Insight into the symbiotic lifestyle of DPANN archaea revealed by cultivation and genome analyses

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Decades of culture-independent analyses have resulted in proposals of many tentative archaeal phyla with no cultivable representative. Members of DPANN (an acronym of the names of the first included phyla Diapherotrites, Parvarchaeota, Aenigmarchaeota, Nanohaloarchaeota, and Nanoarchaeota), an archaeal superphylum composed of at least 10 of these tentative phyla, are generally considered obligate symbionts dependent on other microorganisms. While many draft/complete genome sequences of DPANN archaea are available and their biological functions have been considerably predicted, only a few examples of their successful laboratory cultivation have been reported, limiting our knowledge of their symbiotic lifestyles. Here, we investigated physiology, morphology, and host specificity of an archaeon of the phylum "*Candidatus* Micrarchaeota" (ARM-1) belonging to the DPANN superphylum by cultivation. We constructed a stable coculture system composed of ARM-1 and its original host *Metallosphaera* sp. AS-7 belonging to the order *Sulfolobales*. Further host-switching experiments confirmed that ARM-1 grew on five different archaeal species from three genera—*Metallosphaera*, *Acidianus*, and *Saccharolobus*—originating from geologically distinct hot, acidic environments. The results suggested the existence of DPANN archaea that can grow by relying on a range of hosts. Genomic analyses showed inferred metabolic capabilities, common/unique genetic contents of ARM-1 among cultivated micrarchaeal representatives, and the possibility of horizontal gene transfer between ARM-1 and members of the order *Sulfolobales*. Our report sheds light on the symbiotic lifestyles of DPANN archaea and will contribute to the elucidation of their biological/ecological functions.

DPANN | ARMAN | *Candidatus* Micrarchaeota | symbiotic archaea | thermoacidophiles

Decades of culture-independent analyses have revealed considerable diversity in the archaea that exist in most natural environments. As a result, there have been many tentative proposals of archaeal phyla, most of which have not yet been cultivated (1). Members of DPANN (an acronym of the names of the first included phyla Diapherotrites, Parvarchaeota, Aenigmarchaeota, Nanohaloarchaeota, and Nanoarchaeota), an archaeal superphylum composed of at least 10 of these tentative phyla, inhabit extremely diverse environments (e.g., brackish/freshwater, ocean sediments, hydrothermal vents, acid mine drainage [AMD] sites, hypersaline lakes, permafrost, and the human microbiome) (2). They share small cell and genome sizes and are generally considered obligate symbionts dependent on other microorganisms (3), whereas some of them are suggested to possess a free-living lifestyle [i.e., "*Candidatus* Altiarchaeota" (4) and "*Candidatus* Diapherotrites" (5)]. However, while many draft/complete genome sequences of DPANN archaea are available, in addition to a considerable number of predictions of biological functions, only a few examples of their successful laboratory cultivation have been reported. For instance, "*Candidatus* Nanoarchaeum equitans" (6), "*Candidatus* Nanopusillus acidilobi" (7), and "*Candidatus* Nanoclepta minutus" from the phylum "*Candidatus* Nanoarchaeota"; "*Candidatus* Mancarchaeum acidiphilum" (8) and "*Candidatus* Micrarchaeum sp. A_DKE" (9) from the phylum "*Candidatus* Micrarchaeota"; and "*Candidatus* Nanohaloarchaeum antarcticus" from the phylum "*Candidatus* Nanohaloarchaeota" (10) were reported as cultivated representatives of DPANN archaea.

The phylum *Ca.* Micrarchaeota, one of the DPANN lineages (2, 11), was first discovered in an AMD site on Iron Mountain in 2006 (12). This phylum, along with "*Candidatus* Parvarchaeota" (13), was originally referred to as the archaeal Richmond Mine acidophilic nanoorganism (ARMAN). Although these two phyla had been called the same name "ARMAN," the current phylogeny based on genomic information showed that both lineages are found in association with other archaea (1), indicating their distant relationship. They are known as some of the smallest organisms, with cell volumes as low as 0.009 μm^3, which is close to a

Significance

The DPANN superphylum is a grouping of symbiotic microorganisms categorized based on their genomic contents and a few examples of cultivation experiments. Although the genome information of DPANN archaea is increasing year by year, most of them have remained uncultivated, limiting our knowledge of these organisms. Herein, a thermoacidophilic symbiotic archaeon (ARM-1) from the DPANN superphylum was successfully cultivated and characterized. We determined its physiological, morphological, and genomic characteristics in detail and obtained experimental evidence of the symbiotic lifestyle of this archaeon. Notably, ARM-1 is a symbiotic archaeal strain that showed dependence on a range of host species in a laboratory culture. The results significantly contribute to the true understanding of the physiology and ecology of DPANN archaea.

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The authors declare no competing interest.

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theoretical size limit for life (0.008 μm^3) (14). While *Ca.* Parvarchaeota has only been detected in AMD and hot spring environments, *Ca.* Micrarchaeota has been detected in a variety of natural environments (e.g., AMD sites, hot springs, soil, peat, hypersaline mats, and freshwater) (15). The successful cultivation of *Ca.* Micrarchaeota (with host species) was achieved recently, when three research groups reported that it was cultivated with members of the order *Thermoplasmatales* in the same enrichment cultures (8, 9, 15). In addition, comprehensive phylogenetic analyses revealed some instances of horizontal gene transfer between *Ca.* Micrarchaeota and a member of the order *Thermoplasmatales* (15). These results suggest that *Ca.* Micrarchaeota depends on members of the order *Thermoplasmatales*. However, considering the detection of *Ca.* Micrarchaeota in a variety of environments, it may be possible that different representatives of this phylum have different host requirements. In addition, the existing cultivation systems for *Ca.* Micrarchaeota seem to be difficult to handle, especially with respect to anoxic conditions and slow growth rates (8, 9, 15), which would be a major obstacle for elucidating their symbiotic mechanisms.

Here, we report a coculture system composed of a thermoacidophilic archaeon belonging to the phylum *Ca.* Micrarchaeota (designated ARM-1) and *Metallosphaera* sp. AS-7 belonging to the order *Sulfolobales* from an Indonesian acidic hot spring. Since this coculture system is easy to handle (the system exhibits fast growth, can be cryopreserved, and is easy to maintain under aerobic conditions), we determined the detailed physiological properties of ARM-1 (e.g., temperature/pH ranges, complex substrate/sugar utilization, energy sources, and host specificity) and experimental evidence of its symbiotic lifestyle with *Metallosphaera* sp. AS-7 as well as with the four other members of the order *Sulfolobales*. The results of the coculture experiments provide insights into the physiology and symbiotic lifestyle of this DPANN archaeon.

Results and Discussion

Establishment of a Stable Coculture System. The initial enrichment culture, EnAS_65, was obtained from hot, acidic spring water collected at Kawah Domas hot springs in Tangkuban Perahu, Indonesia. A total of 37 clones were derived from EnAS_65. They were grouped into three operational taxonomic units (OTUs) (Dataset S1) and classified as *Metallosphaera* species (7 clones), *Thermoplasma* species (27 clones), and an archaeon designated ARM-1 (3 clones). However, *Metallosphaera* species and *Thermoplasma* species appeared to be considerably more abundant than ARM-1 on the basis of the total number of clones. Thus, we attempted to enrich ARM-1 from glycerol stocks of EnAS_65 and sought the best culture conditions by applying several different media and incubation temperatures (*SI Appendix, Supplementary Text* and Figs. S1 and S2). Consequently, a coculture system (referred to as EnM) composed of only ARM-1 and *Metallosphaera* species (*Metallosphaera* sp. AS-7), confirmed by genome sequencing as described below, was obtained. This coculture system even showed a significantly higher abundance of ARM-1 than *Metallosphaera* sp. AS-7, which was confirmed by qPCR and microscopy as described below.

Microscopic Observations. In the EnM coculture system, irregular coccus cells 0.7 to 1.4 μm in diameter (mean 0.9 ± 0.2 μm, $n = 20$) and nanosized coccus cells 240 to 440 nm in diameter (mean 320 ± 40 nm, $n = 60$) were observed (Figs. 1 and 2). Fluorescence in situ hybridization (FISH) analysis showed that nanosized cells were derived from ARM-1 (Fig. 1*C*). In the exponential phase, a large number of ARM-1 cells attached to AS-7 cells (Fig. 1*C*) whereas most ARM-1 cells occurred singularly in the stationary phase (Fig. 1*A* and Movie S1). This behavior was similar to that of *Ca*. N. equitans and its host *Ignicoccus hospitalis*, which showed a significantly increased number of free N. equitans cells toward the end of cocultivation (16, 17). Up to 11 ARM-1 cells attaching to a single AS-7 cell were observed. Unlike previous reports of the cultivation of *Ca.* Micrarchaeota A_DKE (9), cell aggregation was rarely observed. Division of ARM-1 cells was occasionally observed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) (Figs. 1 *E* and *F* and 2 *B* and *H*). Dividing ARM-1 cells were observed to either attach to a host cell (Fig. 2 *B* and *H*) or not attach to any host cell (Fig. 1*E*). Since most ARM-1 cells in the exponential phase attach to AS-7 cells, cell division of ARM-1 most likely starts while attaching to host cells. Therefore, the latter observation is probably explained by detachment of dividing ARM-1 cells while in that stage, although we cannot exclude the possibility that some ARM-1 cells may undergo cytokinesis away from the host. Under TEM, the cell-to-cell interaction between ARM-1 and *Metallosphaera* sp. AS-7 in the use of fibrous structures or direct cell-surface connections was observed (Fig. 2 *C–F*). Fibrous structures may contribute to attaching to the cell surface of host organisms or obtaining nutrients from the host. Direct cytoplasmic connections, such as those reported in the phylum *Ca.* Nanoarchaeota (another taxon of DPANN) (18), have not been observed thus far. Both inner and outer membranes were observed in ARM-1 (Fig. 2*A*), which coincides with previous reports for the phylum *Ca*. Micrarchaeota (14). On the other hand, an S layer–like structure, a typical feature of the genus *Metallosphaera* (19), was observed in *Metallosphaera* sp. AS-7 (Fig. 2 *C* and *E*). No flagella were observed for ARM-1 and AS-7 under negative-staining TEM (Fig. 2 *G* and *H*). Extracellular vesicle (EV)–like structures, of which sizes were ∼100 nm in diameter, were occasionally detected near AS-7 cells (Fig. 2*G*). Recently, EVs produced by the thermoacidophilic crenarchaeon *Sulfolobus islandicus* were shown to carry proteins as well as chromosomal and plasmid DNA. Moreover, EVs supported the heterotrophic growth of *S. islandicus* in minimal medium (20), suggesting that EVs can serve as a source of both carbon and nitrogen. If the EV-like structures observed under negative-staining TEM also carry proteins, DNA, or carbon/nitrogen sources, they may possibly provide some nutrients to ARM-1. The electron density of the cytoplasm in ARM-1 cells was higher than that observed in AS-7 cells (Fig. 2 *A–F*), indicating that metabolites were densely packed in the nanosized cells. The existence of both inner and outer membranes may contribute to sustaining the cell structure with densely packed metabolites.

