ARTICLES

Nitrite-driven anaerobic methane oxidation by oxygenic bacteria

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Only three biological pathways are known to produce oxygen: photosynthesis, chlorate respiration and the detoxification of reactive oxygen species. Here we present evidence for a fourth pathway, possibly of considerable geochemical and evolutionary importance. The pathway was discovered after metagenomic sequencing of an enrichment culture that couples anaerobic oxidation of methane with the reduction of nitrite to dinitrogen. The complete genome of the dominant bacterium, named '*Candidatus* Methylomirabilis oxyfera', was assembled. This apparently anaerobic, denitrifying bacterium encoded, transcribed and expressed the well-established aerobic pathway for methane oxidation, whereas it lacked known genes for dinitrogen production. Subsequent isotopic labelling indicated that '*M. oxyfera*' bypassed the denitrification intermediate nitrous oxide by the conversion of two nitric oxide molecules to dinitrogen and oxygen, which was used to oxidize methane. These results extend our understanding of hydrocarbon degradation under anoxic conditions and explain the biochemical mechanism of a poorly understood freshwater methane sink. Because nitrogen oxides were already present on early Earth, our finding opens up the possibility that oxygen was available to microbial metabolism before the evolution of oxygenic photosynthesis.

With the ubiquitous use of fertilizers in agriculture, nitrate (NO_3^-) and nitrite (NO_2^-) have become major electron acceptors in freshwater environments[1]. The feedback of eutrophication on the atmospheric methane (CH_4) budget is poorly understood, with many potential positive and negative feedback loops acting in concert[2]. This previously prompted us to investigate the possibility of anaerobic oxidation of methane coupled to denitrification (reduction of NO_3^- and NO_2^- through nitric oxide (NO) to nitrous oxide (N_2O) and/or dinitrogen gas (N_2)), and microbial communities that perform this process were enriched from two different freshwater ecosystems in The Netherlands[3,4], and recently from mixed Australian freshwater sources by others[5]. All independent enrichment cultures were dominated by the same group of bacteria representing a phylum (NC10) defined only by environmental 16S ribosomal RNA gene sequences[6]. Although many surveys have found these sequences in a variety of aquatic habitats worldwide, reports on the natural activity of these bacteria are scarce (summarized in ref. 4).

Methane is one of the least reactive organic molecules[7]. Aerobic methanotrophs overcome its high activation energy by a reaction with molecular oxygen[8]. Anaerobic sulphate-reducing microbial consortia activate methane by a reversal of its biological production, using a homologue of the methane-releasing enzyme (methyl-coenzyme M reductase) of methanogens[9]. These consortia usually consist of distinct archaea related to methanogens and sulphate-reducing bacteria[10]. Initially, it was hypothesized that anaerobic oxidation of methane coupled to denitrification proceeded in a similar manner, with archaea conducting reverse methanogenesis in association with denitrifying bacterial partners[3]. However, it was subsequently shown that the complete process could also be performed by the bacteria in the total absence of archaea[4,11]. The overall reaction of methane with nitrite (and nitrate) is thermodynamically feasible[3,7]:

$$3CH_4 + 8NO_2^- + 8H^+ = 3CO_2 + 4N_2 + 10H_2O$$

$$(\Delta G^{\circ\prime} = -928\,\mathrm{kJ\,mol^{-1}}\ CH_4)$$

However, so far no known biochemical mechanism has been able to explain the activation of methane in the absence of oxygen or (reversed) methanogens.

Genome assembly from enrichment cultures

We addressed the unknown mechanism of nitrite-dependent anaerobic methane oxidation by metagenomic sequencing of two enrichment cultures described previously: one enriched from Twentekanaal sediment[3,11], here designated 'Twente', and a culture from Ooijpolder ditch sediment[4], designated 'Ooij'. Both enrichments were 70–80% dominated by populations of the same bacterial species (minimum 97.5% 16S rRNA gene identity; for microdiversity in culture 'Ooij' see ref. 4). In the present study we propose to name this species '*Candidatus* Methylomirabilis oxyfera'. The metagenome of culture 'Twente' was

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Table 1 | Databases of genomic, proteomic and transcriptomic data

Enrichment culture	Approach	Molecule type	Amount of data obtained	NCBI-database, accession number
'Twente'	454 pyrosequencing	DNA	90,353,824 nt	Short Read Archive, SRR023516.1
	Illumina sequencing	DNA	196,814,368 nt	Short Read Archive, SRR022749.2
	Assembled genome	DNA	2,752,854 nt	GenBank, FP565575
	Paired-end plasmid (10 kb) sequencing	DNA	16,440,000 nt (trimmed)	Project ID 40193 (linked to FP565575)
	Illumina sequencing	RNA	198,977,152 nt	Gene Expression Omnibus, GSE18535
	nLC LIT FT–ICR MS/MS	Protein	–	Peptidome, PSE127
'Ooij'	Illumina sequencing	DNA	188,099,392 nt	Short Read Archive, SRR022748.2
	nLC LIT FT–ICR MS/MS	Protein	–	Peptidome, PSE128

nLC LIT FT-ICR MS/MS, nanoflow liquid chromatography linear ion-trap Fourier-transform ion cyclotron resonance MS/MS analysis; nt, nucleotides.

obtained by 454 pyrosequencing, Illumina sequencing and paired-end Sanger sequencing of a 10-kilobase (kb) insert plasmid library (Table 1 and Supplementary Fig. 1). Binning based on GC content and coverage indicated that almost 60% of the metagenomic data from pyrosequencing were associated with '*M. oxyfera*'. These data were initially assembled into five scaffolds that could then be joined into a single circular chromosome (2,753 kb; see Supplementary Table 1 for more properties) by long-range PCR amplification.

Consistent with previous work[11], combined metagenomic data contained no evidence for the presence of the archaea that were originally suggested to form a consortium with the dominant bacterium[3]. Out of roughly 365,000 reads obtained by pyrosequencing, only 78 gave a Blast hit with a bacterial 16S or 23S rRNA gene sequence distinct from '*M. oxyfera*', and none matched archaeal sequences (Supplementary Table 2). No other single species constituted a numerically significant part of the overall enriched community to enable the assembly of more than very small (most less than 2 kb) fragments.

