Rare Branched Fatty Acids Characterize the Lipid Composition of the Intra-Aerobic Methane Oxidizer "*Candidatus* Methylomirabilis oxyfera"

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The recently described bacterium "*Candidatus* Methylomirabilis oxyfera" couples the oxidation of the important greenhouse gas methane to the reduction of nitrite. The ecological significance of "*Ca.* Methylomirabilis oxyfera" is still underexplored, as our ability to identify the presence of this bacterium is thus far limited to DNA-based techniques. Here, we investigated the lipid composition of "*Ca.* Methylomirabilis oxyfera" to identify new, gene-independent biomarkers for the environmental detection of this bacterium. Multiple "*Ca.* Methylomirabilis oxyfera" enrichment cultures were investigated. In all cultures, the lipid profile was dominated up to 46% by the fatty acid (FA) 10-methylhexadecanoic acid ($10MeC_{16:0}$). Furthermore, a unique FA was identified that has not been reported elsewhere: the monounsaturated 10-methylhexadecenoic acid with a double bond at the $\Delta 7$ position ($10MeC_{16:1\Delta 7}$), which comprised up to 10% of the total FA profile. We propose that the typical branched fatty acids $10MeC_{16:0}$ and $10MeC_{16:1\Delta 7}$ are key and characteristic components of the lipid profile of "*Ca.* Methylomirabilis oxyfera." The successful detection of these fatty acids in a peatland from which one of the enrichment cultures originated supports the potential of these unique lipids as biomarkers for the process of nitrite-dependent methane oxidation in the environment.

Methane (CH_4) represents globally the second most important greenhouse gas. Methane contributes approximately 20% to the total greenhouse gas budget, with a global warming potential which is about 25 times as strong as that of CO_2 (29). Understanding the sources and sinks of CH_4 is paramount to enabling the development of adequate management strategies that aim to mitigate greenhouse gas emissions and global warming. Methane is one of the least reactive organic molecules, and it was long assumed that methane could only be oxidized aerobically, i.e., with the use of oxygen. However, in recent decades ample evidence has been collected for the occurrence of methane oxidation in anoxic environments. In marine systems, distributions of CH_4 and sulfate in the water column and in sediments first led to the awareness of significant CH_4 consumption in anoxic zones (38, 44). This resulted in the identification of anaerobic oxidation of methane (AOM) coupled to sulfate reduction, and since its first documentation, consortia of archaea and sulfate-reducing bacteria responsible for this process have been studied at many locations (3, 25, 27, 39, 40).

Besides oxygen and sulfate, nitrate or nitrite could theoretically also serve as a suitable, energetically favorable electron acceptor for the oxidation of CH_4. The actual occurrence of AOM coupled to the reduction of nitrate and/or nitrite, however, remained elusive for a long time and was only discovered in 2006 (42). At that time, the methane-oxidizing enrichment culture still comprised both bacteria and archaea, which supported the hypothesis that, similar to what had been found for sulfate-dependent methane oxidation, a bacterial-archaeal consortium accounted for the nitrite-dependent methane-oxidizing activity. Only later did the archaea disappear from the culture (20), possibly influenced by higher nitrite loads (28). Subsequent metagenomic analysis resulted in the complete genome assembly of the bacterium responsible for the nitrite-dependent methane oxidation (19). Despite its anaerobic lifestyle, this microbe is thought to employ an intra-aerobic pathway for methane oxidation by producing its own oxygen from the dismutation of nitric oxide, for which reason it was named "*Candidatus* Methylomirabilis oxyfera" (19).

Human activities not only have led to increased atmospheric methane concentrations but also continue to have a major impact on the global nitrogen cycle. Industrial and agricultural intensification have led to a substantial increase in nitrogen loadings in present-day freshwater and coastal marine environments, leading to environmental problems like eutrophication. Nitrite-dependent methane oxidation thus could comprise an important link between the carbon and nitrogen cycle in various ecosystems, improving the total greenhouse gas balance while alleviating disturbed nitrogen budgets. However, the environmental contribution of "*Ca.* Methylomirabilis oxyfera" remains relatively unexplored. So far, our ability to rapidly identify "*Ca.* Methylomirabilis oxyfera" in the environment relies on molecular techniques (13, 21, 37). Specific primers targeting 16S rRNA and functional genes (i.e., methane mono-oxygenase, *pmoA*) have been developed for the environmental detection of "*Ca.* Methylomirabilis oxyfera," but these may capture only a selection of organisms potentially contributing to nitrite-dependent methane oxidation (13, 21, 37).

