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Supplementary Information for

Calditol-linked membrane lipids are required for acid tolerance in *Sulfolobus acidocaldarius*

Zhirui Zeng, Xiao-Lei Liu, Jeremy H. Wei, Roger E. Summons, and Paula V. Welander

Paula V. Welander
Email: welander@stanford.edu

This PDF file includes:

Supplemental Methods

Molecular Biology Techniques. Oligonucleotides were purchased from Integrated DNA Technologies. Genomic DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen). PCR was performed according to the manufacturer's protocol using Taq DNA polymerase or Phusion high-fidelity DNA Polymerase (New England Biolabs). The GeneJET Plasmid Miniprep Kit (Thermo Scientific) was used for isolation of plasmid DNA from *E. coli*. The GeneJET gel extraction and PCR purification kits were used for purification of DNA fragments during cloning procedures. DNA sequences were confirmed by sequencing at ELIM Biopharm (Hayward, CA).

Plasmids were constructed by sequence and ligation independent cloning (SLIC) (1). For the *saci_1489* deletion construct, 729 bp of the *saci_1489* upstream and 714 bp of the downstream regions were PCR amplified with primers Z001F/R and Z002F/R, respectively, and inserted into the *NcoI* and *BamHI* restriction sites of pSVA407 (2) to yield plasmid pSVA407-*saci_1489*UD. For the *saci_0343* deletion construct, 700 bp of the *saci_0343* upstream and 700 bp of downstream regions were PCR amplified with primers Z004F/R and Z005F/R, respectively, and inserted into the *NcoI* and *BamHI* restriction sites of pSVA407 to yield plasmid pSVA407-saci_0343UD. For the complementation construct, *saci_1489* was PCR amplified from genomic DNA with primers Z003F/R and inserted into the *NcoI* and *XhoI* sites of the self-replicating plasmid pSVA1561 (3) to yield pSVA1561-saci_1489. This plasmid contains a maltose inducible promoter for inducible expression of the cloned gene.

Electrocompetent *E. coli* and *S. acidocaldarius* cells were transformed by electroporation using a Micro-Pulser Electroporator (BioRad) with the program Ec1 (1.8 kv, 1 pulse for a 0.1 cm cuvette). *S. acidocaldarius* competent cells were prepared as described in Wagner et al. (2). Before transformation of *S. acidocaldarius*, all plasmids were methylated through *E. coli* ER1821 bearing the plasmid pM.EsaBC4I, and 1.5 µg of methylated plasmid DNA was used to transform 50 µL of competent cells. Electroporated *S. acidocaldarius* cells were recovered in 1

mL Brock medium supplemented with 0.1% NZ-Amine and 0.2% sucrose at 75°C for 1.5 hour. Cells were plated on the proper selective medium, plates were sealed in plastic bags, and incubated at 75°C for 5-7 days.

Construction of *S. acidocaldarius* mutants. The deletion construct (pSVA407-*saci_1489*UD or pSVA407-*saci_0343*UD) was transformed into the uracil auxotroph *S. acidocaldarius* MW001 and plated on solid medium lacking uracil to select for cells that had integrated the entire plasmid at the locus of interest. Integrants were visualized by spraying the resulting colonies with 5 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). Positive colonies were single colony purified, inoculated into liquid medium, and grown to mid-exponential phase at 75°C. To induce loss of the integrated plasmid and potentially delete the gene of interest, 50 µL of culture were spread on plates containing 10 µg/mL uracil and 100 µg/mL 5-fluoroorotic acid (5-FOA) and incubated at 75°C for 5-7 days. 5-FOA is a fluorinated derivative of a pyrimidine precursor which can be converted to a toxic metabolite, 5-fluorouracil, by the orotidine 5'-phosphate decarboxylase encoded on the selection plasmid (*pyrEF*). Selection on 5-FOA forces the loss of the plasmid from the chromosome and this will produce either the wild type locus or result in the deletion of the gene of interest. Potential *Δsaci_1489* or *Δsaci_0343* mutant colonies were screened by PCR with primers Z006F/R or Z007F/R, respectively, and positive clones were verified by sequencing.

Lipid extractions. Frozen cell pellets were freeze-dried and 30 mg of this lyophilized cell mass was homogenized with a laboratory spatula. An internal quantification standard, C_{46} GDGT (500ng), was added to the ground pellets (4) prior to extracting in a 50 mL Teflon tube by sonicating first in 5 ml of methanol (MeOH) and then in 5 mL of 1:1 (vol/vol) MeOH and dichloromethane (DCM). Each sonication was performed in an ultrasonic bath for 15 min at room temperature, and samples were centrifuged at 4500×g for 10 min after sonication. The two supernatants were combined and filtered through 0.45-µm PTFE filter and dried under a stream

