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Structural identification of ladderane and other membrane lipids of planctomycetes capable of anaerobic ammonium oxidation (anammox)

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The membrane lipid composition of planctomycetes capable of the anaerobic oxidation of ammonium (anammox), i.e. *Candidatus* 'Brocadia anammoxidans' and *Candidatus* 'Kuenenia stuttgartiensis', was shown to be composed mainly of so-called ladderane lipids. These lipids are comprised of three to five linearly concatenated cyclobutane moieties with *cis* ring junctions, which occurred as fatty acids, fatty alcohols, alkyl glycerol monoethers, dialkyl glycerol diethers and mixed glycerol ether/esters. The highly strained ladderane moieties were thermally unstable, which resulted in breakdown during their analysis with GC. This was shown by isolation of a thermal product of these ladderanes and subsequent analysis with two-dimensional NMR techniques. Comprehensive MS and relative retention time data for all the encountered ladderane membrane lipids is reported, allowing the identification of ladderanes in other bacterial cultures and in the environment. The occurrence of ladderane lipids seems to be limited to the specific phylogenetic clade within the *Planctomycetales* able to perform anammox. This was consistent with their proposed biochemical function, namely as predominant membrane lipids of the so-called anammoxosome, the specific organelle where anammox catabolism takes place in the cell.

Recently, identification of the lithotroph 'missing from nature', capable of anaerobic ammonium oxidation (anammox), was reported [1]. Based on 16S rRNA gene phylogeny, *Candidatus* 'Brocadia anammoxidans' and its relative *Candidatus* 'Kuenenia stuttgartiensis' were shown to be deep-branching members of the Order Planctomycetales, one of the major, and perhaps oldest [2], distinct divisions of the Domain Bacteria [1,3,4]. Anammox bacteria derive their energy from the anaerobic combination of the substrates ammonia and nitrite into dinitrogen gas. Anammox bacteria grow exceptionally slowly, dividing only once every two to three weeks. Although initially found in wastewater treatment plants [5], anammox bacteria have now been shown to play an important role in the natural N-cycle in the ocean [6,7]. The anammox bacterium from the anoxic Black Sea, '*Candidatus* Scalindua sorokinii', is phylogenetically distinct (average 16S rDNA sequence similarity of only 85%) from the two other anammox genera [6]. It is, however, closely related to two species of anammox bacteria, *Candidatus* 'Scalindua brodae' and 'Scalindua wagneri', identified in a wastewater treatment plant treating landfill leachate [8].

Anammox catabolism takes place in a separate membrane-bounded intracytoplasmic compartment, the anammoxosome [9]. Hydrazine (N_2H_4) and

Abbreviations
BSTFA, *N,O*-bis-(trimetylsilyl)trifluoroacetamide; CC, column chromatography; DCM, dichloromethane; FAME, fatty acid methyl ester; FID, flame ionization detector; MeOH, methanol; PCGC, preparative capillary gas chromatography; TLF, total lipid fraction.

hydroxylamine (NH_2OH) are the toxic intermediates, and occur as free molecules observed to diffuse into and out of anammox cells [1,10]. Indeed, containment of these chemicals inside the anammoxosome was considered impossible, because both compounds readily diffuse through biomembranes [11]. Recently, we described the discovery of the unprecedented molecular structure of the anammox membrane, which provided an explanation for this biochemical enigma [12]: the anammoxosome membrane is comprised of unique 'ladderane' lipids which form a membrane that is less permeable than normal biomembranes and therefore contains hydrazine, hydroxylamine and protons in the anammoxosome [13]. One of these ladderane structures has recently been confirmed by the chemical synthesis of this unique natural product [14].

In this study we describe in detail the structure of these and other lipids in anammox bacteria and discuss their distributions.

Results

General lipid composition of *Candidatus* 'B. anammoxidans' strain Delft

Figure 1A shows the gas chromatogram of the total lipid fraction (TLF) of a 99.5% pure suspension of *Candidatus* 'B. anammoxidans' isolated via density-gradient centrifugation from a mixed bacterial culture in which 81% of the population consisted of *Candidatus* 'B. anammoxidans' [1]. This represents the lipid characterization of the purest anammox culture available because there is currently no pure culture of any anammox bacterium. In addition to straight-chain and branched fatty acids, this fraction is characterized by the presence of squalene, a number of hopanoids [diploptene, diplopterol, 17β,21β(H)-bishomohopanoic acid, 17β,21β(H)-32-hydroxy-trishomohopanoic acid, 22,29,30-trisnor-21-oxo-hopane] [15] and a series of ladderane lipids.

To rigorously identify these ladderane lipids, a larger batch of our enriched culture in which 81% of the population consisted of *Candidatus* 'B. anammoxidans' was used for fractionation of the lipid extract by TLC. The TLF fraction of this batch was quite comparable in composition with the density-purified *Candidatus* 'B. anammoxidans' fraction (Fig. 1). TLC separation resulted in eight distinct bands (Table 1), which enabled us to obtain pure mass spectra of individual lipids. A further bulk extraction (45 g dry weight of cell material) and preparative separation using column chromatography was used to yield sufficient quantities of highly purified components for further characterization by high-field NMR, hydrolysis and chemical degradation studies.

Hydrocarbons

The TLC hydrocarbon fraction (Table 1) is dominated by diploptene (**1**; for structures see Fig. 2) and, to a

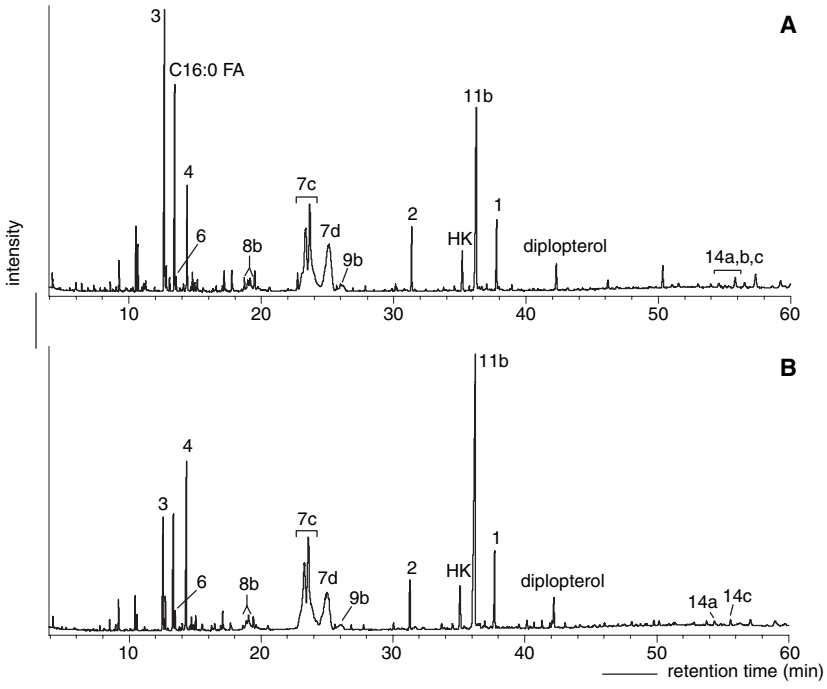


Fig. 1. Gas chromatograms of the TLFs of (A) a 99.5% pure suspension of *Candidatus* 'B. anammoxidans' strain Delft after base hydrolysis of the cell material, and (B) a mixed bacterial culture in which 81% of the population consisted of *Candidatus* 'B. anammoxidans' strain Delft. Fatty acids and alcohols were derivatized to the corresponding methyl esters and trimethylsilyl ethers prior to GC analysis. FA, fatty acid; HK, hopanoid ketone; **1**, diploptene; **2**, squalene; **3**, *iso* hecadecanoic acid; **4**, 10-methylhexadecanoic acid; **6**, 9,14-dimethylpentadecanoic acid. Other numbers refer to structures indicated in Fig. 2.