In addition to genomic approaches, lipid analyses are regularly used to study microbial processes and communities, including the methane cycle. Especially in combination with stable carbon isotope signatures, lipid biomarkers have been successfully used to

demonstrate methanotrophy (coupled to oxygen or sulfate reduction) in various ecosystems (2, 8, 9, 18, 22, 25, 30, 40, 43, 46, 47, 50, 52, 54).

The earliest enrichment culture that was shown to be capable of nitrite-dependent methane oxidation was also screened for its lipid composition (42). However, at that time only a single enrichment culture was available, in which the microbial community still comprised substantial levels of archaea (10 to 15%), which was also confirmed by the presence of archaeol. At present, multiple cultures have been enriched from different environments, the genome of the responsible bacterium has been assembled, archaea members are virtually absent, and no other species comprise a significant part of the community besides "*Ca.* Methylomirabilis oxyfera" (35). Therefore, here we have investigated the potential for new biomarkers for "*Ca.* Methylomirabilis oxyfera" based on its lipid composition. From multiple enrichment cultures of "*Ca.* Methylomirabilis oxyfera" originating from different ecosystems, we determined the typical lipid profile and identified characteristic compounds that may serve as biomarkers for the detection of "*Ca.* Methylomirabilis oxyfera"-like bacteria in the environment. In addition, we investigated environmental samples from a peatland from which one of the "*Ca.* Methylomirabilis oxyfera" enrichment cultures had originally been obtained to test the validity of the potential new lipid biomarkers.

MATERIALS AND METHODS

Sample description. Biomass was obtained from multiple enrichment cultures of "*Ca.* Methylomirabilis oxyfera" and a related, unnamed "*Ca.* Methylomirabilis" species at Radboud University Nijmegen that originated from different environments. The first enrichment culture capable of nitrite-dependent methane oxidation was enriched from the freshwater canal Twentekanaal in the Netherlands (TWK) (42). Since then, several additional cultures have been enriched from different Dutch ecosystems which are examined here: three enrichment cultures originating from sediment from a freshwater ditch in Ooijpolder (Ooij1, Ooij2, and Ooij3) (19, 21); one culture enriched from sludge from a wastewater treatment plant (WWTP) (36); and one, the most recent, culture enriched from the peatland Brunssummerheide (BRH), with a dominant strain different from "*Ca.* Methylomirabilis oxyfera" but within the genus "*Ca.* Methylomirabilis" (59). The enrichment cultures were grown and kept under constant temperature conditions, i.e., 25, 30, 20 to 23, and 25°C for TWK, Ooij (1, 2, and 3), WWTP, and BRH, respectively.

All cultures actively oxidized CH_4 anaerobically with nitrite at the moment of sampling. The most recent enrichment culture, BRH, was sampled over the time of its development (along with increasing CH_4 oxidizing activity) at 11, 14, and 17 months after inoculation. The other cultures had reached and maintained a more or less constant level of activity and enrichment already prior to the first sampling, and they were sampled in two or three replicates over time coinciding with the sampling moments of BRH. Molecular analyses with specific primers targeting 16S rRNA and *pmoA* genes of NC10 phylum bacteria (21, 37) had confirmed the presence of "*Ca.* Methylomirabilis" in all enrichment cultures studied here. The degree of enrichment of the cultures was checked by fluorescence *in situ* hybridization (FISH) analyses with specific probes (42).

In addition to the enrichment culture biomass, samples were taken from the peatland where the inoculum of the most recently enriched culture originated from BRH. A soil profile was sampled from 51- to 102-cm depth, divided into nine sections of 5 to 9 cm (i.e., 51 to 60 cm, 5-cm increments from 60 to 95 cm, and 95 to 102 cm). In this section of the soil profile, a countergradient of methane and nitrate and a peak of abundance of "*Ca.* Methylomirabilis oxyfera"-like bacteria (as measured by quantitative PCR [qPCR]) were observed (59).
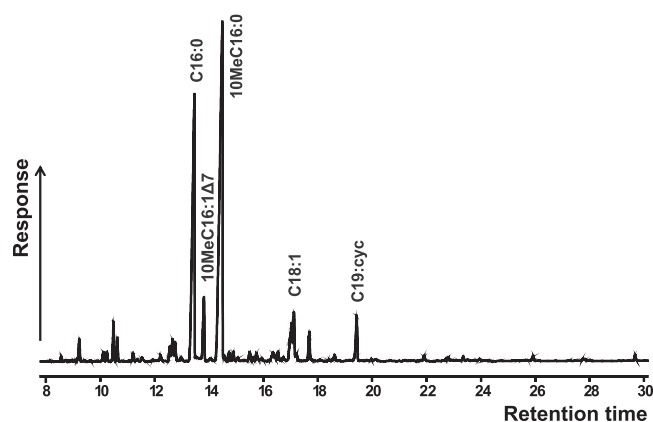

FIG 1 Gas chromatogram of the apolar lipid fraction of the enrichment culture of the nitrite-dependent methane oxidizer "*Ca.* Methylomirabilis oxyfera" Ooij1. Major fatty acids are indicated; cyc denotes a cyclopropyl moiety.

