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ORIGINAL ARTICLE

# Crenarchaeol dominates the membrane lipids of *Candidatus* Nitrososphaera gargensis, a thermophilic Group I.1b Archaeon

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**Analyses of archaeal membrane lipids are increasingly being included in ecological studies as a comparatively unbiased complement to gene-based microbiological approaches. For example, crenarchaeol, a glycerol dialkyl glycerol tetraether (GDGT) with a unique cyclohexane moiety, has been postulated as biomarker for ammonia-oxidizing Archaea (AOA). Crenarchaeol has been detected in *Nitrosopumilus maritimus* and '*Candidatus* Nitrosocaldus yellowstonii' representing two of the three lineages within the Crenarchaeota containing described AOA. In this paper we present the membrane GDGT composition of '*Candidatus* Nitrososphaera gargensis', a moderately thermophilic AOA, and the only cultivated Group I.1b Crenarchaeon. At a cultivation temperature of 46 °C, GDGTs of this organism consisted primarily of crenarchaeol, its regioisomer, and a novel GDGT. Intriguingly, '*Ca.* N. gargensis' is the first cultivated archaeon to synthesize substantial amounts of the crenarchaeol regioisomer, a compound found in large relative abundances in tropical ocean water and some soils, and an important component of the $TEX_{86}$ paleothermometer. Intact polar lipid (IPL) analysis revealed that '*Ca.* N. gargensis' synthesizes IPLs similar to those reported for the Goup I.1a AOA, *Nitrosopumilus maritimus SCMI*, in addition to IPLs containing uncharacterized headgroups. Overall, the unique GDGT composition of '*Ca.* N. gargensis' extends the known taxonomic distribution of crenarchaeol synthesis to the Group I.1b Crenarchaeota, implicating this clade as a potentially important source of crenarchaeol in soils and moderately high temperature environments. Moreover, this work supports the hypothesis that crenarchaeol is specific to all AOA and highlights specific lipids, which may prove useful as biomarkers for '*Ca.* N. gargensis'-like AOA.**
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## Introduction

The importance of Group I Crenarchaeota (that is, those not belonging to the well-known class of Thermoprotei) in the biogeochemical cycling of nitrogen and carbon is becoming increasingly evident, as culture-independent studies reveal the ubiquity and potential activity of these organisms in nature (for example, Francis *et al.*, 2007; Beman *et al.*, 2008; Prosser and Nicol, 2008 and references therein). Environmental analyses demonstrating the presence of abundant and diverse putative Group I Crenarchaeota-associated genes coding for 16S rRNA and the α subunit of ammonia monooxygenase (*amoA*) in the marine water column (Francis *et al.*, 2005), estuarine sediments (Beman and Francis, 2006), sponges (Steger *et al.*, 2008), soils (Leininger *et al.*, 2006) and hot springs (Zhang *et al.*, 2008), indicate that these prevalent Archaea are also predominantly ammonia oxidizers. Archaeal *amoA* copy numbers often exceed those of bacteria, thus suggesting that Archaea may even dominate bacteria in ammonia oxidation under certain conditions (for example, Leininger *et al.*, 2006; Wuchter *et al.*, 2006; Mincer *et al.*, 2007; Martens-Habbena *et al.*, 2009). Despite their apparent ubiquity and potential ecological importance, culturing efforts in concert with these discoveries have resulted in the enrichment and characterization of only a few ammonia-oxidizing Archaea (AOA) to date, together representing three phylogenetic lineages (Könneke *et al.*, 2005;

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de la Torre *et al.*, 2008; Hatzenpichler *et al.*, 2008). The recent enrichment of '*Candidatus* Nitrososphaera gargensis' (Hatzenpichler *et al.*, 2008) has demonstrated the capacity for ammonia oxidation by moderately thermophilic Archaea and extended this capability to the Group I.1b Crenarchaeota, a clade comprised predominantly of 16S rRNA gene sequences recovered from soils.

The core (that is, apolar) component of crenarchaeal cellular membrane lipids, in general, is dominated by glycerol dialkyl glycerol tetraethers (GDGTs) (for example, Figure 1), which may contain multiple cyclopentane moieties (see overviews in Macalady *et al.*, 2004; Schouten *et al.*, 2007c). For a time, a unique GDGT, crenarchaeol, which contains a cyclohexane moiety in addition to its four cyclopentane moieties (Figure 1), was observed in natural samples but not in crenarchaeal cultures until its formal characterization from GDGTs extracted from a co-culture of *Cenarchaeum symbiosum* and the marine sponge *Axinella mexicana* (Sinninghe Damsté *et al.*, 2002). Consequently, it was proposed that the crenarchaeol found so ubiquitously in nature originated from mesophilic Crenarchaeota, a hypothesis supported by confirmation of crenarchaeol synthesis by the marine Group I.1a Crenarchaeon, *Nitrosopumilus maritimus SCM1* (Schouten *et al.*, 2008).

Since then, however, crenarchaeol recovery from hot springs (Pearson *et al.*, 2004; Zhang *et al.*, 2006; Pitcher *et al.*, 2009) and confirmation of crenarchaeol synthesis by the ammonia-oxidizing thermophile (growth temperature of 72 °C), '*Ca.* N. yellowstonii' (ThAOA/HWCG III) (de la Torre *et al.*, 2008), has shown that crenarchaeol is also synthesized by non-mesophilic Crenarchaeota, effectively negating the original hypothesis. Instead, a number of studies have linked the presence of putative AOA to the occurrence of crenarchaeol (Wuchter *et al.*, 2004; Coolen *et al.*, 2007; Schouten *et al.*, 2007a). The significant correlation between GDGT concentrations (including crenarchaeol) and Crenarchaeota Group I.1b *amoA* copy numbers in soil, further indicated that Group I.1b AOA specifically may also synthesize crenarchaeol (Leininger *et al.*, 2006). Taken together, these findings suggest that crenarchaeol is a specific biomarker for AOA (de la Torre *et al.*, 2008).

