



## MICROBIOLOGY

# Evidence for nontraditional *mcr*-containing archaea contributing to biological methanogenesis in geothermal springs

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Recent discoveries of methyl-coenzyme M reductase–encoding genes (*mcr*) in uncultured archaea beyond traditional euryarchaeotal methanogens have reshaped our view of methanogenesis. However, whether any of these nontraditional archaea perform methanogenesis remains elusive. Here, we report field and microcosm experiments based on <sup>13</sup>C-tracer labeling and genome-resolved metagenomics and metatranscriptomics, revealing that nontraditional archaea are predominant active methane producers in two geothermal springs. *Archaeoglobales* performed methanogenesis from methanol and may exhibit adaptability in using methylotrophic and hydrogenotrophic pathways based on temperature/substrate availability. A five-year field survey found *Candidatus* Nezharchaeota to be the predominant *mcr*-containing archaea inhabiting the springs; genomic inference and *mcr* expression under methanogenic conditions strongly suggested that this lineage mediated hydrogenotrophic methanogenesis in situ. Methanogenesis was temperature-sensitive, with a preference for methylotrophic over hydrogenotrophic pathways when incubation temperatures increased from 65° to 75°C. This study demonstrates an anoxic ecosystem wherein methanogenesis is primarily driven by archaea beyond known methanogens, highlighting diverse nontraditional *mcr*-containing archaea as previously unrecognized methane sources.

## INTRODUCTION

Methanogenesis has been widely accepted as a primordial metabolism that has influenced both past and current global climate change (1, 2). Phylogenetic and geological evidence supports the hypothesis that methanogenesis originated in thermal environments such as hydrothermal vents or terrestrial geothermal springs (3–6). Modern hydrothermal ecosystems therefore likely hold clues pertaining to the origin of archaeal methane cycling (7, 8).

Contemporary methanogens populate nearly every anoxic habitat, ranging from near freezing to extreme thermal conditions (9, 10). They share a core metabolism with overlapping routes centered around the methyl–coenzyme M reductase (Mcr) complex, which catalyzes the terminal step in methanogenesis as well as the initial step in anaerobic methane oxidation (9, 11).

Currently, all confirmed methanogens belong to a small number of lineages within the phylum *Euryarchaeota*, known as traditional euryarchaeotal methanogens (9, 12). However, recent metagenomic studies propose methanogen diversity to be vastly underestimated, with terrestrial geothermal springs being a research focus (4, 9, 13, 14). Key methane-metabolizing *mcr* genes have been found in metagenome-assembled genomes (MAGs) not only affiliated with the *Euryarchaeota* order *Archaeoglobales* (4, 15, 16) but also within noneuryarchaeotal lineages from *Thaumarchaeota*, *Aigarchaeota*, *Crenarchaeota* and *Ca. Korarchaeota* (TACK) superphylum such as *Candidatus* Verstraetearchaeota (14, 17), *Ca. Nezharchaeota* (4, 15), *Ca. Korarchaeota* (18), and *Thaumarchaeota* (4, 19). Putative methanogenesis pathways in those newly found MAGs appear to be nontraditional compared to euryarchaeal lineages (4). In addition, these lineages are often overlooked in amplicon-based environmental surveys and remain recalcitrant to cultivation (9, 20). The increase in the number of *mcr*-containing MAGs continues; however, our understanding of these recalcitrant archaea remains nascent and depends exclusively on genome inference rather than empirical evidence (4, 15). This is especially true for *mcr*-containing *Archaeoglobales*, which have been speculated to have several different metabolic lifestyles (4, 15). However, whether and to what extent nontraditional *mcr*-containing archaea contribute to the methane

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cycle remain unknown, as the presence of genes does not equate to actual metabolic activity or ecosystem-level biogeochemical functioning (21). Furthermore, how their metabolic activity responds to changing environmental conditions is poorly understood.

Terrestrial geothermal springs are the primary sources of these nontraditional *mcr*-containing MAGs (4, 13, 15, 17, 18), and such hydrothermal environments were likely more prevalent on Archean Earth than at present due to a higher frequency of volcanism (22, 23). Volcanogenic near neutral-to-alkaline, sulfate-deficient springs have recently been hypothesized to be an alternative site to deep-sea hydrothermal vents for the origin of life (23, 24). While the domination of the biogeochemical carbon cycle by methanogenesis on early Earth is not controversial (1, 25), the prevailing surface temperature on early Earth is often debated and is proposed to range from temperate (0° to 50°C) to hot (60° to 80°C) (26). However, little is known about how methanogenesis responds to temperature changes in ecosystems above 60°C and how this response differs from that of well-studied temperate ecosystems. Such knowledge is crucial to understand the coevolution of methane-cycling ecosystems with Earth's climate because both methanogenic rates and operative pathways are highly temperature dependent (2, 27).