of nitrogen gas (N_2) to yield the “non-hydrolyzed fraction” containing intact polar GDGTs. The remaining cell pellet was resuspended in 10 ml of DCM and transferred to a 40 mL glass vial and dried under N_2. The dried cells were then acid hydrolyzed with 5 mL of 10% hydrochloric acid (HCl) in MeOH (vol/vol) at 70°C for 8 hours. Subsequently, 10 mL of DCM and 10 mL of Nanopure water were added and the mixture was transferred to a 50 mL Teflon tube. The sample was centrifuged for 10 min at 2800×g to separate the organic and inorganic phases. The top layer was removed and the bottom organic layer was dried under N_2 to yield the “acid hydrolyzed fraction” containing the GDCTs and the core GDGTs. For acetylation one aliquot of lipid extract was transferred into a 2 ml vial and dried with a stream of N_2, and then added 100 µL pyridine and 100 µL acetic anhydride to react at 60 °C for 2 hours. The reaction solution was then dried with a stream of N_2 and dissolved again in MeOH for injection.

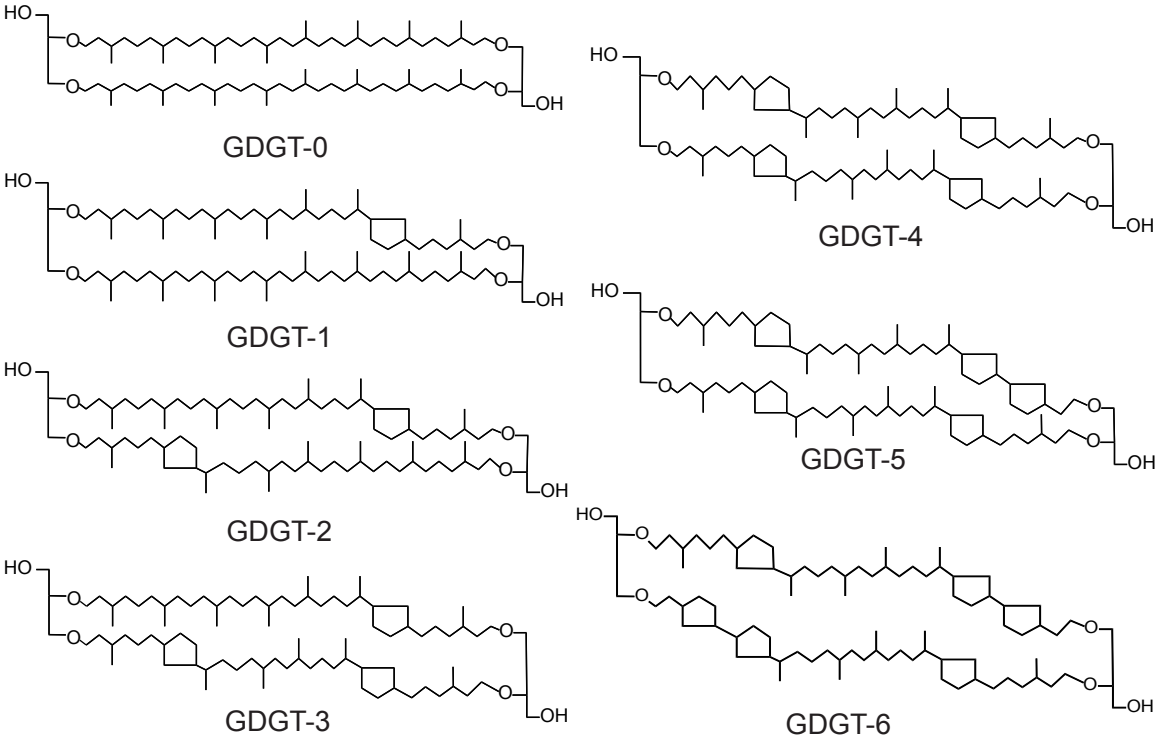

Figure S1. Core GDGT lipid structures with 0 to 6 pentacyclic rings produced by *S. acidocaldarius* and observed in this study.