In addition to crenarchaeol, the regioisomer of crenarchaeol (cf. Sinninghe Damsté *et al.*, 2002) is also abundant in certain settings, particularly in tropical marine environments (Schouten *et al.*, 2002; Kim *et al.*, 2008). However, none of the previously enriched or isolated Crenarchaea produce substantial amounts of the crenarchaeol regioisomer, despite producing crenarchaeol (Sinninghe Damsté *et al.*, 2002; Wuchter *et al.*, 2005; Schouten *et al.*, 2007a, 2008; de la Torre *et al.*, 2008). This discrepancy is also important to investigate as the regioisomer of crenarchaeol is used, together with several other GDGTs, in the $TEX_{86}$ paleothermometer (Schouten *et al.*, 2002) (Figure 1). The $TEX_{86}$ has been shown to correlate well with *in situ* temperatures in sea water enrichment cultures

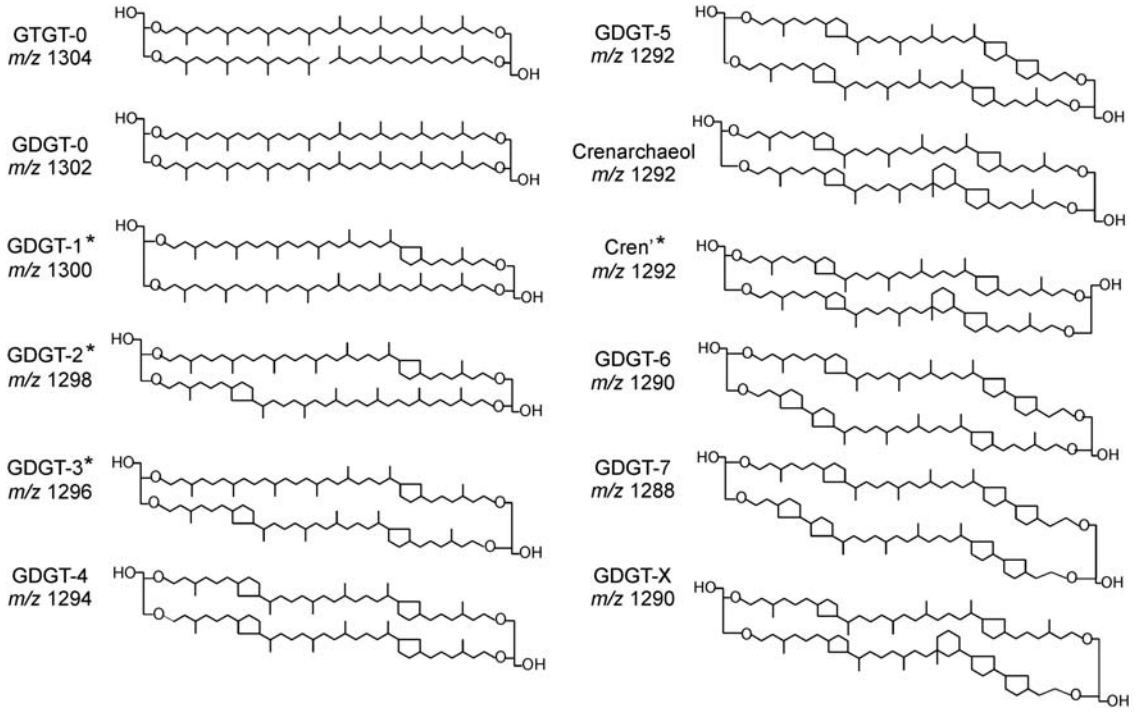


**Figure 1** Structures analyzed in this study: glycerol trialkyl tetraether (GTGT)-0, glycerol dialkyl glycerol tetraethers (GDGTs) 0–7 including crenarchaeol, the crenarchaeol regioisomer (cren') and the new GDGT-X. GDGTs used to calculate the $TEX_{86}$ ratio are indicated by stars.

(Wuchter *et al.*, 2004; Schouten *et al.*, 2007a), as well as the marine water column (Wuchter *et al.*, 2005). However, a calibration of the $TEX_{86}$ by cultivation efforts has not been possible because of the near-lack of the regioisomer in cultures compared with relative abundances observed in natural environments at similar temperatures (cf. Schouten *et al.*, 2007a). Through analysis of the core GDGT and intact polar lipid (IPL)-GDGT composition of '*Ca.* N. gargensis', we have been able to further investigate the phylogenetic distribution of crenarchaeol and its regioisomer in AOA. In addition, we have analyzed lipids of '*Ca.* N. gargensis' cultivated at three growth temperatures to determine whether slight temperature differences result in the modification of its GDGT distribution.

## Materials and methods

*Culture conditions*

The ammonia-oxidizing enrichment culture of '*Candidatus* N. gargensis' (Hatzenpichler *et al.*, 2008) was grown in mineral medium modified from Krümmel and Harms (1982) containing $KH_2PO_4$ (0.4 mM), KCl (1 mM), $MgSO_4$ (0.2 mM), $CaCl_2$ (1 mM) and NaCl (10 mM). In all, 1 ml trace element solution (Ehrich *et al.*, 1995) and a small amount of cresol red were added to the final medium. In contrast to the previous protocol (Hatzenpichler *et al.*, 2008), the substrate concentration was reduced to 0.5 mM $NH_4Cl$ from 2.0 mM. A total of 4.5 l of medium was prepared in 5-l bottles and inoculated with 50 ml of an active preculture. Incubation was carried out in the dark at 46 °C for 3 weeks with moderate stirring (150 r.p.m.). The pH was adjusted to 7.8 and kept constant by daily titration using 15% (w/v) $NaHCO_3$. The consumption of ammonia was regularly measured by test sticks (Merck, Darmstadt, Germany) followed by replenishing the substrate with a 5 mM sterile stock solution. In total, 3–4 mM $NH_4Cl$ were oxidized before cells were harvested by centrifugation (14 000 *g*) and washed in 0.9% NaCl. Several 5-l bottles were collected, and the pellet was stored at −20 °C until final analysis. In the enrichments used in this study, a single archaeal operational taxonomic unit was present, which showed 98–99% 16S RNA gene sequence similarity to '*Ca.* N. gargensis' (Hatzenpichler *et al.*, 2008). In addition, the enrichment also contained a β-proteobacterium and possibly several other bacteria (Hatzenpichler *et al.*, 2008). Cells for the temperature experiment were grown in 3-l flasks with 1.5 l medium without stirring.

