New Insights Into the Polar Lipid Composition of Extremely Halo(alkali)philic Euryarchaea From Hypersaline Lakes

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We analyzed the polar membrane lipids of 13 strains of halo(alkali)philic euryarchaea from hypersaline lakes. Nine belong to the class Halobacteria, representing two functional groups: aerobic polysaccharide utilizers and sulfur-respiring anaerobes. The other four strains represent halo(alkali)philic methanogens from the class Methanomicrobia and a recently discovered class Methanonatronarchaeia. A wide range of polar lipids were detected across the 13 strains including dialkyl glycerol diethers (archaeols), membrane-spanning glycerol tetraethers and diether-based cardiolipins. The archaeols contained a range of core lipid structures, including combinations of C20 and C25 isoprenoidal alkyl chains, unsaturations, and hydroxy moieties. Several diether lipids were novel, including: (a) a phosphatidylglycerolhexose (PG-Gly) headgroup, (b) a N,N,N-trimethylaminopentanetetrol (APT)–like lipid with a methoxy group in place of a hydroxy group on the pentanetetrol, (c) a series of polar lipids with a headgroup with elemental composition of either C12H25NO13S or C12H25NO16S2, and (d) novel cardiolipins containing a putative phosphatidylglycerolphosphate glycerophosphate (PGPGP) polar moiety. We found that the lipid distribution of the 13 strains could be generally separated into two groups, the methanogens (group) and the Halobacteria (class) based on the presence of specific core lipids. Within the methanogens, adaptation to a high or more moderate salt concentration resulted in different ratios of glycerol dialkyl glycerol tetraethers (GDGTs) to archaeol. The methanogen Methanosalsum natronophilum AME2 T had the most complex diether lipid composition of any of the 13 strains, including hydroxy archaeol and macrocyclic archaeol which we surmise is an order-specific membrane adaption. The zwitterionic headgroups APT and APT-Me were detected only in the Methanomicrobiales member Methanocalculus alkaliphilus AMF2 T which also contained the highest level of unsaturated lipids. Only alkaliphilic members of the Natrialbales order contained PGPGP cardiolipins and the PG-Gly headgroup. The four analyzed neutrophilic members of the Halobacteria were characterized by the presence of sulfur-containing headgroups and glycolipids. The presence of cardiolipins with one or more i-C25 alkyl chains, generally
INTRODUCTION

Extremely halophilic euryarchaea from the class Halobacteria, commonly referred to as haloarchaea, have attracted attention both as an object of fundamental research in extremophiles and as producers of extremophilic enzymes with biotechnological potential (Oren, 2002; Begemann et al., 2011; Andrei et al., 2012; Amoozegar et al., 2017). Recently, the knowledge of such euryarchaea has significantly increased by the discovery of anaerobic respiration, the ability to use chitin and cellulose as growth substrates and by the inclusion of relatives outside of the class Halobacteria such as methanogens (Sorokin et al., 2015b,c, 2016a,b, 2017a,b, 2018a, 2019). The recent discovery of extremely halo(alkali)philic methanogenic euryarchaea of the class Methanomethanarchaeia in hypersaline lakes has provided particular insights into the origin of methanogenesis and the strategies methanogens employ to thrive under hypersaline conditions (Sorokin et al., 2017a). A wide range of haloarchaea has been found to exist in hypersaline NaCl-Na2SO4-based lakes with neutral pH and alkaline soda lakes with a previously unrecognized potential to utilize native insoluble cellulososes and chitin as their sole growth substrate (Sorokin et al., 2015c, 2018a, 2019) and the capability of sulfur respiration in salt-saturated media (Sorokin et al., 2016a,b, 2017b, 2018c).

Sustaining optimal membrane function is crucial for all microorganisms in order to maintain cell homeostasis, but is a particular challenge in extreme environments such as hypersaline lakes. The characteristic membrane lipids of haloarchaea form the barrier between the cell's internal functions and the harsh outside environment. In general, the membrane lipids of Archaea are characterized by the presence of ether linkages between a glycerol moiety and isoprene-based alkyl chains. Archaeal membrane lipids include sn-2,3-diphtyanylglycerol diether with two C20 isoprenoid chains (archaeol, AR), extended archaeol (EXT-AR, with a C20 and C25 isoprenoid chain) and tetraethers with two glycerol moieties connected by two C40 isoprenoid chains [glycerol dialkyl glycerol tetraethers (GDGT)]. Membrane-spanning GDGTs can contain up to eight cyclopentane moieties (i.e., GDGT-n, where n is the number of cyclopentane moieties). Membrane-spanning GDGTs have not been detected in members of the class Halobacteria. Archaea also produce bisphosphatidylglycerol diethers, analogous to the diacyl cardiolipins found in Bacteria and Eukarya (Corcelli et al., 2000; Lattanzio et al., 2002; Corcelli, 2009; Angelini et al., 2012; Yoshinaga et al., 2012). The membrane polar lipids of halophilic archaea, mostly from the class Halobacteria, have been characterized in a wide range of studies (Kushwaha et al., 1975; Kates et al., 1993; de Souza et al., 2009; Angelini et al., 2012; Dawson et al., 2012; Lobasso et al., 2015). Phosphatidylglycerophosphate methyl ester (PGP-Me) has often been found to be the dominant polar headgroup. It is thought that the bulky, double charged nature of this headgroup contributes to membrane stability at high salt concentrations (Tenchov et al., 2006). Indeed, Kellermann et al. (2016) observed that for the neutrophilic haloarchaeon Halofexax (H.) volcanii, there was a correlation between the amount of PGP-Me and the concentration of Mg2+ in the media during culturing experiments. Kellermann et al. (2016) hypothesized that the terminal methyl group of PGP-Me acts as an antenna, while the closely positioned, negatively charged neighboring phosphate moieties allow PGP-Me to bind divalent cations [as opposed to monovalent cation bonding to phosphatidylglycerol (PG) or cardiolipins, cf. Figure 9 of Kellermann et al. (2016)]. They suggested that by up- or downregulating the number of negative charges at its surface, a cell can balance membrane permeability under varying salt concentrations. Indeed, the most important membrane adaptation for haloarchaea is low permeability to water, protons and monovalent cations (i.e., Na+ and K+) (Oger and Cario, 2013). Other phoso-based polar headgroups have been detected in haloarchaea in lesser proportions along with some glyco- and sulfo-lipids (Kates, 1992; Koga and Morii, 2005).

In this study the polar lipid composition of 13 strains of halo(alkali)philic euryarchaea (Table 1; Figure 1) isolated...
from different hypersaline lakes was analyzed using High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) and Nuclear Magnetic Resonance (NMR) spectroscopy. Among them are four methanogenic species, of which two (Methanonatronararchaeum (Mn.) thermophilum AMET1T and “Candidatus (Ca.) Methanololarachaeum (Mh.) thermophilum” HMET1) are extremely halophilic methyl-reducers forming a novel class Methanonatronarchaeia (Sorokin et al., 2017a, 2018b), while the other two (Methanososalum (Ms.) natriomobilis AME2T and Methococcus (Mc.) alkalophilus AMF2T) are haloalkaliphilic halobacteria from soda lakes (Sorokin et al., 2015b). Haloalkaliphilic halobacteria from soda lakes are not strictly halophilic and their name is applied to discriminate them from freshwater alkaliarchaeans. Mc. alkalophilus AMF2T can be regarded as a moderately salt-tolerant alkaliarchaeon (growth up to 2 M total Na+), while Ms. natriomobilis AME2T is an extremely salt-tolerant alkaliarchaeon (growth up to 4 M total Na+). Five haloarchaeal isolates are aerobic polysaccharide utilizers: cellulolytic “Halococcoides (Hc.) cellulosivorans” HArcc1T, Natronobiflora (Nb.) cellulostropha AArcel5 and uncharacterized strain AArcel7 and two natronarchaeal chitinolytic strains “Natrarchaeobius (Na.) chitinivorans” AArcht4T and “Na. halalkaliphilus” AArcht-S1T are members of the orders Halobacteriaceae and Natrarchaeales in the class Halobacteria. The last four strains belong to a group of anaerobic sulfur-respiring haloarchaeans. Two haloarchaeal strains comprise two novel genera in the family Halobacteriaceae: Halanaeroarchaeum (Hn.) sulfurireduced HSRT1, which grows exclusively by acetate-dependent sulfur respiration (Sorokin et al., 2016a,b), and Halodesulfurarchaeum (Hd.) formicicum HSR1T, which is capable of utilizing either formate or hydrogen as their electron donor, and elemental sulfur, thiosulfate or dimethylsulfide as electron acceptors (Sorokin et al., 2017b). The two other strains, AArc-Mg and AArc-S1, are alkaliphilic haloarchaeons capable of growth by dissimilatory sulfur reduction [provisionally belonging to a new species “Natronolimnobius (Nl.) sulfurireduced” (Natrarchaeales) and a new genus and species “Halalkaliphilarchaeum (Ha.) desulfuricum” (Haloforaceales)] (Sorokin et al., 2018c). While the dominant lipid groups have been previously reported for a number of these strains, our work reports the full range of dialkyl glycerol diether and tetraether membrane lipids present in the 13 strains, including the in-depth structural characterization of novel compounds.

### MATERIALS AND METHODS

#### Culturing

*M. alkalophilus* AMF2T was grown at 0.6 M total Na+ and 30°C with formate as electron donor and acetate as carbon source and *Ms. natriomobilis* AME2T at 2 M total Na+ with methanol as a sole substrate, both at pH 9.5, as described previously (Sorokin et al., 2015a,b). The other 11 extremely halophilic archaeal isolates were grown at 4 M total Na+, 5 mM KCl and 1 mM Mg2+ until late exponential growth phase. *Mn. thermophilum* AMET1T and “Ca. Mh. thermophilum” HMET1 were grown anaerobically with methanol and formate (50 mM each) and yeast extract as carbon source (100 mg L⁻¹) at 50°C and pH either 9.5 (2 M Na+ as carbonates and 2 M NaCl) or 7.0 (4 M NaCl), respectively (Sorokin et al., 2018b). Sulfur-reducing neutrophilic haloarchaeons were grown anaerobically with sulfur as the electron acceptor and either 10 mM pyruvate (Hn. sulfurireduced HSRT2) or 50 mM formate (Hd. formicicum HSR6T) as the electron donor and 200 mg L⁻¹ of yeast extract as carbon source at 37°C as described by Sorokin et al. (2016a,b, 2017b). Alkaliphilic sulfur reducing haloarchaeons were cultivated anaerobically with sulfur as electron acceptor at 37°C with 10 mM butyrate as the electron donor at pH 9.8 (strain AAarc-Mg) or with 20 mM pyruvate at pH 9.2 (strain AAarc-S1T) (Sorokin et al., 2018c). Aerobic alkaliphilic polysaccharide utilizers were grown either with 10 mM cellobiose at pH 9.5 (strains AAarc5T and AAarc7) or 10 mM N-acetylglucosamine (strains AArcht4T and AArcht-S1T) at pH 9.5 and 9.1, respectively, as described previously (Sorokin et al., 2015c, 2018a, 2019). All cultures were grown in duplicate and the final biomass was pooled together in a single sample for lipid analysis.

