Supplementary materials

Cultivation protocols

Cultivation and growth conditions of Methanopyrus kandleri *and* Methanothermobacter marburgensis

Methanopyrus kandleri and *Methanothermobacter marburgensis* were grown in closed batch cultivation mode under anaerobic conditions with a mixture of molecular hydrogen (H_2) and carbon dioxide (CO_2) containing 20 vol.-% CO_2 in H_2 (H_2/CO_2, 4:1) at an overpressure of 2 bar. Both methanogens were grown in 117 mL serum flasks with a crimped-on rubber stopper (pretreated by boiling ten times for 30 min in fresh ddH_2O; 20 mm, butyl rubber, CLS-3409-14, Chemglass Life Sciences, USA) as reported before [1]. *M. kandleri* was grown at 97 °C shaking at 120 revolutions per minute (rpm) in an water bath (GFL Shaking and Heating Water Bath 1083, Lauda Dr. R. Wobser GmbH & Co. KG, Lauda-Königshofen, Germany) filled with ROTITHERM© fluid, while *M. marburgensis* was grown in a water bath at 65 °C shaking at 120 rpm (GFL Shaking and Heating Water Bath 1083, Lauda Dr. R. Wobser GmbH & Co. KG, Lauda-Königshofen, Germany).

Methanopyrus kandleri *growth medium*

M. kandleri was grown in DSMZ 511 media containing (per 1 L ultrapure, i.e., 18 MΩ water, further referred to as MQ water) 0.25 g NH_4Cl, 0.087 g K_2HPO_4, 0.09 g KH_2PO_4, 11.80 g NaCl, 1.75 g $MgSO_4 \cdot 7H_2O$, 4.5 g $MgCl_2 \cdot 6H_2O$, 0.81 g Na_2SO_4, 0.78 g $CaCl_2 \cdot 2H_2O$, 0.3 g KCl, 10 mL TE solution, 2 mL $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ solution (0.1% w/v), 2 mL $(NH_4)_2Ni(SO_4)_2$ solution (0.1% w/v), 2 mL $Na_2WO_4 \cdot 2H_2O$ solution (0.1% w/v), 10 mL Marine TE, 0.5 mL Na-resazurin solution (0.1% w/v), 1 g $NaHCO_3$, and 10 mL vitamin solution. Fifty mL of medium was then added to 117 mL serum bottles, closed with rubber stoppers (see above) and sealed with crimp caps (20 mm aluminum, Ochs Laborbedarf, Bovenden, Germany). The medium was flushed with 20 vol.-% CO_2 in N_2 (N_2/CO_2, 4:1). The pH was adjusted to 6.5 using HCl and the bottles were autoclaved. After the bottles cooled down, 0.2 mL of $Na_2S \cdot 9H_2O$ (0.5 mol L^{-1}) were added per bottle and the medium was flushed with H_2/CO_2 (4:1). The media was stored in the dark at room temperature.

The TE solution contained (per 1 L of MQ water) 1.5 g nitrilotriacetic acid, 3 g $MgSO_4 \cdot 7H_2O$, 585 mg $MnCl_2 \cdot 4H_2O$, 1 g NaCl, 100 mg $FeSO_4 \cdot 7H_2O$, 180 mg $CoSO_4 \cdot 7H_2O$, 100 mg $CaCl_2 \cdot 2H_2O$, 180 mg $ZnSO_4 \cdot 7H_2O$, 6 mg $CuSO_4$, 20 mg $KAl(SO_4)_2 \cdot 12H_2O$, 10 mg H_3BO_3, 10 mg $Na_2MoO_4 \cdot 2H_2O$, 30 mg $NiCl_2 \cdot 6H_2O$, 0.3 mg $Na_2SeO_3 \cdot 5H_2O$, and 0.4 mg $NaWO_4 \cdot 2H_2O$. The TE solution was autoclaved.

The marine TE solution contained (per 1L of MQ water) 4 g NaBr, 1.8 g $SrCl_2 \cdot 6H_2O$, 0.529 g H_3BO_3, 1 g KI, 100 mg Na-silicate, 0.183 mg NaF, 100 mg KNO_3, and 0.128 mg Na_2HPO_4. The marine TE solution was autoclaved.

The vitamin solution contained (per 1 L of MQ water) 2 mg biotin, 2 mg folic acid, 10 mg pyridoxine-HCl, 5 mg thiamine-$HCl \cdot 2H_2O$, 5 mg riboflavin, 5 mg nicotinic acid, 5 mg D-Ca-pantothenate, 0.1 mg vitamin B12, 5 mg p-aminobenzoic acid, and 5 mg lipoic acid. The vitamin solution was filter-sterilized and kept at 4 °C in the dark.

Methanothermobacter marburgensis *growth medium*

M. marburgensis was grown in minimal medium containing 2.1 g NH_4Cl, 6.8 g KH_2PO_4, 3.6 g Na_2CO_3, 10 mL of 100× TE solution (TES3) in 1000 mL of ddH_2O. Fifty mL of medium was then added to 117 mL serum bottles, closed with rubber stoppers (see above) and sealed with crimp caps as above. The medium was flushed with N_2/CO_2 and the bottles were autoclaved. After anaerobization and autoclaving the medium [1], 0.2 mL of $Na_2S \cdot 9H_2O$ (0.5 mol L^{-1}) were added per bottle and the medium was flushed with H_2/CO_2 (4:1).

