
Supplementary information

Eukaryotes' closest relatives are internally simple syntrophic peptidotrophic archaea

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Supplementary Note 1

The cultivation conditions we devised for culturing new members of *Promethearchaeota*.

As mentioned in the main text, we hypothesized that the sampled biofilm would serve as an ideal source for cultivating 'Heimdallarchaeia' and elucidating their relationship with oxygen. To cultivate them, we used our hypotheses and the following information to guide the establishment of specific cultivation conditions.

1. Indirect provision of energy substrates in primary enrichment cultures

Previous studies have shown that *Promethearchaeota* could be co-enriched with microbial communities performing anaerobic oxidation of methane (AOM) and propionate (AOP)¹⁻³. We hypothesized that members of *Promethearchaeota* can utilize organic matter (probably mainly amino acids/peptides) secreted by microorganisms that consume methane or propionate^{2,4}.

2. Use proteinaceous substrates to further enrich *Promethearchaeota*

Comparative genomics showed that most members of *Promethearchaeota* are capable of anaerobic amino acids/peptides degradation⁵. Therefore, once we confirmed their co-enrichment in cultures amended with methane or propionates, we shifted to proteinaceous substrates (*e.g.*, casamino acids, powdered milk, peptone, and yeast extract) to promote further enrichment.

3. Cultivation at low concentrations of proteinaceous substrates

P. syntrophicum strain MK-D1 is a slow-growing microorganism with extremely low cell yield^{5,6}. Because many slow-growing microorganisms are known to exhibit high affinities for their energy substrates^{7,8}, we hypothesized members of *Promethearchaeota* would similarly possess a high substrate affinity for amino acids and peptides. Therefore, during the initial enrichment phase, we prepared a medium supplemented with low concentrations of amino acids/peptide compounds (0.005%, w/v). Note that *P. syntrophicum* strain MK-D1 could grow at this concentration of casamino acids and 20 amino acids, but scarcely at 0.0005% (w/v)⁵.

4. Addition of antibiotics to enrich archaeal species

We previously employed four antibiotics (*i.e.*, ampicillin, vancomycin, kanamycin, and streptomycin) to enrich *P. syntrophicum* strain MK-D1⁵. In this study, we applied these and other antibiotics to enrich archaeal species more effectively. For example, erythromycin may serve as a promising candidate because a comparative genomic analysis targeting transporters⁹ suggests that *Promethearchaeota* may tolerate it. Ciprofloxacin, azithromycin, cephalothin, and ofloxacin could also act as selective agents for enriching diverse archaeal species, as these antibiotics have been

successfully used to enrich ammonium-oxidizing archaea, haloarchaea and methanogens^{10–15}.

5. Using natural brine as the basal medium for initial enrichment cultures

Establishing cultivation conditions that closely resemble the natural environment is a fundamental strategy for culturing indigenous microorganisms. To maintain conditions as close to *in situ* as possible, we used brine (*i.e.*, gas-associated formation water) obtained anoxically from the gas-production well as the basal medium for the initial enrichment stage. After confirming stable growth of the target archaeon, we shifted to an artificial medium for further enrichment.

6. Adjusting reducing agent concentrations

Some 'Heimdallarchaeia' possess terminal oxidases^{16–20}. Though whether the enzymes support aerotolerance or aerobic respiration remains unclear, 'Heimdallarchaeia' likely have the capacity to help them survive and possibly grow in environments exposed to oxygen. To selectively cultivate these archaea, we either decreased or omitted reducing agents in the culture medium.

Supplementary Note 2

Cultivation of novel 'Hodarchaeales' strain SC1. Since HC1 was confirmed to grow on artificial medium, we expected that other members of *Promethearchaeota* could also be enriched without relying on brine-based medium during the initial enrichment stage. We therefore directly inoculated fresh biofilm sample into artificial media on-site and incubated the culture vials at 20°C, a temperature near that of the sampling site brine (Extended Data Fig. 1c).

After four months of incubation, 16S rRNA gene sequences affiliated with *Promethearchaeales* were detected in an anaerobic medium containing propionate and four antibiotics (*i.e.*, ampicillin, vancomycin, kanamycin, and streptomycin), although their relative abundance was less than 0.04% (Supplementary Table 1). We subsequently repeated subcultures in using the same medium composition twice and additionally detected a small number of sequences belonging to 'Hodarchaeales' (tentatively named HC2). To further enrich these archaea, we transferred them to a medium supplemented with casamino acids (0.005%, w/v), powdered milk (0.005%, w/v), and five antibiotics. After four months of incubation, a new 'Hodarchaeales' population (later strain SC1) appeared, with a relative abundance of 0.55%. This sequence had not been detected in the mother cultures, suggesting that the archaeon was initially a very minor population (Supplementary Table 1).

To further enrich SC1, we continued subcultures in the casamino acids-powdered milk-medium supplemented with the five antibiotics and monitored its growth by qPCR using SC1-specific 16S rRNA gene primers, as well as by iTag analysis of the community composition. During the enrichment process, we also tested three additional antibiotics (*i.e.*, ciprofloxacin, cephalothin, and ofloxacin), to which HC1 was found to be resistant. After 182 days of incubation, SC1 reached a relative abundance of 23% with 10^5 16S rRNA gen copies ml⁻¹ in an anaerobic medium amended with casamino acids, powdered milk, the five antibiotics, and cephalothin (Supplementary Table 1).

To further increase the abundance, growth rate, and cell density of SC1, we added peptone and yeast extract to the medium as additional energy sources and tested the combination of the energy substrates/antibiotics, their concentrations, and cultivation temperatures. After three successive transfers with modified cultivation conditions, we obtained a tri-culture constituting of the target archaeon SC1, the hydrogenotrophic methanogen *Methanocalculus* sp. strain MC3, and the methylotrophic methanogen *Methanobus* sp. strain MLB. This tri-culture has stably been maintained in MK medium amended with casamino acids (0.005%, w/v), powdered milk (0.005%), polypeptone peptone (0.01%, w/v), and yeast extract (0.05%, w/v) at 20°C (Supplementary Table 1). Under these cultivation conditions, SC1 required approximately 60 days to reach maximum growth with a cell density of 10^7 16S rRNA gene copies ml⁻¹ (Fig. 1e). Overall, the time from sampling to the establishment of the tri-culture was approximately three years.

Supplementary Note 3

Taxonomic description for archaeal strains cultured in this study.

Etymology. HC1: The genus name is *Margulisarchaeum* (Mar.gu.lis.ar.chae'um. N.L. neut. n. *archaeum*, an archaeon; N.L. neut. n. *Margulisarchaeum*, an archaeon named for Lynn Margulis, an evolutionary biologist who proposed the endosymbiotic theory, a revolutionary idea in the origin of eukaryotes). The species name is *peptidophilum* (pep.ti.di'phi.lum. N.L. neut. n. *peptidum*, peptide; N.L. masc. adj. suff. *-philus* (from Gr. masc. adj. *philos*), loving; N.L. neut. adj. *peptidophilum*, peptide-loving).

SC1: The genus name is *Flexarchaeum*, (Flex.ar.chae'um. L. masc. past part. *flexus*, bent; N.L. neut. n. *archaeum*, an archaeon; N.L. neut. n. *Flexarchaeum*, a flexible archaeon). The species name is *multiprotrusionum* (mul.ti.pro.tru.si.o'num. L. masc. adj. *multus*, many; N.L. fem. n. *protrusio* (from L. v. *protrudo*, to push out), a protrusion; N.L. gen. pl. n. *multiprotrusionum*, of many protrusions, referring to the numerous membrane-based cellular protrusions).

Locality. Both strains were obtained from biofilms formed on the wall of a gas production well, Chiba Prefecture, Japan.

Diagnosis. Anaerobic, peptides-degrading archaea. Coccoid-shaped cells. Syntrophically grows with hydrogen- and formate-utilizing microorganisms. HC1 reduces nitrate to nitrite. They produce membrane vesicles and membrane-based protrusions.

Supplementary Note 4

Actin-filament and tubulin structures

As also observed for '*L. ossiferum*'²¹, actin filament-like structures were present in HC1 and SC1 and often extended from the cell interior towards the protrusions (Fig. 2j–n, Extended Data Figs. 2m, 3t–w and 4, and Supplementary Videos 1 and 2). Sub-tomogram averaging revealed that these filaments in HC1 closely resemble those previously described in '*L. ossiferum*'²¹ (Extended Data Fig. 5 and Supplementary Note 5), further supporting the presence of conserved actin filament structure within the phylum. In contrast, neither homologous structures nor genes corresponding to the recently discovered tubulin in '*L. ossiferum*'²² were detected in either strain (Supplementary Table 7).

Supplementary Note 5

Analysis of actin filaments in an HC1 cell

One tomogram of HC1 (Fig. 2m, EMD-63317) was selected for further analysis of the filaments in the cell based on the quality of the density of the filaments. The averaged diffraction pattern of the projections (Extended Data Fig. 5a) of the density along the filament tracks exhibited four layer lines (Extended Data Fig. 5b), similar to those of eukaryotic actin filaments²³, at positions corresponding to 45 nm, 23 nm, 7.0 nm, and 6.0 nm. Projection images filtered to retain signals around these four layer lines and the equator²⁴ revealed double-stranded, eukaryotic actin-like features, indicating that the filament architecture resembles that of the eukaryotic actin filament. The predicted crossover repeats and molecular spacing from the averaged diffraction pattern were 45 nm and 32 Å, respectively. The helical rotation between adjacent subunits was estimated to be ± 167 degrees.

The averaged autocorrelation (Extended Data Fig. 5c), computed by varying the rotation in 5-degree steps and shifting along the helical axis in steps of one pixel (6.192 Å), using 12 filaments, exhibited correlation peaks at (190°, equivalent to -170° , 31 Å) and (170°, -31 Å). These results confirm -167° as the correct helical rotation.

Sub-tomogram averaging was performed using RELION 4.0²⁵ and Dynamo 1.1²⁶. The final density map (Extended Data Fig. 4d), reconstructed from 1,491 sub-tomograms ($64 \times 64 \times 64$ voxels), showed a helical rotation and axial rise of -168.4° and $31.4 \text{ \AA}$, respectively—consistent with the diffraction analysis. The resolution of the reconstruction was $19.8 \text{ \AA}$ at the 0.143 FSC threshold (Extended Data Fig. 5e). The filament structure closely resembled that of the eukaryotic actin filament²⁷ and the Loki actin filament²¹, with a slightly longer molecular spacing ($\sim 27.5 \text{ \AA}$ in eukaryotic and Loki actin filaments).

