LETTERS

A microbial consortium couples anaerobic methane oxidation to denitrification

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Modern agriculture has accelerated biological methane and nitrogen cycling on a global scale[1,2]. Freshwater sediments often receive increased downward fluxes of nitrate from agricultural runoff and upward fluxes of methane generated by anaerobic decomposition[3]. In theory, prokaryotes should be capable of using nitrate to oxidize methane anaerobically, but such organisms have neither been observed in nature nor isolated in the laboratory[4–8]. Microbial oxidation of methane is thus believed to proceed only with oxygen or sulphate[9,10]. Here we show that the direct, anaerobic oxidation of methane coupled to denitrification of nitrate is possible. A microbial consortium, enriched from anoxic sediments, oxidized methane to carbon dioxide coupled to denitrification in the complete absence of oxygen. This consortium consisted of two microorganisms, a bacterium representing a phylum without any cultured species and an archaeon distantly related to marine methanotrophic Archaea. The detection of relatives of these prokaryotes in different freshwater ecosystems worldwide[11–14] indicates that the reaction presented here may make a substantial contribution to biological methane and nitrogen cycles.

Global biogeochemical cycles are mainly driven by microorganisms feeding on one-carbon compounds such as methane or carbon dioxide. Each step in the element cycles is catalysed by a specific group of microorganisms. These may or may not be evolutionarily related, but they share a similar lifestyle and so form an 'ecological guild'. Thermodynamic calculations show that most of these guilds have already been discovered (Supplementary Fig. S1), but the microorganisms that couple the anaerobic oxidation of methane (AOM) to denitrification, shown in equations (1) and (2), are considered missing in nature[4–8]:

$$5CH_4 + 8NO_3^- + 8H^+ \rightarrow 5CO_2 + 4N_2 + 14H_2O \quad (\Delta G^{0'} = -765\,kJ\,mol^{-1}CH_4) \tag{1}$$

$$3CH_4 + 8NO_2^- + 8H^+ \rightarrow 3CO_2 + 4N_2 + 10H_2O \quad (\Delta G^{0'} = -928\,kJ\,mol^{-1}CH_4) \tag{2}$$

As AOM coupled to denitrification is possible in theory, both thermodynamically and biochemically (through reverse methanogenesis[15,16]), such microorganisms might in fact exist and consequently our understanding of biogeochemical methane cycling may be incomplete. The lack of experimental evidence for the occurrence of AOM coupled to denitrification is perhaps not surprising, because this process would be expected to occur close to the oxic/anoxic interface in sediments. This interface is generally characterized by steep gradients, occurring within millimetres, masking the process from geochemical detection. Furthermore, laboratory enrichment of the responsible microorganisms could be difficult because of their potentially very slow growth[17].

Here we report the successful enrichment of consortia of microorganisms capable of AOM coupled to denitrification. Anoxic sediment from the Twentekanaal, a canal in the Netherlands, was used as the inoculum for the enrichment culture. This canal contained nitrate concentrations of up to 1 mM and the sediment was saturated with methane, which is typical for freshwater habitats receiving agricultural runoff. A one-litre sample from the upper layer of the sediment was incubated anoxically in the laboratory. Methane was supplied as the only electron donor, and mineral medium containing nitrate (NO_3^-), nitrite (NO_2^-), bicarbonate and trace elements was supplied and removed continuously. Over 16 months of incubation, the influent nitrite concentration was gradually increased to 6 mM, but the actual concentration in the culture medium remained at about 0.1 mM, indicating the growth of a microbial population consuming nitrite. Some nitrate (<1 mM) was also consumed. Methane consumption could not yet be observed experimentally because it was supplied in large excess and any potential conversion remained within the error margin for the CH_4 analysis.

To measure methane consumption, the media and methane supply were stopped and excess methane was removed by flushing with helium gas. The consumption of methane, nitrite and nitrate was now apparent and dinitrogen gas evolved (Fig. 1). Nitrite and nitrate together accounted for all of the produced dinitrogen gas. However, the total denitrification rate ($28.8 \pm 2\,\mu mol\,N_2\,h^{-1}$) was not completely accounted for by methane oxidation ($13.4 \pm 1\,\mu mol\,CH_4\,h^{-1}$) according to equations (1) and (2). Control experiments indicated that this difference was caused by the oxidation of organic compounds from the inoculum or the mineral medium. In these control experiments, the denitrification rate in the absence of methane was $5.5 \pm 0.5\,\mu mol\,N_2\,h^{-1}$, and on methane addition it increased to $21.5 \pm 2\,\mu mol\,N_2\,h^{-1}$. As methane itself was consumed at $22.0 \pm 2\,\mu mol\,CH_4\,h^{-1}$, the stoichiometry of AOM was almost completely consistent with the above equations.