Here, we report an integrated study of methanogenesis in two alkaline, terrestrial geothermal springs located in Tengchong, China. By combining field/microcosm experiments, <sup>13</sup>C-tracer labeling, and genome-centric metagenomics and metatranscriptomics, we provide evidence that methanogenesis in two springs is primarily mediated by nontraditional, *mcr*-containing archaea, particularly at elevated temperatures (≥75°C). We further show community-level responses of methanogenesis to temperature in our microcosms, the methanogenic pathways of these nontraditional methanogens, and discuss the biogeochemical and ecological implications of these results.

## RESULTS

### Hydrochemical attributes, *mcr*-containing archaea, and active methanogenesis in native habitats

The sampling sites were "GuMingQuan Pool" hot spring (GMQP; in situ temperature: 85° to 90°C; pH: 9.6), which belongs to a magmatic geothermal area (Rehai), and the JinZe-3 hot spring (JZ-3; in situ temperature: 65° to 75°C; pH: 8.9), which is from a nonmagmatic geothermal system (Diantan) (for site details, see table S1) (28, 29). The hydrochemical types of these springs are Na-Cl-HCO<sub>3</sub> for GMQP and Na-HCO<sub>3</sub> for JZ-3. Both springs contain abundant dissolved inorganic carbon (DIC) (>10 mM DIC; mainly HCO<sub>3</sub><sup>-</sup>) and have low sulfate levels (0.40 and 0.23 mM for GMQP and JZ-3, respectively) (table S2). While sulfate levels are similar, JZ-3 is deficient in dissolved sulfide (0.17 mg liter<sup>-1</sup>), and levels in GMQP are high (1.55 mg liter<sup>-1</sup>). Dissolved Fe (mainly Fe<sup>2+</sup>) concentrations are 0.02 and 0.27 mg liter<sup>-1</sup> for GMQP and JZ-3, respectively. Total dissolved nitrogen is 0.80 mg liter<sup>-1</sup> in GMQP, while its concentration was below the detection limit in JZ-3. Other terminal electron acceptors, such as nitrate and Fe<sup>3+</sup>, are relatively low (30). According to PHREEQC calculations, the concentrations of dissolved H<sub>2</sub> and CH<sub>4</sub> are close to zero.

From 2017 to 2021, we used genome-centric metagenomics to monitor the temporal dynamics of *mcr*-containing archaea in the sediments of the two springs (Fig. 1A). From these analyses, *mcr*-

containing MAGs were found to be either (i) verified cultivated traditional euryarchaeotal methanogens such as *Methanothermobacter*, *Methanothrix*, and *Methanolinea*; or (ii) nontraditional *mcr*-containing archaea belonging to *Ca. Nezharchaeota*, *Ca. Verstraetearchaeota*, and *Archaeoglobales*. Over the 5-year period, *Ca. Nezharchaeota* were the most abundant *mcr*-containing archaea in both springs, with relative abundances up to 0.18% in JZ-3 and 2.0% in GMQP (on average 0.11 and 1.26%, respectively). Except for *Ca. Nezharchaeota*, only *Ca. Verstraetearchaeota* reached above 0.1% in both communities with the relative abundances up to 0.14 and 0.11% in JZ-3 and GMQP, respectively, whereas all traditional methanogens were at abundances of <0.05%.

