium (III) chloride in hydrochloric acid (1 M) at 90 °C. Nitrite was colorimetrically measured by the Griess reaction as previously described (35). Nitrate concentrations were obtained by subtracting the nitrite concentration from the sum of nitrate and nitrite. Ammonium was measured colorimetrically at 623 nm after a 30-min reaction of 100-µL samples with alkaline phenol catalyzed by sodium nitroprusside at 37 °C (36). For the miniaturized setup (Exetainer incubations), nitrate was quantified with HPLC using a conductivity detector (26). Fe"" was measured using the ferrozine method (37).

Ferrhydrite and birnessite were synthesized according to protocols described previously (23, 38). Briefly, for ferrhydrite, 21 g of ferric citrate (Sigma) was dissolved in 300 mL MilliQ water by vigorous stirring while being heated to 80 °C. After further cooling the solution to room temperature, the pH was quickly adjusted to 8.0 with 10 M NaOH while stirring rigorously for 30 min. The supernatant of the resulting suspension was discarded after centrifuging at 8,000 × g at 4 °C for 1 h, and the pellet was resuspended in 500 mL MilliQ water. After four washing cycles, the pellet was finally resuspended in a small volume of MilliQ water. For birnessite, concentrated HCl (46%) was slowly added to a boiling and rigorously stirring 0.4 M potassium permanganate solution to a final concentration of 0.8 M in furnishing the suspension. After further heating the suspension to 80 °C, the suspension was cooled to room temperature and centrifuged. Washing and resuspension were performed as described for ferrhydrite. Approximate iron and manganese concentrations in the suspensions were determined by measuring the dry weight of an aliquot.

**Fluorescence in Situ Hybridization.** Biomass from the enrichment culture (2 mL) was harvested by centrifugation, fixed, hybridized, and visualized as previously described (26) with additional use of the probe 5'-AAA-FW4-31 (33), specifically targeting AAA.

**ACKNOWLEDGMENTS.** We thank Francisca A. Luesken for starting and initially maintaining the AAA enrichment culture. K.F.E. was supported by a VENI grant from The Netherlands Organization for Scientific Research (863.13.007), B.Z. by the joint program of the Chinese Academy of Science/Royal Dutch Academy of Science (09PhD02), D.S. by the BE-Basic Foundation (fp07.002.7), M.J.M. by the European Research Council (ERC 339980) and the Spinoza Prize, and B.K. by a VENI grant from The Netherlands Organization for Scientific Research (863.11.003) and the European Research Council (ERC 640422).