Applied and Environmental Microbiology

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Appl. Environ. Microbiol. **2004, 70(6):3785. DOI: 10.1128/AEM.70.6.3785-3788.2004.**

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Applied and Environmental Microbiology, June 2004, p. 3785–3788 Vol. 70, No. 6
0099-2240/04/$08.00+0 DOI: 10.1128/AEM.70.6.3785–3788.2004
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Stable Carbon Isotopic Fractionations Associated with Inorganic Carbon Fixation by Anaerobic Ammonium-Oxidizing Bacteria

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Received 28 November 2003/Accepted 4 March 2004

Isotopic analyses of *Candidatus* "Brocadia anammoxidans," a chemolithoautotrophic bacterium that anaerobically oxidizes ammonium (anammox), show that it strongly fractionates against ^{13}C; i.e., lipids are depleted by up to 47‰ versus CO_2. Similar results were obtained for the anammox bacterium *Candidatus* "Scalindua sorokinii," which thrives in the anoxic water column of the Black Sea, suggesting that different anammox bacteria use identical carbon fixation pathways, which may be either the Calvin cycle or the acetyl coenzyme A pathway.

Anaerobic ammonium oxidation (anammox) is the oxidation of ammonium with nitrite as the electron acceptor and dinitrogen gas as the product (for reviews, see references 7 and 8). The process is mediated by obligately anaerobic chemolithoautotrophic bacteria that form a monophyletic cluster inside the *Planctomycetales*, one of the major divisions of the *Bacteria*. So far, four species have been detected and enriched from the biomass of sewage treatment plants: *Candidatus* "Brocadia anammoxidans" (18), *Candidatus* "Kuenenia stuttgartiensis" (13), *Candidatus* "Scalindua wagneri," and *Candidatus* "Scalindua brodae" (14). *Candidatus* "Scalindua sorokinii" was detected in the anoxic water column of the Black Sea (9), providing the first direct evidence for anammox bacteria in the natural environment. Anammox bacteria have a cell compartment known as the anammoxosome, which is the site of anammox catabolism. The lipid bilayer membrane surrounding this anammoxosome contains unusual lipids, so-called "ladderane" lipids, concatenated cyclobutane moieties that are either ether and/or ester linked to the glycerol backbone or occur as free alcohols (e.g., Fig. 1, structures II to IV) (16). The other membranes of anammox bacteria contain lipids typical for planctomycetes in general: iso, normal, and mid-chain methyl hexadecanoic acids (e.g., Fig. 1, structure I).

Anammox bacteria have been shown to be chemoautotrophic organisms (17), but it is still unclear which carbon fixation pathway they use. There are currently four known pathways for CO_2 fixation in microorganisms (see, e.g., references 2 and 3). The Calvin cycle, with ribulose bisphosphate carboxylase as a key enzyme, is operative in many organisms. The 3-hydroxypropionate pathway has been observed in *Chloroflexus aurantiacus* and some archaea. The reverse citric acid cycle, with citrate lyase as a key enzyme, has been found in some sulfate-reducing bacteria and phototrophic bacteria. Finally, the acetyl coenzyme A (acetyl-CoA) pathway, with carbon monoxide dehydrogenase/acetyl-CoA synthase as the indicative enzyme, is detected in many anaerobic microorganisms. In addition to enzyme activities, stable carbon isotopic compositions of total cell material and individual lipids, carbohydrates, and amino acids are often used to infer these biosynthetic pathways in organisms, as the fractionation from the inorganic carbon source to the autotrophic biomass in ^{13}C depends on the biosynthetic pathway used (1, 19, 20, 21).

Here, we determined enzyme activities and studied the stable carbon isotopic fractionations of *Candidatus* "Brocadia anammoxidans" to investigate its carbon fixation pathway. Since this bacterium can be grown only in enrichment cultures, and bulk cell material is thus not solely derived from anammox bacteria, we also determined the isotopic compositions of the specific lipids of this bacterium. Furthermore, the isotopic compositions of ladderane lipids derived from *Candidatus* "Scalindua sorokinii" growing in the anoxic water column of the Black Sea (9) were determined in order to examine the ^{13}C fractionation patterns of anammox bacteria under natural conditions.