Short-read (32 base pairs) Illumina sequencing of culture 'Ooij' revealed that, in contrast with the near-clonal population dominating culture 'Twente' (for single nucleotide polymorphism (SNP) frequency see Supplementary Fig. 2), a more diverse population of '*M. oxyfera*' inhabited this culture, as commonly observed by metagenomic sequencing of microbial populations[12]. In the present case the estimated number of SNPs in culture 'Ooij' was more than threefold that in culture 'Twente'. Because of this microdiversity, assembly of larger contigs for culture 'Ooij' was impossible and the short reads were mapped directly onto the complete genome of '*M. oxyfera*'. Although the two enrichment cultures were dominated by the same species, the sequences were apparently too dissimilar to enable mapping by the currently available approaches[13]. A new mapping algorithm based on iterated Blast searches was therefore developed[14]. This allowed us to construct consensus sequences for genes of the '*M. oxyfera*' populations dominating enrichment culture 'Ooij'. By proteomic detection of peptides predicted from this consensus, the procedure was validated experimentally (Supplementary Table 3). The average identity of the partial genome obtained from enrichment culture 'Ooij' to the complete genome of '*M. oxyfera*' was 91.1% at the DNA level (open reading frames (ORFs) and RNAs), and the SNP frequency among the Ooij populations was at least 3.45% (Supplementary Fig. 2).

To facilitate the mechanistic interpretation of the genomic sequence information, the transcription and expression of predicted genes was investigated by Illumina sequencing of RNA and by liquid chromatography–tandem mass spectrometry (LC–MS/MS) of extracted proteins (Table 1).

Paradoxical predictions from the genome

Both enrichment cultures were grown anoxically and performed methane oxidation coupled to the complete denitrification of nitrite to N_2 (refs 3, 4, 11). We therefore inspected the genome, transcriptome and proteome for homologues of known genes involved in denitrification[15]. '*M. oxyfera*' apparently lacked some genes necessary for complete denitrification (Fig. 1a and Supplementary Table 4). Genes for the reduction of nitrate to nitrite (*narGHJI*, *napAB*), nitrite to NO (*nirSJFD/GH/L*) and NO to N_2O (*norZ* = q*nor*) were present in the genome, and expression as proteins could be demonstrated for Nap, Nir and Nor. However, with the exception of the accessory gene *nosL*, the gene cluster encoding enzymes for the reduction of N_2O to N_2 (*nosZDFY*) was missing. Previous studies have shown that N_2O was not the main product of denitrification but was only produced in trace amounts[4,11]. However, on the basis of the analysis of the data sets outlined in Table 1 and the fact that the '*M. oxyfera*' genome sequence seems complete, we judge it highly unlikely that genes encoding canonical N_2O reductase were overlooked and escaped proper assembly. Because complete denitrification can also be achieved by the combined action of multiple species, we could not yet rule out the possibility that the missing catalytic activity was complemented by other bacteria.

In a similar fashion, we searched for homologues of anaerobic alkane activation enzymes, such as fumarate-adding glycyl-radical enzymes[16] and the methyl-coenzyme M reductase of reverse methanogens[9]. Consistent with the absence of archaea, the metagenome contained no homologue of methyl-coenzyme M reductase. However, alkane-activating glycyl radical enzymes, which had been proposed to activate methane in these organisms[7], were also missing. Instead, the genome did encode the complete pathway for aerobic methane oxidation (Fig. 1b and Supplementary Table 5). This well-known pathway proceeds through methanol (CH_3OH), formaldehyde (CH_2O) and formate (HCOOH) to carbon dioxide (CO_2)[8]. In the

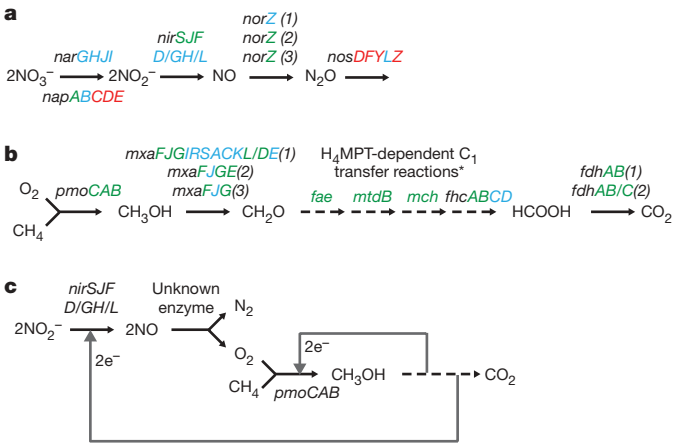

Figure 1 | Significant pathways of *Methylomirabilis oxyfera*. Canonical pathways of denitrification (**a**), aerobic methane oxidation (**b**) and proposed pathway of methane oxidation with nitrite (**c**). *narGHJI*, nitrate reductase; *napABCDE*, periplasmic nitrate reductase; *nirSJFD/GH/L*, nitrite reductase; *norZ*, nitric oxide reductase; *nosDFYLZ*, nitrous oxide reductase; *pmoCAB*, particulate methane monooxygenase; *mxaFJGIRSACKL/DE*, methanol dehydrogenase; *fae*, formaldehyde-activating enzyme; *mtdB*, methylene-tetrahydromethanopterin (H_4MPT) dehydrogenase; *mch*, methenyl-H_4MPT cyclohydrolase; *fhcABCD*, formyltransferase/hydrolase; *fdhABC*, formate dehydrogenase. Genes in red are absent from the genome, those in blue are present in the genome and those genes in green are present in both the proteome and the genome. Asterisk, H_4MPT-dependent reactions involve the intermediates methylene-H_4MPT, methenyl-H_4MPT and formyl-H_4MPT.

first step of this pathway, methane is hydroxylated by a reaction with oxygen, yielding methanol and water. This reaction is catalysed by the enzyme methane mono-oxygenase (MMO). Both metagenomes contained one set of *pmoCAB* genes encoding the particulate (membrane-bound) form of this enzyme complex (pMMO); genes encoding the soluble form were absent. Although the amino-acid sequences were phylogenetically distant from all homologous sequences currently in the databases (Fig. 2 and Supplementary Fig. 3), *pmoA* signature residues and those important for function were well conserved (Supplementary Fig. 4). The complete aerobic methanotrophic pathway was found to be transcribed and expressed in both anaerobic enrichment cultures (Supplementary Table 5), including the complete tetrahydromethanopterin-dependent C_1 transfer module. The phylum NC10 is thus only the fifth phylogenetic group known to harbour this potentially primordial metabolic module[17]. Phylogenetic analysis indicated that '*M. oxyfera*' represents a deeply branching lineage of this C_1 pathway (data not shown).

