Extremophiles (2011) 15:517–528
DOI 10.1007/s00792-011-0382-3

ORIGINAL PAPER

The major lipid cores of the archaeon *Ignisphaera aggregans*: implications for the phylogeny and biosynthesis of glycerol monoalkyl glycerol tetraether isoprenoid lipids

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Received: 27 April 2011 / Accepted: 20 May 2011 / Published online: 1 June 2011

Abstract The lipid cores from *Ignisphaera aggregans*, a hyperthermophilic *Crenarchaeon* recently isolated from New Zealand hot springs, have been profiled by liquid chromatography–tandem mass spectrometry. The distribution revealed includes relatively high proportions of monoalkyl (also known as H-shaped) tetraether cores which have previously been implicated as kingdom-specific biomarkers for the *Euryarchaeota*. Such high expression of monoalkyl tetraether lipids is unusual in the archaeal domain and may indicate that formation of these components is an adaptive mechanism that allows *I. aggregans* to regulate membrane behaviour at high temperatures. The observed dialkyl tetraether and monoalkyl tetraether lipid distributions are similar but not fully concordant, showing differences in the average number of incorporated rings. The similarity supports a biosynthetic route to the ring-containing dialkyl and monoalkyl tetraether lipids via a dialkyl tetraether core containing zero rings, or a closely related structural relative, as an intermediate. Currently, however, the precise nature of the biosynthetic route to these lipids cannot be deduced.

Keywords *Ignisphaera* · Archaea · Tetraether lipids · H-shaped lipids · Isoprenoids · Tandem mass spectrometry

Abbreviations

CID	Collision-induced dissociation
Cp	Cyclopentyl
Da	Daltons
DCM	Dichloromethane
GDD	Glycerol dialkyl diether
GDGT	Glycerol dialkyl glycerol tetraether
GMD	Glycerol monoalkyl diether
GMGT	Glycerol monoalkyl glycerol tetraether
GTGT	Glycerol trialkyl glycerol tetraether
LC–MS/MS	Liquid chromatography–tandem mass spectrometry
$[M+H]^+$	Protonated molecule
APCI	Atmospheric pressure chemical ionisation
ESI	Electrospray ionisation

Introduction

All members of the archaea synthesise complex isoprenoid ether lipids, unique to this domain, which form the structural scaffold of their membranes. The complex lipids typically contain an ether lipid core (e.g. **I–XIII**; Fig. 1), derived from glycerol or calditol, capped by glycosyl and/or modified phosphate head groups (De Rosa and Gambacorta 1988; Sturt et al. 2004). The simplest ether lipid core is archaeol (**I**, Fig. 1), a glycerol dialkyl diether (GDD) in which two C_{20} isopranyl alkanes are ether-linked to a glycerol group which exhibits *sn*-2,3 stereochemistry (Kates 1972). Subsequent additions of head groups

Communicated by A. Driessen.

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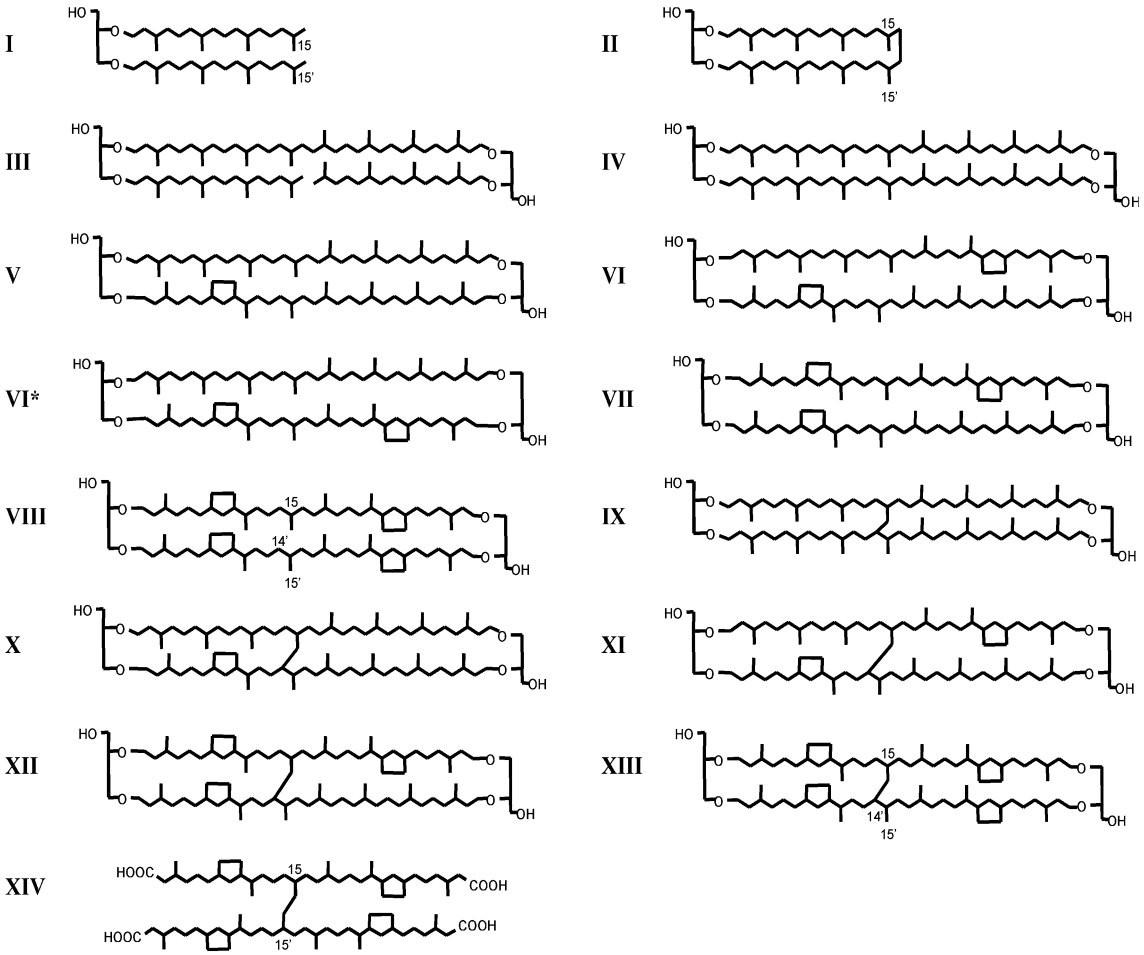

