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Denitrifying bacteria anaerobically oxidize methane in the absence of *Archaea*

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Summary

Recently, a microbial consortium was shown to couple the anaerobic oxidation of methane to denitrification, predominantly in the form of nitrite reduction to dinitrogen gas. This consortium was dominated by bacteria of an as yet uncharacterized division and archaea of the order *Methanosarcinales.* The present manuscript reports on the upscaling of the enrichment culture, and addresses the role of the archaea in methane oxidation. The key gene of methanotrophic and methanogenic archaea, *mcrA*, was sequenced. The associated cofactor F_{430} was shown to have a mass of 905 Da, the same as for methanogens and different from the heavier form (951 Da) found in methanotrophic archaea. After prolonged enrichment (> 1 year), no inhibition of anaerobic methane oxidation was observed in the presence of 20 mM bromoethane sulfonate, a specific inhibitor of MCR. Optimization of the cultivation conditions led to higher rates of methane oxidation and to the decline of the archaeal population, as shown by fluorescence *in situ* hybridization and quantitative MALDI-TOF analysis of F_{430}. Mass balancing showed that methane oxidation was still coupled to nitrite reduction in the total absence of oxygen. Together, our results show that bacteria can couple the anaerobic oxidation of methane to denitrification without the involvement of *Archaea*.

Introduction

In 2006, Raghoebarsing and colleagues obtained an enrichment culture that coupled the anaerobic oxidation of methane (AOM) to denitrification. Freshwater canal sediment was incubated for more than a year in a bioreactor with biomass retention (sequencing batch reactor) until measurable methane and nitrite turnover was obtained. In the enrichment culture, one bacterial phylotype belonging to the candidate division 'NC10' made up ~80% of the population. This division had been defined until then only by environmental sequences (Rappé and Giovannoni, 2003). A smaller fraction of the population (up to 10%) consisted of *Archaea*. These belonged to the order *Methanosarcinales* and were distantly related to *Methanosaeta* and ANME-II (anaerobic methanotrophs) (86–88% 16S rRNA gene identity). Labelling experiments and $\delta^{13}C$ measurements indicated that both of these microorganisms were involved in the AOM, although the bacterial lipids were more strongly labelled (Raghoebarsing *et al.*, 2006). On this basis, it was hypothesized that the process was performed via reverse methanogenesis by an 'ANME archaeon' with electron shuttling to a denitrifying bacterium.

Denitrification is the reduction of nitrate and nitrite to nitrous oxide and dinitrogen gas (Payne, 1973; Knowles, 1982). Many *Bacteria* and *Archaea* have the potential to denitrify (Philippot, 2002), and numerous organic and anorganic compounds can be used as an electron donor for denitrification. Although methane is a favourable electron donor for both nitrate and nitrite reduction (–765 and –928 kJ mol^{-1} CH_4 respectively; Raghoebarsing *et al.*, 2006), experimental evidence for its completely anaerobic oxidation was only found recently.

Though energetically much less favourable, sulfate-dependent AOM has been known for decades from marine sediment profiling and ^{14}C-CH_4 incubation studies (reviewed in Valentine and Reeburgh, 2000; Strous and Jetten, 2004). The hypothesis that it involves methanogens operating in reverse (Zehnder and Brock, 1980) was supported by inhibition studies with the methanogenic inhibitor bromoethane sulfonate (BES; Hoehler *et al.*, 1994), and later by the finding that AOM was performed by methanogen-like archaea (anaerobic methanotrophs, ANME) living in close association with sulfate-reducing *Deltaproteobacteria* (Hinrichs *et al.*, 1999; Boetius *et al.*, 2000). Until now, occurrence and environmental significance of AOM has been demonstrated in numerous sulfate-rich environments, e.g. deep marine gas hydrates (Michaelis *et al.*, 2002) and cold seeps (Orcutt *et al.*,

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2005), shallow marine sediments (Treude *et al*., 2005), terrestrial (Alain *et al*., 2006) and marine mud volcanoes (Niemann *et al*., 2006). In all cases, the presence of one or more of the methane-oxidizing euryarchaeal groups, ANME-I, -II or -III, and the use of sulfate as electron acceptor have been shown. Even though the overall process stoichiometry and the involvement of the ANME archaea are well understood, the biochemical mechanism of sulfate-dependent AOM is still unclear. Environmental genomic studies of ANME archaea support the dominant view that the archaeal partner carries out reverse methanogenesis, but not sulfate reduction (Hallam *et al*., 2004), and that electrons are shuttled to a sulfate-reducing bacterium (SRB). Recently, it was suggested that methanethiol is the archaeal product serving as electron donor for the SRB (Moran *et al*., 2008). On the other hand, the general role of the SRB is still debated, as ANME archaea are sometimes found in the absence of, or without close physical association with the SRB (Orphan *et al*., 2002; Orcutt *et al*., 2005; Treude *et al*., 2005).

In reverse methanogenesis, the methane-activating step is bioenergetically the most difficult, and is thought to be performed by a modified methyl-coenzyme M reductase (MCR) operating in reverse (Hallam *et al*., 2004). MCR contains the nickel-cofactor F_{430} (Friedmann *et al*., 1990), and the only MCR from methanotrophic archaea characterized so far revealed a modification in this otherwise very conserved methanogenic cofactor: MCR purified from a microbial mat naturally enriched in ANME-I archaea had a cofactor F_{430} with an increased molecular mass (Krüger *et al*., 2003). Therefore, it was speculated that, together with the existence of a unique cysteine-rich sequence at the active site region, this modification might change the catalytic properties of the enzyme (Shima and Thauer, 2005). However, it was recently found that MCR from ANME-2 archaea harbours the normal F_{430} and does not contain such a cysteine-rich sequence. (A. Meyerdierks, S. Shima, J. Kahnt and M. Krüger, unpubl. results; cited by Thauer and Shima, 2008). The discovery of another group of putative ANME archaea in the consortium oxidizing methane under denitrifying conditions (Raghoebarsing *et al*., 2006) posed the question, if their characterization would shed light on the biochemical mechanism of AOM.