We were therefore faced with two anaerobic, denitrifying microbial communities that were dominated by the same species, an apparently aerobic methanotroph incapable of complete denitrification. To resolve this puzzle, we investigated whether '*M. oxyfera*' produced N_2 by means of a previously unknown mechanism. Figure 1c shows a possible mechanism that could resolve the conflict between the genetic and experimental evidence; it is based on the conversion of two molecules of NO into O_2 and N_2. This reaction is thermodynamically favourable ($\Delta G^{\circ\prime} = -173.1$ kJ mol^{-1} O_2) but kinetically difficult[18]. No catalyst operating at biologically relevant temperatures (0–100 °C) is known, although for higher temperatures several catalysts (for example copper zeolites) have been developed that decompose NO from industrial and automobile exhaust fumes[19]. The production of oxygen as a metabolic intermediate is not completely new to biology: dismutation of the toxic intermediate chlorite ($ClO_2^- \rightarrow Cl^- + O_2$) by chlorate-reducing bacteria prevents cell damage and yields oxygen for chemo-organotrophic respiration[20], or possibly for mono-oxygenase-dependent biosynthesis[21].

The pathway outlined in Fig. 1c would require only one new enzyme, an 'NO dismutase', to catalyse a thermodynamically feasible reaction and replace N_2O reductase. The oxygen produced would become available to oxidize methane aerobically, explaining the presence of genes for aerobic methane oxidation in the '*M. oxyfera*' genome and the insensitivity of the cultures to oxygen[4]. In this model, the function of the putative quinol-dependent NO reductases (norZ) could be the detoxification of NO rather than respiration, which is consistent with its function in most other known bacteria[22]. Alternatively, together with two multi-copper oxidases encoded in the genome, they are hypothetical candidates for catalysing the oxygen production from NO.

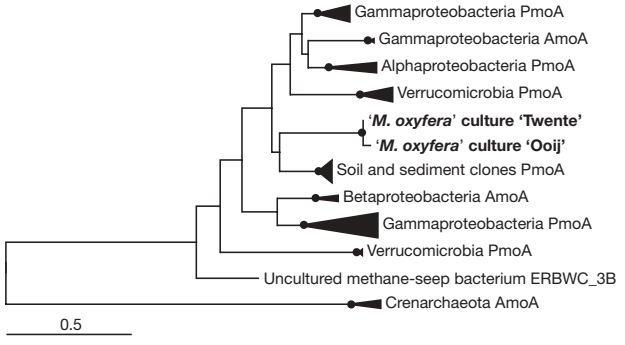

Figure 2 | Phylogeny of '*Methylomirabilis oxyfera*' pmoA protein sequences. Neighbour-joining tree showing the position of enrichment cultures 'Twente' and 'Ooij' (in bold) relative to other pmoA and amoA sequences. The distance tree was computed with the Dayhoff matrix-based method, and bootstrapping of 100 replicates was performed within the neighbour-joining, minimum-evolution, maximum-parsimony and maximum-likelihood evolutionary methods. Bootstrapping results are summarized on the tree, with filled circles representing branch points at which all four methods give greater than 70% support. The scale bar represents 50 amino-acid changes per 100 amino acids. See also Supplementary Fig. 3 for more detailed tree and bootstrap values.

Experimental evidence for the proposed pathway

The operation of the new pathway was addressed experimentally in a series of experiments performed with culture 'Ooij'. Its activity was higher than that of culture 'Twente', and it was less sensitive to experimental handling. To corroborate the coupling of nitrite reduction to methane oxidation, we first incubated 380 ml of the enrichment culture with ^{13}C-labelled methane and with nitrate (2 mM) as the only electron acceptor while monitoring the concentration and isotopic composition of the dissolved gases. With nitrate only, no methane oxidation was detectable and no N_2 was produced. On addition of ^{15}N-labelled nitrite, methane oxidation began and labelled N_2 was formed in stoichiometric amounts (Fig. 3a). Together, these results showed unambiguously that methane oxidation by '*M. oxyfera*' cultures proceeded in the absence of extracellular oxygen: the oxygen concentration remained below the detection limit (0.3 μM), the activity was dependent on the presence of nitrite, and the stoichiometry of the reaction indicated that no electrons were lost to other electron acceptors (theoretical stoichiometry $3CH_4/4N_2$; measured stoichiometry 3/3.87). The experiment also confirmed that denitrification was complete, despite the lack of genes encoding N_2O reductase in the genome of '*M. oxyfera*'. Similar results were obtained after the addition of ^{15}NO (Supplementary Fig. 5). This indicated that NO was an intermediate of '*M. oxyfera*', and the results are consistent with the presence and expression of nitrite reductase.

