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# Archaeal and Bacterial Glycerol Dialkyl Glycerol Tetraether Lipids in Hot Springs of Yellowstone National Park▽

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**Glycerol dialkyl glycerol tetraethers (GDGTs) are core membrane lipids originally thought to be produced mainly by (hyper)thermophilic archaea. Environmental screening of low-temperature environments showed, however, the abundant presence of structurally diverse GDGTs from both bacterial and archaeal sources. In this study, we examined the occurrences and distribution of GDGTs in hot spring environments in Yellowstone National Park with high temperatures (47 to 83°C) and mostly neutral to alkaline pHs. GDGTs with 0 to 4 cyclopentane moieties were dominant in all samples and are likely derived from both (hyper)thermophilic *Crenarchaeota* and *Euryarchaeota*. GDGTs with 4 to 8 cyclopentane moieties, likely derived from the crenarchaeotal order *Sulfolobales* and the euryarchaeotal order *Thermoplasmatales*, are usually present in much lower abundance, consistent with the relatively high pH values of the hot springs. The relative abundances of cyclopentane-containing GDGTs did not correlate with in situ temperature and pH, suggesting that other environmental and possibly genetic factors play a role as well. Crenarchaeol, a biomarker thought to be specific for nonthermophilic group I *Crenarchaeota*, was also found in most hot springs, though in relatively low concentrations, i.e., <5% of total GDGTs. Its abundance did not correlate with temperature, as has been reported previously. Instead, the cooccurrence of relatively abundant nonisoprenoid GDGTs thought to be derived from soil bacteria suggests a predominantly allochthonous source for crenarchaeol in these hot spring environments. Finally, the distribution of bacterial branched GDGTs suggests that they may be derived from the geothermally heated soils surrounding the hot springs.**

Hyperthermophilic archaea thrive at temperatures of >60°C and are found mostly in waters near volcanic areas. Analysis of cultivated hyperthermophilic archaea showed that their membranes are composed predominantly of isoprenoid glycerol dialkyl glycerol tetraethers (GDGTs) with acyclic or cyclic dibiphytanyl chains (e.g., structures I to V and VIII to XI in Fig. 1; Tables 1 and 2). The structural differences of diacyl membrane lipids from nonthermophilic eukaryotes and bacteria, i.e., ether bonds and the formation of a monolayer rather than a bilayer, have been suggested to contribute to the stability of membranes of hyperthermophiles at high temperatures and low pHs (for examples, see references 11, 34, and 64). Thus, it seemed likely that GDGTs were present mostly in extreme environments such as hot springs or hydrothermal vents. However, analyses of environmental samples from nonthermophilic environments such as oceans, lakes, and soils have revealed that GDGTs are abundantly present and structurally diverse (49) and that they are produced not only by archaea but also by some bacteria (72).

Besides GDGTs I to V, which were previously reported to be synthesized by (hyper)thermophilic *Crenarchaeota* and *Euryarchaeota*, compound VI is often a dominant GDGT in the oceans, in lakes, and in river waters and also occurs ubiquitously in soils and peats (16, 33, 43, 49, 51a, 72). In contrast to other GDGTs, GDGT VI contains a unique cyclohexyl moiety and is thought be derived primarily from nonthermophilic group I *Crenarchaeota* and was therefore called crenarchaeol (51a). The unusual cyclohexyl moiety is thought to be an adaptation to the relatively low-temperature environment in which these group I *Crenarchaeota* thrive, since it is not present in (hyper)thermophilic relatives (51a). In cases where the group I *Crenarchaeota* are the dominant source for these GDGTs, such as in most marine environments and certain lake environments, it was shown that the relative distribution of GDGTs I to VI is controlled predominantly by temperature, i.e., with increasing temperature, there is an increasing amount of GDGTs with cyclopentyl moieties (43, 50). A similar phenomenon has been demonstrated for hyperthermophilic archaea (10, 14, 61, 62).

In addition to these archaeal GDGTs, analysis of soils and peats revealed the abundant presence of GDGTs with nonisoprenoid carbon skeletons (GDGTs XII to XVI) (19, 49, 72, 73). Based on the stereochemistry of the glycerol group and their environmental distribution, Weijers et al. (72) showed that they are not derived from archaea but are produced by anaerobic bacteria. A detailed study of a large range of soils revealed that the relative distribution of GDGTs XII to XIV is determined mainly by temperature and pH, i.e., with higher temperature or lower pH, the relative amount of the GDGT with four methyl groups (GDGT XII) increases (74). In contrast, the relative distribution of the bacterial GDGTs containing cyclopentane rings, GDGTs XV and XVI, is determined

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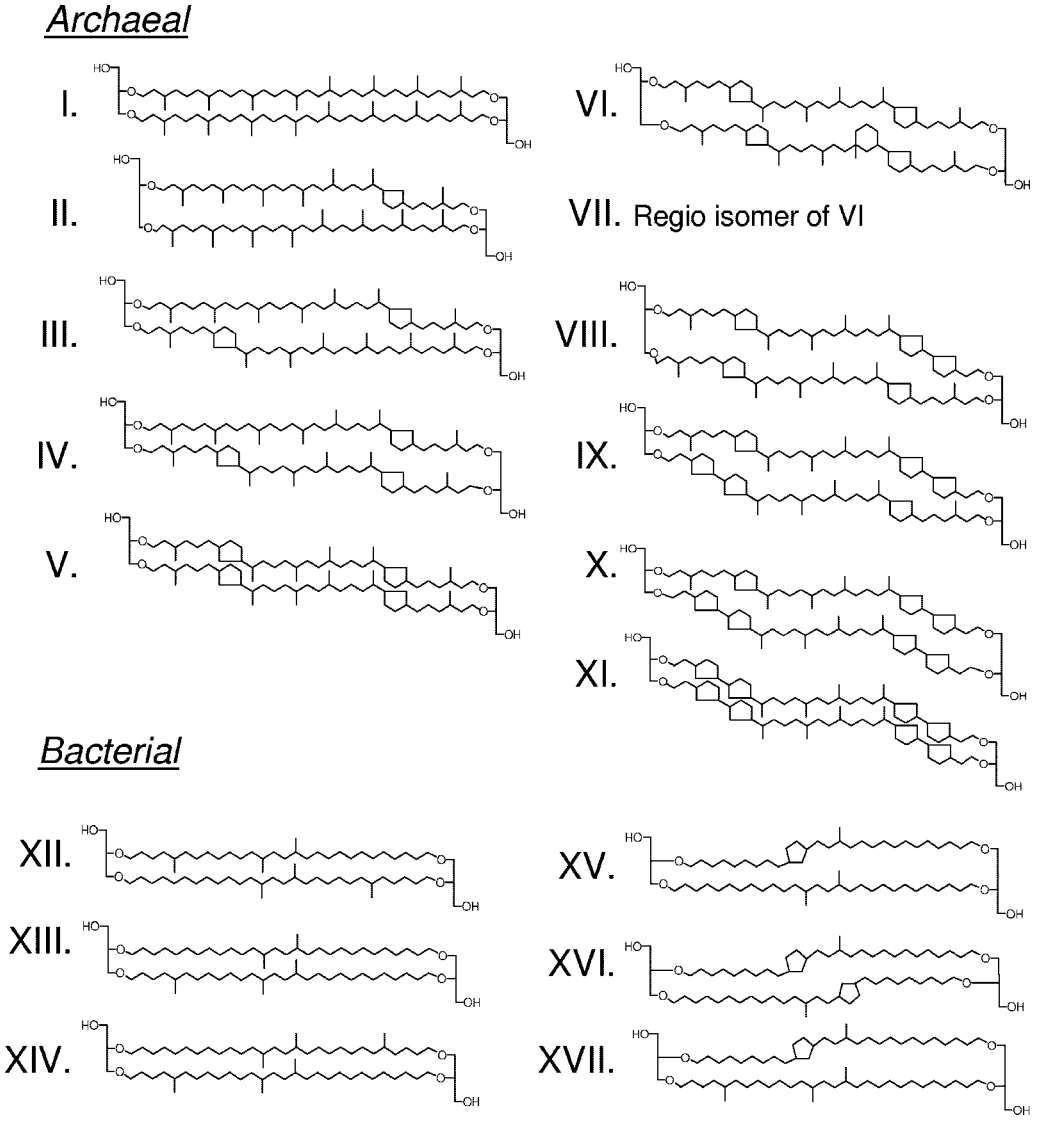