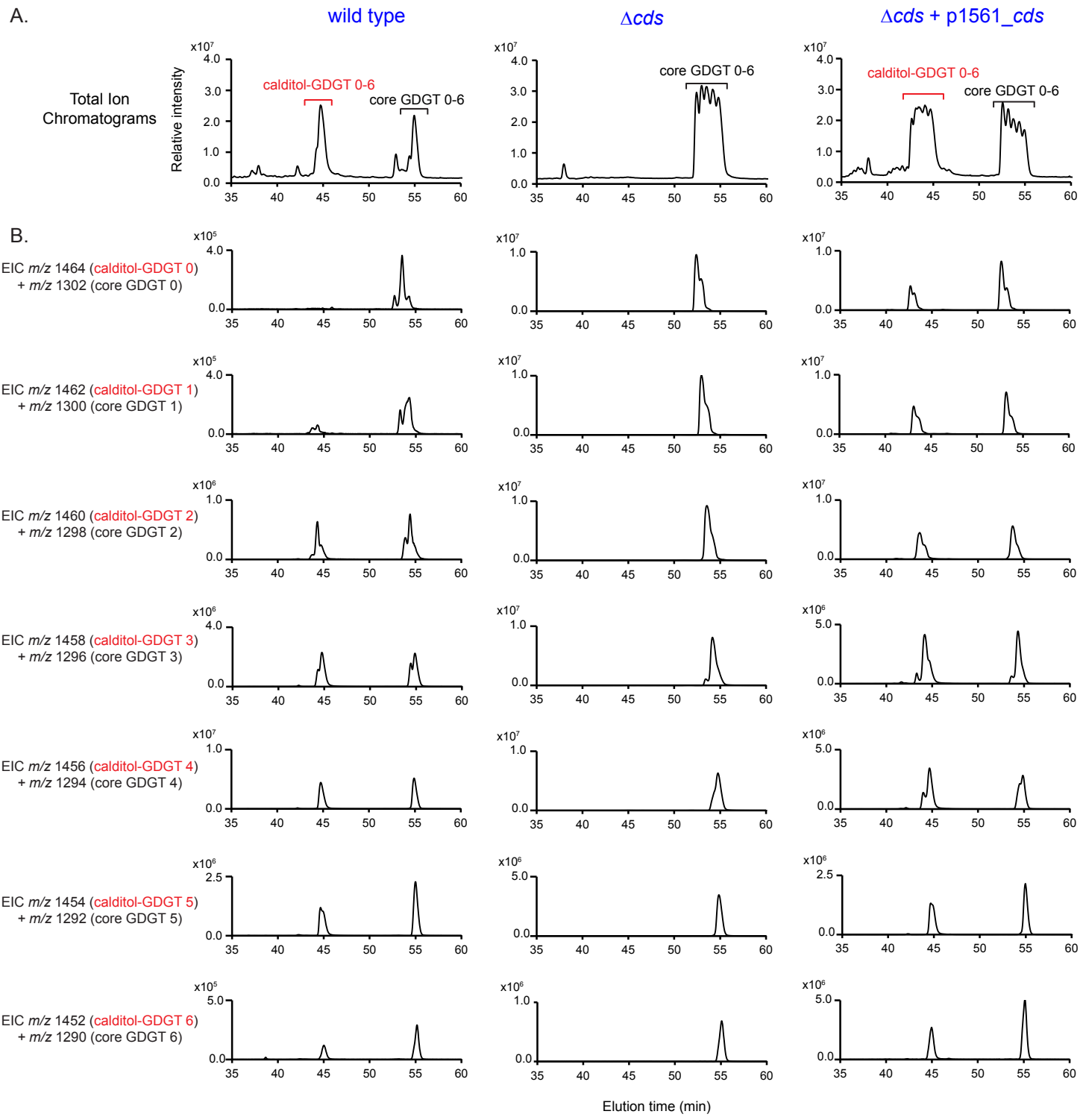

Figure S2. LC-MS analyses of *Sulfolobus acidocaldarius* lipid extracts. (A)Total ion chromatograms and (B) extracted ion chromatograms (EIC) of the acid hydrolyzed fractions from wild type, *Δcds*, and complemented *Δcds* cells. Note that the distribution of cyclized calditol-GDGT and core GDGT in wild type and mutant cells differ significantly. The exact nature of these differences and the reasons for this are currently under investigation.

