Ladderane phospholipids in anammox bacteria comprise phosphocholine and phosphoethanolamine headgroups

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Abstract

Anammox bacteria present in wastewater treatment systems and marine environments are capable of anaerobically oxidizing ammonium to dinitrogen gas. This anammox metabolism takes place in the anammoxosome which membrane is composed of lipids with peculiar staircase-like 'ladderane' hydrocarbon chains that comprise three or four linearly concatenated cyclobutane structures. Here, we applied high-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry to elucidate the full identity of these ladderane lipids. This revealed a wide variety of ladderane lipid species with either a phosphocholine or phosphoethanolamine polar headgroup attached to the glycerol backbone. In addition, *in silico* analysis of genome data gained insight into the machinery for the biosynthesis of the phosphocholine and phosphoethanolamine phospholipids in anammox bacteria.

Introduction

Anammox bacteria affiliated to the order of the *Planctomycetes* are microorganisms of special interest as they are capable of performing the anaerobic conversion of ammonium and nitrite to dinitrogen gas (Strous *et al.*, 1999). This metabolism occurs in the subcellular organelle-like structure known as the anammoxosome, in which the toxic intermediate hydrazine is maintained and which possesses a proton motive force over its membrane (van Niftrik *et al.*, 2004). Phylogenetic studies using the 16S rRNA gene revealed thus far three different genera of anammox bacteria: *Candidatus* 'Brocadia', *Candidatus* 'Kuenenia' and *Candidatus* 'Scalindua'. A representative of the latter genus, '*Candidatus* Scalindua sorokinii,' contributes significantly to the nitrogen cycling in the ocean and its sediments (Kuypers *et al.*, 2003, 2005). Apart from marine ecosystems, the habitats of anammox bacteria also include wastewater treatment plants and freshwater sediments. The anammox process is applied to remove nitrogen in oxygen-limited wastewater (Jetten *et al.*, 2003).

Recently, the membrane lipid composition of anammox bacteria has been studied using gas chromatography-mass spectrometry (GC-MS) and high field nuclear magnetic resonance (NMR) spectrometry, thereby revealing extraordinary core lipid structures (Sinninghe Damsté *et al.*, 2002, 2004). These lipids were found to comprise hydrocarbon chains with three or four linearly concatenated cyclobutane rings, designated [3]- and [5]-ladderanes, respectively, with the [3]-ladderanes condensed to a cyclohexane ring (Fig. 1, substructures a and b). These ladderane structures are either esterified or etherified to a glycerol backbone via an alkyl chain. While the *sn*-2 position of the glycerol backbone is predominantly ether-linked to a C_{20}-[3]-ladderane moiety, a large variety of alcohols and fatty acids are attached at the *sn*-1 position either via an ether or an ester linkage. Molecular modeling indicated that the ladderane lipids surrounding the anammoxosome yield an exceptional rigid biomembrane, implicating that this dense barrier may serve to separate the remainder of the cell from the intermediate hydrazine and to maintain concentration gradients and a proton motive force during slow metabolism (Sinninghe Damsté *et al.*, 2002).

Although the individual structural components of the ladderane lipids have been characterized, much remains unknown about the structural identity of the intact