Lipid analyses. Typically, 50 to 100 ml of the enrichment cultures was harvested and centrifuged, after which the obtained pellets were freeze-dried. For the environmental samples, approximately 5 g of field moist soil per section of the obtained soil core was taken and freeze-dried. The freeze-dried samples were stored dry and frozen ($-20°C$) until further use. Subsamples of the freeze-dried material was saponified with 1N potassium hydroxide (KOH) in methanol (MeOH; 96%) to analyze free and ester-bound lipids. The obtained extracts were methylated with boron trifluoride (BF_3) in MeOH and subsequently separated by column chromatography over activated alumina (Al_2O_3) into apolar and polar fractions with dichloromethane (DCM) and DCM-MeOH (1:1, vol/vol) as the eluent, respectively. The polar fractions were subsequently silylated using bis(trimethylsilyl)trifluoroacetamide (BSTFA) in pyridine at 60°C for 20 min. An aliquot of the apolar fractions was separated into a saturated and an unsaturated fraction by column chromatography over Ag^+-impregnated silica with DCM and ethyl acetate (EtOAc) as the eluent, respectively. Subsequently, an aliquot of the unsaturated fraction (in EtOAc) was hydrogenated using platinum oxide (PtO_2) with a droplet of acetic acid, flushed with hydrogen for 2 h, and stirred overnight. The remainder of the unsaturated apolar fractions was dissolved in hexane and derivatized with dimethyl-disulfide (DMDS) (activated with iodine in diethyl ether at 40°C overnight) to determine the position of the double bond(s) (5, 53).

The total apolar and polar fractions were analyzed by gas chromatography (GC) and subsequently by gas chromatography-mass spectrometry (GC-MS). The fractions after separation of the saturated and unsaturated fraction and after hydrogenation and DMDS adduction were also analyzed by GC and GC-MS. Relative abundance of the fatty acid methyl esters was derived from the (integrated) GC profile of the apolar fraction.