#### Hydrolysis of Biomass and Core Lipid Analysis

In order to remove the headgroups from the intact polar lipids (IPLs) and to obtain the remaining core lipids the freeze-dried biomass was hydrolyzed with HCl/MeOH (1.5 N) by refluxing for 3 h. The hydrolysate was adjusted with aqueous KOH to pH 5, extracted three times with dichloromethane, and dried over Na2SO4.

#### HPLC-MS Analysis of GDGTs and AR

The hydrolyzed biomass extracts were redissolved in hexane/isopropanol (99:1, v:v) and filtered through a 0.45 μm PTFE filter and analyzed using HPLC-atmospheric pressure chemical ionization/mass spectrometry (HPLC-APCI/MS) on an Agilent 1100/Hewlett Packard 1100 MSD instrument equipped with automatic injector and HP-Chemstation software according to Hopmans et al. (2000) with the following modifications. Separation was achieved in normal phase with two Prevail Cyoano columns in series (150 × 2.1 mm; 3 μm) with a linear gradient eluent of hexane:propanol (99.5:0.5, v:v) and a flow rate of 0.2 mL min⁻¹. This remained isocratic for 5 min followed by a linear gradient to 1.8% propanol at 45 min. The injection volume was 10 μL. An external standard of AR:GDGT-0 (1:1, w:w) run in sequence before and after the samples was used to quantify AR and GDGT-0. GDGT-1 was assumed to have the same response as GDGT-0 and was in this way semi-quantified. Hereafter, the concentrations of AR and GDGTs are expressed as percent of their total combined concentration.

#### GC-MS Analysis of Mono and Diethers

Hydrolyzed biomass extracts were methylated with diazomethane in diethyl ether which was removed under a stream of N2 and trimethylsilylated using N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and pyridine (1:1; 20 min at 60°C). Gas chromatography (GC) was performed using a Hewlett Packard 6890 gas chromatograph equipped with an on-column injector and a flame ionization detector (FID). A fused silica capillary column (CP Sil 5 25 × 0.32 mm, df =
### TABLE 1 | Strains of halophilic euryarchaea examined in this study.

| Strain | Species | Growth conditions | pH | Na⁺ M | K⁺ mM | Mg²⁺ mM | T °C | O₂ conditions |
|-------|---------|------------------|----|-------|-------|---------|------|---------------|
| Methanogens | AMET¹ | Methanonatronarchaeum (Mh.) thermophilum | 9.5 | 4.0 | 5.0 | 1.0 | 48 | Anaerobic |
| HMET¹ | “Ca. Methanolitharchaeum (Ml.) thermophilum” | 7.0 | 4.0 | 5.0 | 1.0 | 50 | Anaerobic |
| AME² | Methanosalsum (Ms.) natronophilum | 10 | 3.0 | na | 1.0 | 30 | Anaerobic |
| AMF² | Methanocalculus (Mc.) alkaliphilus | 10 | 0.6 | na | 1.0 | 30 | Anaerobic |
| Anaerobic sulfur reducers | HSR² | Halanaeroarchaeum (Hn.) sulfurireducens | 7.0 | 4.0 | 5.0 | 1.0 | 37 | Anaerobic |
| HSR⁶ | Halodesulfuranarchaeum (Hs.) formicum | 7.0 | 4.0 | 5.0 | 1.0 | 37 | Anaerobic |
| AArc-Mg | “Natronolimnobius (Nl.) sulfurireducens” | 10 | 4.0 | 5.0 | 1.0 | 37 | Anaerobic |
| AArc-Sl | “Halalkaliarchaeum (Ha.) desulfuricum” | 9.1 | 4.0 | 5.0 | 1.0 | 37 | Anaerobic |
| Polysaccharide utilizers | HArcele¹ | “Halococcoides (Hc.) cellulosivorans” | 7.0 | 4.0 | 5.0 | 1.0 | 37 | Aerobic |
| Cellulolytics | AArcele⁵ | Natronobiforma (Nb.) cellulostropha | 9.5 | 4.0 | 5.0 | 1.0 | 37 | Aerobic |
| AArcele⁷ | unpublished | 9.0 | 4.0 | 5.0 | 1.0 | 37 | Aerobic |
| Chitinolytics | AArcht¹ | “Natarchaeobius (Na.) chitinkorans” | 9.5 | 4.0 | 5.0 | 1.0 | 37 | Aerobic |
| AArcht-Sl | “Natarchaeobius (Na.) habalkaiphilus” | 9.3 | 4.0 | 5.0 | 1.0 | 37 | Aerobic |

*Description submitted to IJSEM. Na, not applicable.

For class, order and family of strains see Figure 1.
0.12 μm) with helium as a carrier gas was used. The samples were injected at 70°C. The GC oven temperature was subsequently raised to 130°C at a rate of 20°C min⁻¹, and to 320°C at 4°C min⁻¹. The temperature was then held constant for 15 min. Additionally, all hydrolyzed fractions were analyzed by GC–MS for compound identification. The structural designation of lipids was evaluated by the comparison of their retention times and mass spectral fragmentation patterns with standards and with published spectra (e.g. Pancost et al., 2001). GC–MS was conducted using Thermo Finnigan Trace Ultra GC connected to ThermoFinnigan DSQ MS operated at 70 eV, with a mass range m/z 50–800 and 3 scans s⁻¹ with a silica column (CP Sil-5, 25 × 0.32 mm) and He as the carrier gas at a constant flow rate of 2 mL min⁻¹. The oven temperature program initiated at 70°C, increased first at a rate of 20°C min⁻¹ to 130°C, and next at a rate of 4°C min⁻¹ to the final temperature of 320°C, which was held for 10 min.

GC peak area response (FID) of the mono and diethers was recorded. One of the diethers, AR, had previously been quantified (by HPLC-MS analysis of GDGTs and AR). The two separate hydrolysis-derived ether lipid data sets (GC-MS derived and HPLC-MS derived), were both normalized to AR and combined to give an estimate of the total ether distribution (in percent of the total, Table 2). Combining the data sets involved several assumptions, such as that AR had been conserved equally during the two analyses and that the response of the ethers during GC-MS analysis was uniform. Hence this combined data set should be examined with these caveats in mind.

HPLC-ITMS

For general IPL screening, the IPL extracts were analyzed by high performance liquid chromatography-ion trap mass spectrometry (HPLC-ITMS) according to Sturt et al. (2004) with modifications as described previously (Sinninghe Damsté et al., 2011). The analysis was performed on an Agilent 1,200 series HPLC (Agilent, San Jose, CA), equipped with thermostatted auto-injector and column oven, coupled to an LTQ XL linear ion trap with Ion Max source with electrospray ionization (ESI) probe (Thermo Scientific, Waltham, MA). Separation was achieved on a LiChrospher diol column (250 × 2.1 mm, 5 μm particles; Alltech) maintained at 30°C. The following elution program was used with a flow rate of 0.2 mL min⁻¹: 100% A for 1 min, followed by a linear gradient to 66% A: 34% B in 17 min, maintained for 12 min, followed by a linear gradient to 35% A: 65% B in 15 min, where A = hexane/2-propanol/formic acid/14.8 M NH₃aq (79:20:0.12:0.04, v:v:v:v) and B = 2-propanol/water/formic acid/14.8 M NH₃aq (88:10:0.12:0.04, v:v:v:v). The lipid extract was analyzed by an MS routine where a positive ion scan (m/z 400–2,000) was followed by a data dependent MS² experiment where the base
### Table 2

The distribution of ether lipids released by acid hydrolysis (presented as % of total) across the 13 strains of haloarchaea with three different types of physiology.

| Core lipid | Methanogens | Anaerobic sulfur reducers | Polysaccharide utilizers | Chitinolytics | Cellulolytics |
|------------|-------------|---------------------------|--------------------------|---------------|---------------|
| MetHET1    | AME2        | T                         | HSR2                     | HSR6          | T             |
| AMF2       | T           | HSR1                      | AArc-Mg                  | AArc-Sl       | T             |
| HSL2       | HSR4        | T                         | AArcel1                  | AArcel5       | T             |
| AArcel7    | AArcht4     | T                         | AArc-Sl                  | AArcht-Sl     | T             |
| HArcel1    | T           |                            |                          |               |               |
| AArcel5    | T           |                            |                          |               |               |
| AArcel7    | T           |                            |                          |               |               |
| AArcht4    | T           |                            |                          |               |               |
| AArcht-Sl  | T           |                            |                          |               |               |

#### Ether Lipids

- **Phytanyl glycerol monoethers**
  - sn-1-C: 14, 20
  - sn-2-C: 0.2, 20
  - sn-3-C: 12

- **Extended (sesterterpenyl) glycerol monoethers**
  - sn-1-C: 49
  - sn-2-C: 25

- **Archaeol**
  - sn-1-C: 20
  - sn-2-C: 45
  - sn-3-C: 73

- **Extended archaeol**
  - sn-1-C: 53
  - sn-2-C: 59

- **Macrocyclic archaeol**
  - sn-1-C: 15

- **OH-Archaeol**
  - sn-1-C: 51

#### General Information

- Full strain names given in Table 1.
- sn-n-C: xx, n denotes the sn position of the alkyl chain and xx refers to the number of isoprenoidal carbons. For sn-n-C of the two extended archaeols, n denotes the sn position of the extended alkyl chain (sesterterpenyl).

