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ORIGINAL PAPER

Temperature and pH controls on glycerol dibiphytanyl glycerol tetraether lipid composition in the hyperthermophilic crenarchaeon *Acidilobus sulfurireducens*

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Abstract Cyclization in glycerol dibiphytanyl glycerol tetraethers (GDGTs) results in internal cyclopentane moieties which are believed to confer thermal stability to crenarchaeal membranes. While the average number of rings per GDGT lipid (ring index) is positively correlated with temperature in many temperate environments, poor correlations are often observed in geothermal environments, suggesting that additional parameters may influence GDGT core lipid composition in these systems. However, the physical and chemical parameters likely to influence GDGT cyclization which are often difficult to decouple in geothermal systems, making it challenging to assess their influence on lipid composition. In the present study, the influence of temperature (range 65–81°C), pH (range 3.0–5.0), and ionic strength (range 10.1–55.7 mM) on GDGT core lipid composition was examined in the hyperthermoacidophile *Acidilobus sulfurireducens*, a crenarchaeon originally isolated from a geothermal spring in Yellowstone National Park, Wyoming. When cultivated under defined laboratory conditions, the composition of individual and total GDGTs varied significantly with temperature and to a lesser extent with the pH of the growth medium. Ionic strength over the range of values tested did not influence GDGT composition. The GDGT core lipid ring index was positively correlated with temperature and negatively correlated with pH, suggesting that *A. sulfurireducens* responds to increasing temperature and acidity by increasing the number of cyclopentyl rings in GDGT core membrane lipids.

Keywords Glycerol dibiphytanyl glycerol tetraether · GDGT · Yellowstone · pH · Temperature · Crenarchaea

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Introduction

The membrane core lipids synthesized by Crenarchaeota, as well as by some Euryarchaeota, are composed of glycerol dibiphytanyl glycerol tetraethers (GDGTs) (De Rosa and Gambacorta 1988; De Rosa et al. 1986). In the upper marine water column, marine sediments, and freshwater sediments, the average number of cyclopentyl rings per GDGT correlates with increasing surface water temperature (Powers et al. 2004; Schouten et al. 2002, 2003). In terrestrial systems, other environmental parameters also appear to influence GDGT composition. For example, the distribution and composition of GDGT lipids recovered from terrestrial geothermal springs exhibit varying correlations with

temperature, depending on the subset of environments sampled. In a study from the Great Basin, USA, the average number of rings (ring index) correlated positively with bicarbonate concentration ($R^2 = 0.31$), but did not correlate well with temperature ($R^2 = 0.03$) (Pearson et al. 2004). Similarly, the relative abundances of cyclopentane-containing GDGT lipids recovered from Yellowstone National Park (YNP) geothermal spring mats did not correlate well with temperature or pH (Schouten et al. 2007). In contrast, Pearson et al. (2008) noted a strong correlation between pH and the individual GDGTs crenarchaeol and GDGT-4 in samples collected from a variety of terrestrial hot springs, while weaker correlations were noted between individual GDGTs and temperature (Pearson et al. 2008). The varying results from these studies suggest that other physical or chemical parameters influence the GDGT lipid composition in complex environmental systems, such as geothermal springs. However, individual environmental parameters are often difficult to decouple from other variables in natural environments making it challenging to assess their influence on GDGT composition. This is especially true in geothermal spring environments, where strong thermal and redox gradients present a multiplicity of conditions that may potentially confound interpretation of GDGT lipid profiles (Inskeep and McDermott 2005; Nordstrom et al. 2005; Shock et al. 2005). In addition to environmental considerations, there may be species- or community-specific controls on GDGT lipid composition that have not yet been accounted for due to difficulties in cultivating ecologically representative strains from the natural environment.

Recent results obtained from the euryarchaeon *Thermoplasma acidophilum* indicate that the degree of GDGT cyclization in membrane lipids increases with increasing incubation temperature and decreases systematically with decreasing pH of the cultivation medium (Shimada et al. 2008). However, whether similar pH controls on GDGT cyclization extend to other archaeal phyla has not been determined. Here, we examine the influence of temperature, pH, and ionic strength (IS) on the composition of GDGT lipids in a hyperthermophilic crenarchaeon (*Acidilobus sulfurireducens* str. 18D70) that has been shown previously to be a dominant component of microbial assemblages inhabiting a number of high temperature geothermal springs in YNP, Wyoming (Boyd et al. 2007; Inskeep et al. 2010). Many of these springs have physical and geochemical conditions that are similar to high-temperature geothermal spring environments where community GDGT lipid profiles have been previously determined (Pearson et al. 2008; Schouten et al. 2007), making *A. sulfurireducens* a useful organism for understanding the environmental controls on GDGT lipid composition in these high temperature systems.