The TES3 solution contained 9 g Titriplex I, to which 800 mL ddH_2O was added and pH adjusted to 6.5 with 5 mol L^{-1} NaOH solution, then other components were added, namely 4 g $MgCl_2 \cdot 6H_2O$, 1 g

$FeCl_2 \cdot 4H_2O$, 20 mg $CoCl_2 \cdot 6H_2O$, 120 mg $NiCl_2 \cdot 6H_2O$, and 20 mg $NaMoO_4 \cdot 2H_2O$. The volume was made up to 1000 mL and the pH was adjusted to 7.0 with 1 mol L^{-1} NaOH. The TES3 was autoclaved.

Haloferax volcanii *growth conditions and medium*
H. volcanii was grown as described previously [2] in the medium containing 3 mol L^{-1} NaCl, 150 mmol L^{-1} $MgSO_4$, 50 mmol L^{-1} KCl, 3 mmol L^{-1} $CaCl_2 \cdot H_2O$, 10 nmol L^{-1} $MnCl_2$, 25 mmol L^{-1} Tris-HCl pH 7.2, 0.5% (w/v) tryptone, 0.3% (w/v) yeast extract, and 0.02% (w/v) histidine. The medium was filter sterilized prior to inoculation. The organism was cultivated at 37 °C in an air incubator 170 L ZWYR-2102C (LABWIT Scientific, Burwood East, VIC, 3151, Australia) upon constant orbital shacking at 180 rpm.

Cultivation and growth conditions of Saccharolobus solfataricus *and* Sulfolobus acidocaldarius
S. solfataricus and *S. acidocaldarius* were cultivated in 200 mL long neck bottles containing 50 mL of the cultivation medium at 73 °C upon orbital shaking (100 rpm) in a water bath filled with ROTITHERM© fluid as described before [3]. The cultivation (Brock) medium was prepared from defined stock solutions: Brock solution I (100×) contained 130 g L^{-1} $(NH_4)_2SO_4$, 25 g L^{-1} $MgSO_4 \cdot 7H_2O$, 2 g L^{-1} $FeCl_3 \cdot 6H_2O$, and 3 mL 50% H_2SO_4. Brock solution II (200×) contained 56 g L^{-1} KH_2PO_4 supplemented with trace element stock solutions (10 g L^{-1} each of $MnCl_2$, $ZnSO_4$, $CuCl_2$, $VOSO_4$, Na_2MoO_4, $CoSO_4$, $Na_2B_4O_7$) in the following volumes: 36 mL $MnCl_2$, 4.4 mL $ZnSO_4$, 1 mL $CuCl_2$, 600 µL $VOSO_4$, 600 µL Na_2MoO_4, 200 µL $CoSO_4$, and 90 µL $Na_2B_4O_7$. After autoclaving, the pH was adjusted to 5.0 with 50% (v/v) H_2SO_4. Brock solution III (1000×) consisted of 14 g L^{-1} $CaCl_2 \cdot 2H_2O$. Additional supplements included 20% (w/v) D(+) sucrose and 10% (w/v) tryptone. All stock solutions were prepared separately, sterilized by filtration where appropriate, and stored at 4 °C.
For culture preparation, 50 mL of Brock medium was assembled as follows: 40 mL sterile water, 500 µL Brock solution I, 250 µL Brock solution II, 50 µL Brock solution III, 500 µL 20% sucrose, and 500 µL 10% tryptone. The volume was adjusted to 50 mL with sterile MQ water.

Cultivation and growth conditions of Metallosphera sedula
M. sedula was cultivated under the same cultivation conditions as described above for *S. solfataricus* and *S. acidocaldarius*. However, DSMZ medium 88 was used for cultivation of *M. sedula* and contained per L: 1.30 g $(NH_4)_2SO_4$, 0.28 g KH_2PO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.07g $CaCl_2 \cdot 2H_2O$, 0.02 g $FeCl_3 \cdot 6H_2O$ and 10 mL of 100× Allen's trace element solution.
Allen's trace element solution contained (per L): 180 mg $MnCl_2 \cdot 4H_2O$, 450 mg $Na_2B_4O_7 \cdot 10H_2O$, 22 mg $ZnSO_4 \cdot 7H_2O$, 5 mg $CuCl_2 \cdot 2H_2O$, 3 mg $Na_2MoO_4 \cdot 2H_2O$, 3 mg $VOSO_4 \cdot 2H_2O$, 1 mg $CoSO_4 \cdot 7H_2O$. The media was supplemented with 20 g L^{-1} pyrite and subsequently titrated to a final pH of 2.0 with H_2SO_4 prior to autoclaving and inoculation.
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Supplementary Tables

Table S1. Description of samples/isolates, cultivation temperatures, biomass and lipid yields, and presence of two enzymes, i.e. glycerol-1-phosphate dehydrogenase (G1P, EC 1.1.1.261) and glycerol-3-phosphate dehydrogenase (G3P, EC 1.1 5.3), as per GenBank. Gene accession numbers (GenBank) are quoted if available.