In the present study, the enrichment culture described previously was scaled up and continued, providing more biomass. The MCR gene was partially amplified and the mass of the MCR cofactor F_{430} was determined. Interestingly, while the activity of the culture increased, the archaeal population declined over time until it was no longer detectable, with the 'NC10' bacterium remaining the dominant microorganism in the enrichment. The culture still oxidized methane anaerobically, and this activity was not inhibited by BES, a specific inhibitor of MCR. We conclude that AOM coupled to denitrification proceeded by a biochemical mechanism other than reverse methanogenesis and an archaeal partner was not required.

Results

Development of a microbial community that couples the AOM to denitrification in a continuous culture

A 16 l bioreactor with an external settler for biomass retention (Fig. 1) was inoculated with biomass from the 1.5 l enrichment culture described by Raghoebarsing and colleagues (2006). Fluorescence *in situ* hybridization (FISH) showed that upon inoculation the population was dominated by a bacterium affiliated with the phylum 'NC10' (approximately 80%) and an archaeon of the order *Methanosarcinales* (approximately 10%). Medium containing nitrite and nitrate was supplied continuously, and the activity of the culture was monitored daily by the measurement of nitrite concentration (Fig. 2). For a test phase of 2 months, nitrite was omitted, but methane-oxidizing activity with nitrate alone was very low (data not shown). After that period, nitrite was again added with the medium. It was clearly preferred as an electron acceptor over nitrate, which was only consumed at a rate less than 0.08 mmol day^{-1} in the presence of nitrite. Nitrite-reducing activity remained fluctuating around only 3.5 mmol day^{-1} and showed no general upward trend for more than a year. This indicated either inhibition by products of the

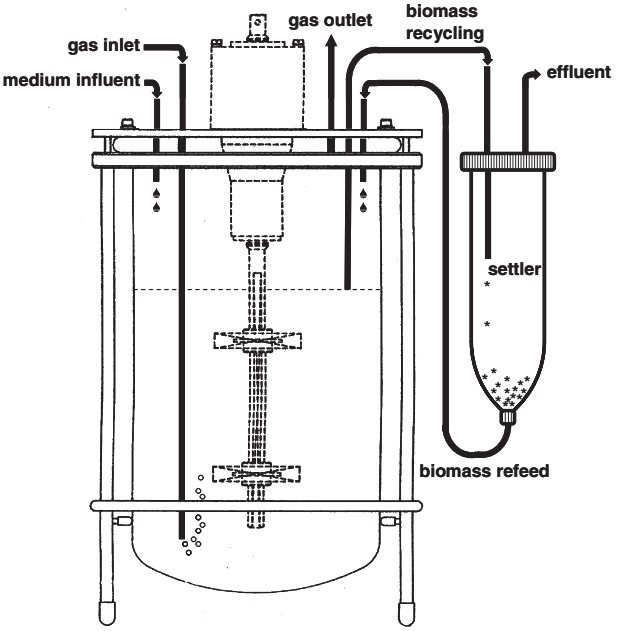

Fig. 1. Continuous culture set-up with biomass retention in an external settler.

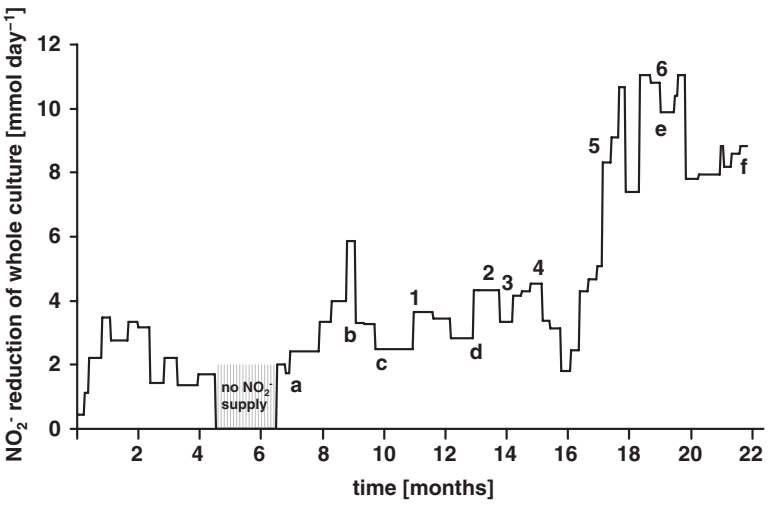

Fig. 2. Development of nitrite-reducing activity in the continuous culture. Numbers indicate additions to or modifications of the medium, letters indicate experiments described in the text. During the period indicated by shading, no nitrite, but only nitrate was supplied to the reactor. 1: addition of 0.1 mM NH_4Cl to the medium; 2: addition of 0.1 mM cysteine to the medium; 3: addition of 1 ml l^{-1} vitamin solution (Balch *et al*., 1979) to the medium; 4: addition of 0.3 g l^{-1} sea salt; 5: addition of 450 ml interstitial water, 6: second addition of 450 ml interstitial water. a: FISH analysis (Fig. 3A); b: DNA extraction for *mcrA* amplification; c: qualitative MALDI-TOF analysis of cofactor F_{430} (Fig. 4); d: FISH analysis (Fig. 3B, *Archaea* decline) and BES inhibition experiment (Fig. 5); e: FISH analysis (Fig. 3C, *Archaea* not detectable) and quantitative MALDI-TOF analysis of cofactor F_{430} (Fig. S1); f: whole-culture batch experiment (Fig. 6).