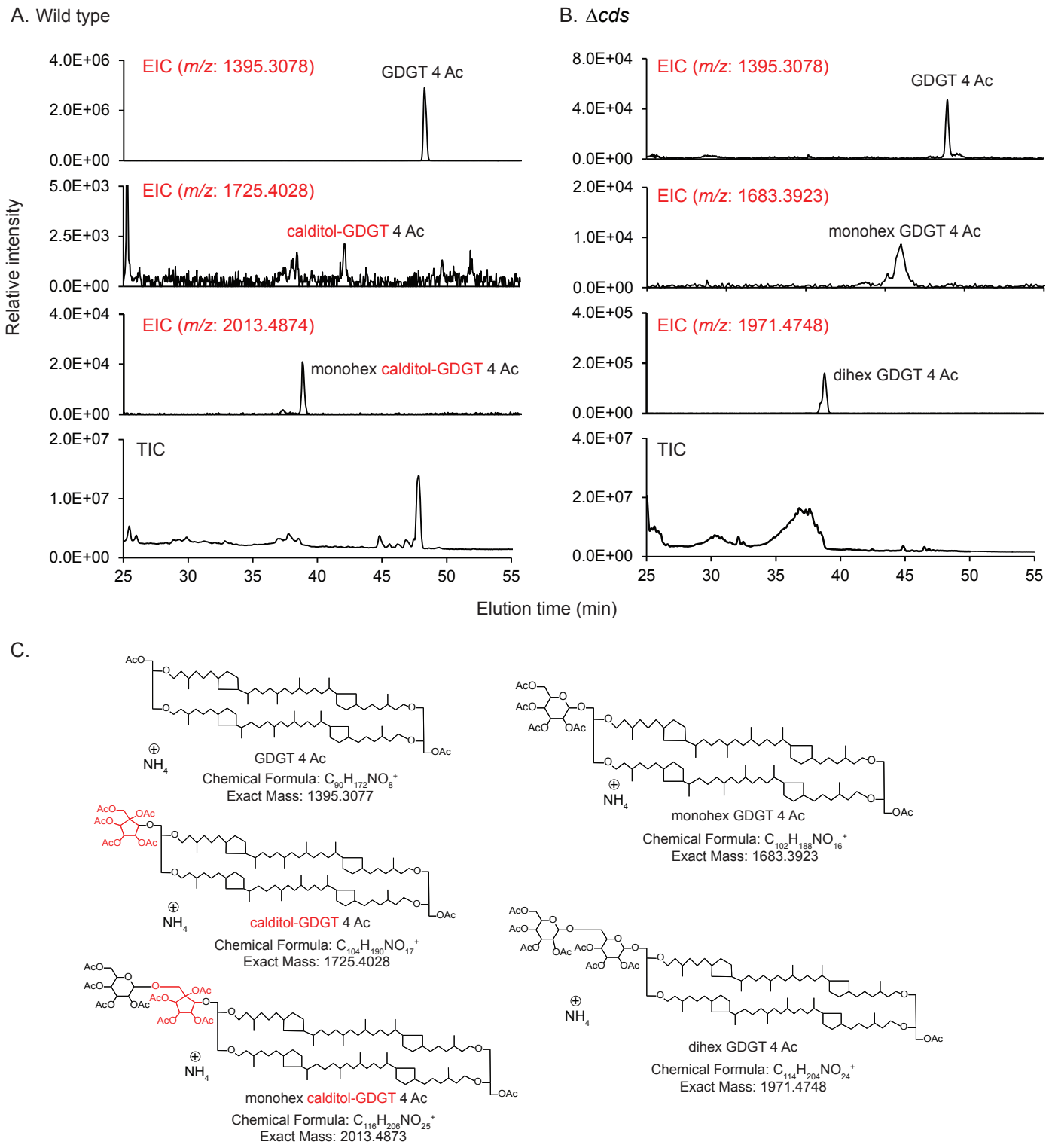

Figure S3. The *S. acidocaldarius cds* mutant produces glycosylated GDGTs. Total ion chromatograms (TIC) and extracted ion chromatograms (EIC) of acetylated non-hydrolyzed fractions from*S. acidocaldarius* (A) wild type and (B) *Δcds* cells showing that both strains glycosylate their core lipids but only the wild type contains calditol. Calditol head groups are distinguished from hexose head groups through acetylation as calditol contains an extra hydroxyl group which increases its mass by 42 Da. (C) Tetraether structures shown in EIC in panels A and B.