To test whether N_2 production by '*M. oxyfera*' proceeded through N_2O as an intermediate, the enrichment culture was incubated with $^{13}CH_4$, nitrate and N_2O, but without nitrite. Under these conditions, neither methane nor N_2O was consumed (Fig. 3b). Consistent with the genomic inventory, N_2O was apparently not a suitable electron acceptor for methane oxidation. Again, methane was oxidized only after the addition of $^{15}NO_2^-$, and almost all (93%) of the label was recovered in N_2. Only a small amount (7%) of the $^{15}NO_2^-$ was converted to N_2O, presumably by community members other than '*M. oxyfera*'. Because a large amount of unlabelled N_2O was present from the start, it can be assumed to have fully penetrated the microbial cells, even those residing in aggregates. For this reason we would expect that if N_2O had been turned over as an intermediate during nitrite reduction by '*M. oxyfera*', most ^{15}N label would have been recovered as N_2O, because the cells would mainly reduce the unlabelled N_2O to N_2. Hence, the ^{15}N label would be 'trapped' in the N_2O 'pool', and definitely so for a model in which the missing genes for N_2O reductase would be complemented by other bacteria in the enrichment culture. However, it is still a possibility that in '*M. oxyfera*' N_2O production and reduction are extremely strictly coupled. Given the absence of genes for a conventional N_2O reductase[23], N_2 production would depend on the presence and activity of an as yet unknown functional analogue. Thus, even in this conservative model it is likely that a novel enzyme produces N_2 in '*M. oxyfera*'.

N_2O reductase is inhibited by acetylene (C_2H_2) at millimolar concentrations[15], and the addition of acetylene would therefore be a straightforward method of providing further evidence for the absence of this enzyme. However, acetylene also inhibits pMMO at much lower concentrations (micromolar range)[24]. Thus because the genomic and proteomic analyses suggested that pMMO was the methane-activating enzyme in '*M. oxyfera*', a complete inhibition of total activity by acetylene would be expected for this organism. Indeed, experiments with culture 'Twente' suggested complete inhibition of methane oxidation activity at concentrations as low as 10 μM acetylene (data not shown).

To provide more evidence for the potential role of pMMO in anaerobic methane oxidation, we used an established assay for

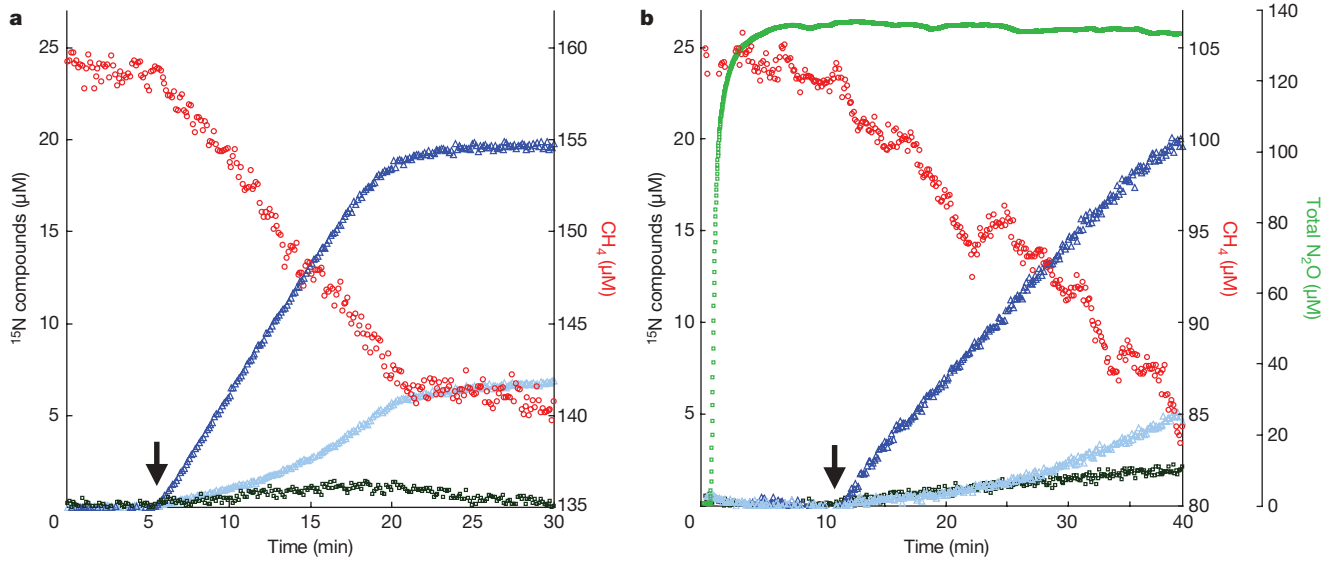

Figure 3 | Coupling of methane oxidation and nitrite reduction in enrichment cultures of '*Methylomirabilis oxyfera*'. Methane is oxidized only after addition of ^{15}N-labelled nitrite (50 μM, arrow), which is converted to ^{15}N-labelled dinitrogen gas in the presence of about 2,000 μM ^{14}N-nitrate (**a**) or 2,000 μM ^{14}N-nitrate and 135 μM ^{14}N-N_2O (**b**). Experiments were performed with 380 ml of anoxic, stirred enrichment culture 'Ooij' (protein content 147 ± 11 mg). Red circles, CH_4; dark blue triangles, $^{15,15}N_2$; light blue triangles, $^{15,14}N_2$; green squares, total N_2O; dark green squares, $^{14,15}N_2O$ and $^{15,15}N_2O$.

pMMO activity, the oxidation of propylene (propene, C_3H_6)[24]. In this assay, pMMO adds one oxygen atom from O_2 to propylene, yielding propylene oxide (propylene epoxide, C_3H_6O). In incubations of enrichment culture 'Ooij' with propylene, formate and oxygen, aerobic pMMO activity occurred at a rate of 0.54 nmol C_3H_6O min^{-1} per mg protein. Next, nitrite was added instead of oxygen. Interestingly, in the presence of nitrite propylene was oxygenated more rapidly (0.94 nmol min^{-1} per mg protein) than in the presence of oxygen. No activity above background levels was detected with N_2O or nitrate. Also in this experiment, the pMMO activity was completely inhibited by 0.15 mM acetylene. To exclude the possibility that contaminating oxygen could explain the observed pMMO activity, we used ^{18}O labelling to trace the oxygen atoms of nitrite into propylene oxide. The direct use of ^{18}O-labelled nitrite proved impossible, because the ^{18}O was quickly exchanged with the unlabelled ^{16}O from water, presumably through the activity of nitrite reductase[25] (Supplementary Fig. 4). To overcome this problem, we generated ^{18}O-labelled nitrite in the incubations themselves by adding ^{18}O-labelled water. In this way, incorporation of the ^{18}O from nitrite into propylene oxide was shown (74–88% originating from nitrite). In line with theoretical expectations, control incubations confirmed that O was not exchanged between water and O_2 or propylene oxide, and that ^{18}O-labelled water did not lead to propylene oxide formation in the absence of nitrite. Control incubations with aerobic methanotrophs were not active with nitrite and did not incorporate ^{18}O from nitrite.