Sample/species	Collection accession number	Reference	Note	Cultivation temperature (°C)	Dry biomass (mg)	Total lipids (mg)	G1P	G3P
Haloferax volcanii	WR340	[2]		37	168.3	4.58	TVT93737.1	TVT95752.1
Metallosphaera sedula	DSM 8348	[3]		73	60.1	2.15	AKV81900.1	AKV80190.1
Methanopyrus kandleri 1	AV19	[4]		97	14.7	0.76	WP_011019598.1	not found
Methanopyrus kandleri 2	AV19	[4]	Dark and slimy pellet	97	34.5	1.38	WP_011019598.1	not found
Methanothermobacter marburgensis	DSM 2133	[5]		65	37.0	1.62	WP_013295808.1	not found
Saccharolobus solfataricus 1		[6]	1st generation from cryostock	73	37.3	1.08	QPG51023.1	QPG49855.1
Saccharolobus solfataricus 2	DSM 1616	[6]	2nd generation from cryostock	73	32.8	0.84	QPG51023.1	QPG49855.1
Sulfolobus acidocaldarius	DSM 639	[6]		73	22.0	1.13	ALU31773.1	ALU32401.1

Table S2. Specific rotation of archaeol isomers obtained from the commercial standard. For chromatogram please see Fig. 4.

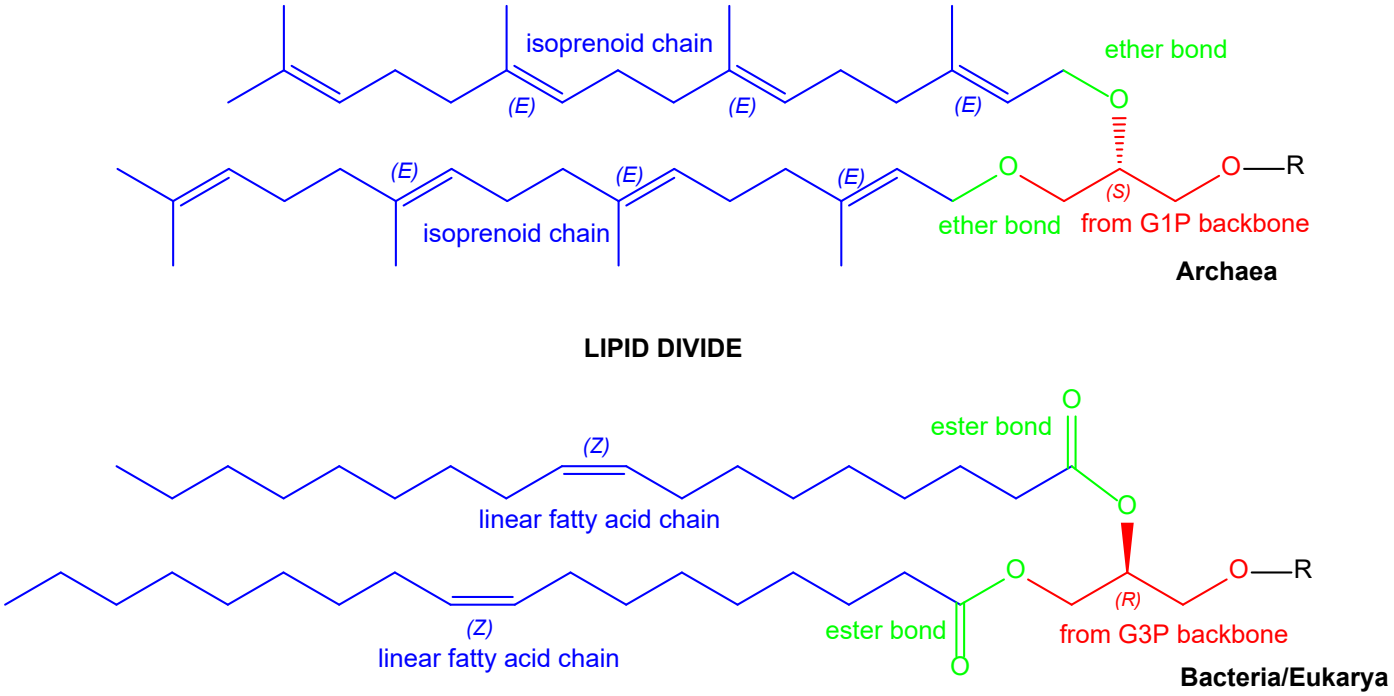
Archaeol	% of all isomers	$[\alpha]_D$	tR (min)
(*S*)-2,3-bis(((3*S*,7*R*,11*R*)-3,7,11,15-tetramethylhexadecyl)oxy)propan-1-ol	1.1	-[a]	31.11
(*S*)-2,3-bis(((3*R*,7*R*,11*R*)-3,7,11,15-tetramethylhexadecyl)oxy)propan-1-ol	10.7	-8.06[b]	31.67
(*R*)-2,3-bis(((3*S*,7*R*,11*R*)-3,7,11,15-tetramethylhexadecyl)oxy)propan-1-ol	4.3	7.19	32.04
(*R*)-2,3-bis(((3*R*,7*R*,11*R*)-3,7,11,15-tetramethylhexadecyl)oxy)propan-1-ol	83.9	8.15[c]	32.66

[a] not measured due to low yield

[b] a value $[\alpha]_D$ -7,1° was published for (*S*)-2,3-bis(((3*R*,7*R*,11*R*)-3,7,11,15-tetramethylhexadecyl)oxy)propan-1-ol [7].