Physiological Properties of ARM-1. The growth temperature and pH range for ARM-1 were 50 to 75 °C and pH 1.5 to 4.5, respectively (*SI Appendix*, Figs. S3 and S4). The highest ARM-1 cell density was observed at 55 °C (∼5.5×10^9 copies of 16S ribosomal RNA [rRNA] gene per milliliter; *SI Appendix*, Fig. S3), while the shortest doubling time was observed at 70 °C (8.4 h; Dataset S2). The qPCR results, showing a significantly larger abundance of ARM-1, were in good agreement with microscopic observations (Fig. 1 and Movie S1). The growth rate of *Ca*. Micrarchaeota (ARMAN) has been thought to be very slow since the number of ribosomes in their cells is quite low (92 per cell on average) (14). In contrast, ARM-1 showed a relatively fast growth rate, sometimes even faster than that of the host species (Dataset S2). This result suggests that protein production occurs highly efficiently because of its nanosized cells, even with a lower number of ribosomes. However, for confirmation, we need to examine the actual ribosome number in ARM-1. Although ARM-1 grew at 75 °C during 4 to 6 d of incubation, the cell density dramatically decreased after day 6 and did not exceed the initial cell density (*SI Appendix*, Fig. S3). The results indicated that ARM-1 is not able to survive at temperatures of 75 °C or higher. Growth of ARM-1 under autotrophic conditions occurred on either S^0 or FeS_2 (*SI Appendix*, Fig. S5). Because none of the known carbon fixation

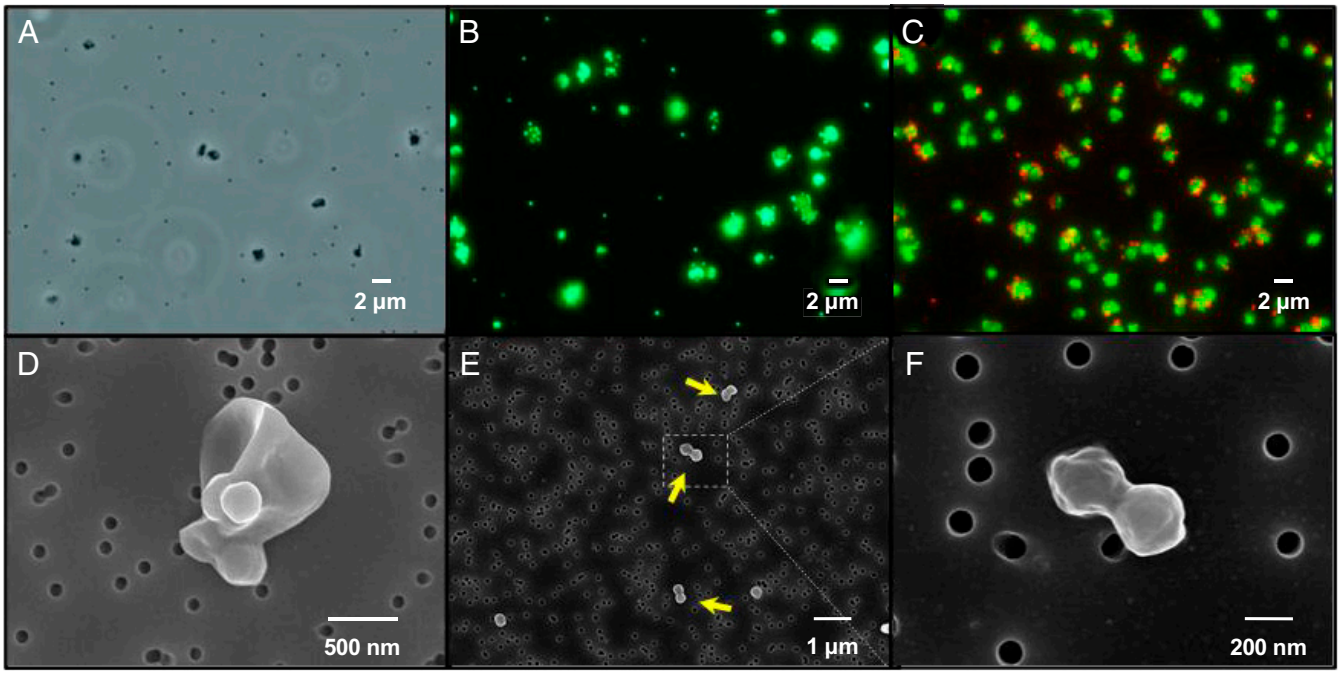

Fig. 1. Microscopic observation of a coculture system composed of ARM-1 and *Metallosphaera* sp. AS-7. (*A*) Cells examined by phase-contrast microscopy in the stationary phase. (*B*) Fluorescence microscopy (stained with SYBR Green I). (*C*) FISH analysis of ARM-1 (red) and AS-7 (green) cells in the exponential phase. (*D*–*E*) SEM. (*D*) ARM-1 cells attached to AS-7 cells. (*E* and *F*) Dividing ARM-1 cells (yellow arrows).

pathways were found in the ARM-1 genome, as described below, ARM-1 possibly utilizes organic substances that are autotrophically synthesized by *Metallosphaera* sp. AS-7, which possesses a carbon dioxide assimilation pathway called the 3-hydroxypropionate/4-hydroxybutyrate cycle (21). Growth of ARM-1 under heterotrophic conditions (in the presence of AS-7) occurred on yeast extract, casamino acids, peptone, tryptone, glucose, and sucrose (*SI Appendix*, Fig. S5). These substrates also supported the growth of pure cultures of AS-7 (*SI Appendix*, Fig. S6). The growth of ARM-1 on complex substrates was remarkably faster than that on the sugars (Dataset S2). Although faster growth might occur simply due to the better growth of the host (which may produce more necessary nutrients for ARM-1), the results suggested that nucleic acids, peptides, polypeptides, amino acids, vitamins, or other constituents in the complex substrates are important factors for the growth of ARM-1. Similar with that of *Ca.* N. equitans (16, 17), in most cases, the growth of ARM-1 stopped when the host entered the mid- to late-stationary phase (*SI Appendix*, Figs. S3–S5), implying that "actively growing" host cells rather than dead cells are important for the growth of ARM-1. Furthermore, ARM-1 did not grow without the addition of host species (Fig. 3), indicating that they are not free-living organisms but rather obligate symbionts. The filtrate of AS-7 culture did not support the growth of ARM-1.

Growth of ARM-1 on Various *Sulfolobales* Strains. We found that the growth of ARM-1 was also supported by the following members of the order *Sulfolobales*: *Acidianus brierleyi* DSM1651^T, *Metallosphaera sedula* DSM5348^T, *Metallosphaera hakonensis* DSM7519^T, and *Saccharolobus solfataricus* DSM1616^T (Fig. 3). On the other hand, no growth of ARM-1 was observed on *Sulfolobus acidocaldarius* DSM639^T, *Sulfuracidifex metallicus* DSM6482^T, *Sulfodiicoccus acidiphilus* HS-1^T, *Sulfurisphaera ohwakuensis* TA-1^T, or *Thermoplasma* sp. AS-7 (*SI Appendix*, Fig. S7).

Ca. Micrarchaeota has been thought to depend on cell wall–deficient archaea of the order *Thermoplasmatales* since its discovery (8, 9, 13–15). However, our coculture experiments showed that ARM-1 grew on at least five different *Sulfolobales* species, each of which was isolated from a different acidic hot spring (22–26). In addition, ARM-1 did not grow on *Thermoplasma* sp. AST-1 isolated from the same acidic hot spring (*SI Appendix*, *Supplementary Text* and Fig. S7). These results suggested that host specificities in *Ca.* Micrarchaeota are not limited to a specific taxon. Given the diverse microorganisms existing in natural environments, the use of many hosts should confer greater advantages than the use of a single specific host, which might be a survival strategy of this archaeon. ARM-1 could have the ability to obtain its essential nutrients from a variety of host organisms in its habitat. Notably, having a range of hosts is particularly different from other cultivated DPANN archaea such as *Ca*. Nanoarchaeota, which is known to have a specific host (6, 7, 27).

Genome Analyses. A total of 122,606 long reads (1,185,209,857 bp) were obtained from a PacBio RS II sequencer. After filtering the long reads by Filtlong, a total of 25,155 long reads of high quality (500,009,373 bp) were obtained. A total of 2,231,542 short reads (1,087,167,027 bp) were also obtained from an Illumina MiSeq sequencer. After coassembly of the long and short reads, two complete circular genomes having 814,439 bp (for ARM-1) and 2,464,819 bp (for *Metallosphaera* sp. AS-7), respectively, were obtained. Each of the genomes contained only a single copy of 16S rRNA. No contigs possessing other 16S rRNA genes were detected, indicating that only two species existed in the coculture system. The genome of ARM-1 has 849 protein coding sequences (CDSs), 3 rRNAs, and 46 transfer RNAs (tRNAs), with a GC content of 34.1%, while that of *Metallosphaera* sp. AS-7 has 2,976 CDSs, 2 rRNAs, and 47 tRNAs, with a GC content of 47.1% (Dataset S3).