Fig. 1 Structures discussed in the text. For tetraether cores, only isomers in which the glycerol groups are oriented antiparallel to one-another are represented. The position of the covalent link between isoprenoid chains in GMGT lipids is not established conclusively and, consequently, its placement, based on previous reports of the structures (Morii et al. 1998; Schouten et al. 2008b; Knappy et al. 2009), is tentative

followed by hydrogenation of the geranylgeranyl chains yield complex lipids containing **I** as the core. In some archaea, two complex lipids based on **I** can undergo an intermolecular dimerisation creating a diglycerol-linked pair of C_{40} isoprenoid hydrocarbons (Nemoto et al. 2003). There is debate as to whether the geranylgeranyl chains in the core lipids involved in this coupling are fully saturated or contain remnant double bonds (Eguchi et al. 2003; Koga and Morii 2007). Nevertheless, there is an apparent lack of specificity for the orientation of the GDD precursors prior to condensation; it appears that the coupling of the precursors is equally favourable whether the two glycerol groups are initially aligned parallel or antiparallel to one-another (Gräther and Arigoni 1995). Subsequent dephosphorylation yields the antiparallel isomer **IV**, a glycerol dialkyl glycerol tetraether (GDGT) lipid core, along with its parallel isomer (not shown in Fig. 1). A glycerol trialkyl glycerol tetraether (GTGT) lipid core, **III**, has also been observed in a number of thermophilic and mesophilic archaea (De Rosa and Gambacorta 1988; Hopmans et al. 2000; Uda et al. 2000; de la Torre et al. 2008; Schouten et al. 2008a, b) and is presumably formed when the dimerisation does not proceed to completion following formation of the first C–C bond. Interestingly a glycerol monoalkyl diether (GMD), macrocyclic archaeol (**II**), has been identified in *Methanocaldococcus jannaschii* (formerly *Methanococcus jannaschii*), an isolate from a deep-sea hydrothermal vent (Comita and Gagosian 1983; Comita et al. 1984; Sprott et al. 1991). As *M. jannaschii* also synthesises GDD **I** (Comita and Gagosian 1983; Comita et al. 1984; Sprott et al. 1991), it is conceivable that GMD **II** may be formed via an intramolecular coupling between two terminal methyl groups on the opposing phytanyl chains of a single GDD lipid. A direct biosynthetic link between **I** and **II** has yet to be established conclusively.

An additional adaptation of the membrane lipids utilised by some archaea is the incorporation of cyclopentyl (Cp) moieties within the aliphatic chains of the tetraether lipid

cores (e.g. **V–VIII**, Fig. 1). Thermophilic and hyperthermophilic members of both the *Euryarchaeota* and *Crenarchaeota* can synthesise tetraether lipids containing between zero and eight Cp rings in total, with up to four rings per biphytanyl chain (De Rosa and Gambacorta 1988; Schouten et al. 2007). Likewise, mesophilic and moderately thermophilic members of the *Thaumarchaeota* synthesise tetraether lipids containing up to five Cp rings in total (DeLong et al. 1998; Damsté et al. 2002; Schouten et al. 2008a; Pitcher et al. 2010; Park et al. 2010), although some structures produced by these organisms (e.g. crenarchaeol, the characteristic lipid for the phylum; Damsté et al. 2002) also contain a cyclohexyl ring. Interestingly, despite the large number of structures possible from combination of two ring-containing biphytanes, only a handful of lipids have been identified to date, both in environmental samples and archaeal cultures. This restriction appears to be due to controls on the positions in which the rings are incorporated within the alkyl chains (De Rosa et al. 1980a). Further limitation is attributable to the small number of combinations of the alkyl chains that appear to be adopted, with all tetraether molecules definitively identified exhibiting biphytanyl pairs that differ by, at most, two Cp rings (De Rosa and Gambacorta 1988; Thurl and Schäfer 1988; Schouten et al. 2000; Pancost et al. 2001). The apparent absence of Cp ring-containing relatives of **I**, and occurrence of ring-containing derivatives of **II–IV** (De Rosa and Gambacorta 1988; Pancost et al. 2001; Stadnitskaia et al. 2003; de la Torre et al. 2008), suggests that ring formation occurs either simultaneous to, or following generation of C_{40} isoprenoid chains during biosynthesis. A mechanism involving α methyl–δ methylene cyclisation within a coupled biphytanyl chain has been proposed (Weijers et al. 2006a), but has yet to be confirmed either in vitro or in vivo.

Recently, structures of a further type, glycerol monoalkyl glycerol tetraether (GMGT) lipids which contain C_{80} isoprenoid hydrocarbons and are often referred to as H-shaped lipids, have been identified in thermophilic representatives of the *Euryarchaeota* (**IX–XIII**; Fig. 1). A structural relative of **IV**, lipid **IX**, in which the two biphytanyl chains are conjoined by a C–C covalent link, was first identified in the methanogen *Methanothermus fervidus*, although the precise position of the link was only assigned tentatively (Morii et al. 1998). Additional structural variation in monoalkyl tetraether lipids has subsequently been observed in *Aciduliprofundum boonei*, a thermophilic *Euryarchaeon* that inhabits deep sea hydrothermal vents (Reysenbach et al. 2006). This archaeon was shown to synthesise GDGT lipids, lipid **IX** and a series of structures which were identified as GMGT lipids containing 1–4 Cp rings, although the structures of the GMGT lipids were not assigned fully (Schouten et al. 2008b). Here we report the di- and tetraether core lipid profile of a hyperthermophilic *Crenarchaeon*, *Ignisphaera aggregans*, which synthesises significant proportions of GMGT lipids.

Materials and methods

Organism

Ignisphaera aggregans (strain AQ1.S1^T) is a novel archaeal species recently isolated from hot springs in New Zealand (Niederberger et al. 2006). It is a hyperthermophile that is viable at temperatures up to 98°C, with an optimum growth temperature of 92–95°C. Distinct genes identified in the 16S rRNA analysis of *I. aggregans* indicate that it belongs to the *Crenarchaeota* (more specifically, within the order *Desulfurococcales*) and that it possibly represents a novel genus within this kingdom. *I. aggregans* was cultivated in 1 L of growth medium at 95°C as described previously (Niederberger et al. 2006). Cells were harvested after 4 days, lyophilised, and the resultant pellet was stored at −20°C.