microorganisms or the deficiency of an unknown growth factor. For that reason the medium flow rate was varied (to increase the washout of potentially toxic by-products of the process), and the following additions to the medium were tested: 0.1 mM NH_4Cl (after 11 months, as an alternative nitrogen source), 0.1 mM cysteine (after 13.5 months, as an alternative sulfur source), 1 ml l^{-1} vitamin solution (after 14 months, according to Balch *et al*., 1979) and 0.3 g l^{-1} sea salt (after 15 months, as a complex mixture of potentially missing trace elements). The additions were included in the medium preparation from the specified time onward, but did not show clear effects on overall activity (Fig. 2). After 17 and 19 months, 450 ml anaerobic, filtered, but not sterile interstitial water from freshwater ditch sediment was added, and activity increased. Presence of electron donors for nitrite reduction in the interstitial water as a cause for this positive effect can be excluded, as no stimulation of denitrifying activity was detectable in batch incubations without methane (data not shown).

Monthly FISH analysis of the culture showed that at all times at least 70% of the biomass belonged to bacteria of the 'NC10' phylum, which had already been prevalent in the inoculum (Fig. 3A–C). These bacteria were thin rods, approximately 1 μm long and 0.3 μm wide, with the DNA concentrated in the centre of the cell as judged by the localization of the DNA-binding DAPI stain (pictures not shown). Also, in the first 10 months, the archaea of the order *Methanosarcinales* found in the primary enrichment remained enriched to up to 10% of cells (Fig. 3A). These coccus-shaped cells were always present in round clusters of 3–6 μm diameter. After 10 months, they gradually became less abundant, and their fluorescence less bright. After 12 months, they could only occasionally be found with FISH (< 1% of cells, Fig. 3B), and were no longer detectable from month 15 onwards.

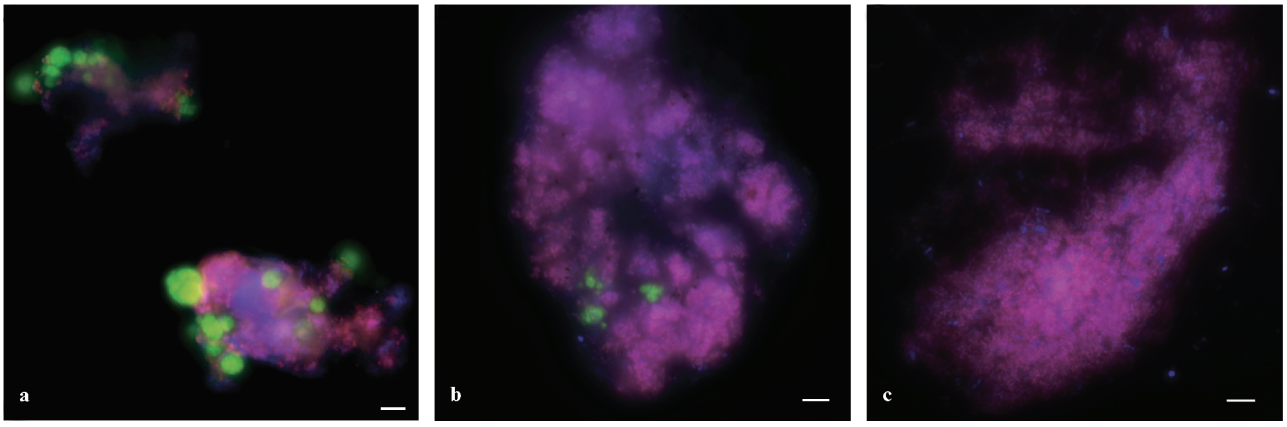

Fig. 3. Fluorescence *in situ* hybridization of biomass from the bioreactor at different times: (A) after 7 months, (B) after 13 months and (C) after 19 months of enrichment. Epifluorescence micrographs taken with 1000× magnification after hybridization with probes S-*-DBACT-0193-a-A-18 or S-*-DBACT-1027-a-A-18 (Cy3, red), probe S-D-Arch-0915-a-A-20 (Fluos, green) and a mixture of EUB I-III and V (Cy5, blue). 'NC10' bacteria appear pink due to double hybridization with both specific and general bacterial probes. Scale bar = 5 μm.

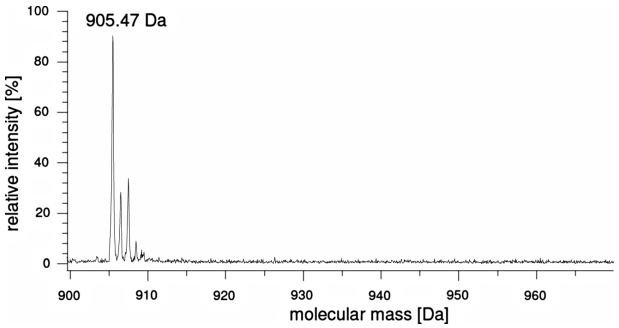

Fig. 4. MALDI-TOF mass spectrum of biomass from the enrichment culture after 10 months showing the presence of cofactor F_{430} with a monoisotopic molecular mass of 905 Da.

McrA *gene and cofactor* F_{430} *detection and quantification*

After 9 months, when the archaeal population still accounted for about 10% of the biomass, PCR amplification and phylogenetic analysis of a 760 bp fragment of the *mcrA* gene that encodes the α-subunit of the MCR revealed the presence of two similar genes [95.6% identity on amino acid (aa) level]. They formed a monophyletic group together with some environmental sequences (87–99% identity on aa level) within the *Methanosarcinales*, and were distantly related to both the *mcrA* group e supposedly corresponding to ANME-IIa (70–75% identity; Hallam *et al*., 2003; Alain *et al*., 2006) and cultivated *Methanosaeta* and *Methanosarcina* species (64–70% identity on aa level).