The enrichment culture used nitrite in preference to nitrate as the substrate for denitrification—for experiments in which nitrite was depleted in the presence of both methane and nitrate, AOM ceased, and resumed only when nitrite was re-introduced 2 h after depletion. However, during longer incubation periods in the absence of nitrite (10–20 h), AOM restarted and was then coupled to nitrate consumption. This suggests that the enrichment culture could adapt to nitrate, and that both nitrite and nitrate were suitable substrates for AOM

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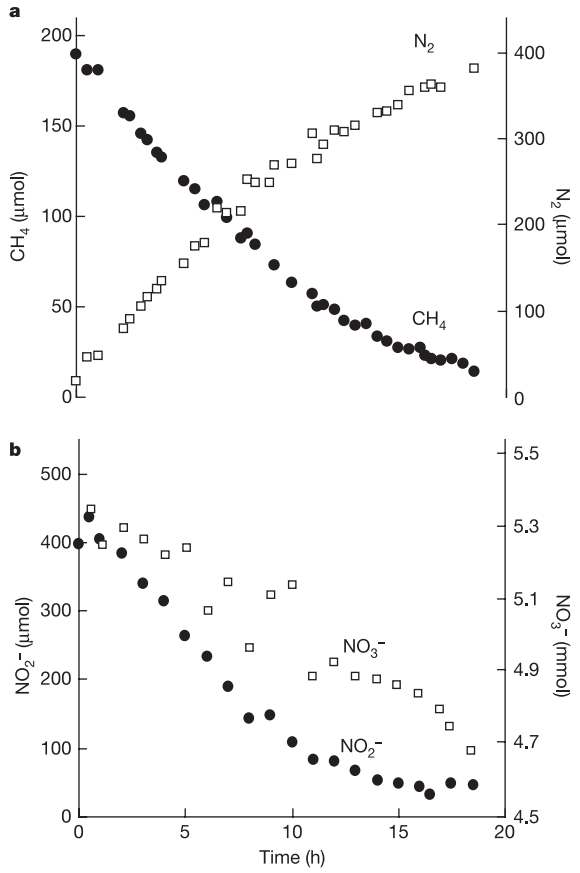

Figure 1 | AOM is coupled to the denitrification of nitrite by the enrichment culture after 16 months of enrichment. a, **b**, The total amounts of methane, dinitrogen gas, nitrate and nitrite present in the culture vessel are indicated. The initial concentrations of these compounds in the culture liquid were 6.0 μM, 0.30 μM, 3.6 mM and 0.24 mM, respectively. During this experiment, the total amount of protein in the enrichment culture was 100 mg.

(equations (1) and (2)). The apparent affinity constant for methane was very high (less than 0.6 μM, Fig. 1). The affinity of sulphate-dependent AOM for methane is four orders of magnitude lower[18] (affinity constant >16 mM). The addition of 2 mM sulphate to the enrichment culture neither stimulated nor inhibited AOM.

Together, these experiments show unambiguously that methane can be oxidized anaerobically in this system, and that this oxidation is coupled to denitrification. The participation of oxygen in this process can be fully excluded. First, we measured oxygen levels continuously in the culture liquid and periodically in the headspace, but did not detect any (detection limit 80 p.p.m.). Second, all of the detected dinitrogen gas in the headspace of the culture was accounted for by the consumption of nitrite and nitrate. Had air leaked into the culture, more dinitrogen gas would have been detected in the headspace than was predicted by the reaction stoichiometry. Third, the stoichiometry of methane consumption coupled to denitrification was in good agreement with equations (1) and (2). Had oxygen been involved, much less nitrite or nitrate would have been consumed per mol of methane. Finally, no methane was consumed as the enrichment culture adapted from nitrite to nitrate (see above).