Anammox samples. Anammox bacterial cells were grown in enrichment cultures in an anaerobic sequencing batch reactor as described previously (17). *Candidatus* "Brocadia anammoxidans" strain Delft was enriched from an anaerobic wastewater treatment plant in Rotterdam, The Netherlands. Particulate organic matter for lipid analyses was collected in the western basin of the Black Sea (site 7605 [42°30.71′N, 30°14.69′E] and site 7620 [42°55.56′N, 30°03.65′E]) during the R/V *Meteor* cruise in December 2001. Material was collected at several depths by in situ filtration of large volumes (~1,000 liters) of

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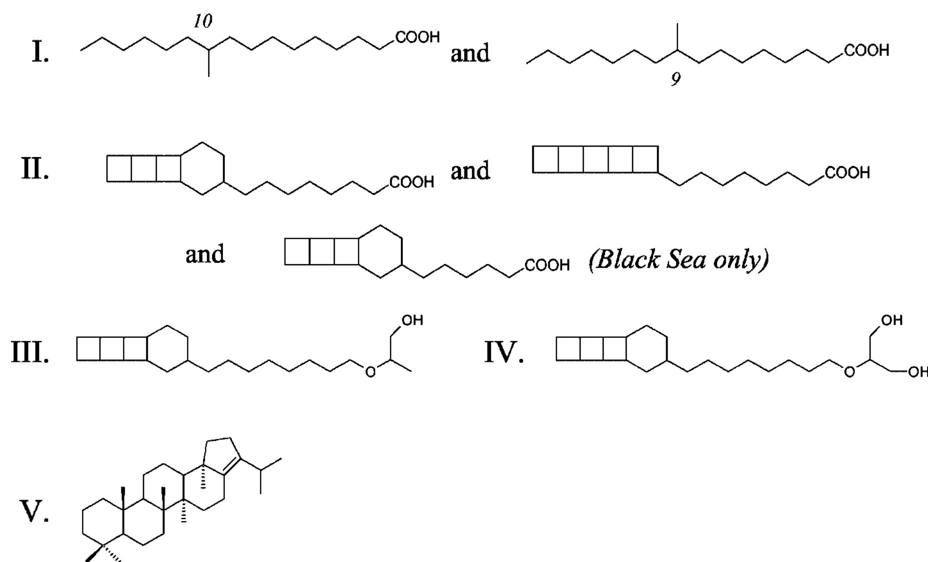

FIG. 1. Structure of anammox lipids, i.e., branched fatty acids (I), ladderane fatty acids (II), ladderane glycol ether (III), ladderane glycerol ether (IV), and hop-17(21)-ene (V), present in the enrichment culture of *Candidatus* "Brocadia anammoxidans."

water through 292-mm-diameter precombusted glass fiber filters (nominal pore size, 0.7 μm) with in situ pumps. Water samples for dissolved inorganic carbon (DIC) analysis were obtained by a pumpcast-CTD system and were killed off with $HgCl_2$.

Enzyme assay conditions. *Candidatus* "Brocadia anammoxidans" cells from either fluidized bed reactors (two samples) or from a sequencing batch reactor (also two samples) were washed twice with a solution containing 50 mM Tris-HCl (pH 7.8), 1 mM dithiothreitol, and 2 mM $MgCl_2$. They were then suspended anaerobically inside a glove box (95% N_2, 5% H_2) in 4 ml of buffer and passed four times through a French pressure cell at 4°C. Unbroken cells and debris were removed by centrifugation in gas-tight tubes at 40,000 × *g*. The supernatant was used as cell extract and contained about 12 mg of protein per ml. Assays were carried out at least in triplicate (standard deviation, less than 15%) in anaerobic quartz cuvettes as described previously (5, 6, 12), with enzymes or extracts of the appropriate reference organisms used as controls (Table 1).

Lipid and isotope analysis. Lipids were analyzed by gas chromatography (GC), GC-mass spectrometry (MS), and GC–isotope ratio-monitoring MS (irmMS) as described previously (15).

Analysis of the $\delta^{13}C$ of total DIC in the medium was performed by headspace analysis of 0.5 to 1 ml of water that had reacted with H_3PO_4 for at least 1 h at room temperature. The headspace was subsequently analyzed 10 times with a ThermoFinnigan Gas Bench II coupled to a Delta^{PLUS} irmMS system, with typical standard deviations of 0.1‰. Stable carbon isotope ratios were determined relative to lab standards calibrated with NBS-18 carbonate (International Atomic Energy Agency [IAEA]). Analysis of the $\delta^{13}C$ of total DIC in the Black Sea water column was performed by headspace analysis of 2 ml of water after the addition of 100 μl of H_3PO_4 and equilibration at 40°C for 2 h. The headspace was subsequently analyzed six times by using a Multiflow system connected to an Isoprime irmMS system, with typical standard deviations of 0.1 to 0.2‰. Stable carbon isotope ratios were determined relative to lab standards calibrated on NBS-19 carbonate and CO-8 (IAEA).