The metabolic capability of ARM-1 was predicted on the basis of annotation information (Fig. 4 and Dataset S4). ARM-1 has a complete tricarboxylic acid (TCA) cycle (*SI Appendix*, Fig. S8) and mevalonate (MVA) pathway. The MVA pathway was the eukaryote type (*SI Appendix*, Fig. S9), which is rare in archaea

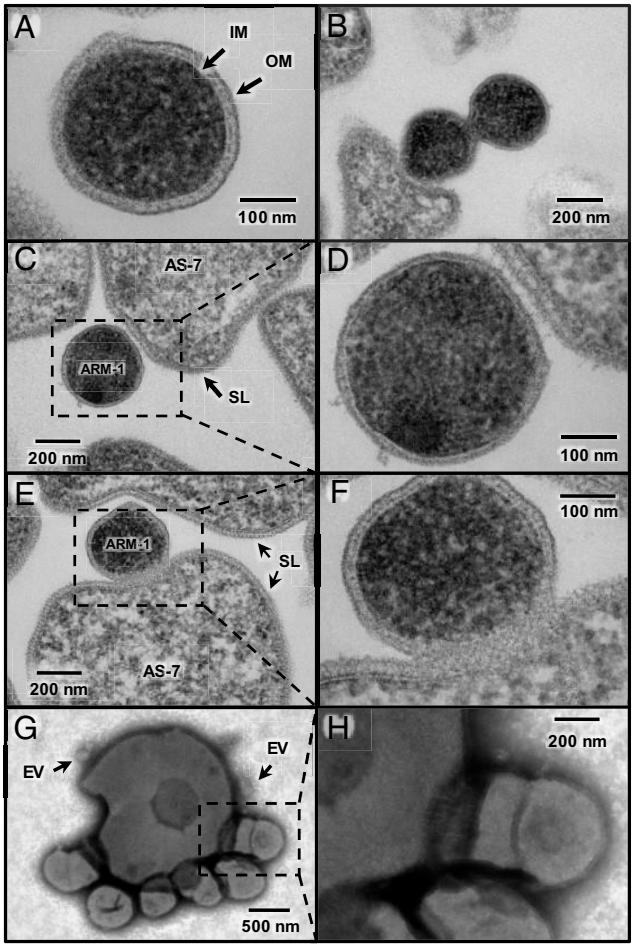

Fig. 2. TEM of a coculture system composed of ARM-1 and *Metallosphaera* sp. AS-7. (*A*) A single ARM-1 cell. (*B*) Dividing ARM-1 cells attached to a host cell. (*C* and *D*) Cell-to-cell interaction using fibrous structures. (*E* and *F*) Direct cell-surface connection. (*G* and *H*) Negative staining of ARM-1 and AS-7. IM, inner membrane; OM, outer membrane; SL, S layer–like structure; EV, extracellular vesicle–like structure.

but detected in the order *Sulfolobales*, to which host organisms of ARM-1 belong (28). The existence of the TCA cycle, a nearly complete respiratory chain (complexes I, II, IV, and V), and genes for oxidative stress (*cydA*, *sod*, and *ahpC*) were in good agreement with the aerobic lifestyle of ARM-1, which differed from a previous report that suggested a microaerobic/anaerobic lifestyle of this taxon (15). Most genes for glycolysis, gluconeogenesis, the pentose phosphate pathway, carbon/nitrogen fixation, sulfur/nitrogen assimilation, and amino acid/nucleotide/cofactor/vitamin biosynthesis were absent, consistent with the extreme dependency of ARM-1 on the host organisms. A number of transporter genes and archaea-type flagellum genes (*flaJ*, *flaI*) (29) may contribute to obtaining metabolites produced from host organisms. ARM-1 may have the capability of protein and RNA degradation since genes for protein degradation (cysteine/metallo/serine/threonine peptidases) and RNA degradation (*rrp41*, *rrp42*, *rrp4*, *csl4*) were found, possibly contributing to the acquisition of amino acids and nucleotides. Genes for cell division (*minD*, *ftsZ*, *sepF*), cell-wall construction (S layer–like protein), and DNA replication (*polD*, *pcn*, *rpa*, etc.) indicate that the observation of dividing ARM-1 cells under SEM and TEM was not a methodological artifact (Figs. 1 and 2).

Phylogenetic analyses of the 16S rRNA genes (*SI Appendix*, Figs. S10 and S11) indicated that ARM-1 and *Metallosphaera* sp. AS-7 belong to the phylum *Ca*. Micrarchaeota and the genus *Metallosphaera*, respectively. The 16S rRNA gene sequence similarities and average amino acid identity (AAI) values between ARM-1 and other cultivated representatives of *Ca*. Micrarchaeota were 80.3 to 81.5 and 43.6 to 44.1%, respectively (Datasets S5 and S6), suggesting that ARM-1 represents a novel order of this phylum (30). The 16S rRNA gene of ARM-1 contained a 522-bp insertion sequence at position 1052 (*Escherichia coli* position 1093) encoding a hypothetical protein (*SI Appendix*, Fig. S12 and Dataset S4). The amino acid sequence of the protein showed 25.4% similarity to "hypothetical protein UNLARM2_0643" (National Center for Biotechnology Information [NCBI] accession no. EET90204), which also exists in the 16S rRNA gene of "*Ca.* Micrarchaeum acidiphilum" ARMAN-2 as an insertion (13). No significant homology with any other protein sequence was found. The function of the protein as well as whether it is spliced out from mature 16S rRNA are currently uncertain. To our knowledge, the insertion sequence in the 16S rRNA gene has been reported in the phyla *Crenarchaeota* (the orders *Desulfurococcales* and *Thermoproteales*) (31), "*Ca.* Aigarchaeota" (32), and *Ca.* Micrarchaeota (ARMAN) (12). Coincidentally, an insertion sequence at *E. coli* position 1093 was also reported in several members of the phyla *Crenarchaeota* and "*Ca.* Aigarchaeota" (31), suggesting that this position somehow tends to have insertion sequences in certain archaeal lineages by unknown mechanisms. Although the insertion sequence is uncommon in Bacteria, several members of the family *Beggiatoaceae* in the phylum *Proteobacteria* have an insertion sequence in the 16S rRNA gene (33).

The AAI value between *Metallosphaera* sp. AS-7 and its closest relative, *M. sedula*, was 87.7%, suggesting that AS-7 represents a novel species of the genus *Metallosphaera*. The genome tree constructed for ARM-1 showed topology similar to that of the maximum-likelihood (ML) tree of the 16S rRNA gene (Fig. 5 and *SI Appendix*, Fig. S10), indicating that ARM-1 is the most deep-branching lineage among cultivated representatives of *Ca.* Micrarchaeota [ARM-1, A_DKE, and Mia14 (8, 9)], although other clades composed of metagenome assembled genomes (MAGs) derived from groundwater and freshwater sediments were located more deeply in the genome tree. The genome tree indicates that ARM-1 retains more ancestral characteristics than A_DKE and Mia14, suggesting the thermophilic and acidophilic origins of these organisms. The genome size of ARM-1 was 0.14 to 0.2 Mbp smaller than that of A_DKE and Mia14, with many fewer CDSs (Dataset S3), suggesting that gene acquisitions occurred in A_DKE and Mia14 during the course of evolution. The recently described new member of the phylum *Ca.* Micrarchaeota, "*Ca.* Fermentimicrarchaeum limneticum" (34), derived from a freshwater lake metagenome, was distantly placed in other clades composed of MAGs originating from various environments (Fig. 5).

The results of BLASTP searches among ARM-1 and cultivated micrarchaeal representatives (A_DKE and Mia14) (8, 9) were visualized by the Circos program (Fig. 6). The total numbers of bidirectional best hits between ARM-1/A_DKE, ARM-1/Mia14, and Mia14/A_DKE were 491, 459, and 583, respectively (Datasets S7, S8, and S9), consistent with the topologies of the phylogenetic trees, in which Mia14 and A_DKE were more closely clustered (Fig. 5 and *SI Appendix*, Fig. S10). A total of 414 genes were determined to be core genes (Dataset S10), from which 286 were assigned to clusters of orthologous groups (COG) functional categories (Fig. 7). The 286 genes were mainly composed of COG functional categories of J (translation, ribosomal structure, and biogenesis), L (replication, recombination, and repair), O (posttranslational modification, protein turnover, and chaperones), C (energy production and conversion), and K (transcription). On the other hand, 311 to 324 genes were found to be unique genes in