*Core lipid analysis*

Acid hydrolysis was carried out on freeze-dried biomass of cultivated '*Ca.* N. gargensis' to cleave polar headgroups. Biomass was refluxed in 2 ml of 5% HCl in methanol (MeOH) for 3 h. The cooled solution was adjusted to pH 5 with 2 N KOH:MeOH (1:1 v/v). Bidistilled water was added to a final ratio of $H_2O$:MeOH (1:1 v/v) and this mixture was washed three times with dichloromethane (DCM). The DCM fractions were collected and dried over $Na_2SO_4$. The extract was dissolved in hexane:propanol (99:1 v/v) filtered over a 0.4 μm PTFE filter before analysis by high-performance liquid chromatography (HPLC) atmospheric pressure chemical ionization mass spectrometry (HPLC-Atmospheric Pressure Chemical Ionization (APCI)/mass spectrometry (MS)).

*HPLC-APCI/MS analysis*

Archaeal GDGTs were analyzed using a modified procedure from Hopmans *et al.* (2000) and Schouten *et al.* (2007b). Archaeal GDGTs were detected with full scan analysis from *m/z* 900–1400 and the relative abundance determined by integration of the peak areas of their $[M+H]^+$ and $[M+H+1]^+$ ions.

*Identification of a novel GDGT-'X'*

Individual GDGTs of '*Ca.* N. gargensis' were isolated by semi-preparative HPLC according to the procedure described by Smittenberg *et al.* (2002). The fraction enriched in GDGT-X was subjected to ether bond cleavage as described by Hoefs *et al.* (1997) and analyzed by gas chromatography/mass spectrometry (GC/MS) for biphytanes. GC/MS analysis was carried out on a Thermofinnigan TRACE GC equipped with a fused silica capillary column (25 m × 0.32 mm) coated with CP Sil-5 (film thickness 0.12 μm) and helium as the carrier gas. Sample was dissolved in hexane and injected at 70 °C. Subsequently the oven was programmed to increase to 130 °C at 20 °C $min^{-1}$ and then to 320 °C at 4 °C $min^{-1}$, where it was held for 10 min. The GC was coupled with a Thermofinnigan DSQ quadrupole MS with an ionization energy of 70 eV scanning a mass range of *m/z* 50–800 at three scans per second.

*IPL analysis*

Intact polar lipids were extracted from freeze-dried biomass using a modified Bligh and Dyer technique (Bligh and Dyer, 1959). A known volume of single-phase solvent mixture of MeOH:DCM:phosphate buffer (2:1:0.8 v/v/v) was added to the sample in a centrifuge tube and placed in an ultrasonic bath for 10 min. The extract and residue were separated by centrifuging at 2500 r.p.m. for 5 min and the solvent mixture collected in a separate flask (three times). The DCM and phosphate buffer were added to the single-phase extract to give a new ratio of MeOH:DCM:phosphate buffer (1:1:0.9 v/v/v) and to induce phase separation. The extract was centrifuged at 2500 r.p.m. for 5 min. DCM phase was collected in a round-bottom flask and the methanol:phosphate buffer phase was washed two

additional times with DCM. The combined DCM phases were reduced under rotary vacuum and evaporated to dryness under a stream of $N_2$. Residual biomass was re-extracted using 5% trichloroacetic acid instead of phosphate buffer according to Sturt *et al.* (2004), and analyzed separately. This extract contained the same IPLs identified in the first extraction, but in much lesser amounts.

*HPLC-electrospray ionization (ESI)/MS*
Intact polar lipid-glycerol dialkyl glycerol tetraethers were analyzed according to conditions described previously (Schouten *et al.*, 2008) as modified from Sturt *et al.* (2004). For the analysis an Agilent (Palo-Alto, CA, US) 1100 series LC equipped with a thermostatted auto-injector was coupled to a Thermo TSQ Quantum EM triple quadrupole MS equipped with an Ion Max source with ESI probe. Detection was achieved using positive ion ESI/MS by scanning mass range *m/z* 1000–2000.

## Results

*Core lipids*
High-performance liquid chromatography-APCI/mass spectrometry analysis of the GDGTs released from acid-hydrolyzed whole-cell biomass from '*Ca.* N. gargensis', grown at its optimal growth temperature of 46 °C, revealed that it produces predominantly crenarchaeol and the crenarchaeol regioisomer (Figure 2b). GDGTs 0–4 were also present, but in very low abundances relative to crenarchaeol and its regioisomer (Table 1). In addition, a GDGT with a $[M+H]^+$ of *m/z* 1290 (that is, six rings or double bonds) was present, which eluted earlier than GDGT-6 known to occur in (hyper)thermophilic Archaea, suggesting that it represented a novel core membrane lipid (henceforth referred to as 'GDGT-X').

Tentative identification of GDGT-X was carried after isolation with semi-preparative HPLC and treatment with $HI/LiAlH_4$ to release the carbon skeletons of the GDGT. GC/MS analysis revealed the presence of two $C_{40}$ isoprenoidal hydrocarbons present in approximately equal abundance, indicating that together they comprised the intact GDGT-X. The first eluting isoprenoid had an MS and retention time identical to that of a biphytane with two cyclopentane moieties (de Rosa and Gambacorta, 1988; Hoefs *et al.*, 1997; Schouten *et al.*, 1998), with characteristic fragment ions at *m/z* 97, 125, 165 and 194, and an $M^+$ ion of *m/z* 558 (Figure 3a). The second eluting compound had a mass spectrum with fragment ions at *m/z* 95, 151, 163 and 261, and a fragment of *m/z* 539, likely corresponding to an $M^+$-15 ion (Figure 3b). The latter would be in agreement with a biphytane carbon skeleton containing four cyclic moieties; the mass spectrum of this compound is quite similar to that of the biphytane containing two cyclopentane moieties and a cyclohexane moiety present in crenarchaeol (Schouten *et al.*, 1998), except that the fragment ions 165 and 263 are now 2 Da lower. These fragments would be consistent with the same biphytane carbon skeleton present in crenarchaeol, but containing an additional cyclopentane moiety at the end of the alkyl chain. Indeed, it is well-known that hyperthermophilic Crenarchaeota make cyclopentane moieties at this position of the biphytane carbon skeleton when grown at high temperatures (de Rosa and Gambacorta, 1988). Based on this, the structure of GDGT-X was tentatively identified as being nearly identical to that of crenarchaeol but with an additional cyclopentane moiety (Figure 1).