Table 1. Major compound classes of the lipid extract of *Candidatus* 'Brocadia anammoxidans' strain Delft. ND, not determined; these lipids were less abundant in the lipid extract of the large batch.

TLC band	R_f	Compound class	Composition	Corresponding reparative CC fraction	Amount (%)[a]
1	0.85–0.97	Hydrocarbons	Diploptene (**1**), squalene (**2**)	CC1	4
2	0.76–0.85	Fatty acid methyl esters	normal, branched and ladderane fatty acids (**3**–**8**) methyl esters	CC3	15
3	0.67–0.76	Ketones	17β-22,29,30-trisnor-21-oxo-hopane		ND
4	0.55–0.59	Alcohols	Diplopterol		ND
5	0.48–0.55	Glycerol diethers, Alcohols	**13a-g**, **9**, **10**	CC5	25[b]
6	0.41–0.48	Glycerol ether/esters	**14a-g**	CC5	25[b]
7	0.16–0.23	Glycerol monoether	**11a**	CC7	14
8	0.04–0.08	Glycerol diethers and ether/esters[c]			
		Bacteriohopanetetrol	**13a-g, 14a-g**		ND

[a] By weight, in percentage of total extract based on the preparative column chromatographic separation using a large batch of cell material. [b] Together withTLC fraction 6. [c] These are thought to represent glycerol diethers and ether/esters with polar end groups which have subsequently been hydrolysed during work-up.

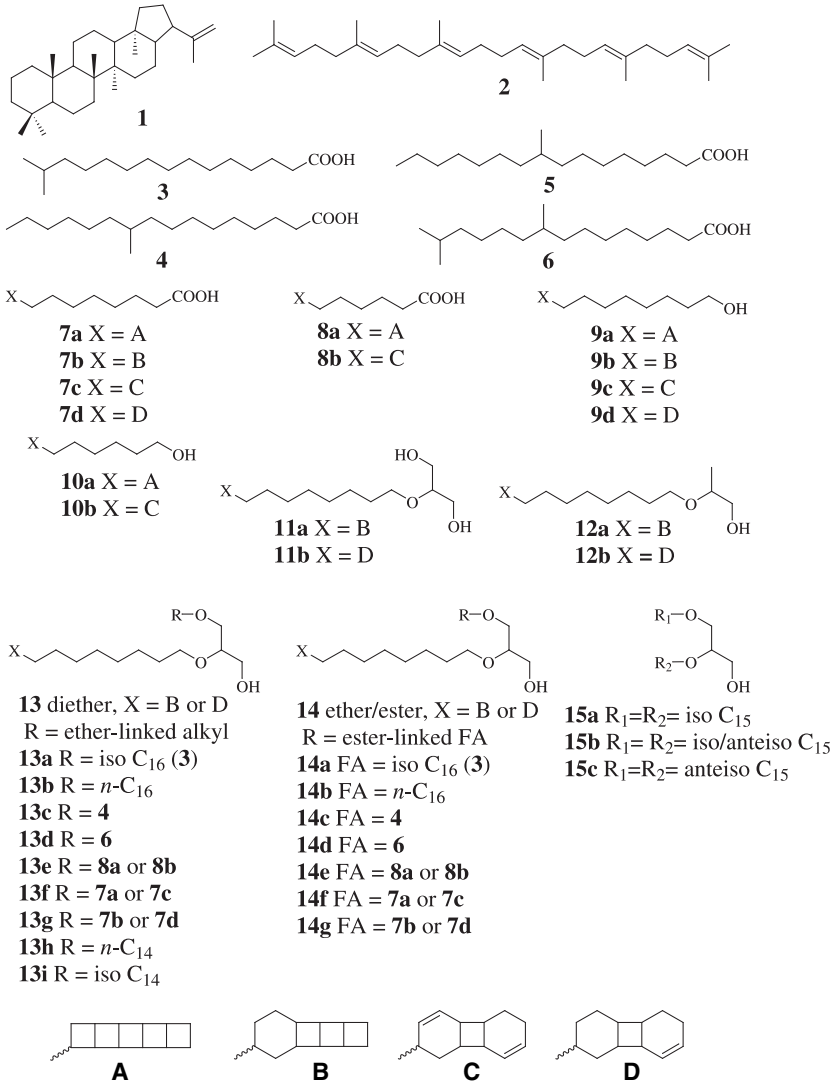


Fig. 2. Structures of annammox bacterial lipids. The three dimensional structures of the [5]- and [3]-ladderane moieties (A and B, respectively) are reported elsewhere [12].

lesser extent, squalene (**2**). Both lipids are widespread in the bacterial domain of life.

Fatty acids

These lipids represent a substantial fraction (Table 1) of the extract and are comprised of a set of conventional straight-chain fatty acids (i.e. saturated and unsaturated straight-chain fatty acids, branched fatty acids) and so-called ladderane fatty acids. Fatty acids common to bacteria include: *n*-C_{14}, *n*-C_{15}, *n*-C_{16}, *n*-C_{17}, *n*-C_{18}, *i*-C_{14}, *i*-C_{15}, *i*-C_{16}, *i*-C_{17}, *i*-C_{18}, *ai*-C_{15}, *ai*-C_{17} and monounsaturated *n*-C_{16}, *n*-C_{17}, *n*-C_{18}, *n*-C_{19}. The relatively high abundance of the 14-methylpentadecacanoic acid (*i*-C_{16}) (**3**) is not often seen in bacteria. More unusual branched fatty acids are the 10-methylhexadecanoic acid (**4**) and 9,14-dimethylpentadecanoic acid (**6**). They were identified on the basis of relative retention times and mass spectral data (Fig. 3A,C). 10-Methylhexadecanoic acid has been reported before in other planctomycetes [16].

In addition to these fatty acids, the chromatogram of this fraction showed some broad peaks eluting slightly later than the other fatty acids. These peaks are also well represented in the chromatograms of the TLFs (Fig. 1). The molecular ions in the mass spectra of these peaks (Fig. 4A,B) revealed molecular masses of 316 and 318 Da, suggesting C_{20} fatty acids with five and four rings or double bonds, respectively. Hydrogenation of the TLC fraction did, however, not result