In theory, two mechanisms could explain this result: first, the direct use of NO by pMMO, or second, the production of oxygen by pMMO or a separate enzyme followed by consumption of the produced oxygen by pMMO (Fig. 1c). The divergent position of the '*M. oxyfera*' pMMO (Fig. 2) may argue for the first possibility; however, most residues typical for pMMOs were conserved (Supplementary Fig. 4). The 3:8 methane:nitrite stoichiometry observed renders the second possibility more likely, because apparently not all NO is reduced through pMMO. With the second possibility the remaining oxygen (25%) may be consumed by terminal oxidases.

We addressed these two possibilities experimentally by measuring the production of ^{18}O-labelled oxygen in the incubations with propylene. Indeed, a small amount of labelled oxygen was released in anoxic incubations with propylene, nitrite and ^{18}O-labelled water (0.15 nmol min^{-1} per mg protein; Fig. 4). In control incubations of aerobic methanotrophs in the presence of ^{18}O-labelled water and ^{18}O-labelled nitrite, no labelled oxygen was detectable, ruling out chemical or non-specific reactions with pMMO. In addition, production of oxygen by enzymes for the detoxification of reactive oxygen species, for example catalase or superoxide dismutase, is unlikely, because reactive oxygen species are not known to be produced in the absence of oxygen, even when NO is present[26]. Furthermore, these enzymes are presumably more active in aerobically grown methanotrophs, which did not produce oxygen from nitrite. When methane was used instead of propylene, no oxygen was released by '*M. oxyfera*'. This may be explained by the roughly 2–3-fold higher enzymatic rates

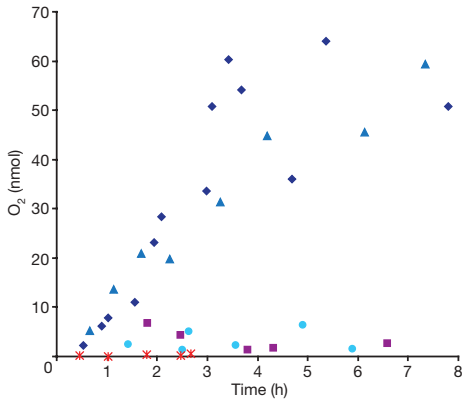

Figure 4 | Oxygen production from nitrite in '*Methylomirabilis oxyfera*'. Whole cells of enrichment culture 'Ooij' were incubated in buffer containing nitrite and 25% ^{18}O-labelled water, leading to 90% O exchange within 30 min. Total oxygen production from this indirectly labelled $N^{18}O_2^-$ was inferred from the measured concentration of $^{16,18}O_2$ and $^{18,18}O_2$ in the helium headspace with the following additions: propylene (dark blue diamonds), propylene and acetylene (blue triangles), methane (purple squares) and oxygen (light blue circles). Anaerobic control incubations of *Methylosinus acidophilus* (red asterisks) with ^{18}O-labelled nitrite did not produce measurable amounts of oxygen. Cells were concentrated to obtain similar maximum rates of propylene oxidation activity; 1.15 nmol min^{-1} (with NO_2^-, 1.22 mg of protein) for '*M. oxyfera*', and 1.68 nmol min^{-1} (with O_2, 0.046 mg of protein) for *M. acidophilus*.

with methane (Fig. 3 and ref. 4) than with propylene; the lower activity of pMMO with propylene allowed some oxygen to escape.

Conclusions and perspectives

We have described the discovery of a new 'intra-aerobic' pathway of nitrite reduction. This pathway resembles the original proposal for the mechanism of denitrification. After its discovery in the nineteenth century, it was generally assumed that denitrification proceeded by the production of oxygen from nitrate, which could subsequently be used for respiration (reviewed in ref. 27). It therefore seems that an old hypothesis may have been brushed aside too easily.

The new pathway of 'intra-aerobic denitrification' is not necessarily restricted to methane-oxidizing bacteria: with all currently available assays for denitrification the process would either be overlooked (for example with the acetylene-block technique) or lumped together with conventional denitrification (for example with isotope pairing). Under dynamic oxic/anoxic conditions and with recalcitrant substrates (aromatic compounds, alkanes and alkenes) the process may certainly offer ecological advantages.

The production of oxygen from nitrogen oxides is also of interest for ordering the evolution of metabolic pathways on early Earth, which are mostly believed to have proceeded from reduced (for example fermentation) to oxidized (for example aerobic respiration) pathways[28,29]. In this model, the pathway presented here may have evolved to exploit newly formed pools of nitrogen oxides after the oxygenation of the atmosphere. Alternatively, on the basis of enzyme phylogenies, respiration has been discussed as a primordial pathway[30,31] that originally depended on nitrogen oxides that were most probably present on early Earth[31], although whether they were quantitatively important is under debate[28,32]. Our study adds a new aspect to this debate, because it is tempting to speculate that intra-aerobic denitrification may have preceded oxygen production by photosynthesis, or extended the niches for the evolution of aerobic pathways in a still predominantly anaerobic environment before the Great Oxidation Event about 2.45 Gyr ago[33]. The intra-aerobic pathway presented here would have enabled microorganisms to thrive on the abundant methane in the Archaean atmosphere[34] without direct dependence on oxygenic photosynthesis, causing ^{13}C-depleted sedimentary carbon that has so far been attributed to aerobic methanotrophs[35].