[c] various authors have published the following $[\alpha]_D$ for (*R*)-2,3-bis(((3*R*,7*R*,11*R*)-3,7,11,15-tetramethylhexadecyl)oxy)propan-1-ol, i.e., +7,1° [8], +8,5° [9], +9.68 [10], or +8,4° [11].

Supplementary Figures



R = polar head including a phosphoric acid ester and carbohydrate, choline, ethanolamine, etc.

Fig. S1. Structures of core lipids of Archaea and Bacteria/Eukarya, defined as lipids lacking a polar head group including a phosphoric acid ester.

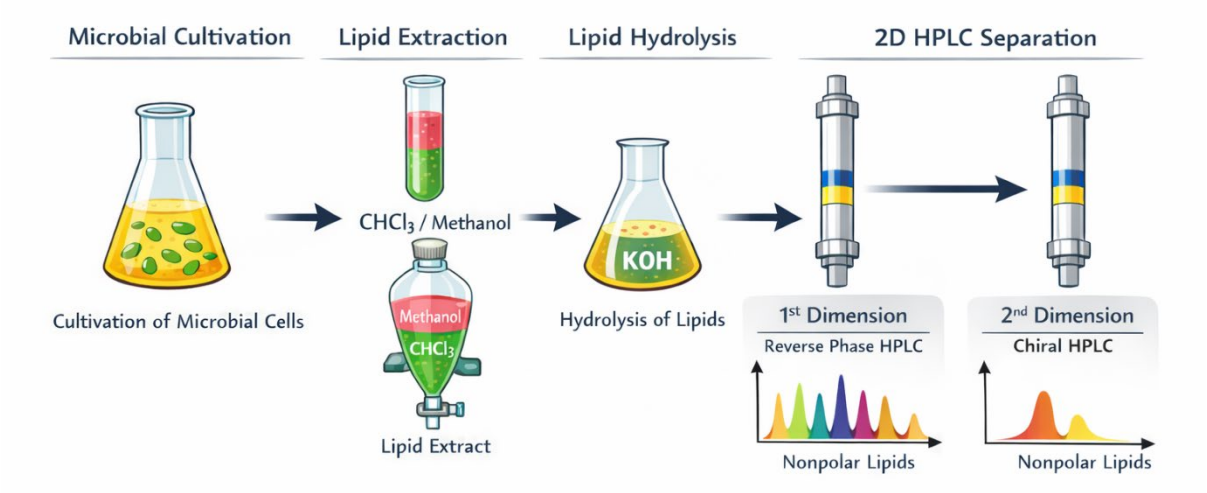


Fig. S2. Step-by-step lipid analysis process. Microbial lipids were extracted from cultivated cells using a chloroform/methanol-based protocol and subsequently hydrolyzed under alkaline conditions to obtain core lipids. The hydrolyzed lipid fraction was analyzed using two-dimensional HPLC, combining reverse-phase separation with chiral HPLC. This approach enabled efficient separation and characterization of nonpolar lipids, including stereochemical resolution. The image was created in BioRender.com.