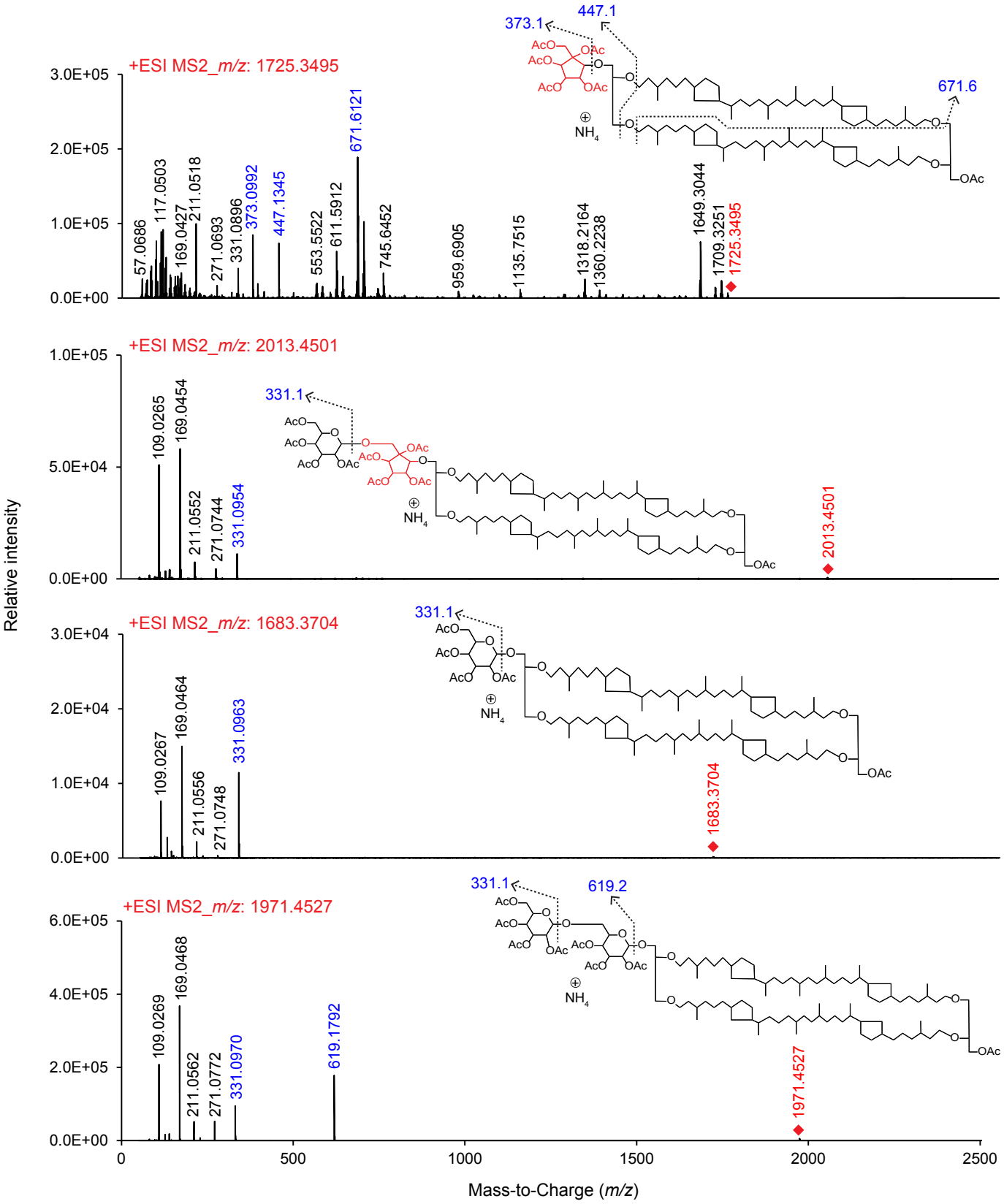

Figure S4. Mass spectra of acetylated calditol-GDGTs and hexose-GDGTs. MS/MS spectra of acetylated glycosidic GDGT-4 and calditol-GDGT-4 showing the characteristic fragments for their identification.

Table S1. Proteins identified in *S. acidocaldarius* genome with a radical SAM motif (Pfam04055)

IMG Gene ID	IMG Locus Tag	IMG Annotation
638197012	Saci_0071	conserved Archaeal protein
638197176	Saci_0240	conserved Archaeal protein
638197231	Saci_0296	predicted Fe-S oxidoreductases
638197244	Saci_0309	lipoic acid synthetase
638197278	**Saci_0343**	**radical SAM domain protein**
638197279	**Saci_0344**	**radical SAM domain protein**
638197309	Saci_0374	conserved Archaeal protein
638197631	Saci_0703	conserved Archaeal radical SAM superfamily protein
638197889	Saci_0962	universally conserved protein
638197968	Saci_1044	conserved Archaeal protein
638198063	Saci_1141	conserved Archaeal protein
638198248	Saci_1331	conserved protein
638198392	Saci_1479	cyclic pyranopterin monophosphate synthase (MoaA)
638198402	**Saci_1489**	**conserved Archaeal protein**
638198497	Saci_1585	conserved Archaeal protein
638198516	Saci_1604	conserved Archaeal protein
638198543	Saci_1632	conserved Archaeal protein
638198693	Saci_1785	conserved Prokaryal protein

Table S2. Sequences used to generate Cds phylogenetic tree

IMG Locus Tag	IMG Genome ID	Genome Name	GenBank Accession	NCBI Bioproject Accession	NCBI Biosample Accession
TB_LI09_3DRAFT_10005913	3300000229	Groundwater microbial communities from subsurface biofilms in sulfidic aquifier in Frasassi Gorge, Italy, sample from two redox zones-LI09_3	_	PRJNA336827	SAMN05518261
JGI2160J19893_100096772	3300001855	Marine sediment microbial communities from White Oak River estuary, North Carolina - WOR-2-8_12	–	PRJNA366768	SAMN06268482
Ga0114920_100221603	3300009528	Deep subsurface microbial communities from South Pacific Ocean to uncover new lineages of life (NeLLi) - Chile_00310 metaG	–	PRJNA405477	SAMN07631026
Ga0114921_101774722	3300009150	Deep subsurface microbial communities from South Atlantic Ocean to uncover new lineages of life (NeLLi) - Benguela_00093 metaG	_	PRJNA367444	SAMN06267295
Ga0172363_100121925	3300013130	Sediment microbial communities from Lake Kivu, Rwanda - Sediment s2_kivu2a2	_	PRJNA404428	SAMN07630773
Ga0105105_102127021	3300009009	Freshwater sediment microbial communities from Prairie Pothole Lake near Jamestown, North Dakota, USA - PPLs Lake P8 Core (1) Depth 1-3cm September2015	_	PRJNA365086	SAMN06264427
Ga0129327_101115071	3300013010	Freshwater to marine salinity gradient microbial communities from Chesapeake Bay, USA - CPBay_Spr_31_0.8_DNA	–	PRJNA364792	SAMN06264995
Ga0099843_1258431	3300007155	Iron oxide microbial mat communities from Yellowstone National Park, Wyoming, USA - ECH_C_top_diel_T=5 metaT (Metagenome Metatranscriptome)	_	PRJNA365453	SAMN06264516