An AlphaFold3²⁸ prediction based on the HC1 actin homolog sequence produced a monomer structure nearly identical to eukaryotic actin²⁹, except for an N-terminal extension (yellow in Extended Data Fig. 5f, g). A fitted model based on this prediction matched the reconstructed density well, including the N-terminal extension, and showed a similar arrangement to that of the eukaryotic actin filament. The slightly longer molecular spacing is likely due to the N-terminal extension, which is absent in both Loki actin (UYP47028.1) and eukaryotic actin sequences.

Supplementary Note 6

Ferritin-like granular structures

Intracellular granular structures were found in HC1 and SC1, albeit less abundant in SC1 (Fig. 2k–n, and Extended Data Figs. 2h–j and 3t–w, Supplementary Videos 1 and 2). These granules in HC1 exhibited a wide range of diameters, 11.7 to 46.1 nm , with an average of $24.8 \pm 5.3 \text{ nm}$ (Supplementary Fig. 3). In the transcriptome and proteome, we find high expression of two proteins that can form granules in this size range: ribosomes and ferritin(-like) proteins³⁰. Among these, ferritin is detected among the top three highest expressed proteins in the transcriptome, at a gene expression level 1.77 times that of the sum of all ribosomal proteins (Supplementary Table 5). These findings suggest that a large proportion of the observed granular structures are likely to be ferritin. However, cryo-scanning transmission electron microscopy (STEM) electron energy loss spectroscopy (EELS) analysis failed to detect a distinct iron signal from the cells (Supplementary Fig. 4), which may be due to limited iron incorporation, technological sensitivity limits, or ferritin existing in an apo-form under the culture conditions used.

Supplementary Note 7

Filaments on the protrusions surface

The HC1 and SC1 protrusions were discovered to have filaments on their surfaces (Fig. 2d, e, and Extended Data Figs. 2e, f, m, n and 3g–j, o, p). These filaments were helical,

often short but variable in length (~125 nm and ~1.5 μ m for HC1 and SC1), thicker than pili and archaella but comparable to flagella (15–20 nm and 20–25 nm in diameter for HC1 and SC1), and equipped with round structures at the tip (approximately 50 nm in diameter for HC1 and 55 nm for SC1) (Extended Data Figs. 2f, m, n, and 3o, p). HC1 and SC1 cells are often observed attached to their partner methanogens via their protrusions, and these filaments tend to be present at the interface (Fig. 2d, e, and Extended Data Fig. 3i, j). While the nature or function of these filaments remain unknown, these observations suggest that they may help mediate physical attachment and symbiotic interactions with partner organisms. Notably, prokaryotic filament structures—flagella, pili, and archaella—have been repeatedly demonstrated to play critical roles in syntrophic interactions (that is, partner recognition and electron transfer)^{31–33}. Albeit without filaments, protrusion-mediated attachment to syntrophic partners has also been observed for *P. syntrophicum* MK-D1^{5,6} and *L. ossiferum*²¹.

Supplementary Note 8

Peptide quantification

We used an UPLC-based analysis to quantify the consumption of macromolecules in the culture media and, as another approach, we tested the Pierce Quantitative Fluorometric Peptide Assay kit (Thermo Fisher Scientific). However, due to inconsistent measurement reproducibility, we discontinued its use. According to the manufacturer's instructions, amino acid-containing buffers are incompatible with this assay, suggesting that the cysteine employed as a reducing agent in the culture media may have interfered with the measurements.

Supplementary Note 9

Abbreviations in Figure 4

Pep, extracellular peptidase; Opp, oligopeptide permease; Fd, ferredoxin; Q, quinone; QH₂, quinol; Put, putrescine; ADMA, asymmetric dimethylarginine; DMA, dimethylamine; Fmd, formamide; Suc, succinate; Ac, acetate; Pr, propionate; Glut, glutarate; BCAA, branched-chain amino acid; BCFA, branched-chain fatty acid; MT, methanethiol; Fo, formate, HPA, 2-hydroxyphenylacetate; HGA, homogentisate; ANT, anthranilate; 4OT, 4-oxalocrotonate; FixC, electron-bifurcating flavoprotein transmembrane oxidoreductase subunit; NDH₂, type 2 NADH:Q oxidoreductase; Nuo*, type 1 NADH:Q oxidoreductase (or complex I) lacking NAD-binding module NuoEFG; Cbd?, cytochrome bd oxidase-like protein; Cox, cytochrome c oxidase (or complex IV);

cy, cytochrome c; Fdh, formate dehydrogenase; NfnAB, NADH-dependent reduced ferredoxin:NADP⁺ oxidoreductase; Hdr, heterodisulfide reductase.

Supplementary Note 10

Glycoproteome analysis of strains HC1 and SC1

Protein diversity

Our glycoproteome analysis using *N*-glycosylation open search identified 786 and 926 different proteins from strain HC1 grown on 0.005% and 0.05% yeast extract, respectively, representing approximately 12.0% and 14.1% of its total coding sequence (CDS). For strain SC1, 1,436 different proteins were identified, constituting about 26.2% of its total CDS. These proportions, similar to another member, *P. syntrophicum* MK-D1, where 19.1% of CDS was detected³⁴, are significantly lower compared to the approximately 50% previously reported for *Thermoproteota* archaea in our earlier analysis³⁵. The lower proportion of detected proteins in *Promethearchaeota* archaea may be attributed to their larger genome sizes and higher gene numbers, which may result from increased gene duplications and reduced gene losses, unlike other archaeal lineages¹⁷.

Throughout the two cultivation conditions, high normalized spectral abundance factors (NSAF) values were observed in HC1 for hypothetical proteins (KTGHC1_P0390 and KTGHC1_P6317), a trehalose/maltose transport system substrate-binding protein (KTGHC1_P2505), peptide/nickel transport system substrate-binding proteins (KTGHC1_P4283 and KTGHC1_P5849), and DNA-binding protein (KTGHC1_P0859). Similarly, in SC1, peptide/nickel transport system proteins (KTGSC1_P0547 and KTGSC1_P0136) exhibited high NSAF values, along with other hypothetical proteins. Similar substrate-binding proteins (SBPs) were abundantly detected in *P. syntrophicum* MK-D1³⁴, suggesting the common significance of these SBPs in the nutritional strategies of *Promethearchaeota* archaea.

Protein N-glycosylation

The structure of archaeal *N*-glycans, including the composition and sequence of monosaccharides, provides important insights into the phylogenetic relationships among archaeal species. Protein *N*-glycosylation of HC1 and SC1 was examined as previously described^{34,35}. Strain HC1 revealed a single predominant modification value of 1233.48 Da in both cultivation conditions. In contrast, modifications at 1308.55 Da were abundant in strain SC1, suggesting a different glycan structure. Many glycoproteins, including substrate-binding proteins and hypothetical proteins, were identified (Supplementary

Table 8), all undergoing *N*-glycosylation at asparagine residues within the conserved N-X-S/T sequon. This glycosylation pattern, where X represents any amino acid except proline, is common across archaea, bacteria, and eukaryotes. Similar to previously reported archaeal S-layer proteins, S-layer proteins of HC1 (KTGHC1_P6247) and SC1 (KTGSC1_P0196) were *N*-glycosylated, primarily in the vicinity of the N-terminus (Supplementary Table 8 and Extended Data Fig. 8).

The MS/MS spectra of the glycopeptides from HC1 consistently identified unusual potential oxonium ions of sugars at 160.10, 245.11, and 291.12 Da. The MS/MS analysis revealed a probably linear glycan consisting of 290.11-244.11-176.03-160.07-159.10-204.06 (Extended Data Fig. 8a). Archaeal protein *N*-glycosylation is known for its diverse array of linking sugars³⁶. An unusual linking sugar with an *m/z* of 290.11 Da, potentially HexNAc modified with serine (theoretical mass, 290.1114 Da), was previously reported as the linking sugar in the hyperthermophilic archaeon *Pyrodictium abyssi*, although the possibility of it being a different atypical sugar cannot be excluded³⁴. Additionally, 244.11 Da mass may represent Hex(NAc)₂, as reported in the linking sugar of *Pyrobaculum calidifontis*³⁷. The identification of two HexNAc variants at the reducing end suggests that the *N*-glycan of HC1 possesses a chitobiose core, similar to those found in *Thermoproteota* archaea and eukaryotes³⁶. The masses of 176.03 Da and 160.07 Da are potentially uronic acid and deoxy-methyl hexose, respectively. Uronic acids and methylated sugars were previously reported in *N*-glycans of various *Methanobacteriota* archaea³⁶. The 204.06 Da mass may correspond to acetyl-hexose. No candidate structure was identified for the mass of 159.10 Da, suggesting it may be an unknown component. In summary, the glycan structure of HC1 exhibits reducing ends characteristic of *Thermoproteota* and eukaryote glycans, middle parts of *Methanobacteriota* glycans, and unique non-reducing ends with unknown sugar components.

Similarly, the MS/MS spectra of the glycopeptides from SC1 consistently identified unusual potential oxonium ions of sugars at 186.08, 245.11, and 277.10 Da. The MS/MS analysis revealed a potentially linear glycan consisting of 244.11-244.11-115.06-276.10-185.07-244.22 (Extended Data Fig. 8b). As the glycan detected in HC1, the 244.11 Da mass may represent Hex(NAc)₂. No candidate structure was identified for the mass of 115.06, 185.07, and 276.10 Da, suggesting they may be unknown components. The glycan structure of strain SC1 features reducing ends characteristic of *Thermoproteota* and eukaryote glycans, with middle parts and non-reducing ends that include unknown sugar components. Previous glycoproteomic analysis identified a glycan structure in *P. syntrophicum* that lacks a chitobiose core³⁴.

In summary, glycan structure similarities were observed between HC1 and SC1, and the reducing end contained a structure shared with eukaryotes and *Thermoproteota* (a group of archaea most closely related to the *Promethearchaeota*). Although a previous study has reported that *P. syntrophicum* MK-D1 possesses glycan structures that are significantly different from those of eukaryotes³⁴, the current study suggests that *P. syntrophicum* MK-D1 may be an exceptional case and glycan structural similarities have been confirmed between eukaryotes and 'Heimdallarchaeia'.