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**UHPLC-HRMS**

Additional analysis of selected extracts was carried out using an ultra-high performance liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS) method (Besseing et al., 2018) in order to determine the accurate mass of certain unknown compounds. An Agilent 1290 Infinity I UHPLC equipped with thermostatized auto-injector and column oven, coupled to a Q Exactive Orbitrap MS with Ion Max source with heated electrospray ionization (HESI) probe (Thermo Fisher Scientific, Waltham, MA) was used. Separation was achieved on an YMC-Triart Diol-HILIC column (250 × 2.0 mm, 1.9 μm particles, pore size 12 nm; YMC Co., Ltd, Kyoto, Japan) maintained at 30°C. The eluent composition and flow rate were the same as described above for the Agilent 1200/LTQ XL ion trap HPLC-MS system although the elution program was adjusted thus: 100% A, followed by a linear increase to 30% B at 20 min, followed by a 15 min hold, and a further increase to 60% B at 50 min. Positive ion ESI settings were: capillary temperature, 275°C; sheath gas (N₂) pressure, 35 arbitrary units (AU); auxiliary gas (N₂) pressure, 10 AU; spray voltage, 4.0 kV; probe heater temperature, 275°C; S-lens 70 V. Target lipids were analyzed with a mass range of m/z 350–2,000 (resolving power 70,000 at m/z 200), followed by data-dependent tandem MS² (resolving power 17,500), in which the 10 most abundant masses in the mass spectrum were fragmented successively (stepped normalized collision energy 15, 22.5, 30; isolation width 1.0 m/z). The Q Exactive was calibrated within a mass accuracy range of 1 ppm using the Thermo Scientific Pierce LTQ Velos ESI Positive Ion Calibration Solution. During analysis dynamic exclusion was used to temporarily exclude masses (for 6 s) in order to allow selection of less abundant ions for MS².
Compound Isolation and NMR Analysis

Specific IPLs were isolated from Bligh-Dyer extracts of *M. alkaliphilus* AMF2T biomass using an Agilent Technologies (Santa Clara, CA) 1100 series HPLC with a thermostatted autoinjector, column oven, and a Foxy Jr. fraction collector (Teledyne Isco, Lincoln, NE, USA). Aliquots of filtered Bligh-Dyer extract (200 µL) were injected onto a LiChromspher DIOL column (250 mm x 10 mm, 10 µm; Alltech, Deerfield, IL), all fractions were eluted using the identical gradient and mobile phase composition described above for the HPLC-ITMS analysis, but at a flow rate of 3 mL/min. The column effluent was collected in 3 mL fractions. Fractions from semi-preparative HPLC were screened for the presence of the target compound with flow injection analysis using HPLC-ITMS as described above, and the fractions containing the target compound were pooled. For further purification the combined fractions were re-injected and column effluent was collected in 15 s fractions.

\[N,N,N',N'-\text{trimethyl aminopentanetetrol} (\text{APT})-\text{AR}\] and an unknown lipid (referred to below as **Ia**) were dissolved in 500 µL 99.9% CDCl₃ at concentrations of 3.0 and 0.8 mg mL⁻¹, and NMR experiments were performed at 298 K on a Bruker 900-MHz Avance III spectrometer equipped with 5 mm TCI cryoprobe, running under TOPSPIN 3.2. One-dimensional (1D) ¹H and ¹³C-decoupled 1D ¹³C and Distortionless Enhancement by Polarization Transfer (DEPT)-135 experiments were recorded with spectral widths/offsets of 20 ppm/4.7 ppm, 200 ppm/100 ppm, and 200 ppm/100 ppm and with 16,384 (16k), 4,096 (4k), and 4,096 (4k) complex points, respectively. 12- by 12-ppm 2D correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), and nuclear Overhauser effect spectroscopy (NOESY) experiments were performed with 512 by 256 complex points (by 200 for the NOESY) and an offset frequency of 4.7 ppm. Mixing times were 60 and 120 ms for TOCSY and 250 ms for NOESY experiments. (¹H, ¹³C)-heteronuclear single-quadrature correlation spectroscopy (HSQC) and (¹H, ¹³C)-heteronuclear multiple-bond correlation spectroscopy (HMBC) experiments were recorded with spectral widths/offsets of 12/4.7 ppm for protons, 200/100 ppm for ¹³C HSQC (512 x 64 complex points) and 150/75 ppm for the HMBC (1k x 80 complex points). All spectra were calibrated with respect to internal residually protonated CHCl₃ at 7.24 ppm (¹H) and 77.0 ppm (¹³C).

Genomic and Phylogenetic Analyses

Partial 16S rRNA gene sequences of the strains mentioned in the manuscript and others were obtained from the Silva 128 release (https://www.arb-silva.de/). A phylogenetic tree of 16S rRNA gene sequences was inferred with the Neighbor-Joining method (Saitou and Nei, 1987) in MEGA6 (Tamura et al., 2013). The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor, 1969). The analysis involved 29 nucleotide sequences with a total of 1,185 positions in the final dataset.

Putative polypropenyl synthase coding genes were identified in the available genomes of the studied strains along with a selection of other archaea with PSI-BLAST (Position-Specific iterated BLAST) searches at the protein level (www.ncbi.com) using two iteration steps using the annotated geranylgeranyl disphosphate synthase of *Aeropyrum (A.) pernix* [accession number Q9UWR6.1; (Tachibana et al., 2000)] as query sequence and limiting the search to hits with percentage of identity higher than 20% and e-value <1 × 10⁻³⁰. Putative polypropenyl synthases were aligned by Muscle (Edgar, 2004) in the MEGA6 software (Tamura et al., 2013) and edited manually. Phylogenetic reconstruction was performed by maximum likelihood in the MEGA 6 software (Tamura et al., 2013) based on the JTT matrix-based model (Jones et al., 1992). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories, +G parameter = 1.5942). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 4.2308% sites). The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 49 amino acid sequences. There was a total of 421 positions in the final dataset.

RESULTS

Thirteen strains of haloarchaea isolated from hypersaline soda lakes were compared for the composition of their membrane lipids. The strains were separated into three groups based on their physiology: 4 methanogens, 5 polysaccharide utilizers and 4 sulfur-respiring haloarchaea (Table 1).

Core Lipid Composition

The ether lipids detected by GC-MS and HPLC-APCI/MS were all normalized to AR and combined to give the estimated distribution of all hydrolysis-derived ether core lipids (Table 2). In 12 of the 13 strains, AR was either a dominant (73–88%) or major (20–58%) component, whereas in one polysaccharide utilizing, moderately alkaliphilic strain, AArcT7, AR was only present as a minor component (12%). Two forms of EXT-AR in both C₂₀–₂₅ and C₂₅–₃₀ configurations (with different sn2 and sn3 arrangements) were detected. *M. natronophilum* AMET₂T was found to contain hydroxy archaeols (OH-AR; 15%) and the macrocyclic AR (MAR; 6%), neither of which were detected in any other strains. Four different phytanyl glycerol monoethers were detected, the sn2- and sn3- alkyl glycerol monoether, in both the C₂₀ and C₂₅ forms. These did not constitute more than 16% of the diethers, except in the case of moderately alkaliphilic chitinolytic strain "*Na. haloalkaliphilus"* AArcht-SI₇, where C₂₅ (extended) sn-3-alkyl glycerol ether was present as 49% of the diether fraction. We have interpreted the four alkyl glycerol monoethers as artifacts that are formed during acid hydrolysis from diether lipids containing unsaturated alkyl moieties (de Souza et al., 2009) as confirmed by the IPL measurements (see section IPLs). GDGT-0 and GDGT-1 were detected in the methanogenic strains, but not in the members of *Halobacteria*. GDGT-0 accounted for 59 and 79% of the total core lipids in the two extremely halophilic methanogens from the class *Methanotromonarchaeia*, alkaliphilic *Mn. thermophilum* AMET₁T and neutrophilic "*Ca. Mh. thermophilum*" HMET₁, while GDGT-1 was 7% in the former but absent in the latter. In
the two haloalkaliphilic members of the class *Methanomicrobia*, *Ms. natronophilum* AME2\(^T\) and *M. alkaliphilus* AMF2\(^T\), GDGT-0 accounted for 17 and 15% while GDGT-1 was present at 1 and <0.1%, respectively.

**IPLs**

Analysis by HPLC-ITMS (e.g., Figure 2) and UHPLC-HRMS of the Bligh-Dyer extracts of the 13 strains resulted in the identification of a wide range of IPLs, including novel compounds. We describe these in three sections: diethers, ether analogs of cardiolipins and GDGTs.

**Diether IPLs**

Diether IPLs detected by HPLC-ITMS (see Table S1 for all species detected, structures in Figure 3) contained the following polar headgroups: phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylglycerophosphate (PGP), and its methyl ester (PGP-Me), APT (with tri-, di- and mono-N-methylations), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylglycerosulfate (PGS), and dihexose (2Gly, also known as DGA-1 in e.g., Kates [1992]). The core component of the IPLs included AR, EXT-AR, OH-AR, and MAR (also detected by GC-MS analysis), unsaturated (Uns) forms of both AR and EXT-AR (with between 1 and 8 unsaturations) and OH-AR (with between 1 and 5 unsaturations). The diether IPLs (Table S1) were examined in terms of their MS peak area response, which is generally dependent on polar group ionization and therefore do not reflect the absolute quantities of the different lipids. Besides these known diether species, also a number of novel diether lipids (I–III, Figure 2) were detected. Their identification using combinations of MS\(^2\) fragmentation, accurate mass and NMR spectroscopy is described below.