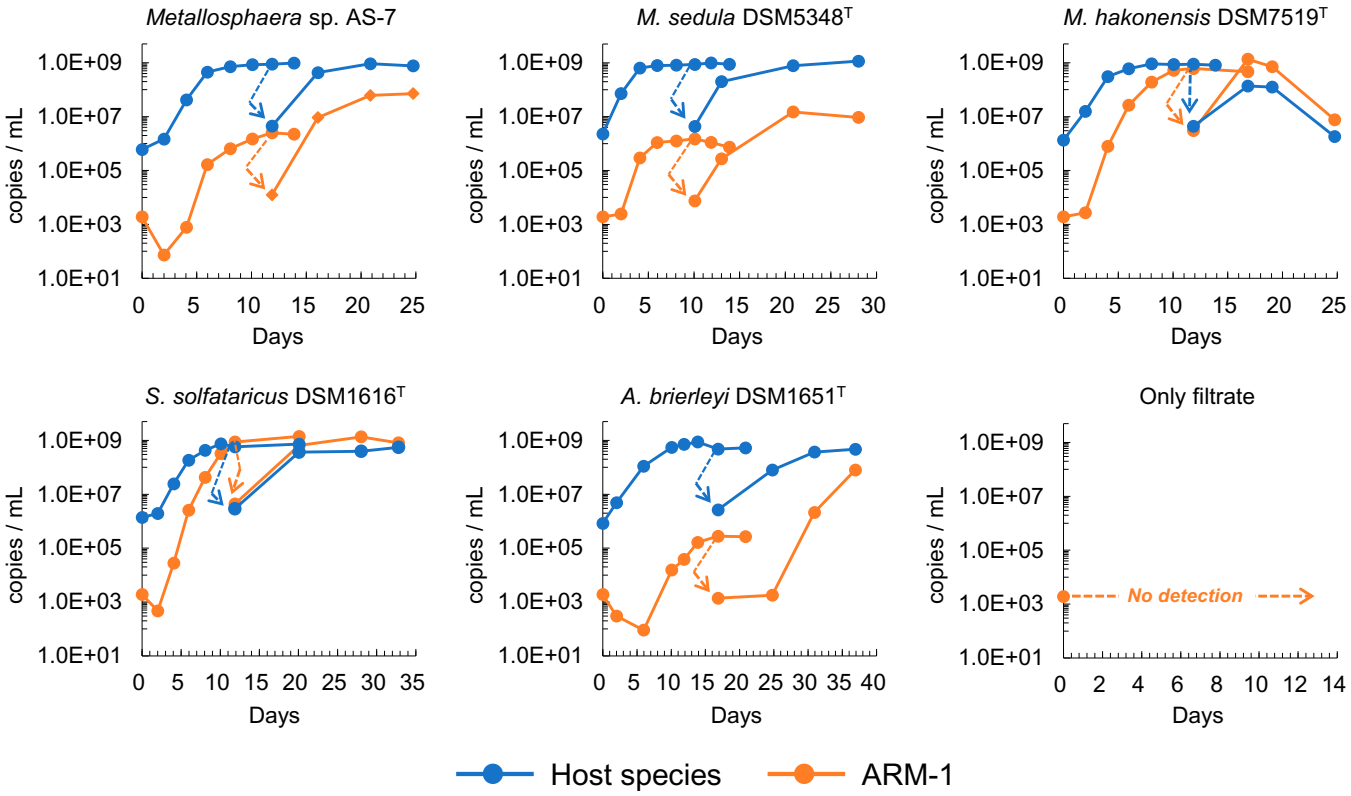

Fig. 3. Growth curves of ARM-1 on various thermoacidophilic species. The *x* axis indicates the copy number of the 16S rRNA gene calculated by qPCR. Subcultivation was performed at the points indicated by arrows. For other archaeal species that did not support the growth of ARM-1, see *SI Appendix*, Fig. S7.

each representative, and most of them were not assigned to any COG functional category (Fig. 7 and Datasets S11, S12, and S13). The genes for reverse gyrase (*rgy*), CRISPR-associated proteins (*cas1* to *7* with CRISPR repeats), and succinyl-CoA synthetase (*sucC* and *sucD*) were identified in the unique genes of ARM-1. The gene *rgy* is known as a genomic signature for hyperthermophiles (and extreme thermophiles) (35), which is in good agreement with the unique thermophilic characteristics of ARM-1 among cultivated micrarchaeal species. Of note, the gene *rgy* has not been reported in other members of the phylum *Ca*. Micrarchaeota. The existence of CRISPR-associated genes with CRISPR repeats indicates that ARM-1 was previously exposed to a virus attack in its habitat and possesses an antiviral defense system. Recently, the CRISPR-Cas9 system was discovered in Archaea from a MAG of the phylum *Ca*. Micrarchaeota (36). On the other hand, the CRISPR-Cas system found in ARM-1 was not CRISPR-Cas9 (class 2); it appeared to be class 1 (subtype I-B) based on the organization of the *cas* genes (37, 38). Since *sucC* and *sucD* are essential components of a complete TCA cycle and were not found in other cultivated micrarchaeal representatives, we conducted phylogenetic analyses of these two genes to investigate where the genes originated from. In the phylogenetic trees of each gene, they were placed in the cluster composed of members of the order *Sulfolobales* (*SI Appendix*, Figs. S13 and S14). This finding suggests that *sucC* and *sucD* of ARM-1 were possibly acquired by horizontal gene transfer from members of the order *Sulfolobales*. Chen et al. reported that only 1 MAG out of 29 MAGs of *Ca.* Micrarchaeota contained *sucC* and *sucD* (15), indicating that these genes are rare in this taxon. The existence of *sucC* and *sucD*, allowing ARM-1 to have a complete TCA cycle, possibly contributes to the faster growth of ARM-1 than that of other cultivated micrarchaeal representatives.

Conclusions

Here, we report the detailed physiological, morphological, and genomic characteristics of an archaeon of the phylum *Ca.* Micrarchaeota in the DPANN superphylum (referred to as ARM-1), expanding our knowledge of the symbiotic lifestyles of DPANN archaea. At least five different host species were able to support the growth of ARM-1, suggesting that some DPANN archaea in natural environments also have a wider range of host dependency. Genomic analyses showed inferred metabolic capabilities, common/unique genetic contents of ARM-1 among cultivated micrarchaeal representatives, and the possibility of horizontal gene transfer between ARM-1 and members of the order *Sulfolobales*.

Based on the experimental data presented above, we surmise that the general life cycle of ARM-1 is as follows: 1) ARM-1 cells generally occur singularly (after cell division) until they find appropriate host cells; 2) ARM-1 cells attach to "actively growing" host cells and obtain necessary nutrients through unknown mechanisms; 3) after obtaining necessary nutrients, the cells start cell division on a host cell; and 4) after cell division, the daughter cells start to seek other host cells in their habitat.

We believe that our coculture system is easier to handle than existing coculture systems reported for *Ca.* Micrarchaeota (the system exhibits fast growth, can be cryopreserved/regenerated, and is easy to maintain under aerobic conditions). This coculture system, deposited in the Japan Collection of Microorganisms (JCM) and Indonesian Culture Collection (InaCC), will certainly contribute to a greater understanding of the symbiotic lifestyles of DPANN archaea. Finally, based on physiological, morphological, phylogenetic, and genomic analyses, we propose *Microcaldus variisymbioticus* (phylum: *Microcaldota*; class: *Microcaldia*; order: *Microcaldales*; family: *Micracardaceae*) for

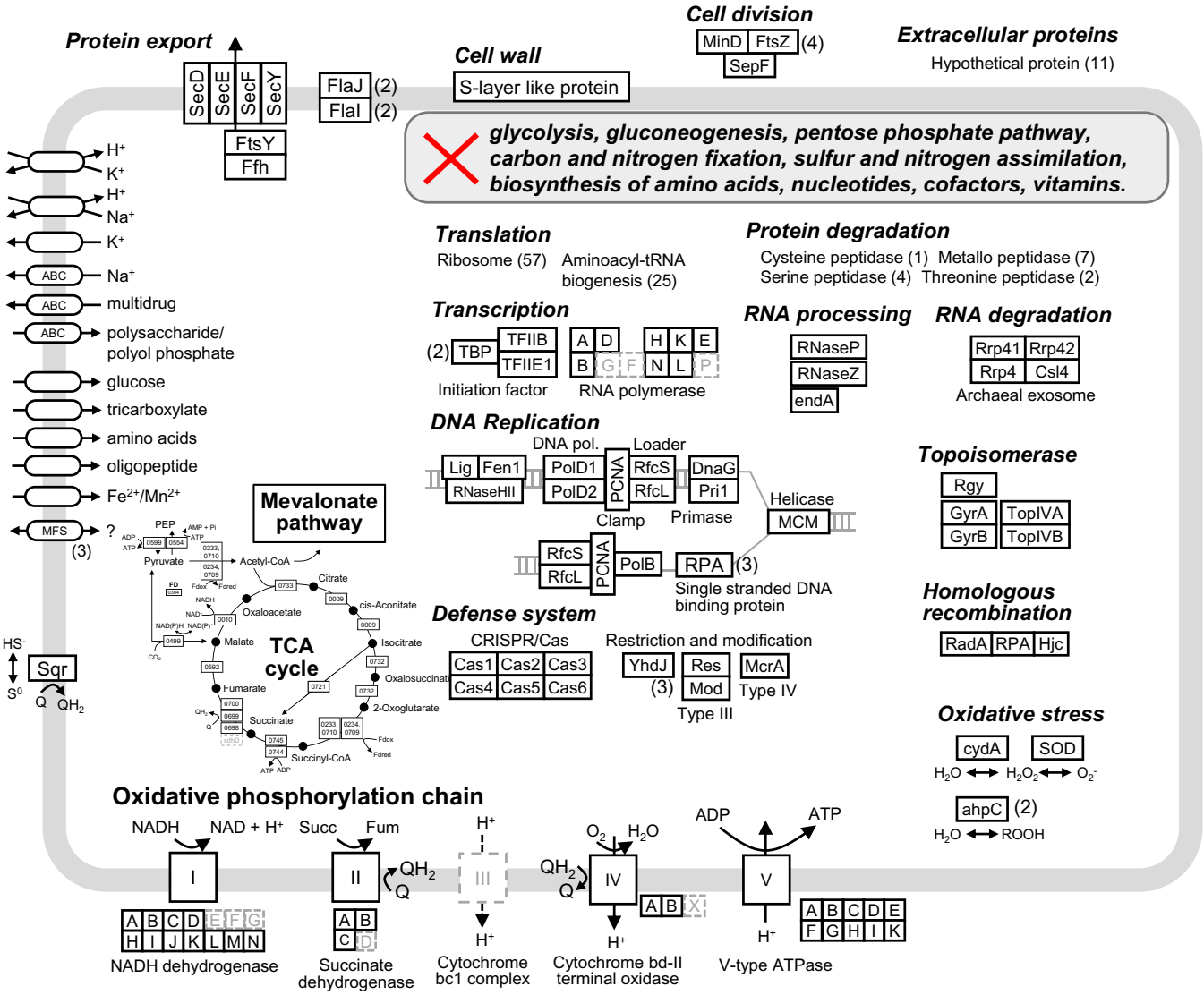

Fig. 4. Overview of the potential metabolic capability of ARM-1 inferred from genomic information. Metabolic pathways were constructed based on the annotation of predicted genes (Dataset S4). Gray indicates the lack of a gene. The total number of genes is indicated in parentheses near the gene name. See *SI Appendix*, Figs. S8 and S9 for the TCA cycle and mevalonate pathway.

strain ARM-1 as described below. Following the suggested emendations of rules 8, 15, and 22 in the International Code of Nomenclature of Prokaryotes (39, 40), we propose anew phylum, *Microcaldota*, rather than the phylum *Micrarchaeota*, which was used as the *Candidatus* status. For strain AS-7, we tentatively propose "*Metallosphaera javensis*" to accommodate it, based on its 16S rRNA gene sequence similarity and AAI value compared with that of *M. sedula*.