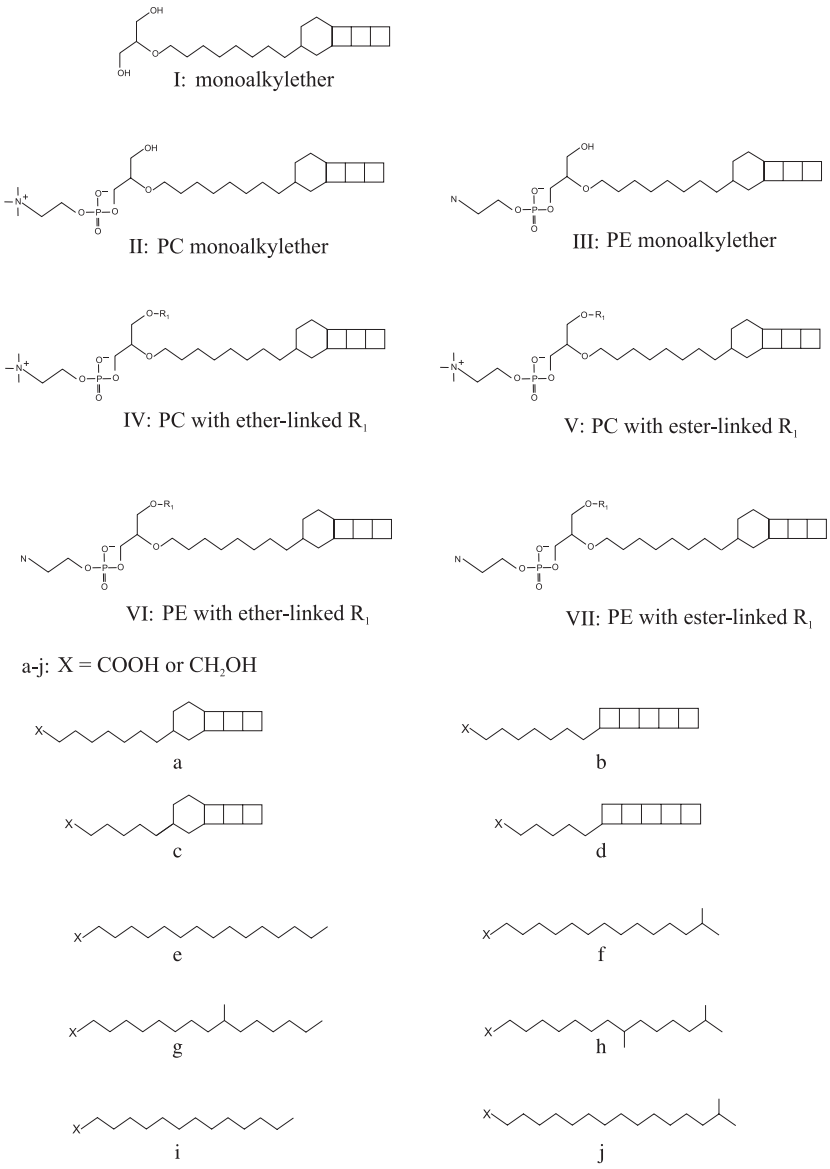

Fig. 1. Structures of anammox ladderane lipids. The molecular structure of ladderane monoalkyl glycerol ether lipid is depicted (I). Structures II and III represent the phosphocholine and phosphoethanolamine monoalkyl glycerol ether lipids, respectively. The general structure of phosphocholine diether and phosphocholine ester/ether ladderane lipids is shown by IV and V, respectively. The general structure of phosphoethanolamine diether and phosphoethanolamine ester/ether ladderane lipids is shown by VI and VII, respectively. Other structures detected in this study are: (a) C_{20}-[3]-ladderane; (b) C_{20}-[5]-ladderane; (c) C_{18}-[3]-ladderane; (d) C_{18}-[5]-ladderane; (e) *n*-C16; (f) *iso*-C16; (g) 10-methylhexadecane; (h) 9,14-dimethylpentadecane, (i) *n*-C14; (j) *iso*-C17.

phospholipids as they reside in the membrane, in particular the nature of the headgroups. To elucidate the full structure of the intact ladderane lipid species, we have analyzed the intact phospholipids by high-performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). We demonstrated that phosphocholine (PC) and phosphoethanolamine (PE) are the major headgroup moieties of ladderane lipids and that the molecular diversity of the anammox lipid species is predominantly accomplished by the wide variety of hydrocarbon tails at the *sn*-1 position of the glycerol backbone.

Materials and methods

Cell cultures

Anammox cell material was obtained from an oxygen limited waste water treatment plant of Paques B.V. (Balk, the Netherlands), converting nitrite and ammonium to dinitrogen gas. Fluorescence *in situ* hybridization (FISH) analysis confirmed the dominance of anammox bacteria in the reactor.

