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 (10MeC16:0). Furthermore, a unique FA was identified that has not been reported elsewhere: the monounsaturated 10-methylhexadecenoic acid with a double bond at the Δ7 position (10MeC16:1Δ7), which comprised up to 10% of the total FA profile. We propose that the typical branched fatty acids 10MeC16:0 and 10MeC16:1Δ7 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 (CH₄) 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 CO₂ (29). Understanding the sources and sinks of CH₄ 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 CH₄ and sulfate in the water column and in sediments first led to the awareness of significant CH₄ 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 CH₄. 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 overall 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 (Ooi1, Ooi2, and Ooi3) (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, Ooi1 (1, 2, and 3), WWTP, and BRH, respectively.

All cultures actively oxidized CH₄ 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 CH₄ oxidizing activity) at 11, 14, and 17 months after inoculation. The other samples were taken over the time of its development (along with increasing CH₄ oxidizing activity) in various ecosystems (2, 8, 9, 18, 22, 25, 30, 40, 43, 46, 47, 50, 52, 54).

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 3 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 were 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 (BF₃) in MeOH and subsequently separated by column chromatography over activated alumina (Al₂O₃) 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 (PtO₂) 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 addition 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 Ooi1. 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) (C₁₆ to C₂₃). The most abundant FA was 10-methyl-hexadecanoic acid (10MeC₁₆:0) followed by hexadecanoic acid (C₁₆:0). In addition, GC-MS analyses revealed the presence of a previously unknown component, which eluted at a retention time between those of C₁₆:0 and 10MeC₁₆:0.
Figure 2 presents the mass spectra of the most abundant FA of the lipid profile, identified as a 10MeC₁₆:₀ (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 mono-unsaturated C₁₇ fatty acid. This compound was indeed recovered in the unsaturated fraction, and after hydrogenation it was converted to 10MeC₁₆:₀ FA, as confirmed by GC-MS. The mass spectrum of the unknown component is consistent with monounsaturated 10MeC₁₆:₀ 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, 10MeC₁₆:₀ 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 (C₁) and the methyl group (at C₁₀). GC-MS analyses after dimethyl-disulfide (DMDS) adduction resulted in diagnostic fragments of m/z 187, 189, and 157, representing \(\omega C₁₀\Delta C₇\), and \(\Delta C₇-32\) fragments, respectively (Fig. 2C), thus locating the double bond at the \(\Delta 7\) position. Consequently, this unknown FA was identified as 10-methyl-hexadec-7-enoic acid, or 10MeC₁₆:₁₇.

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, 10MeC₁₆:₀ and C₁₆:₀, comprised approximately 35 to 46% and 18 to 26% of the total fatty acids, respectively. The unique FA 10MeC₁₆:₁₇ 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
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 (C18:1; 38.6%) and longer saturated even-carbon-number FAs (C20:0 to C28:0; 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 10MeC16:0 and 10MeC16:1/H90047 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 10MeC16:0 and 10MeC16:1/H90047. Lipid analyses revealed the presence of the FA 10MeC16:0 throughout the 51- to 102-cm soil profile (Fig. 4). The FA 10MeC16:1/H90047 was identified in the sections from 70 to 90 cm. At their highest concentrations, the 10MeC16:0 and 10MeC16:1/H90047 FA comprised approximately 2.2 and 0.5% of the total fatty acids, respectively (Fig. 4). Relative to the C16:0 FA, the 10MeC16:0 and 10MeC16:1/H90047 FA were present at approximately 10 and 3% of the amount of C16:0. Together with the characteristic FA 10MeC16:0 and 10MeC16:1/H90047, 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 C17 fatty acids 10MeC16:0 and 10MeC16:1/H90047 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 10MeC16:1/H90047 has been reported before (10MeC16:1/H90047 [2]), but the nitrite-dependent “Ca. Methylomirabilis” bacteria provide the only account of 10MeC16:1/H90047. Interestingly, lipid analyses of the first available nitrite-dependent meth-
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 archaeal 16S rRNA gene signature that dominates the metabolic activity of the community, besides “Methylomirabilis oxyfera” (35).

Our new data reveal the presence of the characteristic fatty acids 10MeC16:0 and 10MeC16:1,7 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 10MeC16:0 and 10MeC16:1,7 became more abundant while the abundance of C18:1 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 10MeC16:0 FA to 10MeC16:1,7 FA remains more or less the same. Intrinsic 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 10MeC16:0 and 10MeC16:1,7 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 10MeC16:1,7 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 temperature are relatively high compared to those in the environment, the relative abundance of the novel unsaturated 10MeC16:1,7 FA is more likely to be higher rather than lower in the environment than in our enrichment cultures.

Its high abundance denotes that the 10MeC16:0 fatty acid is a characteristic component of the “Ca. Methylomirabilis oxyfera” lipids, but it is not exclusive to “Ca. Methylomirabilis oxyfera.” 10MeC16:0 FA has been proposed to be characteristic of sulfate-reducing bacteria (SRB) of the genera Desulfo bacter 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, 10MeC16:0 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 10MeC16:0 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 10MeC16:0 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 10MeC16:0 FA became significantly enriched in 13C after incubation with 13C-CH4 (11). Based on the high abundance of 10MeC16:0 FA in “Ca. Methylomirabilis oxyfera” identified in our study, we speculate that the presence and 13C labeling of this FA

FIG 4 Profile of the proposed biomarker lipids together with cell numbers of Methylomirabulis and methane and nitrate concentrations in a peatland (Brunssummerheide, the Netherlands). (A) Methane (CH4) and nitrate (NO3−) 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 10MeC16:0 and 10MeC16:1,7 as percentages of the total fatty acids recovered from the apolar fraction after lipid extraction (note that values for 10MeC16:1,7 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).
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 nitrate/nitrite-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_{16:1ω2} 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_{16:1ω2} in environmental samples. Nevertheless, we were able to successfully detect both of the FA 10MeC_{16:0} and 10MeC_{16:1ω2} 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_{16:1ω2} FA both peak between 80 and 90 cm depth in the soil profile. Correspondingly, in this zone countergradients of methane and nitrate for these methane-oxidizing bacteria. However, the likely low abundance of “Ca. Methylomirabilis oxyfera” enrichment cultures originated validated the potential of these lipids as biomarkers. These new lipid biomarkers can complement genomic techniques to study the diverse players in the methane cycle.

ACKNOWLEDGMENTS

We thank Franciska Luesken and Mingliang Wu for access to the enrichment culture biomass and Gis van Dijk, Christian Fritz, and Alfons Smolders for collaboration on the Brunssummerheide samples.

This research was funded by a personal Rubicon grant to D.M.K. from the Netherlands Science Foundation (NOW; project 825.10.018) and the Darwin Centre for Biogeoosciences in support of D.M.K. and K.F.E. (projects 142.16.3071 and 142.16.3072, publication number DW-2012-1006). B.Z. is financially supported by a CAS-KNAW grant and M.S.M.J. by the ERC (grant 232937).

REFERENCES


Lipid Biomarkers for “Ca. Methylomirabilis oxyfera”
22. Freeman KH, Hayes JM, Trendel JM, Albrecht P.
23. Havelsrud OE, Haverkamp THA, Kristensen T, Jakobsen KS, Rike AG.