YNPsite14_CeleraDRAFT_106210	2022920007	Hot spring microbial communities from One Hundred Springs Plain, Yellowstone National Park, Wyoming, USA - YNP14 OSP Spring	–	PRJNA337118	SAMN05518158
Ga0080003_10059782	3300005859	Hot spring microbial communities from Joseph's Coat, Yellowstone National Park, USA - JC2_E (SPADES assembly)	–	PRJNA366310	SAMN06268698
YNP1_75530	2014031002	Hot spring microbial communities from Yellowstone National Park, Wyoming, USA - YNP1 Alice Springs, Crater Hills	–	PRJNA337120	SAMN05518659
Ga0079042_10009966	3300006181	Hot spring microbial mat communities from Yellowstone National Park, Wyoming, USA - ECH_B nyco_MetaG	–	PRJNA375678	SAMN06343748
Ga0079041_10002292	3300006857	Hot spring microbial mat communities from Yellowstone National Park, Wyoming, USA - ECH_B host_MetaG	–	PRJNA375677	SAMN06343747
Ga0167615_10056683	3300013009	Extremophilic microbial mat communities from Yellowstone National Park, USA - BED_Mat_host_9_15 (v2)	–	PRJNA367343	SAMN06267146
Ga0182010_100210611	3300014490	Permafrost microbial communities from Stordalen Mire, Sweden - 611E1M metaG	–	PRJNA406790	SAMN07631786
Ga0187772_100080051	3300018085	Tropical peat soil microbial communities from peatlands in Department of Meta, Colombia - 0116_SJ02_MP15_20_MG	–	PRJNA443517	SAMN08776832
Ga0187780_1000007610	3300017973	Tropical peat soil microbial communities from peatlands in Department of Meta, Colombia - 1015_Q2_SP10_20_MG	–	PRJNA443525	SAMN08777027
Ga0187770_100660741	3300018090	Tropical peat soil microbial communities from peatlands in Department of Meta, Colombia - 0116_SJ02_MP02_20_MG	–	PRJNA443515	SAMN08776833

JGI24147J26652_10017966	3300002103	Arctic peat soil from Barrow, Alaska - Barrow Graham LP Incubations 11-33A	_	PRJNA330169	SAMN05422026
JGIcombinedJ26865_10164291	3300002347	Arctic peat soil from Barrow, Alaska - NGEE Surface sample 415-1 deep-072012 (NGEE Surface samples 415 (1, 2, 3 deep-072012) AP id is 1030520, ASSEMBLY_DATE=20131219)	–	PRJNA330294	SAMN05421916
JGI24721J44947_100034043	3300002966	Hot spring thermophilic microbial communities from Obsidian Pool, Yellowstone National Park, USA - OP-RAMG-01	–	PRJNA366136	SAMN06268369
JGI20128J18817_100000859	3300001684	Hot spring microbial communities from Joseph's Coat, Yellowstone National Park, USA - JC2_E	_	PRJNA366310	SAMN06268698
Ga0180298_10149462	3300014869	Subseafloor sediment microbial communities from Guaymas Basin, Gulf of California, Mexico - Guay13, Core 4488-10 , 4-6 cm	–	PRJNA406717	SAMN07632361
Ga0116200_102257641	3300010332	Marine hydrothermal vent microbial communities from Guaymas Basin, Gulf of California to study Microbial Dark Matter (Phase II) - Marker 14 Mat core 4571-4 3-6 cm metaG	_	PRJNA365032	SAMN06264874
Ga0164310_101961912	3300014913	Subseafloor sediment microbial communities from Guaymas Basin, Gulf of California, Mexico - Guay1, Core 4569-9, 0-3 cm	–	PRJNA368383	SAMN06269071
Ga0190329_100035017	3300021494	Hydrothermal vent sediment bacterial communities from Southern Trench, Guaymas Basin, Mexico - 4872-04-2-3_MG	–	PRJNA445005	SAMN08777168
Ga0080003_100005160	3300005859	Hot spring microbial communities from Joseph's Coat, Yellowstone National Park, USA - JC2_E (SPADES assembly)	–	PRJNA366310	SAMN06268698
Ga0080003_10166501	3300005859	Hot spring microbial communities from Joseph's Coat, Yellowstone	–	PRJNA366310	SAMN06268698