**Novel diether lipids Ia and Ib**

Two of the alkaliphilic polysaccharide utilizing strains, *Nb. cellulositropha* AArcel5\(^T\) and strain AArcel7 contained lipids (e.g., Ia and Ib, Figure 2) with parent ions appearing in MS spectra at \(m/z\) 1039.7 and 969.7, respectively. For both parent ions, upon MS\(^2\) fragmentation, a loss of 162 Da (loss of hexose) was observed, generating product ions at \(m/z\) 877.8 (Figure 4A) and 807.8 (Figure 4C), respectively. These product ions match the \(m/z\) value of the protonated molecules ([M+H]\(^+\); Table S1) of PG-EXT-AR and PG-AR, respectively. Indeed, upon further fragmentation in MS\(^3\), product ions representing the EXT-AR (\(m/z\) 723.7, Figure 4B) and AR (\(m/z\) 653.6, Figure 4D) core lipids were observed. Therefore, we identified these lipids as phosphatidylglycerohexose-EXT-AR (PG-Gly-EXT-AR, Ia) and phosphatidylglycerohexose-AR (PG-Gly-AR, Ib). This headgroup combined with a diacyl core lipid has been described in halophilic gammaproteobacterium of the genus *Halomonas* (Giordano et al., 2007), while a very similar diether phosphoglycolipid attached to a sulfated sugar moiety was identified in the alkaliphilic haloarchaeon *Natrononomonas (N.) mooolapensis* (Hoffmann et al., 2015). The position of the sugar of both the bacterial and archaeal glycosylated-PG described in the literature were determined by NMR to be at the 2-glycero position (Giordano et al., 2007; Hoffmann et al., 2015). In our study the MS\(^3\) fragmentation pattern could not confirm whether the hexose was at the 1- or 2-glycero position, but the similarity of the PG-Gly spectra
FIGURE 3 | Structures of known polar headgroups and core lipids described in this study. Polar headgroups: PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PGP, phosphatidylglycerophosphate; PGP-Me, phosphatidylglycerophosphate methyl ester; PGS, phosphatidylglycerosulfate; trimethyl APT, N,N,N-trimethyl aminopentanetetrol (for other methylations see Figure 5); 2Gly, dihexose. Dialkyl glycerol diether core lipids; AR, archaeol; EXT-AR, extended archaeol; MAR, macrocyclic archaeol; OH-AR, hydroxy archaeol; Uns, unsaturated. Glycerol dialkyl glycerol tetraether core lipids (GDGTs, where \( n \) is the number of cyclopentane moieties).

with those of 1-(6-sulfo-D-glcp/galf-\( \beta \)-1,2-glycero)-phospho-2,3-diphytanylglycerol found in \( N. \) moolapensis (Hoffmann et al., 2015) provides an indication that they both have same structural arrangement.

**Novel N,N,N-trimethyl APT derivatives**

The dominant but unknown IPL in \( Mc. \) alkaliphilus AMF2\( ^{\text{T}} \) (IIa, Figure 2) exhibited a parent ion at \( m/z \) 923.0 during HPLC-ITMS analysis. Upon UHPLC-HRMS analysis it was found to have an accurate mass of 922.7816 (Table S2). Upon UHPLC-HRMS fragmentation, product ions at \( m/z \) 288.1205, 270.1100, 208.1544, and 190.1438 were formed (Figure 5A; Table S2). Both the parent ion and all the main fragments produced are offset by 14 Da compared to the parent ion and fragments of \( N,N,N \)-trimethyl APT-AR ([M+H]\(^+\) \( m/z \) 908.7666, Table S2). These fragment ions of \( N,N,N \)-trimethyl APT-AR (Figure 5B) can be interpreted as follows: \( m/z \) 274.1037 is the intact \( N,N,N \)-trimethyl APT headgroup, \( m/z \) 256.0953 is a loss of \( \text{H}_2\text{O} \) from the \( m/z \) 274.1037 ion, \( m/z \) 194.1399 is the headgroup without the phosphate moiety, and \( m/z \) 176.1284 represents a loss of \( \text{H}_2\text{O} \) from the \( m/z \) 194.1399 ion. The mass difference between fragments of \( N,N,N \)-trimethyl APT and of IIa was always in the range 14.0145–14.0168 Da which indicates that IIa contains an additional CH\(_2\) group (14.0151 Da) in its headgroup, likely by conversion of one of the alcohol groups into a methoxy group, i.e., \( N,N,N \)-trimethyl aminopentanemethoxytriol (APT-Me). Based
The phytanyl chains were identified in comparison with those identical AR and IPL IIa (Table S3) proton atoms, including those of the polar headgroup (N\textsuperscript{+}-trimethyl APT-Me derivatives IIa-c) without the phosphate moiety, and m/z 190.1438 is the H\textsubscript{3}O from the m/z 208.1544 ion. A series of mono- to octa- Uns-AR with a N,N,N-trimethyl APT-Me headgroup (HPLC-ITMS MS\textsuperscript{2} fragmentations (Table S1) with a decreasing intensity of the product ion arising from loss of one alkyl chain, depending on the number of unsaturations. The minor unknowns Ib, IIc were identified using UHPLC-HRMS as N,N-dimethyl (m/z 908.7661, Figure 5C) and N-monomethyl APT-Me-AR (m/z 894.7506, Figure 5E), analogous to N,N-dimethyl APT-AR (m/z 894.7510, Figure 5D) and N-monomethyl APT-AR (m/z 880.7349, Figure 5F).

In order to confirm the identification of the novel N,N,N-trimethyl APT-Me derivatives IIa-c and to assess the position of the Me group on the pentanetetrol moiety, the most abundant IPL of this series, IIa, was purified using semi-preparatory HPLC in order to carry out analysis by NMR spectroscopy. The putative N,N,N-trimethyl APT-AR was also purified and analyzed by NMR spectroscopy for comparison. Both N,N,N-trimethyl APT-AR and IPL IIa exhibited similar NMR spectra with almost identical \textsuperscript{13}C- and \textsuperscript{1}H-NMR shifts for most of carbon and proton atoms, including those of the polar headgroup (Table S3). The \textsuperscript{13}C- and \textsuperscript{1}H-shifts associated with the glycerol unit and the phytanyl chains were identified in comparison with those described for GDGTs (Sinninghe Damsté et al., 2002) and are not presented here. The \textsuperscript{13}C- and \textsuperscript{1}H-NMR shifts reported by Ferrante et al. (1987) for N,N,N-trimethyl APT are similar to ours and the few differences that occur can all be accounted for by the differences in solvent and experimental conditions used (e.g., \textsuperscript{2}H\textsubscript{2}O and benzene-\textsubscript{d}6/methanol-\textsubscript{d}4 vs. CD\textsubscript{3}C\textsubscript{3} used in this study). Whereas, the N,N,N-trimethyl APT headgroup exhibits 6 carbon signals for its polar headgroup, that of the putative N,N,N-trimethyl APT-Me IPL exhibited seven carbon signals, confirming that the additional 14 Da was due to an additional C atom and its associated protons. This additional C atom (C\textsubscript{7}; Table S3) was determined by a DEPT experiment to be either a primary or tertiary carbon. HSQC analysis provided evidence for a single signal at 3.36 ppm, indicative of a primary methyl group. Furthermore, its \textsuperscript{13}C shift at 59 ppm agrees with a methoxy group and hence it was assigned to be an N,N,N-trimethyl APT-Me. The carbon and associated proton signals of three carbon atoms (C\textsubscript{1}, C\textsubscript{2}, C\textsubscript{6}) of the N,N,N-trimethyl APT-Me headgroup were almost identical to those of N,N,N-trimethyl APT-AR (Table S3), which provides evidence the additional CH\textsubscript{3} group (C\textsubscript{7}) was bound to an oxygen atom at either C\textsubscript{3}, C\textsubscript{4}, or C\textsubscript{5}. The HMBC spectrum provided confirmation that C\textsubscript{7} was in fact bound to the oxygen at C\textsubscript{6} as the protons at C\textsubscript{7} interacted most strongly with those of C\textsubscript{6} (see structure insert Table S3).

Further confirmation of the structure from the HMBC spectrum were the cross peaks between the protons of C\textsubscript{6} with those of C\textsubscript{1}, and those of C\textsubscript{1} with both C\textsubscript{6} and C\textsubscript{3} and from the COSY spectrum that indicated that the protons of C\textsubscript{2} were J-coupled with C\textsubscript{3}. 

![Figure 4](https://example.com/figure4.png) | HPLC-ITMS\textsuperscript{2} and MS\textsuperscript{2} spectra with inserts of structure with fragmentations indicated for (A,B) PG-Gly-EXT-AR and (C,D) PG-Gly-AR. i-C\textsubscript{25}, C\textsubscript{25} isoprenoid chain; i-C\textsubscript{25}, C\textsubscript{25} isoprenoid chain.
**Diethers with novel sulfur-containing headgroups (IIIa-d)**

Four unknown IPLs with two closely related headgroups were detected in four strains (all neutrophilic) distributed across all three archaeal metabolic groups, namely in methanogenic *Ca. Mh. thermophilum* HMET1, cellulolytic *Hc. cellulosivorans* HArcel1, and sulfur-respiring *Hd. formicicum* HSR6 and *Hn. sulfurireducens* HSR2. During HPLC-ITMS analysis, two compounds with the same headgroup, IIIa, and IIIb produced [M+NH$_4$]$^+$ ions at m/z 1091.5 and 1161.5, and two with a closely related headgroup (IIIc and IIId) produced [M+NH$_4$]$^+$ at m/z 1171.4 and 1241.4 (Figure 6). Structural identification using UHPLC-HRMS resulted in the partial elucidation of the structure of these unknown headgroups (accurate masses of the parent ions and fragments are given in Table S4). The MS$^2$ spectrum (Figure 6A) of IIIa possessing a [M+NH$_4$]$^+$ of m/z 1091.7936 ion exhibited a loss of SO$_3$ and NH$_3$ from the [M+NH$_4$]$^+$ to
produce a fragment ion at m/z 994.8116. The ion at m/z 832.7605 was interpreted as the loss of hexose \( \text{C}_6\text{H}_{10}\text{O}_5 \) (162.0523 Da) from the ion at m/z 994.8116. A further loss of \( \text{C}_6\text{H}_{10}\text{O}_3 \) from the m/z 832.7605 ion produced the most abundant product ion at m/z 653.6800 representing the AR core lipide). The fragment ion m/z 373.3675 was assigned as a phytanyl moiety attached to a glycerol moiety \( \text{C}_{23}\text{H}_{49}\text{O}_3 \). The elemental composition of this ion, together with its MS\(^2\) fragmentation allowed us to propose a putative structure of IIIa as having a headgroup with elemental composition \( \text{C}_{12}\text{H}_{25}\text{NO}_3\text{S} \) and an AR core (Figure 6A; Table S4). The second IPL with the same headgroup, IIIb, possessed a \([\text{M}+\text{NH}_4]^+\) ion at m/z 1161.8720 and exhibited similar losses in its MS\(^2\) spectrum (Figure 6B and Table S4): loss of \( \text{SO}_3\) and \( \text{NH}_3 \) from the \([\text{M}+\text{NH}_4]^+\) to produce a fragment ion at m/z 1064.8916, sequential losses of hexose and a \( \text{C}_6\text{H}_{10}\text{O}_3 \) group to produce ions at m/z 902.8398 and 723.7582, respectively. The smaller fragment ions were assigned a phytanyl moiety attached to a glycerol moiety \( \text{m/z} \ 373.3676, \text{C}_{23}\text{H}_{49}\text{O}_3 \) and the corresponding \( \text{C}_25 \) isoprenoid alkyl chain attached to a glycerol moiety \( \text{m/z} \ 443.4470, \text{C}_{25}\text{H}_{50}\text{O}_3 \). Based on accurate mass determination of fragments and losses the IIIb compound was identified as having a \( \text{C}_{12}\text{H}_{25}\text{NO}_3\text{S} \) headgroup with an EXT-AR core.