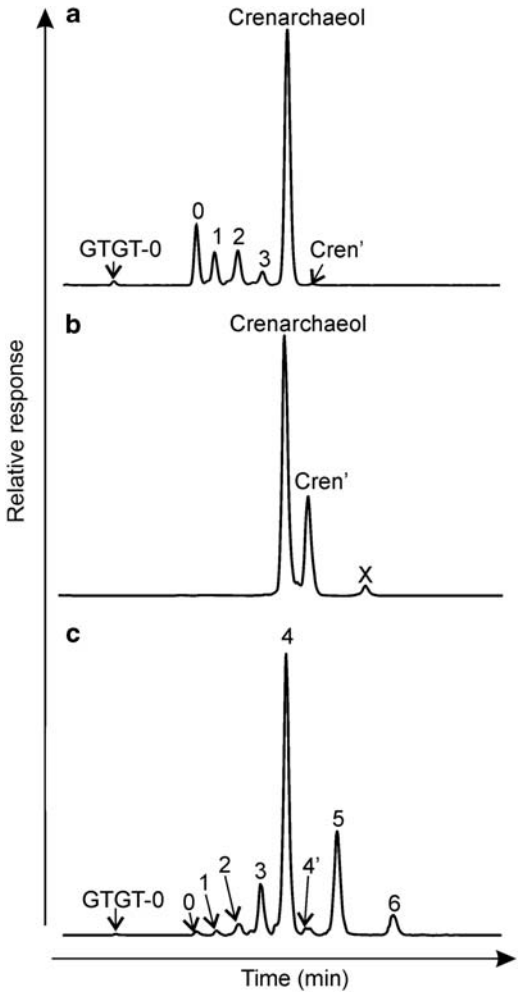


**Figure 2** High-performance liquid chromatography (HPLC-APCI) base peak chromatograms showing glycerol dialkyl glycerol tetraethers (GDGTs) derived from acid-hydrolyzed biomass of (**a**) *Nitrosopumulis maritimus SCM1* (Schouten *et al.*, 2008), (**b**) '*Candidatus* Nitrososphaera gargensis' grown at 46 °C and (**c**) *Sulfolobus solfataricus* P2 strain DSM1617 (Ellen *et al.*, 2009). Peak labels: glycerol trialkyl tetraether (GTGT)-0 is labeled as such; other peaks follow GDGT designations according to Figure 1 (e.g., '1' = GDGT-1).

To test whether '*Ca.* N. gargensis' would adjust its GDGT composition when grown at different

**Table 1** Relative abundance of GDGTs recovered from acid-hydrolyzed biomass of the original enrichment culture material, and '*Candidatus* Nitrososphaera gargensis' grown at 42, 46 and 50 °C

| *Growth temperature (°C)* | *GDGT relative abundance* | | | | | | | | | *$TEX_{86}$* | *$TEX_{86}$-temp (°C)* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *0* | *1* | *2* | *3* | *4* | *4′* | *cren* | *cren′* | *X* | | |
| 46[a] | 0.10 | 0.12 | 0.15 | 0.32 | 2.2 | 6.2 | 65 | 21 | 4.5 | 0.994 | 45.1 |
| 42 | 0.32 | 0.38 | 0.47 | 1.0 | 1.1 | 2.1 | 69 | 24 | 1.6 | 0.985 | 44.6 |
| 46 | 0.12 | 0.14 | 0.17 | 0.38 | 2.0 | 8.3 | 65 | 19 | 4.2 | 0.993 | 45.1 |
| 50 | 0.10 | 0.13 | 0.17 | 0.38 | 1.5 | 4.1 | 70 | 21 | 2.3 | 0.994 | 45.1 |

Abbreviations: cren, crenarchaeol; cren′, crenarchaeol regioisomer; GDGT, glycerol dialkyl glycerol tetraether; $TEX_{86}$, TetraEther Index of tetraethers consisting of 86 carbon atoms.
GDGT numbers correspond to structures shown in Figure 1.
[a]Original enrichment culture used to innoculate media incubated at 42, 46 and 50 °C (below).

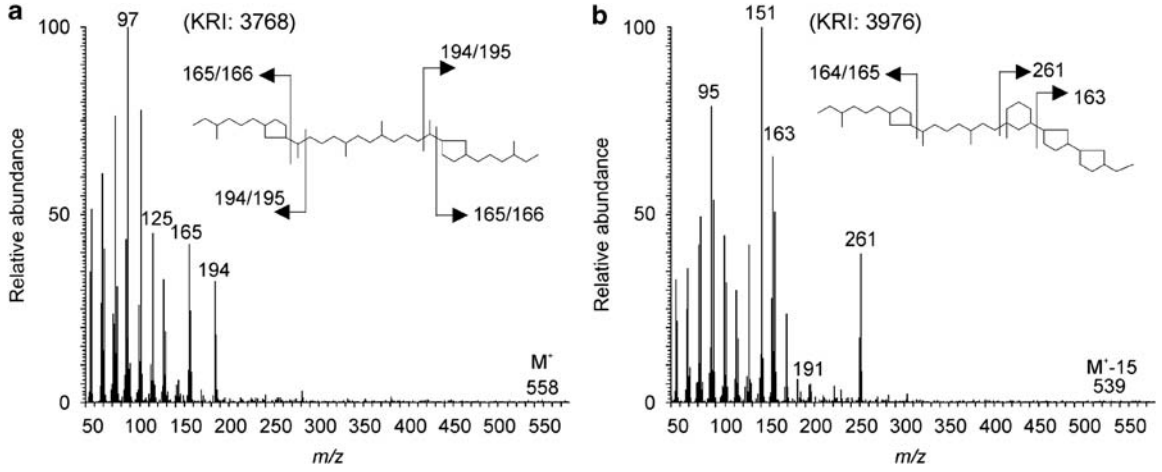


**Figure 3** (**a**, **b**) Mass spectra corresponding to biphytanes released from a prepared glycerol dialkyl glycerol tetraether (GDGT) fraction enriched in GDGT-X after treatment with HI/$LiAlH_4$. Kirchman retention indices (KRI) are indicated in brackets.

temperatures, media was inoculated with the original enrichment culture and incubated at 42, 46 and 50 °C for 3 weeks. Acid hydrolysis of biomass harvested during the end of logarithmic phase growth revealed that, at all the three temperatures crenarchaeol and its regioisomer remained the most abundant GDGTs, with significant amounts of GDGT-X. There were also notable amounts of GDGT-4 and an unknown isomer of GDGT-4 in the 46 °C culture, which were less abundant in the original enrichment culture. Furthermore, at all growth temperatures, '*Ca.* N. gargensis' continued to produce only minor amounts of GDGTs 0–3, each comprising $\leqslant$2% of total GDGTs, and barely detectable amounts of GTGT-1 and GDGT-6 (data not shown). The relative abundances of GDGTs 0–3 were slightly higher at 42 °C than at 46 and 50 °C. In contrast, the relative abundances of GDGTs 4 and X, were highest at 46 °C (Table 1).