'*Candidatus* Methylomirabilis oxyfera'

Etymology. *methyl* (modern Latin): the methyl group; *mirabilis* (Latin): astonishing, strange; *oxygenium* (Latin): oxygen; *fera* (Latin): carrying, producing. The name alludes to the substrate methane, which is oxidized by a surprising combination of pathways, involving oxygen as an intermediate.

Locality. Enriched from freshwater sediments of the Twentekanaal and Ooijpolder ditches, The Netherlands.

Properties. Methane-oxidizing and nitrite-reducing bacterium of the candidate division NC10. Grows anaerobically, but produces oxygen for the aerobic oxidation of methane. Reduces nitrite to dinitrogen gas without a nitrous oxide reductase. Gram-negative rod with a diameter of 0.25–0.5 μm and a length of 0.8–1.1 μm. Mesophilic with regard to temperature and pH (enriched at 25–30 °C and pH 7–8). Slow growth (doubling time 1–2 weeks).

METHODS SUMMARY

Molecular methods. DNA from both enrichment cultures was isolated as described[36], with modifications, and used as a template for pyrosequencing[37], construction of a 10-kb insert plasmid library (enrichment culture 'Twente') and single-end Illumina sequencing (both cultures). Coding sequences of the closed genome were predicted, and automatic functional annotation was performed with MaGe[38]. Selected annotations (Supplementary Tables 4 and 5) were confirmed manually.

Short reads resulting from Illumina sequencing of enrichment culture 'Ooij' were mapped onto the completed genome of '*M. oxyfera*' (culture 'Twente') with an algorithm based on iterated Blast searches and validated by comparison with proteomics results (ref. 14 and Supplementary Table 3).

Protein extracts from both enrichment cultures were separated by SDS–PAGE, trypsin-digested and analysed by LC–MS/MS[39]. Mass spectrometric data files were searched against a database of predicted '*M. oxyfera*' protein sequences.

Total RNA from enrichment culture 'Twente' was reverse-transcribed and sequenced by the single-end Illumina technique.

Activity experiments. All incubations were performed at 30 °C and pH 7.3 with cell suspensions from culture 'Ooij'[4]. For measurement of nitrogenous intermediates, 380 ml containing 2 mM NO_3^- and 100 μM CH_4 was incubated anaerobically while measuring the concentration and isotopic composition of CH_4, O_2 and nitrogenous gases with microsensors[40–42] and membrane-inlet mass spectrometry. N_2O, $^{15}NO_2^-$ and ^{15}NO were added as specified. Propylene oxidation and oxygen production assays were performed with 0.5 ml of tenfold concentrated cells. NO_2^-, formate and gases were added in combinations specified in Results. ^{18}O-labelled NO_2^- was generated in '*M. oxyfera*' incubations by O-equilibration with 25% ^{18}O-labelled water in the medium[25]. For *Methylosinus acidophilus* (DQ076754) controls, ^{18}O-labelled NO_2^- was used in addition to ^{18}O-labelled water. Propylene oxide in headspace samples was quantified by gas chromatography[11], and formation of $^{18}O_2$ and incorporation of ^{18}O into propylene oxide was determined by coupled gas chromatography–mass spectrometry as described[4], with modifications.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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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 Contributions Genome sequencing and assembly from enrichment culture 'Twente' was performed by D.L.P., E.P., S.M. and J.W. M.S., E.M.J.-M., K.-J.F. and H.S. performed the sequencing and initial assembly of sequence from enrichment culture 'Ooij'. Mapping of sequences from enrichment culture 'Ooij' to 'Twente' was performed by B.E.D. and M.S. B.E.D. and E.P. performed SNP and coverage analyses. Genome annotation and phylogenetic analysis were conducted by M.K.B. H.J.M.O.d.C. provided support with alignments. Sample preparation for proteome analysis was performed by M.K.B. and M.W., with LC–MS/MS and protein identification performed by J.G. and H.J.C.T.W. Material for transcriptome analysis was prepared by T.v.A. and F.L., with sequencing performed by E.M.J.-M., K.-J.F. and H.S. Continuous cultures were set up and maintained by K.F.E. and K.T.v.d.P.-S. Experiments for nitrogenous intermediates were designed and performed by K.F.E., M.M.M.K., F.S., D.d.B. and J.Z., and those for methane activation were designed and performed by K.F.E. Pilot experiments were conducted by K.F.E., F.L., M.K.B., K.T.v.d.P.-S, T.A. and M.S. K.F.E., M.K.B., M.S.M.J. and M.S. conceived the research. K.F.E., M.K.B. and M.S. wrote the paper with input from all other authors.

Author Information Sequencing and proteomic data are deposited at the National Centre for Biotechnology Information under accession numbers FP565575, SRR023516.1, SRR022749.2, GSE18535, SRR022748.2, PSE127 and PSE128. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to K.F.E. (k.ettwig@science.ru.nl) or M.S. (mstrous@mpi-bremen.de).

METHODS

DNA preparation. DNA from both enrichment cultures was isolated as described[36], with modifications. After incubation of biomass in DNA extraction buffer for 2 h, 0.7 vol. of phenol/chloroform/3-methylbutan-1-ol (25:24:1, by volume) was added and the mixture was incubated for 20 min at 65 °C. The aqueous phase was recovered by centrifugation and another treatment with phenol/chloroform/3-methylbutan-1-ol was performed. The aqueous phase was then mixed with an equal volume of chloroform/3-methylbutan-1-ol (24:1, v/v) and the DNA was precipitated and cleaned as described[36].