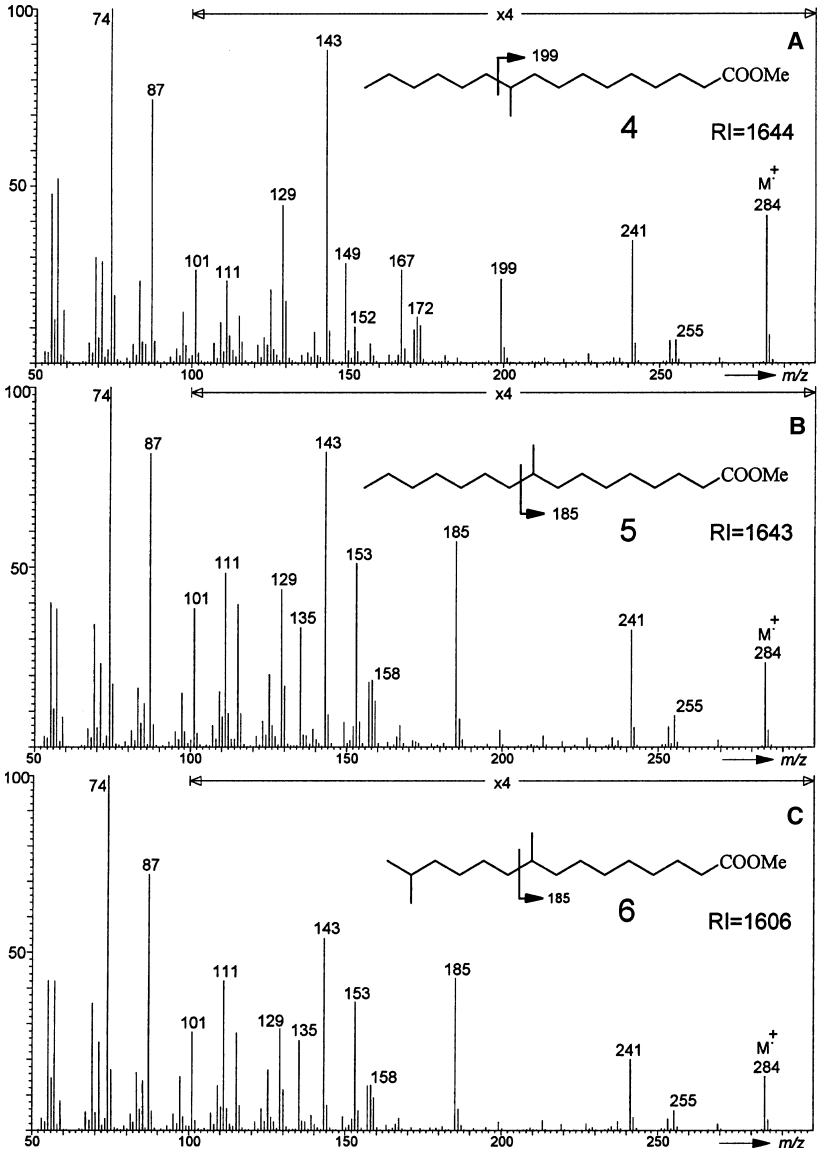


Fig. 3. Mass spectra (corrected for background) of (A) 10-methylhexadecanoic acid (**4**) methyl ester, (B) 9-methylhexadecanoic acid (**5**) methyl ester, and (C) 9,14-dimethylpentadecanoic acid (**6**) methyl ester.

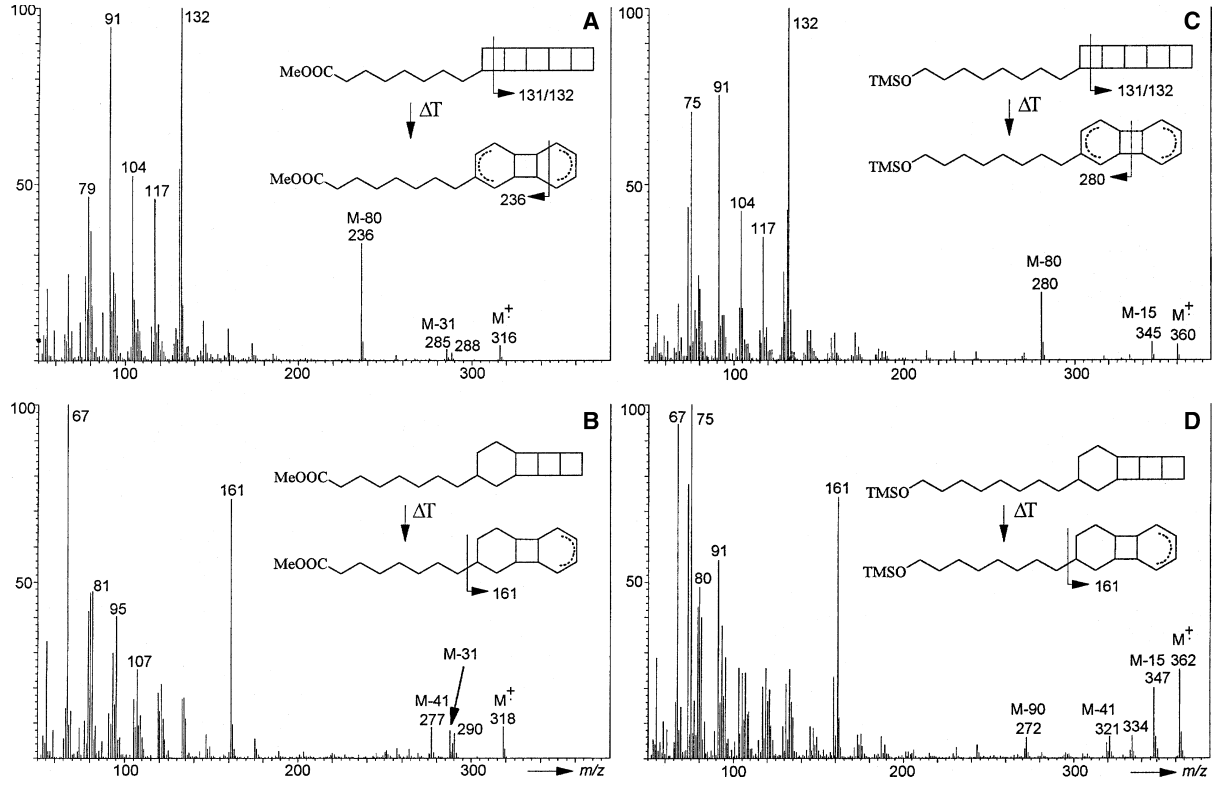
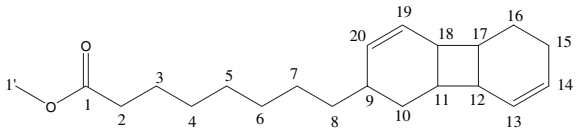


Fig. 4. Mass spectra (corrected for background) of (A) [5]-ladderane FAME (**7a**), (B) [3]-ladderane FAME (**7b**), (C) [5]-ladderane alcohol (**9a**) as TMS ether derivative, and (D) [3]-ladderane alcohol (**9b**) as TMS ether derivative. The structures of the original lipids are indicated in the spectra but it should be noted that the mass spectra reflect their thermal degradation products formed during GC analysis (see text).

in a shift of the molecular mass, indicating that no double bonds were present. As the mass spectra were difficult to interpret, one of these components was isolated by HPLC from the large batch of cell material and its structure was determined by high-field NMR spectroscopy [12]. Its structure (**7a**) is comprised of five linearly concatenated cyclobutanes substituted by a heptyl chain, which contained a carboxyl moiety at its ultimate carbon atom. All rings were found to be fused by *cis*-ring junctions, resulting in a staircase-like arrangement of the fused butane rings (designated A; Fig. 2), defined as [5]-ladderane [17]. This assignment is in good agreement with the obtained mass spectrum (Fig. 4A; in fact, this represents the spectrum of its thermal degradation products, see below) because most characteristic fragments can be explained.