Ether core lipid extraction

Ether lipid cores were extracted via refluxing the *I. aggregans* cell pellet in 4.8 M methanolic HCl (50 mL) for 4 h at 100°C, followed by liquid–liquid extraction with three 25 mL portions of dichloromethane (DCM). The isolated organic fraction was concentrated to a small volume in vacuo, passed through a plug of anhydrous Na_2SO_4 and reduced to dryness in vacuo. The residue was reconstituted in a minimum amount of DCM and loaded onto a column of activated alumina (approx. 7 × 50 mm), which had been dry-packed in a Pasteur pipette and pre-washed with hexane/DCM (9:1 vol/vol). Apolar components were removed by washing with three bed volumes of hexane/DCM (9:1 vol/vol) and the ether lipids were eluted in three bed volumes of DCM/methanol (1:1 vol/vol). The purified lipids were reduced to dryness under a stream of nitrogen and dissolved, by sonication for 2 min, in hexane/isopropanol (99:1 vol/vol) prior to atmospheric pressure chemical ionisation (APCI) mass spectrometry or in methanol/DCM/10 mM ammonium acetate (2:1:0.8 vol/vol/vol) prior to electrospray ionisation (ESI) mass spectrometry.

Mass spectrometry and liquid chromatography–tandem mass spectrometry

Direct infusion mass spectrometry of the lipid extract from *I. aggregans* was performed using an HCTultra ETD II ion trap mass spectrometer (Bruker Daltonics; Coventry, UK), equipped with either an APCI source or an ESI source, in

each case operated in positive ion mode. The HCT operational parameters for APCI were set at: nebulizer gas (N_2) pressure 15 psi; drying gas (N_2) flow 8 L min^{-1}; drying gas temperature 325°C; vaporiser temperature 450°C; capillary voltage −3,500 V. Ion optic parameters were optimised for tetraether lipids via tuning on the protonated molecule ($[M+H]^+$) of **IV** (*m/z* 1301.8), as found in a direct infusion of a portion of a lipid extract from *M. thermautotrophicus* (Knappy et al. 2009). The HCT parameters for ESI were set at: nebulizer gas pressure 8 psi; drying gas flow 4 L min^{-1} and temperature 300°C; capillary voltage −4,500 V. Irrespective of the ionisation source used, the mass spectral scan range was fixed at *m/z* 50–3,000.

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) was achieved, via modifications to previous methods (Hopmans et al. 2000; Knappy et al. 2009), using an Ultimate 3000 rapid separation liquid chromatograph (Dionex; Sunnyvale, USA) coupled to the HCTultra ETD II ion trap. Lipid separation was achieved on a Waters Spherisorb S5-NH_2 column (4.6 × 250 mm, 5 μm; Elstree, UK), using a binary solvent system (A = hexane, B = isopropanol) operated at a flow rate of 1 ml min^{-1}. The gradient elution programme comprised a 5-min isocratic period with 1% B followed by a linear ramp to 2% B at 61.25 min and, finally, by a second linear ramp to 15% B at 90 min. Following each analysis, the column was backflushed for 10 min with 25% B to remove retained polar material and reconditioned with 1% B for 15 min prior to subsequent injection. The injection volume was held at 5 μL for all analyses. Ionisation was afforded by APCI, as described above. The mass spectral scan range was routinely set at *m/z* 900–1,500 to identify previously reported tetraether lipids (De Rosa and Gambacorta 1988; Schouten et al. 2008b), but was extended to *m/z* 50–2,000 to investigate the expression of diether lipids in *I. aggregans*. Online MS/MS spectra were generated following collision-induced dissociation (CID) of the most abundant ion observed in each mass spectral scan. The isolation width was set to a 3 *m/z* unit window with the maximum accumulation time set at 40 ms and fragmentation amplitude fixed at 2.0 V (SmartFrag© not activated).

Data processing

All MS ions are discussed using their *m/z* values as measured by the ion trap mass spectrometer. Product ions in MS/MS and the losses of neutral molecules that generated these ions are discussed using their nominal *m/z* and mass values as assigned from the MS/MS spectra, respectively. The nominal values can be up to 1.4 *m/z* less than the values measured on the ion trap instrument used in these studies on account of the substantial mass sufficiency in tetraether lipids and the product ions generated from them during CID (Knappy et al. 2009).

To allow for the sub-unit resolution of the ion trap instrument employed, reconstructed mass chromatograms were generated by selecting all ions within ±0.5 *m/z* units of the target *m/z* value. Lipid relative abundances were calculated from individual ion chromatogram LC–MS peak areas, with ionisation efficiencies assumed to be constant for all of the lipids.

Results and discussion

Structural identifications

Tetraether lipids of *I. aggregans* were assigned by LC–MS/MS analysis of a methanolytic extract of harvested cells (scan range *m/z* 900–1,500), the base peak chromatogram exhibiting peaks within two main groups (Fig. 2a). Lipids of this structural type can be recognised from the isotope peak abundances of the protonated molecules in the APCI spectra (Hopmans et al. 2000) and can be further classified as GTGT, GDGT and GMGT lipids based on the MS/MS spectrum obtained for each following CID (Knappy et al. 2009). Thus, all tetraether lipids show prominent ions arising from losses of up to 184 daltons (Da), attributed to expulsion of small neutral molecules such as water and C_3H_4O (Fig. 3). Those ions dominate the MS/MS spectra of the GMGT lipids (Fig. 3b) as the bridging carbon–carbon bond between the isoprenoid chains prevents loss of either chain. The GDGT lipids, on the other hand, also show abundant ions in the range *m/z* 550–750 (Fig. 3a), arising from losses involving one or other of the isoprenoid chains. The MS/MS spectra of GTGT lipids show ions arising from the former processes together with prominent ions in the range *m/z* 900–1,050, arising from losses involving one of the C_{20} isoprenoid chains (Knappy et al. 2009).

The two main groups of peaks in the base peak chromatogram (Fig. 2a) were identified from their MS/MS spectra as GDGT and GMGT lipids, the former being the earlier eluting species. The major GDGT lipids (**IV**–**VIII**) contain 0–4 Cp rings and were identified by comparison of MS/MS spectra with those of known lipids present in extracts from members of the genus *Sulfolobus* (Knappy 2010). These structures have been identified in a number of thermophilic and hyperthermophilic organisms and have been fully characterised (De Rosa and Gambacorta 1988). Extraction of mass chromatograms corresponding to the protonated molecules of the major GDGT lipids allowed the recognition of a series of minor lipids (**V′**–**VIII′**) eluting slightly earlier than their corresponding major isobar (Fig. 2b). The MS/MS spectra of the minor components