Acidilobus sulfurireducens couples the oxidation of peptides to the reduction of elemental sulfur (S°) under anoxic conditions and grows over a temperature range of 61–89°C (optimum of 81°C) and a pH range of 2.0–5.5 (optimum of 3.0). Growth yields of *A. sulfurireducens* grown under optimal conditions are low (2.1×10^7 cells mL^{-1} or ~100 mg biomass L^{-1}) (Boyd et al. 2007), likely due to low free energy associated with coupling organic carbon oxidation to sulfur reduction at high temperature (<95 kJ mol^{-1} at 85°C) (Amend and Shock 2001). Despite difficulties in cultivating and obtaining sufficient biomass for biochemical analyses (Boyd et al. 2007), our examination of GDGT lipid profiles in membranes of *A. sulfurireducens* cultivated under defined laboratory conditions indicates a central role for both temperature and pH in controlling the composition of total and individual GDGT lipids and provides new insight into the role of these parameters in controlling the distribution of GDGTs in natural systems.

Materials and methods

To examine the role of the pH of the growth medium on the composition of GDGT lipids, *A. sulfurireducens* was cultivated in peptone-elemental sulfur (PS) medium incubated at 81°C with the pH adjusted to 3.0, 3.5, 4.0, 4.5, and 5.0 as previously described (Boyd et al. 2007). The pH of growth media was buffered using sodium citrate buffer (10 mM final concentration). The influence of temperature on GDGT lipid composition was assessed in PS medium with the pH adjusted and buffered to 3.0 with sodium citrate (10 mM final concentration). The influence of ionic strength (IS) on the GDGT lipid composition was determined in modified PS medium containing reduced concentrations of NH_4Cl (0.1 g L^{-1}), KCl (0.03 g L^{-1}), $CaCl_2{\cdot}2H_2O$ (0.03 g L^{-1}), $MgSO_4{\cdot}7\,H_2O$ (0.03 g L^{-1}), and KH_2PO_4 (0.05 g L^{-1}). Following pH adjustment to 3.0, the electrical conductivity (EC) of growth media was adjusted through additions of NaCl. IS was calculated according to the formula: $IS = 0.0127 \times EC_{298}$, where EC is the electrical conductivity measured at 298°K. Medium pH was not buffered in IS treatments.

Growth curves were constructed for each cultivation condition using both cell density and sulfide concentration as previously described (Boyd et al. 2007). Triplicate 2-l cultures were harvested for the pH and temperature treatments, whereas only a single 2-l culture was harvested for the ionic strength experiments. Replicates for IS experiments were not performed since preliminary investigations indicated that IS over the range tested did not influence GDGT composition (see results and discussion). Cultures were harvested during mid-exponential growth phase, and

biomass was first treated to remove S° using carbon disulfide as previously described (Boyd et al. 2007).