RESULTS

Enrichment cultures. Figure 1 shows a representative gas chromatogram of the apolar fraction of "*Ca.* Methylomirabilis oxyfera" culture Ooij1. The apolar fraction comprised the majority of the total lipids, as analyses of the polar fraction did not reveal any other major lipids (data not shown). The lipids were identified by GC-MS analyses and comparison to established spectra of known compounds. The lipid profile was dominated by relatively short-chain fatty acids (FA) (C_{14} to C_{19}). The most abundant FA was 10-methyl-hexadecanoic acid ($10MeC_{16:0}$), followed by hexadecanoic acid ($C_{16:0}$). In addition, GC-MS analyses revealed the presence of a previously unknown component, which eluted at a retention time between those of $C_{16:0}$ and $10MeC_{16:0}$.
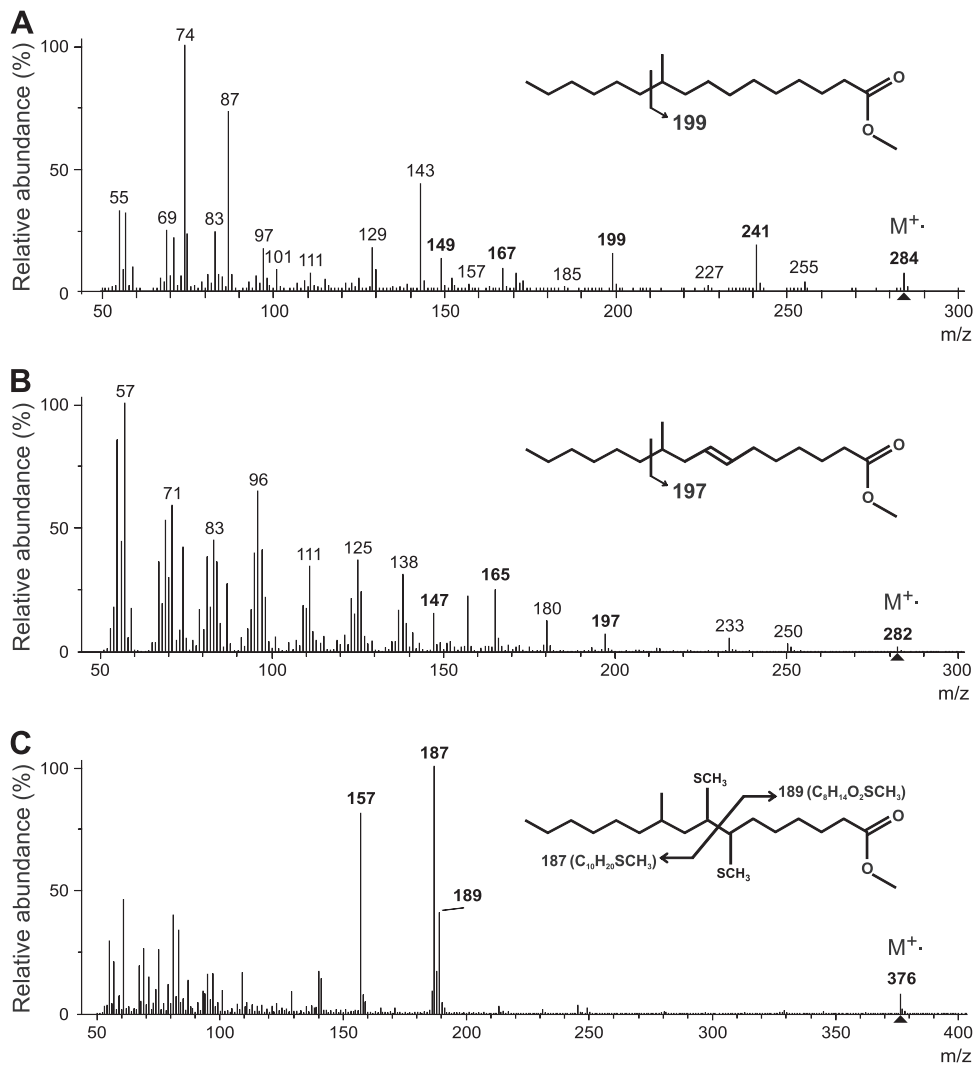
FIG 2 Mass spectra of the fatty acids (FAs) 10MeC$_{16:0}$ (A) and 10MeC$_{16:1\Delta7}$ (B) (analyzed as methyl ester derivatives), two key components of the lipid profile of enrichment cultures of *Methylomirabilis oxyfera*. The 10MeC$_{16:1\Delta7}$ FA thus far has been reported only in these enrichment cultures. (C) The position of the double bond in the 10MeC$_{16:1\Delta7}$ FA was deduced from the mass spectrum of the FA methyl ester after DMDS adduction.

Figure 2 presents the mass spectra of the most abundant FA of the lipid profile, identified as a 10MeC$_{16:0}$ (48) and the unknown compound observed in "*Ca.* Methylomirabilis oxyfera" enrichment lipid extracts. The spectrum of the unknown compound (Fig. 2B) showed a molecular ion at *m/z* 282, suggesting a monounsaturated C$_{17}$ fatty acid. This compound was indeed recovered in the unsaturated fraction, and after hydrogenation it was converted to 10MeC$_{16:0}$ FA, as confirmed by GC-MS. The mass spectrum of the unknown component is consistent with monounsaturated 10MeC$_{16}$ FA: diagnostic fragment ions were observed from the preferential cleavage between the 10th and 11th C atom of the acyl chain at *m/z* 197, 165, and 147, all shifted by two Daltons relative to the equivalent fragment ions in the mass spectrum of its saturated counterpart, 10MeC$_{16:0}$ FA (showing diagnostic fragment ions at *m/z* 199, 167, and 149, respectively). This identified the position of the double bond between the carboxyl carbon (C$_1$) and the methyl group (at C$_{10}$). GC-MS analyses after dimethyldisulfide (DMDS) adduction resulted in diagnostic fragments of *m/z* 187, 189, and 157, representing ωC$_{10}$, ΔC$_7$, and ΔC$_7$-32 frag-

ments, respectively (Fig. 2C), thus locating the double bond at the $\Delta7$ position. Consequently, this unknown FA was identified as 10-methyl-hexadec-7-enoic acid, or 10MeC$_{16:1\Delta7}$.

The lipid profile of "*Ca.* Methylomirabilis oxyfera" enrichment cultures Ooij2, Ooij3, and WWTP was similar overall to the distribution described for Ooij1. Based on FISH analyses of the total microbial community, these enrichment cultures were found to be dominated by "*Ca.* Methylomirabilis oxyfera" by approximately 70 to 80% without any other single species making up a significant amount of the bacterial community (35). The relative abundances of the major FAs in the different enrichment cultures are given in Table 1. The most abundant FAs, 10MeC$_{16:0}$ and C$_{16:0}$, comprised approximately 35 to 46% and 18 to 26% of the total fatty acids, respectively. The unique FA 10MeC$_{16:1\Delta7}$ made up 4 to 10% of the total FA profile. Repeated sampling over time of a single enrichment culture showed only minor changes in the FA profile.