After 10 months MALDI-TOF analysis of biomass from the enrichment culture revealed the presence of the cofactor F_{430} associated with the methanogenic enzyme MCR. Of the two known forms, only the well-described variety from methanogens with a molecular mass of 905 Da (Friedmann *et al*., 1990) was found, and not the heavier modification (951 Da, Krüger *et al*., 2003) associated with ANME I archaeal methanotroph communities (Fig. 4).

After 19 months, the aforementioned method failed to detect cofactor F_{430}. By applying ^{15}N-labelled F_{430} as an internal standard, the concentration of F_{430} was determined to be less than 0.67 pmol mg^{-1} protein (Fig. S1). Assuming that the archaea in the culture had a methyl coenzyme MCR with a molecular weight of 303 kDa and with two molecules of F_{430} bound to it (Friedmann *et al*., 1990), and MCR making up 10% of cell protein (Rouvière and Wolfe, 1987), it can be concluded that they accounted for less than 0.1% of whole-culture protein.

Inhibition experiment with bromoethane sulfonate (BES)

The enrichment culture responded very sensitively to manipulations, and simple batch incubations after transfer to culture flasks (40–80 ml liquid volume in 60–120 ml serum bottles capped with black butyl rubber stoppers, with and without pH buffer) showed very little (< 10% of continuous culture) or no activity (data not shown). Reproducible results, with activities in the same range as the continuous culture, were obtained after pumping biomass from the enrichment culture into 300 ml subculture bottles (see *Experimental procedures*). The addition of BES (final concentration 10 mM) to a subculture oxidizing methane and reducing nitrite had no measurable effect on the activity, neither had another addition of the same dose (Fig. 5).

Demonstration of activity of the culture after disappearance of Archaea

The activity of the whole culture did not decrease after the apparent disappearance of the *Archaea*, but nitrite reduction reached a level on average nearly three times higher than before (9.1 ± 1.3 mmol nitrite reduced per day, Fig. 2, months 18–22). A batch experiment with the whole culture after 22 months demonstrated the simultaneous consumption of methane and the reduction of nitrite and nitrate to dinitrogen gas and minor amounts of N_2O in the total absence of oxygen (Fig. 6). The consumption of nitrite and nitrate was matched by the recovery of gaseous nitrogen compounds, indicating that no significant amount of air could have entered the system.

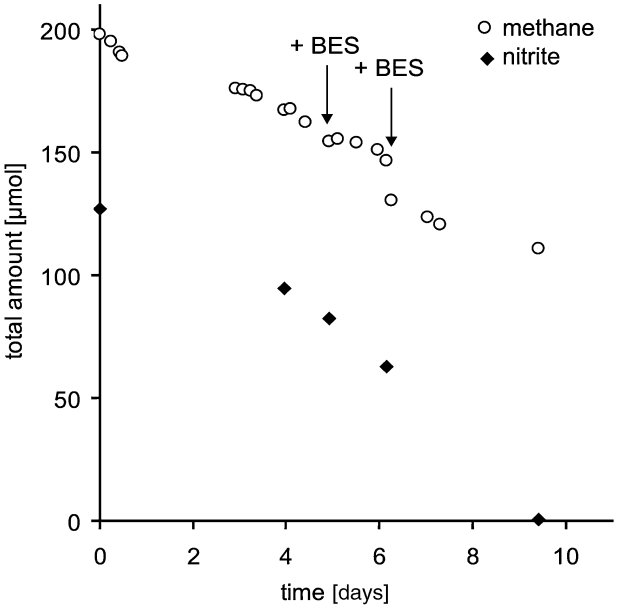

Fig. 5. Lack of inhibition by BES addition on the consumption of methane and nitrite in a 220 ml subculture after 13 months of enrichment. Arrows indicate the addition of BES to final concentrations of 10 and 20 mM respectively. Nitrite was measured by HPLC using the conductivity detector, methane with a GC-FID.