Proposal of New Taxa.

Description of *Microcaldus* gen. nov. *Microcaldus* (Mi.cro.cal'dus Gr. masc. adj. mikros, small; L. masc. adj. caldus, hot; N.L. masc. n. *Microcaldus*, a thermophilic small-size microorganism).

Cells are cocci, 240 to 440 nm in diameter. Obligate symbiont. It grows under aerobic conditions in thermoacidophilic environments. The type species is *Microcaldus variisymbioticus*.

Description of *Microcaldus variisymbioticus* sp. nov. *Microcaldus variisymbioticus* (va.ri.i.sym.bi.o.ti'cus L. masc. adj. varius, various; N.L. masc. adj. symbioticus, living together; N.L. masc. adj. variisymbioticus, living symbiotically with various microorganisms).

Grows under aerobic conditions with the following members of the order *Sulfolobales*: *A. brierleyi*, *M. sedula*, *M. hakonensis*, *M. javensis*, and *S. solfataricus*. Growth occurs at 50 to 75 °C and pH 1.5 to 4.5 in the presence of host organisms. Grows on complex substrates (yeast extract, tryptone, peptone, casamino acids) and sugars (glucose, sucrose) in the presence of host organisms. No growth occurs without host organisms. The DNA GC content is 34.1%. The type strain is ARM-1 (=JCM 33787 =InaCC Co.001). The strain was isolated from a terrestrial acidic hot spring in Indonesia.

Description of *Microcaldaceae* fam. nov. *Microcaldaceae* (Mi.cro.cal.da.ce'ae N.L. masc. n. *Microcaldus*, type genus of the family; suff. *-aceae*, ending to denote a family; N.L. fem. pl. n. *Microcaldaceae*, the family of the genus *Microcaldus*).

The description of the family *Microcaldaceae* is the same as that of the genus *Microcaldus*. The type genus is *Microcaldus*.

Description of *Microcaldales* ord. nov. *Microcaldales* (Mi.cro.cal.da'les N.L. masc. n. *Microcaldus*, type genus of the order; suff. *-ales*, ending to denote an order; N.L. fem. pl. n. *Microcaldales*, the order of the genus *Microcaldus*).

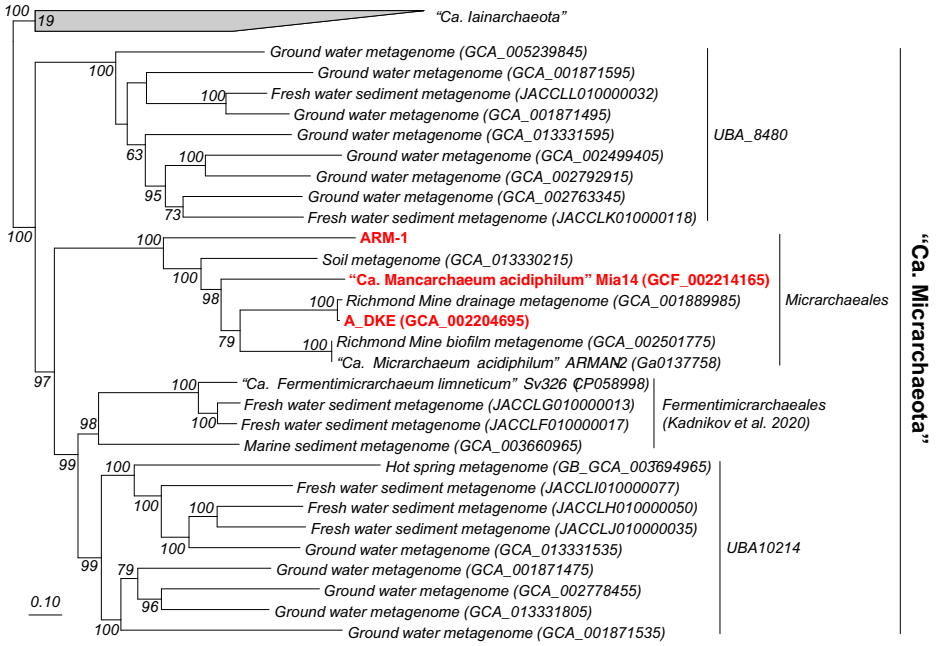

Fig. 5. Genome tree based on amino acid sequences of archaeal marker genes. GenBank accession numbers and JGI ID codes are indicated. Bootstrap values higher than 50% are indicated at each node. Cultivated representatives are indicated in red. Taxonomy shown is according to the Genome Taxonomy Database.

The description of the family *Microcaldales* is the same as that of the genus *Microcaldus*. The type genus is *Microcaldus*.

Description of *Microcaldia* class. nov. *Microcaldia* (Mi.cro.cal'dia N.L. masc. n. *Microcaldus*, type genus of the type order *Microcaldales*; suff. *-ia*, ending to denote a class; N.L. neut. pl. n. *Microcaldia*, the class of the order *Microcaldales*).

The description of the class *Microcaldia* is the same as that of the genus *Microcaldus*. The type order is *Microcaldales*.

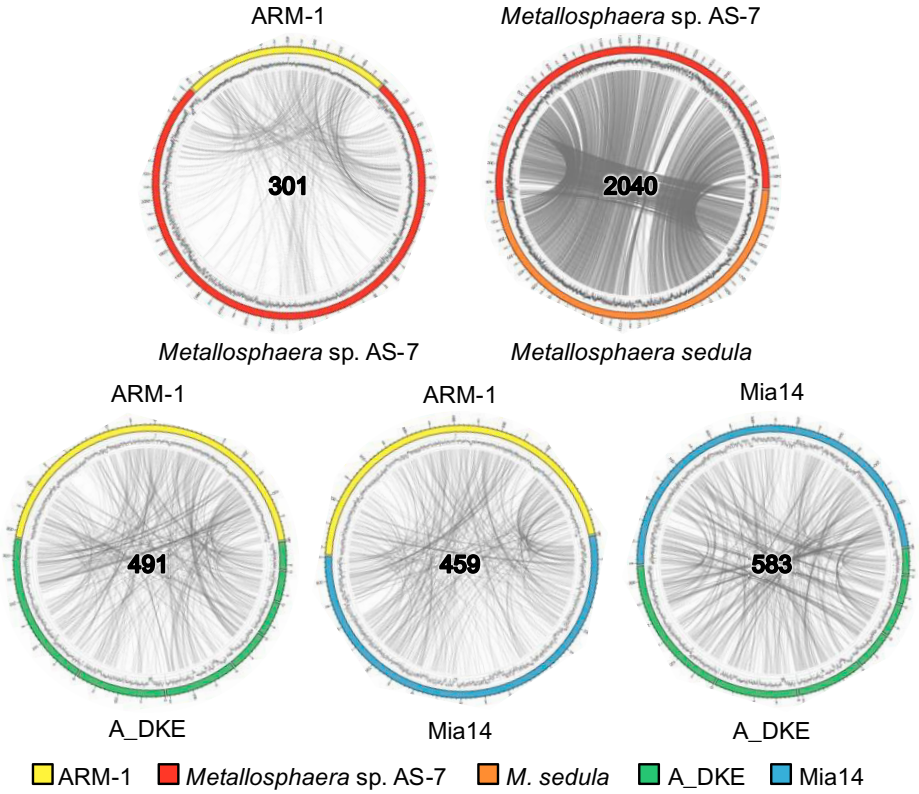

Fig. 6. Circos plots of ARM-1, *Metallosphaera* sp. AS-7, *M. sedula* DSM5348^T, and cultivated representatives in the phylum *Ca*. Micrarchaeota. Rings from outside to inside: genomic coordinates; GC content; lines in the center indicate amino acid sequences with significant similarity (*E* value: 1E-10, BLASTP) between individual genomes (Datasets S7, S8, S9, and S14). The total number of lines is indicated in the center.

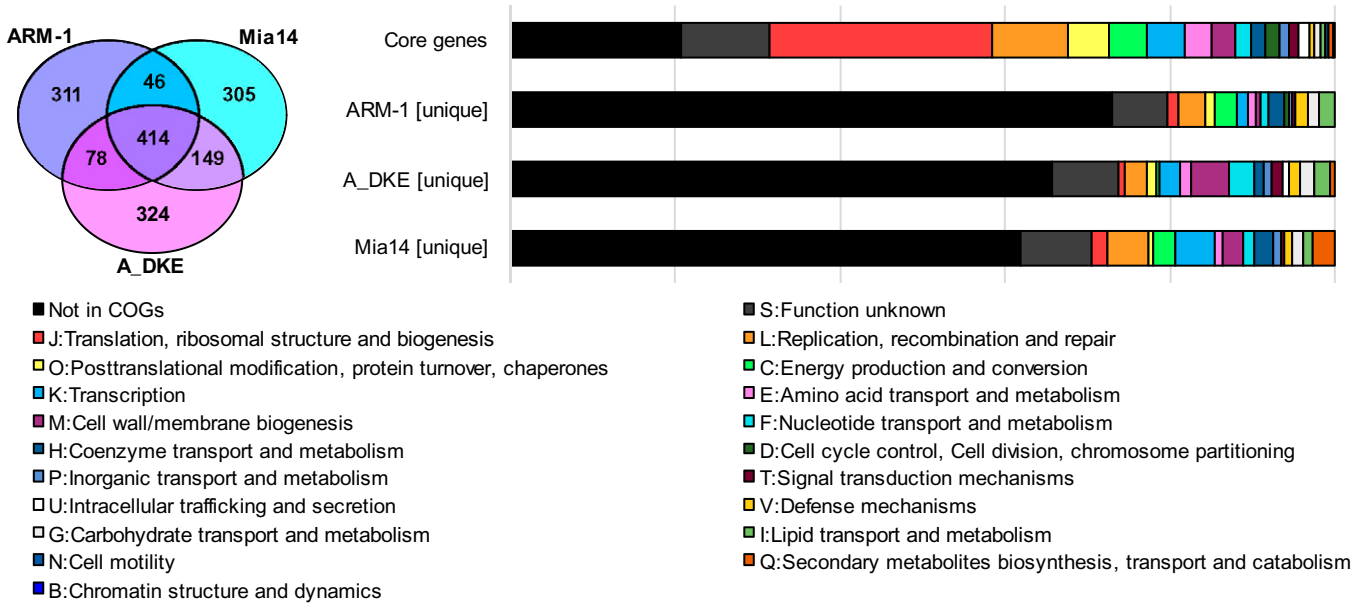

Fig. 7. Core and unique genes of cultivated representatives of *Ca.* Micrarchaeota and its COG assignment.