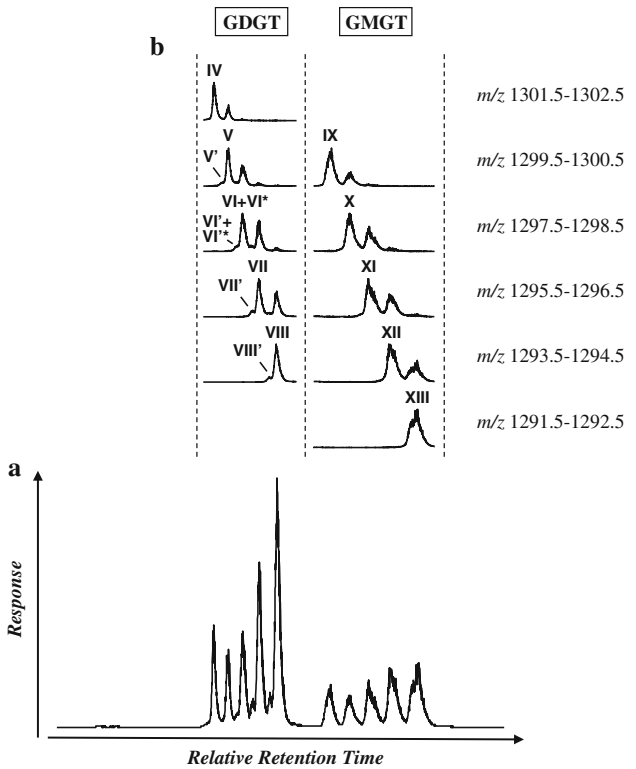

Fig. 2 Partial LC–MS ion chromatograms for the lipid extract from *I. aggregans*: **a** base peak chromatogram; **b** individual ion chromatograms for tetraether lipids

confirmed them as GDGT lipids. Hence, they most likely represent regioisomers of the major lipids.

The independent loss of the two component alkyl chains of GDGT lipid cores during MS/MS enables their designation according to the number of rings present in each. In lipids where the two chains are not identical, the chain containing the fewer rings is lost more readily. For example, the MS/MS spectrum of **VII** (Fig. 3a) exhibits a product ion at *m/z* 739, formed from loss of a biphytadiene containing one Cp ring (−556 Da), that is more abundant than that at *m/z* 741, which is formed from loss of a biphytadiene containing two Cp rings (−554 Da). Similarly, CID of **VI** generates product ions formed from loss of alkyl chains containing one cyclopentyl ring (*m/z* 741; −556 Da), indicating the presence of one ring in each C_{40} chain. Interestingly, however, additional product ions formed from losses of alkyl chains containing zero (*m/z* 739; −558 Da) and two (*m/z* 743; −554 Da) Cp rings are also evident in the resulting MS/MS spectrum (Fig. 4). A GDGT lipid containing this combination of alkyl chains has been reported in *T. tenax* (Thurl and Schäfer 1988) and also in a number of aquatic sediments following chemical degradation of isolated tetraether fractions to the component isoprenoid chains (Schouten et al. 2000; Pancost et al. 2001). As such, in addition to **VI**, *I. aggregans* most likely produces a structural isomer, **VI***, which has a zero ring–two ring isoprenoid hydrocarbon pairing and co-elutes

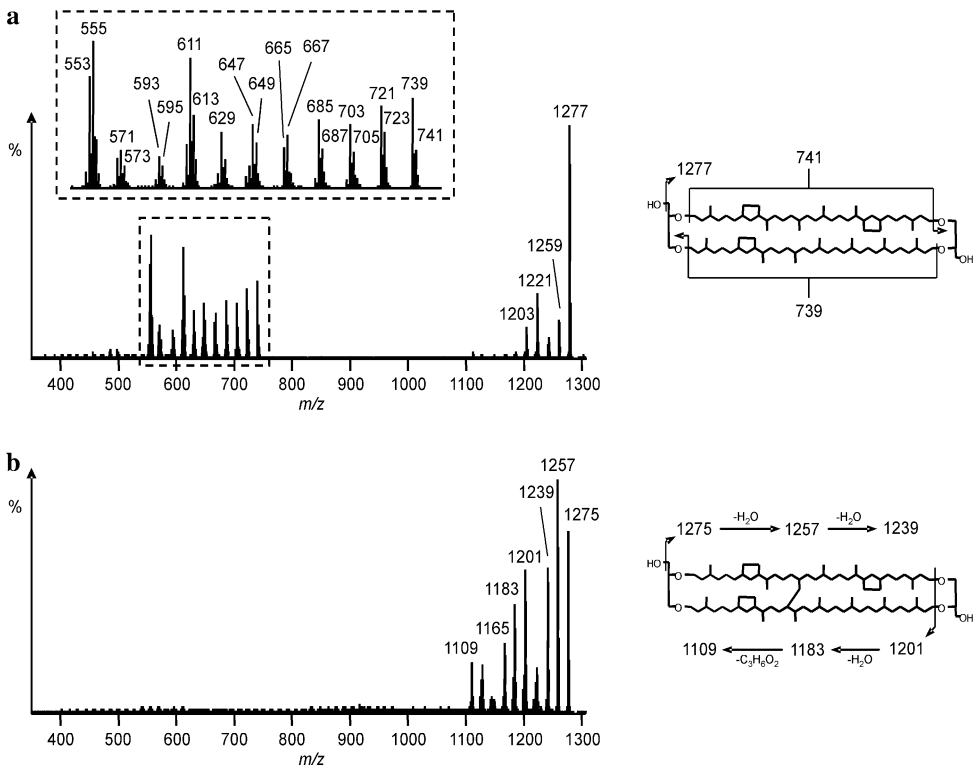

Fig. 3 Characteristic MS/MS spectra of: **a** GDGT **VII** ($[M+H]^+$ m/z = 1,295.8) and **b** GMGT **XII** ($[M+H]^+$ m/z = 1,293.8), both of which contain three cyclopentyl rings. Characteristic fragmentations for each lipid are shown. Product ions are labelled with nominal *m/z* values