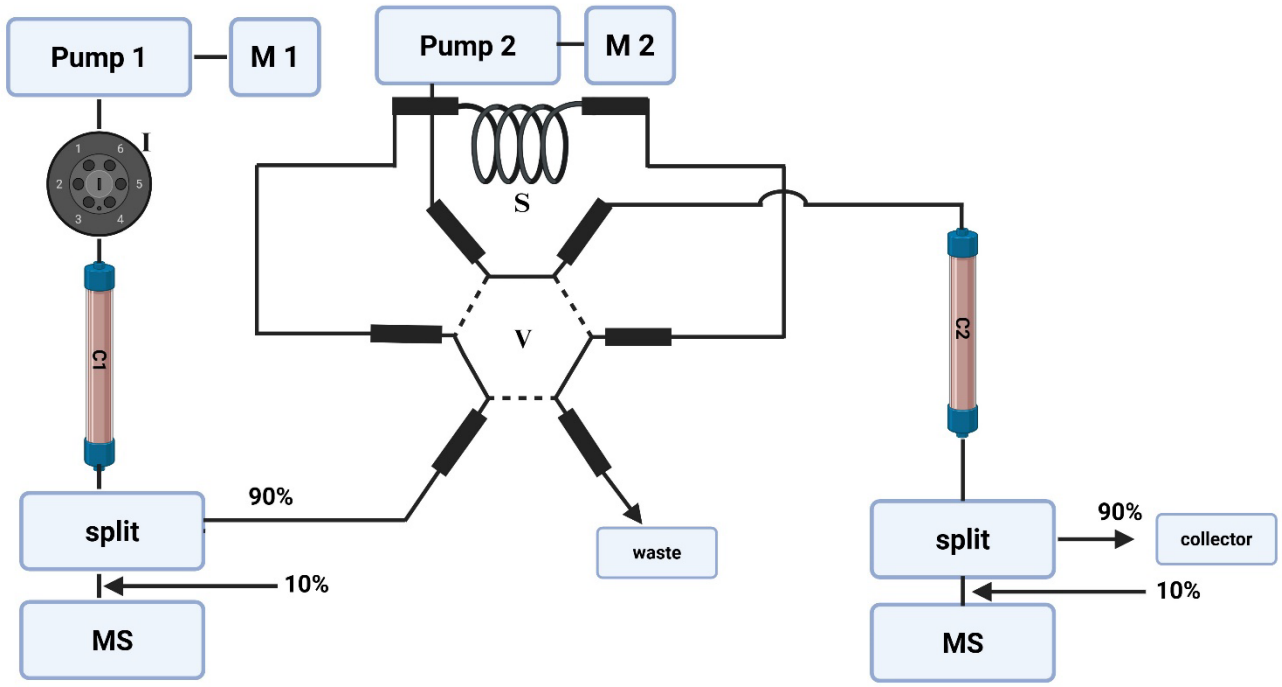


Fig. S3. A schematic diagram of the automatic column-switching HPLC system used in this study is shown. The solid and dotted lines indicate the switch positions of valve A and B, respectively. The abbreviations used are as follows: C1, three Luna Omega columns (C18); C2, two Astec Cyclobond™ I 2000 DMP chiral LC columns (3,5-dimethyl-phenyl-carbamate modified β-cyclodextrin); I, injector, S; sample loop (200 µL); M1, ACN with 5 mmol L^{-1} $AcNH_4$ and iPrOH with 5 mmol L^{-1} $AcNH_4$ (gradient); M2, hexane and iPrOH (97:3 v:v with 10 mmol L^{-1} $AcNH_4$). The image was created in BioRender.com.

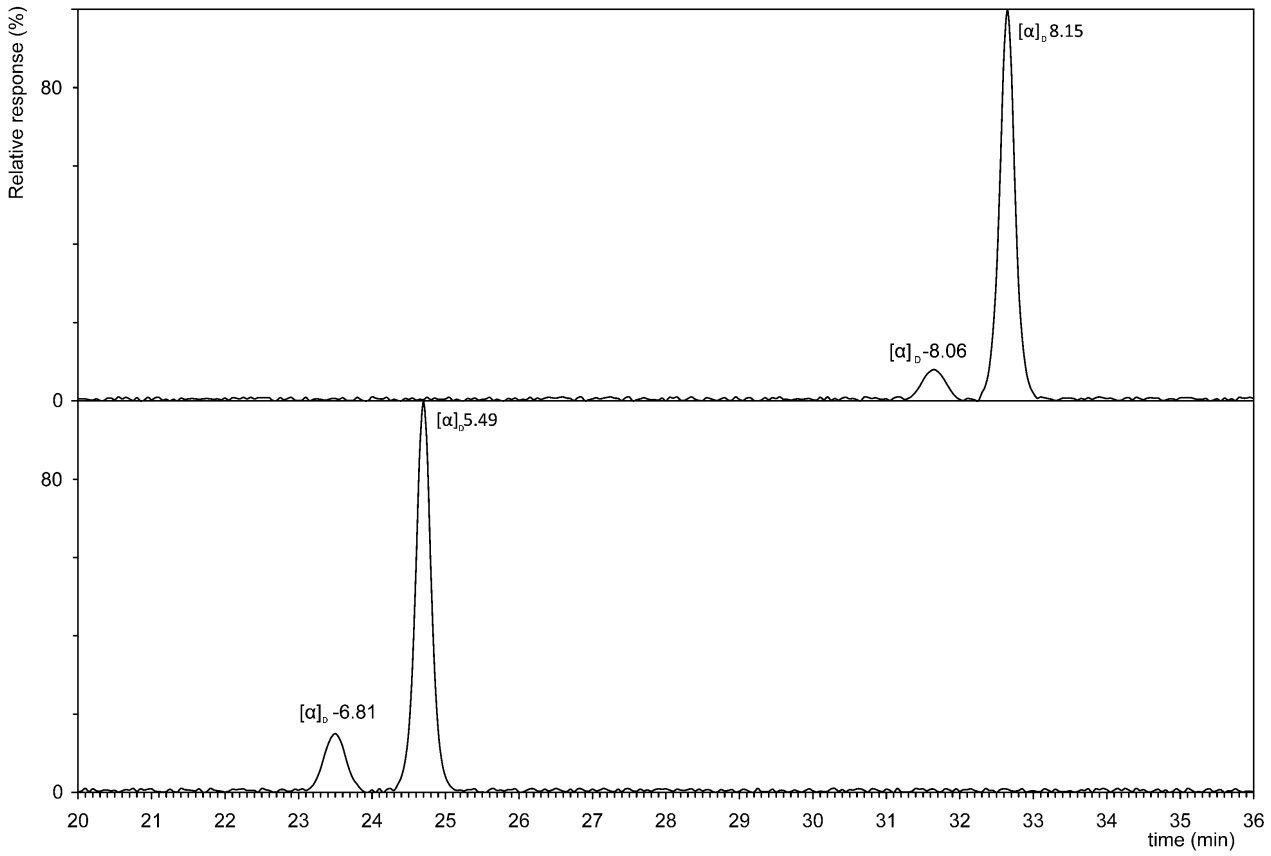


Fig. S4. Chiral chromatography of archaeols (tR 31.67 min and tR 32.66 min) and extended archaeols (tR 23.52 min and tR 24.71 min) including specific rotation ([α]D) values of all four isomers.