Biomass was subjected to lipid extraction as previously described (Pearson et al. 2004; Zhang et al. 2006), and a second treatment was used to remove residual co-extracted S°. Here, 1 g of small copper shavings was added to the total lipid extract (TLE) in a glass bottle containing 5 mL of dichloromethane (DCM). The solution was mixed by gentle shaking followed by incubation at room temperature for 8 h to allow the elemental sulfur and copper to react. Samples and DCM were transferred to 30 mL Teflon tubes and were centrifuged (3,000*g*, 1 min). Following centrifugation, the organic DCM phase (total lipid extract) was collected and concentrated using a nitrogen gas stream. A solution of 5% HCl in methanol was used for converting intact polar lipids to core lipids in the total extract. Finally, total core lipids were detected using LC–MS as previously described (Pitcher et al. 2009). Core GDGTs were dissolved in hexane/isopropanol (99:1, v/v) and separated by high performance liquid chromatography (HPLC). Core GDGTs were resolved on a Prevail cyano column heated to 30°C on an Agilent 1200 HPLC system. Mass spectrometric identification and quantification were achieved using an ion trap mass spectrometer coupled to the HPLC by an atmospheric pressure chemical ionization (APCI) interface. Diethers were not quantified in this study. Targeting tetraether archaeal lipids, ion scans were set to *m*/*z* 1,200–1,500. Quantification was based on peak intensities in the mass chromatogram of the M^+ ions using *Sulfolobus acidocaldarius* GDGT standards. The standard error of mean (SEM) of GDGT relative abundances associated with replicate injections of *S. acidocaldarius* standards ranged from 0.01 to 0.65% of total peak area, with smaller SEMs associated with less abundant GDGTs and larger SEMs associated with more abundant GDGTs, resulting in a linear relationship between average GDGT abundance and the associated SEM ($R^2 = 0.93$) (data not shown). Each biological replicate was analyzed once. The detection threshold for GDGTs using these methods was 0.1 μg per compound injected. GDGTs identified by LC–MS are reported according to the nomenclature of Schouten et al. (2003) and as modified in Pearson et al. (2008). Importantly, GDGT-4′ is an isomer of GDGT-4 (four cyclopentyl rings), GDGT-5′ is an isomer of GDGT-5 (five cyclopentyl rings), and neither GDGT-5 nor 5′ is crenarchaeol (four cyclopentyl rings plus one cyclohexyl ring) (Sinninghe Damsté et al. 2002). This conclusion follows from the fact that GDGT-4 and 4′ do not co-elute with GDGT-5 and 5′ (Fig. 1). In contrast, crenarchaeol typically co-elutes with GDGT-4, and its abundance can be estimated from linear interpolation of authentic mass spectra of GDGT-4 and crenarchaeol (here the abundance was zero). The weighted average number of GDGT rings per lipid molecule (ring index, RI) was calculated according to the formula: RI = [%GDGT-1 + 2 × (%GDGT-2) + 3 × (GDGT-3) + 4 × (%GDGT-4 + %GDGT-4′) + 5 × (%GDGT-5 + %GDGT-5′) + 6 × (%GDGT-6) + 7 × (%GDGT-7)]/100 as previously described (Pearson et al. 2004).

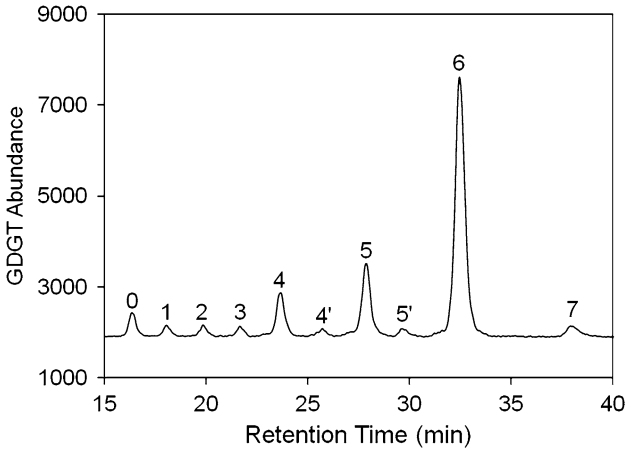

Fig. 1 HPLC–MS chromatogram of GDGTs for a culture of *A. sulfurireducens* cultivated at 81°C and a pH of 3.0. GDGTs are reported according to the nomenclature of Schouten et al. (2003) and as modified in Pearson et al. (2008), and correspond with those presented in Table 1

Linear regressions of all replicate ring indices as a function of pH and incubation temperature were performed. The relative abundance of lipids from all replicate treatments were used to calculate Spearman rank correlation coefficients (ρ) and *P* values using the 'cor.test' function within the base R core package (http://www.r-project.org/contributors.html). The relative abundance of lipids from replicate treatments were used to construct Euclidean distance matrices describing the dissimilarity of the lipid profiles between treatments. In addition, Euclidean distance matrices were constructed to describe the dissimilarity of pH and temperature between treatments. Euclidean dissimilarity matrices were constructed using the R package vegan (http://vegan.r-forge.r-project.org/). The relationships between dissimilarity matrices describing lipid profiles and pH or incubation temperature were evaluated using Mantel tests computed from 999 permutations with the R package vegan.