FIG. 1. Structures of GDGTs present in the hot springs of Yellowstone National Park.

mainly by pH, i.e., with higher pH, the relative amounts of GDGTs XV and XVI decrease (74).

From the above discussion, it is clear that GDGTs in low-temperature environments (<30°C) are structurally diverse and can be produced by multiple organisms. Remarkably, relatively little attention has been paid to the GDGT distribution in high-temperature environments such as hot springs, from which many of the hyperthermophilic archaea known to produce GDGTs have been isolated. Ward et al. (67) analyzed the distribution of biphytanyl moieties released by chemical degradation of GDGTs in a few hot spring microbial mats from Yellowstone National Park, found biphytanes likely derived from GDGTs I to V, and attributed them to methanogenic and thermoacidophilic archaea. Pancost et al. (41) found GDGTs I to V and VIII to XI in sinters from hot springs (75 to 82°C) in New Zealand and attributed these to hyperthermophilic archaea. Recently, Pearson et al. (42) and Zhang et al. (78) have reported the distribution of GDGTs in a number of neutral to alkaline hot spring microbial mats. They surprisingly also reported, besides GDGTs I to IX, crenarchaeol in various relative amounts. Pearson et al. (42) and Zhang et al. (78) attributed this to a more widespread occurrence of crenarchaeol, not just in the nonthermophilic group I *Crenarchaeota*. This assumption was based on the fact that no 16S rRNA gene sequences from group I *Crenarchaeota* were retrieved from these hot springs (42). Furthermore, the amount of crenarchaeol relative to that of GDGT I was at a maximum in hot springs with temperatures of ca. 40 to 50°C, leading Zhang et al. (78) to suggest that crenarchaeol-producing organisms had a thermophilic growth optimum. Therefore, Zhang et al. (78) concluded that crenarchaeol biosynthesis may have evolved within the moderate thermophiles and thus could be older than the major marine expansion of group I *Crenarchaeota*, thought to have occurred during the Cretaceous period (27, 51a). However, this latter view is at odds with a hypothesis presented earlier (51a) which argued that crenarchaeol biosynthesis was a key step in the expansion of the marine mesophilic *Crenarchaeota*.

TABLE 1. Occurrence and distribution of GDGTs in *Euryarchaeota*[a]

| Order or species | Temp (°C) | pH | GDGTs (%) | GDGT isomer(s) | Reference(s) or source |
|---|---|---|---|---|---|
| *Thermoplasmatales* | | | | | |
| *Ferroplasma "acidarmanus"* | 37 | 1.0 | 100 | I–V | 34 |
| *Ferroplasma acidophilum* | 37 | 1.7 | 100 | I–V | 34 |
| *Thermoplasma acidophilum* | 30–60 | 1–2 | 95 | I–V | 28 |
| *Thermoplasma volcanium* | 45–62 | 1.5 | 100 | I–V, VIII | 62 |
| *Picrophilus oshimae* | 60 | 0.7 | 100 | I–V, VIII | 48 |
| *Picrophilus torridus* | 45–62 | 1.3 | 100 | I–V, VIII–XI | 62 |
| DHVE2-cluster | | | | | |
| *Aciduliprofundum boonei* | 70 | 4.5 | 100 | I–V | 45 |
| *Thermococcales* | | | | | |
| *Thermoccus aggregans* | ~80 | 7 | ~50 | I–V, tr VIII | 6 |
| | | | 46 | I | 56 |
| *Thermoccus celer* | 88 | 5.8 | 46 | I | 56 |
| *Thermoccus coalescens* | 87 | 6.5 | 63 | I | 26 |
| *Thermoccus barophilus* | 85 | 7 | ND | | 35 |
| *Thermoccus hydrothermalis* | 79 | 6.8 | 63 | I | 32 |
| *Palaecoccus ferrophilus* | 83 | 6 | >80 | I | 56, 57 |
| *Pyrococcus woesei* | 102 | 6 | Minor | I | 30 |
| *Pyrococcus furiosus* | 85 | 7 | 69 | I | 56 |
| *Archaeoglobales* | | | | | |
| *Archaeoglobus fulgidus* | 60–95 | 5.5–8 | Major | I | 60 |
| *Archaeoglobus profundus* | | | Major | I | 60 |
| *Ferroglobus placidus* | 85 | 7 | Present | NR | 15 |
| *Methanopyrales* | | | | | |
| *Methanopyrus kandleri* | 97 | 6.5 | Minor | I–V | van der Meer et al., unpublished |
| *Methanosarcinales* | | | | | |
| *Methanosarcina barkeri* | 35 | 6.7 | Minor | I | 17 |
| *Methanosarcina mazei* | 35 | 6.7 | ND | | 11 |
| *Methanolobus bombayensis* | 35 | 7 | Minor | I | Schouten et al., unpublished |
| *Methanococcoides burtonii* | 20–23 | 7–7.8 | ND | | 39 |
| *Methanobacteriales* | | | | | |
| *Methanothermus fervidus* | 88 | 6.5 | 91 | I | 38 |
| *Methanobacterium ruminantium* | 37 | 6.7 | 28 | I | 29 |
| *Methanobacterium thermoautotrophicum* | 65 | 6.7 | 56 | I | 29 |
| *Methanobrevibacter smithii* | 35 | | 8 | I | 53 |
| *Methanosphaera stadtmanae* | 35 | 6.7 | 40 | I | 53 |
| *Methanococcales* | | | | | |
| *Methanococcus jannaschii* | 6 | 85 | ND | | 8 |
| *Methanococcus voltae* | 20–45 | 6.5–8 | ND | | 13 |
| *Methanococcus igneus* | | | Minor | I | 60 |
| *Methanomicrobiales* | | | | | |
| *Methanospirillum hungatei* | 30–45 | 7 | 50 | I | 52 |
| *Halobacteriales* | 0–40 | 6–7 | ND | | 25 |
| ANME-cluster | | | | | |
| ANME-1 | 5–35 | ~7 | 100 | I–V | 4 |