		National Park, USA - JC2_E (SPADES assembly)			
Ga0080006_11902124	3300005861	Ferric oxide microbial mat and aquatic microbial communities from Rainbow Spring, Yellowstone National Park, USA - RS3B (SPADES assembly)	_	PRJNA366317	SAMN06268705
OneHSP_7476CDRAFT_10003425	3300000342	Ferrous microbial mat communities from One Hundred Spring Plain, Yellowstone National Park, USA - T=74-76	–	PRJNA336650	SAMN05518653
Ga0079044_10017752	3300006855	Hot spring microbial mat communities from Yellowstone National Park, Wyoming, USA - ECH_C host_MetaG	–	PRJNA375680	SAMN06343750
Ga0040879_1048251	3300003730	Thermal spring microbial communities from Beowulf Spring, Yellowstone National Park, Wyoming, USA - Beowulf (BE_D) (Metagenome Metatranscriptome)	_	PRJNA365740	SAMN06267756
JGI20133J14441_100012826	3300001340	Ferric oxide microbial mat and aquatic microbial communities from Rainbow Spring, Yellowstone National Park, USA - RS3B	–	PRJNA366317	SAMN06268705
JGI20133J14441_100004255	3300001340	Ferric oxide microbial mat and aquatic microbial communities from Rainbow Spring, Yellowstone National Park, USA - RS3B	–	PRJNA366317	SAMN06268705
YNP_08_myell_20060	2502894001	*Metallosphaera archaeon*	_	_	_
Ga0128346_103192	2681813019	*Sulfolobus* sp. JCM 16833	_	_	PRJDB820
ST1392	638154519	*Sulfolobus tokodaii* 7, JCM 10545	BAB66459	_	PRJNA246
Aboo_0737	646564501	*Aciduliprofundum boonei* T469	YP_003483110	_	PRJNA38403
MflrDRAFT_02178	2518645542	*Methanoflorens stordalenmirensis*	_	_	_
Ga0128335_100238	2681813032	*Sulfolobus metallicus* DSM 6482, JCM 9184	–	_	PRJDB806
Ga0172927_111534	2728369534	*Sulfolobus* sp. A20	_	_	PRJNA339201
Ga0199187_11746	2740892001	*Acidianus manzaensis* YN-25	_	_	PRJNA380321

CM19_05805	2574180086	*Candidatus* Acidianus copahuensis ALE1	–	–	PRJNA240874
Ga0180371_11429	2721755441	Unclassfied Crenarchaeota ZZQ bin_1	–	–	–
ASUL_01974	2588254065	*Sulfolobales archaeon* AZ1	–	–	PRJNA198460
Mcup_0901	650716051	*Metallosphaera cuprina* Ar-4	YP_004409490	–	PRJNA65497
Ahos_1265	650716001	*Acidianus hospitalis* W1	YP_004458446	–	PRJNA60875
AciM339_0328	2508501109	*Aciduliprofundum* sp. MAR08-339	–	–	PRJNA73039
NAG2ff85r04_00864	2524023239	NAG2_ff85-r04	–	–	–
A474_10477	2514885039	*Sulfolobales* sp. HSU1	–	–	–
Myr05_01008	2551306706	*Metallosphaera yellowstonensis* r05	–	–	–
Ga0100943_111650	2645727535	*Metallosphaera sedula* ARS120-2	–	–	PRJNA289699
Ga0081668_111464	2648501262	*Sulfolobus solfataricus* SULA	–	–	PRJNA279935
Ga0126086_11463	2684622731	*Sulfolobus acidocaldarius* NG05B_CO5_10	–	–	PRJNA304349
Ga0180374_106163	2721755443	*Sulfolobus* sp. ZZQ bin_3_2nd	–	–	–
–	–	*Candidatus* Marsarchaeota G1 archaeon BE_D (taxid:1978158)	PSN85745.1	PRJNA362583	SAMN06639946
–	–	*Candidatus* Marsarchaeota G2 archaeon OSP_D (taxid:1978157)	PSN91903.1	PRJNA362583	SAMN06639947
–	–	*Candidatus* Marsarchaeota G2 archaeon ECH_B_SAG-G16 (taxid:1978167)	PSN97909.1	PRJNA362583	SAMN06639953
–	–	*Candidatus* Korarchaeota archaeon	PMB77276.1	PRJNA419931	SAMN08107309

Table S3. Ecological characteristics of metagenomes with Cds hits

Ecosystem Category	Ecosystem Subtype	Ecosystem Type	Habitat	No. of Cds hits
Aquatic	Alkaline	Non-marine Saline and Alkaline	Hypoxic/sulfidic aquatic	1
	Coastal	Marine	Freshwater to marine saline gradient	5
	Groundwater	Freshwater	Groundwater	1
	Hot (42-90^{o}C)	Thermal springs	Acidic hot water	3
			Ferric mat	4
			Hot spring	97
			Hypersaline mat	16
			Microbial mats	14
			Thermal spring	21
	Hydrothermal vents	Marine	Marine sediment	21
	Hypersaline	Non-marine Saline and Alkaline	Hot spring	13
	Lake	Freshwater	Anoxic zone freshwater	2
			Sediment	8
	Oceanic	Marine	Deep subsurface	2
			Marine sediment	2
	Unclassified	Non-marine Saline and Alkaline	Ferric oxide microbial mat	7
			Ferrous mat	21
	Wetlands	Freshwater	Bog	11
			Peatland	123
Terrestrial	Unclassified	Soil	Peat	18
			Arctic peat soil	69
			Permafrost	2
			Soil	13
	Wetlands	Soil	Bog	2
			Fen	11
			Tropical peatland	25