Supplementary Note 11

Cellular responses to increased peptide concentrations in MK-D1 and SC1

P. syntrophicum MK-D1 and SC1 demonstrated behavior similar to HC1 when cultured at higher peptide concentrations. For MK-D1, when the substrate 0.05% casamino acids was replaced with 0.05% yeast extract, the doubling time shortened only slightly (from 14 days to 11 days)⁶. Meanwhile, the cell size nearly doubled (from 0.3–0.75 μm to 0.7–1.5 μm) and the number of protrusions per cell increased substantially (from a maximum of 8 to 14) (Extended Data Fig. 9). As for SC1, when the yeast extract concentration was raised to 0.5%, its cell size increased by approximately 1.5 times while the cell yield remained unchanged (Extended Data Fig. 9 and Supplementary Table 11).

Supplementary Note 12

Codon utilization and expression of enzymes associated with anaerobic peptidotrophy and aerotolerance

Enzymes related to anaerobic peptidotrophy (extracellular peptidases, glutamate dehydrogenase, glycine dehydrogenase, 2-oxoacid:ferredoxin oxidoreductases, and hydrogenases) had codon utilization similar to ribosomal proteins, putting them in the top 25% among HC1's proteins (Fig. 3f; Supplementary Table 5). Supporting the validity of the approach, transcriptomics and proteomics both confirm that all the above genes/proteins were highly expressed by HC1 (Supplementary Table 5). Meanwhile, genes encoding cytochrome *c* oxidase, cytochrome *bd* oxidase-like protein, complex III cytochrome *b* (without cytochrome *c/f* or electron carrier copper proteins), type II NADH:quinone oxidoreductase, and nitrate reductase were all predicted to have low expressivity. Transcriptomics and proteomics detected low expression of these genes/proteins in anaerobic culture conditions despite the absence of oxygen and nitrate. This is in line with our inference that the genes likely support anaerobic peptidotrophy with aerotolerance.

Supplementary Note 13

A potential role for slow growth in the emergence of eukaryotic cells

The slow growth rate conserved among all cultured *Promethearchaeota* members could have theoretically played a critical role in the rise of eukaryotes. More specifically, we suspect the property may have contributed to closing several major gaps between FECA/archaea and eukaryotes: (i) absence/presence of a mitochondrion (endosymbiont), (ii) membrane lipid composition (ether vs ester lipids^{38,39}), (iii) cell size, and (iv) genome composition. Firstly, assuming FECA somehow endogenized the protomitochondrion, for FECA to retain the endosymbiont, it must have had a growth rate slower than the protomitochondrion. In addition, the protomitochondrion very likely possessed the ability to respire oxygen and this would have given it an energetic advantage to grow faster than FECA. The endogenized protomitochondrion would have also died faster than the host, based on the well-established linear relationship between growth rate and death rate⁴⁰. Theoretically, this could have led to accumulation of dead protomitochondrion cells in the host cell and, thus, protomitochondrion-derived bacterial ester lipids and bacterial genetic material. Given that ether and ester lipids can mix^{41,42} and the host archaeon likely grew slowly, the archaeon's membranes' ether lipids could have been slowly and potentially passively replaced by ester lipids. Moreover, the accumulation of protomitochondrion-derived lipids could have passively increased the host's size. This may help explain (i) why the eukaryote membrane is composed of bacteria-like ester lipids yet is a single layer and lacks a peptidoglycan layer (unlike Gram-negative bacteria and the [proto]mitochondrion) and (ii) how the protoeukaryote cell size may have began to increase. Next, regarding the bacterial genetic material release from protomitochondrion death, such exposure to intracellular foreign genetic material may have made the host archaeon prone to acquisition of bacterial genes—gene transfer from an endosymbiont to the host organism is a common phenomenon. This process may have contributed to high enrichment of bacteria-derived genes in the host genome and perhaps increased evolutionary rates (that is, the endosymbiont may accumulate mutations faster given its higher cell replication rate).

Supplementary Methods

Culturing. During the initial enrichment stage of HC1, we used filter-sterilized brine as the basal medium. The brine was collected in a 5-L glass bottle filled with water from the gas-production well (Extended Data Fig. 1) and immediately sealed with a butyl rubber stopper and screw cap to maintain anoxic conditions. The bottle was brought back to the laboratory under refrigerated conditions in the dark. Upon arrival, the brine was filtered through a 0.22- μ m-pore-size polyethersulfone filter unit (Corning) in an anaerobic chamber (Coy Laboratory Products) equipped with a KOACH T 500-F tabletop air filtration system (KOKEN Ltd). Using a 50-ml syringe attached to a 22-gauge needle, 20 ml of the filtered brine was dispensed into sterile anoxic 50-ml serum glass bottles prepared ahead of time (sealed with butyl rubber stoppers and aluminum caps, purged with N₂/CO₂ gas (80:20, v/v), and autoclaved). The filtered-sterilized brine was used as a basal medium from the primary to the third subculture. After the fourth subculture, we used an artificial KTG3 medium as the basal medium. The composition of the KTG3 medium was based on the chemical analysis of the brine water⁴³ (Extended Data Fig. 1) and was as follows (per liter): 1.64 g MgCl₂·6H₂O, 0.38 g CaCl₂·2H₂O, 17.64 g NaCl, 0.38 g KCl, 0.5 g NH₄Cl, 0.136 g KH₂PO₄, 2.3 g NaHCO₃, 10 ml SrCl₂ solution, 10 ml NaF/LiCl solution, 1 ml trace element solution, 2 ml Se/W solution, 0.5 ml resazurin solution, 1 ml vitamin solution, and 25 ml Na₂S/cysteine solution. The composition and preparation of all the solutions were as previously described⁴⁴. The artificial medium was purged with N₂/CO₂ gas (80:20 v/v), and the pH was adjusted to 7.0 at 25°C.

The purity of HC1 was routinely examined by microscopy and iTag analysis. Purity was further verified by whole genome shotgun sequencing, which only detected the sequences of HC1 and *Methanocalculus* MC2. The preceding “mother” enrichment cultures of the HC1 and MC2 co-culture, three microbial populations had persisted: *Mariniphaga*, *Alcanivorax*, or *Methanobolus*. Based on separate isolation of these strains, we had confirmed medium compositions they grow under: (i) Marine Broth 2216; (ii) 1/5 Marine Broth 2216 plus 0.5% sodium pyruvate; and (iii) KTG3 medium supplemented with 5 mM methanol, respectively (all at 30°C; see below). Using this information, we tested whether we could detect any growth of these organisms in subcultures from the HC1 and MC2 co-culture—that is, ensure that the three above populations had been rejected. In addition, to test for any other contaminating organotrophic organisms, we also incubated subcultures in the following media at 10°C, 20°C, 30°C, 37°C, and 55°C: (i) thioglycolate medium (BD Difco) and (ii) KTG3 medium supplemented with 1 mM sucrose, 1 mM glucose, 1 mM fructose, 1 mM xylose, and 0.01% (w/v) yeast extract. We did not detect any growth of contaminating bacteria/archaea after two months of

incubation in all five media. We also used PCR with bacterial 16S rRNA gene primer pairs 27F/907R^{45,46} and EUB338*/1490R^{45,47–49} and HCR-FISH with 16S rRNA-targeted oligonucleotide probe targeting bacteria EUB338⁴⁷ to verify that no contaminating bacteria are present in the HC1 and MC2 co-cultures.

For the cultivation of other *Promethearchaeota* members that may not have been selected for using the enrichment procedure, we used a basal medium that successfully cultured *P. syntrophicum* strain MK-D1 (MK medium)⁴⁴. SC1 was obtained from a culture using MK medium amended with propionate and antibiotics (Supplementary Table 1). After obtaining a stable culture of SC1, we performed HCR-FISH and PCR in the same way as for the purity check of HC1, but no bacterial signals were detected, suggesting that SC1 is a pure tri-culture consisting of only three archaeal species. We also confirmed the absence of microbial growth other than SC1 in the following media at 10°C, 20°C, 30°C, 37°C, and 55°C for 3 months of incubation: (i) thioglycolate medium (BD Difco); and (ii) MK medium supplemented with 1 mM sucrose, 1 mM glucose, 1 mM fructose, 1 mM xylose, and 0.01% (w/v) yeast extract.

To isolate *Mariniphaga* and *Alcanivorax* from the HC1 enrichment culture, we used Marine Broth 2216 and 1/5 Marine Broth 2216 plus 0.5% sodium pyruvate, respectively, and cultured at 30°C. The isolated bacterial strains were used to test their susceptibility to antibiotics (Supplementary Table 2).

Methanocalculus sp. strain MC2 was isolated from an HC1 pure co-culture using the KTG3 basal medium supplemented with H₂ (ca. 1.5 kPa in the headspace of the culture bottle), acetate (1 mM), yeast extract (0.01%, w/v), and coenzyme M (0.5 mM) at 30°C. To obtain a pure culture, colonies were isolated through two successive roll-tube cultivations under the same growth conditions. A single colony was picked and transferred to fresh medium for further cultivation. The purity of the isolate was confirmed microscopically and by sequencing. Both the 16S rRNA gene and whole genome sequences of the isolate were identical to those of strain MC2 originally present in the pure co-culture with HC1, confirming successful isolation of the target methanogen.

To examine whether HC1 can grow under low-oxygen conditions in the presence of aerobic bacteria, co-cultivation experiments were performed with *Mariniphaga* sp. strain PC or *Alcanivorax* sp. strain AC, both of which were isolated during the enrichment of HC1. Cultures were grown in KTG13 medium without addition of reducing agents for 20 days. The cultures were subsequently transferred three times under the same conditions. The aerobic bacteria were added only at the start of the first cultivation.

Observation of membrane vesicles using and thin filaments using TEM

For observation of HC1's membrane vesicles via negative-staining technique, the same TEM was used. The preparation of samples for this observation was performed according to the following procedure. Briefly, approximately 880 ml of HC1 culture was filtered through a polyvinylidene difluoride (PVDF) membrane with a pore size of 0.45 μm (Merck Millipore) on a clean bench. The filtered culture liquid was subsequently concentrated using Centricon Plus-70, Amicon Ultra-15 and Ultra-0.5 centrifugal filtration units with a molecular weight cutoff of 100 kDa (Merck Millipore). The concentrated sample was fixed with 2.5% (w/v) glutaraldehyde for 1.5 h. An aliquot of the fixed sample was absorbed onto formvar/carbon-coated copper grids overnight, stained with 2% uranyl acetate, and observed by the same TEM at an acceleration voltage of 200 kV.