The $\delta^{13}C$ values of the biomass were determined by elemental analysis-irmMS using a Carlo Erba Flash elemental analyzer coupled to a ThermoFinnigan Delta^{PLUS} irmMS system,

TABLE 1. Enzyme activities in cell extracts of *Candidatus* "Brocadia anammoxidans" and control organisms

Enzyme (EC no.)	*Candidatus* "Brocadia anammoxidans" sp act (nmol/min/mg of protein)	Control sp act (nmol/min/mg of protein)	Control source[a]
CO dehydrogenase (EC 1.2.99.2)	<1	230	UASB sludge
		3,780	*Methanotrix soehngenii*
ATP citrate (pro-3S) lyase (EC 3.1.2.16)	<1	130	*Desulfobacter*
Ribulose bisphosphate carboxylase (EC 4.1.1.39)	<1	65	*Nitrosomonas europaea*
3-Hydroxy propionate dehydrogenase (EC 1.1.1.59)	<1	7,800	Lactate dehydrogenase
Malate dehydrogenase (EC 1.1.1.37)	52	160	*Paracoccus denitrificans*
Nitrate reductase (EC 1.7.99.4)	34	120	*Paracoccus denitrificans*

[a] UASB, upflow anaerobic sludge blanket.

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TABLE 2. Stable carbon isotope data and fractionation factors obtained from biomass of *Candidatus* "Brocadia anammoxidans" strain Delft and particulate organic matter from the Black Sea[a]

	$\delta^{13}C$ (‰ vs VPDB)								ε (‰ vs VPDB)[c]		
	DIC	CO_2[a]	Biomass	I	II[b]	III	IV	V	biomass–II	CO_2–II	CO_2–IV
Reactor 1 (days)											
34	−27.5	−35.6	−53.5	ND	ND	ND	ND	ND	ND	ND	ND
Reactor 2 (days)											
1	−24.3	−32.4	−48.7	−66	−65	−67	ND	ND	17	34	
8	−24.2	−32.3	−52.6	−74	−73	ND	−75	ND	22	44	47
58	−24.6	−32.7	−47.9	−70	−63	−70	−74	−69	16	32	45
70	−24.6	−32.7	−54	ND	−64	−75	−76	−69	11	34	46
86	ND	ND	−56.8	−76	−71	−73	−74	−70	15	NA	
Black Sea (m depth)											
90	−1.3[d]	−12.3	NA	ND	−58	ND	ND	ND	NA	49	ND
95	−1.4[d]	−12.4	NA	ND	−58	ND	ND	ND	NA	49	ND
100	−1.4	−12.4	NA	ND	−55	ND	ND	ND	NA	45	ND

[a] Calculated according to an equation described previously (10). The temperatures were 35°C in cultures and 8.3°C in the Black Sea. For structures of anammox lipids (I to V), see Fig. 1. ND, not determined; NA, not applicable, VPDB, Vienna Pee-Dee Belemnite.
[b] Since ladderane fatty acids are known to disintegrate during GC analysis, all the different fatty acid methyl esters containing cyclobutane rings were summed.
[c] ε biomass-ladderanes = $\{[(1{,}000 + \delta^{13}C \text{ biomass})/(1{,}000 + \delta^{13}C \text{ ladderanes})] - 1\} \times 1{,}000$.
[d] Inferred from $\delta^{13}C$ DIC measured at 80- and 100-m depths.

with reproducibility ranging from 0.1 to 0.7‰. Stable carbon isotope ratios were determined by using lab standards calibrated on NBS-22 oil (IAEA).

Enzyme activities. Strictly anaerobically prepared cell extracts of *Candidatus* "Brocadia anammoxidans" were used to determine the key enzymes of the four known CO_2 fixation pathways (Table 1). However, all assays showed activity of less than 1 nmol per min per mg of protein in the extracts, while the activity of control organisms was at least 65 nmol per min per mg of protein under the same conditions. A standard housekeeping enzyme like malate dehydrogenase showed a good level of activity (52 nmol per min per mg of protein). The enzyme potentially involved in providing reduced equivalents to the CO_2 fixation pathway, nitrate/nitrite oxidoreductase, was present at 34 nmol per min per mg of protein, indicating that cell extract preparations did not compromise the observed activities. Furthermore, the CO_2 fixation rate should have been on the order of 4 nmol per min per mg of protein (17), which should have been detectable in concentrated extracts. This lack of CO_2-fixing enzyme activities in cell extracts prompted us to investigate the stable isotopic fractionation patterns of anammox bacteria.