IIIc and IIId produced similar MS\(^2\) spectra to IIIa and IIIb (Figures 6C,D and Table S4), but IIIc and IIId contained an additional \( \text{SO}_3\)H group (based on UHPLC-HRMS\(^2\) fragmentation, Table S4) on the polar headgroup. Hence, they were assigned as a \( \text{C}_{12}\text{H}_{25}\text{NO}_4\) headgroup with either an AR (Figure 6C) or EXT-AR (Figure 6D) core. The exact position(s) of the \( \text{SO}_3\)H group(s) could not be established. Biomass was not available in great enough quantity to carry out the compound isolation that would be required for NMR analysis.

Ether Analogs of Cardiolipins

Ether analogs of cardiolipins, with two dialkyl glycerol diether cores connected by a polar moiety, were detected in four of the 13 strains (Table 3, Table S1). Two of these cardiolipins were identified as bisphtatidylglycerols (BPGs) containing two AR cores (Figure 7A) and a BPG with an AR and an EXT-AR core (Figure 7B). These IPLs have been reported previously in other haloarchaea (Lattanzio et al., 2002; Corcelli, 2009; Angelini et al., 2012; Lobasso et al., 2015). Three novel tetraether cardiolipins (IVA) were found in three polysaccharide utilizing alkaliphilic haloarchaeal strains, "Na. chitinivorans" AArch1\(^4\), Nb. cellulosithrophi AARc5\(^7\) and AARc7. They exhibited \([\text{M}+\text{H}]^+\) masses (m/z 1676.4, 1746.5, 1816.6) identical to those of a series of unknown cardiolipins from the cell membrane of two species of alkaliphilic haloarchaea from the alkaliphilic genus Natronococcus that were previously reported by Angelini et al. (2012). While Angelini et al. (2012) postulated these were glycocardiolipins, their MS\(^2\) spectra did not support the presence of sugar groups. One of our three novel tetraether cardiolipins (IVA) exhibited a \([\text{M}+\text{H}]^+\) at m/z 1676.4. Its MS\(^2\) spectrum (Figure 7C) contained product ions at m/z 1396.0, consistent with a loss of a phytanyl chain (280 Da) and at m/z 1023.7 (loss of AR, 653 Da). The most abundant ion at m/z 869.7 is also present in the MS\(^2\) spectrum of AR-PGP-AR (Figure 7A).

In the case of AR-PGP-AR the m/z 869.7 represents a loss of AR (653 Da; Figure 7A), whereas for IVA it represents a loss of 807 Da. This additional 154 Da within the polar bridge of the cardiolipin can be explained by an additional glycerophosphate moiety. Hence, we have assigned IVA as containing a putative phosphatidylglycerophosphate glycerophosphate (PGPGP) polar group (Figure 7C). Based on previous nomenclature (e.g., Lattanzio et al., 2002; Corcelli, 2009; Angelini et al., 2012), the novel cardiolipins would be termed bisphtatidylglycerol glycerophosphates (BPGPGPs). A similar tetraether cardiolipin (IVB) exhibited a \([\text{M}+\text{H}]^+\) at m/z 1746.5, 70 Da larger than IVA. This difference in mass suggests the presence of an EXT-AR core instead of an AR core. Indeed, the product ions in the MS\(^2\) spectrum arising from fragmentation of this ion mirrored the losses seen for IVA cardiolipin with addition of losses associated with an EXT-AR chain (Figure 7D). These were: m/z 1466.0 (loss of a phytanyl chain, 280 Da), m/z 1023.7, and 1093.8 (loss of AR, 653 Da and EXT-AR, 723 Da, respectively) and four ions at m/z 807.8, 869.7, 877.8, and 939.7, which represent the AR and EXT-AR containing halves of the parent ion, fragmented on either side of the central phosphate group (Figure 7D). Furthermore, the most dominant product ions, at m/z 869.6 and 939.7 were also the dominant ions in the MS\(^2\) spectrum of PG-PG-AR, EXT-AR (Figure 7B). The \([\text{M}+\text{H}]^+\) of the third novel cardiolipin (IVC) was 140 Da larger than the first, at m/z 1816.6, in agreement with the presence of two EXT-ARs instead of a single AR-group. The product ions in its MS\(^2\) spectrum are interpreted as follows: m/z 1536.2: loss of a phytanyl chain of 280 Da, m/z 1093.8: loss of EXT-AR of 723 Da, two ions at m/z 877.8 and 939.7, representing the two halves of the parent ion when fragmented at either side of the central phosphate group (Figure 7E). As for compounds IIIa-d, NMR analysis was not carried out as there was not sufficient biomass for compound isolation.

GDGT-Based IPLs

We detected membrane-spanning GDGTs in 4 out of the 13 examined halo(alkali)philic euryarchaea, all methanogens (Table 3). These included 2Gly-GDGT-0, PG-GDGT-0-PE, PG-GDGT-1-PG, and PG-GDGT-0-PE, identified by comparison of their masses and their diagnostic fragmentation patterns in MS\(^2\) with those described in the literature (Rossel Cartes, 2009; Yoshinaga et al., 2011).

DISCUSSION

A wide range of intact polar dialkyl glycerol diethers, membrane-spanning GDGTs and cardiolipins were identified in 13 strains of halo(alkali)philic archaea in this study (cf. Table S1). Many of the intact polar diethers detected have been described previously in other haloarchaea. However, some IPLs described in this study have not, to the best of our knowledge, been previously reported. These include an APT-Me polar headgroup, a series of cardiolipins with a PGPGP polar group, a PG-Gly headgroup with an AR core, and AR and EXT-AR combined with headgroups containing a hexose, a \( \text{C}_6\text{H}_{10}\text{NO}_5 \) sugar with either one (IIIa,b) or two (IIIc,d) sulfate groups.
Factors Determining Lipid Composition

We examined the distribution of membrane lipids across the 13 strains and examined what factors determined their lipid composition. Within the scope of this project it was not possible to carry out controlled culture experiments with varying growth conditions. Hence, here we are limited to hypothesizing on the factors controlling the lipid distributions at fixed growth conditions optimal for each examined strain. Previous studies have examined extensively the impact of phylogeny and physiology on the membrane lipid composition of archaea (Koga et al., 1998; Koga and Nakano, 2008; Elling et al., 2015; Kellermann et al., 2016). It is known that low permeability to water, protons and monovalent cations (i.e., Na\(^+\) and K\(^+\)) is the most important membrane adaption.
FIGURE 7 | HPLC-ITMS² spectra of cardiolipins with putative structures and fragmentations. (A) PGP-AR, AR, (B) PGP-AR, EXT-AR, (C) PGPG-AR, AR, (D) PGPG-AR, EXT-AR, and (E) PGPG-EXT-AR, EXT-AR. i-C<sub>20</sub>, C<sub>20</sub> isoprenoid chain; i-C<sub>25</sub>, C<sub>25</sub> isoprenoid chain.
for haloarchaea (Oger and Cario, 2013). The adaption of haloarchaeal membranes to salt, temperature, oxygen levels, and UV light examined experimentally by Kellermann et al. (2016) allowed them to hypothesize about the function of e.g., different polar headgroups (PG, PGP-Me, and cardiolipins) and core lipid composition. For example, they proposed that by changing the proportion of these polar headgroups, and hence the number of negative charges at its membrane surface, a haloneutrophil can actively balance membrane permeability under varying salt concentrations.

Hereafter, we examine specific lipids and groups of lipids and discuss the possible factors they are associated with, including the phylogenetic relationships between the strains (cf. Figure 1 and Table 1), the three different metabolic processes the 13 strains carry out (methanogenesis, polysaccharide utilization and sulfur respiration) and possible lipid adaptions associated with growth conditions. We have summarized the characteristic lipids associated with these separate factors in Figure 8 and we discuss them in detail in the following sections.

**Characteristic Lipids of the Methanogens**

The only characteristic shared by all four methanogens, and not by the other nine strains (all Halobacteria), was the presence of membrane-spanning GDGT lipids (Table 2). This is to be expected since GDGTs have been identified in a wide range of archaea including methanogenic euryarchaea, but not so far in the Halobacteria class [cf. summary tables in Koga et al. (1998) and Schouten et al. (2013)]. PG-GDGT-0-PG and PG-GDGT-1-PG were found in three of the four methanogenic strains (Tables 2, S1). 2Gly-GDGT-0 was only detected in neutrophilic “Ca. Mh. Thermophilum” HMET1 (Table S1). 2Gly-GDGTs, including 2Gly-GDGT-0, have been found previously in a wide range of environments (but not in soda lakes), including peat bogs, a suboxic water column and various marine benthic ecosystems (Lipp et al., 2008; Rossel et al., 2008; Lipp and Hinrichs, 2009; Schubotz et al., 2009; Liu et al., 2010; Yoshinaga et al., 2012; Gibson et al., 2013), and while 2Gly-GDGT-0 has been identified in cultures of Methanobacterium thermoautotrophicum (Koga et al., 1993), it has also been found in Thaumarchaeota (Schouten et al., 2008; Pitcher et al., 2011). As we discuss further in section Neutrophilic strains, the presence of this sugar headgroup may relate to the neutrophilic nature of HMET1 (cultured at pH 7, Table 1). PG-GDGT-0-PE was only found in Mn. thermophilum AMET1 and grown optimally at moderately thermophilic conditions in addition to salinity and pH extremes. PG-GDGT-PE has been previously described in two methane-rich seep environments, where it was specifically associated with anaerobic methane-oxidizing archaea of the ANME-1 cluster (Rossel et al., 2011).