For observation of thin filaments on the surface of SC1' protrusions, an unfixed SC1 cell sample was used. The sample was placed on a carbon-coated grid for 1 min at 25°C, rinsed with PBS, and then stained with 2% phosphotungstic acid solution for 1 min. The solution was then removed from the grid, the sample was allowed to dry before TEM images were acquired using a JEM1010 EM (JEOL) equipped with a FastScan-F214(T) charge-coupled device camera (TVIPS). The resulting filament image was analyzed using RELION 3.0²⁵. The image was segmented as 64 $\times$ 64 pixel squares with 90% overlap, yielding 404 images that were subsequently subjected to 2D classification.

Cryo-EM using a CRYO ARM 300 II

A 1.5 μl aliquot of HC1 culture was applied on a glow-discharged holey carbon grid (Quantifoil R 1.2/1.3 Cu grid, Quantifoil MicroTools GmbH). The grid was automatically blotted at 22 °C and 80% humidity, and plunged into liquid ethane using a Leica EM GP2 (Leica Microsystems). The frozen grid was mounted onto a liquid-nitrogen cryo-specimen holder and loaded into a CRYO ARM 300 II (JEOL), equipped with a cold-field emission electron gun operating at 300 kV and an omega-type in-column energy filter (energy slit width: 30 eV). Images were recorded using a K3 direct detection camera (GATAN) at nominal magnification from $\times 4,000$ to $\times 15,000$, corresponding to an imaging resolution of 3.3–12.4 Å per pixel, with the total dose under 10 electrons per Å² using a low-dose system. Tilt series were collected automatically in a range of $\pm 60^\circ$ at 2° increments using SerialEM v4.2.0 beta (<http://bio3d.colorado.edu/SerialEM>)⁵⁰. The total electron dose on the specimen per tilt series was maintained under approximately 90 electrons per Å² to minimize radiation damage. Tilt series alignment was performed using gold fiducials, and tomograms reconstruction was carried out using the IMOD v4.9.12⁵¹.

Cryo-STEM-EELS analysis

A 1.5 μ l of culture liquid was applied on a glow-discharged Quantifoil R1.2/1.3 Cu grid (Quantifoil MicroTools). The grid was automatically blotted at 22°C and 80% humidity and plugged into liquid ethane using a Leica EM GP2 (Leica Microsystems). Cryo-STEM-EELS imaging was performed on a JEM-F200 transmission electron microscope (JEOL) equipped with a CEFIED energy filtering and imaging device (CEOS) and an ELA electron-counting detector (DECTRIS). The microscope was operated at 200 kV accelerating voltage with a probe current of 120 pA. Spectrum-imaging data were acquired over a 173×149 -pixel raster using a dwell time of 1 ms pixel⁻¹. Five consecutive frames were recorded and subsequently aligned and summed to improve the signal-to-noise ratio. The energy dispersion was set to 0.75 eV channel⁻¹. All datasets were processed by exponential background subtraction, and principal-component noise filtering before quantitative analysis.

Sub-tomogram averaging of actin filaments

Filament tracks generated using AMIRA 3D Pro (Thermo Fisher Scientific; Fig. 2n) were refined by fitting a cylindrical model with a diameter of 8 nm. Three-dimensional regions along the refined tracks were extracted (40×40 voxels around the track) and straightened²³. The central layer with thickness of 12 voxels was used to generate projection images. An averaged diffraction pattern was then calculated from the projections of 91 straightened filament maps.

To determine the three-dimensional structure of the filament within the tomogram, 2,512 sub-tomograms ($64 \times 64 \times 64$ voxels) were extracted along the filament tracks using RELION 4.0²⁵. A low-pass filtered actin filament map at 6 nm resolution, generated from 2ZWH.pdb²¹, was used as the initial model (<https://www.rcsb.org/structure/2ZWH>). A final structure at 3 nm resolution was obtained through two rounds of 3D classification, followed by 3D refinement of 388 selected sub-tomograms. A longer filament map ($128 \times 128 \times 128$ voxels) was subsequently constructed from the refined map and used as the template for template matching (Supplementary Fig. 11).

Template matching (Supplementary Fig. 12) was performed using Dynamo 1.1²⁶. Initially, a template matching step was conducted using a wall model ($40 \times 40 \times 40$ voxels) with a thickness of 6 pixels to identify and exclude cell membranes. Membrane regions with high cross-correlation values were selected through segmentation using ChimeraX software⁵². These regions were then masked by setting the surrounding density values to zero. A second round of template matching was then performed using the

filament density as the template, resulting in the extraction of 10,246 sub-tomograms based on cross-correlation values.

The extracted sub-tomograms were initially divided into six classes via 3D classification. Two sets, each comprising two classes, were selected for further refinement. A second round of 3D classification was performed on each set, and one class showing an obvious subunit arrangement from each was selected and merged. Sub-tomograms located outside of the cell were manually removed, and a final 3D refinement was carried out (Supplementary Fig. 11).

A predicted subunit model based on the actin amino acid sequence of HC1 (KTGHC1_P0909) was generated using AlphaFold²⁸ and fitted into the density map as a rigid body. Seven subunits were assembled to construct the filament model. Molecular dynamics flexible fitting (MDFF) was then performed using ISOLDE⁵³, an extension of ChimeraX⁵², with distance restraints applied to each subunit (excluding the N-terminal extension). Final refinement was performed using Phenix⁵⁴ and coot⁵⁵.

Immunofluorescence staining using lokiactin-specific antibodies.

For western blotting, archaeal cultures (270 ml of HC1, and 80 ml of SC1) were concentrated to about 5 ml using a 0.22- μ m-pore-size polyethersulfonate (PES) filter unit (Corning) on a clean bench. The concentrated cell suspensions were further concentrated by centrifugation at 20,400 $\times$ g for 10 min at 4°C. The collected cell samples were washed once with their respective basal media without energy substrates (e.g., peptone and yeast extract) and then denatured in SDS sample buffer containing 1% (v/v) dithiothreitol at 97°C for 5 min. The samples were run on Super Sep Ace 10–20% precast polyacrylamide gel (Fujifilm), transferred to PVDF membranes, and blocked with Blocking One (Nacalai tesque). The blots were probed with respective primary antibodies and secondary antibody (goat anti-rabbit IgG (H+L) HRP conjugate). Signals of the blots were detected by chemiluminescence with ECL Select reagent (Cytiva). The yeast (*Saccharomyces cerevisiae*) lysate used as the negative control was prepared in accordance with a previous report⁵⁶.

For immunofluorescence staining, cells were fixed under anaerobic conditions by adding a mixture of formaldehyde (methanol-free, formaldehyde solution, 16% w/v, Electron Microscopy Sciences) and glutaraldehyde (distilled grade, 50% solution, TAAB Laboratories Equipment Ltd) directly to the fully grown cultures to final concentrations of 4% (w/v) formaldehyde and 0.2% (w/v) glutaraldehyde for 2 h at room temperature in the dark. The fixed cells were then concentrated by centrifugation at 20,400 $\times$ g for 10 min at 4°C and immobilized on poly-L-lysine-coated 8-well chamber slides. The slide samples

were subsequently permeabilized with 0.1% Triton X-100 and blocked with 3% BSA. After blocking, the cells were probed with respective primary antibodies (1:500 diluted) and secondary antibodies diluted either 1:100 (goat anti-rabbit Alexa Fluor 555, Abcam) for observation under a BX53 Olympus epifluorescence microscope or 1: 200 (goat anti-rabbit abberior STAR 580, Abberior) for observation with a STELLARIS STED confocal microscope (Leica). The samples were counterstained with SYBR Green-I (Thermo Fisher Scientific) and mounted with Prolong Diamond (Thermo Fisher Scientific) prior to observation. Images were acquired using a STELLARIS STED confocal microscope (Leica) with a $\times 100/1.4$ NA oil-immersion objective at $6 \times$ zoom, line scanning frequency of 400 Hz, and line average of 3. Fluorescence from the secondary antibodies was acquired using stimulated emission depletion (STED) mode with 587 nm for excitation and the depletion at 775 nm, while SYBR Green-I-derived fluorescence was acquired in confocal mode. Through all immunofluorescence staining procedures, pipetting was performed gently to minimize physical stress on the cells and prevent the detachment of cellular protrusions. Although SPY505-DNA (Spirochrome) was initially considered for DNA staining, we opted not to use it because it also stained well non-cell materials such as medium-derived precipitates.

Genome, transcriptome, proteome and glycoproteome analyses

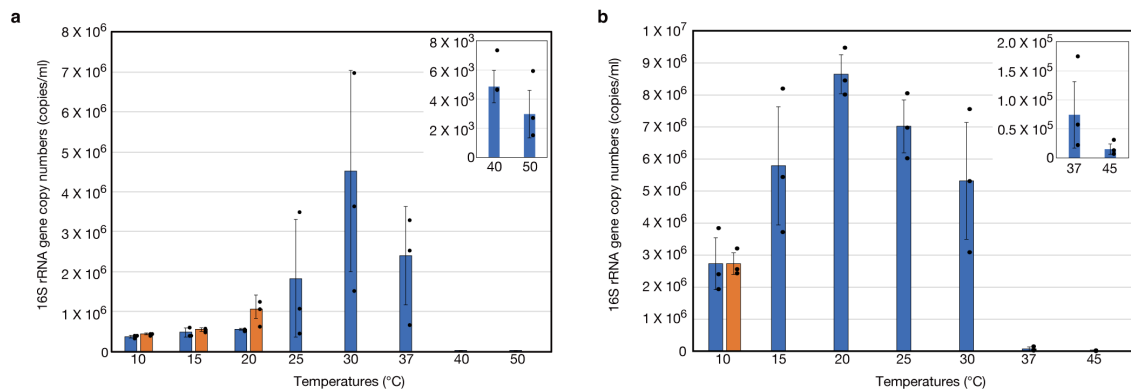
For genome analysis, the mate-paired library with an average insert size of 3 kb was prepared with Nextera Mate Pair Library Preparation kit (Illumina). Raw short reads were trimmed using Trimmomatic ver. 0.39⁵⁷ to remove adapters and low-quality bases (Phred score < Q20) with a minimum read length of 100 bp. Linker sequences in mate-paired reads were removed using NextClip v1.3.1⁵⁸. Short-read assemblies were generated with CLC Genomic Workbench v23 (Qiagen) and SPAdes v3.15.3⁵⁹. The resulting assemblies were manually merged and optimized to produce longer contigs. Scaffolding was performed with BESST v2.2.8⁶⁰ for mate-pair and LRScaf v1.1.8⁶¹ for long reads. Gaps within the scaffolds were filled with short reads using GapFiller v1.10⁶².