Description of *Microcaldota* phylum. nov. *Microcaldota* (Mi.cro. cal.do'ta N.L. masc. n. *Microcaldus*, type genus of the class *Microcaldia*; suff. *-ota*, ending to denote a phylum; N.L. neut. pl. n. *Microcaldota*, the phylum of the class *Microcaldia*).

Obligate symbiont. Extremely small cocci. Known host organisms are members of the order *Thermoplasmatales* or the order *Sulfolobales*. Grows under aerobic, microaerobic, or anaerobic conditions. A common characteristic of cultivated representatives is acidophilicity (8, 9). The environmental DNA of this taxon has been detected in AMD sites, hot springs, soil, peat, hypersaline mats, and freshwater (15). The type genus is *Microcaldus*.

Materials and Methods

Sample Collection and Initial Enrichment Culture. Hot spring water (62 °C, pH 3.3) was collected at Kawah Domas hot springs in Tangkuban Perahu, Indonesia (6° 45′ 41″ S 107° 37′ 32″ E), and transported to the laboratory at ambient temperature. A 100-μL aliquot of the sample was inoculated into 10 mL of modified Brock's basal salt (MBS) medium (41) supplemented with yeast extract (1 g/L) and elemental sulfur (5 g/L) (MBSYS). The pH was adjusted to 3.0 with 50% H_2SO_4. Incubation was performed at 65 °C under aerobic conditions. The growth of microorganisms was monitored according to the optical density at 600 nm (OD_{600}). When growth entered the stationary phase, 100 μL of the culture was transferred to fresh medium in the same manner as described above, and subcultivation was repeated four times (this enrichment culture is referred to as EnAS_65). Subsequently, 1 mL of the enrichment culture was centrifuged (15,880 × *g*, 5 min, 25 °C), and the supernatant was removed. The resulting cell pellet was stored at −25 °C until DNA extraction was carried out for microbial community analysis. Aliquots of EnAS_65 supplemented with 10% glycerol were stored at −80 °C for further cultivation experiments.

Microbial Community Analysis of the Initial Enrichment Culture. Microbial DNA was extracted from cell pellets obtained from EnAS_65 using Extrap Soil DNA Plus Kit version 2 (Nippon Steel and Sumikin Eco-Tech). Microbial community analysis (16S rRNA gene clone analysis) was conducted using a previously described method (42) with some modifications to the PCR cycle, as follows: initial denaturation step at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 40 s, and a final extension step at 72 °C for 2 min [primers A340F and A1000R (43)]. In brief, archaeal 16S rRNA genes amplified by PCR were cloned into the pT7Blue T-vector (Novagen), transformed into *E. coli* DH5α cells, and subjected to blue/white selection to construct clone libraries. Approximately 600 bp of the 5′ region of each inserted 16S rRNA gene was sequenced. Clones with >99% sequence similarity were grouped into OTUs.

Establishment of a Stable Coculture System Composed of ARM-1 and *Metallosphaera* sp. AS-7. The procedure for obtaining the stable coculture system composed of ARM-1 and *Metallosphaera* sp. AS-7 is summarized in *SI Appendix*, Fig. S1 (for details, see *SI Appendix, Supplementary Text*). First, the microbial community of EnAS_65 was regenerated from a glycerol stock using the same conditions as the initial enrichment culture, and then it was subcultivated four times. Second, S^0 was removed from the MBSYS medium (MBSY), followed by subcultivation seven times. Third, the incubation temperature was decreased from 65 to 55 °C. This enrichment culture is referred to as EnAS_55. Eventually, a stable coculture system composed of ARM-1 and *Metallosphaera* sp. AS-7 was obtained via the following procedure: 1) EnAS_55 was filtered using a sterilized syringe filter (pore size: 0.45 μm; material: polyethersulfone); 2) the filtrate, which was found to still contain many nanosized cells (10^6 to ~10^7 cells per milliliter), was added to the MBSY medium at a ratio of 2:8 (F-MBSY); 3) a pure culture of *Metallosphaera* sp. AS-7 in the stationary phase was inoculated into F-MBSY at a ratio of 1:200, followed by incubation at 55 °C (the resulting coculture system is referred to as EnM); and 4) using EnM instead of EnAS_55 and *Metallosphaera* sp. AS-7, procedures 1 to 3 were repeatedly conducted for 2 y (*SI Appendix*, Fig. S1*G*).

Microscopic Observations. The morphology of microbial cells in the coculture system EnM was examined by phase-contrast microscopy (BX53; Olympus), fluorescence microscopy (BX51; Olympus), epifluorescence microscopy (BX63; Olympus), SEM (JSM-7500F; JEOL), and TEM (JEM-1400Plus; JEOL). For standard fluorescence microscopy, cells fixed with 1% glutaraldehyde were washed with Tris·HCl (pH 8), mixed with a 100-fold dilution of SYBR Green I at a ratio of 7:3, and examined. FISH was performed as previously described (44) with some modifications. The FISH probes used for ARM-1 and AS-7 were designed manually (Dataset S16). The probes were labeled with either Texas red or 6-carboxyfluorescein at their 5′ end. Cells were fixed with 2% paraformaldehyde for 1 h, washed in phosphate-buffered saline (PBS), and resuspended in PBS before spotting 15 μL onto a silane-coated glass slide. The spotted sample was dried, incubated in 100 μL of 0.25 N HCl solution for 30 min at room temperature, rinsed in distilled water, and dehydrated with ethanol. Hybridization was carried out with the probes (final concentration 1 pmol/μL each) in buffer (0.9 M NaCl, 20 mM Tris·HCl, pH 7.5) at 46 °C overnight. After hybridization, the slide was washed in buffer (0.9 M NaCl, 20 mM Tris·HCl, pH 7.5) at 48 °C for 20 min, lightly rinsed in distilled water, and enclosed with mounting medium (SlowFade Gold Antifade Reagent; Life Technologies). The fluorescence signals were detected with an epifluorescence microscope (BX63; Olympus) fitted with filter sets specific for Texas red and fluorescein. For SEM, cells from EnM were fixed with 1% glutaraldehyde for 30 min at room temperature. A total of 200 μL of the fixed culture was filtered through a 0.1-μm pore size filter (Isopore, GTTP; Merck Millipore). Cells trapped on the filter were washed by soaking in 300 mL of sterilized Milli-Q water for 3 min. This washing step was repeated. After washing, the filter was flash-frozen by pressing a small copper block precooled to −100 °C using a

cooling unit (FDC10; SUN Technologies). The cells on the filter were immediately dehydrated by freeze drying using a freeze dryer (FD-6510; SUN Technologies). The filter was then attached to carbon double-sided tape and pasted on a carbon stub. Sputter coating with osmium (5 s) was carried out using an osmium coater (HPC-1SW; Vacuum Device). The sputter-coated sample was analyzed at an accelerating voltage of 5 kV. TEM was performed by Tokai-EMA based on rapid freezing and freeze fixation methods as follows: Cells were sandwiched with the copper disk and rapidly frozen in liquid propane at −175 °C; the sample was freeze-substituted with 2% glutaraldehyde, 1% tannic acid, and 2% distilled water in ethanol at −80 °C for 2 d; three dehydrations (30 min each) were performed with ethanol; the sample was washed with propylene oxide twice (30 min each), then incubated in a 70:30 mixture of propylene oxide and resin (Quetol-812; Nissin EM) for 1 h; overnight volatilization of propylene oxide was conducted; the sample was transferred to fresh 100% resin and polymerized at 60 °C for 48 h; the polymerized resins were ultrathin-sectioned at 70 nm with a diamond knife using an ultramicrotome (Ultracut UCT; Leica) and sections were mounted on copper grids; the grids were stained with 2% uranyl acetate at room temperature for 15 min and washed with distilled water; secondary staining was performed with lead stain solution (Sigma-Aldrich) at room temperature for 3 min; the grids were imaged by a transmission electron microscope (JEM-1400Plus; JEOL) at an acceleration voltage of 100 kV; and digital images were taken with a charge-coupled device camera (EM-14830RUBY2; JEOL). Negative-staining TEM was also performed by a Tokai-EMA as follows: The coculture sample was adsorbed to formvar film–coated copper grids and stained with 2% phosphor tungstic acid solution (pH 7.0) for a few seconds, and the grids were imaged under a transmission electron microscope (JEM-1400Plus; JEOL) at an acceleration voltage of 100 kV.

Physiological Characterization of ARM-1. Unless otherwise stated, cultivation experiments were conducted at 60 °C and pH 3.0 under aerobic conditions. To determine the growth temperature/pH range, ARM-1 with *Metallosphaera* sp. AS-7 was cultivated using MBSY medium with the following temperature/pH ranges: 40 to 80 °C at intervals of 5 °C, and pH 1.0 to 4.5 at intervals of 0.5. The growth of ARM-1 under autotrophic conditions was examined using MBSs (41) supplemented with either S^0 or FeS_2 (both at 5 g/L). Growth on the following complex substrates and sugars as a sole carbon source was examined as follows at a final concentration of 0.1% (weight/volume): yeast extract, casamino acids, peptone, tryptone, glucose, sucrose, lactose, and maltose. A 50-μL aliquot of EnM in stationary phase was inoculated into 10 mL of each medium, followed by incubation for up to 50 d. Two hundred microliters of each culture was regularly sampled for qPCR analysis to evaluate the growth of ARM-1 and *Metallosphaera* sp. AS-7.