Sample preparation

Freeze dried cell material was used to isolate intact polar lipids according to the method of Bligh & Dyer (1959). The

total lipid fractions were analyzed by HPLC-ESI-MS/MS and MALDI-TOF-MS. In addition, the extract was acid hydrolyzed for GC/MS analysis as described previously (Sinninghe Damsté *et al.*, 2005).

HPLC-ESI-MS/MS analysis

Intact ladderane lipids were analyzed according to Sturt *et al.* (2004) with some modifications. An Agilent 1100 series LC (Agilent, San Jose, CA) was used, equipped with thermostatted autoinjector and column oven, coupled to a Thermo TSQ Quantum Ultra EM triple quadrupole mass spectrometer equipped with an Ion Max source with electrospray ionization (ESI) probe (Thermo Electron Corporation, Waltham, MA). Separation was achieved on an Inertsil diol column (250 mm × 2.1 mm, 5 μm particles; Alltech Associates Inc., Deerfield, IL) maintained at 30 °C. In accordance with Sturt *et al.* (2004), the following linear gradient was used with a flow rate of 0.2 mL min^{-1}: 100% A to 35% A: 65% B over 45 min, maintained for 20 min, then back to 100% A for 20 min to re-equilibrate the column, where A = hexane/2-propanol/formic acid/14.8 M NH_{3aq} ratios 79 : 20 : 0.12 : 0.04 [volume in volume in volume in volume, v/v/v/v] and B = 2-propanol/water/formic acid/14.8 M NH_{3aq} ratios 88 : 10 : 0.12 : 0.04 (v/v/v/v). For MS detection, source parameters were optimized using loop injections of standard phospholipids (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-*rac*-glycerol, 1,2-dipalmitoyl-*sn*-glycero-3-phosphate, and soya L-α-phosphatidylinositol, Avanti Polar Lipids, Alabaster, AL) into a stream of 0.2 mL min^{-1} of eluent A. ESI settings were as follows: capillary temperature 250 °C, sheath gas (N_2) pressure 49 (arbitrary units), auxiliary gas (N_2) pressure 21 (arbitrary units), spray voltage 4.2 kV, and source CID − 14 V. The total lipid extract was analyzed by an MS routine where a positive ion scan (*m/z* 250–1000) was followed by a data-dependent MS/MS experiment where the base peak of the mass spectrum was fragmented [collision energy 20 V, collision gas (argon) 0.8 m Torr]. Phosphocholine lipid species were monitored by parental ion scanning for *m/z* 184, whereas phosphoethanolamine lipid species were detected by neutral loss scanning for *m/z* 141.

MALDI-TOF-MS analysis

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was performed on a Biflex III instrument (Bruker, FRG) operating in the reflectron mode. A small volume (0.5 μL) of the total lipid extract was spotted on a steel target plate. After drying a second layer (0.5 μL) of matrix solution (10 mg 2,5-dihydroxybenzoic acid in 1 mL methanol with 10 μL of 10% trifluoroacetic acid) was added. Triton X-100 was used for calibration (Ishida *et al.*, 2002).

Results and discussion

GC-MS analysis of anammox cell material

To date, no pure culture of any anammox bacterium is available. In the present study, we investigated anammox biomass obtained from a waste treatment nursery reactor in Balk, the Netherlands. The relative abundance of anammox cell material was determined by FISH microscopy using probe KST1273 targetting *Kuenennia stuttgartiensis* (Schmid *et al.*, 2000), showing that *c.* 70% of the microbial population comprised anammox cells.

To gain insight into the composition of the basic lipid structures in the anammox cell material, the total lipid extract was subjected to GC-MS analysis after acid hydrolysis. Similar to previous studies (Sinninghe Damsté *et al.*, 2005), we observed the bacterial membrane rigidifiers squalene, diploptene, diplopterol and a hopanoid ketone (Fig. 2). In addition, palmitic acid (*n*-C16) (e, for structures, see Fig. 1), and the branched fatty acids 14-methylpentadecanoic (*iso*-C_{16}) (**f**) and 10-methylhexadecanoic acid (g) were detected. Other components were represented by the three broad peaks that comprised either the [3]-ladderane or the [5]-ladderane alkyl moiety (a, b and d). In agreement with our previous work (Sinninghe Damsté *et al.*, 2005), the most prominent peak in the GC trace represented the hydrolyzed *sn-2*-monoalkyl glycerol ether lipid (I, Figs 1 and 2). Furthermore, glycerol diether lipids with a 14-methylpentadecanoic or 10-methylhexadecanoic tail were detected. As the observed lipids distribution is almost identical to that of the 99.5% pure suspension of *Candidatus*