Coding sequences (CDSs) were predicted with Prodigal v2.6.3⁶³. Deduced amino acid sequences were subjected to BLASTP search against the NCBI non-redundant protein database and the KEGG GENES database, and functional annotations were manually assigned based on the KEGG Orthology database. Prediction of rRNA and non-coding RNA genes was performed with Rfam v13.0⁶⁴, and tRNA genes were identified using tRNAscan-SE v1.3.1⁶⁵. For strain HC1, these annotations were further verified using CD-Search⁶⁶ with conserved domain database (CDD) version 3.21⁶⁷ based on a cutoff of e-value of $1e-5$. COX1 and COX3 were also annotated using HMMER v3.4 (hmmsearch

with default settings and an e-value cutoff of $1e-5$) (hmmer.org) and HMM profiles available from the Pfam protein families database⁶⁸. NiFe hydrogenases were classified using HydDB with default settings⁶⁹.

RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions and subsequently concentrated with the RNA Clean & Concentrator-5 Kit (Zymo). RNA quality and quantity were assessed using an Agilent 2100 Bioanalyzer system with an RNA Pico kit (Agilent Technologies). Library preparation was performed using TruSeq Stranded mRNA Library Prep Kit and TruSeq RNA CD Index Plate (Illumina). The constructed cDNA library was sequenced on an Illumina HiSeqX platform. Raw RNA-seq reads were processed using Trimmomatic ver. 0.39⁵⁷ to trim low-quality and adapter sequences. The trimmed reads were aligned to the reference genome using Bowtie2 v2.3.5.1⁷⁰, and gene expression levels were quantified with featureCounts v1.6.385⁷¹ using the corresponding gene annotations. Gene expression counts were normalized using the Python package RNAnorm v2.1.0 (<https://github.com/genialis/RNAnorm>).

For proteomic analysis, HC1 was cultured for 30 days in a casamino acids-peptone-yeast extract medium at two different yeast extract concentrations (0.005% and 0.05% [w/v]), using the same parent culture as the inoculum. SC1 was cultured for 50 days in a casamino acids-powdered milk-polypeptone peptone-yeast extract medium. *P. syntrophicum* MK-D1 was cultured for 105 days in a casamino acids-powdered milk medium.

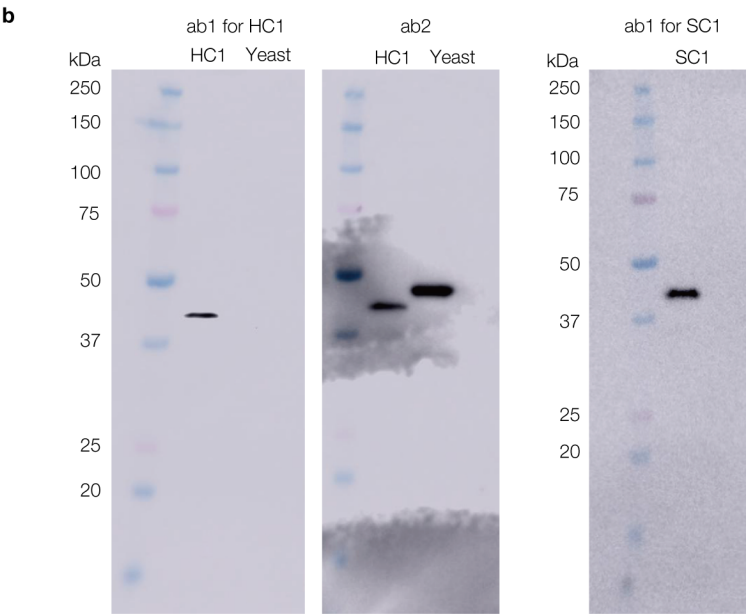


Supplementary Fig. 1 | Effect of temperature on growth of cultured archaea. a, HC1 cultures. Blue and orange bars show the 16S rRNA gene copy numbers after 32 and 62 days of incubation, respectively. Cultures incubated at 10°C, 15°C, and 20°C were monitored for a total of 62 days and then analyzed by qPCR. **b**, SC1 cultures. Blue and orange bars show the 16S rRNA gene copy numbers after 57 and 113 days of incubation, respectively. Cultures incubated at 10°C were monitored for a total of 113 days and then analyzed by qPCR. Data are mean $\pm$ s.d. of triplicate determinations. Each data point is shown as a dot.

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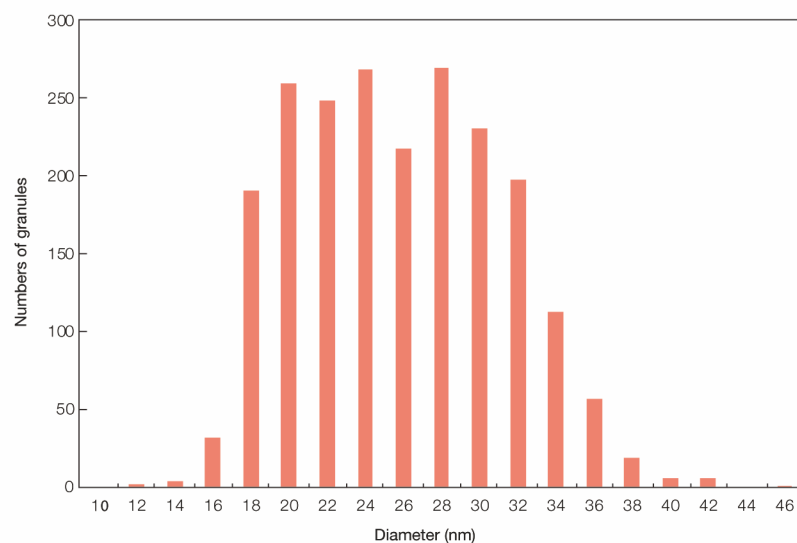
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SC1	-----MSEVSGKPLVIDNGTGLSKNGYAGEDQPRTVFPTLV	36
MK-D1	-----MVVEYDFLAAPLVVDNGTGLSKNGFAGEDQPRSVFPTLI	40
L.oss	-----MDWLAAPLVVDNGTGLSKNGFAGEDQPRSVFPTLI	36
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HC1	GYPKYQGIMSDVDHYVREYYVGEEAMNLRGVLKLMYPIEHGTVSDWDAMEKIWHYTFHND	120
SC1	GYPKYQGIMSDVDHYVREYYVGEEAMNLRGVLKLMYPIEHGTVDWDAMEKIWHYTFHND	96
MK-D1	GYPKYTSIMTDVEHYTREYYIGEEALQLKGVLKLMFPVEHGVIEDWMAMEKIWHYTFYTD	100
L.oss	GYPKYTSIMTDVEHYTREYYIGEEALQLKGVLKLMFPVEHGVIEDWMAMEKIWHYTFYTD	96
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HC1	LRVNPADHPVLLTEPPMNPESNKLKMAEILFETFNVPFHVSMQAVLSLYASGRGTGLV	180
SC1	LRVNPSDHPVLLTEPPMNPDTNRQKMAEILFNTFNVPAMYVSMQAVLSLYASGRGTGLVI	156
MK-D1	LRVDPSEHPVLLTEAPLNPRPNREKMAEIMFETFNVPALYVAMQAVLSLYASGRGTGCVI	160
L.oss	LRVDPSEHPVLLTEAPLNPRPNREKMAEIMFETFNVPALYVAMQAVLSLYASGRGTGCVI	156
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HC1	DAGDGVVHIVPIYEGFSVSHAIRIDLGRDLTAHLQKLLMARGHKFVSSAEKEIVRDIK	240
SC1	DAGDGVHIVPIYEGFAISHAIDIRIDIAGRDITHHLQKLLMARGHKFVSSAEKEIVRDIK	216
MK-D1	DIGDGVSHVPIYEGFALSHASRLDLGRDITTYLQRLLRQKGFSTTSAEKEIVRDIK	220
L.oss	DIGDGVSHVPIYEGFALSHASRLDLGRDITTYLQRLLRQKGFSTTSAEKQIVRDIK	216
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HC1	ERLCYVALEPEKELKLAEKHQGMEKQYILPDGQTITVGVERFKAPEALFNPAMVGKEDDP	300
SC1	ERLCYVALEPGKEMKLAEKHQGMEKQYILPDGNTITVGSERFMAPEAFNPALLGKEEA	276
MK-D1	EKLCYIALDPEKEIMLSKKVAGMEKVYMLPDGETITVSVVERFLAPECFNPNPSVIGKELEP	280
L.oss	EKLCYVALDPEKEIMLSKKVAGMEKVYMLPDGETITVSVVERFLAPECFNPNPSVIGKELEP	276
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HC1	LDDAIFGAVQACDIDLRKELYQNIVLSGGTTFMFPGLKERLHKELTELVPENVEIRIVAPP	360
SC1	LDDAIFSSVSSCDIDLRKELYQNIVLSGGSTMFPGLKERLHKELTELVPENVEIRIVAPP	336
MK-D1	LDDVICSAIKQCDVLDLRDLYGNIVLSGGSTMYPGLKERLTKEIKEQIPESVDVKIAPP	340
L.oss	LDDVICTAIAKACDVLDLRDLYGNIVLSGGSTMYPGLKERLTKEIKEQIPESVDVKIAPP	336
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HC1	ERRYSVWIGGSILASLKTFFQRLWISKNEYQESGGQAIKRLGF	402
SC1	ERRYSVWIGGSILSSLKTFFQRLWISKKEYEEQGVTAVKRAI-	377
MK-D1	ERMYSVWIGGSILSSLKTFFQRMWVTRREYKEIGPSIIHRCF-	381
L.oss	ERMYSVWIGGSILSSLKTFFQRMWVTRREYKEMGPQVIHRCF-	377