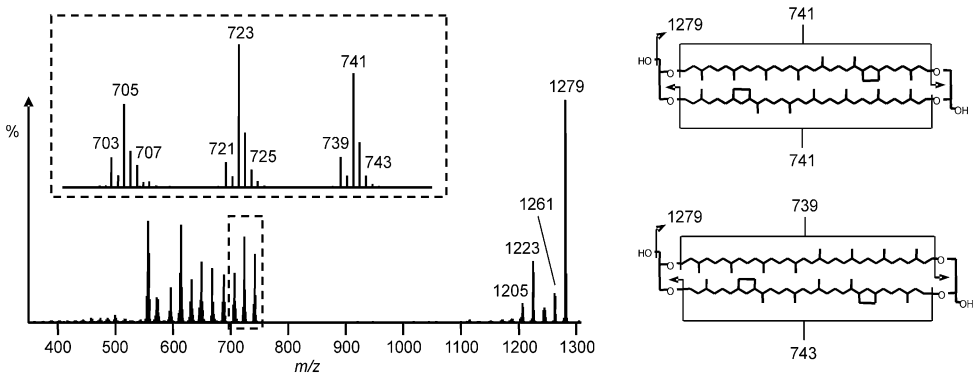

Fig. 4 MS/MS spectrum generated following CID of co-eluting isomers GDGT **VI** (which contains a 1 ring, 1 ring isoprenoid chain combination) and GDGT **VI*** (which contains a 0 ring, 2 ring isoprenoid chain combination), with $[M+H]^+$ m/z = 1,297.8. Characteristic fragmentations for each lipid are shown. Product ions are labelled with nominal m/z values

under the chromatographic conditions employed. The MS/MS spectrum of **VI′** similarly suggests that it is accompanied by a co-eluting isomer, **VI′***, and that the two isomers also differ in the number of rings in each chain, one having a single Cp ring in each of the isoprenoid chains and the other having both rings in one chain and none in the other.

The GMGT structure **IX** has been identified in a number of (hyper)thermophilic archaea, including *M. fervidus* (Morii et al. 1998), *Methanothermobacter thermautotrophicus* (Morii et al. 1998; Knappy et al. 2009), *A. boonei* (Schouten et al. 2008b) and four strains of the order *Thermococcales* (Sugai et al. 2004), and has also been identified in sediments in a number of non-thermophilic environments (Schouten et al. 2008c). The assignment of the lipid in *I. aggregans* was supported by comparison of the MS/MS spectrum with that of the component identified previously in *M. thermautotrophicus* (Knappy et al. 2009). Due to the higher operational energy of the HCT ion trap, several new product ions were identified that were not easily observed in the MS/MS spectrum of **IX** recorded using a Finnigan MAT LCQ ion trap (Knappy et al. 2009). These include an ion formed from loss of 184 Da which is most likely a fully deoxygenated C_{80} carbocation containing three terminal double bonds. Other product ions formed during the higher energy CID of **IX** are less easily assigned and may relate to dissociations in which part of the C_{80} hydrocarbon core is lost from the structure. Further verification of the presence of **IX** in *I. aggregans* was provided by analysis of the *M. thermautotrophicus* extract before and after spiking with a small amount of the *I. aggregans* extract; the doped sample showed an enhancement of the peak for **IX** in relation to the un-doped sample.

The MS and MS/MS spectra of the other major GMGT lipids (**X**–**XIII**) are analogous to those of **IX**. In particular, product ions are only observed in the m/z range 1,050–1,300. The greater prominence of the product ions arising from losses of small neutral molecules (e.g. m/z 1,257 and 1,201 in the MS/MS spectrum of GMGT **XII**; Fig. 3b) than for the corresponding ions in the spectrum of GDGT **VII** (m/z 1,259 and 1,203; Fig. 3a) most likely reflects the more limited dissociation pathways available for GMGT lipids. The m/z values of the protonated molecules indicate that they also contain 1–4 Cp rings. Unfortunately, the absence of product ions arising from losses of isoprenoid chains from GMGT lipids limits the structural information provided for these lipids by MS/MS. As such, co-eluting GMGT structural isomers, if present, are not distinguishable using this method. These structures were tentatively assigned in the thermoacidophilic *Euryarchaeon A. boonei* (Schouten et al. 2008b), the only other source currently recognised.

To screen for diether lipid cores, which exhibit $[M+H]^+$ at m/z values outside the detection range used in the initial analyses, an extended mass spectral scan range (m/z 50–2,000) was employed during a repeat LC–MS/MS analysis of the *I. aggregans* extract. The reconstructed ion chromatogram for m/z 653–654.5 indicates the presence of an early eluting lipid core with $[M+H]^+$ at m/z 653.5, consistent with that expected for GDD **I** (Fig. 5a). The observed retention time relative to that of **IV** (Fig. 5b) is consistent with previous LC–MS analyses of samples which contained both lipids (Mancuso et al. 1986; Turich et al. 2007; Reigstad et al. 2008; Weijers et al. 2009). The identity of the component was confirmed as GDD **I** by the presence of the product ion at m/z 373, formed by loss of phytene (Gattinger et al. 2003; Turich et al. 2007), during CID (Fig. 5c). No components consistent with macrocyclic diether lipid **II** (theoretical $[M+H]^+$ m/z = 651.7) were identified, suggesting that this lipid core is not synthesised by *I. aggregans* when grown at 95°C.

Some archaea produce complex lipids which are capped with modified phosphate groups containing amino functionality (Koga et al. 1993; Koga and Morii 2005), head groups which are known to be resistant to cleavage via acidic methanolysis (Koga and Morii 2006). To investigate whether the methanolysed lipid extract from *I. aggregans* contains lipid cores which remain trapped as amino group-bearing phospholipids, a portion was analysed using direct infusion mass spectrometry (scan range m/z 50–3,000).

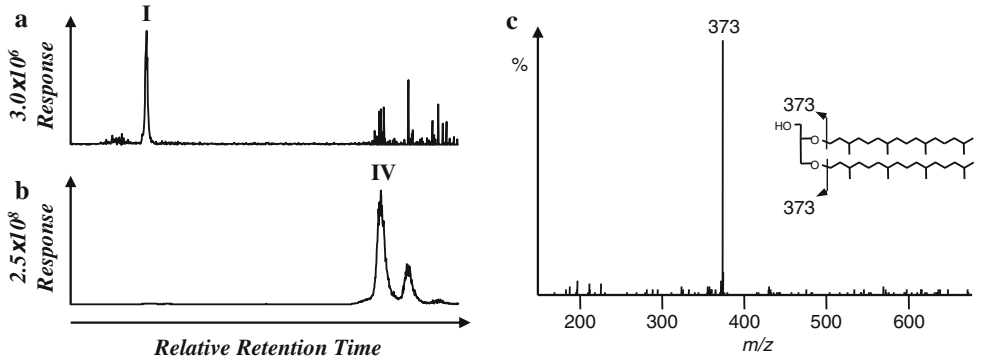

Fig. 5 Reconstructed ion chromatograms for **a** *m/z* 653–654.5 and **b** *m/z* 1,301.5–1,302.5 following LC–MS/MS analysis of the *I. aggregans* extract using an extended scan range (*m/z* 50–2,000). The relative retention times of GDD **I** and GDGT **IV** are illustrated. **c** MS/MS spectrum of GDD **I** ($[M+H]^+$ $m/z = 653.5$). Characteristic fragmentations for the lipid are shown

Irrespective of whether ionisation was effected by APCI or by ESI (an alternative technique suitable for ionisation of complex polar lipids; Sturt et al. 2004), the mass spectra obtained provided no evidence for remnant complex polar lipids of any type.