To examine the potential for in situ methanogenesis in GMQP, we leveraged static respiration chamber measurements (figs. S1 and S2) and <sup>13</sup>C-labeling microcosm assays. JZ-3 is a large man-made concrete-lined rectangular pool, and its water depth precludes deployment of the chamber. On the basis of the accumulation of CH<sub>4</sub> in the sampling chamber, in situ CH<sub>4</sub> flux was calculated to be 8.0 μmol m<sup>-2</sup> day<sup>-1</sup>. In microcosm assays, short-term methanogenesis rates, which were quantified on the basis of the sediment mass, incubation time, gas accumulation, and headspace volume following the methods of Chen *et al.* (27), were as low as ~0.02 μg C g<sup>-1</sup> sediment day<sup>-1</sup>, and neither amendments of hydrogenotrophic (H<sub>2</sub> and H<sub>2</sub>/HCO<sub>3</sub><sup>-</sup>) nor methylotrophic precursors (MeOH) substantially stimulated CH<sub>4</sub> accumulation over 30 days at 85°C (Fig. 1B). Those results suggest that temperature could be a limiting factor to both pathways in GMQP spring. However, the enrichment of <sup>13</sup>C CH<sub>4</sub> increased from ~1% in unlabeled microcosms to 3% for <sup>13</sup>C-HCO<sub>3</sub><sup>-</sup>-amended microcosms and 15% for <sup>13</sup>C-MeOH-amended microcosms within 24 hours of incubation (Fig. 1C). The lower <sup>13</sup>C enrichment of CH<sub>4</sub> in <sup>13</sup>C-HCO<sub>3</sub><sup>-</sup> cultures compared to the <sup>13</sup>C-MeOH series was attributed to pre-existing unlabeled DIC (11.25 mM, mainly HCO<sub>3</sub><sup>-</sup>) in the native geothermal waters. Methane production from unlabeled DIC may also explain the relatively stable <sup>13</sup>C content of CH<sub>4</sub> despite a slight increase in CH<sub>4</sub> concentration on day 10. Together, these results from field and microcosm incubation experiments demonstrate active hydrogenotrophic and methylotrophic methanogenesis in GMQP.

### Community-level responses of methanogenesis from in vitro microcosm incubations

While temperature appears to be a limiting factor for methanogenesis in GMQP microcosm assays at in situ temperatures (Fig. 1D), in vitro JZ-3 sediment plus geothermal water incubations without substrates showed higher rates of CH<sub>4</sub> production at similar in situ temperature ranges (65° to 75°C) without exogenous substrates (figs. S3 to S5). After an initial 30-day 65°C temperature-stabilization period, subsequent methanogenesis assays showed that CH<sub>4</sub> and CO<sub>2</sub> levels in JZ-3 rapidly reached maxima of 31 and 608 μM within 48 hours, respectively (fig. S5, A and B). When the temperature of these microcosms was increased from 65° to 75°C and then incubated for 30 days at 75°C, methanogenesis assays showed that CH<sub>4</sub> and CO<sub>2</sub> concentrations reached 5 and 334 μM after 48 hours, respectively (fig. S5, A and B). Stoichiometrically, the CH<sub>4</sub>:CO<sub>2</sub> ratio was higher at 65°C relative to 75°C, suggesting that CH<sub>4</sub> production was more sensitive than CO<sub>2</sub> production to temperature increases.

Similar stepwise temperature-gradient incubations (65°C/75°C; 85°C/75°C) were then implemented to probe methanogenic activity in GMQP sediments (see Materials and Methods