In contrast to these stable enrichments, distinct changes in the profile over time were observed for the BRH culture that was
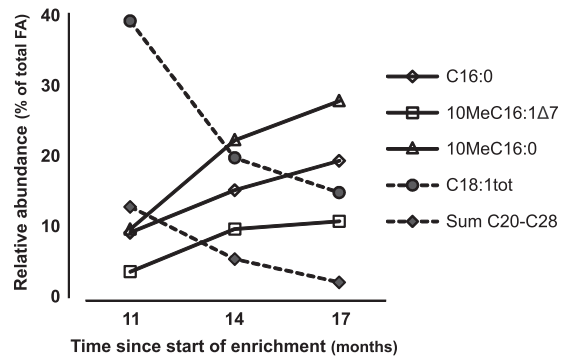
FIG 3 Changes over time in the relative contribution of key FAs of *Methylomirabilis* enrichment culture BRH. During the depicted period since the start of enrichment, the degree of enrichment and activity of methane oxidation coupled to nitrite reduction in the culture increased.

Figure 3 axes: y-axis, Relative abundance (% of total FA) (0–40); x-axis, Time since start of enrichment (months) (11, 14, 17). Legend: $C_{16:0}$; $10\text{MeC}_{16:1\Delta7}$; $10\text{MeC}_{16:0}$; $C_{18:1tot}$; Sum C_{20}–C_{28}.

TABLE 1 Approximate degree of enrichment in "Ca. Methylomirabilis oxyfera" and relative abundance of fatty acids in the lipid profile of the "Ca. Methylomirabilis oxyfera" enrichment cultures[f]

Enrichment culture	% enrichment in "Ca. Methylomirabilis oxyfera"[a]	$C_{14:0}$	$10\text{MeC}_{14:0}$	$isoC_{15}$	aiC_{15}	$isoC_{16}$	$C_{16:1tot}$	$C_{16:0}$	$10\text{MeC}_{16:1\Delta7}$	$10\text{MeC}_{16:0}$	$C_{18:1tot}$	$C_{18:0}$	C_{19cyc}	$C_{20:0}$	$C_{22:0}$	$C_{24:0}$	$C_{26:0}$	$C_{28:0}$	Sum	Branched[e]
		Relative abundance (%) of:																		
Ooij1[b]	70–80	0.8	0.4	1.8	1.0	1.1	2.5	26.2	45.9	6.2	1.9	5.2							97.2	56.0
Ooij2[b]	70–80	0.6	0.8	2.3	1.0	3.9	2.0	19.0	45.6	6.7	2.3	5.8							96.6	62.3
Ooij3[b]	70–80	0.5	0.7	1.3	2.4	2.6	3.2	17.8	34.9	4.8	3.0	18.0							96.6	50.9
WWTP[b]	70–80	1.5	0.9	2.3	1.5	4.3	5.8	21.1	37.8	8.6	1.4	2.3							97.6	58.3
TWK[c]		0.8		2.3			6.8	11.4	28.9	14.3	2.8	12.7							85.5	42.9
BRH 11 m[d]	15–25			2.3	0.8	0.5	13.2	8.9	3.4	9.4	38.6	3.1	4.1	3.2	2.2	2.6	2.6	1.9	95.6	15.8
BRH 14 m[d]	45–55	1.0	1.0	1.3	1.1	0.5	16.6	14.9	9.4	21.9	19.4	2.2	2.3	2.2	1.4	0.9	1.1	1.1	96.5	36.4
BRH 17 m[d]	65–75	0.8		0.7	0.7	0.7	14.0	19.0	10.5	27.4	14.5	3.1	2.2	0.9	0.5			0.7	95.5	42.7

[a] Approximate enrichment of the culture based on FISH analyses.
[b] Averaged data of replicate lipid analyses; $n = 3$ for Ooij1 and Ooij3 and $n = 2$ for Ooij2 and WWTP.
[c] Data are from Raghoebarsing et al. (42).
[d] 11 m, 14 m, and 17 m denote 11, 14, and 17 months of enrichment, respectively.
[e] Branched FA as a percentage of the sum of FA (in the preceding column).
[f] Compounds comprising <0.5% of the total FA are omitted.