Cocultivation Experiments of ARM-1 with Various Thermoacidophilic Species. In addition to *Metallosphaera* sp. AS-7 and *Thermoplasma* sp. AST-1 (methods for isolation are described in *SI Appendix, Text*), the following thermoacidophilic type strains belonging to different genera were cocultivated with ARM-1: *A. brierleyi* DSM1651^T, *M. sedula* DSM5348^T, *M. hakonensis* DSM7519^T, *S. solfataricus* DSM1616^T, *S. acidocaldarius* DSM639^T, *S. metallicus* DSM6482^T, *Sd. acidiphilus* HS-1^T, and *Sp. ohwakuensis* TA-1^T. All strains were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) except for *Sd. acidiphilus* HS-1^T and *Sp. ohwakuensis* TA-1^T, which were originally isolated in our laboratory.

Before cocultivation, a pure culture of each strain was precultivated in MBSY medium (pH 3.0), with the exception of *S. metallicus* DSM6482^T, which was precultivated in MBSYS medium (pH 3.0). All strains were subcultivated at least three times before performing the cocultivation tests. Pure ARM-1 cells were collected from EnM by filtration two times using a sterilized syringe filter (pore size: 0.45 μm; material: polyethersulfone). An aliquot of each pure culture (50 to 200 μL) and 2 mL of the filtrate containing ARM-1 cells were inoculated into 8 mL of cultivation medium. As a negative control, 2 mL of the filtrate was inoculated into 8 mL of MBSY medium (pH 3.0) without the inoculation of any of the strains. All incubations were conducted at 60 °C under aerobic conditions. When growth entered the stationary phase, subcultivation was conducted using 50 μL of each culture as the inoculum in 10 mL of fresh medium. Aliquots of 200 μL of each coculture were regularly sampled for qPCR analysis.

Quantification of Growth. To quantify the growth of each archaeal species in the cocultures, qPCR was performed using an Mx3000P qPCR System (Agilent Technologies). Microbial DNA from the cocultures was extracted using a DNA extraction machine (magLEAD 6gC; Precision System Science) with DNA extraction reagent (MagDEA Dx SV; Precision System Science). The PCR mixture contained 12.5 μL of TB Green Premix Ex Taq II (Takara Bio), 0.75 μL of forward and reverse primers (10 μM), and 2 to 11 μL of DNA template. The final volume was adjusted to 25 μL with sterilized Milli-Q water. The PCR cycling conditions were as follows: initial denaturation at 95 °C for 30 s, followed by 35 cycles of 95 °C for 5 s, 60 °C for 30 s, and 72 °C for 30 s. Fluorescence data were collected at the end of each 60 °C plateau. At the end of the PCR program, dissociation curve analysis was performed from 60 to 95 °C. Standard curves for quantifying the growth of each strain were developed as follows: The target sequence (123 to 155 bp of 16S rRNA gene) derived from each strain by standard PCR was ligated to a pMD20 T-vector (Takara Bio), which was then transformed into *E. coli* DH5α; plasmid DNA containing each target sequence was extracted from *E. coli* DH5α cells using a NucleoSpin Plasmid QuickPure Kit (Macherey-Nagel); plasmid DNA was treated with the restriction enzyme *Pst*I (Takara Bio); and qPCR for the standard curve was conducted using serially diluted plasmid DNA as a template. The copy number of plasmid DNA was calculated on the basis of DNA quantity (ng), DNA size (bp), and Avogadro's number ($1\ \text{mol} = 6.022 \times 10^{23}$ molecules). Primers for qPCR (Datasets S16 and S17) were designed using NCBI/Primer-BLAST (45) or Primer3 software (46).

Genome Analyses. Microbial cells of ARM-1 and *Metallosphaera* sp. AS-7 were collected by centrifugation (15,880 × *g*, 10 min, 4 °C) from the coculture system EnM. Genomic DNA was extracted from the cells using Genomic-Tip 500/G (QIAGEN). To obtain long reads, the genomic DNA was sent to Macrogen and sequenced by a PacBio RS II sequencer. The obtained long reads were filtered using Filtlong version 0.2.0 (https://github.com/rrwick/Filtlong). To obtain short reads, shotgun sequencing was conducted by an Illumina MiSeq sequencer with a QIAseq FX DNA Library Kit (QIAGEN) and MiSeq Reagent Kit version 3 (600 cycles, 300-bp paired-end reads; Illumina). The short and long reads were coassembled using Unicycler version 0.4.8 (47). Annotation of genome sequences was carried out using Prokka (ver. 1.13) (48), RAST server (ver. 2.0) (49), DFAST (ver. 1.1.0) (50), Kyoto Encyclopedia of Genes and Genomes pathway tools (51), eggNOG (ver. 4.5.1) (52), hmmscan against Pfam (53), and MEROPS (54). The annotated data obtained by these programs were manually curated. Subcellular localization of each protein encoded in the genome sequences was predicted using PSORTb 3.0 (55).

A phylogenetic tree of the 16S rRNA gene was constructed as follows. Related sequences with ARM-1 and *Metallosphaera* sp. AS-7 were collected from the NCBI GenBank and Joint Genomics Institute's Integrated Microbial Genomes (JGI-IMG) databases (only sequences >1,300 bp were included in the final dataset). Alignment of these sequences was performed using MUSCLE (56). Poorly aligned regions of the alignment were removed by trimAl with the automated1 option (57) and manually checked. An MLtree was constructed using RAxML (21) with the GTRGAMMA model. Bootstrap support values were calculated with 1,000 replicates. For a genome tree, amino acid sequences of 122 archaeal marker genes were obtained using GTDB-Tk version 0.3.3 (58, 59). In the same manner as described above, the obtained sequences were aligned and trimmed, and then an ML tree was constructed with the PROTGAMMALG model. The tree data were visualized in the ARB software package (60). Secondary structures of 16S rRNA of ARM-1 and an insertion sequence found in the 16S rRNA were predicted by R2DT (61) and RANfold (62), respectively.

Genomic comparison of ARM-1 and cultivated representatives in the phylum *Ca*. Micrarchaeota [A_DKE and Mia14 (8, 9)] was conducted by BLASTP (*E* value cutoff: 10^{-10}; coverage cutoff: 70%). The BLASTP results (bidirectional best hits) were visualized using the Circos program (63). Based on the BLASTP results, we determined core and unique genes among the cultivated representatives of *Ca*. Micrarchaeota, and these genes were classified into COG categories using eggNOG version 4.5.1 (34). Phylogenetic analyses of some unique genes of ARM-1 were conducted with the top 100 related sequences collected from the NCBI GenBank database by BLASTP with the Nucleotide database in the same manner as described above. The AAI was calculated using the Kostas laboratory server (enve-omics.ce.gatech.edu/ani/). Genomic comparison of ARM-1 and *Metallosphaera* sp. AS-7 was investigated in the same way.

Culture Deposition. The coculture composed of strains ARM-1 and AS-7 was deposited in the Japan Collection of Microorganisms as JCM33787 and Indonesian Culture Collection as Co.001.

Data Availability. Sequence data of 16S rRNA genes supporting the findings of this study have been deposited in GenBank under accession nos. LC490573–LC490578. Genomic sequences of ARM-1 and *Metallosphaera* sp. AS-7 with raw read data have been deposited under the following accession nos.: AP024486 (ARM-1 genome), AP024487 (AS-7 genome), DRR248897 (PacBio RS II long reads), and DRR248898 (Illumina MiSeq short reads). All study data are included in the article and/or supporting information.