Because the cyclobutane ring is already quite strained, and this certainly holds for the [5]-ladderane moiety composed of five linearly concatenated cyclobutane rings, the thermal lability of this fatty acid may explain the broad peak when this component is analysed with capillary GC. Indeed, the isolated ladderane fatty acid **7a** isolated by HPLC showed a similar broad peak when analysed by GC. When this component was analysed with a longer GC column (i.e. 60 m), the broad peak was resolved in several peaks with mass spectra almost identical to each other and the mass spectrum of the broad peak (Fig. 4A). This suggested that, indeed, the [5]-ladderane moiety is thermally unstable and that this component transforms during GC analysis into thermally more stable degradation products. To prove this, these products were isolated using preparative GC and the fractions obtained were studied using 1D and 2D ^{1}H NMR spectroscopy. This revealed that the ^{1}H NMR spectra of the products are all different from its precursor and all contain four olefinic protons, probably indicating breakdown of cyclobutane rings. The most abundant ($\approx$ 0.3 mg) and purest of the degradation products was further studied by high-resolution NMR spectroscopy to fully elucidate its structure and was identified as **7c** (Table 2). Its structure shows that it is indeed a thermal degradation product of the [5]-ladderane fatty acid. Cleavage and internal proton shifts of bonds between C-10 and C-19 and C-13 and C-16 of the [5]-ladderane moiety (designated A) lead to a moiety comprised of one cyclobutane ring with two condensed cyclohexenyl groups (C). This transformation results in a release of the

Table 2. Proton and carbon NMR data of one of the thermal degradation products of the ladderane fatty acid **7a**.

C-number[a]	Proton shift (p.p.m)	Carbon shift (p.p.m.)[b]				COSY correlations
		Primary	Secondary	Tertiary	Quaternary	
						
1	–				180	NA
2	2.33 (t, 2H)		33.8			H3
3	1.65 (bt, 2H)		24.7			H2, H4
8	1.53 (m, 2H)		35.0			H7, H9
9	1.88 (m, 1H)			36.3		H8, H10, H10′
10	1.78 (m, 1H)		32.0			H10′, H11
	2.02 (bt, 1H)					H9, H10, H18[c]
11	1.69 (bt, 1H)			43.6		H10, H12, H18
12	2.07 (m, 1H)			43.1		H11, H17, H13[d]
13	5.56 (dd, 1H)			129.4		H12, H14, H15[c]
14	5.68 (ddd, 1H)			126.7		H12[c], H13, H15
15	2.15 (m, 2H)		26.0			H13, H16, H16′, H12[c], H13[c]
16	1.86 (m, 1H)		28.7			H15, H16′, H17
	1.45 (m, 1H)					H15, H16, H17
17	1.75 (m, 1H)			35.2		H12, H16, H16′, H18[d], H11[c]
18	3.01 (bdd, 1H)			32.5		H11, H17, H19, H10″[c], H20[c]
19	5.64 (dd, 1H)			130.5		H18, H20, H17[c]
20	5.58 (d, 1H)			132.8		H19, H17[c,d], H18[c,d]
1′	3.69 (s, 3H)	51.2				None

[a] Signals for carbons C-4 to C-7 were not determined. [b] As determined by a HMBC experiment. [c] Long-range correlation. [d] Weak correlation.

internal steric strain of the [5]-ladderane moiety. The mass spectrum shown in Fig. 4A, thus, in fact represents that of a mixture of its thermal stabilization products.

The second broad peak (Fig. 1A), eluting slightly later than the thermal decomposition products of the [5]-ladderane fatty acid **7a**, possesses a molecular mass 2 Da higher. A fraction, isolated by HPLC, containing 25% of this component (the remaining part being **7a** and **8a**) was also studied by NMR spectroscopy. Its NMR spectrum showed strong similarities with that of the ladderane glycerol monoether **11a** (see below). The ring system (designated B) is comprised of three condensed cyclobutane and one cyclohexane moieties substituted by a heptyl chain, which contained a carboxylic moiety at its ultimate carbon atom, resulting in structure **7b**. Structurally and stereochemically it is almost identical to the [5]-ladderane fatty acid **7a**, except that two cyclobutane rings in A are transformed in a cyclohexyl ring by removal of the bond between C-13 and C-16, leading to the [3]-ladderane moiey B. The characteristic fragment ions in its mass spectrum (Fig. 4B) can be explained with this structural assignment. The [3]-ladderane fatty acid **7b** is evidently also not thermally stable, resulting in thermal stabilization during GC analysis and the broad peak shape. The fraction subjected to preparative GC to study the thermal degradation of the [5]-ladderane fatty acid **7a** (see above) also contained small amounts of the [3]-ladderane fatty acid **7b**, which enabled to provide a clue on its thermal stabilization products. The ^{1}H NMR spectrum of the product related to [3]-ladderane fatty acid **7b** was indeed different from the one after isolation by HPLC at ambient temperature; it clearly revealed the presence of two olefinic protons, suggesting that two cyclobutane rings were transformed into one cyclohexene ring (e.g. **7d** but the small amounts obtained precluded rigorous identification), analogous to the thermal degradation of [5]-ladderane fatty acid **7a**. Again, the mass spectrum presented (Fig. 4B) is, thus, derived from its thermal stabilization product(s).

The smaller broad peak eluting before the thermal stabilization products of the [5]- and [3]-ladderane fatty acids **7a** and **7b** (Fig. 1A) shows a mass spectrum similar to that of the mixture of thermal stabilization products of the [5]-ladderane fatty acid **7a** apart from the fact that the *m/z* values of the molecular ion and some of the characteristic ions are 28 Da lower. This indicates that this component **8a** represents a homologue with two carbon atoms less in the side-chain but with an identical [5]-ladderane moiety.

In our earlier publication [12], we reported the ladderane fatty acids as methyl esters. Subsequently, extraction of the cell material with pure dichloromethane (instead of a methanol/dichloromethane gradient) revealed that methylation of the fatty acids occurred during the extraction procedure, possibly by the methanol used in the normal extraction procedure.

Ladderane alcohols

Ladderane alcohols with structures (**9a–b**, **10a–b**) similar to those of ladderane fatty acids (**7a–b**, **8a–b**) were identified and occur in smaller relative amounts (Fig. 1). Examples of their mass spectra are depicted in Fig. 4C,D and show characteristics similar to those of ladderane fatty acids. Again the chromatographic peaks are broad, likely resulting from the formation of thermal stabilization products (e.g. **9c–d**, **10c–d**) during GC analysis.