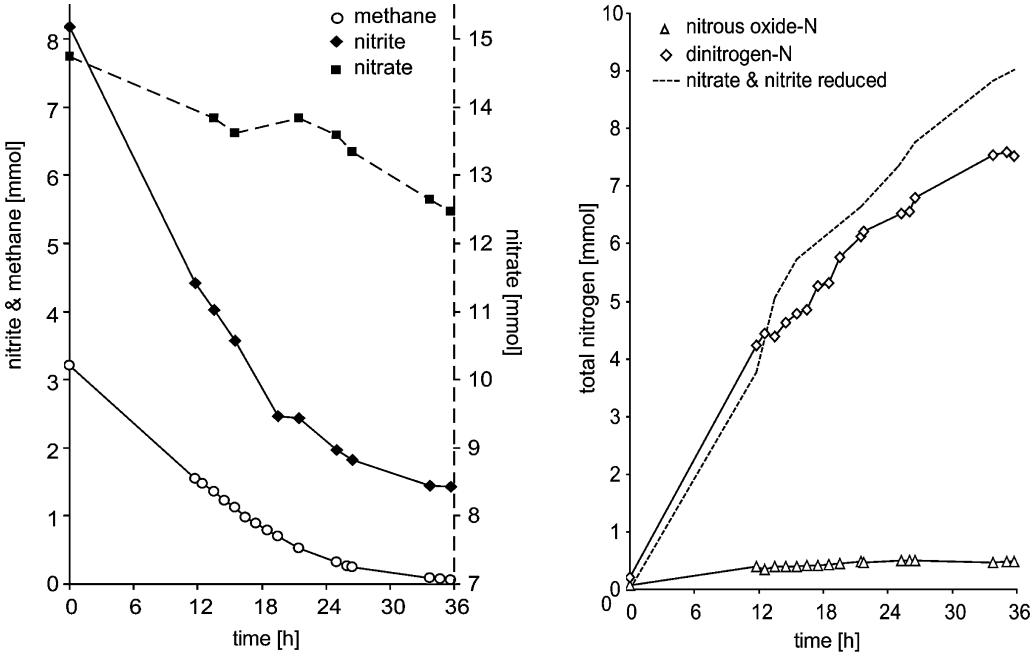

Fig. 6. Coupling of methane oxidation and denitrification in the enrichment culture after 22 months of enrichment. At the start of the experiment, concentrations of methane, nitrite and nitrate in solution were 0.39, 0.63 and 1.13 mM; total amounts in liquid and headspace together are given in the figure. The culture contained 1130 mg protein in 13 l (±40 mg). Left: Consumption of methane, nitrate and nitrite. Nitrate and nitrite were separated using HPLC and quantified by a UV detector. Right: Production of dinitrogen and nitrous oxide gas and calculated amount of nitrite and nitrate reduced.

Methane and nitrite reduction rates were 1.7 and 3.7 nmol min^{-1} (mg protein)$^{-1}$ respectively.

Discussion

The key conclusion of the presented data is that, unlike previously hypothesized, *Archaea* are not essential for the AOM with nitrite as electron acceptor. Both the lack of inhibition by the specific inhibitor BES and the disappearance of the archaea from the culture (as demonstrated by several independent methods) point to a biochemical mechanism other than the symbiosis of a 'reverse methanogen' with a denitrifying bacterium.

In reverse methanogenesis, MCR is thought to be the methane-activating enzyme (Krüger *et al*., 2003; Hallam *et al*., 2004). Although failure of the methyl-coenzyme M analogue BES to inhibit AOM has been observed in two previous studies, one with marine sediments (Alperin and Reeburgh, 1985) and one with lake water (Iversen *et al*., 1987; did not observe inhibition of methanogenesis by 20 mM BES either), most studies do report a potent inhibition at concentrations of 1–5 mM BES (Zehnder and Brock, 1980; Hoehler *et al*., 1994; Nauhaus *et al*., 2005). In sediment incubations, degradation of BES by SRB can easily explain a lack of inhibition. However, in the present study degradation could be excluded, as BES was visible and stable on the high-performance liquid chromatography (HPLC) chromatograms used to determine nitrate and nitrite. Additionally, the BES solution was tested on *Methanosarcina barkeri* cultures growing on methanol, with 20 mM final concentration resulting in an immediate and total inhibition of methane production.

The lack of inhibition is in line with the gradual disappearance of the *Archaea* from the culture, as shown by FISH (Fig. 3). The accuracy of FISH counts was constrained by growth of microorganisms in dense cell clusters, small size of the 'NC10' bacterial cells and patchy distribution of archaeal cell clusters. It was nevertheless obvious that *Archaea* became increasingly rare and, finally, apparently extinct, while the 'NC10' bacteria remained the dominant phylotype in the culture. To back these results, the essential cofactor F_{430} of MCR was quantified at the time when no archaeal cells could be detected anymore by FISH. The MALDI-TOF method using ^{15}N-labelled F_{430} as internal standard did reveal minute amounts of F_{430} in the culture [< 0.67 pmol (mg protein)$^{-1}$]. However, assuming typical amounts of MCR in archaeal cells, these values corresponded to < 0.1% of protein originating from methanotrophic or methanogenic archaea. As F_{430} is a very stable molecule that could persist in dead cells trapped in biofilm aggregates (Diekert *et al*., 1980; Pfaltz *et al*., 1982), the value of less than 0.1% might still be an overestimate. The decline of the archaeal population was accompanied by

an increase in the nitrite consumption rate of the culture (Fig. 2), making it clear that bacteria alone were responsible for the observed activity. Although methane consumption could not be monitored continuously, the high methane consumption rate measured in the whole-culture batch experiment (see below) indicated that nitrite consumption did not become uncoupled from methane oxidation after the decline of the archaeal population. This experiment, performed after 22 months, clearly showed the simultaneous disappearance of methane and nitrite and the formation of dinitrogen gas (Fig. 6). The oxygen concentration was always under the detection limit of the electrode used, and even if this could be due to its rapid consumption, the diffusion of a significant amount of air into the culture could still be excluded, as this would have been apparent from an increase in dinitrogen gas concentration. Opposed to that, dinitrogen gas development (7.5 mmol N_2-N) was fully accounted for by nitrite and nitrate reduction (9 mmol).

Nitrite was preferred over nitrate as an electron acceptor both in the continuous culture and in the above experiment, consistent with the observations of Raghoebarsing and colleagues (2006). In the linear phase of the batch experiment (21 h) the ratio of NO_2^- to NO_3^- consumption was 5:1. If all the nitrite reduction in this period (5.75 mmol) was coupled to methane oxidation according to the following equation:

$$3CH_4 + 8NO_2^- + 8H^+ \rightarrow 3CO_2 + 4N_2 + 10H_2O \quad (1)$$

then nitrite accounted for 80% of total methane oxidation (2.68 mmol). The remaining 20% of methane oxidation could be explained by nitrate reduction (1.1 mmol) with the following stoichiometry:

$$5CH_4 + 8NO_3^- + 8H^+ \rightarrow 5CO_2 + 4N_2 + 14H_2O \quad (2)$$

leaving 0.35 mmol NO_3^- consumption uncoupled to methane oxidation. This denitrification might be due to decay processes leading to internal production of substrates for denitrifiers or due to contamination of the demineralized water and chemicals used in the medium with trace amounts of organic electron donors. Measurement inaccuracies also could be a possible explanation, as nitrite and nitrate consumption together account for slightly more nitrogen than recovered as N_2 and N_2O (Fig. 6).