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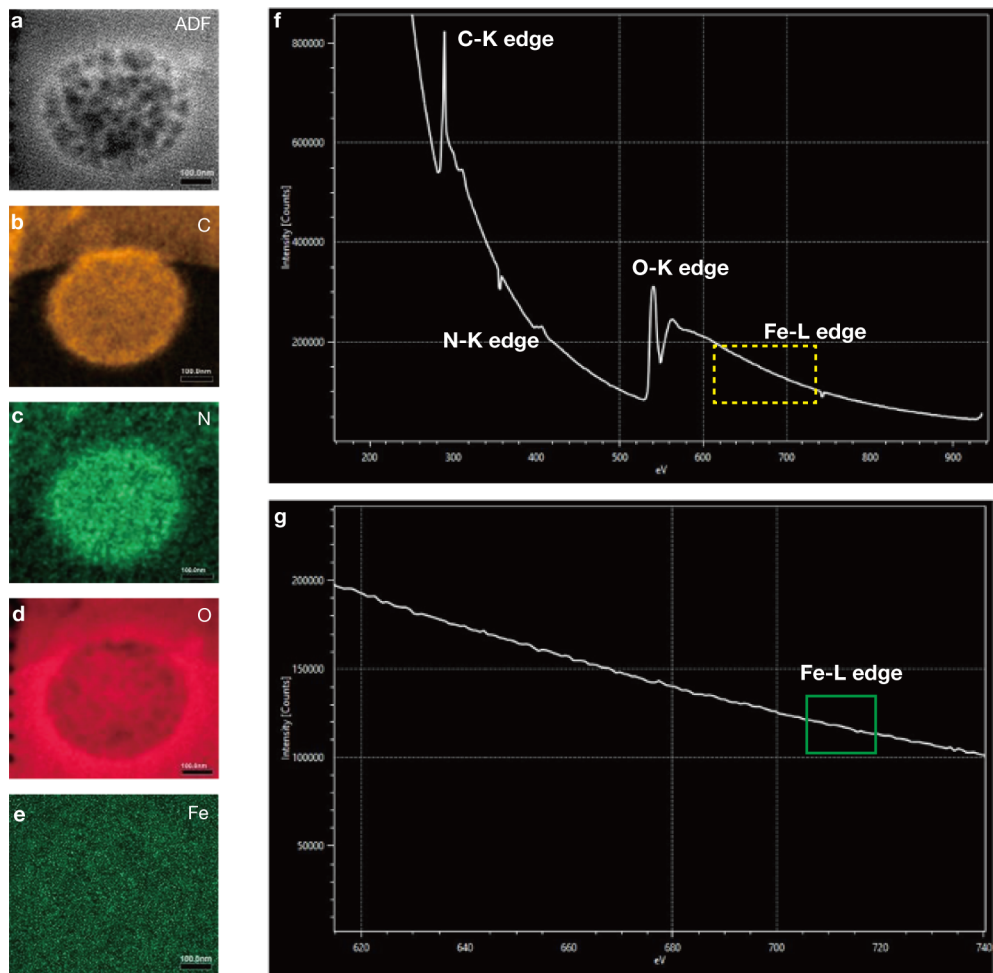
Supplementary Fig. 2 | Lokiactin amino acid sequence of cultured archaea and confirmation of their expression using western blotting analysis. a, Multiple sequence

alignment for lokiactin sequences of cultured *Promethearchaeota* strains. Sequences were aligned with Clustal Omega provided EMBL-EBI as a web service (<https://www.ebi.ac.uk/jdispatcher/msa/clustalo>). The regions colored in light blue showed that the peptides were used for creating antibodies (*i.e.*, ab1 for HC1, ab1 for SC1, and ab2 for both strains). The region highlighted in yellow in the HC1 sequence corresponds to the N-terminal extension predicted by the AlphaFold3 model (see also Extended Data Fig. 5f, g). **b**, Western blotting analysis of lokiactin-specific antibodies. Yeast lysate was used as a negative control during the western blotting analysis using HC1 cultures.

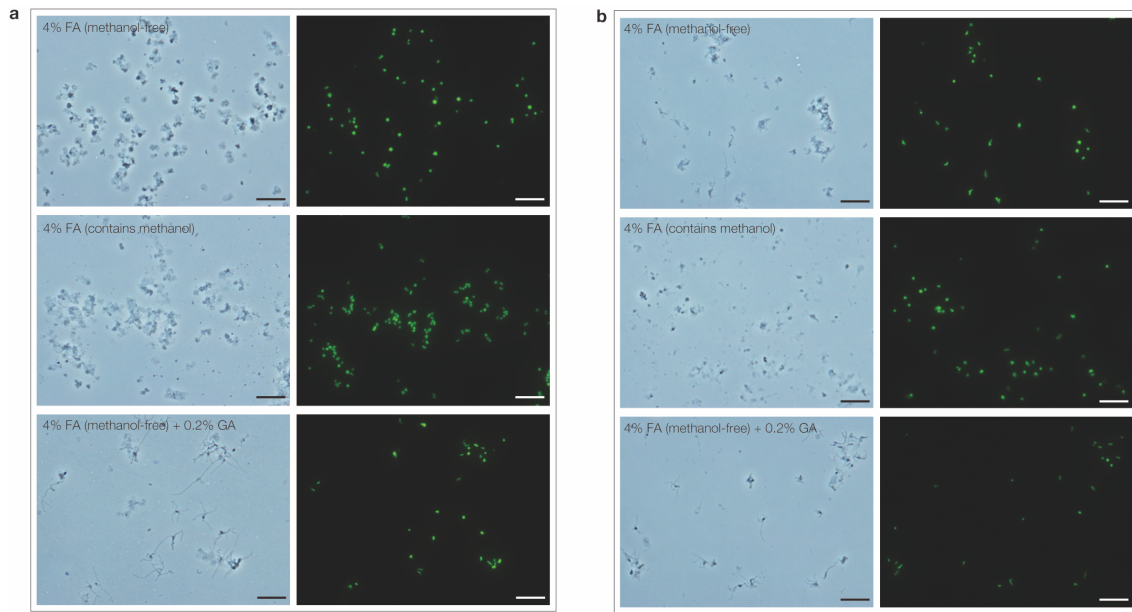


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612 **Supplementary Fig. 3 | Size distribution of granular structures in an HC1 cell.** A
613 total of 2,127 granular structures depicted in Fig. 2l were quantified using AMIRA 3D
614 Pro software.



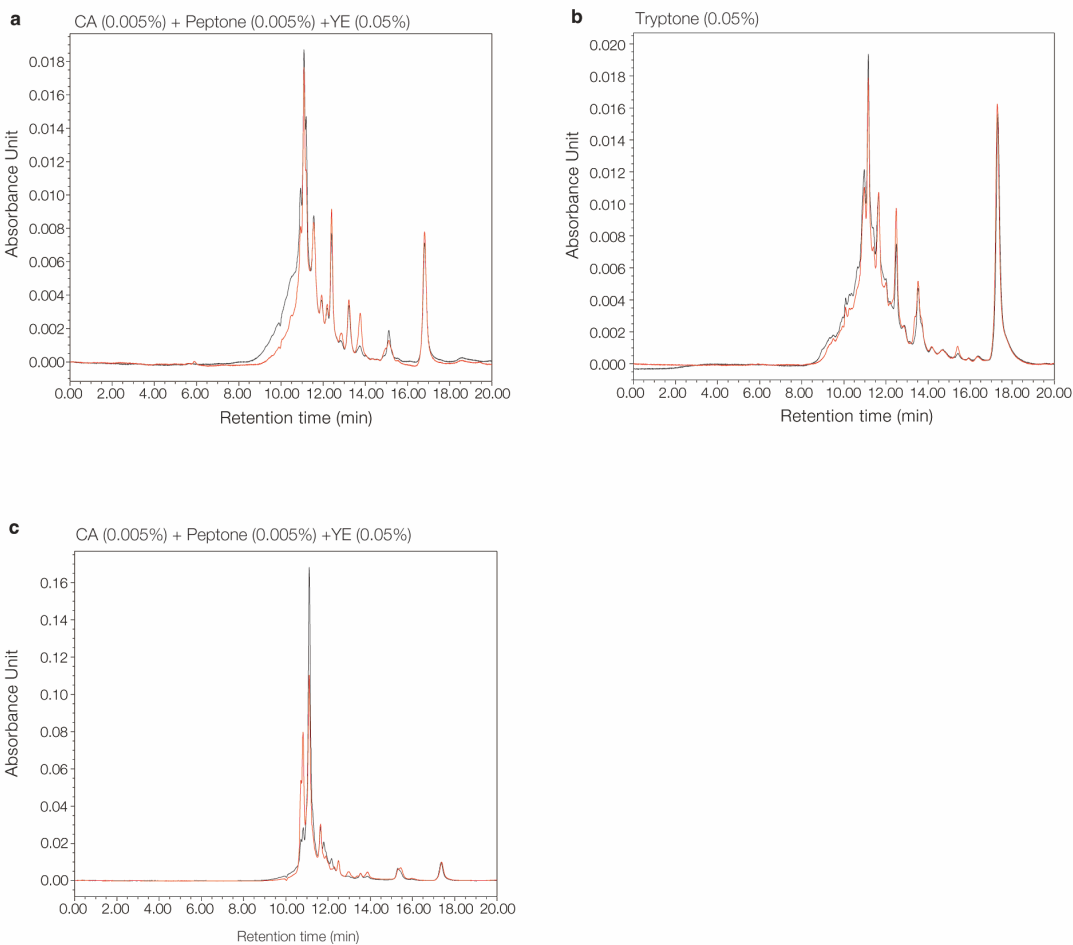
Supplementary Fig. 4 | STEM-EELS analysis of HC1. **a–e**, STEM images of a representative HC1 cell, including an annular dark field (ADF) image (**a**) and corresponding elemental maps for carbon (**b**), nitrogen (**c**), oxygen (**d**), and iron (**e**). Scale bars, 100 nm. **f**, Overview of the EELS spectrum, with the Y-axis showing intensity (count) and the X-axis indicating energy loss (eV). **g**, Enlarge view of the dashed yellow box in panel (**f**). The green box indicates the typical range of Fe-L edges. This analysis was performed on 5 cells of HC1, all of which exhibited similar results.



Supplementary Fig. 5 | Microscopy images of chemically fixed cells of HC1 and SC1.

a, Fixed cells of HC1 culture. **b**, Fixed cells of SC1 culture. Fixing solution names (formaldehyde [FA], and glutaraldehyde [GA]) are indicated for each panel. The fluorescence images on the right side show cells stained with SYBR Green I. Each fluorescence image is in the same field as the image on the left. Prior to the fixation, we confirmed that HC1 and SC1 cells produced many protrusions in these cultures using phase contrast microscopy. The experiments with HC1 and SC1 cultures were each independently repeated three times with similar results. Scale bars, 10 μm .