Distributions and significance

The major GDGT lipid cores of *I. aggregans* ($GDGT_{maj}$ = **IV**, **V**, **VI**+**VI***, **VII** and **VIII**) incorporate up to four cyclopentyl rings, with minor, earlier eluting structural isomers of each ring-containing lipid ($GDGT_{min}$ = **V′**, **VI′**+**VI′***, **VII′** and **VIII′**) also being present. GDGT lipids containing Cp rings are expressed in both (hyper)thermophilic *Euryarchaeota* and *Crenarchaeota* (De Rosa and Gambacorta 1988; Schouten et al. 2007), in *Thaumarchaeota* isolated from marine and hot spring environments (Damsté et al. 2002; de la Torre et al. 2008; Schouten et al. 2008a; Pitcher et al. 2010; Park et al. 2010) and in aquatic sediments (e.g. Schouten et al. 2002; Powers et al. 2004; Kim et al. 2008), terrestrial soils (Weijers et al. 2006b) and peats (Weijers et al. 2004, 2006a) deposited under a variety of environmental conditions. In most of these cases, the degree of incorporation of Cp rings in tetraether lipids is temperature dependent, generally increasing with increasing growth temperature (De Rosa et al. 1980b; Uda et al. 2001, 2004; Schouten et al. 2002; Powers et al. 2004; Kim et al. 2008; Lai et al. 2008; Shimada et al. 2008; Boyd et al. 2011). In the case of (hyper)thermophilic archaea, it has been suggested that this reflects adaptation to produce structures that pack more tightly in the membrane (Gabriel and Chong 2000) and, accordingly, which regulate membrane behaviour, e.g. fluidity or proton permeability (Gabriel and Chong 2000; Gliozzi et al. 2002). Other factors, including ambient pH (Shimada et al. 2008; Boyd et al. 2011), the presence (or absence) of oxygen during growth (Trincone et al. 1989) and whether heterotrophic or autotrophic growth is adopted (Thurl and Schäfer 1988), are also known to influence the proportions of different ring-containing tetraether lipids produced in some archaea. It is likely that ring-incorporation in GDGT lipid cores is used in a similar fashion by *I. aggregans* to moderate physicochemical properties of the membrane. Although the locations of the rings in lipids **VI′** and **VI′*** have been delimited to particular isoprenoid chains, full structural determination for these components was not obtained. Similarly, elucidation of the structures of **V′**, **VII′** and **VIII′** will require further work. The orientation of the two glycerol groups with respect to one-another in the GDGT structures could not be directly confirmed by LC–MS/MS. Nevertheless, all GDGT lipids in *I. aggregans* have been designated as the antiparallel isomers in accordance with previous reports of their structures (e.g. De Rosa and Gambacorta 1988). Notably, however, in all three archaeal species for which the GDGT glycerol orientations have been explicitly determined (Gräther and Arigoni 1995) the parallel isomers have been identified in approximately equal abundance to their antiparallel counterparts. Consequently, the co-occurrence or even exclusivity of parallel GDGT isomers in *I. aggregans* must also be considered as possibilities. Given that the ratios of $GDGT_{min}$ to $GDGT_{maj}$ isomers containing the same number of Cp rings (0.1–0.14) are each substantially less than 1:1, it is unlikely that the major and minor isomerism is the result of differing glycerol orientations. This assertion is supported by the absence of a minor isomer of GDGT **IV**. Hence, if both parallel and antiparallel isomeric forms are present simultaneously, they must co-elute during LC–MS/MS. It is noteworthy, however, that a lipid found in *Sulfolobus solfataricus*, which elutes slightly later than GDGT **VIII** during normal phase LC–MS and has been proposed as its parallel isomer (Damsté et al. 2002), is not produced by *I. aggregans*. GDD **I** was identified as a minor lipid core component in the organism, present in approximately 0.4%

abundance relative to GDGT **IV**. GTGT **III**, on the other hand, could not be identified in the *I. aggregans* extract, although this does not preclude the possibility that the organism may synthesise this lipid when grown under different culture conditions.

In addition to GDD and GDGT lipids, *I. aggregans* also produces GMGT lipids containing 0–4 Cp rings (**IX–XIII**). Structural isomerism of the GMGT lipids, as was observed for the GDGT lipids, was not evident in any of the chromatograms. This is partly due to limitations in the information provided by LC–MS/MS, namely the lack of specific information regarding the isoprenoid chain compositions, meaning that co-eluting GMGT structural isomers would not be distinguished. In particular, it seems likely that GMGT **XI** would be accompanied by an additional isomer in which the two rings in the structure are located in the same isoprenoid chain (i.e. a zero–two ring chain combination), mirroring the isomerism observed in GDGTs **VI** and **VI***. Further structural profiling is required to confirm this and, consequently, we report **XI** as a single isomer. The structures shown for GMGT lipid cores (Fig. 1) are tentative assignments based on the previously reported structures of **IX** (Morii et al. 1998; Schouten et al. 2008b; Knappy et al. 2009) and **X–XIII** (Schouten et al. 2008b). In these structures, the positions of the Cp rings are assumed to match those in GDGT lipids containing the same number of rings. The covalent bond joining the isoprenoid chains is ascribed to a link between a C-15 methyl on one chain and a C-14′ methylene on the other chain. It is important to note that these previous assignments are founded on the best structural fit to the spectroscopic and spectrometric data obtained for **IX** (Morii et al. 1998) and are not conclusive. A series of C_{80} tetraacids containing 4–8 Cp rings, potential transformation products of GMGT lipids, identified in naphthenate deposits in crude oil piping (Lutnaes et al. 2006) have been fully characterised by nuclear magnetic resonance spectroscopy (Lutnaes et al. 2007). In particular, the tetraacid containing four rings (**XIV**; Fig. 1) has been shown to possess the rings in positions along the isoprenoid chains that match those observed in fully characterised GDGT **VIII** (De Rosa and Gambacorta 1988; Lutnaes et al. 2007). Furthermore, the covalent link between the isoprenoid chains in the tetraacid was found to be between a C-15′ methyl and a C-15 methyl group. Given that the C-15′ methyl group in one GDD lipid couples intermolecularly to the C-15 methyl in a different GDD lipid during formation of GDGT lipids (Koga and Morii 2007; Nemoto et al. 2003) and C-15′ and C-15 methyl groups may couple intramolecularly during formation of GMD lipids, it is plausible that C-15′ and C-15 methyl groups in a GDGT lipid may also intramolecularly couple to give a GMGT lipid. As such, GMGT **XIII** may have a structure resembling tetraacid **XIV** as opposed to the structure shown in Fig. 1. By association, a C-15′ methyl to C-15 methyl bond may be the chain-spanning covalent link in GMGTs **IX–XII**. In either case, more rigorous structural analysis of the GMGT lipids is needed. Although antiparallel forms are, again, assumed for each of the GMGT lipid cores, the occurrence or co-occurrence of parallel forms should also be regarded as distinct possibilities. As discussed for the GDGT lipids, if both parallel and antiparallel GMGT isomeric forms are present, they are not fully chromatographically resolved from each other during LC–MS/MS.