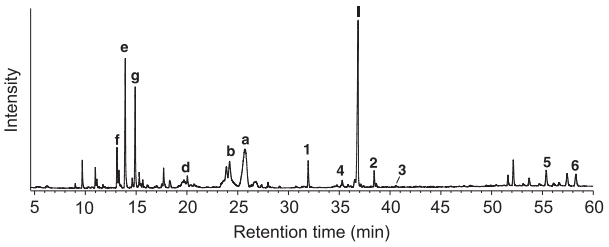

Fig. 2. Basic lipid structures of anammox cell material determined by gas chromatography-mass spectrometry (GC-MS). The total lipid extract of anammox bacteria obtained from waste water treatment plants was acid hydrolyzed before GC-MS, thereby identifying: (1) squalene; (2) diploptene; (3) diploterol; (4) hopanoid ketone; (a) C_{20}-[3]-ladderane; (b) C_{20}-[5]-ladderane; (d) C_{18}-[5]-ladderane; (e) *n*-C16; (f) 14-methylpentadecanoic acid; (g) 10-methylhexadecanoic acid; (I) mono alkyl glycerol ether with [3]-ladderane; (5) ether/ester glycerol with C_{20}-[3]-ladderane and *iso*-C16; (6) ether/ester glycerol with C_{20}-[3]-ladderane and 10-methylhexadecanoic acid. Numbers refer to structures in Fig. 1.