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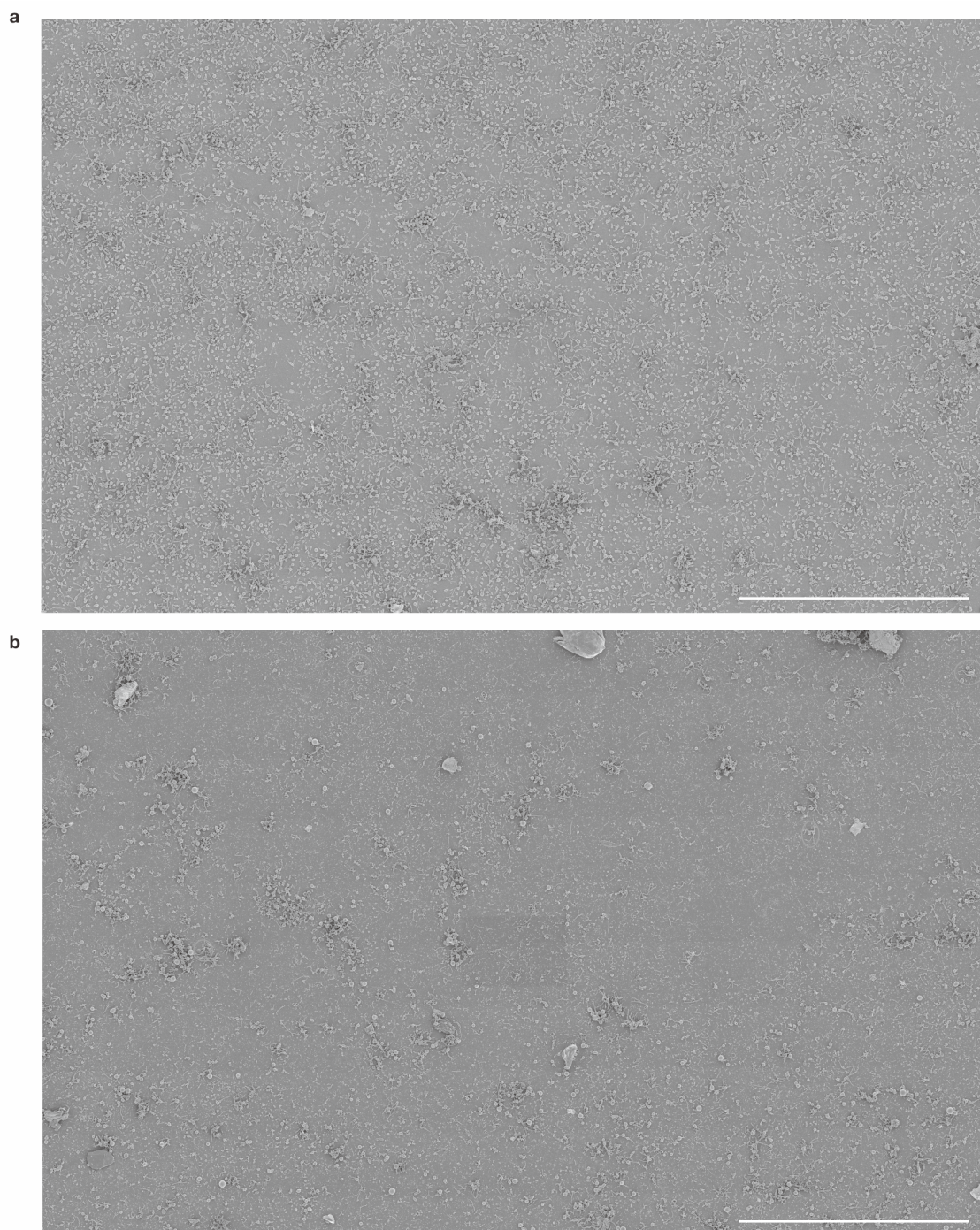
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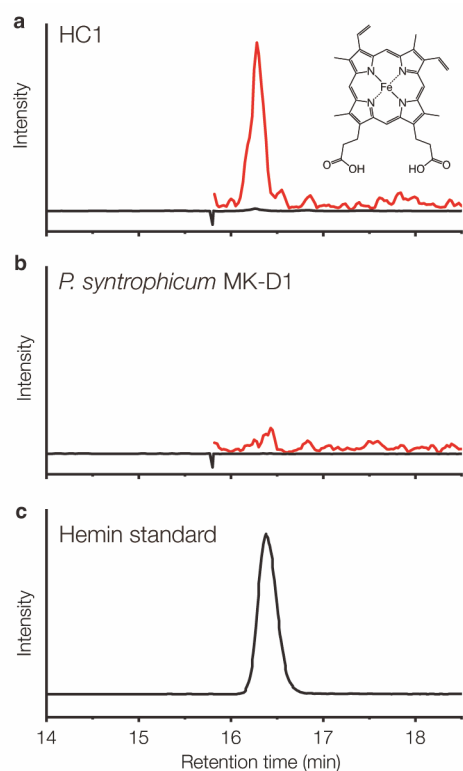
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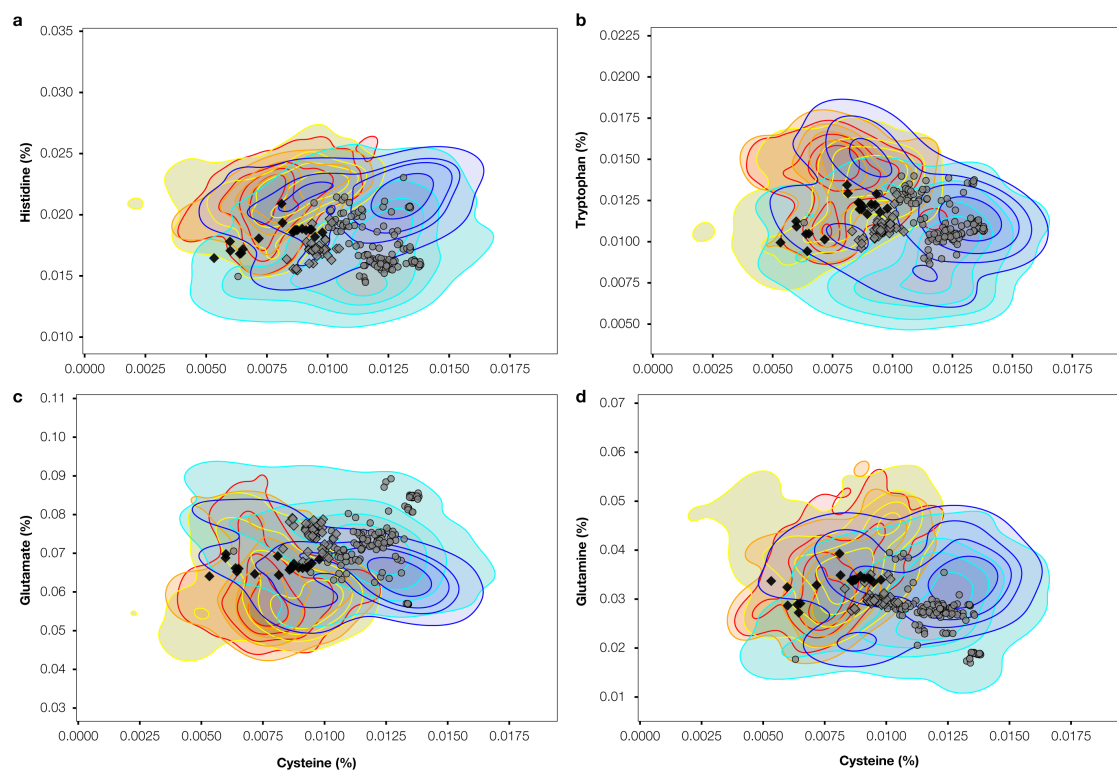
Supplementary Fig. 6 | UPLC-based detection of high molecular weight compounds in HC1 and SC1 cultures. a,b, HC1 cultures fed with casamino acids, peptone, and yeast extract (0.05%, w/v) (a), and tryptone (0.05%). **c,** SC1 culture fed with casamino acids, peptone and yeast extract (0.005%, 0.005%, and 0.05%, respectively). Black and red lines show before and after cultivation, respectively.



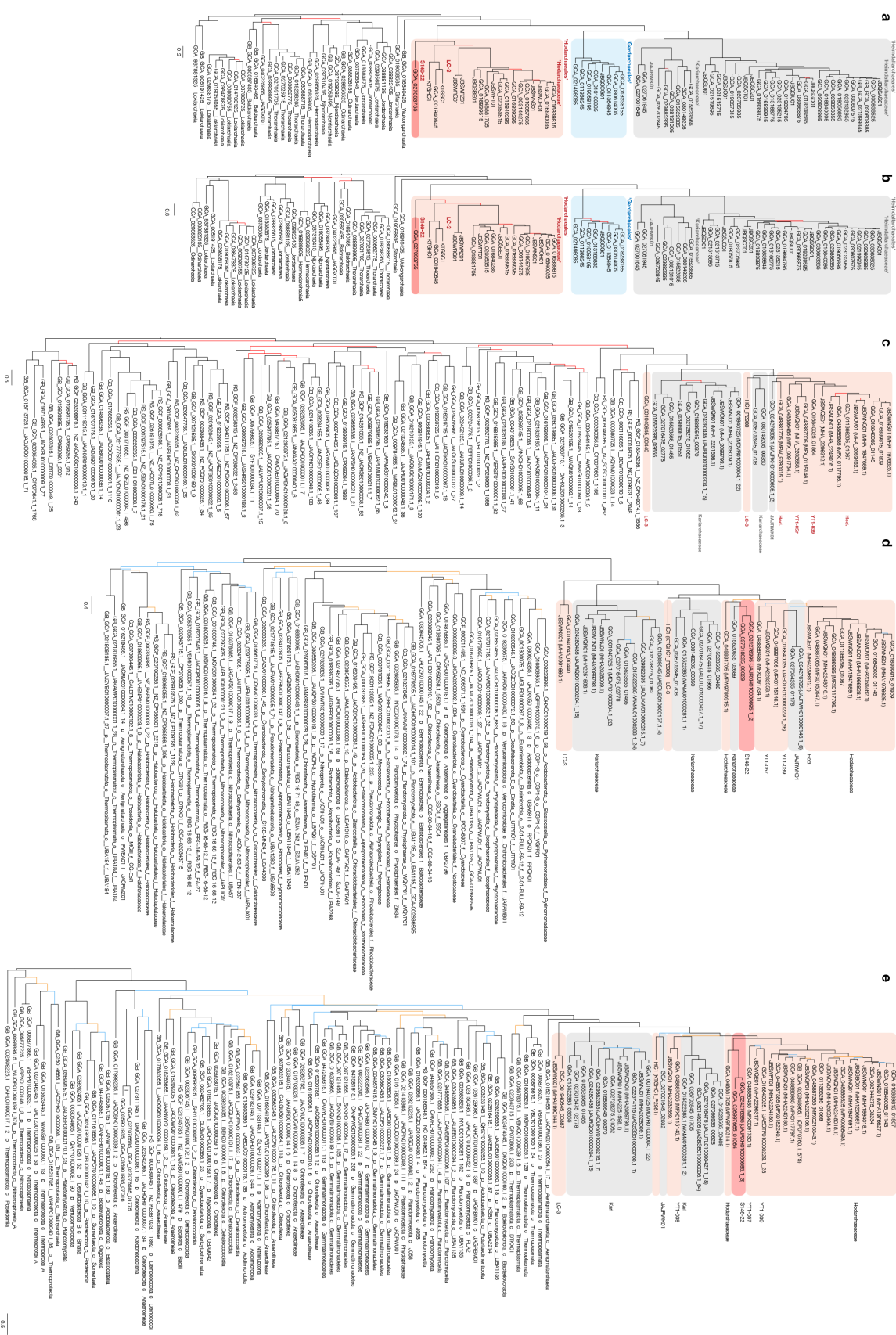
Supplementary Fig. 7 | Wide-area SEM images of HC1. a, b, HC1 cultured with 0.005% yeast extract (**a**) and 0.05% yeast extract (**b**). The culture media also contained 0.005% casamino acids and 0.005% peptone. Scale bars, 80 μ m. High-resolution versions of these images and segmentation images, and data set have been deposited on figshare (<https://doi.org/10.6084/m9.figshare.28379207>).