Mono alkyl glycerol ethers

TLC separation resulted in one band dominated (92% by GC) by one component. This could be repeated using preparative column chromatography with the

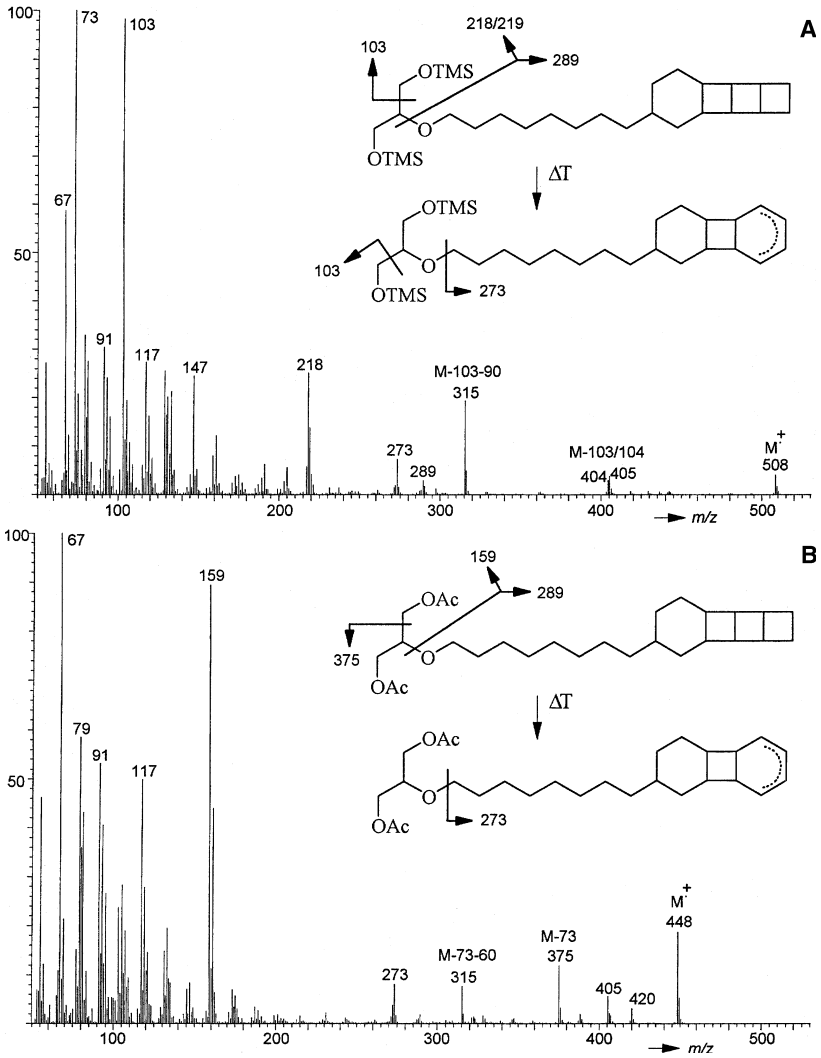


Fig. 5. Mass spectra (corrected for background) of the [3]-ladderane 2-alkyl glycerol monoether **11a** as (A) TMS ether derivative, and (B) acetate derivative. The structure of the original lipid is indicated in the spectra but it should be noted that the mass spectrum reflects its thermal degradation product formed during GC analysis (see text).

large batch of cell material, resulting in a fraction (CC7) almost exclusively consisting of one component (97% pure by GC). This component was, on basis of its mass spectrum after both silylation and acetylation (Fig. 5A,B, respectively), identified as an *sn*-2 glycerol monoalkyl ether with a C_{20} alkyl chain containing four rings or double bonds. Hydrogenation indicated that it did not contain any double bonds. Ether bond cleavage with HI and subsequent reduction of the formed iodide with $LiAlH_4$ [18] resulted in the generation of a C_{20} hydrocarbon containing four rings. The exact structure (**11a**) of glycerol ether was elucidated with high-field NMR spectroscopy [12]. The ladderane moiety is identical to that of ladderane fatty acid **7b**, i.e. composed of three linearly concatenated cyclobutane rings with a condensed cyclohexane ring (Fig. 2, moiety B). Although its peak shape in the gas chromatogram is substantially less broad than those of mixtures of thermal stabilization products of ladderane fatty acids **7c** and **7d** (Fig. 1A), it is likely that during GC analysis **11a** is transformed into thermal stabilization products (e.g. **11b**) analogous to what happens with ladderane fatty acid **7b**. However, because **11a** and **11b** are less volatile, the transformation is complete and has not resulted in a substantial loss of chromatographic resolution, probably because the transformation took place when **11a** was still focused at the beginning of the capillary column.

Small amounts of a component similar to glycerol monoether **11a** but lacking one of the OH groups (**12a**) was identified based on its mass spectrum. It occurs in relatively small amounts in strain Dokhaven of *Candidatus* 'B. anammoxidans'.

Glycerol diethers and mixed glycerol ether/esters

The last part of the chromatogram of the TLF shows a complex mixture (Fig. 1A) of compounds which were identified as 1,2-di-*O*-alkyl *sn*-glycerols (**13**) and 1-acyl-2-*O*-alkyl *sn*-glycerols (**14**). They were concentrated in a fraction obtained by column chromatography (CC5), which enabled to study their structure in detail. Base hydrolysis of this fraction resulted in the removal of some of these components (Fig. 6) and the generation of substantial amounts of the ladderane *sn*-2 mono alkyl glycerol ether **11a** and smaller amounts of the regular [iso-C_{16} (**3**), *n*-C_{16}, 10-methyl hexadecanoic acid (**5**) and 9,14-dimethyl pentadecanoic acid (**6**)] and ladderane (predominantly **7a**) fatty acids. The components that could be hydrolysed are thus likely glycerol ether/esters, which contain at the *sn*-2 position a [3]-ladderane moiety whereas they contain at the *sn*-1 position an ester bound ladderane or regular fatty acid.

The cluster of peaks that were not affected by base hydrolysis (Fig. 6B) represent dialkyl glycerol diethers (**13**), characterized by a base peak ion at *m/z* 131 in their mass spectra [19,20]. All mass spectra also contained fragment ions at *m/z* 273 and 315 (Fig. 7A,C), also prominent in the mass spectrum of the [3]-ladderane alkyl glycerol monoether **11a** (Fig. 4A), indicating that all diethers have this structural element in common. The identity of the second ether-bound alkyl side-chain

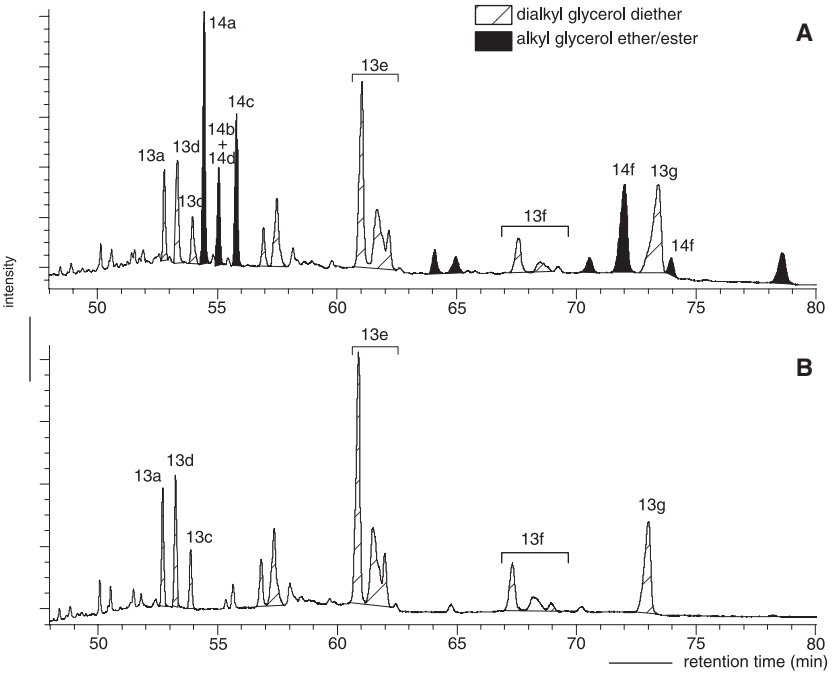


Fig. 6. Partial GC traces (reflecting the isothermal part of the temperature program) of fraction CC5 (fraction 5 obtained by preparative column chromatography of the large batch of cell material) of the extract of *Candidatus* 'B. anammoxidans' strain Delft containing the 1,2-di-*O*-alkyl *sn*-glycerols and 1-*O*-alkyl, 2-acyl, *sn*-glycerols (A) before and (B) after base hydrolysis. Components are indicated with numbers relating to structures indicated in Fig. 2.