*Intact polar lipids*

High-performance liquid chromatography-electrospray ionization/mass spectrometry analysis of the Bligh and Dyer extract of '*Ca.* N. gargensis' showed four peaks with single molecular ions and two major peak clusters with IPLs that were not baseline separated (Figure 4). Based on their mass spectra, resulting from in-source fragmentation, six IPLs were tentatively identified (Table 2, Figure 4).

The first peak (1) was identified as crenarchaeol with a glycosidically bound hexose headgroup (Figure 4). The dominant ions included 1454, 1471 and 1476, representing the $[M+H]^+$ of the IPL, ammonium ($[M+NH_4]^+$) and sodium ($[M+Na]^+$) adducts, respectively. An additional dominant ion at *m/z* 1292 indicated that crenarchaeol or the crenarchaeol regioisomer was the core GDGT of this polar lipid.

The second peak (2) appeared to consist of a single IPL with a protonated molecule at *m/z* 1629 Da ($[M+NH_4]^+$ and $[M+Na]^+$ at *m/z* 1647 and 1652, respectively) and fragments at *m/z* 1292 and 1454 corresponding to crenarchaeol and crenarchaeol with a monohexose group. This suggests that this IPL is a monohexose crenarchaeol with an additional but unknown headgroup of 176 Da; however, it was not possible to determine the structure of this additional headgroup based on MS. Similar intact polar GDGTs with a headgroup of 176 Da have, to the

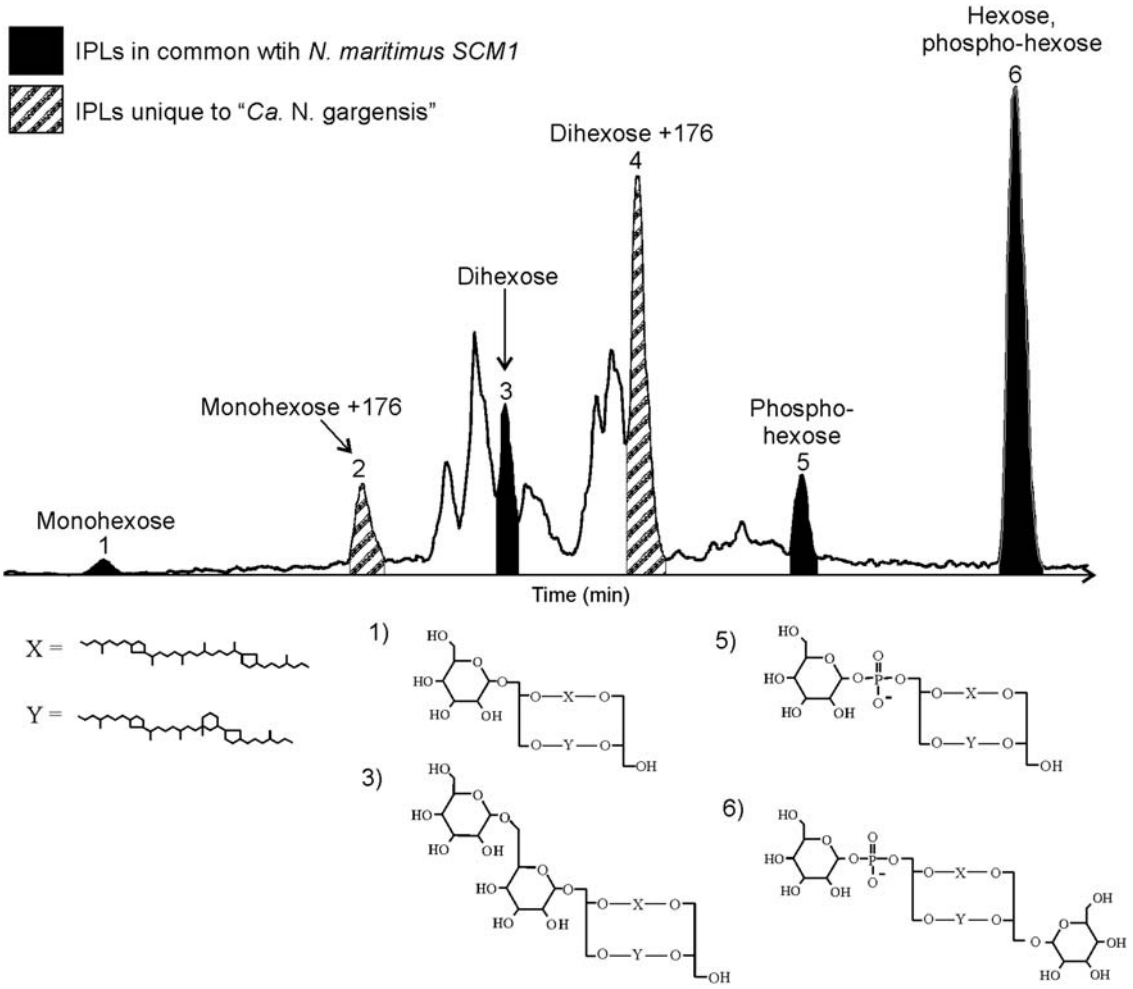


**Figure 4** High-performance liquid chromatography-electrospray ionization/mass spectrometry (HPLC-ESI/MS)-base peak chromatogram of intact polar lipids (IPL) extracted from '*Candidatus* Nitrososphaera gargensis'. Numbered peaks correspond to structures described in Table 2 and tentative structures drawn below the chromatogram (with the exception of IPLs eluting in peaks 2 and 4, which were not drawn because of the unknown nature of the 176 Da moiety). Stars indicate unknown compounds with fragment ions below *m/z* 1200 (i.e., not GDGT-based).