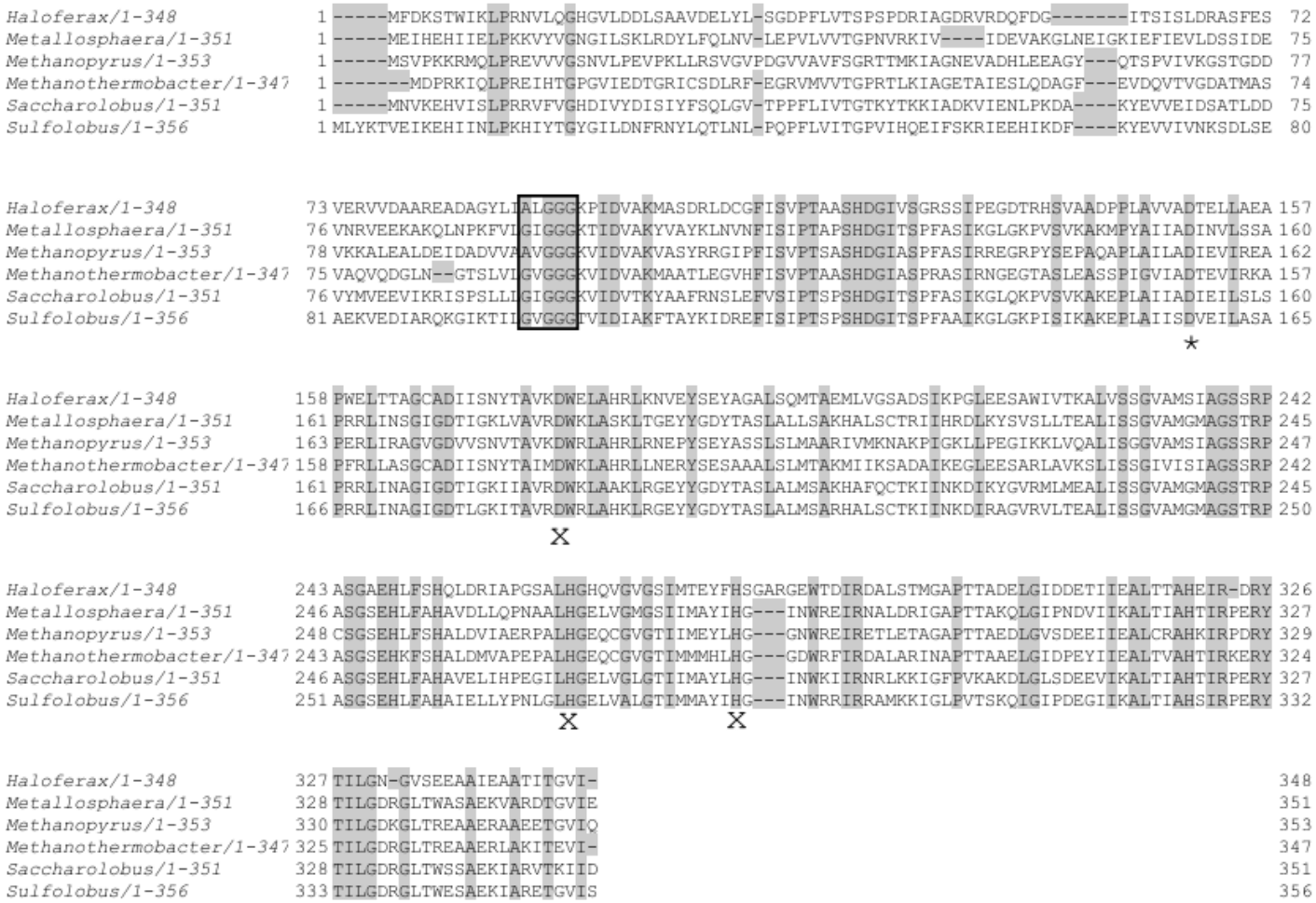
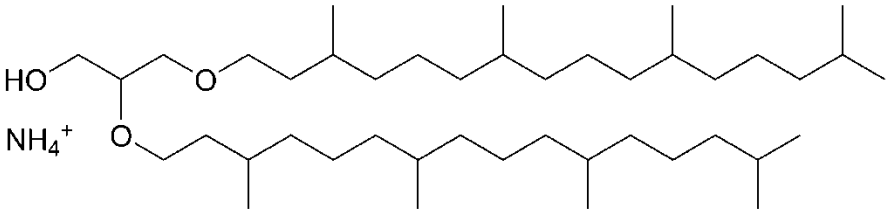
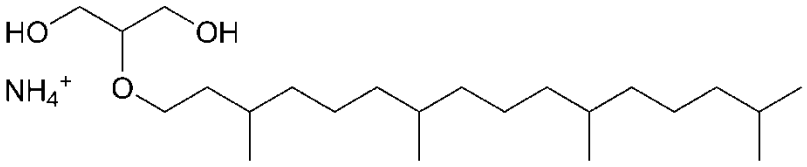


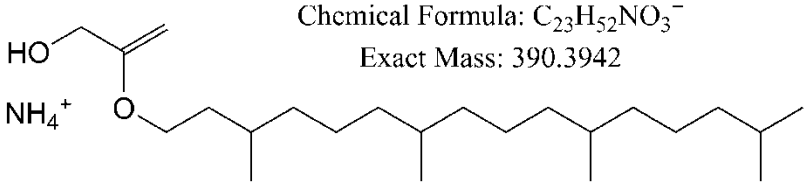
Fig. S5. Multiple sequence alignment of identified glycerol-1-phosphate dehydrogenases from the organisms used in this study. Grey-highlighted amino acids indicate conserved residues. Squared residues represent the Rossmann fold, which facilitates NADH/NADPH coenzyme binding. X-marked residues indicate Zn^{2+}-binding sites, while star-marked residues denote substrate-binding positions.