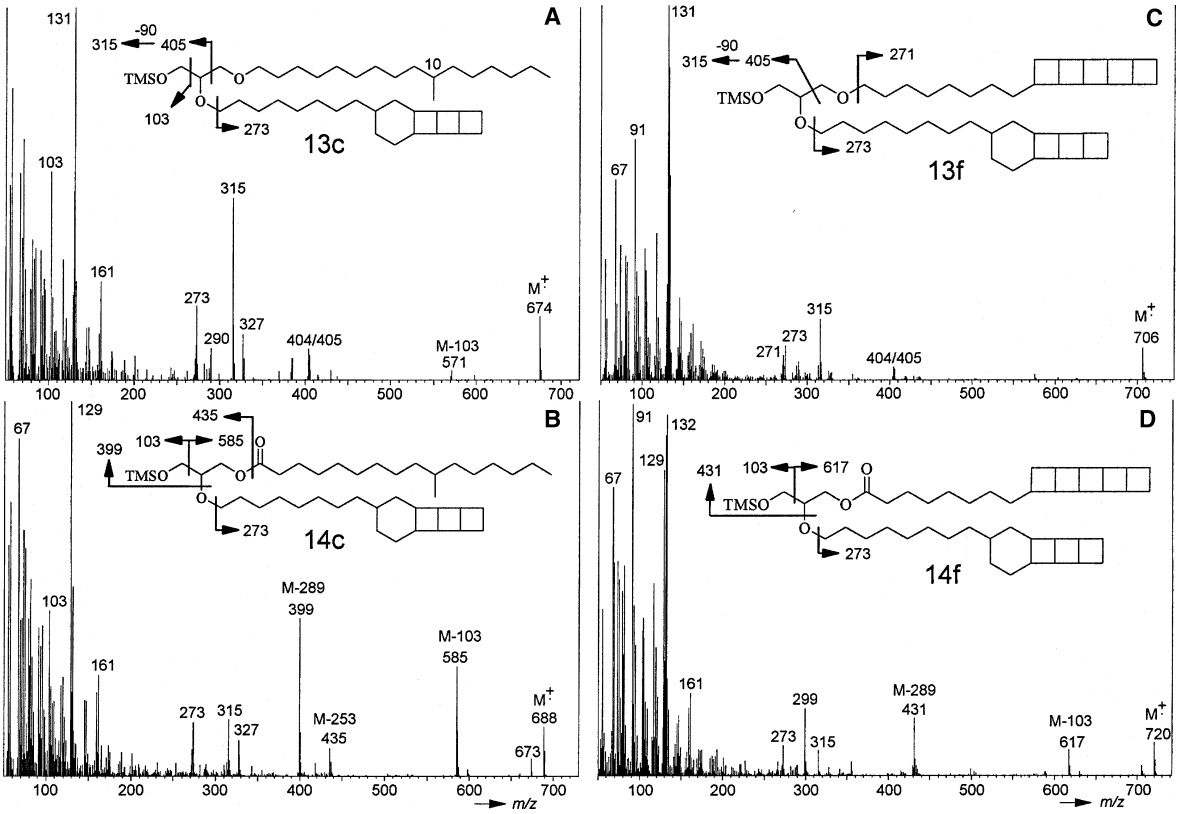


Fig. 7. Mass spectra (corrected for background) of ladderane dialkyl glycerol diethers **13c** (A) and **13f** (C) and the corresponding glycerol mixed ether/esters **14c** (B) and **14f** (D), all analysed as TMS derivatives. The structure of the original lipid is indicated in the spectra but it should be noted that the mass spectrum reflects its thermal degradation product formed during GC analysis (see text).

was established by the molecular mass, other specific fragment ions in the mass spectrum and the relative retention time. In this way two type of dialkyl glycerol diethers were identified: one containing two ladderane moieties (**13e**–**13 g**) and the other containing one ladderane moiety and one acyclic, branched or normal alkyl group (**13a**–**13d**) (Fig. 6). This latter 'mixed'-type glycerol diether has previously been reported in the biomass of an anaerobic wastewater plant, where annamox bacteria belonging to the *Scalindua* genera comprised 20%. In that case, a mixed ladderane dialkyl glycerol diether, in which the second alkyl chain was comprised of an n-C_{14} moiety, was unambiguously identified by isolation and high-field 2D NMR studies [21]. The mass spectra and relative retention time data of the diethers reported here are consistent with those of the unambiguously identified 'mixed' diether. The glycerol diethers containing two ladderane moieties (**13e**–**13 g**) are always represented by more than one peak in the chromatogram (Fig. 6A). This is likely due to the fact that several isomers of thermal stabilization products were formed during GC analysis.

Smaller amounts of di-*O*-pentadecyl glycerol diether (**15a**–**c**) were also encountered, especially in the strain Dokhaven (see below). They were identified on basis of comparison of mass spectral data published previously [19]. Measurement of their relative retention time data indicated that the ether-bound pentadecyl chains are branched (iso or anteiso).

The mass spectra of the 1-acyl-2-*O*-alkyl *sn*-glycerols contain a characteristic fragment ion at *m/z* 129 and the loss of [3]-ladderane alkyl ether (M – 289) and acyl fragments (Fig. 7B,D). Together with the molecular mass (determined from the molecular ion in the mass spectra) and the distribution of the fatty acids released upon base hydrolysis, this resulted in the structural assignment of these components. Again these components are comprised of two groups, i.e. one containing two ladderane moieties (**14e**–**14g**) and the other containing one ladderane moiety and one acyclic, branched or normal alkyl group (**14a**–**14d**).

If cells of the culture were extracted with a modified Bligh and Dyer extraction method to be able to identify glycerol diethers and ester/ethers with polar head

groups, GC/MS analysis after acid hydrolysis of the most polar subfraction of this extract (i.e. the group of lipids with polar head groups) indicated that a substantial part of the glycerol diethers and ester/ethers did indeed contain a polar head group.

Lipid compositions of other planctomycete cultures

The culture of *Candidatus* 'B. anammoxidans' strain Dokhaven contained essentially the same lipids as that of *Candidatus* 'B. anammoxidans' strain Delft (cf. Figs 1 and 8A) albeit in slightly different relative quantities. One peculiar difference was that the dominant branched fatty acid in the strain Dokhaven is the 9-methylhexadecanoic acid instead of the 10-methylhexadecanoic acid in strain Delft. In *Candidatus* 'K. stuttgartiensis' the ladderane lipids were less abundant. In fact, we were only able to detect ladderane lipids after acid hydrolysis of the residue after extraction (Fig. 8B). This may relate to the polar head groups attached to the ladderane glycerol backbone.

Two planctomycetes, *Pirellula marina* and *Gemmata obscuriglobus*, phylogenetically distantly related to the anammox bacteria [1], were also examined for the presence of ladderane membrane lipids and were shown not to contain these characteristic molecules.