**Table 2** Summary of the main ions in the mass spectra of the intact polar lipids of '*Candidatus* Nitrososphaera gargensis' shown in Figure 4.

| *Peak* | *Fragments* | *$[M+H]^+$* | *$[M+NH_4]^+$* | *$[M+Na]^+$* | *Headgroup* |
|---|---|---|---|---|---|
| 1 | 1292 | 1454 | 1471 | 1476 | Monohexose |
| 2 | 1292, 1454 | 1630 | 1647 | 1652 | Monohexose+176 |
| 3 | 1292 | 1616 | 1633 | 1638 | Dihexose |
| 4 | 1292, 1454 | 1792 | 1809 | 1814 | Dihexose+176 |
| 5 | None | 1534 | 1556 | 1578 | Phospho-hexose |
| 6 | 1534 | 1696 | 1713 | 1718 | Hexose, phospho-hexose |

best of our knowledge, not yet been reported elsewhere. The molecular weight of the headgroup is 14 Da higher than that of a hexose moiety, suggesting that it is possibly a methylated hexose moiety. However, further identification by isolation and NMR techniques is needed to confirm this hypothesis.

Most peaks in the cluster-containing peak 3 (indicated by stars, Figure 4) contained ions with *m/z* ratios below 1200 in their mass spectra, suggesting that they did not contain GDGTs as core lipids. This was not surprising as the culture was an enrichment containing multiple species of bacteria in addition to '*Ca.* N. gargensis'. The GDGT-based IPL eluting in Peak 3 itself gave a mass spectrum identical to that described previously for diglycosidic GDGTs (Schouten *et al*, 2008), with $[M+H]^+$, $[M+NH_4]^+$ and $[M+Na]^+$ ions corresponding to

*m/z* values of 1616, 1633 and 1638, respectively. Most peaks in the next eluting cluster (Figure 4) consisted mainly of unknown compounds, again unrelated to GDGTs as they had fragment ions all below 1200. The IPL eluting in peak 4, with an $[M+H]^+$ at *m/z* 1792, had an MS suggestive of diglycosidic crenarchaeol with an additional unknown headgroup of 176 Daltons. Likely, this unknown headgroup is the same as that found for the IPL represented by peak 2.

The MS of the IPL eluting in peak 5 (Figure 4) is in agreement with that of phospho-hexose crenarchaeol based on the *m/z* values of 1534, 1556 and 1578, which corresponded to the $[M+H]^+$, $[M+NH_4]^+$ and $[M+Na]^+$ ions, respectively (Table 2). This compound has been reported in *N. maritimus SCM1* (Schouten *et al.*, 2008). Finally, the IPL in the last eluting peak (6) (Figure 4) had a mass spectrum suggestive of crenarchaeol with a glycosidically bound hexose headgroup and a phospho-hexose headgroup with *m/z* values of 1696, 1713 and 1718 corresponding to the $[M+H]^+$, $[M+NH_4]^+$ and $[M+Na]^+$ ions, respectively (Figure 3, Table 2). This IPL has also been described previously for *N. maritimus SCM1* (Schouten *et al.*, 2008).

## Discussion

*Phylogenetic occurrence of crenarchaeol*

Our results show that '*Candidatus* N. gargensis', a moderately thermophilic, ammonia-oxidizing Group I.1b Crenarchaeon, synthesizes crenarchaeol as its main core membrane GDGT. This expands the phylogenetic distribution of crenarchaeol synthesis to include, in addition to the marine Group I.1a Crenarchaeota (Sinninghe Damsté *et al.*, 2002; Schouten *et al.*, 2008) and the ThAOA/HWCG III clade (de la Torre *et al.*, 2008), the soil Group I.1b Crenarchaeota, which all belong to the monophyletic lineage of AOA recently described by Prosser and Nicol (2008) (cf. Figure 5). In addition, our findings confirm previous circumstantial evidence for crenarchaeol synthesis by Group I.1b Crenarchaeota based on the ubiquitous presence of crenarchaeol in soils (Weijers *et al.*, 2006), and the significant correlation linking ammonia-oxidizing soil Archaea to crenarchaeol synthesis (Leininger *et al.*, 2006). Our results signify that all cultivated representatives of Group I Crenarchaeota to date synthesize crenarchaeol, thus providing support for the suggested specificity of crenarchaeol to Archaea involved in ammonia oxidation (de la Torre *et al.*, 2008). Crenarchaeol has also been detected at sites of archaeal ammonia oxidation in the marine water column of the coastal North Sea (Wuchter *et al.*, 2006), the Black Sea (Coolen *et al.*, 2007), Icelandic hot springs (Reigstad *et al.*, 2008), Nevada and California hot springs (Pearson *et al.*, 2004; Zhang *et al.*, 2006; Pitcher *et al.*, 2009) and agricultural