sampled over the time course of enrichment (Table 1 and Fig. 3). Sampling over time coincided with increased activity and degree of enrichment of the culture. FISH analyses estimated the abundance of "*Ca.* Methylomirabilis" in the culture to be approximately 15 to 25, 45 to 55, and 65 to 75% after 11, 14, and 17 months of enrichment, respectively (Table 1). At the earliest sampling moment after 11 months of enrichment, the lipid profile from the BRH reactor contained considerable amounts of monounsaturated octadecenoic acids ($C_{18:1}$; 38.6%) and longer saturated even-carbon-number FAs ($C_{20:0}$ to $C_{28:0}$; totaling 12.5%). These FAs likely are derived from plant tissue (e.g., plant waxes or sphingolipids) in the original sample from the environment (16). At later sampling times, these FAs had become much less abundant (totaling 14.5 and 1.9%, respectively, after 17 months), and the relative contribution of the typical FA $10\text{MeC}_{16:0}$ and $10\text{MeC}_{16:1\Delta7}$ had increased from 9.4 and 3.4% after 11 months of enrichment to 27.4 and 10.5% after 17 months, respectively (Table 1 and Fig. 3).

Environmental samples. Samples from a 51- to 102-cm soil core from the Brunssummerheide peatland were examined for the presence of the FA $10\text{MeC}_{16:0}$ and $10\text{MeC}_{16:1\Delta7}$. Lipid analyses revealed the presence of the FA $10\text{MeC}_{16:0}$ throughout the 51- to 102-cm soil profile (Fig. 4). The FA $10\text{MeC}_{16:1\Delta7}$ was identified in the sections from 70 to 90 cm. At their highest concentrations, the $10\text{MeC}_{16:0}$ and $10\text{MeC}_{16:1\Delta7}$ FA comprised approximately 2.2 and 0.5% of the total fatty acids, respectively (Fig. 4). Relative to the $C_{16:0}$ FA, the $10\text{MeC}_{16:0}$ and $10\text{MeC}_{16:1\Delta7}$ FA were present at approximately 10 and 3% of the amount of $C_{16:0}$. Together with the characteristic FA $10\text{MeC}_{16:0}$ and $10\text{MeC}_{16:1\Delta7}$, all other major FA observed in the lipid profile of "*Ca.* Methylomirabilis oxyfera" enrichment cultures were also present throughout the 50- to 100-cm soil profile.

DISCUSSION

The two C_{17} fatty acids $10\text{MeC}_{16:0}$ and $10\text{MeC}_{16:1\Delta7}$ characterize the lipid profile of the "*Ca.* Methylomirabilis oxyfera" enrichment cultures. To the best of our knowledge, the latter so far has only been observed in "*Ca.* Methylomirabilis oxyfera" enrichment cultures. One other unsaturated $10\text{MeC}_{16:1}$ has been reported before ($10\text{MeC}_{16:1\Delta8}$ [2]), but the nitrite-dependent "*Ca.* Methylomirabilis" bacteria provide the only account of $10\text{MeC}_{16:1\Delta7}$. Interestingly, lipid analyses of the first available nitrite-dependent meth-
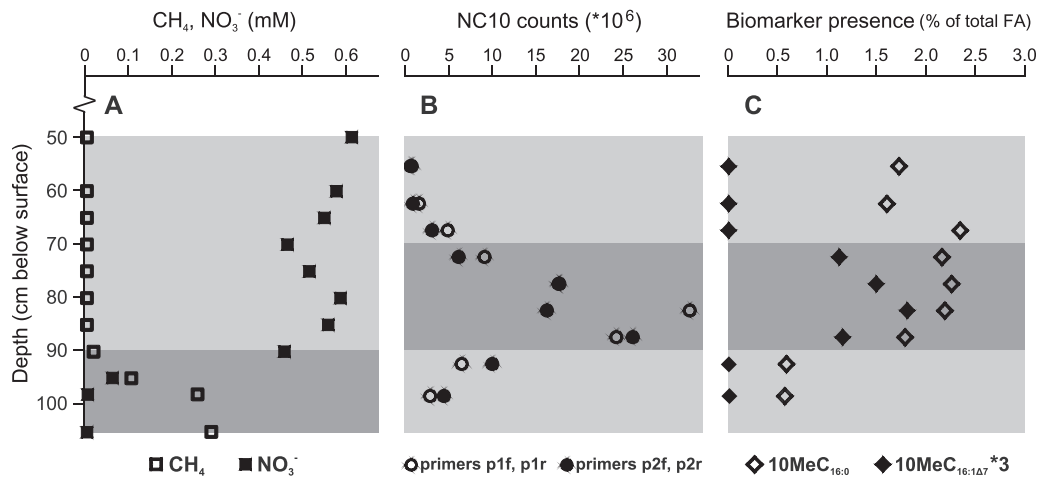
FIG 4 Profile of the proposed biomarker lipids together with cell numbers of *Methylomirabilis* and methane and nitrate concentrations in a peatland (Bruns-summerheide, the Netherlands). (A) Methane (CH_4) and nitrate (NO_3^-) concentrations throughout the profile (June 2010). (B) Cell numbers per g wet soil obtained by qPCR analysis with two independent primer sets targeting the 16S rRNA gene of "*Ca.* Methylomirabilis"-like bacteria (data are from Zhu et al. [59]). (C) Relative abundance of $10MeC_{16:0}$ and $10MeC_{16:1\Delta7}$ as percentages of the total fatty acids recovered from the apolar fraction after lipid extraction (note that values for $10MeC_{16:1\Delta7}$ are multiplied by three) (data are from this study). The darker gray bars indicate the methane-nitrate countergradient (A), and just above that is the peak in NC10 abundance (B) and the peak in abundance of the proposed biomarker lipids (C).