Although it is clear from the presented data that *Archaea* are dispensable for AOM with nitrite as electron acceptor, their role in the initial enrichment is still an intriguing question. Both methanogenesis and reverse methanogenesis at the high redox potential of denitrifying conditions are surprising given the requirement of very reduced conditions (around –600 mV intracellularly) for the functioning of the MCR (Shima and Thauer, 2005). It could still be possible that the archaea in the initial enrichment (Raghoebarsing *et al*., 2006) did oxidize methane in syntrophy with denitrifying bacteria (not necessarily the dominant 'NC10' phylotype) or without a partner. This possibility was supported by the facts that they were enriched over a period of 16 months and that archaeal lipids were clearly depleted in ^{13}C (–67‰) in comparison with the supplied methane (–27‰). In this case, their disappearance would likely be due to a lost competition with the dominant bacteria after change from the sediment-containing sequencing batch reactor used initially to the continuous culturing system described in the present paper. Also the additions to the medium (NH_4Cl, cysteine, vitamins and sea salt), though not sufficient to influence overall activity, might have shifted the competitive advantage towards the bacteria. On the other hand, the archaea may have been methanogenic, as the ^{13}C-CH_4-labelling experiment performed with the initial enrichment culture (Raghoebarsing *et al*., 2006) suggested. In that experiment the archaea showed only a minor incorporation of ^{13}C from methane into archaeal lipids (+18‰ within 6 days), whereas some bacterial lipids were strongly labelled (+4426‰). The small amount of ^{13}C incorporated into archaeal lipids might result from using waste products, intermediates or dead cells as substrates for methanogenesis. It has since been suggested (Thauer and Shima, 2006) that the low affinity constant for methane reported there (< 0.6 μM) was not consistent with MCR being the methane-activating enzyme accounting for the bulk of methane oxidation. A low affinity constant was confirmed in the present study. From the data presented here, an affinity constant for methane of approximately 5 μM could be determined, which is in the same order of magnitude as in field studies reporting circumstantial evidence for the occurrence of AOM coupled to denitrification in an anoxic aquifer (9 μM, Smith *et al*., 1991). In contrast, the affinity of AOM with sulfate as electron acceptor was in the mM range (Nauhaus *et al*., 2002), consistent with a different initial enzymatic step.

When it was discovered that the MCR cofactor F_{430} of the methanotrophic ANME-I archaea had an unusual mass (951 versus 905 Da, Krüger *et al*., 2003), it was speculated that this modification was involved in the reversal of the enzyme function, from methane formation to methane activation. The archaea from the culture described here did not have this modified cofactor F_{430}, but the conventional, 'methanogenic' type. Circumstantial evidence, however, indicates that also ANME-II archaea harbour the conventional version (A. Meyerdierks, S. Shima, J. Kahnt and M. Krüger, unpubl. results, reported in Thauer and Shima, 2008). Therefore, the type of cofactor does not seem to be of predictive value for the enzyme function. Also, neither of the *mcrA* sequences obtained in this study could be clearly assigned to a group of well-characterized methanogens or methanotrophs. Instead,

together with environmental sequences, they formed their own distinct group that is slightly closer related to the *mcr* group e supposedly belonging to ANME-IIa (Hallam *et al*., 2003; Alain *et al*., 2006) than to the methanogenic genera *Methanosarcina* and *Methanosaeta*, corresponding to the 16S rRNA gene-derived phylogeny (Raghoebarsing *et al*., 2006). Recently, first physiological data were reported for members of a group of environmental *mcrA* sequences (e.g. EU276007) clustering with the sequences presented in this study. These organisms, enriched from Tibetan wetlands, were shown to be versatile methanogens, able to use methanol, acetate, hydrogen and TMA as substrates (Zhang *et al*., 2008).

Unfortunately, due to their disappearance from the enrichment culture, the physiology of the archaea found in the earlier stages of the culture remains elusive. However, the cultivation set-up described in the present study proved successful to sustain and increase the biomass of bacteria presumably oxidizing methane without a partner. This opens up the way to explore the biochemical mechanism employed by these bacteria.