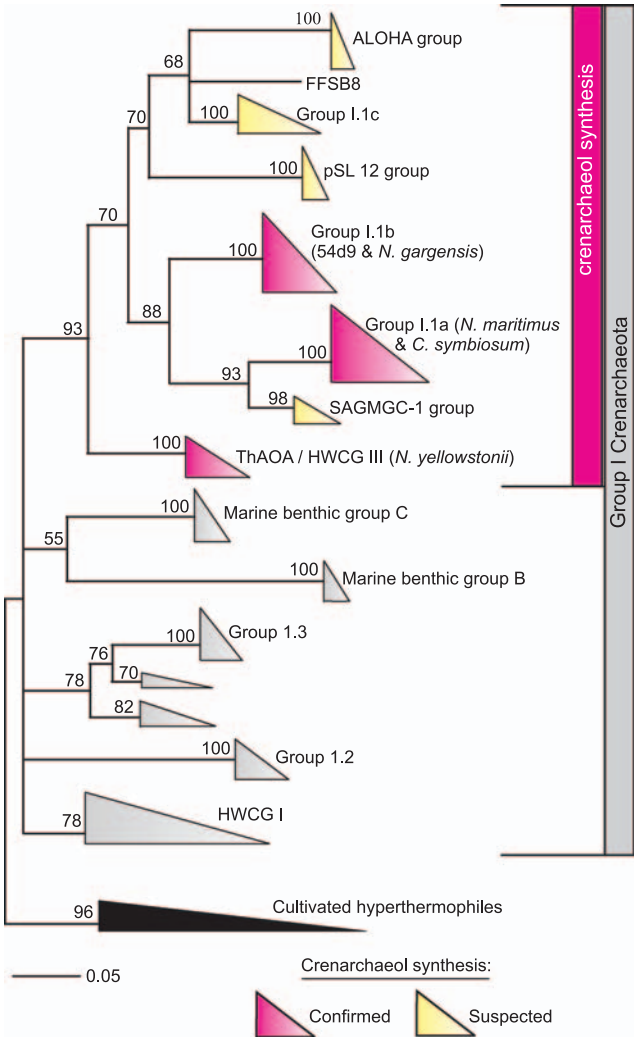


**Figure 5** 16S rRNA gene-based phylogeny of the Crenarchaeota redrawn from Prosser and Nicol highlighting confirmed and suspected crenarchaeol synthesizers within the 'Group I' Crenarchaeota based on the potential widespread distribution of archaeal ammonia-oxidization within this monophyletic clade and its hypothesized link to crenarchaeol synthesis.

soils (Leininger *et al.*, 2006). Although Wuchter *et al.* (2006) and Leininger *et al.* (2006) found a strong correlation between crenarchaeol abundance and *amoA* copy numbers, but because of a lack of cultured representatives from Group I Crenarchaeota falling outside the recognized AOA, it yet cannot be unambiguously concluded that crenarchaeol synthesis is actually restricted to AOA.

Our results differ from those of *N. maritimus* and other AOA enrichment cultures in that the GDGT composition of '*Candidatus* N. gargensis' is dominated by crenarchaeol and the crenarchaeol regioisomer (cf. Figures 2a and b). All previously analyzed AOA contained substantial amounts of other GDGTs in addition to crenarchaeol, most notably GDGT-0 in *N. maritimus SCM1* (Schouten *et al.*, 2008) and *C. symbiosium* (Sinninghe Damsté *et al.*, 2002), and large amounts of glycerol trialkyl glycerol tetraethers

synthesized by '*Ca.* N. yellowstonii' (de la Torre *et al.*, 2008). These large differences in tetraether distribution between the different organisms may be due to the different optimal growth temperatures of individual isolates/enrichment cultures, which range from 28 °C for *N. maritimus SCM1* to 72 °C for '*Ca.* N. yellowstonii'. Indeed, cultivation of '*Ca.* N. gargensis' at temperatures lower than its optimal growth temperature resulted in a slight increase in GDGT-0. Alternatively, these differences in composition may reflect genetic differences affecting membrane lipid biosynthesis between the phylogenetic clusters I.1a, I.1b and ThAOA/HWCG III. The later hypothesis implies that differences in the Crenarchaeota community composition between environmental samples may have a notable effect on the composition of environmental GDGT assemblages.

*Specific GDGTs for phylogenetic clusters in Group I Crenarchaeota*

The core lipids of '*Ca.* N. gargensis' contain a unique GDGT-X (Figures 1 and 2). The addition of a cyclopentane moiety is homologous to thermophilic temperature adaptations observed in other cultivated hyperthermophiles (for example, *Sulfolobus*), which increase the number of cyclopentane rings in their GDGTs as temperature increases. Indeed, this GDGT is not found in the mesophilic *N. maritimus SCM1*, but it was also not reported to be present in the thermophilic '*Ca.* N. yellowstonii' by de la Torre *et al.* (2008). The relative amount of GDGT-X also did not increase when '*Ca.* N. gargensis' was grown at higher temperatures. Thus, GDGT-X may be unique to (thermophilic) Group I.1b Crenarchaeota rather than an adaptation to elevated growth temperature.

'*Ca.* N. gargensis' also synthesizes two IPL headgroups containing moieties corresponding to 176 Da (Figure 4, peaks 2 and 4). To the best of our knowledge, such a moiety has not been reported as yet for cultures or in natural environments. Schouten *et al.* (2008) reported IPLs with an unknown headgroup of 180 Da in *N. maritimus SCM1*, which is not present in '*Ca.* N. gargensis'. On the other hand, '*Ca.* N. gargensis' also synthesizes some IPLs with headgroups identical to those synthesized by *N. maritimus SCM1* (Schouten *et al.*, 2008); IPLs with a monohexose, dihexose and hexose-phosphohexose moieties (Figure 4) are common to both species, thereby indicating that these IPLs may become useful biomarkers for broad AOA screens. Direct detection of some crenarchaeal IPLs (that is, containing crenarchaeol) has been achieved in deep sea sediments using HPLC-ESI/MS (Sturt *et al.*, 2004; Biddle *et al.*, 2006). They found IPLs containing crenarchaeol with hexose and dihexose moieties, but not with a phospo-hexose moiety that is present in both *N. maritimus SCM1* and '*Ca.* N. gargensis'. It is interesting to note that they also found GDGTs with an unknown headgroup of 180 Da, similar to that synthesized by *N. maritimus SCM1*. This might indicate a specific contribution of Group I.1a Crenarchaeota in deep-sea sediments, although they did not recover any Group I.1a Crenarchaeal 16S rRNA gene sequences from their clone libraries. An alternative explanation is that archaeal members derived from, for example, Marine Benthic Group B, which were well-represented in those recovered sequences, synthesize similar IPL headgroups and possibly crenarchaeol. Again, cultivated representatives of sedimentary Archaea are needed to confirm this.