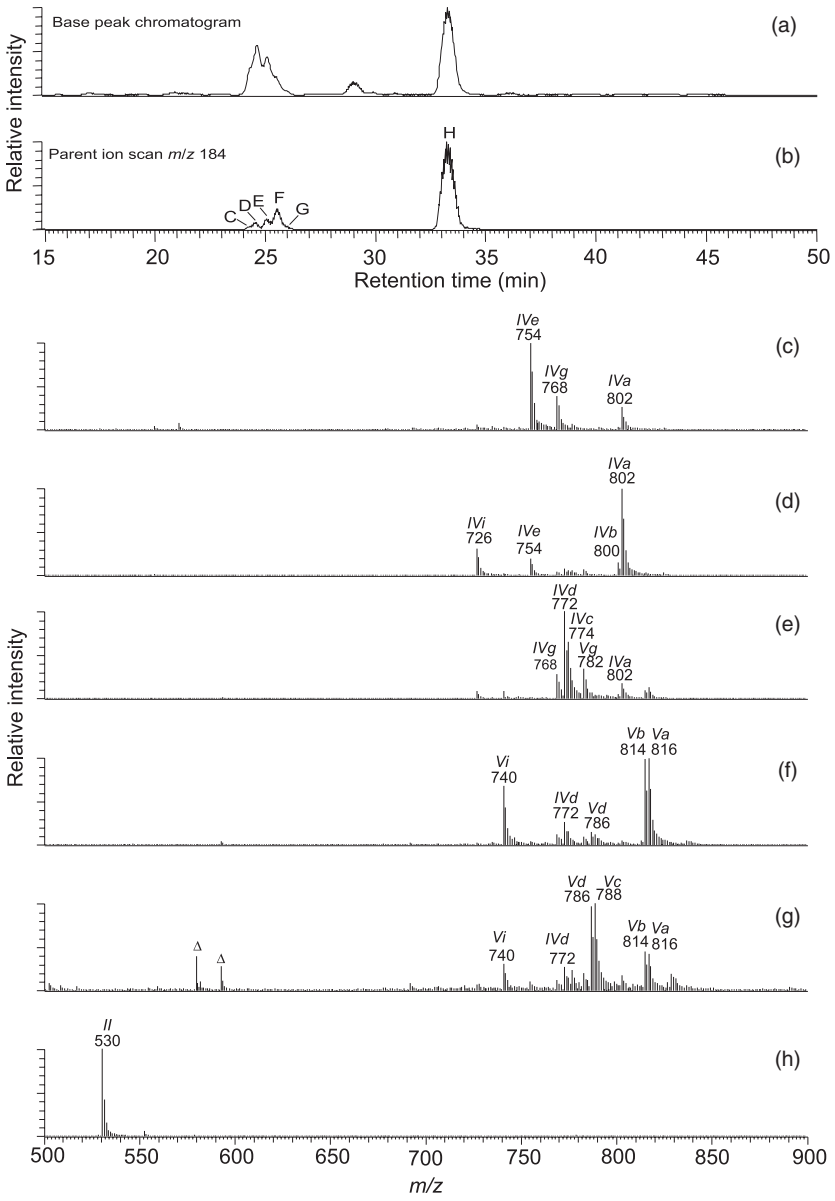

Fig. 3. High performance liquid chromatography-ion base chromatogram and electrospray ionization tandem mass spectrometry analysis of the total lipid extract of anammox cells for phosphocholine lipid species. (a) Full scan analysis of the base peak chromatogram of a total anammox lipid extract. (b) Base peak mass chromatogram obtained by parent ion scanning for *m/z* 184. (c–h) Mass chromatograms of $[M+H]^+$ ions of the identified ladderane phosphocholine lipids. For more details, see Results section. For structures, see Fig. 1. The Δ-marked peaks denote unknown components.

'B. anammoxidans' cells (Sinninghe Damsté *et al.*, 2005), we concluded that this material allows us to investigate intact phospholipids in anammox bacterial cells.

Identification of intact anammox lipids

To determine the structural identity of the intact ladderane lipid species, the anammox lipid extract was subjected to HPLC-ESI-MS/MS (Sturt *et al.*, 2004). The most prominent signal in the base peak chromatogram was observed at 33 min (Fig. 3a). Full scan MS analysis showed that this peak corresponds to a single component with *m/z* 530, most likely the $[M+H]^+$ ion of the component, whereas MS/MS analysis revealed a typical fragment with *m/z* 184, characteristic for a phosphocholine lipid headgroup (Fig. 3b) (Brügger *et al.*, 1997; Sturt *et al.*, 2004). Based on this, the molecular ion and the results obtained by GC-MS, we identified the major component as the C_{20}-[3]-ladderane mono-alkylether with a phosphocholine headgroup moiety (II, Fig. 1).

The second most intense signal in the HPLC base peak chromatogram appeared at ~25 min (Fig. 3a). Both full scan MS and MS/MS analysis by parental ion scanning for *m/z* 184 indicated that this cluster of peaks is composed of a mixture of phosphocholine lipid species (Figs 3a and b). Based on the molecular masses, the typical fragment with