Experimental procedures

Enrichment of microorganisms

Microorganisms were grown in continuous culture in a 16 l glass bioreactor (Applikon, the Netherlands; liquid volume 13 l) inoculated with biomass from an enrichment culture anaerobically oxidizing methane (Raghoebarsing *et al*., 2006). The culture was stirred at 200 r.p.m., the temperature was controlled at 30°C and medium was continuously supplied (0.2–0.8 l day^{-1}). The liquid volume in the culture vessel was maintained by a level-controlled effluent pump, and biomass was retained within the system by an external settler (Fig. 1). The medium contained (per litre): $KHCO_3$ 1.25 g, KH_2PO_4 0.05 g, $CaCl_2{\cdot}2H_2O$ 0.3 g, $MgSO_4{\cdot}7H_2O$ 0.2 g, $FeSO_4$ 0.0025 g, Na-EDTA 0.0025 g, $NaNO_3$ 0.255 g (3 mM) and trace element solution 0.5 ml. The trace element solution contained (g l^{-1}): EDTA 15, $ZnSO_4{\cdot}7H_2O$ 0.43, $CoCl_2{\cdot}6H_2O$ 0.24, $MnCl_2{\cdot}4H_2O$ 0.99, $CuSO_4$ 0.25, Na_2MoO_4 0.242, $NiCl_2{\cdot}6H_2O$ 0.19, SeO_2 0.067, H_3BO_3 0.014 and $Na_2WO_4{\cdot}2H_2O$ 0.050. After 1 year of enrichment, the EDTA in medium and trace element solution was omitted and trace metals and iron were supplied in an acidic solution containing Fe, Zn, Co, Mn, Cu, Ni, B and an alkaline solution containing Se, W and Mo at the concentrations specified above. The medium was continuously sparged with a mixture of Ar/CO_2 (95:5). The culture was continuously sparged with CH_4/CO_2 (95:5 v/v, purity > 99.995%, Air Liquide, the Netherlands) at a flow rate of 10 ml min^{-1} as carbon and energy source and to maintain anaerobiosis. The CO_2 concentration was sufficient to buffer the culture liquid in a pH range of 7.3–7.6 as monitored by a pH electrode. Absence of oxygen was monitored by a Clark-type oxygen electrode. To prevent growth of photosynthetic organisms, the culture vessel was wrapped in black foil and black tubing with low oxygen permeability (Viton and Norprene, Cole Parmer, USA) was used. Nitrite concentration in the culture was maintained between 0 and 1 mM by varying the flow rate (0.2–0.8 l day^{-1}) and the nitrite concentration (3–15 mM) of the influent medium, dependent on the nitrite-reducing activity of the culture. For 2 months (Fig. 2), nitrite was omitted and medium containing only 10 mM nitrate as electron acceptor was supplied, but as methane-oxidizing activity in a batch test was much slower with only nitrate than that with nitrite (data not shown), the medium was again prepared with nitrite as the main electron acceptor. Further modifications of the medium composition are specified in the results sections. The demineralized water for medium preparation was autoclaved, but the culture was not operated aseptically. Interstitial water was extracted by centrifugation from freshwater ditch sediment collected in the Ooijpolder, the Netherlands, filtered through a paper filter and made anaerobic by sparging with dinitrogen gas before addition to the reactor.

Fluorescence in situ *hybridization*

Biomass (2–4 ml culture liquid) was harvested monthly from the enrichment culture, centrifuged, washed with phosphate-buffered saline (PBS; 10 mM Na_2HPO_4/NaH_2PO_4 pH 7.2 and 130 mM NaCl) and fixed for 2–3 h with 3% (w/v) paraformaldehyde in PBS on ice. After washing with PBS, samples were stored in PBS/ethanol (1:1) at –20°C.

For microscopy, fixed biomass was spotted in 10 µl volumes onto the wells of Teflon-coated microscope slides and embedded in 0.1% agarose (w/v). After sequential dehydration in 50%, 80% and 96% ethanol for 3 min, each probe was hybridized for 1.5 h at 46°C in hybridization buffer containing 900 mM NaCl, 2 mM Tris/HCl pH 8.0, 0.2‰ sodium dodecyl sulfate, 40% formamide and a combination of the following oligonucleotide probes: S-*-DBACT-0193-a-A-18 and S-*-DBACT-1027-a-A-18 (dominant bacteria affiliated with the 'NC10' phylum; Raghoebarsing *et al*., 2006); a mixture of EUB I-III and V (most *Bacteria*; Daims *et al*., 1999), S-D-Arch-0915-a-A-20 (most *Archaea*; Stahl and Amann, 1991). For image acquisition, a Zeiss Axioplan 2 epifluorescence microscope equipped with a CCD camera was used together with the Axiovision software package (Zeiss, Germany). Percentages of phylogenetic groups were estimated based on visual inspection of at least one complete well.

Amplification and phylogenetic analysis of the mcrA *gene*

After 9 months of enrichment, DNA was extracted from 2 ml of concentrated biomass withdrawn from the bottom of the external settler. A procedure based on phenol/chloroform/isoamylalcohol modified from Juretschko and colleagues (1998) was used. From the initial enzymatic steps described therein only a proteinase K digest was performed. An approximately 760-bp-long fragment of the *mcrA* gene was PCR-amplified using the ME1 and ME2 primers (Hales *et al*., 1996) with an annealing temperature of 55°C. A PCR product of the correct length was cloned into *E. coli* with the pGEM T-easy cloning vector system (Promega). Plasmid DNA was isolated from overnight cultures of single white colonies using

the FlexiPrep kit (Amersham Biosciences) and digested with the EcoRI restriction enzyme. For each restriction type, two purified plasmid DNA samples were sequenced using both the M13 forward and reverse primers. Sequences were translated and aligned to close BLAST hits in BioEdit using the muscle algorithm (Edgar, 2004) followed by manual adjustments. For calculating the percentage of amino acid identities additional reference sequences were added to the alignment, and calculations were based on 157–260 positions. Sequences have been submitted to GenBank (accession numbers EU495303 and EU495304).

Analytical procedures

Nitrate and nitrate. Nitrite concentration in the enrichment culture was estimated daily with Merckoquant test strips (0–80 mg l^{-1}; Merck, Germany). Medium and culture liquid concentrations of nitrate and nitrate were occasionally verified by colorimetric measurements as described previously (Kartal *et al*., 2006). In batch experiments, nitrate and nitrite were measured with HPLC on a Hewlett Packard 1050 system. 10 µl of liquid sample was injected with an autosampler. A 30 mM sodium hydroxide solution was used as the liquid phase at a flow rate of 1.5 ml min^{-1}. Anions were isocratically separated on a 4×250 mm Ionpac AS11-HC (Dionex, UK) column at 30°C and detected using the built-in UV detector or a CD25 conductivity detector (Dionex, UK).

Gaseous nitrogen compounds and methane. Gas samples of 100 µl volume were withdrawn with gas-tight glass syringes (Hamilton, Switzerland) through a rubber septum on top of the reactor or the incubation bottle, respectively. Dinitrogen gas and nitrous oxide were measured on an Agilent 6890 series gas chromatograph (Agilent, USA) equipped with a Porapak Q column, a molecular sieve (Hewlett Packard, USA) and a thermal conductivity detector working at 200°C. The carrier gas was helium at a flow rate of 21 ml min^{-1}. The injection temperature was 125°C, the oven temperature 65°C. The inlet area of the GC was flushed with helium to minimize entry of N_2 from air.