To date, GMGT lipids have only been identified in cultivated members of the *Euryarchaeota* (Morii et al. 1998; Sugai et al. 2004; Schouten et al. 2008b; Knappy et al. 2009) or in marine and lacustrine sediments (Schouten et al. 2008c). This has prompted a tentative suggestion that these lipids may act as biomarkers for *Euryarchaeota* in the environment (Schouten et al. 2008c). The identification of lipids **IX–XIII** in *I. aggregans* indicates that this assumption must be re-assessed. Members of both the *Euryarchaeota* and the *Crenarchaeota* are able to incorporate isoprenoid cross-linked tetraether lipids into their cell membranes and, consequently, **IX–XIII** cannot be considered as kingdom-specific biomarkers in the interpretation of environmental lipid profiles.

GMGT lipids contribute nearly 39% to the total core lipid in *I. aggregans*, as calculated from the measured abundances of known lipids **I**, **IV–XIII** and their minor isomers only. To the best of our knowledge, this represents one of the largest expressions of GMGT lipids in cultivated archaea reported to date, with only *Thermococcus waiotapuensis*, grown at 85°C, known to produce a higher proportion within its lipid cores (48.9%; Sugai et al. 2004). The proportions of macrocyclic GMD lipids (Sprott et al. 1991) and/or membrane-spanning GDGT lipids (Sprott et al. 1991; Lai et al. 2008; Matsuno et al. 2009) have been observed to increase relative to GDD lipids in several archaea when grown at increasing temperatures. Such adaptations are attributed to incorporation of more rigid structures, thought to help to limit membrane fluidity at elevated temperatures (Sprott et al. 1991). Similarly, it has been suggested that isoprenoid cross-linking in archaeal tetraether lipids may confer more rigidity to the cell membrane, preventing lysis even at very high temperatures (Schouten et al. 2008b). As such, the high levels of GMGT lipids expressed in *I. aggregans* may indicate an adaptation of lipid biosynthetic pathways in this organism to allow survival under the extreme thermal stresses present in the hot springs from which it originates. It is unlikely, however, that this is the sole reason for the elevated GMGT levels observed in this archaeon. Different members of the order *Thermococcales* show variable proportions of GDGT and GMGT lipids despite being grown at the same

temperature (85°C; Sugai et al. 2004). Furthermore, the percentage composition of GMGT cores observed in each of the eight (hyper)thermophilic organisms in which they have been conclusively identified to date does not appear to correlate to either the viable temperature range or maximum viable growth temperature of the organisms (Table 1). In addition, the identification of GMGT **IX** in sediments from low temperature marine environments (<35°C; Schouten et al. 2008c) indicates that GMGT lipids are not exclusive to thermophilic archaea. These observations suggest that taxon-specific differences among archaeal species may also place constraints on the extent of isoprenoid cross-linking in archaeal membrane lipids.

The distribution of major GDGT lipids in *I. aggregans* grown at 95°C indicates that GDGT structures containing higher numbers of cyclopentyl rings are synthesised in preference to less heavily cyclised structures (Fig. 6a). One exception is the GDGT **IV**, which is found in greater abundance in *I. aggregans* than GDGT **V** despite containing fewer (i.e. zero) Cp rings. Notably, however, **IV** has been postulated to be an intermediate in the formation of ring-containing lipids **IV**–**VIII** (Weijers et al. 2006a). As such, the prominence of **IV** may be representative of only partial conversion to **V**–**VIII** during lipid biosynthesis. A similar trend is observed for the $GDGT_{min}$ isomers **V′**–**VIII′**, which are also found in increasing abundance as ring number increases (Fig. 6a). Interestingly, however, the proportion of each minor GDGT to its major structural isomer is not constant, increasing with ring number up to structures with three rings, followed by a decrease in the ratio for the structures containing four rings. The incongruence of these ratios may suggest that the organism can actively control the proportions of the different $GDGT_{min}$ isomers expressed, although it should be noted that the differences in the ratios, where identified, are fairly small.

The distribution of ring-containing GMGT lipids in the *I. aggregans* extracts generally mirrors that of the GDGT lipids, with the more heavily cyclised structures being produced in the highest relative abundance (Fig. 6a). Again, the only exception is for the lipid containing zero rings, **IX**, which is found in greater abundance than expected. The close analogy between the abundances of GDGT and GMGT lipids suggests that they may be intrinsically linked by a biosynthetic pathway. Similarly, a previous lipid profile of *A. boonei* showed that GMGT:GDGT ratios for lipids containing the same number of rings were approximately constant regardless of the ring number (Schouten et al. 2008b). This led the authors to suggest that GDGT lipids may be the biosynthetic precursors to the GMGT lipids (Schouten et al. 2008b). The absence of macrocyclic diether **II** in both *I. aggregans* and *M. thermautotrophicus* (Comita and Gagosian 1983, Comita et al. 1984), organisms which both express lipids **I**, **IV** and **IX** (Knappy et al. 2009), strongly implies that **IX** is formed directly from **IV** (or an unsaturated precursor thereof) and not via cross-linking of a molecule of **I** with **II**. If the isoprenoid chains in ring-containing GDGT lipids **V**–**VIII** were ligated via a similar biosynthetic pathway, GMGT lipids **X**–**XIII** would be formed. If, however, this were the case then minor structural isomers of GMGTs **X**–**XIII**, formed from **V′**–**VIII′**, would also be expected in *I. aggregans* and such structures were not observed. It remains possible that the isomers are present but obscured by co-elution with the major GMGT lipids reported above.