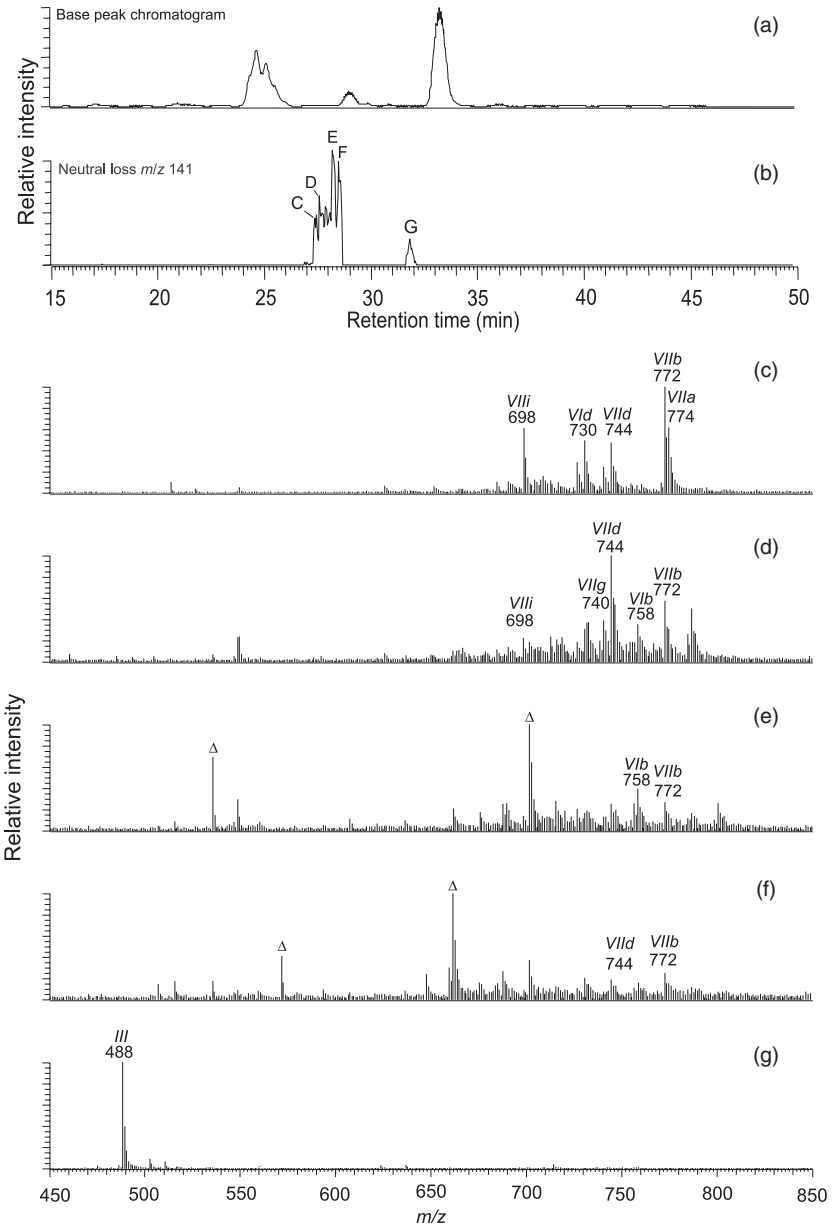

Fig. 4. High performance liquid chromatography electrospray ionization tandem mass spectrometry analysis of the total lipid extract of anammox cells for phosphoethanolamine lipid species. (a) Full scan analysis of the base peak chromatogram of a total anammox lipid extract. (b) Base peak mass chromatogram obtained by neutral loss scanning for *m/z* 141. (c–g) Mass chromatograms of $[M+H]^+$ ions of the identified ladderane phosphoethanolamine lipids. For more details, see Results section. For structures, see Fig. 1. The Δ-marked peaks denote unknown components.

m/z 184 and the core lipids identified by GC-MS and high field NMR (Sinninghe Damsté *et al*., 2005), we tentatively assigned a variety of phosphocholine lipids with the C_{20}-[3]-ladderane moiety at the *sn*-2 position. In peak C of the parent ion mass chromatogram (Fig. 3b), phosphocholine diether lipids with an *n*-C16 (IVe) and 10-methylhexadecanoic tail (IVg) were identified (Fig. 3c). Phosphocholine diethers in peak D eluted shortly after these components and possess either an etherified myristic (n-C_{14}) (IVi), C_{20}-[3]-ladderane (IVa) or C_{20}-[5]-ladderane (IVb) tail (Fig. 3d). Other diether phosphocholine lipids were observed in peak E and include the C_{18}-[3]-ladderane (IVc), C_{18}-[5]-ladderane (IVd) and 10-methylhexadecanoic acid (IVg), as well as the glycerol ester/ether lipid with a 10-methylhexadecanoic acid (Vg) tail (Fig. 3e). In peak F, more phosphocholine species with ether- and ester-linked tails were found (Fig. 3f). This peak comprises predominantly lipids with an *n*-C14 (Vi), C_{20}-[3]-ladderane (Va) or C_{20}-[5] ladderane (Vb) moiety at the s*n*-1 position. Glycerol ester/ether lipids with a C_{18}-[3]-ladderane (Vc) or C_{18}-[5]-ladderane chain (Vd) were identified in peak G (Fig. 3g). Remarkably, we did not observe any diacyl phosphocholine species by parental ion scanning for *m/z* 184, suggesting that these lipids are either absent

in anammox bacterial membranes or present below the detection limit.