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1. C. J. Castelle, J. F. Banfield, Major new microbial groups expand diversity and alter our understanding of the tree of life. *Cell* **172**, 1181–1197 (2018).
2. N. Dombrowski, J.-H. Lee, T. A. Williams, P. Offre, A. Spang, Genomic diversity, lifestyles and evolutionary origins of DPANN archaea. *FEMS Microbiol. Lett.* **366**, fnz008 (2019).
3. L. Li *et al.*, Comparative genomics provides insights into the genetic diversity and evolution of the DPANN superphylum. *mSystems* **6**, e0060221 (2021).
4. J. T. Bird, B. J. Baker, A. J. Probst, M. Podar, K. G. Lloyd, Culture independent genomic comparisons reveal environmental adaptations for Altiarchaeales. *Front. Microbiol.* **7**, 1221 (2016).
5. N. H. Youssef *et al.*, Insights into the metabolism, lifestyle and putative evolutionary history of the novel archaeal phylum 'Diapherotrites.' *ISME J.* **9**, 447–460 (2015).
6. H. Huber *et al.*, A new phylum of Archaea represented by a nanosized hyperthermophilic symbiont. *Nature* **417**, 63–67 (2002).
7. L. Wurch *et al.*, Genomics-informed isolation and characterization of a symbiotic Nanoarchaeota system from a terrestrial geothermal environment. *Nat. Commun.* **7**, 12115 (2016).
8. O. V. Golyshina *et al.*, 'ARMAN' archaea depend on association with euryarchaeal host in culture and in situ. *Nat. Commun.* **8**, 60 (2017).
9. S. Krause, A. Bremges, P. C. Münch, A. C. McHardy, J. Gescher, Characterisation of a stable laboratory co-culture of acidophilic nanoorganisms. *Sci. Rep.* **7**, 3289 (2017).
10. J. N. Hamm *et al.*, Unexpected host dependency of Antarctic Nanohaloarchaeota. *Proc. Natl. Acad. Sci. U.S.A.* **116**, 14661–14670 (2019).
11. C. Rinke *et al.*, Insights into the phylogeny and coding potential of microbial dark matter. *Nature* **499**, 431–437 (2013).
12. B. J. Baker *et al.*, Lineages of acidophilic archaea revealed by community genomic analysis. *Science* **314**, 1933–1935 (2006).
13. B. J. Baker *et al.*, Enigmatic, ultrasmall, uncultivated Archaea. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 8806–8811 (2010).
14. L. R. Comolli, B. J. Baker, K. H. Downing, C. E. Siegerist, J. F. Banfield, Three-dimensional analysis of the structure and ecology of a novel, ultra-small archaeon. *ISME J.* **3**, 159–167 (2009).
15. L. X. Chen *et al.*, Metabolic versatility of small archaea Micrarchaeota and Parvarchaeota. *ISME J.* **12**, 756–775 (2018).
16. R. J. Giannone *et al.*, Life on the edge: Functional genomic response of *Ignicoccus hospitalis* to the presence of *Nanoarchaeum equitans*. *ISME J.* **9**, 101–114 (2015).
17. U. Jahn *et al.*, *Nanoarchaeum equitans* and *Ignicoccus hospitalis*: New insights into a unique, intimate association of two archaea. *J. Bacteriol.* **190**, 1743–1750 (2008).
18. T. Heimerl *et al.*, A complex endomembrane system in the archaeon *Ignicoccus hospitalis* tapped by *Nanoarchaeum equitans*. *Front. Microbiol.* **8**, 1072 (2017).
19. A. Klingl, S-layer and cytoplasmic membrane—Exceptions from the typical archaeal cell wall with a focus on double membranes. *Front. Microbiol.* **5**, 624 (2014).
20. J. Liu *et al.*, Archaeal extracellular vesicles are produced in an ESCRT-dependent manner and promote gene transfer and nutrient cycling in extreme environments. *ISME J.* **15**, 2892–2905 (2021).
21. I. A. Berg, D. Kockelkorn, W. Buckel, G. Fuchs, A 3-hydroxypropionate/4-hydroxybutyrate autotrophic carbon dioxide assimilation pathway in Archaea. *Science* **318**, 1782–1787 (2007).
22. G. Huber, C. Spinnler, A. Gambacorta, K. O. Stetter, *Metallosphaera sedula* gen, and sp. nov. represents a new genus of aerobic, metal-mobilizing, thermoacidophilic Archaebacteria. *Syst. Appl. Microbiol.* **12**, 38–47 (1989).
23. S. Takayanagi *et al.*, *Sulfolobus hakonensis* sp. nov., a novel species of acidothermophilic archaeon. *Int. J. Syst. Bacteriol.* **46**, 377–382 (1996).
24. W. Zillig *et al.*, The *Sulfolobus*-"*Caldariella*" group: Taxonomy on the basis of the structure of DNA-dependent RNA polymerases. *Arch. Microbiol.* **125**, 259–269 (1980).
25. A. Segerer, A. Neuner, J. K. Kristjansson, K. O. Stetter, *Acidianus infernus* gen. nov., sp. nov., and *Acidianus brierleyi* comb. nov.: Facultatively aerobic, extremely acidophilic thermophilic sulfur-metabolizing archaebacteria. *Int. J. Syst. Bacteriol.* **36**, 559–564 (1986).
26. C. L. Brierley, J. A. Brierley, A chemoautotrophic and thermophilic microorganism isolated from an acid hot spring. *Can. J. Microbiol.* **19**, 183–188 (1973).
27. E. St John *et al.*, A new symbiotic nanoarchaeote (*Candidatus* Nanoclepta minutus) and its host (*Zestosphaera tikiterensis* gen. nov., sp. nov.) from a New Zealand hot spring. *Syst. Appl. Microbiol.* **42**, 94–106 (2019).
28. H. Hayakawa *et al.*, Modified mevalonate pathway of the archaeon *Aeropyrum pernix* proceeds via *trans*-anhydromevalonate 5-phosphate. *Proc. Natl. Acad. Sci. U.S.A.* **115**, 10034–10039 (2018).
29. S. V. Albers, K. F. Jarrell, The archaellum: An update on the unique archaeal motility structure. *Trends Microbiol.* **26**, 351–362 (2018).
30. K. T. Konstantinidis, R. Rosselló-Móra, R. Amann, Uncultivated microbes in need of their own taxonomy. *ISME J.* **11**, 2399–2406 (2017).
31. Z. J. Jay, W. P. Inskeep, The distribution, diversity, and importance of 16S rRNA gene introns in the order Thermoproteales. *Biol. Direct* **10**, 35 (2015).
32. T. Nunoura *et al.*, Insights into the evolution of Archaea and eukaryotic protein modifier systems revealed by the genome of a novel archaeal group. *Nucleic Acids Res.* **39**, 3204–3223 (2011).
33. V. Salman, R. Amann, D. A. Shub, H. N. Schulz-Vogt, Multiple self-splicing introns in the 16S rRNA genes of giant sulfur bacteria. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 4203–4208 (2012).
34. V. V. Kadnikov *et al.*, Metabolic diversity and evolutionary history of the archaeal phylum "*Candidatus* Micrarchaeota" uncovered from a freshwater lake metagenome. *Appl. Environ. Microbiol.* **86**, e02199-20 (2020).
35. P. Forterre, A hot story from comparative genomics: Reverse gyrase is the only hyperthermophile-specific protein. *Trends Genet.* **18**, 236–237 (2002).
36. D. Burstein *et al.*, New CRISPR-Cas systems from uncultivated microbes. *Nature* **542**, 237–241 (2017).
37. K. S. Makarova, F. Zhang, E. V. Koonin, SnapShot: Class 1 CRISPR-Cas systems. *Cell* **168**, 946–946.e1 (2017).
38. K. S. Makarova, F. Zhang, E. V. Koonin, SnapShot: Class 2 CRISPR-Cas systems. *Cell* **168**, 328–328.e1 (2017).
39. A. Oren, D. R. Arahal, R. Rosselló-Móra, I. C. Sutcliffe, E. R. B. Moore, Emendation of rules 5b, 8, 15 and 22 of the International Code of Nomenclature of Prokaryotes to include the rank of phylum. *Int. J. Syst. Evol. Microbiol.* **71**, 21–23 (2021).
40. C. T. Parker, B. J. Tindall, G. M. Garrity, International Code of Nomenclature of Prokaryotes. *Int. J. Syst. Evol. Microbiol.* **69** (1A), S1–S111 (2019).
41. N. Kurosawa *et al.*, *Sulfurisphaera ohwakuensis* gen. nov., sp. nov., a novel extremely thermophilic acidophile of the order Sulfolobales. *Int. J. Syst. Bacteriol.* **48**, 451–456 (1998).
42. H. D. Sakai, N. Kurosawa, Exploration and isolation of novel thermophiles in frozen enrichment cultures derived from a terrestrial acidic hot spring. *Extremophiles* **20**, 207–214 (2016).
43. S. Gantner, A. F. Andersson, L. Alonso-Sáez, S. Bertilsson, Novel primers for 16S rRNA-based archaeal community analyses in environmental samples. *J. Microbiol. Methods* **84**, 12–18 (2011).
44. P. L. Bond, J. F. Banfield, Design and performance of rRNA targeted oligonucleotide probes for in situ detection and phylogenetic identification of microorganisms inhabiting acid mine drainage environments. *Microb. Ecol.* **41**, 149–161 (2001).
45. J. Ye *et al.*, Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* **13**, 134 (2012).
46. A. Untergasser *et al.*, Primer3—New capabilities and interfaces. *Nucleic Acids Res.* **40**, e115 (2012).
47. R. R. Wick, L. M. Judd, C. L. Gorrie, K. E. Holt, Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Comput. Biol.* **13**, e1005595 (2017).
48. T. Seemann, Prokka: Rapid prokaryotic genome annotation. *Bioinformatics* **30**, 2068–2069 (2014).
49. R. K. Aziz *et al.*, The RAST server: Rapid annotations using subsystems technology. *BMC Genomics* **9**, 75 (2008).
50. Y. Tanizawa, T. Fujisawa, Y. Nakamura, DFAST: A flexible prokaryotic genome annotation pipeline for faster genome publication. *Bioinformatics* **34**, 1037–1039 (2018).
51. H. Ogata *et al.*, KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res.* **27**, 29–34 (1999).
52. J. Huerta-Cepas *et al.*, eggNOG 4.5: A hierarchical orthology framework with improved functional annotations for eukaryotic, prokaryotic and viral sequences. *Nucleic Acids Res.* **44**, D286–D293 (2016).
53. R. D. Finn, J. Clements, S. R. Eddy, HMMER web server: Interactive sequence similarity searching. *Nucleic Acids Res.* **39** (suppl. 2), W29–W37 (2011).
54. N. D. Rawlings *et al.*, The MEROPS database of proteolytic enzymes, their substrates and inhibitors in 2017 and a comparison with peptidases in the PANTHER database. *Nucleic Acids Res.* **46**, D624–D632 (2018).
55. N. Y. Yu *et al.*, PSORTb 3.0: Improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. *Bioinformatics* **26**, 1608–1615 (2010).
56. R. C. Edgar, MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **32**, 1792–1797 (2004).
57. S. Capella-Gutiérrez, J. M. Silla-Martínez, T. Gabaldón, trimAl: A tool for automated alignment trimming in large-scale phylogenetic analyses. *Bioinformatics* **25**, 1972–1973 (2009).
58. D. H. Parks *et al.*, A standardized bacterial taxonomy based on genome phylogeny substantially revises the tree of life. *Nat. Biotechnol.* **36**, 996–1004 (2018).
59. P.-A. Chaumeil, A. J. Mussig, P. Hugenholtz, D. H. Parks, GTDB-Tk: A toolkit to classify genomes with the Genome Taxonomy Database. *Bioinformatics* **36**, 1925–1927 (2019).
60. W. Ludwig *et al.*, ARB: A software environment for sequence data. *Nucleic Acids Res.* **32**, 1363–1371 (2004).
61. B. A. Sweeney *et al.*, R2DT is a framework for predicting and visualising RNA secondary structure using templates. *Nat. Commun.* **12**, 3494 (2021).
62. A. R. Gruber, R. Lorenz, S. H. Bernhart, R. Neuböck, I. L. Hofacker, The Vienna RNA websuite. *Nucleic Acids Res.* **36**, W70–W74 (2008).
63. M. Krzywinski *et al.*, Circos: An information aesthetic for comparative genomics. *Genome Res.* **19**, 1639–1645 (2009).