We investigated the incorporation of methane into microbial biomass by analysing the membrane lipids of the enrichment culture. We detected a single archaeal and several bacterial biomarkers (Table 1). The archaeal biomarker sn2-hydroxyarchaeol, found in methanogens[19] and ANME-2 methanotrophic Archaea[20], was the only biomarker substantially depleted in ^{13}C compared to methane. This change indicates that the carbon in this marker compound originated from methane. When ^{13}C-labelled methane was supplied to the culture, incorporation into sn2-hydroxyarchaeol was observed after six days. Notably, the bacterial biomarkers were labelled more rapidly and substantially than the archaeal biomarker (Table 1). These results are comparable to those obtained for a similar ^{13}C-labelling experiment with a consortium performing sulphate-dependent AOM[21], in which rapid and substantial incorporation of ^{13}C-labelled methane was noted in the lipids of sulphate-reducing bacteria, but minor ^{13}C incorporation was observed in archaeal lipids only after prolonged incubation (>300 days). Our biomarker data thus indicate that a consortium consisting of an archaeon and a bacterium is responsible for AOM coupled to denitrification.

To determine the phylogenetic identity of the members of this consortium, we isolated genomic DNA from the biomass in the enrichment culture and constructed bacterial and archaeal 16S ribosomal RNA gene libraries. Sequence analysis of the bacterial clone library showed one dominant group of sequences clustering inside a subdivision with no other cultivated species (Fig. 2a and Supplementary Fig. 2a). This subdivision was distant from all other bacterial subdivisions (sequence identity less than 85%). Similar

Table 1 | Incorporation of methane into the bacterial and archaeal biomarkers of the enrichment culture

Biomarker compound	Amount (%)	Origin	Enrichment culture (t = 16 months)	After labelling with ^{13}C methane	
				3 days	6 days
			$\delta^{13}C$ (‰ versus VPDB)	$\delta^{13}C$ (‰ versus VPDB)	$\delta^{13}C$ (‰ versus VPDB)
Methane			−40.7		
Bicarbonate			−24.6		
$C_{14:0}$ fatty acid	0.8	Bacteria	−26.1	+2,300	+4,400
Iso-$C_{15:0}$ fatty acid	2.3	Bacteria	−31.0	+125	+189
$C_{16:1}$ (Δ^9) fatty acid	6.8	Bacteria	−43.7	+4,300	−†
$C_{16:0}$ fatty acid	11.4	Bacteria	−30.5	+370	+580
10-me-$C_{16:1}$ (Δ^7) fatty acid	5.5	Bacteria	−37.9	−‡	−‡
10-me-$C_{16:0}$ fatty acid	28.9	Bacteria	−38.5	−36.4	−36.5
$C_{18:0}$ fatty acid	2.8	Bacteria	−31.4	+310	+380
$C_{18:1}$ ($\Delta^{11} + \Delta^{10}$) fatty acid	14.3	Bacteria	−35.8	+900	−†
$C_{19cycloprop.}$ fatty acid	12.7	Bacteria	−38.0	+290	+420
Diplopterol	4.1	Bacteria	−46.6	+380	−†
Sn2-hydroxyarchaeol	2.2	Archaea	−67.0	−76	−49

The table shows the relative abundances and stable carbon isotopic composition of the major lipids from the enrichment culture and the incorporation of ^{13}C into these compounds after 3 or 6 days of incubation with ^{13}C-labelled methane ($\delta^{13}C = [(^{13}C/^{12}C)_{sample}/(^{13}C/^{12}C)_{standard}] - 1$). For the archaeal compound from the enrichment culture, the large depletion in ^{13}C indicates that its carbon is derived from (^{13}C-depleted) methane. The minor but significant incorporation of ^{13}C into this compound during labelling ($\Delta\delta^{13}C = -49 - (-67) = +18‰$) suggests slow growth. VDPB, Vienna Pee Dee Belemnite.

†$\delta^{13}C$ after 6 days was not determined for some lipids already substantially enriched in ^{13}C after 3 days.

‡$\delta^{13}C$ could not be determined owing to low abundance of the compound and co-elution.

sequences from this subdivision have previously been retrieved from the denitrifying zone of sediments from Lake Biwa in Japan[12] and from contaminated groundwater in the United States[13]. The archaeal clone library contained a single sequence, which was only distantly related to the AOM Archaea of group 2 (ANME-2 (refs 9, 22), 86–87% identity) and cultivated methanogens (86–88% identity, Fig. 2b and Supplementary Fig. 2b). The highest similarity was found with archaeal clone sequences obtained from freshwater sediments from Lake Michigan in the United States[14] and contaminated soils in Japan[11].