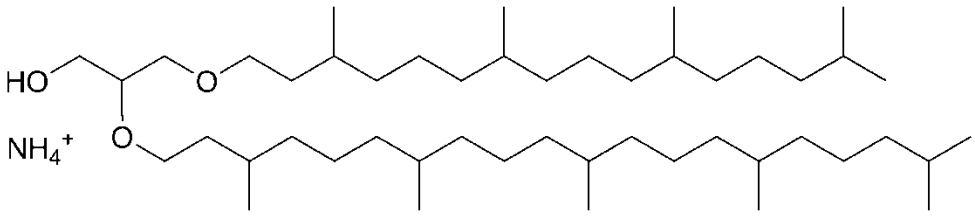
Chemical Formula: $C_{43}H_{92}NO_3^+$
Exact Mass: 670.7072



Chemical Formula: $C_{23}H_{52}NO_3^-$
Exact Mass: 390.3942



Chemical Formula: $C_{23}H_{50}NO_2^+$
Exact Mass: 372.3836



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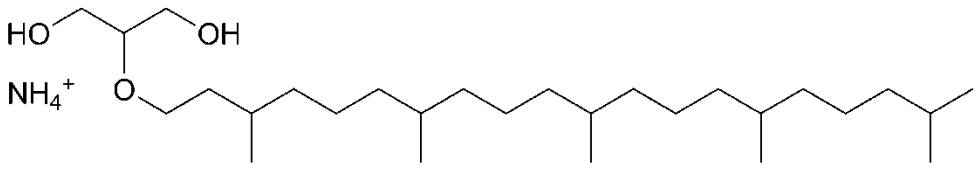
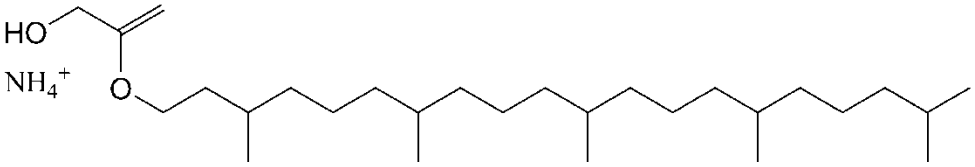


Chemical Formula: $C_{28}H_{62}NO_3^+$
Exact Mass: 460.4724



Chemical Formula: $C_{28}H_{60}NO_2^+$
Exact Mass: 442.4619

Fig. S6. Predicted structure of archaeol (C20-C20-AR, uppermost row) and its fragment ions, rows 2 and 3 from the top, quoting exact masses and summary chemical formulas. The lower half shows extended archaeol (C25-C20-AR, 4th row from the top) and its fragment ions quoting the same information as above.