[a] NR, not reported; ND, not detected; tr, trace.

The relatively few studies done on the occurrence and distribution of GDGT in hot spring microbial mats and their sometimes surprising results prompted us to study in more detail the occurrence and distribution of GDGTs in a number of hot spring environments. We analyzed microbial mats and sediments from several hot springs in Yellowstone National Park, as a number of the archaea known to synthesize GDGTs have been isolated from these hot springs (e.g., 5, 20, 77) and archaeal 16S rRNA gene sequences related to them have also been found in samples from some of the springs (for examples, see references 1, 37, and 44). These hot springs had a relatively large range of temperatures (47 to 83°C) and were mostly of neutral to alkaline pH range (6.0 to 8.9), enabling us to study the impact of these environmental parameters on the relative distribution of GDGTs.

TABLE 2. Occurrence and distribution of GDGTs in *Crenarchaeota*[a]

| Order or species | Temp (°C) | pH | GDGTs (%) | GDGT isomer(s) | Reference or source |
|---|---|---|---|---|---|
| *Desulfurococcales* | | | | | |
| *Desulfurococcus mobilis* | 85 | 6 | | I | 31 |
| *Ignicoccus* sp. | 95 | | | I | 23 |
| *Pyrolobus fumarii* | 106 | 5.5 | | I | 3 |
| *Thermosphaera aggregans* | 85 | 6.5 | | I–V | 20 |
| "*Caldisphaerales*" | | | | | |
| *Caldisphara lagunensis* | 45–80 | 2–5 | 100 | I + "cyclopentane containing" | 22 |
| *Thermoproteales* | | | | | |
| *Thermoproteus tenax* | 85 | 5.5 | 95 | I–V, VIII | 59 |
| *Thermoproteus neutrophilus* | 85 | 6.5 | 63 | NR | 29 |
| *Pyrobaculum aerophilum* | 100 | 7 | 95 | I–V | 65 |
| *Pyrobaculum islandicum* | | | 99 | I–V | 60 |
| *Caldivirga maquilingensis* | 83 | 4 | 100 | "Cyclopentane containing" | 21 |
| *Vulcanisaeta distributa* | 65–89 | 3.5–5 | 100 | I + "cyclopentane containing" | 22 |
| *Sulfolobales* | | | | | |
| *Sulfolobus solfataricus* | 70–85 | 3–4 | 100 | I–V, VIII–XI | 11 |
| *Sulfolobus acidocaldarius* | 70 | 2–3 | 100 | I–V, VIII–XI | 11 |
| *Sulfolobus shibatae* | 76 | 3–4 | 100 | I–V, VIII–XI | 55 |
| *Metallosphaera hakonensis* | 70 | 3 | 100 | I + "cyclopentane containing" | 58 |
| *Metallosphaera sedula* | 75 | 2.8 | Major | I–V, VIII | 63 |
| Group I.1A | | | | | |
| *Nitrosopumilus maritimus* | 30 | 7 | 100 | I–VII | Schouten et al., unpublished |

[a] NR, not reported; ND, not detected; tr, trace.

## MATERIALS AND METHODS

**Samples.** The microbial mats and sediments sampled are listed in Table 3 together with selected environmental parameters of the hot spring waters. Typically, mat and sediment samples were taken with solvent-rinsed spatulas, kept frozen after sampling, and freeze-dried prior to lipid analysis. Hot spring water nutrients were measured spectrophotometrically using an autoanalyzer system, a Bran and Luebbe TRAACS 800+. Sulfide was trapped as zinc sulfide in the field directly after sampling by adding zinc acetate to the spring water. Sulfide concentrations were measured against a standard series in the laboratory using the colorimetric methylene blue method (7).

**Lipid extraction.** Hot spring samples were typically extracted by sonication with methanol (twice), methanol-dichloromethane (DCM) (1:1, vol/vol; twice), and finally DCM (twice). The supernatants were collected, and after evaporation of the solvents, the extracts were dried over a small column filled with sodium sulfate. An aliquot of the total lipid extract was directly fractionated into apolar and polar fractions using a small column with activated alumina and using hexane-DCM (9:1, vol/vol) and DCM-methanol (1:1, vol/vol) as eluents, respectively. For samples from Calcite Springs and Obsidian Pool, another aliquot of the total extract was hydrolyzed by refluxing the samples for 1 h in 1 M HCl-methanol. The hydrolyzed extract was neutralized using 1 M NaOH in methanol and transferred using DCM to a separatory funnel. The DCM layer was washed against water (thrice). The DCM layer was dried using a sodium sulfate column to yield the hydrolyzed extract. The hydrolyzed extract was separated into apolar and polar fractions as described above. The polar fractions were condensed by rotary evaporation, dried further under nitrogen, dissolved in hexane-isopropanol (99:1, vol/vol), and filtered using a polytetrafluoroethylene 0.4-μm filter prior to injection in the high-performance liquid chromatograph-mass spectrometer (HPLC-MS).