Table S4. Strains used in this study

Strains		**Genotype**	**Sources or reference**
Escherichia coli	DH10B	F⁻ *endA1 recA1 galE15 galK16 nup GrpsL ΔlacX74* Φ80lacZΔM15 *araD139 Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC)λ⁻*	D.K. Newman (Caltech)
	ER1821	F⁻*glnV44 e14⁻(McrA⁻) rfbD1 relA1 endA1 spoT1 thi-1 Δ(mcrC- mrr)114::IS10*	New England Biolabs
Sulfolobus acidocaldarius	MW001	*ΔpyrE* (uracil auxotrophic parent strain)	(2)
	ZZsa001	MW001 + pSVA1561	This work
	ZZsa002	MW001 *Δsaci_0343*	This work
	ZZsa003	MW001 *Δsaci_1489*	This work
	ZZsa004	MW001 *Δsaci_1489* + pSVA1561	This work
	ZZsa005	MW001 *Δsaci_1489* + pSVA1561-*saci_1489*	This work

Table S5. Plasmids used in this study

Plasmids	Description	Source or reference
pSVA407	*S. acidocaldarius* deletion plasmid	(2)
pSVA1561	*S. acidocaldarius* expression plasmid	(2)
pSVA407-*saci_1489*UD	Deletion plasmid containing *saci_1489* upstream and downstream regions for *saci_1489* deletion. The upstream and downstream regions were amplified by PCR with primers Z001F/R, and Z002F/R, respectively, and cloned into the *NcoI* and *BamHI* sites of pSVA407 plasmid.	This work
pSVA407-*saci_0343*UD	Deletion plasmid containing *saci_0343* upstream and downstream regions for *saci_0343* deletion. The upstream and downstream regions were amplified by PCR with primers Z004F/R, and Z005F/R, respectively, and cloned into the *NcoI* and *BamHI* sites of pSVA407 plasmid.	This work
pSVA1561-*saci_1489*	*Δsaci_1489* mutant complementation plasmid. The *saci_1489* gene was amplified by PCR with primers Z003F/R, and cloned into the *NcoI* and *XhoI* site of pSVA1561 plasmid.	This work

Table S6. Primers used in this study

Primers	Sequence (5' to 3')	Notes
Z001F	CCCGACGTCGCATGCTCCCGGCCGCATTTATCAAGTTATCAAAAACAATT	*saci_1489* upstream cloning forward
Z001R	ATTTGGTTAGGCTCTTCTATTCATAATTTAAGAAACCTC	*saci_1489* upstream cloning reverse
Z002F	AAATTATGAATAGAAGAGCCTAACCAAATGTATTTTTAT	*saci_1489* downstream cloning forward
Z002R	TACTAGAACTGCTCAAACCTAGGTCAGAGTAACTAATTCAGACGTAAACGC	*saci_1489* downstream cloning reverse
Z003F	ATAAATAATTACGTGATTAAGTTAACCATGAATAGAGATATTAAAGCGTTTAGA	Complementation *saci_1489* forward
Z003R	CCCTTAATGGTGATGATGGTGATGTTAGGCTCTACCTAAAAATCTCC	Complementation *saci_1489* reverse
Z004F	CCCGACGTCGCATGCTCCCGGCCGCGAGATAAGGGGATAATACTCTCAGG	*saci_0343* upstream cloning forward
Z004R	AATATACATGGACATAGTCATGAAACACCACCTGA	*saci_0343* upstream cloning reverse
Z005F	TGGTGTTTCATGACTATGTCCATGTATATTCCTACTGGTT	*saci_0343* downstream cloning forward
Z005R	TACTAGAACTGCTCAAACCTAGGTCAGAGATTGGTTTATTTTCCAAAACC	*saci_0343* downstream cloning reverse
Z006 F	ATGACAAGAAAAACAAAGAAGATTTA	*saci_1489* deletion check forward
Z006R	CGCAATAAAGGATTTCCTAGAT	*saci_1489* deletion check reverse
Z007F	GTGCCTTTAATTGTAAACATTGTG	*saci_0343* deletion check forward
Z007R	AAGTCTATTAAGTCTCCCTCTTCCA	*saci_0343* deletion check reverse

SI Appendix References

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4. Huguet C, Hopmans EC, Febo-Ayala W, Thompson DH, Damste JSS, & Schouten S (2006) An improved method to determine the absolute abundance of glycerol dibiphytanyl glycerol tetraether lipids. *Organic Geochemistry* 37(9):1036-1041.