Full scan MS analysis of the peak at ~29.5 min revealed a single component with *m/z* 488 (Fig. 4a). The corresponding MS/MS analysis demonstrated the loss of a neutral fragment with *m/z* 141 (Fig. 4b), diagnostic for a phosphoethanolamine lipid headgroup (Brügger *et al.*, 1997; Sturt *et al.*, 2004). Based on this and the molecular ion, we assigned this peak the C_{20}-[3]-ladderane phosphoethanolamine mono-alkylether (III, Fig. 1). The total anammox lipid extract was further explored for phosphoethanolamine lipid species by neutral loss scanning for *m/z* 141. The mass chromatogram and corresponding mass spectra revealed a variety of diether and ester/ether phosphoethanolamine species. We tentatively identified both etherified and esterified linked [5]-ladderane attached to the *sn*-1 position of the glycerol backbone (VIb VId, VIIb and VIId). In addition, ester-linked C_{20}-[3]-ladderane (VIIa), 10-methylhexadecanoic acid (VIIg) and *iso*-C14 (VIIi) were observed. In contrast to phosphocholine lipids, the chromatographic separation between diether and ester/ether phosphoethanolamine species was less clear.

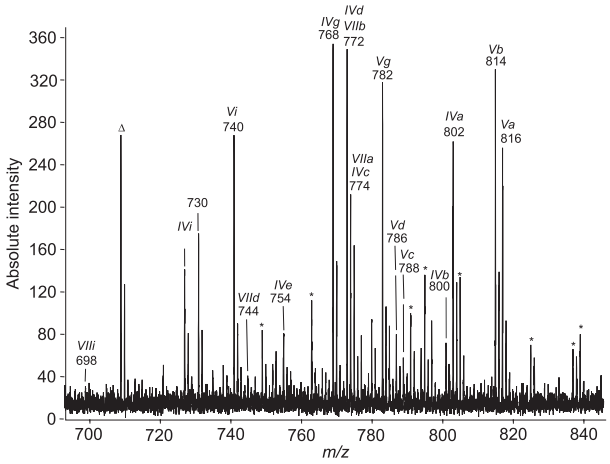

Fig. 5. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry spectrum of the total lipid extract of anammox cells showing the diether and ester/ether specific mass range (*m/z* 680–845). The peaks are assigned in accordance with the lipid structures depicted in Fig. 1. The asterisk marks denote $[M+Na]^+$ ions, and the Δ-marked peak denotes an unknown component.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis of the total lipid extract resulted in the confirmation of the presence of the phospholipids described above (Fig. 5). Most peaks represent the $[M+H]^+$ ions, but also several $[M+Na]^+$ ions were observed. The most dominant peaks in the spectrum are the phosphocholine diether lipids with a 10-methylhexadecanoic (IVg) and C_{18}-[5]-ladderane (IVd) tail. Other observed phosphocholine diether lipids include IVa, IVb, IVc, IVe, IVi. The glycerol ester/ether phosphocholine lipids Va, Vb, Vc, Vd, Vi, Vg were also detected by MALDI-TOF-MS, as well as several phosphoethanolamine lipids (VId, VIIa, VIIb, VIIc,

Table 1. Genes with known homologues involved in phosphoethanolamine and phosphocholine phospholipid biosynthesis