**Instrumental analysis.** GDGT analyses were performed as described by Hopmans et al. (18) using an Agilent (Palo Alto, CA) 1100 series HPLC-MS equipped with an autoinjector and Chemstation chromatography manager software. Separation was achieved on a Prevail Cyano column (2.1 by 150 mm, 3 μm; Alltech, Deerfield, IL), maintained at 30°C. Injection volumes ranged from 1 to 20 μl. GDGTs were eluted isocratically with 99% hexane and 1% isopropanol for

TABLE 3. Samples studied and selected environmental parameters from hot springs in Yellowstone National Park[a]

| Hot spring | Area | Sample | Date (day-mo-yr) | Temp (°C) | pH | $SO_4$ (mM) | $S^{2-}$ (mM) | $NH_4^+$ (mM) | Predominant microbe(s) | Reference(s) |
|---|---|---|---|---|---|---|---|---|---|---|
| Obsidian Pool | Mud Vulcano | Sediment | 18-9-2000 | 75 | 6 | ~0.4 | ~1 | ~15 | *Aquificales*/*Proteobacteria* | 1 |
| Calcite Springs | Tower-Roosevelt | Black filaments | 18-9-2000 | 83 | 7.8 | 6–31 | ND | ~1700 | *Aquificales* | 44 |
| Nymph Creek | Norris Geyser Basin | Algal mat | 26-8-1997 | 47 | 2.6 | ND | ND | ND | *Cyadinium* | 67, 68 |
| New Pit | Mammoth Springs | Anoxygenic phototroph mat | 26-8-1997 | 55 | 6.5 | 7.1 | 54 | 32 | *Chromatium*/*Chloroflexus* | 68 |
| Octopus Spring | Lower Geyser Basin | Cyanobacterial mat | 27-8-1997 | 60 | 8.3 | 0.21 | BDL | 0.6 | *Synechococcus*/*Roseiflexus* | 68, 70 |
| Mushroom Spring | Lower Geyser Basin | Cyanobacterial mat | 22-8-1999 | 61 | 8.1 | 0.20 | BDL | 0.6 | *Synechococcus*/*Roseiflexus* | 68, 70 |
| | Lower Geyser Basin | Cyanobacterial mat | 22-8-1999 | 50 | 8.5 | ND | BDL | ND | *Synechococcus*/*Roseiflexus* | 68, 70 |
| Bath Lake Vista | Mammoth Springs | Anoxygenic phototroph mat | 28-8-1997 | 57 | 6.2 | 5.6 | 133 | 49 | *Chloroflexus*-like bacteria | This study |
| | | Cyanobacterial mat | 28-8-1997 | 54 | ND | ND | 40 | ND | *Synechococcus*/*Chloroflexus* | This study |
| | | Cyanobacterial mat | 28-8-1997 | 53 | 6.6 | 5.7 | 43 | 48 | *Synechococcus*/*Chloroflexus* | This study |

[a] ND, not determined; BDL, below detection limit.

5 min and then a linear gradient to 1.8% isopropanol for 45 min. The flow rate was set at 0.2 ml/min. After each analysis, the column was cleaned by back-flushing hexane-propanol (90:10, vol/vol) at 0.2 ml/min for 10 min.

Detection was achieved using positive-ion atmospheric-pressure chemical ionization of the eluent. Conditions for the Agilent 1100 atmospheric-pressure chemical ionization-MS were as follows: nebulizer pressure, 60 lb/in$^2$; vaporizer temperature, 400°C; drying gas ($N_2$) flow, 6 liters/min, and temperature, 200°C; capillary voltage, −3 kV; corona, 5 μA (~3.2 kV). The conditions for GDGT analysis were optimized by injection of cholesterol or by a GDGT I standard. GDGTs were detected by mass scanning from *m/z* 950 to 1450. Relative abundances of GDGTs were quantified by integration of the peak area of the $[M+H]^+$ and $[M+H+1]^+$ ions using Excalibur software. Crenarchaeol (nominal mass of protonated molecule = 1,292 Da) coelutes with GDGT V (nominal mass of protonated molecule = 1,294 Da), and thus, its isotope peaks (the $[M+H+2]^+$ and $[M+H+3]^+$ ions) can potentially interfere with that of the $[M+H]^+$ and $[M+H+1]^+$ ions of GDGT V, leading to an overestimation of GDGT V (see reference 71). However, as crenarchaeol is usually only a minor component compared to GDGT V and its isotope peaks are <33% of its molecular $[M+H]^+$ and $[M+H+1]^+$ ions, the overestimation of the relative abundance of GDGT V is minor in this case.

The methylation index of branched tetraethers (MBT) and the cyclization ratio of branched tetraethers (CBT) quantify the relative abundance of methyl groups and cyclopentyl moieties, respectively, in branched GDGTs (74). They are calculated as follows:

$$\mathrm{MBT} = \frac{[\mathrm{XIV}] + [\mathrm{XV}] + [\mathrm{XVI}]}{[\mathrm{XII}] + [\mathrm{XIII}] + [\mathrm{XIV}] + [\mathrm{XV}] + [\mathrm{XVI}]}$$

$$\mathrm{CBT} = -\log\left(\frac{([\mathrm{XVII}] + [\mathrm{XV}])}{([\mathrm{XIII}] + [\mathrm{XIV}])}\right)$$

The Roman numerals correspond to structures in Fig. 1. The calibration equations used to calculate pH and annual mean air temperatures from the CBT and the MBT (74) are $\mathrm{CBT} = 3.33 - (0.38 \cdot \mathrm{pH})$ and $\mathrm{MBT} = 0.122 + (0.187 \cdot \mathrm{CBT}) + (0.020 \cdot \mathrm{MAT})$.

## RESULTS AND DISCUSSION

**Distribution and origin of archaeal GDGTs (I to V and VIII to XI).** HPLC-MS analysis of the hot spring samples revealed that all contained isoprenoid GDGTs, indicating that archaea are present across a range of hot spring environments. In general, GDGTs I to V were the most abundant (e.g., Fig. 2A to C; Table 4), with GDGT V usually dominating, except for the mats from Bath Lake Vista, where GDGT I dominated (Table 4). These GDGTs are likely derived from (hyper)thermophilic *Crenarchaeota* and *Euryarchaeota*, which are known to synthesize these lipids in culture (Tables 1 and 2) and for which closely related 16S rRNA gene sequences have been found in a number of these hot springs. For example, the archaeal diversity in Obsidian Pool is large and 16S rRNA gene sequences were found related to all orders of the *Crenarchaeota* (1, 37), *Korarchaeota* (1), and sequences related to the euryarchaeotal order *Archaeoglobales* (1). Indeed, the isoprenoid GDGTs in Obsidian Pool are composed of GDGTs I to V and VIII to X, which are synthesized by cultivated species from these orders (Tables 1 and 2). For Calcite Springs, 16S rRNA gene sequences have been found related to *Korarchaeota* and to the crenarchaeotal orders *Desulfurococcales* and *Thermoproteales* (44). The GDGTs synthesized by these archaea, mainly GDGTs I to V, are indeed abundant in Calcite Springs, but we also detected GDGTs VIII to X, suggesting that there may be other archaeal species present as well. Finally, one archaeal 5S rRNA gene sequence has been found in biomass that developed on glass fiber filters incubated in the source waters of Octopus Spring (54), but due to the small database of 5S rRNA gene sequences at that time, it was unclear to which order or even kingdom these sequences belonged. Blank et al. (2) failed to obtain archaeal 16S rRNA gene sequences from sinters at the edge of the Octopus Spring source pool despite using archaebacterium-specific primers for PCR. A number of studies have shown that methanogenesis occurs in cyanobacterial mats in Octopus Spring, suggesting that methanogenic archaea must be present (46, 47, 66). However, these archaea produce predominantly GDGT I (Table 1). Thus, the presence of GDGTs II to V suggests that other archaea besides methanogenic archaea are present as well, and they likely have escaped detection by molecular biological techniques up to now possibly because 16S rRNA gene studies have focused on sequences of predominant phototrophs (70) and because some archaea (e.g., methanogens) are at the top of the mat food chain and thus are much less abundant (68).