We used both the bacterial and archaeal 16S rRNA gene sequences to design specific probes for fluorescence *in situ* hybridization (FISH). Samples collected over the 16-month period of enrichment were now hybridized with these probes. In samples from the first three months, only occasional, single cells tested positive. Over time, both the bacterium and the archaeon became increasingly enriched, until they were the dominant microorganisms in the culture. After 16 months, approximately 10% of 4,6-diamidino-2-phenylindole (DAPI)-stained cells consisted of Archaea, all of which hybridized with the specific probe targeting the dominant archaeal sequence. The remainder of the culture consisted of bacteria, of which approximately 80% hybridized with the three specific probes targeting the dominant bacterial sequence (Fig. 2). As shown in Fig. 2, the Archaea were generally present as clusters inside a matrix of bacterial cells. The ratio of bacterial to archaeal cells (approximately 8:1) was different from the ratio reported for sulphate-dependent AOM[18] (approximately 2:1). This difference might be explained by the higher energy yield of denitrification compared to sulphate reduction. α-, β- and γ-proteobacteria together made up <5% of the community. The sulphate reducers known to be involved in sulphate-dependent AOM[10] were not detected, consistent with our observation that sulphate was not converted in the culture.

The nitrite-dependent AOM rate was 140 μmol CH_4 per g protein per hour (Fig. 1), corresponding to approximately 0.4 fmol CH_4 per cell per day for the Archaea in our enrichment culture. For sulphate-dependent AOM, a similar rate has been reported for the archaeal partner (0.7 fmol CH_4 per cell per day)[18]. This indicates that for AOM coupled to denitrification, the archaeal growth rate could be extremely low, with a doubling time in the order of several weeks, consistent with the long duration of the enrichment procedure.

To our knowledge this is the first report of archaeal AOM coupled to bacterial denitrification. In the 1970s, Mason[8] discredited earlier studies that pure cultures of methanotrophic bacteria were able to denitrify using methane as the sole carbon and energy source. Instead, it was established experimentally that methanotrophs can oxidize methane aerobically to methanol or acetate at low oxygen concentrations, and that the methanol or acetate can subsequently be used to drive denitrification[23–26]. Thus, the reaction presented here defines a new microbial guild with a potential contribution to biogeochemical cycling that has so far been overlooked. The recovery of related 16S rRNA gene sequences from different habitats and locations[11–14] indicates that this process may contribute significantly