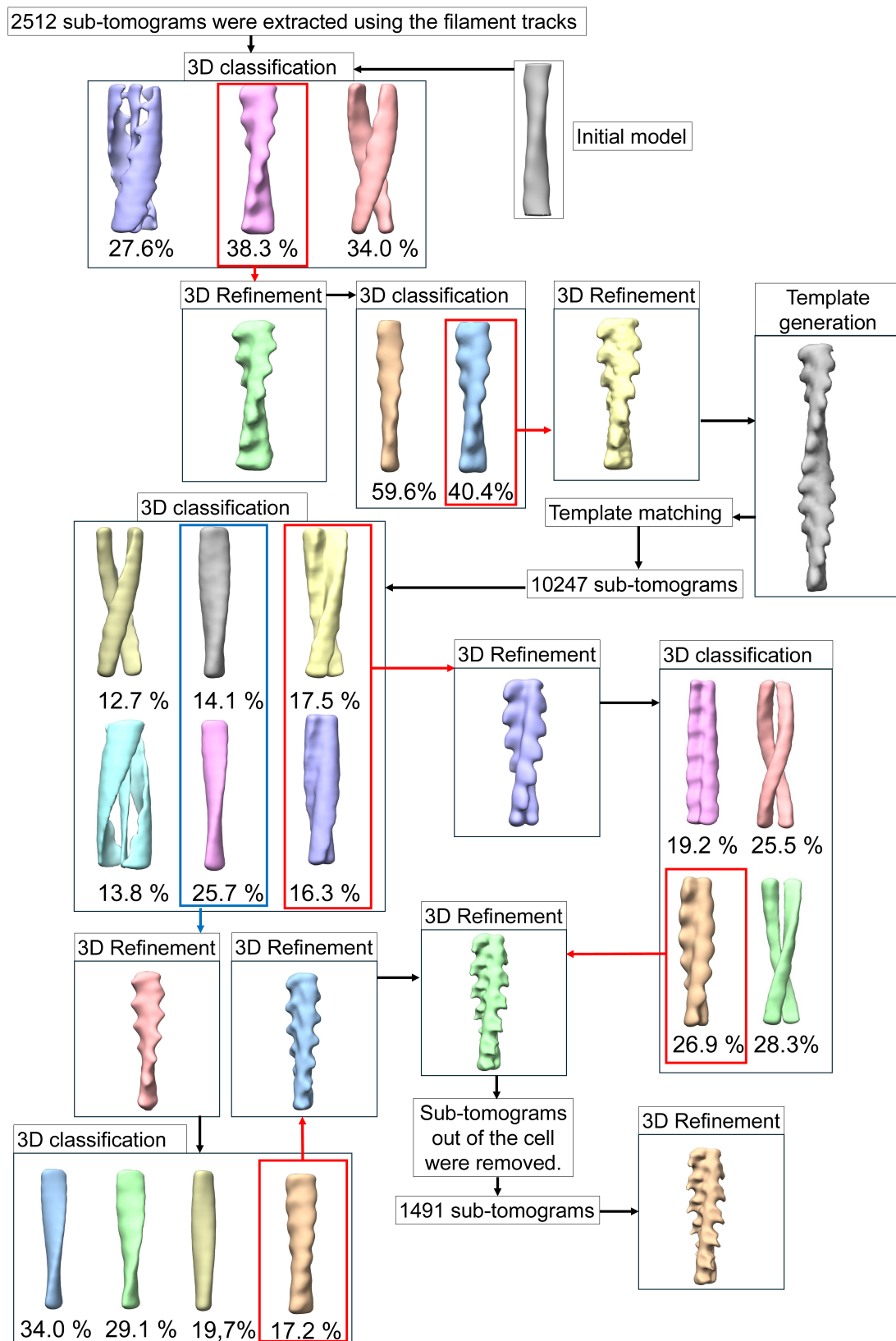
Supplementary Fig. 8 | Chromatograms of heme B. **a**, HC1. **b**, *P. syntrophicum* MK-D1. **c**, Standard material of heme B in oxidized form (hemin; $\geq 97.0\%$ purity, Sigma-Aldrich). The y-axes for **(a)** and **(b)** are shown on the same scale. Each measurement was performed in triplicate, yielding consistent results across all samples.



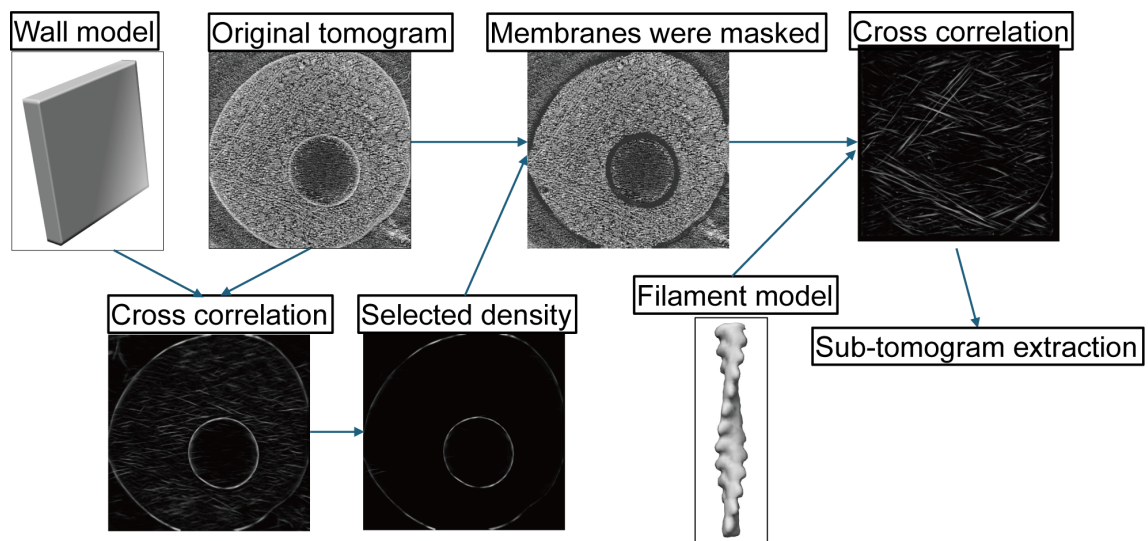
Supplementary Fig. 9 | Amino acid frequencies the genome-encoded proteomes of isolates and *Promethearchaeota* genomes. Kernel density contours indicate the distributions of obligate aerobes, diazotrophic (Nif-harboring) obligate aerobes, facultative (an)aerobes, obligate anaerobes, and cytochrome c oxidase (COX)-harboring obligate anaerobes (excluding *Promethearchaeota*). 'Heimdallarchaeia' and other *Promethearchaeota* genomes are shown as points (diamonds and circles, respectively).



Supplementary Fig. 10 | Maximum-likelihood estimations of the phylogenies of 'Heimdallarchaeia' and cytochrome *c* oxidase. **a, b,** Maximum-likelihood estimation of the phylogeny of *Promethearchaeota* based on a concatenated alignment of 47 marker genes using IQTREE with **(a)** the Poisson+UDM0064LCLR model or **(b)** the optimal model among C10, C20, C30, C40, C50, and C60 (+LG+G+F) selected by ModelFinder (LG+C60+G+F) and ultrafast bootstrap support (1000 iterations). Branches with support less than 95 are indicated in red. **c,** Maximum-likelihood estimation of the phylogeny of the Cox complex based on a concatenated alignment of COX subunits 1, 2, and 3 and tree construction using IQTREE with the optimal model among C10, C20, C30, C40, C50, and C60 (+LG+G+F) selected by ModelFinder. Ultrafast bootstrap support (1000 iterations) is shown. Branches with support less than 95 are indicated in red. **d, e,** Maximum-likelihood estimation of the phylogeny of COX1 (**d**) and COX3 (**e**) using IQTREE and optimal models selected as described above. Branch support was calculated using 100 bootstrap replicates and BOOSTER. Branches with support ≥ 75 and < 85 are colored orange and those < 75 are colored blue. Sequences belonging to 'Heimdallarchaeales' and 'Hodarchaeales' are colored gray and red respectively. The families that the sequences derive from are indicated ('Hodarchaeales' is abbreviated as "Hod."). Sequences from 'Hodarchaeales' families YT1-039 and YT1-059 were included in COX1 and COX3 analysis but not the species tree (Fig. 5g) due to the absence of high-quality MAGs.



Supplementary Fig. 11 | Schematic diagram of the sub-tomogram averaging flow.



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