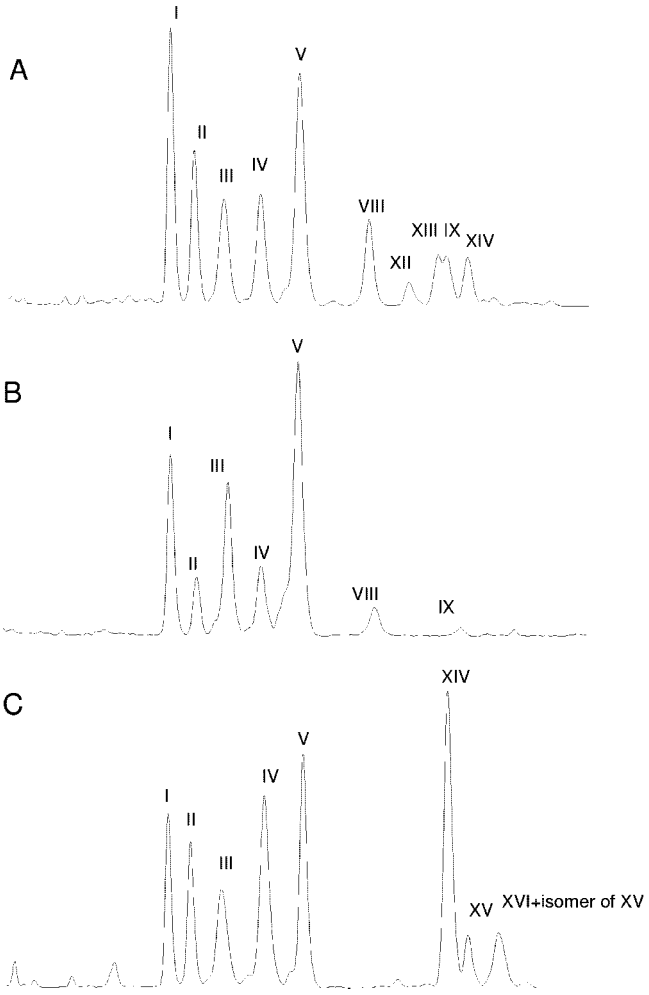


FIG. 2. Base peak chromatograms of HPLC-MS analysis of hot spring samples showing the distributions of GDGTs in Obsidian Pool (temperature, 75°C; pH 6) (A), Calcite Springs (temperature, 83°C; pH 7.8) (B), and Mushroom Spring (temperature, 50°C; pH 8.5) (C).

Surprisingly, GDGTs IX to XI, which contain five to seven cyclopentane rings, are usually in low abundance or sometimes even absent (for examples, see Fig. 2; Table 4), despite the fact that they have been found to be synthesized by a number of hyperthermophilic *Crenarchaeota* (Table 2). This suggests that

TABLE 4. GDGT composition in total lipid extracts of microbial mats and sediments from hot springs in Yellowstone National Park

| Hot spring or extract | Temp (°C) | pH | % GDGTs[a] | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Isoprenoid | | | | | | | | | | | Nonisoprenoid | | | | |
| | | | I | II | III | IV | V | VI | VII | VIII | IX | X | XI | XII | XIII | XIV | XV | XVI |
| Obsidian Pool | 75 | 6 | 18 | 10 | 11 | 11 | 23 | 0.4 | ND | 8 | 5 | ND | ND | 2 | 5 | 4 | 0.8 | 0.1 |
| Obsidian Pool extract[b] | | | 23 | 13 | 13 | 11 | 20 | 0.5 | ND | 4 | 2.4 | ND | ND | 3 | 6 | 4 | ND | ND |
| Calcite Springs | 83 | 7.8 | 17 | 9 | 12 | 13 | 31 | 0.7 | ND | 7 | 3 | ND | ND | 3 | 3 | 2 | ND | ND |
| Calcite Springs extract[b] | | | 17 | 6 | 19 | 10 | 39 | 0.4 | ND | 5 | 2 | ND | ND | 0.3 | 0.5 | 0.5 | ND | ND |
| Nymph Creek | 47 | 2.6 | 10 | 7 | 17 | 19 | 33 | 0.3 | ND | 1 | 2 | ND | ND | ND | 12 | 1 | ND | ND |
| New Pit | 55 | 6.5 | 8 | 1 | 3 | 3 | 20 | 5 | ND | 5 | 1 | 0.4 | ND | 9 | 10 | 29 | 4 | 1 |
| Octopus Spring | 60 | 8.3 | 13 | 12 | 13 | 18 | 13 | 0.7 | ND | 0.2 | ND | ND | ND | 1 | 0.8 | 23 | 5 | 2 |
| Mushroom Spring | 61 | 8.1 | 2 | 2 | 4 | 10 | 18 | 0.5 | ND | ND | ND | ND | ND | 9 | 5 | 47 | 3 | ND |
| | 50 | 8.5 | 10 | 9 | 11 | 19 | 17 | 0.7 | ND | 0.2 | ND | ND | ND | 0.7 | 0.6 | 21 | 9 | 1 |
| "Bath Lake Vista" | 57 | 6.2 | 52 | 1 | 5 | 2 | 9 | ND | ND | 2 | ND | ND | ND | 1 | 1 | 27 | ND | ND |
| | 54 | ND | 56 | 1 | 1 | 0.9 | 5 | ND | ND | 3 | ND | ND | ND | 6 | ND | 28 | ND | ND |
| | 53 | 6.6 | 46 | 3 | 2 | 1 | 5 | 1 | ND | 3 | ND | ND | ND | ND | 13 | 26 | ND | ND |

[a] Roman numerals refer to the structures in Fig. 1. ND, not detected.
[b] Extract released after acid hydrolysis of residual biomass left after lipid extraction.