Discussion

To the best of our knowledge, the ladderane lipids are the first natural products identified with the extremely strained linearly concatenated cyclobutane moieties. Bacterial membrane lipids are known to contain cyclopropane [22], cyclohexane and cycloheptane rings [23], and thermophilic [24] and mesophilic [25] archaea produce glycerol dialkyl glycerol tetraethers with cyclopentane and cyclohexane moieties. However, cyclobutane moieties are not common in nature. Miller and Schulman [17] performed theoretical studies on linearly concatenated ladderanes and indicated their very strained nature. Our study confirms this finding because the ladderane fatty acids are thermally labile and cannot be analysed intact by GC. This complicates their analysis in bacterial cultures and we are currently developing a method using HPLC coupled to MS to overcome this problem. Our previous study [12] indicated that HPLC does not result in structural modification of the ladderane lipids.

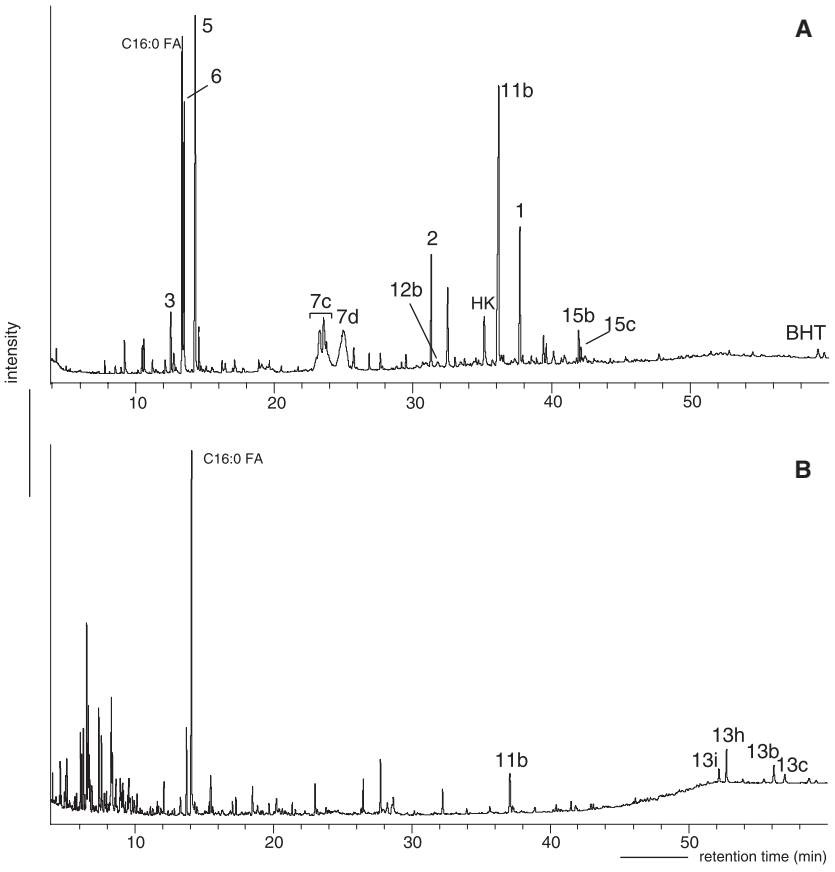


Fig. 8. Gas chromatograms of (A) the TLF of a 99.5% pure suspension of *Candidatus* 'B. anammoxidans' strain Dokhaven, and (B) the TLF after acid hydrolysis of the residue of the cell material of *Candidatus* 'K. stuttgartiensis' after lipid extraction and base hydrolysis. Fatty acids and alcohols were derivatized to the corresponding methyl esters and TMS ethers prior to GC analysis. Numbers refer to structures indicated in Fig. 2. FA, fatty acid; HK, hopanoid ketone.

The natural occurrence of these strained ladderane membrane lipids indicates that they must fulfil a special function in the cells of the anammox bacteria. We have previously investigated the location of the ladderane lipids in the cell membrane by enrichment of intact anammoxosomes from cells of *Candidatus* 'B. anammoxidans' strain Dokhaven [12]. Lipid analysis showed a strong enrichment in ladderane lipids in the enriched anammoxosome fraction: the characteristic branched fatty acids (i-C_{16}, 9-methyl hexadecanoic acid and 9,14-dimethyl pentadecanoic acid), which have also been reported in other planctomycetes [16], were completely absent. This suggests that these lipids predominantly comprise the outer membrane, whereas the ladderane lipids are part of the membrane of the anammoxosome. Modelling studies [12] have indicated that a membrane composed of ladderane lipids could form a denser membrane than a conventional membrane composed of diacyl glycerols. This dense membrane is thought to contain the toxic intermediate of the anammox reaction, hydrazine, in the anammoxosome and thus be essential for the functioning of anammox bacteria. In addition, the relatively impermeable ladderane membrane is thought to be able to generate and maintain a proton motive force for ATP synthesis [26]. That planctomycetes not capable of anammox and not containing an anammoxosome, such as *Gemmata* and *Pirellula*, do not produce ladderane lipids is in good agreement with the idea that ladderane lipids are essential for performing the anammox reaction. Evidence from different sources indicates that also the third genus of anammox bacteria, *Candidatus* 'Scalindua', although not yet available in enrichment culture, also produces ladderane lipids [6,8,21]. In summary, our data show that there is a phylogenetically distinct group in the planctomycetes that is equipped with a unique set of membrane lipids which enable them to perform anammox.

Our data also show that the molecular composition of the ladderane lipids is complex. We identified ladderane fatty acids, fatty alcohols, glycerol monoethers and diethers, and mixed glycerol ether/esters. In addition, glycerol diethers and ether/esters were identified with one ladderane moiety and one alkyl chain and even glycerol diethers with two alkyl chains. Apparently, a mix of ladderane (and perhaps other) membrane lipids is required to fulfil the physical requirements of the membrane of the anammoxosome. The presence of the ether-linkages in the membrane lipids (linking the lipids to the glycerol backbone) is somewhat unexpected in members of the Bacteria. Ether linkages were once thought to be the hallmark for the Domain Archaea [27]. But although glycerol diethers are rare amongst bacteria, they have been detected in thermophiles [28–30] and in sulfate-reducing bacteria of a microbial consortium capable of the anaerobic oxidation of methane [31,32]. Mixed glycerol ether/esters were found in deep-branching thermophilic bacteria such as *Aquifex* [30] and *Thermotoga* [33,34], in two mesophilic sulfate-reducing bacteria [35], and in a member of the propionibacteria [36]. Glycerol ethers do occur in the Domain Bacteria but seem to be most abundant in, but are not limited to, species representing early branches in the bacterial domain in the tree of life based on rRNA genes. This would be consistent with the recently suggested position of the planctomycete phylum closest to the root of the bacterial domain in the phylogenetic tree of life [2].

The biosynthesis of the ladderane lipids would require a unique set of enzymes to be able to put together such a strained molecule. At present, we can only speculate about the biosynthetic route as no obvious intermediates were detected. There is a close structural resemblance between ladderane lipids containing moiety A and B (Fig. 2); it only requires one additional C-C bond in the cyclohexyl moiety of B (by removal of specific hydrogen atoms) to form the [5]-ladderane moiety A. This provides a hint to the possible biosynthetic route involved. Perhaps, a C_{12} macrocycle is formed by ring closure at C-9 and C-20 of a C_{20} polyunsaturated fatty acid. The cyclobutane rings could subsequently be formed by C-C bond formation requiring a special and as yet unknown enzyme. Biosynthesis of the [3]-ladderane moiety would then require one cyclization step less than the [5]-ladderane moiety. Additional work is required to test this hypothesis.