ane-oxidizing enrichment culture (TWK) already showed these remarkable lipids (42). However, at that time the microbial community in the culture still comprised substantial levels of archaea (10 to 15%), which was confirmed by the presence of the typical archaeol. At present, multiple cultures have been enriched from different ecosystems, and after prolonged enrichment these cultures have become 70 to 80% enriched in "*Ca.* Methylomirabilis oxyfera," while archaea are virtually absent (confirmed by FISH analyses and the absence of archaeol) and no other species comprises a significant part of the community besides "*Ca.* Methylomirabilis oxyfera" (35).

Our new data reveal the presence of the characteristic fatty acids $10MeC_{16:0}$ and $10MeC_{16:1\Delta7}$ in all investigated "*Ca.* Methylomirabilis" enrichment cultures. Moreover, we could successfully detect both FA in environmental samples from a field site from which one of the enrichment cultures originated (Fig. 4). In the stable enrichment cultures, these two fatty acids together make up more than 40% of the total lipids (Table 1). The change over time in the fatty acid composition of the BRH enrichment culture shows that both $10MeC_{16:0}$ and $10MeC_{16:1\Delta7}$ became more abundant while the abundance of $C_{18:1}$ and the longer-chain fatty acids decreased (Table 1 and Fig. 3). This reflects the increased enrichment of the culture and demonstrates that both of these FA are key constituents of the lipid profile of "*Ca.* Methylomirabilis oxyfera." This is further supported by the observation that, over the time of development, the ratio of the abundance of $10MeC_{16:1\Delta7}$ FA to $10MeC_{16:0}$ FA remains more or less the same. Intrinsically, analyses of enrichment culture biomass rather than pure cultures will not provide the pure lipid profile of the organism of interest but will always include lipids of the members of the side community. We believe, however, that the abundance of the fatty acids $10MeC_{16:0}$ and $10MeC_{16:1\Delta7}$ in all enrichment cultures from different origins and their increase over the time of development clearly show that the anaerobic nitrite-reducing methanotroph "*Ca.* Methylomirabilis" is the most likely source organism of these fatty acids.

As temperature can affect the degree of saturation of fatty acids (10), the question arises of whether the unprecedented $10MeC_{16:1\Delta7}$ will be a significant fatty acid of "*Ca.* Methylomirabilis" under all environmental conditions. The setup of our study did not allow us to thoroughly test this for "*Ca.* Methylomirabilis oxyfera." However, the ratio of saturated to unsaturated FA is generally thought to increase with temperature. As our enrichment temperatures are relatively high compared to those in the environment, the relative abundance of the novel unsaturated $10MeC_{16:1\Delta7}$ FA is more likely to be higher rather than lower in the environment than in our enrichment cultures.

Its high abundance denotes that the $10MeC_{16:0}$ fatty acid is a characteristic component of the "*Ca.* Methylomirabilis oxyfera" lipids, but it is not exclusive to "*Ca.* Methylomirabilis oxyfera." $10MeC_{16:0}$ FA has been proposed to be characteristic of sulfate-reducing bacteria (SRB) of the genera *Desulfobacter* and *Desulfobacula*, where it is found to make up various amounts of approximately 5 to 25% of the total lipids (4, 15, 32, 33, 45, 51). Besides these sulfate reducers, $10MeC_{16:0}$ FA is also occasionally, but generally in much smaller amounts, found in other bacteria; the literature reports its presence in several actinobacteria (6, 12, 23, 31, 41, 56, 58), in anammox bacteria and other planctomycetes (48, 49), in an iron-reducing *Geobacter* species (34, 57), in a *Marinobacter* species (55), and in the marine denitrifier *Pseudomonas nautica* (14). Several studies report the presence of $10MeC_{16:0}$ FA in relation to the occurrence of anaerobic methane oxidation (AOM) coupled to sulfate reduction (1, 2, 7, 17, 26, 39). One study on the methane-oxidizing community in landfill cover soils reports remarkably large amounts of $10MeC_{16:0}$ FA of up to 16% of the total extracted phospholipid fatty acids. However, this distinct fatty acid was not discussed to be characteristic for the (methane-oxidizing) bacterial population, despite the fact that this $10MeC_{16:0}$ FA became significantly enriched in ^{13}C after incubation with ^{13}C-CH_4 (11). Based on the high abundance of $10MeC_{16:0}$ FA in "*Ca.* Methylomirabilis oxyfera" identified in our study, we speculate that the presence and ^{13}C labeling of this FA