either archaea capable of synthesizing GDGTs VIII to XI are in low abundance or that environmental conditions were such that GDGTs I to V were preferentially synthesized compared to GDGTs VIII to XI (see reference 67). Important environmental controls on the distribution of these GDGTs are likely temperature and pH (see references 13, 33, 62). Culture studies, for example, have shown that with increasing growth temperature, (hyper)thermophilic *Crenarchaeota* and *Euryarchaeota* synthesize increasing amounts of GDGTs containing more cyclopentane moieties (61, 62). To investigate this for our hot spring samples, we calculated the relative degree of cyclization (see reference 62) for the archaeal GDGTs:

$$\text{Degree cyclization} = \frac{[\text{II}] + 2[\text{III}] + 3[\text{IV}] + 4[\text{V}] + 5[\text{VIII}] + 6[\text{IX}] + 7[\text{X}] + 8[\text{XI}]}{[\text{I}] + [\text{II}] + [\text{III}] + [\text{IV}] + [\text{V}] + [\text{VIII}] + [\text{IX}] + [\text{X}] + [\text{XI}]}$$

Cross-plotting this cyclization ratio in hot spring samples against temperature and pH results in a scatter plot and no significant correlation (Fig. 3A and B, respectively). This suggests that there is no dominant control of temperature or pH on the archaeal GDGT distribution. Thus, it is more likely that other environmental factors have an impact as well and that there may also be genetic (i.e., species-based) control of the distribution of the common archaeal GDGTs. Uda et al. (62) analyzed biphytane carbon skeletons for a number of species in the order *Thermoplasmatales* and found that all individual species linearly increased their relative numbers of cyclopentane rings with temperature (Fig. 3A). However, the degree of cyclization of the released biphytane carbon skeletons varied from species to species (Fig. 3A). Thus, it is not surprising that our hot spring data show no apparent correlation with temperature, as the archaeal communities in the chemically different hot springs (Table 3) are likely to be dissimilar. Interestingly, in the case where we sampled a chemically similar environment, i.e., Octopus Spring (Table 3), there is an increase in the degree of cyclization from 2.4 at 50°C to 3.1 at 60°C, consistent with the results of Ward et al. (67).

Another complicating factor in determining the environmental impact on GDGT distribution is that GDGTs VIII to XI seem to have a more-restricted occurrence in the *Archaea* and are present predominantly in the orders *Sulfolobales* and *Thermoplasmatales* (Tables 1 and 2). The members of these orders also happen to be archaea which optimally grow at low pH (i.e., <4). In contrast, most other archaea which live at much higher pH synthesize predominantly GDGT I (Table 1 and 2). Since nearly all of the hot springs we investigated have a pH of >6, it is not surprising that GDGTs VIII to XI are in relatively low abundance. Indeed, the relative degree of cyclization on Nymph Creek, an acidic hot spring, is higher (3.1) than that of the neutral Octopus Spring hot spring (2.4), while temperatures are relatively similar, i.e., 47 versus 50°C (Table 3). Ward et al. (67) also found a substantially higher degree of cyclization in biphytane carbon skeletons from Nymph Creek than in other neutral hot spring environments. It has recently been suggested that membranes composed of monolayer-forming GDGTs are essential to survival in strongly acidic conditions (see reference 33). Our results and a review of the literature on lipids of cultivated archaea (Tables 1 and 2) also suggest that the biosynthesis of a large number of cyclopentane rings within these GDGTs is essential for living in hot acidic environments. Indeed, it has been shown that liposomes composed of GDGTs I to V and VIII to XI have a low proton permeability even at temperatures up to 80°C, in contrast to liposomes of diethers and GDGT I (12, 64).

Finally, GDGT I may also be predominantly sourced from (hyper)thermophilic *Crenarchaeota* of the order *Thermococcales*, *Euryarchaeota* of the order *Archaeoglobales*, and methanogenic archaea. These archaea synthesize predominantly GDGT I (Table 1) and do not seem to produce GDGTs containing cyclopentane moieties, with the exception of *Methanopyrus kandleri*, which synthesizes GDGTs II to V in low abundance (M. T. J. van der Meer, S. Schouten, and J. S. Sinninghe Damsté, unpublished results) and uncultivated methanotrophic *Euryarchaeota* from the ANME-1 cluster, which synthesize GDGTs II to V (4, 40, 51). If there is a substantial contribution of GDGT I from these *Crenarchaeota* and *Euryarchaeota*, then this will also obscure any relation between the relative number of cyclopentane moieties and

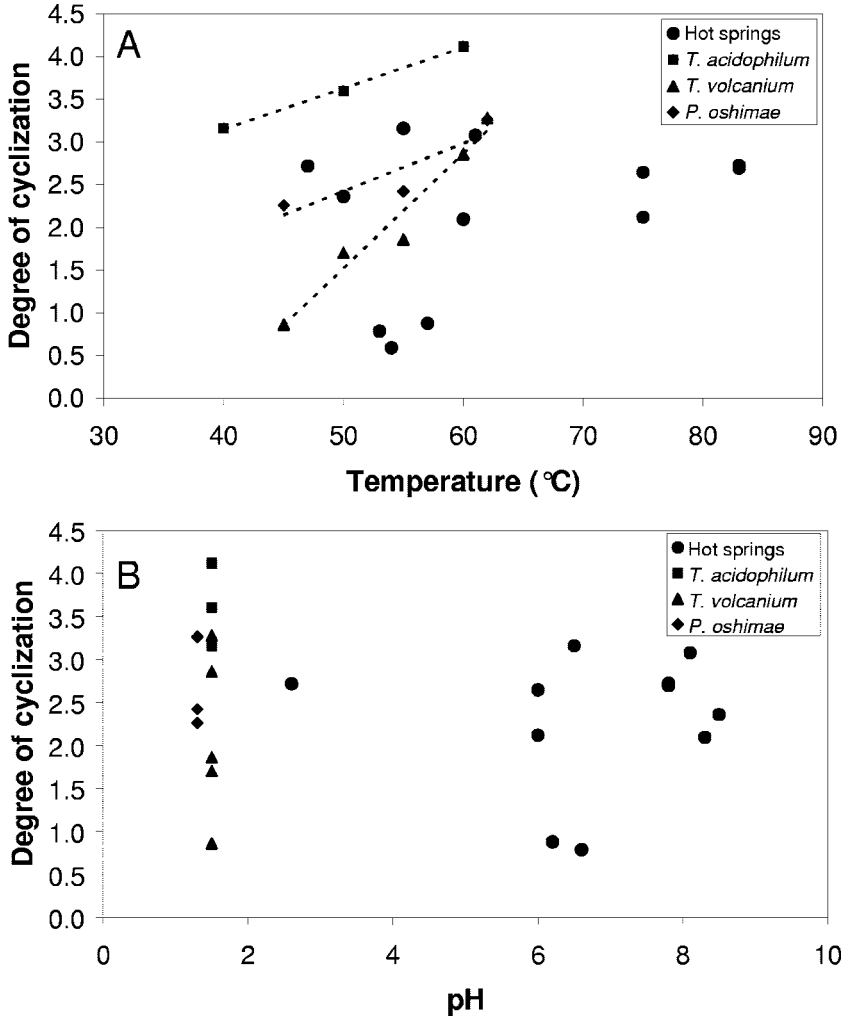


FIG. 3. Cross plot of the relative number of cyclopentane moieties in GDGTs I to V and VIII to XI in the hot springs (this study) and cultivated archaea (*Thermoplasma acidophilum*, *Thermoplasma volcanium*, and *Picrophilus oshimae*) (from reference 62) against the temperature (A) and pH (B) of the hot springs. Broken lines, increase in degree of cyclization with temperature for cultivated archaea.