Within the methanogens there was a division between the two classes based on the ratio of GDGTs to AR (cf. Figure 8). The two members of Methanonatronarchaeia (“Ca. Mh. thermophilum” HMET1 and Mn. thermophilum AMET1T) contained more GDGT than AR (GDGT0+GDGT1/AR = 4 and 2, respectively) while the two members of the class Methanomicrobia (Ms. natronophilum AME2T and Mc. alkalophilus AMF2T) contained more AR than GDGTs (ratio values 0.4 and 0.2, respectively). This difference may be an adaption to external salt concentration: while the two Methanonatronarchaeia are extremely halophilic methanogens (both cultured at 4 M Na+), the Methanomicrobia are haloalkaliphiles, in that Ms. natronophilum AME2T is an extremely salt-tolerant alkaliphile (cultured at 2 M total Na+), while Mc. alkalophilus AMF2T is a moderately salt-tolerant alkaliphile (cultured at 0.6 M total Na+). The presence of GDGTs in the membrane of methanogens may be associated with the reduction of wasteful ion cycling under chronic energy stress conditions (Valentine, 2007), as tetraether-containing membranes are less permeable to ions than purely diether-based membranes (Mathai et al., 2001; Shinoda et al., 2005).

The polar headgroup PS was present in alkaliphilic members of Methanonatronarchaeia (Mn. thermophilum AMET1T) and Methanosarcinales (Ms. natronophilum AME2T), but it was absent in any members of the Halobacteria. This polar headgroup has been described previously in a wide range of methanogenic archaea (Koga et al., 1993, 1998; Koga and Morii, 2005), and its presence in Ms. natronophilum AME2T, a member of Methanosarcinales, and absence in Mc. alkalophilus AMF2T, belonging to Methanomicobia, agrees with the findings of Koga et al. (1998). While PI was only detected in Methanosarcinales members Ms. natronophilum AME2T, this polar headgroup has been identified across a wide range of phyla and is not specific to methanogenic euryarchaea (Koga et al., 1993; Koga and Morii, 2005; Oger and Cario, 2013). However, the combination of the PI headgroup with the OH-AR and Uns(1-5)-OH-AR cores (cf. Table S1) is much less common. IPLs containing OH-AR and Uns(n)-OH-AR have been detected in deep-sea cold seep sediments (Yoshinaga et al., 2011), in the crenarchaeon Sulfolobus (S.) acidocaldarius (Spratt et al., 1997), the methanogen Methanosarcina (M.) barkeri (Nishihara and Koga, 1991) and in the extreme cold-adapted methanogen Methanococoides (M.) burtonii (Nichols et al., 2004). M. barkeri, M. burtonii, and Ms. natronophilum AME2T are all members of the Methanosarcinales order, which suggests that OH-AR and Uns(n)-OH-AR may be characteristic of this order rather than being related to e.g., cold adaption.

Two cardiolipins based on a PGP polar group and generally termed BPGs (Corcelli, 2009; Angelini et al., 2012) were detected only in “Ca. Mh. thermophilum” HMET1 from the class Methanonatronarchaeia (Table 3). To the best of our knowledge BPGs have only been described to date in haloarchaea from the order Halobacteriales (Lattanzio et al., 2002; Corcelli, 2009; Angelini et al., 2012; Lobasso et al., 2015; Kellermann et al., 2016; although not in the Halobacteriales investigated in this study), but not before in any methanogenic members of the Euryarchaeota. Our results indicate that synthesis of BPGs is not restricted to the Halobacteriales. Indeed, Yoshinaga et al. (2012) detected novel lyso-cardiolipins (with only two isoprenoid chains) in surface sediments of methane-charged deep-ocean seeps which are usually dominated by uncultured anaerobic methanotrophic (ANMEs) archaea related to methanogenic euryarchaea, in particularly from the order Methanosarcinales (Knittel and Boetius, 2009). It is interesting to note that unlike the other 3 methanogens, and the majority of the other strains (cf. section Neutrophilic strains), “Ca. Mh. thermophilum” HMET1...
TABLE 3 | Distribution of different classes of intact polar lipids (% of total peak area response) for the 13 strains of haloarchaea.

| IPL sType | Headgroup | Methanogens | Anaerobic sulfur reducers | Polysaccharide utilizers | Cellulolytics | Chitinolytics |
|-----------|-----------|-------------|---------------------------|-------------------------|---------------|-------------|
|           |           | AMET<sup>T</sup> | HMET<sup>T</sup> | AME2<sup>T</sup> | AMF2<sup>T</sup> | HSR<sup>T</sup> | HSR6<sup>T</sup> | AArc-Mg | AArc-Si<sup>T</sup> | HArCel<sup>T</sup> | AArcM<sup>T</sup> | AArc<sup>T</sup> | AArch4<sup>T</sup> | AArch5<sup>T</sup> | AArch-Si<sup>T</sup> |
| Dether    | Illa-c    | 9            | 17                        | 14                      | 16            | 1            | 3            |          |          |          |          |          |          |          |          |          |
|           | 2Gly      | 29           | 2                         | 6                       | 0.1           | 1            | 3            |          |          |          |          |          |          |          |          |          |
|           | PGP       | 0.2          | 5                         | 1                       | 1             | 11           | 1            |          |          |          |          |          |          |          |          |          |
|           | PG        | 28           | 40                         | 23                      | 4             | 20           | 1           | 1          |          |          |          |          |          |          |          |          |
|           | PE        | 1            | 1                         | 2                       | 3             |              |              |            |          |          |          |          |          |          |          |          |
|           | PS        | 8            | 5                         |                        |               |              |              |            |          |          |          |          |          |          |          |          |
|           | APT-Me (Ila-c) | 58       |                          |                         |               |              |              |            |          |          |          |          |          |          |          |          |
|           | APT       | 23           |                          |                         |               |              |              |            |          |          |          |          |          |          |          |          |
|           | PGS       | 8            | 22                        | 4                       | 4             | 46           | 49          | 67         | 68        |          |          |          |          |          |          |          |
|           | PGP-Me    | 30           | 3                         | 30                      |               | 8            | 22          | 46         | 49        | 67        | 68        |          |          |          |          |          |
|           | Pi        | 38           |                          |                         |               |              |              |            |          |          |          |          |          |          |          |          |
|           | PG-Gly (Ia,b) | 8        |                          |                         |               |              |              |            |          |          |          |          |          |          |          |          |
| Cardiolipin | PGP (BPG) | 6            |                          |                         |               |              |              |            |          |          |          |          |          |          |          |          |
|           | PGP-GP (Ma-c) | 1        |                          |                         |               |              |              |            |          |          |          |          |          |          |          |          |
|           | GDGT      | 8            |                          |                         |               |              |              |            |          |          |          |          |          |          |          |          |
|           | 2Gly      | 30           | 1                         | 13                      |               |              |              |            |          |          |          |          |          |          |          |          |
|           | 2PG       | 2            |                          |                         |               |              |              |            |          |          |          |          |          |          |          |          |
|           | PG/PE     | 2            |                          |                         |               |              |              |            |          |          |          |          |          |          |          |          |

Full strain names given in Table 1. IPLs were examined in terms of their peak area response from HPLC-ITMS analysis and thus the proportions reported do not necessarily reflect the quantitative relative abundance. PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PGP, phosphatidylglycerophosphate; PGP-Me, phosphatidylglycerophosphate methyl ester; PGS, phosphatidylglycerosulfate; APT, aminopentanetetrol; APT-Me, aminomethoxypentanetriol; PG-Gly, phosphatidylglycerohexose; 2Gly, dihexose; PGP-GP, phosphatidylglycerophosphate glycerophosphate; BPG, bisphosphatidylglycerol; GDGT, glycerol dialkyl glycerol tetraether (where n is the number of cyclopentane moieties). For data handling the three methylation of APT-Me-AR and the three methylations of APT-AR were combined as one group. For both APT-Me-AR and APT-AR the N,N,N-trimethyl form was the most abundant (minimum of total peak area 94% for APT-Me-AR and 96% for APT-AR), the N,N-dimethyl APT-Me-AR and APT-AR were present in a low relative abundance (maximum of total peak area 6% for APT-Me-AR and 2.5% for APT-AR) while the N-monomethyl APT-Me-AR and APT-AR were the lowest in relative abundance (maximum of total peak area <0.5% for APT-Me-AR and 1.5% for APT-AR).
is a haloneutrophile, being cultured at pH 7. Previously, the haloneutrophile *H. volcanii* has been shown experimentally to adapt to low Mg\(^{2+}\) levels (as per the culturing conditions here, e.g., 1 mM Mg\(^{2+}\)) by upregulating cardiolipin levels, in order to counterbalance the absence of “cardiolipin-surrogates” that form at higher Mg\(^{2+}\) concentrations, between the Mg\(^{2+}\) cations and two anionic PG lipids (Kellermann et al., 2016).

**Characteristic Lipids of the Halobacteria**

The absence of membrane-spanning GDGT lipids in *Halobacteria* has been hypothesized to have evolved to effectively respire using mechanisms acquired from bacteria through horizontal gene transfer (Kellermann et al., 2016). The authors suggest that the specific structure of haloarchaeal bilayer membranes has evolved to provide optimal balance between preventing ion permeability and maintaining sufficient fluidity for respiratory electron transport.