Sequencing methods. We performed 454 pyrosequencing on total genomic DNA from enrichment culture 'Twente' with the Roche Genome Sequencer FLX system by Keygene N.V.[37]. Sequencing of the single-end Illumina samples was performed with total genomic DNA from both enrichment cultures, as described. The samples were prepared in accordance with the manufacturer's protocol (Illumina). Subsequent sequencing was conducted on a genome Analyser II (Illumina). A 10-kb insert plasmid library of total genomic DNA from enrichment culture 'Twente' was constructed; clones were picked and bi-directionally sequenced by using standard protocols. Sequence data resulting from the plasmid library and pyrosequencing were combined, and a preliminary global assembly, performed with both Phrap[43] and Newbler (454 Life Sciences) assembly softwares, resulted in five scaffolds containing seven contigs. Gaps were closed and the genome was made circular with a combination of several methods, such as sequencing of a transposon-shotgun library of plasmids overlapping the assembly gaps (with 3,229 validated sequences obtained), and PCR amplification and subsequent sequencing of regions between scaffold ends (297 validated sequences). Identified in the genome were 47 transfer RNAs representing all amino acids, 3 small RNAs and 1 ribosomal RNA operon, as well as all of the 63 conserved clusters of orthologous groups, confirming the completeness of the genome. Gene prediction was performed with AMIGene software. Coding sequences were predicted (and assigned a unique identifier prefixed with 'DAMO'), and automatic functional annotation was performed as described previously with the MaGe system[38]. Annotations for selected genes (Supplementary Tables 4 and 5) were confirmed manually.

Phylogenetic analysis of PmoA and AmoA protein sequences. An alignment of selected bacterial and archaeal PmoA and AmoA amino-acid sequences was generated with MEGA version 4 (http://www.megasoftware.net), and conservation of important residues was judged by comparison with refs 44, 45. There were a total of 178 amino-acid positions in the final data set. A distance tree was generated in MEGA, using the neighbour-joining method. Bootstrap values of 100 replicates were generated for neighbour-joining, minimum evolution and maximum parsimony, with the Dayhoff matrix-based method. PhyML 3.0 (www.atgc-montpellier.fr/phyml/) was used to generate maximum-likelihood bootstrap values.

Proteomics. A cell-free extract from the biomass of enrichment culture 'Twente' was prepared by concentrating a sample of the biomass in 20 mM phosphate buffer and bead-beating for 2 min. The supernatant was then boiled for 10 min in sample buffer. For a cell-free extract from enrichment culture 'Ooij', a sample of the biomass was concentrated in 20 mM phosphate buffer containing 1 mM phenylmethylsulphonyl fluoride, protease inhibitor cocktail and 1% SDS, and the sample was boiled for 7 min. Both cell-free extracts were then loaded on an SDS–PAGE gel, prepared with standard methods, with about 50 μg protein per lane. After separation of proteins and staining with colloidal Coomassie blue, the gel lane was cut into four slices, each of which was destained by three cycles of washing with, successively, 50 mM ammonium bicarbonate and 50% acetonitrile. Protein reduction, alkylation and digestion with trypsin were performed as described[39]. After digestion, the samples were desalted and purified as described[46]. Sample analysis by LC–MS/MS was performed with an Agilent nanoflow 1100 liquid chromatograph coupled online through a nano-electrospray ion source (Thermo Fisher Scientific) to a 7-T linear ion-trap Fourier transform ion cyclotron resonance mass spectrometer (Thermo Fisher Scientific). The chromatographic column consisted of a 15-cm fused silica emitter (PicoTip Emitter, tip 8 ± 1 μm, internal diameter 100 μm; New Objective) packed with 3-μm C_{18} beads (Reprosil-Pur C_{18} AQ; Dr Maisch GmbH)[47]. After the peptides had been loaded on the column in buffer A (0.5% acetic acid), bound peptides were gradually eluted with a 67-min gradient of buffer B (80% acetonitrile, 0.5% acetic acid). First, the concentration of acetonitrile was increased from 2.4% to 8% in 5 min, followed by an increase from 8% to 24% in 55 min, and finally an increase from 24% to 40% in 7 min. The mass spectrometer was operated in positive-ion mode and was programmed to analyse the top four most abundant ions from each precursor scan by using dynamic exclusion. Survey mass spectra (m/z 350–2,000) were recorded in the ion cyclotron resonance cell at a resolution of $R = 50{,}000$. Data-dependent collision-induced fragmentation of the precursor ions was performed in the linear ion trap (normalized collision energy 27%, activation $q = 0.250$, activation time 30 ms). Mass spectrometric datafiles were searched against a database containing the predicted protein sequences from the '*M. oxyfera*' genome and known contaminants, such as human keratins and trypsin. Database searches were performed with the database search program Mascot version 2.2 (Matrix Science Inc.). To obtain factors for the recalibration of precursor masses, initial searches were performed with a precursor-ion tolerance of 50 p.p.m. Fragment ions were searched with a 0.8-Da tolerance and searches were allowed for one missed cleavage, carbamidomethylation (C) as fixed modification, and deamidation (NQ) and oxidation (M) as variable modifications. The results from these searches were used to calculate the m/z-dependent deviation, which was used to recalibrate all precursor m/z values. After recalibration of the precursor masses, definitive Mascot searches were performed with the same settings as above, but with a precursor-ion tolerance of 15 p.p.m. In addition, reverse database searches were performed with the same settings. Protein identifications were validated and clustered with the PROVALT algorithm to achieve a false-discovery rate of less than 1% (ref. 48).

Transcriptomics. Total cell RNA was extracted from enrichment culture 'Twente' biomass with the RiboPure-Bacteria Kit (Ambion) in accordance with the manufacturer's instructions. An additional DNase I treatment was performed (provided within the RiboPure-Bacteria Kit). RNA quality was checked by agarose-gel electrophoresis, and the RNA concentration was measured with a Nanodrop ND-1000 spectrophotometer (Isogen Life Science). First-strand cDNA was generated with RevertAid H Minus M-MuLV Reverse Transcriptase (Fermentas Life Sciences), in accordance with the manufacturer's protocol. Second-strand cDNA synthesis was performed with RNase H (Fermentas Life Sciences) and DNA polymerase I (Fermentas Life Sciences) in accordance with the manufacturer's protocol. Purification of the double-stranded DNA for sequencing was performed with the Qiaquick PCR Purification Kit (Qiagen) in accordance with the manufacturer's instructions, and single-end Illumina sequencing was performed as described above.