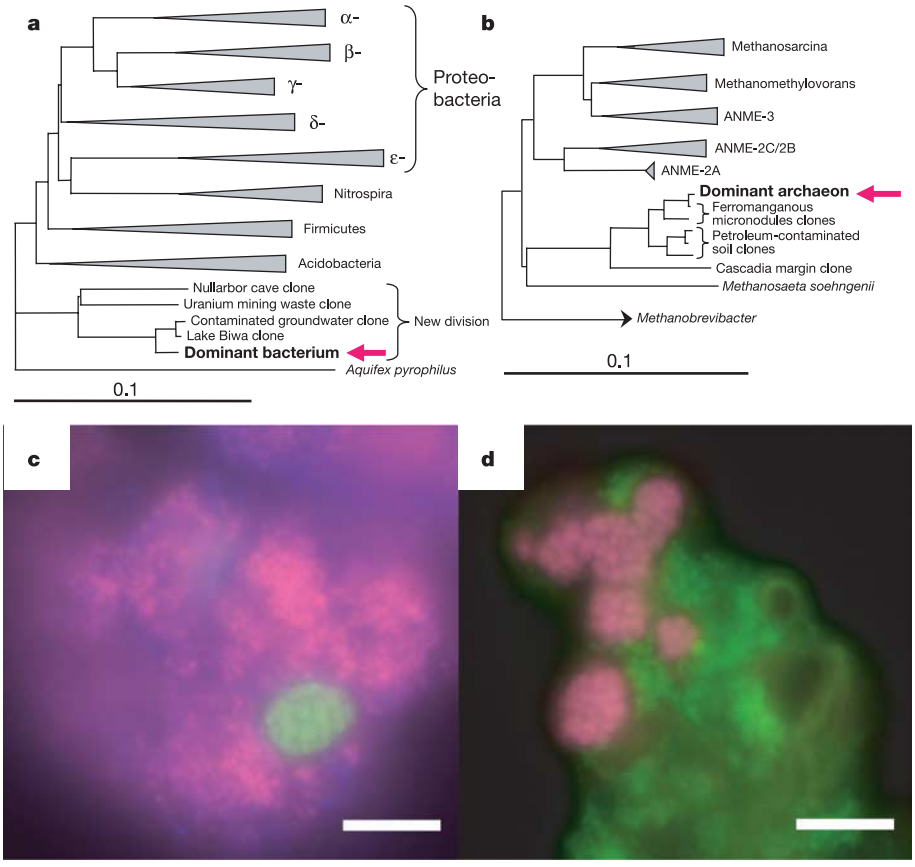

Figure 2 | Phylogeny and fluorescence *in situ* detection of the archaeal and bacterial members of the consortium mediating AOM coupled to denitrification. **a**, **b**, Consensus trees of the dominant bacterial (**a**) and archaeal (**b**) 16S rRNA sequences of the enrichment culture. Scale bars indicate 10 base substitutions per 100 bases. See also Supplementary Fig. 2 for more detailed trees, bootstrap values and treeing methods. **c**, Epifluorescence micrograph after hybridization with the general bacterial probe EUBmix (blue), the specific bacterial probe DBACT-193 (red) and the specific archaeal probe DARCH-872 (green). The bacterial partner is pink, as it hybridizes with both the general and specific bacterial probes. **d**, Epifluorescence micrograph after hybridization with the general archaeal probe ARCH915 (blue), the specific archaeal probe DARCH-872 (red) and the general bacterial probe EUBmix (green). The archaeal partner is pink because it hybridizes with both the general and specific archaeal probes. Scale bars, 5 μm. See Methods for specification of probes.

to methane oxidation, and could potentially counteract worldwide increases in methane production associated with intensive agriculture. With biomarkers and probes for the responsible microorganisms now available, this possibility can be addressed.

METHODS

Sampling. Samples for inoculation were taken from the sediment of the Twentekanaal (52° 11′ 04″ N and 6° 24′ 40″ E, The Netherlands). Samples were collected from the top 15 cm of the sediment at 1 m water depth. At the time of sampling, the methane concentration at 15 cm sediment depth was 0.8 mM. The nitrate concentration in the water column was 0.1 mM. Cultivation and analytical methods are described in the Supplementary Information.

Calculations. The values of the Gibbs energy changes reported for equations (1) and (2) were calculated for standard conditions (25 °C, pH 7). The affinity constant of a microbial conversion is the substrate concentration at which the conversion rate is half of the maximum conversion rate. In this case the affinity constant for methane was estimated from the slope of the methane consumption in Fig. 1.

Methane incorporation and lipid analysis. Aliquots (60 ml) of the enrichment culture were anaerobically transferred to 120-ml serum bottles with an atmosphere of 90% argon and 10% ^{13}C-labelled methane. The bottles were incubated on a shaker at 30 °C and then used for lipid analysis after three or six days. Lipids were ultrasonically extracted after freeze-drying and analysed by gas chromatography/mass spectrometry and isotope ratio gas chromatography/mass spectrometry as described previously[27]. $^{13}CO_2$ was measured as the end product of AOM, using a GC-isotope ratio mass spectrometer (ThermoFinnigan Delta Plus).

16S rRNA gene sequence analysis and FISH. Chromosomal DNA from 1-ml reactor biomass was isolated and used as a template for polymerase chain reaction (PCR) amplification of 16S rRNA genes. PCR was performed using general bacterial primers (616F and 630R; ref. 27) and general archaeal primers (AR20F and AR958R; ref. 28). Cloning, sequencing and phylogenetic analyses were performed as described previously[27]. On the basis of the bacterial and archaeal 16S rRNA gene sequences, new oligonucleotide probes were designed. The bacterial probes were S-*-DBACT-0193-a-A-18 (5′-CGCTCGCCCCC TTTGGTC-3′), S-*-DBACT-0447-a-A-18 (5′-CGCCGCCAAGTCATTCGT-3′) and S-*-DBACT-1027-a-A-18 (5′-TCTCCACGCTCCCTTGCG-3′), and the archaeal probe was S-*-DARCH-0872-a-A-18 (5′-GGCTCCACCCGTTG TAGT-3′). We also used the general archaeal probe S-D-ARCH-0915-a-A-20, the general bacterial probe EUBmix and probe DSS658 for sulphate reducers[10]. FISH was performed as described previously[27]. Formamide concentrations used in FISH experiments varied between 20% and 40%.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Information The 16S rRNA gene sequences have been deposited in GenBank under accession numbers DQ369741 (archaeal sequence) and DQ369742 (bacterial sequence). Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to M.S. (m.strous@science.ru.nl).