While diether lipids of the four methanogenic strains contained no EXT-AR (with the exception of an EXT-AR cardiolipin in *Ca. Mh. thermophilum* HMET1, see section Biosynthesis of EXT-AR), many of the analyzed *Halobacteria* strains showed a high proportion of EXT-AR in agreement with the other cultured genera from the class (*Tindall et al.*, 1984; *Teixidor et al.*, 1993; *Tachibana*, 1994; *Xu et al.*, 1999, 2001; * Gibson et al.*, 2005; *Xue et al.*, 2005; *Wang et al.*, 2007; *Minegishi et al.*, 2010). This absence of diether EXT-AR in methanogens is in agreement with previous studies (e.g., *Koga et al.*, 1998). HPLC-ITMS analysis (Table S1) revealed that the *Halobacteria* core lipids also contained Uns-EXT-AR. The presence of EXT-AR has been suggested to provide a “zipper” like effect between the two layers of the membrane, interlinking the C\(_{25}\) isoprenoid of the “outward” facing lipid, with those of the “inward” facing lipids (*De Rosa et al.*, 1982) providing increased membrane stability. Kellermann et al. (2016) hypothesized that in the case of alkaliphilic haloarchaea (six of the nine *Halobacteria* strains examined here) the EXT-AR zipper configuration within the bilayer is not compatible with membrane-shielding PG-cation interactions (as seen in neutrophilic haloarchaea). Therefore, alkaliphilic haloarchaea with high levels of EXT-AR would need to upregulate the amount of PGP-Me, to provide an alternative mode of Mg\(^{2+}\) buffering at the membrane surface. Indeed, in agreement with this idea, PGP-Me was the dominant headgroup (46–68%, Table 3) across the *Halobacteria* and has been described previously as a distinguishing feature of haloarchaea (*Kushwaha et al.*, 1981; *Kates*, 1992; *Kates et al.*, 1993; *Sprott et al.*, 1997; *Lattanzio et al.*, 2002; *Oren*, 2002; *Gibson et al.*, 2005; *Tenchov et al.*, 2006; *Xu et al.*, 2007; *Angelini et al.*, 2012; *Oger and Cario*, 2013; *Meador et al.*, 2014; *Kellermann et al.*, 2016). During culturing experiments with neutrophilic *H. volcanii*, a correlation between the amount of PGP-Me and the Mg\(^{2+}\) concentration was observed (Kellermann et al., 2016) and was hypothesized to relate to the closely positioned, two negatively charged phosphate moieties which allow binding to divalent cation to reduce the permeability of monovalent cations under high salt concentrations [cf. Figure 9 of Kellermann et al. (2016)]. As stated above, six of the nine *Halobacteria* strains examined were haloalkaliphiles, while three were haloneutrophiles and all nine were cultured at very low Mg\(^{2+}\) concentrations (1 mM). Kellermann et al. (2016) suggested that in order to maintain membrane stability at low Mg\(^{2+}\) concentrations, haloalkaliphiles adapt by upregulating cardiolipins in place of PG or PGP-Me, while haloalkaliphiles utilize the combined EXT-AR and PGP-Me approach described above. However, in our study we did not see a difference between the neutrophilic and alkaliphiles haloarchaea in terms of the relative amounts of cardiolipin, PG and PGP-Me or EXT-AR. Therefore, it is the Mg\(^{2+}\) concentration which is more important in this case, rather than the pH. It should be noted however that at high pH only low levels of Mg\(^{2+}\) remain in solution.
Characteristic Lipids of the Methanomicrobia

While the two *Methanonatronarchaeia* and *M. alkaliphilus* AMF2\(^T\) all exhibited relatively common diether core lipid compositions (AR and Uns-AR), *Ms. natronophilum* AME2\(^T\) had the most complex diether lipid composition of any of the thirteen strains: based on GC-MS analysis (Table 2) it contained 45% AR, 15% OH-AR, and 6% MAR. The 16% of alkyl glycerol monoethers represents the Uns-AR and Uns-OH-AR identified in intact form by LC-ITMS (Tables S1, S5). The presence of OH-AR alongside AR is considered characteristic of the *Methanosarcinales* order to which *Ms. natronophilum* AME2\(^T\) belongs (Koga et al., 1998). The hydroxylation of AR to produce OH-AR has been postulated to have an effect on membrane stability, possibly increasing the polar surface area of the membrane or by creating hydrophilic pockets (Sprott et al., 1990). While MAR is not considered to be a general characteristic of the *Methanosarcinales* order, it has been previously reported in certain genera of the order, such as extremely halophilic *Methanohalobium* (Koga et al., 1998). The presence of MAR phospholipids has been shown to improve membrane impermeability to protons and solute leakage and membrane stability in methanogenic archaea (Koga et al., 1998) in a similar way to tetraether lipids. This structural effect is thought to explain its accumulation in the hyperthermophilic methanogen *Methanococcus jannaschii* [where it can account for up to 85% of the core lipids; (Sprott et al., 1991; Dannenmuller et al., 2000)], and other members of the *Methanococcales* order (Koga et al., 1998). The same might be true for extremely halophilic methanogens. As discussed above, *Ms. natronophilum* AME2\(^T\) is an extremely salt-tolerant alkaliophile (cultured at 3 M total Na\(^+\)) and the MAR and OH-AR core lipid adaptions of this strain may be order- or species-specific adaptions to high salt and high pH conditions.

Two closely related polar headgroups (tri, di, and mono N-methyl), APT and APT-Me were detected only in the single *Methanomicrobiales* member *M. alkaliphilus* AMF2\(^T\). While (tri, di- and mono) N-methyl APT-Me are all novel compounds, trimethyl APT has been described in a range of methanogenic euryarchaea, including the members of the order *Methanomicrobiales*, but, in agreement with our findings, not in the order *Methanosarcinales* (Ferrante et al., 1987; Koga et al., 1993, 1998; Koga and Morii, 2005). While APT has been considered previously a characteristic lipid headgroup of methanogenic archaea (Koga et al., 1993), our finding suggests it may be specific at the order level. It would be expected that the zwitterionic nature of these two headgroups would mean that they provide an overall neutral charge at the membrane surface and hence would not bind to cations in the same manner as negatively charged (anionic) lipids, e.g., PGP-Me (cf. section Characteristic lipids of the Halobacteria). This predominantly neutral membrane surface may have evolved to adapt to the conditions encountered by *M. alkaliphilus* AMF2\(^T\) of moderate salt (as per the culturing conditions 0.6 M Na\(^+\), 1 mM Mg\(^{2+}\)) and high pH (9.5–10). It is of note that Szekely et al. (2011) found that, when modeling interactions between lipid membranes and various ions, while divalent ions do bind to zwitterionic headgroups, the presence of at least one double bond in the lipid tail is sufficient to prevent this divalent ion adsorption. The authors suggested that this was due to the looser packing of unsaturated lipids, allowing free rotation of the headgroup. It is of note therefore that *M. alkaliphilus* AMF2\(^T\), contained a high amount of unsaturated diether lipids of all 13 strains examined (12% alkyl glycerol monoethers formed during acid hydrolysis from unsaturated diether lipids, Table 2). This suggests that the presence of high levels of zwitterionic headgroups and of unsaturated lipids in AMF2\(^T\) are interrelated membrane adaptions.

It should be noted that the other member of the *Methanomicrobia* class, *Ms. natronophilum* AME2\(^T\), also contained a high proportion of Uns-ARs (16%, Table 2), whereas in all other classes it was <5%. Dawson et al. (2012) studied the effect of salinity on AR saturation in four strains of haloarchaea and found that the degree of unsaturation increased with increasing salinity of growth medium as well as with the strains' increasing salinity optimum. They hypothesized that increased AR unsaturation is a physiological adaptation to both maintain membrane fluidity and reduce ion-permeability as salinity increases. Kellermann et al. (2016) observed for *H. volcanii* an increase in the degree of unsaturation with increasing Na\(^+\) levels primarily within the bulk IPl PG-AR (although they noted that total AR did not exhibit the same trend in unsaturation, which they suggested was due to impaired growth performance at both the low and high ends of the salt concentration range). However, a higher salt concentration does not explain the high level of AR unsaturation in the two *Methanomicrobia* as both *Ms. natronophilum* AME2\(^T\) and *M. alkaliphilus* AMF2\(^T\) were cultured at the lowest salt concentrations of all 13 strains (2 M and 0.6 M total Na\(^+\)). This suggests that AR unsaturation in response to salt concentrations is a class specific adaption, manifested at lower salt concentrations by these *Methanomicrobia* than the *Methanomicrobiaceae* and Halobacteria, which are all more extreme halophiles.

Lipids of the Natrialbales

A group of lipids only seen in members of the order *Natrialbales* included three PGPGP cardiolipins, one with two AR cores, one with an AR and an EXT-AR core and the final one with two EXT-AR cores (Table S1). These PGPGP cardiolipins were present in alkaliophilic polysaccharide utilizing haloarchaea "*Na. chitinivorans*" AArch4\(^T\), *Nb. cellulosirostrata* AArcel5\(^T\) and AArcel7, but not in the less alkaliophilic "*Ha. desulfuricum*" AArc-Sf\(^T\). These PGPGP cardiolipins may be the same as unknown cardiolipins, postulated to be glycoscardiolipins, detected previously in two species of alkaliophilic haloarchaea from the order *Halobacteria* (Angelini et al., 2012). If this is the case then PGPGP cardiolipins may be characteristic of the alkaliophilic members of *Halobacteria* growing at low Mg background. It has been previously suggested, based on results from culturing experiments with the neutrophilic *Halobacteria* member *H. volcanii* that at low Mg\(^{2+}\) concentration, the membrane upregulates the amount of PGP cardiolipin, to assure membrane integrity [cf. Figure 9 of Kellermann et al. (2016)]. At high Mg\(^{2+}\) levels, *H. volcanii* upregulated PGP-Me to increase
Mg$^{2+}$ binding at its closely positioned phosphate moieties (Kellermann et al., 2016). Thus, it may be possible that PGPGP headgroups could be orientated in a way that neighboring phosphate moieties are tethered via divalent cations, as per PGP-Me-AR, while also benefitting from the additional structural integrity of the cardiolipin structure.

The polar headgroup assigned as PG-Gly was only detected in two alkaliphilic Natrialbales (Nb. cellulositropho AArcel5 and AArcel7) but in no other orders. The PG-Gly headgroup with a diacyl core has been described in halophilic gammaproteobacteria of the genus Halomonas (Giordano et al., 2007), where it was postulated that the bulky headgroup would enhance steric protection through hydrogen bonding via glycosyl headgroups to ensure the osmotic stability of the cellular membrane under high-salt conditions. Furthermore, a very similar phosphoglycolipid with a sulfated sugar moiety was identified in halophilic halaoarchaeon N. moalopenis from the Halobacteriales order (Hoffmann et al., 2015). The presence of this headgroup in both extremely halophilic archaea and moderately halophilic bacteria further supports the hypothesis that this headgroup provides additional osmotic stability.

**Neutrophilic Strains**

Cellulolytic “Hc. cellulosivorans” HArcelT and sulfur-respiring *Hd. formicicum* HSR6T and *Hn. sulfurireducens* HSR2T of the order Halobacteriales all contained the PGS headgroup, not seen in any of the other 10 strains as well as the novel sulfur-containing headgroup (IIIa-d), which was also seen in methanogenic “Ca. Mh. thermophilum” HMET1. The presence of these sulfur containing lipids may be a distinctive characteristic associated with these Halobacteriales members or may relate to the neutrophilic nature of these extreme halophiles. Indeed, while nine strains out of 13 examined were alkaliphilic, the remaining four neutrophilic strains (cf. Table 1): methanogenic “Ca. Mh. thermophilum” HMET1, sulfur-respiring *Hd. formicicum* HSR6T and *Hn. sulfurireducens* HSR2T and aerobic polysaccharide utilizing “Hc. cellulosivorans” HArce1T were all cultured at pH 7. PGS was only detected in three of these strains (not in HMET1) and not in any of the alkaliphilic strains (Table 3). Indeed, PGS has been reported as a characteristic component in the other neutrophilic haloarchaea, such as the members of *Halofax* and *Halorubrum lacusprofundi* from the Halofaxales (Kates, 1992; Gibson et al., 2005) and *Halomicrobium mukohataei* from the Halobacteriales (Oren et al., 2002).