Experimental procedures

Cultures

Cells were grown in sequencing batch reactors as described elsewhere [37], enabling the efficient retention of biomass of these slowly growing bacteria. The anammox bacteria were physically purified from the enriched retentostat cultures by an optimized Percoll density gradient centrifugation [38]. *Candidatus* 'B. anammoxidans' strain Delft was enriched from an anaerobic wastewater treatment reactor from Gist Brocades, Delft, the Netherlands. *Candidatus* 'B. anammoxidans' strain Dokhaven was enriched from an anaerobic wastewater treatment plant in Rotterdam, the Netherlands. *Candidatus* 'K. stuttgartiensis' was also enriched from the later wastewater treatment plant.

Lipid analysis

Cells and enrichment fractions were ultrasonically extracted with methanol (MeOH), MeOH/dichloromethane (DCM) (1 : 1, v/v), and DCM (×3). An aliquot (≈ 1 mg) of the combined extracts was methylated with diazomethane in diethyl ether, filtered over a small pipette filled with silica with ethyl acetate as the eluent, and silylated with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) in pyridine at 60 °C for 15 min. These derivatized total lipid fractions were analysed with GC and GC-MS.

Polar lipid analysis

Cells were extracted with a DCM/MeOH/H_2O (1/2/0.8, v/v/v) mixture and subsequently DCM and H_2O were added to obtain a DCM layer. The obtained DCM extract was subsequently separated by column chromatography on silicagel-60 in a DCM, an acetone and a methanol fraction [39]. The DCM fraction was, after adding an internal standard (6,6-d_2−3-methyleicosane), silylated with BSTFA and analysed by GC. An internal standard was added to an aliquot of the acetone-fraction and analysed by GC and GC/MS after silylation. The remaining part of the acetone fraction was hydrolysed with 5% (v/v) HCl/MeOH by refluxing for 3 h. Subsequently, the hydrolysate was neutralized with KOH (pH 6) and extracted with DCM, dried over $NaSO_4$, silylated with BSTFA and analysed by GC. The methanol fraction was, after adding an internal standard, hydrolysed with 5% (v/v) HCl/MeOH, neutralized, extracted with DCM, dried over $NaSO_4$, silylated with BSTFA and analysed by GC and GC/MS. Glycerol diethers and ether/esters were quantified in all fractions by integration of appropriate peak areas.

TLC

An aliquot (≈ 5 mg) of the extract was methylated with diazomethane and separated by TLC (Merck, Kieselgel 60; 0.25 mm) according to Skipski [40]. The obtained bands were scraped off and extracted with ethyl acetate (ultrasonically, ×3). The TLC fractions 4–8 (Table 1) were silylated with BSTFA in pyridine at 60 °C for 15 min and all fractions were analysed by GC and GC/MS.

Hydrogenation

The fatty acid methyl ester (FAME) fraction obtained after TLC was hydrogenated (PtO_2) in ethyl acetate with a few droplets of acetic acid for 2 h and stirred for one night. After evaporation of the ethyl acetate the sample was cleaned over a small pipette with Na_2CO_3 and $MgSO_4$ in DCM and analysed by GC.

HI/LiAlH4 treatment

Cleavage of ether bonds with HI and subsequent reduction of the formed iodides was performed as described previously [18].

Isolation of ladderane lipids

For isolation of lipids the extract (54 mg) of a large batch (45 g dry weight) of *Candidatus* 'B. anammoxidans' (strain Delft) was separated with column chromatography (Al_2O_3; 7 × 1.3 cm, V_0 = 5 mL) by elution with 10 mL hexane/DCM, 9 : 1 v/v (CC1), 10 mL hexane/DCM, 8 : 2 v/v (CC2), 10 mL hexane/DCM, 7 : 3 v/v (CC3), 10 mL hexane/DCM, 6 : 4 v/v (CC4), 10 mL hexane/DCM, 1 : 1 v/v (CC5), 10 mL DCM (CC6) and 13 mL methanol/DCM, 1 : 1 v/v (CC7). The ladderane glycerol monoether was obtained in pure form in the most polar fraction CC7. Preparative HPLC was carried out on the methyl ester fraction (CC3) to isolate the ladderane fatty acid methyl esters [12]. This resulted in two fractions containing ladderanes, one containing [5]- and [3]-ladderane FAMEs (1.8 mg) in 3 : 1 ratio (as determined by GC) and the other (1.0 mg) in an 85 : 15 ratio. These fractions were first studied individually by ^{1}H NMR and COSY and subsequently combined for further study by high-field NMR. Preparative GC was used to isolate thermal degradation products from this combined mixture of ladderane FAMEs. An aliquot of fraction CC5 was subjected to base hydrolysis (0.5 M KOH in methanol) under reflux for 1 h.

Preparative capillary GC (PCGC)

PCGC was performed on a HP 6890 gas chromatograph equipped with a Gerstel temperature programmable injector, a 60 m × 0.25 mm i.d. CP-SIL 5CB capillary column (d.f. = 0.25 μm) and a Gerstel preparative fraction collection system cooled with a cryostatic bath at 16 °C. Details of the trapping procedure have been described elsewhere [41]. Samples were dissolved in ethyl acetate and injected at 70 °C. The oven temperature was rapidly raised to 220 °C (20 °C·min^{-1}) and further programmed at 2 °C·min^{-1} to 260 °C and then at 8 °C·min^{-1} to 320 °C. Four hundred and fifty injections were performed to trap sufficient material.

NMR spectroscopy

Isolated lipids were solved in $CDCl_3$. NMR spectroscopy was performed on a Varian Unity Inova 500 (Palo Alto, CA, USA), a Bruker DRX600 and a Bruker AV-750 (Rheinstetten, Germany) spectrometer equipped with an SWBB probe, an inverse TBI-Z probe with a pulsed field gradient (PFG) accessory, and a BBI-zGRAD probe, respectively. All experiments were recorded at 300 K in

$CDCl_3$. Proton and carbon chemical shifts were referenced to internal $CDCl_3$ (7.24/77.0 p.p.m). In the 2D ^{1}H-^{13}C COSY the number of complex points and sweep widths were 2000 points/6 p.p.m. for ^{1}H and 512 points/150 p.p.m. for ^{13}C. In the 2D ^{1}H-^{1}H COSY the number of complex points and sweep widths were 2000 points/5.5 p.p.m. Quadrature detection in the indirect dimension was achieved with the time-proportional-phase-incrementation method. The data were processed with VARIAN or NMR SUITE software packages. After apodization with a 90 shifted sinebell, zero filling to 512 real points were applied for the indirect dimensions. For the direct dimensions zero filling to 4000 real points, Lorentz transformations were used.

GC and GC/MS

GC was performed using a Fisons GC8000 instrument, equipped with an on-column injector and a flame ionization detector (FID). A fused silica capillary column (25 m × 0.32 mm) coated with CP Sil5 (d.f. 0.12 μm) was used with He as carrier gas. The samples were injected at 70 °C and the oven temperature was programmed to 130 °C at 20 °C·min^{-1} and then at 4 °C·min^{-1} to 320 °C, at which it was held for 10 min. GC/MS was performed on a HP5890 gas chromatograph interfaced with a VG Autospec Ultima mass spectrometer operated at 70 eV with a mass range of *m/z* 40–800 and a cycle time of 1.7 s (resolution 1000). The gas chromatograph was equipped with a fused silica capillary column same as described for GC. The carrier gas was helium. The same temperature programme as for GC was used.

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