Results and discussion

Influence of incubation temperature on GDGT composition

The GDGTs with masses (*m*/*z*) ranging from 1,302 (0 rings) to 1,288 (7 rings) were identified in cultures of *A. sulfurireducens* cultivated over a temperature range of

Table 1 Relative abundance of GDGTs in lipid extracts of *A. sulfurireducens* cultivated under various growth conditions

Fraction of total isoprenoid GDGTs[a]												
Temp (°C)	pH	IS	GDGT-0 1302[b]	GDGT-1 1300[b]	GDGT-2 1298[b]	GDGT-3 1296[b]	GDGT-4 1294[b]	GDGT-4′ 1294[b]	GDGT-5 1292[b]	GDGT-5′ 1292[b]	GDGT-6 1290[b]	GDGT-7 1288[b]
65	3.0	44.9	1.6	0.3	0.8	1.1	16.0	0.6	40.0	0.6	38.5	0.5
			(1.0)	(0.3)	(0.5)	(1.0)	(5.4)	(0.5)	(3.7)	(0.6)	(9.4)	(0.4)
70	3.0	44.9	2.8	1.2	1.0	1.2	13.1	4.7	33.6	1.2	40.4	0.7
			(1.8)	(1.1)	(1.1)	(1.6)	(3.6)	(6.0)	(7.6)	(1.2)	(12.3)	(0.7)
75	3.0	44.9	2.5	0.5	0.8	1.0	14.0	0.5	26.2	0.3	52.4	1.8
			(2.2)	(0.9)	(0.9)	(1.0)	(10.7)	(0.6)	(18.1)	(0.6)	(24.8)	(2.3)
81	3.0	44.9	2.0	0.5	0.6	0.4	3.0	0.3	10.4	0.9	75.2	6.6
			(2.5)	(0.8)	(1.0)	(0.9)	(3.0)	(0.8)	(3.8)	(1.2)	(12.2)	(5.7)
81	3.0	44.9	2.0	0.5	0.6	0.4	3.0	0.3	10.4	0.9	75.2	6.6
			(2.5)	(0.8)	(1.0)	(0.9)	(3.0)	(0.8)	(3.8)	(1.2)	(12.2)	(5.7)
81	3.5	43.3	1.6	BD	BD	BD	4.8	BD	16.8	BD	72.3	4.4
			(1.4)				(3.4)		(9.5)		(8.6)	(5.7)
81	4.0	43.1	7.5	BD	BD	1.5	10.6	0.2	22.4	0.3	55.9	1.6
			(12.0)			(2.5)	(9.2)	(0.4)	(9.4)	(0.5)	(20.9)	(2.1)
81	4.5	43.1	8.9	BD	BD	BD	6.0	BD	23.0	BD	63.0	1.0
			(7.8)				(4.0)		(5.0)		(19.0)	(1.0)
81	5.0	43.1	9.8	BD	BD	BD	2.2	1.0	19.4	1.0	65.7	0.9
			(16.4)				(0.7)	(1.1)	(5.9)	(1.1)	(21.9)	(0.8)
81	3.0	10.1	BD	BD	BD	BD	0.5	0.3	8.0	BD	87.7	3.4
81	3.0	15.0	BD	BD	BD	BD	0.4	0.2	8.0	BD	88.0	3.3
81	3.0	22.3	BD	BD	BD	BD	0.3	0.1	8.2	BD	86.8	4.6
81	3.0	27.8	BD	BD	BD	BD	0.7	0.2	7.7	BD	85.3	6.0
81	3.0	38.0	BD	BD	BD	BD	0.5	0.2	10.2	BD	85.3	3.8
81	3.0	55.7	BD	BD	BD	BD	0.4	0.1	6.9	BD	90.5	2.1

Standard deviation among replicates is indicated in parentheses below the average GDGT abundance, when applicable

BD below detection (detection limit = 0.1 μg per compound injected)

[a] Numerals refer to GDGT structures as reported in Schouten et al. (2003)

[b] Mass-to-charge ratio (*m*/*z*)