Isotope analysis. In an initial experiment, an enrichment culture of the anammox bacterium *Candidatus* "Brocadia anammoxidans" was grown for 34 days. DIC and biomass analyses showed a large difference in ^{13}C contents, with the cell material being depleted of ^{13}C by 26‰ compared to the inorganic carbon, suggesting a large isotopic fractionation by the anammox bacterium (Table 2). To further investigate this finding, an enrichment culture consisting of about 80% of the anammox bacterium *Candidatus* "Brocadia anammoxidans" was grown for 86 days (Table 2). The DIC present in the medium remained constant at −24.4‰ ± 0.2‰, but the collected cell material was isotopically more variable and ranged from −47.9 to −56.8‰, with no clear visible trend (Table 2). These results show that, similar to our initial experiment, the cell material is strongly depleted in ^{13}C compared to CO_2; i.e., the isotopic fractionation between cell material and CO_2, expressed as ε, ranges between 11 and 22‰. This fractionation is similar to the range observed for organisms when either the Calvin cycle or the acetyl-CoA pathway is used but is much larger then fractionations observed for organisms when the reverse tricarboxylic acid cycle or the 3-hydroxypropionate pathway is used (2, 19, 20, 21).

Confirmation of the large ^{13}C fractionation by anammox bacteria was obtained by analyzing lipids (Fig. 1) known to be exclusively derived from these bacteria (17) (Table 2). Values that were highly ^{13}C depleted were observed for anammox lipids compared to CO_2, showing that indeed the anammox bacterium strongly fractionates against ^{13}C. This result suggests that this anammox bacterium uses either the Calvin cycle or the acetyl-CoA pathway. However, the large difference in ^{13}C contents of ladderane lipids compared to those of CO_2 (up to 47‰) (Table 2) is beyond the maximum range of fractionations (ca. 40‰) (4, 11, 15) observed for lipids biosynthesized by organisms using the Calvin cycle. This tentatively suggests that anammox bacteria may use the acetyl-CoA pathway, which is known to sometimes yield larger fractionations in ^{13}C than the Calvin cycle (11). The absence of CO dehydrogenase/acetyl-CoA synthase in cell extracts of *Candidatus* "Brocadia anammoxidans" indicates that the assay for this enzyme still has to be optimized, although great care was taken to ensure the strict anaerobic conditions generally necessary to detect this enzyme (5).

To investigate the isotopic fractionation of anammox bacteria under natural conditions, we analyzed the isotopic compositions of ladderane lipids (Fig. 1, structure II) present at several depths in the anoxic water column of the Black Sea (9). Molecular investigations have shown that these lipids originate from a different anammox bacterium, provisionally named *Candidatus* "Scalindua sorokinii," which has a 12.4% sequence difference in the 16S rRNA gene compared to *Candidatus* "Brocadia anammoxidans" (9). As observed for the enrichment culture of *Candidatus* "Brocadia anammoxidans," the lipids of *Candidatus* "Scalindua sorokinii" are strongly depleted in ^{13}C (Table 2), and the fractionation compared to CO_2

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is even larger (up to 49‰) than that observed for the same lipids in the *Brocadia* enrichment culture (up to 47‰). This result suggests not only that anammox bacteria also strongly fractionate against ^{13}C under natural conditions but also that the autotrophic carbon fixation pathways within the diverse group of anaerobic ammonium-oxidizing bacteria are similar.

Conclusions. Our study of the stable carbon isotopic compositions of lipids and the biomass of anaerobic ammonium-oxidizing bacteria shows that they possess a carbon fixation pathway which strongly fractionates against ^{13}C. The results are consistent with either the use of the Calvin cycle or perhaps more likely, based on the large ^{13}C depletions, the acetyl-CoA pathway.

We thank L. van Niftrik and B. Maas for laboratory cultivation of anammox biomass. C. Hanfland and the AWI (Bremerhaven) provided the in situ pumps, G. Lavik (MPI) provided assistance with the pumps, and G. Klockgether (MPI) assisted with extractions.

We thank the crew of the R/V *Meteor* for collaboration and the Romanian and Turkish authorities for access to their national waters.

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