*Distribution and occurrence of the crenarchaeol regioisomer*

The crenarchaeol regioisomer has, until now, been reported in very low (that is, $<5\%$ of total GDGTs) amounts in cultured/enriched Group I Crenarchaeota representatives. Neither *N. maritimus* nor *C. symbiosum* synthesize substantial amounts of the regioisomer in culture (Schouten *et al.*, 2008; Sinninghe Damsté *et al.*, 2002), and only trace amounts were observed in '*Ca.* Nitrosocaldus yellowstonii' when grown at 72 °C (de la Torre *et al.*, 2008). Similarly, only small amounts of the regioisomer were found in marine Group I.1a archaeal enrichments obtained from the North Sea (Wuchter *et al.*, 2004) and the Indian Ocean (Schouten *et al.*, 2007a) when cultivated over a range of temperatures (5–35 °C and 25–40 °C, respectively). Trace amounts of crenarchaeol regioisomer detected in these cultures and enrichments show that these Group I Crenarchaeota are indeed capable of synthesizing the crenarchaeol regioisomer; however, only appear to do so to a small degree.

Despite its limited production in cultured Crenarchaeota until now, the crenarchaeol regioisomer is abundant in some natural environments, particularly in tropical marine waters and sediments as well as in lake sediments, though its abundance rarely exceeds ca. 15% of the corresponding crenarchaeol concentration (Schouten *et al.*, 2002; Wuchter *et al.*, 2005; Kim *et al.*, 2008). In some tropical soils, however, the relative abundance of the crenarchaeol isomer is much higher and approaches ca. 40% of the local crenarchaeol concentration (Weijers *et al.*, 2006). Members of the soil Group I.1b crenarchaeota with a GDGT composition similar to that of '*Ca.* N. gargensis' are likely responsible for this unusual environmental GDGT pattern. A potential contribution of Group I.1b Crenarchaeota to the increased crenarchaeol regioisomer abundances in tropical marine waters and sediments appears less parsimonious, as sequences affiliated with Group I.1b are not often reported in open marine environments. For example, Herfort *et al.* (2007) and Coolen *et al.* (2007) only found 16S rRNA gene sequences related to marine Group I Crenarchaeota in the North Sea and the Black Sea water columns, respectively. It should,

however, be noted that Group I.1b sequences have indeed been recovered from some marine sediments (for example, Park *et al.*, 2008; Sahan and Muyzer, 2008) and corals (Beman *et al.*, 2007). Thus, three plausible explanations exist for the high regioisomer abundance in tropical marine waters: (i) culture conditions used to enrich or cultivate marine Group I.1a Crenarchaeota till now have simply not been effective at stimulating production of the crenarchaeol regioisomer, (ii) previously overlooked Group I.1b AOA are responsible for the regioisomer production in tropical marine waters or (iii) large differences in crenarchaeol regioisomer production exist among individual Group I.1a species. The latter two hypotheses are supported by Shah *et al.* (2008), who present evidence supporting alternative biological or geographical origins of crenarchaeol regioisomer, which was isolated from a mixed sedimentary GDGT assemblage.

Our results also have implications for $TEX_{86}$ paleothermometry, as paleoreconstructions of sea surface temperatures using $TEX_{86}$ rely on the inclusion of the crenarchaeol regioisomer in the calculated $TEX_{86}$ ratio. If regioisomer synthesis is not only controlled by temperature, but also by the occurrence of specific phylogenetic clusters of Crenarchaeota producing enhanced amounts of the regioisomer, then this may complicate the use of the $TEX_{86}$. Such a scenario may explain part of the scatter observed in the $TEX_{86}$-temperature calibration (Kim *et al.*, 2008). At this point, however, it is impossible to evaluate which potential shifts in $TEX_{86}$-temperature estimates will be caused by shifts in crenarchaeal populations in the natural environment, as such community shifts are potentially influenced by a wide variety of factors including optimal growth temperatures, substrate availability, presence of competitors and predators, and species biogeography.

Our results with '*Ca.* N. gargensis' may be used to test the $TEX_{86}$ at high temperatures, although we note that '*Ca.* N. gargensis' is a moderately thermophilic organism isolated from a non-marine environment. A $TEX_{86}$ value of 1 would (linearly) extrapolate to a temperature of 45.4 °C using the Kim *et al.* (2008) calibration. This occurs when the crenarchaeol regioisomer is highly dominating over GDGTs 1–3, such as what is observed for '*Ca.* N. gargensis'; the $TEX_{86}$-derived temperature calculated from our biomass grown at 46 °C was 45.1 °C (cf. Table 1). There was no notable difference in the relative GDGT abundances when '*Ca.* N. gargensis' was grown at 46 or 50 °C, but at 42 °C the increase in the relative abundance of GDGTs 1–3 resulted in a slightly lower, but analytically robust, $TEX_{86}$ (Table 1). The $TEX_{86}$ empirical relationship predicts a more pronounced increase in GDGTs 1–3 than we observed—that is, shifting from 46 to 42 °C should correspond to a $TEX_{86}$ decrease of 0.050 (that is, from 0.99 to 0.94). The measured $TEX_{86}$ decrease of only 0.008 signifies that factors in addition to temperature influence the membrane GDGT distribution at high (and likely low) temperatures, rendering the GDGT-temperature relationship nonlinear in these regions. Nevertheless, our results indicate that temperature does indeed exert some influence over the relative abundance of these GDGTs among individual species within the Group I.1b, similar to the phenomena observed by incubation experiments with Group I.1a Crenarchaeota (Wuchter *et al.*, 2004; Schouten *et al.*, 2007a).

Ultimately, the confirmation of crenarchaeol synthesis within the soil Group I.1b Crenarchaeota further supports the hypothesis that crenarchaeol is synthesized by all Archaea carrying out ammonia oxidation. However, as no cultivated representatives outside the recognized AOA lineages of the Group I Crenarchaeota are available at this moment, it cannot yet be evaluated how widespread crenarchaeol synthesis is among this group. '*Ca.* N. gargensis' is the first AOA for which the production of large amounts of the crenarchaeol regioisomer has been demonstrated, suggesting that Group I.1b Crenarchaeota are a likely source of the documented elevated regioisomer abundances in some natural environments. Furthermore, the unique lipids (a new GDGT and IPL with new headgroups) synthesized by '*Ca.* N. gargensis' may prove useful in future studies as biomarkers for Group I.1b Crenarchaeota or a subclade of this group. Screening more crenarchaeal enrichment cultures, as they become available for their IPLs will determine if indeed this is the case. In contrast, environmental screening for IPLs common to *N. maritimus* and '*Ca.* N. gargensis' (for example, crenarchaeol with a hexose and phosphohexose headgroup) may prove useful for the general detection of AOA.

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