observed in the aforementioned study is indicative of the involvement of "*Ca.* Methylomirabilis"-like microorganisms in those ecosystems.

The association of the signature lipid 10MeC$_{16:0}$ with both sulfate- and nitrite-dependent AOM may obscure the diagnostic value of this FA as a biomarker for either bacterial process. In studies where the presence of this FA is used to substantiate the involvement of sulfate reducers in methane oxidation, a potential role of "*Ca.* Methylomirabilis oxyfera"-like bacteria thus far has gone unnoticed. This would not pose a major concern as long as the two processes are thought to occur in separated environments. AOM coupled to sulfate reduction has mainly been studied in marine ecosystems, while all "*Ca.* Methylomirabilis oxyfera" enrichment cultures enriched thus far have been from freshwater areas. Only one study has reported the potential presence of "*Ca.* Methylomirabilis oxyfera"-like bacteria in marine sediments based on genomic analyses, but this was only based on 16S rRNA reads and no similarity with functional genes (for methane monooxygenase) of "*Ca.* Methylomirabilis oxyfera" could be found in the metagenome (24). The contribution of "*Ca.* Methylomirabilis oxyfera" to methane oxidation in a nitrite/nitrate-limited and sulfate-rich environment may be insignificant and vice versa. However, some ecosystems may be suitable for the cooccurrence of both types of AOM, for example, estuaries that are influenced by sulfate-rich seawater and that simultaneously experience high nitrogen inputs from river water.

Fortunately, the unprecedented finding of the fatty acid 10MeC$_{16:1\Delta7}$ in "*Ca.* Methylomirabilis" offers a unique biomarker for these methane-oxidizing bacteria. However, the likely low abundance of "*Ca.* Methylomirabilis oxyfera" in a complex microbial community may complicate the detection of this single FA 10MeC$_{16:1\Delta7}$ in environmental samples. Nevertheless, we were able to successfully detect both of the FA 10MeC$_{16:0}$ and 10MeC$_{16:1\Delta7}$ in environmental samples. The observed presence of the characteristic lipids in the field samples corresponds with qPCR analyses on the same soil core: abundance of NC10 phylum bacteria (to which "*Ca.* Methylomirabilis" belongs) based on qPCR data and the presence of the 10MeC$_{16:0}$ and 10MeC$_{16:1\Delta7}$ FA both peak between 80 and 90 cm depth in the soil profile. Correspondingly, in this zone countergradients of methane and nitrate are observed, indicating the activity of anaerobic methanotrophs (Fig. 4) (59). The 10MeC$_{16:0}$ and 10MeC$_{16:1\Delta7}$ FA made up approximately 2.2 and 0.5% of the total FA in the environmental samples, which, compared to their quantities in enrichment cultures (35 to 46% and 4 to 10%, respectively), could represent an approximate maximum abundance of "*Ca.* Methylomirabilis" of about 4 to 5% of the community. This is in good accordance with the share of NC10 phylum bacteria in the total bacterial community of 3 to 8% at a depth of 80 to 90 cm as assessed by qPCR (59). We propose that the combined search for the unique FA 10MeC$_{16:1\Delta7}$ and the foremost abundant 10MeC$_{16:0}$ FA provide a strong tool to study the presence and significance of "*Ca.* Methylomirabilis oxyfera"-like bacteria in the environment.

Conclusion. The characteristic fatty acids 10MeC$_{16:0}$ and 10MeC$_{16:1\Delta7}$ may offer a useful tool in studying "*Ca.* Methylomirabilis oxyfera" and the process of nitrite-dependent methane oxidation in the environment. The successful detection of these fatty acids in samples from the ecosystem from which one of the "*Ca.* Methylomirabilis oxyfera" enrichment cultures originated validates the potential of these lipids as biomarkers. These new lipid biomarkers can complement genomic techniques to study the diverse players in the methane cycle.

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