Enzyme name	EC number	Gene	Open reading frame	Amino-acid length	Mass (kDa)	Best blast hit	E-value
Diacylglycerol kinase	2.7.1.107	*dgkA*	0558	117	13	*Haemophilus ducreyi*; AE017154 (40% identity; 64% similarity)	3.00E-12
Phosphoglycerol transferase	2.7.8.20	*mdoB*	0559	710	82	*Vibrio vulnificus*; CMCP6 (identity 27%; similarity 42%)	2.00E-19
Phosphatidate cytidylyltransferase	2.7.7.41	*cdsA*	2654	281	30	*Parachlamydia* UWE25; BX908798_320 (identity 34%; similarity 50%)	2.00E-22
CDP-diacylglycerol-serine *O*-phosphatidyltransferase	2.7.8.8	*pssA*	1887	242	30	*Burkholderia mallei* ATCC 23344; CP000010_1586 (identity35%; similarity 54%)	4.00E-21
Phosphatidylserine decarboxylase	4.1.1.65	*psd*	0640	225	25	*Geobacter sulfurreducens*; PSD_GEOSL (identity 46%; similarity 62%)	3.00E-35
Phosphatidylethanolamine *N*-methyltransferase	2.1.1.17	*pemT/ pmtA*	1440	208	23	*Azoarcus* sp. EbN1; CR555306_3817 (38% identity; 57% similarity)	4.00E-33
Phosphatidylethanolamine *N*-methyltransferase	2.1.1.17	*pemT/ pmtA*	3916	272	29	*Rhodoferax ferrireducens* DSM 15236 EA039786 (identity 42%; similarity 57%)	2.00E-43

The assembled genome from *Kuenenia stuttgartiensis* (made available by Genoscope, Evry, France) was blasted for genes involved in biosynthesis of phosphoethanolamine and phosphocholine lipid headgroups. Blast hits are indicated for each gene identified and were obtained using the GenBank/NCBI and the KEGG genes databases (http://www.genome.jp).

VIId) (Fig. 5). Furthermore, upon separation by differential centrifugation (Sinninghe Damsté *et al.*, 2002) no difference was observed in the phospholipid content between the cytoplasmic and the anammoxosome membrane (data not shown).

Whereas phospholipids with a phosphocholine headgroup are a very abundant membrane components in most eukaryotes, its occurrence in prokaryotic cells is not common. Based on the available genomic databases, it has been reported that *c.* 10% of all bacteria possess a machinery to produce phosphocholine lipids (Sohlenkamp *et al.*, 2003). Here, we reconstructed the *in silico* biosynthetic pathway for the phosphoethanolamine and phosphocholine headgroups of the anammox phospholipids from the genome assembly of *K. stuttgartiensis*, a closely related anammox bacterium (Jetten *et al.*, 2003) (Table 1). The synthesis starts from 1,2 diacyl-*sn*-glycerol which is phosphorylated by a diacylglycerol kinase (orf0558) and phosphoglycerol transferase (orf0559) to yield 1,2 diacyl-*sn*-glycerol-3-phosphate. The phosphorylated intermediate is than activated by a CDP-diacylglycerol synthase (orf2654) and converted into phosphatidyl-L-serine by a CDP-diacylglycerol phosphotransferase (orf1887). Subsequently, this serine intermediate is decarboxylated yielding a phosphoethanolamine moiety by the gene product of orf0640 (phosphatidyl-L-serine decarboxylase). Finally, this ethanolamine intermediate can be repeatedly methylated yielding phosphocholine by the consecutive action of methyltransferases encoded by orf1440 and orf3916. Hence, as all the genes required could be identified in *K. stuttgartiensis*, the deduced pathway leading to the headgroup formation of ladderane phospholipids in anammox bacteria may be identical to known phospholipid routes in other bacterial species.

Taken together, in the present study both HPLC-ESI-MS/MS and MALDI-TOF-MS techniques revealed that anammox bacteria possess intact ladderane membrane lipid with phosphocholine and phosphoethanolamine polar headgroups. The molecular diversity of these unprecedented membrane phospholipids is enhanced by a wide range of hydrocarbon chains that are either ether- or ester-linked at the *sn*-1 position of the glycerol backbone. Further research is now focused on determining the mechanical properties and tightness of anammox membranes, in order to understand how the intact ladderane phospholipids are able to form extraordinary dense membrane barriers and limit diffusion of metabolites in living microbial cells.

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