Short-read sequence mapping. Reads resulting from the Illumina sequencing of enrichment culture 'Ooij' were mapped onto the complete genome of '*M. oxyfera*' (enrichment culture 'Twente') with an algorithm based on iterated Blast searches[14]. In brief, the 32-nt Illumina reads were mapped to the 'Twente' reference genome by composing a majority-vote consensus. This initial consensus assembly was then taken to remap all short reads iteratively, improving the assembly coverage and bringing the genome closer to the consensus of the sequenced population 'Ooij'. Three different programs were used to map the short reads to the reference genome (Maq, Blast and MegaBlast), and with each program at least 12 iterations were constructed. For Blast and MegaBlast several different word lengths were also tried, ultimately yielding a total of 87 potential consensus genomes for the 'Ooij' culture Illumina reads (see Supplementary Table in ref. 14). To test empirically which of these assemblies best described the '*M. oxyfera*' 'Ooij' population, the ORF coordinates from the complete 'Twente' genome were mapped to each of these assemblies (the reads were mapped without gaps, so the genomic coordinates are identical) and all ORFs were translated into protein. Subsequently, the peptides obtained by LC–MS/MS (see above) were mapped to these translated ORFs with Mascot (see above). It was found that the Blast-based assembly with a word length of 8 could explain the largest number of peptides (Supplementary Table 3). As the number of peptides reached a plateau after seven iterations, we chose iteration 7 as the optimal sequence for the '*M. oxyfera*' 'Ooij' consensus genome. Positions at which SNPs occurred were identified by mapping the Illumina reads of the two enrichment cultures to their respective genomes with Maq (default settings). Sites with a polymorphic consensus were designated as SNP sites.

Activity measurements. All activity experiments were performed at 30 °C and pH 7.3 (10 mM MOPS) with whole cells from enrichment culture 'Ooij'. For measurement of nitrogenous intermediates (Fig. 3), 380 ml of enrichment culture containing 147 ± 11 mg of protein, about 2 mM NO_3^- (to maintain the redox potential) and 100 μM CH_4 were incubated in a modified Schott glass bottle without headspace and stirred with a glass-coated magnet (Supplementary Fig. 7). Microsensors for O_2 (ref. 41), N_2O (ref. 40) and NO (ref. 42), prepared and calibrated as described previously, were inserted into the culture through Teflon-coated rubber. The concentration and isotopic composition of methane and nitrogenous gases were measured in liquid withdrawn through a sintered glass filter by membrane-inlet mass spectrometry, with a quadrupole mass spectrometer (GAM 200; IP Instruments). To compensate for the liquid loss, the setup was coupled to a helium-flushed medium reservoir. N_2O was added in gas-saturated anaerobic medium, and ^{15}N-labelled NO_2^- as anaerobic stock solution. The ^{15}NO stock solution was prepared by adding H_2SO_4 to a solution containing ^{15}N-labelled NO_2^- and KI, and capturing the evolved NO gas in anaerobic water[42].

Propylene oxidation and oxygen production assays (Fig. 4) were performed (at least in duplicate) with 0.5 ml of tenfold concentrated whole cells from enrichment culture 'Ooij' in 3-ml Exetainers (Labco). Biomass was centrifuged anaerobically, washed once and resuspended in anaerobic, MOPS-buffered

(pH 7.3, 10 mM) medium[4] without NO_2^- and NO_3^-. Control incubations were performed with the aerobic, methanotrophic α-Proteobacterium *Methylosinus acidophilus* in NO_3^--free M2 medium[49]. Samples were preincubated anaerobically for at least 4 h with a helium headspace containing 6% CH_4 to deplete a cellular store of electron acceptors. Pilot experiments had shown that rates of propylene oxidation with and without added electron acceptors were otherwise identical for up to 5 h. After removal of the CH_4 from the headspace by six cycles of vacuum and helium (0.5 bar) supply, a combination specified in the Results section of the following salts (as anaerobic stock solutions) and gases (purity 99% or greater) were added: ^{15}N-labelled NO_2^- (3 mM final concentration), O_2 (6.5% in headspace, resulting in about 84 μM in solution), formate (5 mM final concentration), propylene (16% in headspace), acetylene (0.4% in headspace, resulting in 150 μM). $N^{18}O_2^-$ was not added directly to '*M. oxyfera*' incubations because the ^{18}O in it was quickly equilibrated with O in water by the activity of nitrite reductase[25], leading to 96% equilibration with O in NO_2^- within 30 min (Supplementary Fig. 6). Instead, unlabelled NO_2^- was used in combination with medium containing 25% ^{18}O-labelled water (more than 97% ^{18}O; Cambridge Isotope Laboratories). For control incubations of *M. acidophilus*, ^{18}O-labelled NO_2^- (produced as described[50] and checked by mass spectrometry after conversion to N_2O as described[51]) was used in addition to ^{18}O-labelled water, because no nitrite reductase activity can be expected. Samples were horizontally incubated at 30 °C on a shaker (250 r.p.m.). Headspace samples were taken every 30–60 min with a gas-tight glass syringe. Propylene oxide and CH_4 were quantified by gas chromatography as described previously[11], with increased temperatures of oven (150 °C), injection port and detector (180 °C). Oxidation rates were calculated from the linear part of the graphs ($R^2 \geq 0.9$), using at least three measuring points. Samples not exceeding the rate of controls without added electron acceptor (see above) were considered negative.

Formation of $^{18}O_2$ was measured by coupled GC–MS as described previously[4], detecting the masses 32–36 Da. Calibration for small amounts of oxygen was performed with known amounts of $^{18,18}O_2$ in helium and the $^{16,18}O_2$ and $^{18,18}O_2$ content of air, taking into account the average isotopic composition of atmospheric O_2. The lower limit for the accurate quantification of $^{18,18}O_2$ was 0.1 nmol ml^{-1}. Air contamination was minimized by flushing the inlet area of the gas chromatograph with helium, and the measured values of $^{16,18}O_2$ and $^{18,18}O_2$ were corrected for their abundance in contaminating air, assessed by the amount of $^{16,16}O_2$ and $^{14,14}N_2$. Incorporation of ^{18}O into propylene oxide was measured with a modification of the above method at a higher column temperature (150 °C) and detecting the masses 58–60 Da.

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