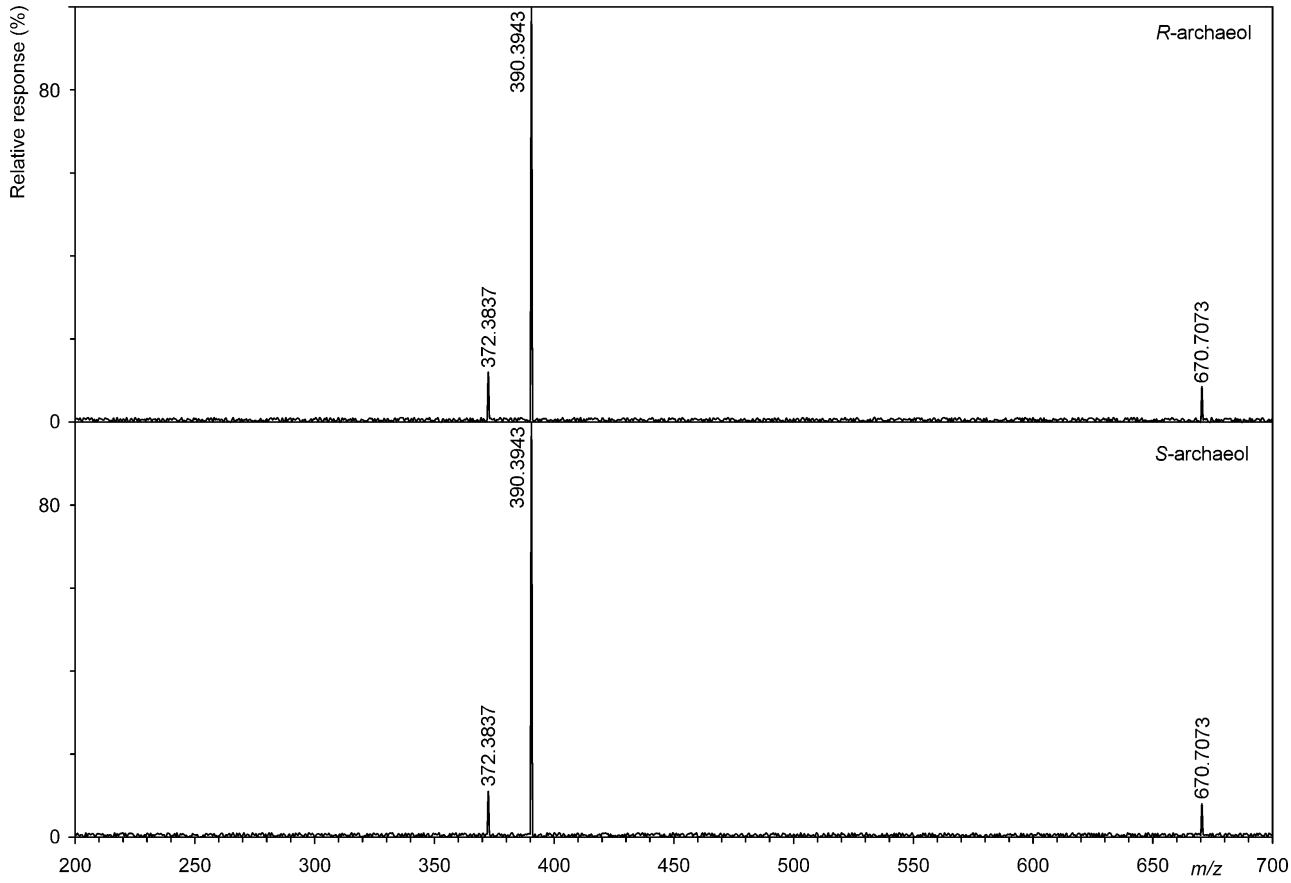


Fig. S7. Tandem MS of *R*-archaeol and *S*-archaeol.

Supplementary references

[1] R.-S. Taubner, S.K.-M.R. Rittmann, Method for indirect quantification of CH_4 production via H_2O production using hydrogenotrophic methanogens, Front. Microbiol. 7, (2025), 532. https://doi.org/10.3389/fmicb.2016.00532.

[2] K. Winter, J. Born, F. Pfeifer, Interaction of haloarchaeal gas vesicle proteins determined by split-GFP, Front. Microbiol. 9, (2018) 1897. doi: 10.3389/fmicb.2018.01897.

[3] G. Huber, C. Spinnler, A. Gambacorta, K.O. Stetter, *Metallosphaera sedula* gen. nov. and sp. nov. represents a new genus of aerobic, metal-mobilizing, thermoacidophilic Archaebacteria, Syst. Appl. Microbiol. 12, (1989) 38-47. https://doi.org/10.1016/S0723-2020(89)80038-4.

[4] R. Huber, M. Kurr, H.W. Jannasch, K.O. Stetter, A novel group of abyssal methanogenic archaebacteria (*Methanopyrus*) growing at 110 °C, Nature 342, (1989) 833–834. https://doi.org/10.1038/342833a0.

[5] A. Wasserfallen, J. Nölling, P. Pfister, J. Reeve, E. Conway de Macario, Phylogenetic analysis of 18 thermophilic *Methanobacterium* isolates supports the proposals to create a new genus, *Methanothermobacter* gen. nov., and to reclassify several isolates in three species, *Methanothermobacter thermautotrophicus* comb. nov., *Methanothermobacter wolfeii* comb. nov., and *Methanothermobacter marburgensis* sp. nov. Int. J. Syst. Evol. Microbiol. 50, (2000) 43–53. https://doi-org.d360prx.biomed.cas.cz/10.1099/00207713-50-1-43.

[6] E. Wimmer, I.A. Zink, C. Schleper. Reprogramming CRISPR-mediated RNA interference for silencing of essential genes in Sulfolobales. In: Ferreira-Cerca, S. (eds) Archaea. Methods in Molecular Biology, vol 2522, (2022). Humana, New York, NY. https://doi.org/10.1007/978-1-0716-2445-6_11.

[7] H. Morii, T. Eguchi, Y. Koga, In vitro biosynthesis of ether-type glycolipids in the methanoarchaeon *Methanothermobacter thermautotrophicus*, J. Bacteriol. 189, (2007) 4053–4061. https://doi.org/10.1128/JB.01875-06.

[8] C.A.A. van Boeckel, P. Westerduin, J. H. van Boom, Synthesis of two purple-membrane glycolipids and the glycolipid sulfate *O*-(β-d-glucopyranosyl 3-sulfate)-(1→6)-*O*-α-d-mannopyranosyl-(1→2)-*O*-α-d-glucopyranosyl-(1→1)-2, 3-di-*O*-phytanyl-sn-glycerol, Carbohydr. Res. 133 (1984) 219-234. https://doi.org/10.1016/0008-6215(84)85200-3.

[9] A.J. Hancock, M. Kates, A sulfate ester of phosphatidyl glycerol (diether analogue) from *Halobacterium cutirubrum*, Chem. Phys. Lipids 8, (1972) 87-90. https://doi.org/10.1016/0009-3084(72)90046-1.

[10] S. Régnier, E. Lam, V. Vasquez, C.F. Martinez-Farina, F.C. Stark, G. Agbayani, L. Deschatelets, R. Dudani, B.A. Harrison, B. Akache, M.J. McCluskie, U.D. Hemraz, Effect of chiral purity on adjuvanticity of archaeol-based glycolipids, J. Med. Chem. 65 (2022) 8332-8344. https://doi.org/10.1021/acs.jmedchem.2c00072.

[11] C. Joo, T. Shier, M. Kates, Characterization and synthesis of mono- and diphytanyl ethers of glycerol, J. Lipid Res. 9, (1968) 782-788. https://doi.org/10.1016/S0022-2275(20)42693-8.