65–81°C at a constant medium pH of 3.0 (Fig. 1; Table 1). Crenarchaeol, a common component of GDGT lipids from hot springs in the Great Basin, Nevada (Pearson et al. 2004), was not detected in membrane extracts of YNP isolate *A. sulfurireducens* when cultivated over this temperature range. The composition of total GDGT lipids was significantly correlated with incubation temperature (Mantel $R^2 = 0.53$, $P < 0.01$). Interestingly, the abundance of individual GDGT lipids 0–7 exhibited differing degrees of correlation with incubation temperature (Table 2). With the exception of GDGT-5, the abundances of GDGTs with 0–5 cyclopentyl rings were negatively correlated with incubation temperature (Table 2); however, only GDGT-4 varied with incubation temperature in a statistically significant manner (Spearman $\rho = -0.76$, $P < 0.01$). In contrast, the abundance of GDGT-6 and GDGT-7 varied positively with incubation temperature, both of which were statistically significant (Table 2). Therefore, GDGTs with <5 cyclopentyl rings are likely to be preferred components of the cell membrane at lower cultivation temperatures, while GDGT-6 and GDGT-7 are preferred at higher incubation temperatures. Importantly, the relative abundance of GDGT-4 did not vary significantly at incubation temperatures of 65, 70, or 75°C, but did vary significantly at 81°C. In contrast, GDGTs with six and seven cyclopentyl rings decreased in abundance systematically when the incubation temperature was lowered from 81 to 65°C. Thus, GDGTs with six and seven cyclopentyl rings respond over a large range of incubation temperatures (65–81°C), whereas the response of GDGT-4 is restricted to a narrower temperature range between 75 and 81°C. Incubation temperature is thus likely to be a significant predictor of the abundance of GDGT-5′, GDGT-5, and GDGT-7 over a wide range of temperatures, whereas

the abundance of GDGT-4 is likely only to be predictable at incubation temperatures >75°C.

A plot of the average number of cyclopentyl rings per GDGT molecule (ring index) as a function of cultivation temperature yielded a positive trending relationship ($R^2 = 0.33$) (Fig. 2a). This relationship is in agreement with previous work conducted with the euryarchaeon *T. acidophilum* (Shimada et al. 2008; Uda et al. 2001) and the crenarchaeon *Sulfolobus solfataricus* (formerly *Caldariella acidophila*) (De Rosa et al. 1980), both of which exhibited similar increases in cyclization with increasing cultivation temperature. Increased cyclization is thought to increase the packing density of the lipid (Gabriel and Chong 2000; Gliozzi et al. 1983) which in turn is thought to decrease the permeability of the membrane in a process analogous to bacteria increasing the saturation of acyl lipids in response to increasing temperatures (van de Vossenberg et al. 1999).

Table 2 Spearman rho coefficient (ρ) and P values for individual and total GDGT lipids as a function of incubation temperature or medium pH

Lipid	Temperature		Medium pH	
	Spearman's ρ	P value	Spearman's ρ	P value
GDGT-0	−0.18	0.52	0.09	0.77
GDGT-1	−0.15	0.59	NA	NA
GDGT-2	−0.27	0.33	NA	NA
GDGT-3	−0.49	0.06	0.04	0.91
GDGT-4	−0.76	<0.01	0.25	0.39
GDGT-4′	−0.42	0.11	0.53	0.05
GDGT-5	0.05	0.87	0.25	0.39
GDGT-5′	−0.75	<0.01	0.49	0.07
GDGT-6	0.73	<0.01	−0.41	0.14
GDGT-7	0.76	<0.01	−0.60	0.02
Total GDGTs	0.53*	<0.01*	0.03*	0.36*

NA not applicable

* Mantel R^2 and P value

Influence of pH on GDGT composition

GDGTs 0–7 were synthesized by *A. sulfurireducens* when cultivated in media ranging in pH from 3.0 to 5.0 at a constant incubation temperature of 81°C; however, GDGT-0 and GDGT-1 were only detected in membranes of cells cultivated at pH 3.0 (Table 1). While the composition of total GDGT lipids was not significantly correlated with the pH of the growth medium (Mantel $R^2 = 0.03$, $P = 0.36$), the abundance of several individual GDGT lipids varied as a function of the pH of the growth medium. The abundance of GDGTs containing five or fewer cyclopentyl rings exhibited positive correlations with pH, suggesting that these lipids are preferred at higher pH. Importantly, only GDGT-4′ varied significantly with pH (Spearman $\rho = 0.53$, $P = 0.05$) although a strong correlation was also noted between pH and GDGT-5 (Spearman $\rho = 0.49$, $P = 0.07$). In contrast, the abundance of GDGTs containing six and seven cyclopentyl rings varied inversely with pH, with the abundance of GDGT-7 exhibiting a significant correlation (Spearman $\rho = -0.60$, $P = 0.02$) and the abundance GDGT-6 exhibiting a strong correlation (Spearman $\rho = -0.41$, $P = 0.14$) (Table 2). Thus, pH is a significant predictor of the abundance of GDGT-4′ and GDGT-7, and to a lesser extent GDGT-5′ and GDGT-6.