environmental parameters. Thus, taken together, our data suggest that the different GDGTs are sourced by different populations of archaea in hot springs of different chemistries and that this will obscure any impact of environmental parameters on their distribution. This readily explains the lack of correlation between GDGT distribution and temperature in hot springs (42; this study). Temperature has a greater influence on the distributions of GDGTs in marine and some lake environments because archaeal diversity is lower there, i.e., predominantly group I *Crenarchaeota* (50, 75, 76). Nevertheless, even there, care has to be taken, as this temperature signature can be obscured by the input of other populations of archaea with different GDGT distributions, such as methanotrophic archaea (Table 2; 40) and terrestrial archaea (reference 73).

**Occurrence and distribution of nonisoprenoid GDGTs (XII to XVI).** All the hot spring samples we studied contained substantial amounts of GDGTs XII to XVI, ranging from 8% to 64% of all GDGTs (Table 4). These GDGTS are ubiquitously found in low-temperature environments such as soils (73) and peats (72) and are thought to be synthesized by mesophilic anaerobic bacteria (72). In soils, they are the dominant GDGTs (73), while in marine, river, and lake environments, they are generally in much lower abundance than are isoprenoid GDGTs (16, 19). Their relative abundance in the latter environments correlates well with the relative input of soil-derived organic matter (16, 24, 36), and they are thus likely of allochthonous origin. Similarly, the nonisoprenoid GDGTs in our hot springs may derive largely from the surrounding soils via runoff through precipitation. Indeed, Ward et al. (69) found $C_{29}$ sterols of allochthonous origin in anoxic microbial mats of Octopus Spring and New Pit. The most-dominant nonisoprenoid GDGT is, in general, GDGT XIV, although in a few hot springs GDGT XIII is somewhat more abundant than this GDGT (Table 4; see also below).

Weijers et al. (74) have shown that in soils the distribution of bacterial GDGTs is correlated with mean annual air temperatures and pH. For selected hot springs, i.e., where all the bacterial GDGT isomers could be quantified, we calculated the MBT and CBT of nonisoprenoid tetraethers (see reference 74). Using these indices and the empirical correlation equa-

TABLE 5. MBTs, CBTs, and pH and temperature estimates derived from selected mats

| Hot spring | In situ temp (°C) | Air temp (°C)[a] | In situ pH | MBT | CBT | pH calculated | Temp calculated (°C) |
|---|---|---|---|---|---|---|---|
| Obsidian Pool | 75 | ~1 | 6 | 0.40 | 0.96 | 6.2 | 5 |
| Octopus Spring | 60 | ~2 | 8.3 | 0.94 | 0.65 | 7.1 | 35 |
| New Pit | 55 | ~2 | 6.5 | 0.64 | 1.0 | 6.1 | 17 |
| Mushroom Spring | 61 | ~2 | 8.1 | 0.79 | 1.2 | 5.7 | 22 |
| | 50 | | 8.5 | 0.96 | 0.36 | 7.8 | 38 |

[a] Mean annual air temperature. Data are from the Western Regional Climate Center (www.wrcc.dri.edu).

tions with air temperature and pH as described by Weijers et al. (74) (see Materials and Methods), we estimated the pH and temperature at which the GDGT-lipid-producing bacteria were living (Table 5). The results show that for Obsidian Pool, the CBT and MBT suggest that the bacterial GDGTs XII to XVI were biosynthesized at relatively cold temperatures, close to the mean annual air temperature of the surrounding area. This is consistent with the hypothesis of a predominantly allochthonous source for these GDGTs. In contrast, temperatures reconstructed from bacterial GDGT distributions in New Pit, Mushroom, and Octopus Springs suggest that the temperatures were much higher than the mean annual air temperatures by up to 35°C (Table 5). Possibly, this indicates that a minor part of the nonisoprenoid GDGTs is synthesized in situ in these hot springs. Alternatively, and perhaps more likely, these GDGTs may be derived mainly from bacteria living in soils located close to the hot springs, which are relatively warmer than soils further removed from the geothermal springs. Future analysis of the soils surrounding these hot springs should reveal whether the anaerobic bacteria producing GDGTs XII to XVI are present at these elevated temperatures.

**Distribution and origin of crenarchaeol (GDGT VI).** For most sediments, we found a relatively low abundance (<1% of all GDGTs) of GDGT VI, crenarchaeol, with the exception of the New Pit hot spring, where crenarchaeol represented ca. 5% of all GDGTs (Table 4). The presence of crenarchaeol in neutral to alkaline hot springs is consistent with previous reports (42, 78). To compare our GDGT distributions with those of Zhang et al. (78), we calculated the ratios of crenarchaeol to the sum of crenarchaeol and GDGT I. The ratios were mostly below 0.1, with the exception of that for the New Pit hot spring, where it was 0.39. These ratios are substantially lower than those of most of the hot spring samples examined by Zhang et al. (78), who reported ratios ranging from 0.1 up to 1.0. When we plotted the crenarchaeol/GDGT I ratio obtained from our hot spring samples (Fig. 4) and from marine surface sediments (49, 50; S. Schouten et al., unpublished results) against temperature, we obtained a different scatter plot than that of Zhang et al. (78) (Fig. 4A). For marine sediments, the ratio of crenarchaeol to crenarchaeol and GDGT I linearly increases between 5 and 25°C, as reported previously (50). However, this ratio does not show an apparent correlation with temperature for our hot springs, and there is seemingly no "thermophilic temperature optimum" present (see reference 78). Similarly, there is no strong correlation of this ratio with the pH of the hot springs (Fig. 4B). The reason for the discrepancies between our results and those of Pearson et al. (42) and Zhang et al. (78) is at present not clear. The distribution of GDGTs in their hot spring samples is quite different than that in ours; e.g., in our case GDGT V is often dominating. Crenarchaeol and GDGT V coelute but are separated by 2 daltons in their protonated molecule mass, 1,292 versus 1,294 Da, respectively, and this will lead only to an overestimation of GDGT V in the case of high crenarchaeol abundance and not vice versa (see Materials and Methods and reference 71). Another difference between our study and those of Pearson et al. (42) and Zhang et al. (78) is that those authors specifically examined the acid-hydrolyzed glycolipid fraction of their Bligh-Dyer-method-extracted mats, while in our case we directly analyzed the GDGT fraction of the whole extract of the hot spring mats and sediments obtained by DCM-methanol extraction. Possibly, GDGTs may have escaped our detection, as they were still bound as glycolipids. However, when we performed acid hydrolysis on sample residues from Calcite Springs and Obsidian Pool left after extraction, we did not obtain substantially higher amounts of crenarchaeol (Table 4). Thus, it seems that we did not miss a large pool of sequestered crenarchaeol, at least in these samples.