Another characteristic of the neutrophilic strains was the presence of glycolipids. All four neutrophilic strains contained the 2Gly polar headgroup with an AR core, which was only detected in trace levels in two alkaliphiles, *Mc. alkaliphilus* AMF2T and *Nb. cellulositropho* AArcel5T (Table 3). Similarly, the lipid composition of “Ca. Mh. thermophilum” HMET1 was dominated by 2Gly-GDGT-0 whereas the three alkaliphilic methanogens only produced GDGTs with phospho-based polar headgroups (Tables 3, S1). This supports the assertion that glycolipids are absent or present only in trace amounts in membranes of alkaliphilic representatives of extremely halophilic Euryarchaeota (Oger and Cario, 2013; Siliakus et al., 2017).

**Biosynthesis of EXT-AR**

Low levels of an IPL cardiolipin with an EXT-AR core in ’Ca. Mh. thermophilum’ HMET1 (Table S1; supported by the presence of EXT-AR in hydrolyzed core lipids, Table 2), is an interesting result as, of the 8 known orders of methanogens, only a representative of *Methanomassiliicoccales* has been described to date as containing EXT-AR (Koga and Morii, 2005; Becker et al., 2016). Indeed, the analyzed strains in the class Methanomicrobia (*Ms. natronophilum* AME2T and *Mc. alkaliphilus* AMF2T) did not contain any EXT-AR, whereas all analyzed *Halobacteridae* did (cf. Tables 2, S5). These results led us to the hypothesis that the close relationship between the novel class-level lineage Methanomonararchaeia and the class Halobacteria (Sorokin et al., 2017a, cf. Figure 1) means that they share the same genetic capability to produce EXT-AR. It should be noted, however, that EXT-AR was only present in low levels in “Ca. Mh. thermophilum” HMET1 (Tables 2, S1) and that *Mn. thermophilum* AMET1T, also a member of the *Methanomonararchaeia*, did not contain any EXT-AR. It is possible that the presence of EXT-AR in HMET1 and not AMET1T is related to culture conditions as the medium for HMET1 contained 4 M NaCl, while that of AMET1T contained 2 M Na$^+$ as NaCl and 2 M Na$^+$ as carbonates. Hence the osmotic pressure in case of AMET1T was much lower, since sodium carbonates, as weak electrolytes, impose roughly two time less osmotic pressure than the strongly electrolytic NaCl (Robinson and Macaskill, 1979). Dawson et al. (2012) noted previously that, while the presence of EXT-AR seems to be in part taxonomic, an increase in its abundance is advantageous for haloarchaea as it decreases membrane ion-permeability. As described above, the presence of EXT-AR has been suggested to provide a “zipper” like effect between the two layers of the membrane providing increased membrane stability (De Rosa et al., 1982). It has also been found that C$_{25}$-C$_{25}$ EXT-AR liposomes are thicker than C$_{20}$-C$_{20}$ AR liposomes and have relatively lower permeability (Gmajner et al., 2011), so a similar effect could be expected of the C$_{25}$-C$_{25}$ EXT-AR detected in this study.

In order to examine the genetic capability to produce EXT-AR we examined the polyprenyl synthases (belonging to the prenyltransferase family) involved in the elongation of isoprenoids (Liang et al., 2002) in the genomes of the two members of *Methanomonararchaeia* class along with eight haloarchael isolates from this study plus additional archaeal species, including a single-cell genome sequence, from Nereus Deep in the Red Sea, belonging to a putatively novel family within the archaeal candidate division SA1 (Ngugi and Stingl, 2018), to which the two members of *Methanomonararchaeia* also belong (Figure 9). Homology search analysis revealed multiple putative polyprenyl synthases in the analyzed genomes. Specific amino acid (AA) residues of the polyprenyl synthase genes are known to determine the length of the isoprenoid chain. Specifically, the fifth amino acid before the First Aspartate-Rich Motif (FARM) has been found to determine the length of the formed isoprenoid chain. For the case of the C$_{25}$ isoprenoid chains of the EXT-AR, also the eighth and 11th amino acids are important in the determination of the C$_{25}$ extension (Liang et al., 2002). Figure 9 illustrates the relationships between the different polyprenyl
FIGURE 9 | Phylogenetic tree of putative polyprenyl synthases involved in isoprenoid elongation. Phylogenetic reconstruction was performed by maximum likelihood as indicated in the methods. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The nature of the amino acids at the 11th, 8th, and 5th position before the aspartate-rich motif is given in square brackets e.g., [AA

11

, AA

8

, AA

5

]. Bulky amino acids such as phenylalanine (F) block the length of the isoprenoids at C

20

, while smaller amino acids such as serine (S) allow for elongation to C

25

and beyond (Ohnuma et al., 1996; Liang et al., 2002).

Many of the examined archaeal genomes encode multiple putative polyprenyl synthases, generally separated in function by the nature of the specific FARM amino acids described above (Figure 9). A major cluster of synthases, which we assigned as long chain polyprenyl synthases (Figure 9), are all characterized by a P/L/G (proline

11

, leucine

8

, glycine

5

) FARM motif. Indeed, replacement of bulky F at the fifth position with a smaller amino acid residue such as serine (S) has been described as the most effective way to elongate beyond the C

20

length. Another point to note is that A. pernix is known to produce exclusively a C

25

, C

25

AR (Ohnuma et al., 1999). However, it only contained one putative polyprenyl synthase, which clustered with the short chain polyprenyl synthases (Figure 9), most closely with acidophilic S. acidocaldarius which does not produce EXT-AR (Liang et al., 2002). Conversely, S. acidocaldarius contains the bulkier L in the eighth position. The clustering of these genes together, despite this difference, suggests that the polyprenyl synthase of A. pernix was originally a short chain polyprenyl synthase which underwent certain amino acid replacements, but overall remains most closely related to the other short chain polyprenyl synthases. Interestingly, although the methanogen Methanomassilicococcus luminyensis has been described as containing EXT-AR (Becker...
et al., 2016), based on its very bulky, chain-limiting FARM motif (I₁₁, I₈, F₅) as pointed out in Figure 9, this would not be expected.

A third cluster of polyprenyl synthases contained the three members of the archaeal candidate division SA1: the only two members of Methanonatronarchaeia presently known in culture, “Ca. Mh. thermophilum” HMET1 and Mn. thermophilum AMET1 and a single-cell genome sequence from Nereus Deep in the Red Sea. All three only contained one putative polyprenyl synthase with two Is, and the relatively bulky F at the 11th, 8th, and 5th positions, a motif associated with short chain polyprenyl synthase. However, these putative polyprenyl synthases cluster far apart from all the other short chain polyprenyl synthases and cluster closer to the long chain polyprenyl synthases (Figure 9). While this conflicting motif and clustering provide no specific evidence that this is the gene involved in the production of EXT-AR containing cardiolipins in Methanonatronarchaeia, it does raise the possibility that these novel extremophilic methanogens utilize a different isoprenoid elongation mechanism than the other extremely halophilic euryarchaeota examined here. Perhaps their isolated phylogenetic position has afforded them alternative pathways to adapt their membranes to hypersaline conditions using EXT-AR. However, as EXT-AR was only present in low levels in ‘Ca. Mh. thermophilum’ HMET1 (Tables 2, S1) and was not present in Mn. thermophilum AMET1, it does not appear to be a significant membrane adaptation under the culturing conditions examined in this study.

CONCLUSIONS

Analyzing a large set of novel halo(alkali)philic representatives of Euryarchaeota, novel diether-based lipids were detected including a PG-Gly headgroup combined with a diether core, an APT-like lipid with an additional methoxy group in place of a hydroxy group on the pentanetetrol and finally headgroups with elemental composition of either C₁₂H₂₅NO₁₃S or C₁₂H₂₅NO₁₃S₂. Furthermore, novel cardiolipins were detected in three strains: a putative PGPGP polar group bridging the diether components. The presence of cardiolipins with EXT-AR in one of the Methanonatronarchaeia strains was unusual, as only one other methanogenic archaeal order has been reported to produce EXT-AR. Both Methanonatronarchaeia strains contain a specific polyprenyl synthase which clustered separately from those of the haloarchaea. This may indicate the potential of these strains to synthesize EXT-AR.

We compared the distribution of membrane lipids across the 13 strains to examine what factors determined lipid composition, including the phylogenetic relationships between the strains, the three different metabolic processes they carry out and adaptations associated with the pH. In general we found that the lipid distribution of the 13 strains could be generally separated into two, the methanogens (group) and the Halobacteria (class) based on the presence of specific core lipids. Within the methanogen group adaption to high or moderate salt concentration resulted in the extremely halophilic class Methanonatronarchaeia containing more GDGTs than AR, while the less halophilic members of the class Methanomicobia contained more AR than GDGTs. The methanogen Ms. natronophilum AME2 had the most complex diether lipid composition of any of the 13 strains, including OH-AR and MAR which we surmise is an order-specific membrane adaption. The zwitterionic lipids APT and APT-Me headgroups were detected only in the Methanomicobia member M. alkaliphilus AMF2, which also contained the highest proportion of unsaturated lipids. Many of the Halobacteria strains contained a high proportion of the EXT-AR core and PG-P-Me headgroup in agreement with the other cultured genera from the class. Within the Halobacteria only alkaliphilic members of the Natrialbales order contained PGPGP cardiolipins and the PG-Gly headgroup. The four neutrophilic strains belonging to Halofaracales and Halobacteriales orders were characterized by the presence of sulfur-containing headgroups and glycolipids.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

AUTHOR CONTRIBUTIONS

NB carried out data analysis and wrote manuscript. DS isolated and cultured archaea. EH oversaw analytical methods and carried out data analysis. MK and WR carried out extractions and data analysis. LV carried out homology search and phylogenetic analyses. HW carried out NMR analysis and JS conceived and supervised the project.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00377/full#supplementary-material
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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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