A plot of the average ring index as a function of increasing pH of the cultivation medium reveals a negative trending relationship ($R^2 = 17$) (Fig. 2b). An increase in the ring index with increasing acidity is in agreement with indirect evidence presented in other studies which suggested that GDGT cyclization in Crenarcheota increases with decreasing pH (Baker-Austin and Dopson 2007; Golyshina and Timmis 2005; Macalady et al. 2004; Schouten et al. 2007; van de Vossenberg et al. 1998a, b; Yamauchi et al. 1993). However, the data presented here provide controlled experimental evidence that supports such a relationship. Interestingly, GDGT cyclization in the euryarchaeon *T. acidophilum* has been reported to decrease with decreasing pH of the cultivation medium (Shimada

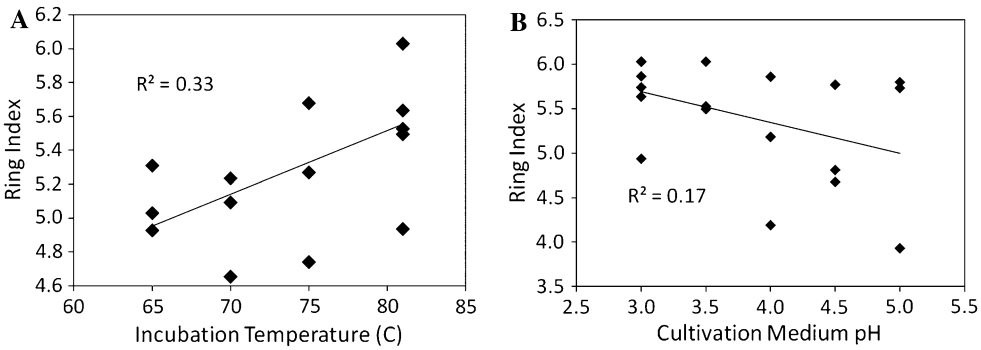

Fig. 2 GDGT ring index as a function of incubation temperature (**a**) and the pH of growth medium (**b**). Three replicates were performed for each cultivation conditions with the exception of cultures incubated at 81°C and pH 3.0 for which six replicates were performed

et al. 2008). This may be due to species- and/or phylum-specific differences in the mechanism by which archaea respond to increasing acidity. Further examination of the influence of pH on the GDGT composition of other Archaeal lineages is warranted.

Influence of IS on GDGT composition

Membranes of *A. sulfurireducens* contained GDGT lipids with masses ranging from 1,300 (1 ring) to 1,288 (7 rings) when cultivated at IS ranging from 10 to 56 mM (Table 1). However, significant correlations between individual lipids or the GDGT ring index and ionic strength were not identified (data not shown). Further examination of the influence of IS on GDGT cyclization at concentrations greater than 55 mM will require the use of organisms that tolerate a wider range of IS.

Conclusions

The present study shows that temperature and pH exert independent control on GDGT lipid composition in the crenarchaeon *A. sulfurireducens* cultivated under controlled laboratory conditions. The data help to explain why in systems such as the marine environment, where temperature, but generally not pH, varies significantly, GDGT lipid composition is a reasonable proxy for temperature (Schouten et al. 2002). Similar arguments for one-variable control might be true for other environments in which one parameter greatly dominates other physical variables. However, in many Earth surface environments, multiple parameters vary simultaneously, and many abiological factors such as temperature, pH, ionic strength, or pressure as well as biological factors such as community composition may together control GDGT lipid composition. Thus, multiple varying parameters may preclude the establishment of a strong biophysical correlation between GDGT lipid composition and a single environmental variable that influences membrane structure and function. In support of such a notion is the result in the present study indicating that GDGTs with <5 cyclopentyl rings are likely to be preferred components of the lipid membrane of *A. sulfurireducens* when cultivated at lower temperature and at higher pH, whereas GDGTs with >6 cyclopentyl rings are preferred components of the membrane when cultivated at higher temperature and more acidic pH. Thus, *A. sulfurireducens* adapts to a range of temperatures and pH, albeit to a lesser extent, by modulating the abundance of cyclopentyl rings in their membrane GDGT lipids. A further extension of this work would be to co-vary temperature and pH to determine if the combination of these environmental parameters better predicts the relative abundance of cyclopentyl rings in GDGTs than that predicted by each parameter alone.

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