The relationship between the GMGT and GDGT lipids has the potential to reveal biosynthetic relationships

Table 1 Summary of the organisms in which GMGT lipids have been identified to date, including the proportion of GMGT lipid as a percentage of the total core lipid found in each case

Organism	Kingdom	Growth temp (viable range)/°C	GMGTs	GMGT (%)	References
Methanothermus fervidus	*Eury*	84 (65–97)	**IX**	31	Stetter et al. 1981; Koga et al. 1993; Morii et al. 1998
Pyrococcus horikoshii	*Eury*	85 (80–102)	**IX**	34.0	Sugai et al. 2004
Thermococcus celer	*Eury*	85 (75–97)	**IX**	15.5	Sugai et al. 2004
Thermococcus guaymasensis	*Eury*	85 (56–90)	**IX**	12.0	Sugai et al. 2004
Thermococcus waiotapuensis	*Eury*	85 (60–90)	**IX**	48.9	Sugai et al. 2004
Aciduliprofundum boonei	*Eury*	70 (55–75)	**IX**–**XIII**	NR	Reysenbach et al. 2006; Schouten et al. 2008b
Methanothermobacter thermautotrophicus	*Eury*	70 (40–75)	**IX**	0.4[a]	Zeikus and Wolfe 1972; Morii et al. 1998; Knappy et al. 2009
Ignisphaera aggregans	*Cren*	95 (85–98)	**IX**–**XIII**	38.8	Niederberger et al. 2006; current study
Aquatic sediments	*Eury Cren*?	<35	**IX**	0–6.5[a]	Schouten et al. 2008c

Eury Euryarchaeota; *Cren* Crenarchaeota; *NR* not reported

[a] Diether lipids were not quantified during the lipid profiling undertaken in these studies. As such, the values quoted in each case are the percentages of the total tetraether lipid only

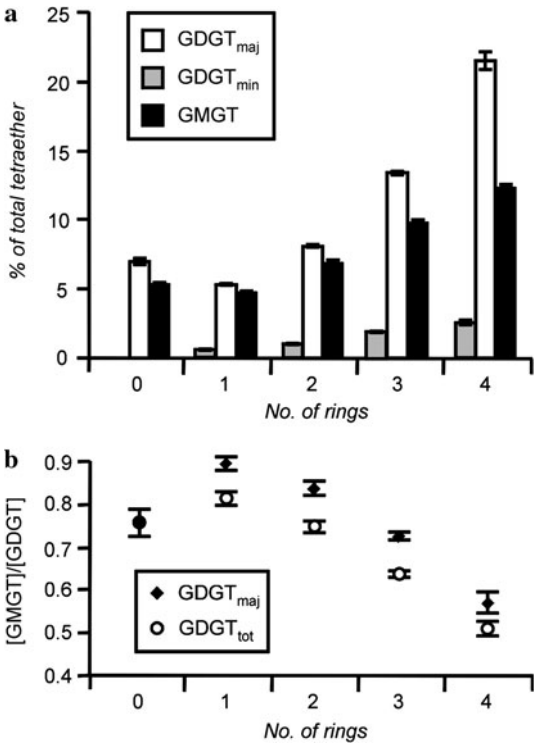

Fig. 6 **a** Relative proportions (as a percentage of the total tetraether lipid) of major GDGT isomers ($GDGT_{maj}$), minor GDGT isomers ($GDGT_{min}$) and GMGT lipid cores in *I. aggregans*. **b** Ratios of GMGT lipids to $GDGT_{maj}$ lipids (*black diamonds*) and total GDGT lipids ($GDGT_{tot} = GDGT_{maj} + GDGT_{min}$; *open circles*) which contain the same number of cyclopentyl rings. *Error bars* show $\pm 1\sigma$ from the mean of analytical triplicates

between the two core lipid families. The ratios of GMGT to $GDGT_{maj}$ and to total GDGT lipid ($GDGT_{tot} = GDGT_{maj} + GDGT_{min}$) for each ring number in *I. aggregans* (Fig. 6b) cover both possibilities: either that minor GMGT isomers are absent or that they are masked by co-elution. With the exception of **IX**:**IV**, the magnitude of GMGT:GDGT for both measures decreases as ring number increases. Thus, if GMGT lipids do indeed originate from GDGT lipids, the number of rings in the precursor GDGT must be a limiting factor on the degree of its conversion to its GMGT counterpart. Alternatively, it is conceivable that GMGT lipids **X–XIII** are formed by sequential incorporation of Cp rings into **IX**, or an unsaturated precursor of this lipid, akin to the route proposed for the formation of GDGTs **V–VIII** from **IV** (Weijers et al. 2006a). The lipid distribution observed in *I. aggregans* would also be consistent with this second scheme provided that the enzyme or enzyme suite involved in Cp ring-incorporation showed lower activity on **IX** than on **IV**. It is also possible that both proposed biosynthetic pathways or other routes may operate simultaneously to produce Cp ring-containing GMGTs. Irrespective of the route taken, the GMGT lipid profile of *I. aggregans* appears to be inconsistent with that of *A. boonei*, where no reduction in GMGT proportions for higher ring numbers was noted (Schouten et al. 2008b). On the basis of the limited evidence obtained from these two organisms, it remains difficult to assign a particular biosynthetic route to GMGTs **X–XIII** at the present time.

Conclusions

I. aggregans grown at 95°C biosynthesises both diether and tetraether isoprenoid lipid cores for its cellular membrane, which contains one of the most complex distributions of the latter reported in cultivated archaea to date. The source of such complexity arises from incorporation both of cyclopentane rings and isoprenoid chain cross-links in the tetraether lipid cores. It is presumed that both structural elements are used to regulate specific physicochemical properties of the cell membrane, particularly fluidity, at the high growth temperatures in which the organism thrives. No explanation is currently available to account for the inclusion of two mechanisms likely to influence membrane rigidity. The presence of GMGT lipids in *I. aggregans* is the first confirmation of this lipid core type in a *Crenarchaeote*, and indicates that these components cannot be used as markers for *Euryarchaeota* in environmental samples. The monoalkyl lipids are almost certainly biosynthetically derived from dialkyl tetraether lipids, although the precise pathway to these compounds still needs to be established. In any case, *I. aggregans* represents an ideal organism for further study of isoprenoid lipid biosynthesis, particularly in terms of variations introduced into the membrane lipids in response to external stresses. This organism should also prove to be a key species in assessing the potential of GMGT lipids as biomarkers for thermophilic archaea in hot spring environments.

Acknowledgments The authors acknowledge support for this study by a scholarship from the University of York. James Chong (University of York, UK; *M. thermautotrophicus*) and Michael Danson (University of Bath, UK; *S. solfataricus* and *S. shibatae*) are thanked for provision of the cellular material used to tune the mass spectrometer and verify the GDGT lipid assignments in *I. aggregans*, respectively. Two anonymous reviewers are also thanked for their constructive comments.

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