Apparently then, in the hot springs we studied, there must be controls on the relative abundance of crenarchaeol other than those suggested by Zhang et al. (78). It is known that crenarchaeol is also synthesized by mesophilic soil *Crenarchaeota* (33, 71, 73), and since the terrestrial hot springs are evidently in close contact with surrounding soils, crenarchaeol may potentially be of allochthonous origin. We therefore compared its abundance with that of the bacterial GDGTs XII to XIV, which are hypothesized to be predominantly of allochthonous origin (see above). When the relative abundance of crenarchaeol against GDGT I is plotted against the relative abundance of GDGTs XII to XIV against GDGT I, then it is obvious that the highest relative abundance of crenarchaeol is associated with the highest relative abundance of GDGTs XII to XIV (Fig. 4C). This suggests that in our hot spring samples, crenarchaeol is likely of allochthonous origin. Consistent with this suggestion is the lack of substantial amounts of the crenarchaeol regioisomer. This isomer strongly increases in abundance with temperature, and in marine environments with temperatures of >25°C, it comprises ca. 10% of crenarchaeol abundance (50, 75). The lack of crenarchaeol isomer thus suggests that crenarchaeol was synthesized at mesophilic temperatures, i.e., the surrounding soils of the hot springs, and that it was carried through, e.g., rain runoff, into the hot spring.

**Conclusions.** Analyses of hot spring environments of Yellowstone National Park revealed that bacterial and archaeal GDGTs occur ubiquitously. Most of the isoprenoid GDGTs are sourced in situ by diverse consortia of (hyper)thermophilic *Crenarchaeota* and *Euryarchaeota*, suggesting that they are widespread in hot spring systems. Crenarchaeol, derived from

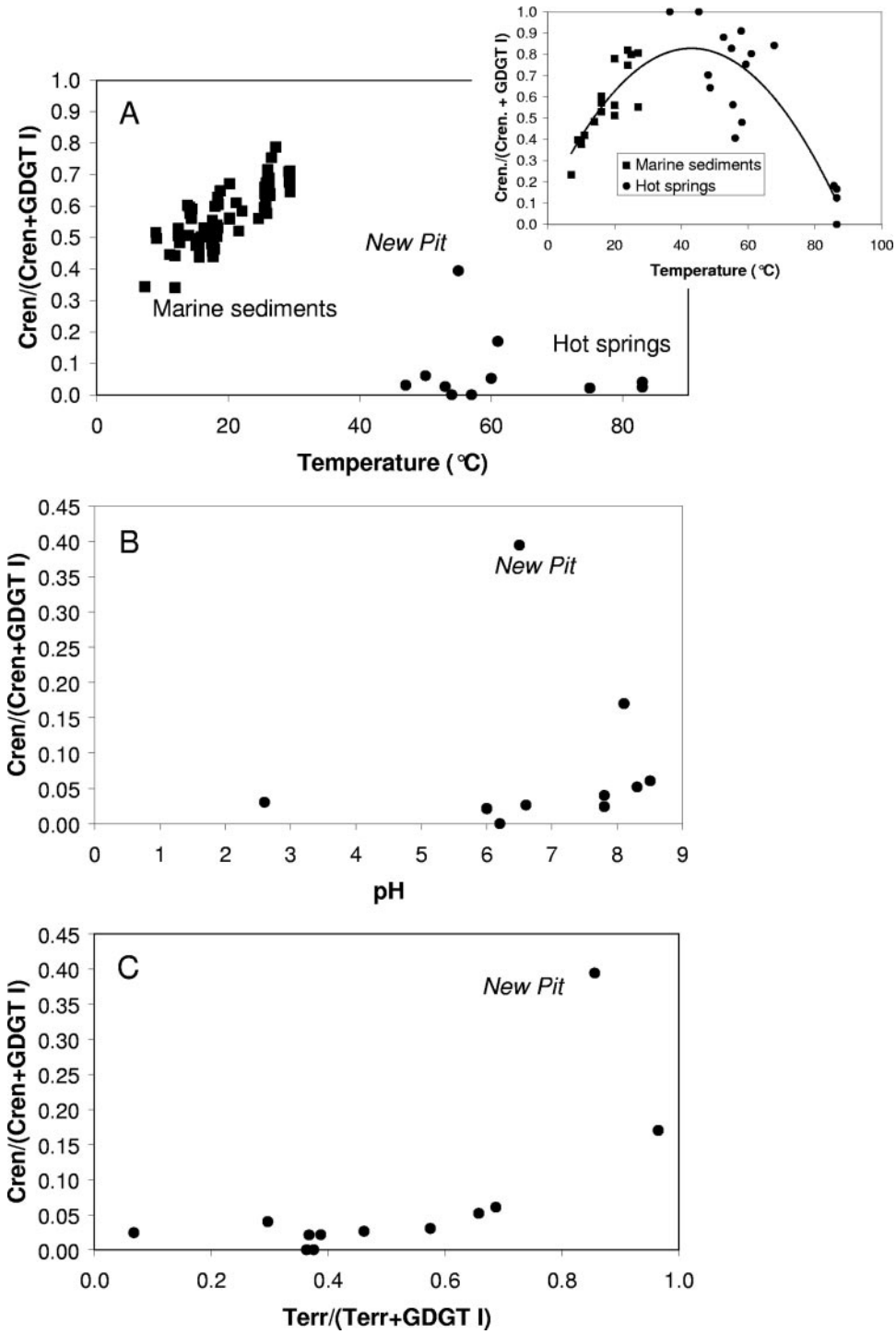


FIG. 4. (A and B) Cross plots of the relative ratio of crenarchaeol to GDGT I against temperature (A) and pH (B); (C) relative ratio of the major terrestrial GDGTs (XII to XIV) to GDGT I. The inset in panel A shows a plot of the ratio of crenarchaeol to GDGT I against the temperature of the hot springs studied by Zhang et al. (78) and the marine sediments of Zhang et al. (78) and Schouten et al. (49).

group I C*renarchaeota*, is present in relatively low abundance and, we hypothesize, is of allochthonous origin from the surrounding soils, similar to the bacterial GDGTs. Similarly, in some of the hot springs, the bacterial GDGTs are likely derived from closely surrounding soils which have been geothermally heated.

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