Methane was measured on a HP 5890 gas chromatograph equipped with a Porapak Q column (80/100 mesh) and a flame ionization detector (Hewlett Packard, USA). The carrier gas was helium at a flow rate of 28 ml min^{-1}. The injection and detection temperature was 150°C, the oven temperature 120°C. The gases were quantified by comparing the peak areas to those of a commercial calibration gas mixture (Air liquide, precision ±2%) and self-made standards.

Total protein. Total protein content was measured after sonication or bead-beating of 2–4 times concentrated cell suspensions with the bicinchoninic acid (BCA) assay (Pierce, USA), according to the manufacturer's instructions. Briefly, the Biuret method producing Cu^{+1} ions is combined with the subsequent reaction of these ions with BCA yielding a coloured complex colorimetrically detectable at 562 nm. Bovine serum albumine was used as a standard.

Identification and quantification of cofactor F_{430}. After 10 months of enrichment, 2 ml culture liquid was pelleted at 10 000 *g* and re-suspended in 50 µl matrix solution containing 10 mg ml^{-1} α-cyano-4-hydroxy-cinnamic acid in a 50:50 mixture of acetonitrile and 0.1% (v/v) trifluoroacetic acid (Farhoud *et al*., 2005). The mixture was spotted on a target plate and analysed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS, Biflex III, Bruker, Germany).

After 19 months of enrichment, the above-mentioned method failed to detect cofactor F_{430} anymore. In order to quantify trace amounts of cofactor F_{430}, highly concentrated culture liquid was spiked with ^{15}N-labelled cofactor F_{430} as an internal standard. Specifically, culture liquid was pelleted at 10 000 *g*, re-suspended in water to a concentration of 6 mg ml^{-1} protein and mixed 1:1 with matrix solution [50% saturation of α-cyano-4-hydroxy-cinnamic acid in a solution of 70% acetonitrile, 30% H_2O and 0.1% trifluoroacetic acid (v/v)]. After ultrasonication for 5 min at room temperature, the mixture was supplemented with a MCR standard containing ^{15}N-labelled F_{430} to final concentrations of 2, 4 and 8 nM, subsequently spotted on a target plate and analysed by MALDI-TOF MS (Ultraflex, Bruker, Germany). ^{15}N-labelled cofactor F_{430} was extracted from *M. thermoautotrophicum* grown with $^{15}NH_4^+$ as sole nitrogen source. Incorporation of ^{15}N increases the molecular mass of cofactor F_{430} resulting in a main peak at 911 Da compared with 905 Da in unlabelled cofactor. Comparison of peak height allowed estimation of the maximum concentration of F_{430} in the sample.

Batch incubation experiments

Inhibition experiment. Bromoethane sulfonate (Sigma-Aldrich, Germany) inhibition experiments were carried out after 13 months of enrichment in a subculture consisting of a 300 ml glass bottle capped with a modified stainless steel plate with three ports, held in place by a screw cap. One port was closed with a septum allowing gas-tight sampling of the headspace, the other two were connected to Norprene tubes (Cole-Parmer, USA) reaching into the bottle for liquid supply and withdrawal. After being made anoxic by thorough flushing with a mixture of Ar/CO_2 (95:5), culture liquid from the enrichment culture was being pumped through at a flow rate of 17.5 ml min^{-1}. The gas headspace of the bottle was set at 80 ml by the height of the effluent tube. The subculture bottle was stirred by a magnetic stirrer and kept at 30°C. After *c.* 24 h (accounting for at least 110 volume changes) it was disconnected from the main culture vessel by closing the influent and effluent tubes. By flushing with Ar/CO_2 (95:5) methane concentration in the headspace was decreased to about 6%, and gas samples of 100 µl were measured 3–4 times daily. Once a day a 1 ml liquid sample was withdrawn, centrifuged and the supernatant stored at –20°C until analysis of nitrite. After 119 and 148 h, BES was added from anaerobic 2 M stock solution to final concentrations of 10 and 20 mM respectively. The data shown are from one experiment, two separate ones carried out yielded essentially the same result.

Whole-culture batch experiment. To measure methane consumption and development of gaseous nitrogen compounds in the continuous culture, medium and gas supply were stopped and headspace methane concentration decreased

to *c.* 2.5% by flushing with Ar/CO_2 (95:5). At each sampling time, headspace gas samples of 100 µl each were taken for immediate analysis by gas chromatography (see above), and 1 ml liquid sample was centrifuged and the supernatant stored at –20°C until analysis. Assuming that liquid and gas phase were in equilibrium, the total amount of gaseous compounds in the culture vessel was calculated based on the measured headspace concentration and the solubility coefficients for methane, nitrous oxide and dinitrogen gas at 30°C in water (Wilhelm *et al*., 1977). Rates of methane and nitrite consumption were calculated from the linear part of the respective graphs (11.75–19.5 h).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Estimation of the F_{430} content of concentrated cell suspension (protein concentration 6 mg ml^{-1}) by MALDI TOF MS using ^{15}N-labelled F_{430} as an internal standard. Unlabelled F_{430} has a monoisotopic mass of 905 Da, fully ^{15}N-labelled F_{430} a mass of 911 Da. (1) ^{15}N-labelled standard (34 nM) only; (2) cell suspension without added standard; (3) cell suspension with 8 nM 15 N-labelled standard; (4) cell suspension with 4 nM ^{15}N-labelled standard; (5) cell suspension with 2 nM ^{15}N-labelled standard. Peak height comparison shows that the sample contains approximately 2 nM F_{430}.

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