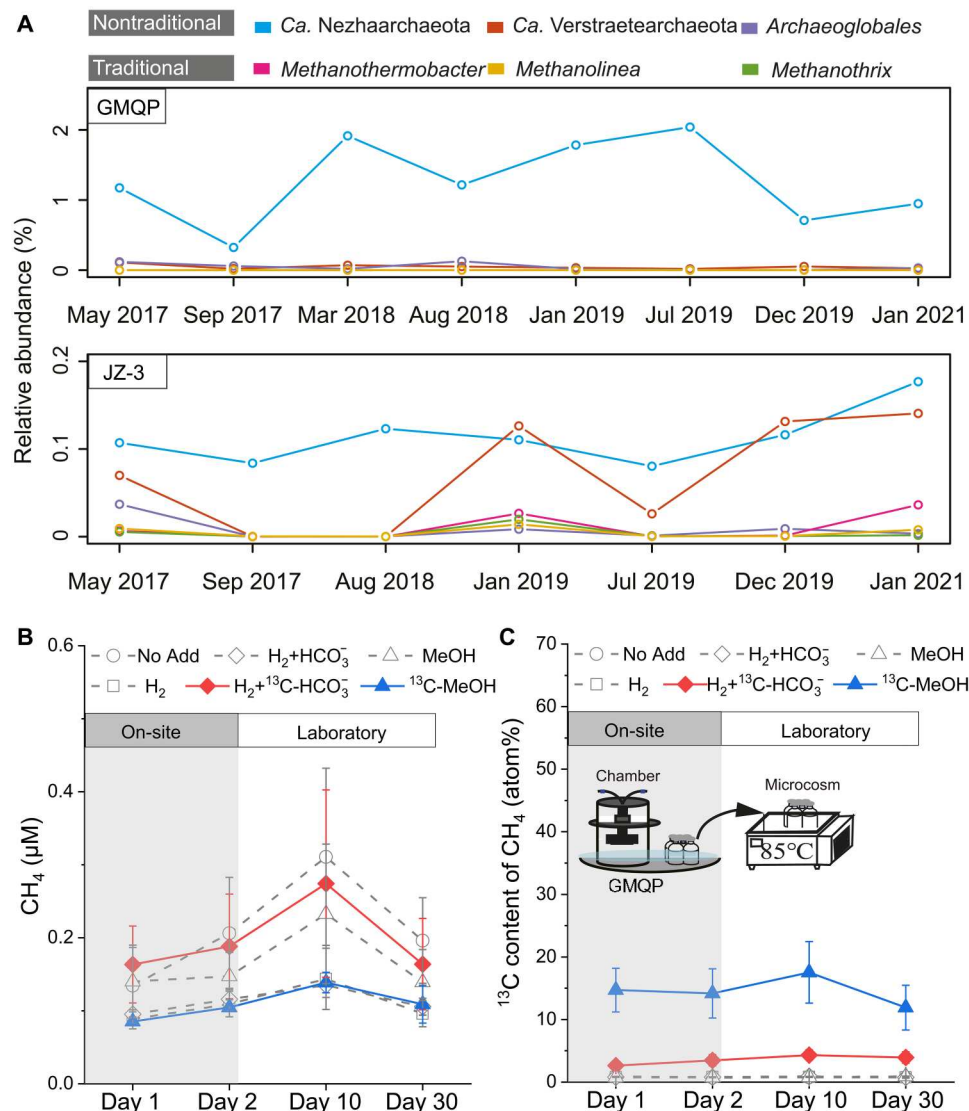


**Fig. 1. Field investigations of methanogenesis in two geothermal springs [“GuMingQuan Pool” hot spring (GMQP) and JinZe-3 hot spring (JZ-3)] located in the Tengchong volcanogenic geothermal area (China).** (A) Temporal dynamics of methanogenic communities from 2017 to 2021 as revealed by genome-centric metagenomics. The *mcr*-containing metagenome-assembled genomes (MAGs) at these sampling times were generated and grouped on the basis of phylogenetic trees of key diagnostic protein sequences for methane metabolism (McrABG). The relative abundance of each MAG in a sample was calculated as the proportion of reads recruited for a given MAG against all reads in the metagenomic data. (B and C) Microcosm incubations for the dynamics of methane formation rate measured by the change in <sup>13</sup>C-CH<sub>4</sub> content (GMQP) after the microcosms were spiked with hydrogenotrophic or methylotrophic methanogenic precursors (~6% H<sub>2</sub> and/or 10 mM precursors). Microcosms were first incubated on-site before being transferred to the laboratory under an in situ temperature of 85°C. SDs reflect methane formation from three microcosms (*n* = 3). No Add, no addition; MeOH, methanol. For CO<sub>2</sub> dynamics in these microcosm incubations, see fig. S13.

and fig. S6 for details). At the end of the temperature-stabilization period (30 days, without substrates), the accumulated CH<sub>4</sub> levels in microcosms were substantially higher at 65°C than at 85°C (49.4 versus 0.2 μM; table S3). Subsequently, the methanogenic precursors CO<sub>2</sub>, MeOH, acetate (Ac), and methylamine (MeNH<sub>3</sub><sup>+</sup>), along with combinations of these substrates with H<sub>2</sub>, were incubated with GMQP sediment slurries. The 85°C incubations failed to produce methane even when they were cooled to 75°C, and CH<sub>4</sub> levels remained at ~0.2 μM throughout. In contrast, the 65°C-incubated cultures gave varying responses to methanogenic precursors. These results demonstrated that biological methanogenesis was

sensitive to temperature, with incubation at lower than in situ temperatures stimulating methanogenic activity in the GMQP microcosms. Only H<sub>2</sub>/CO<sub>2</sub>, H<sub>2</sub>/MeOH, and MeOH stimulated methane formation; acetate had no stimulating effect, and MeNH<sub>3</sub><sup>+</sup> had an inhibitory effect (Fig. 2, A and B). The addition of H<sub>2</sub> at 65°C stimulated methane formation from 12 hours onward and was 56-fold greater than control without substrate amendments [no addition (No Add)] (Fig. 2A). Compared with H<sub>2</sub>, MeOH alone stimulated CH<sub>4</sub> production slowly and produced fivefold less CH<sub>4</sub> at day 30 than the H<sub>2</sub> treatment (Fig. 2A). The addition of H<sub>2</sub> + MeOH strongly stimulated methane formation, similar to the addition of

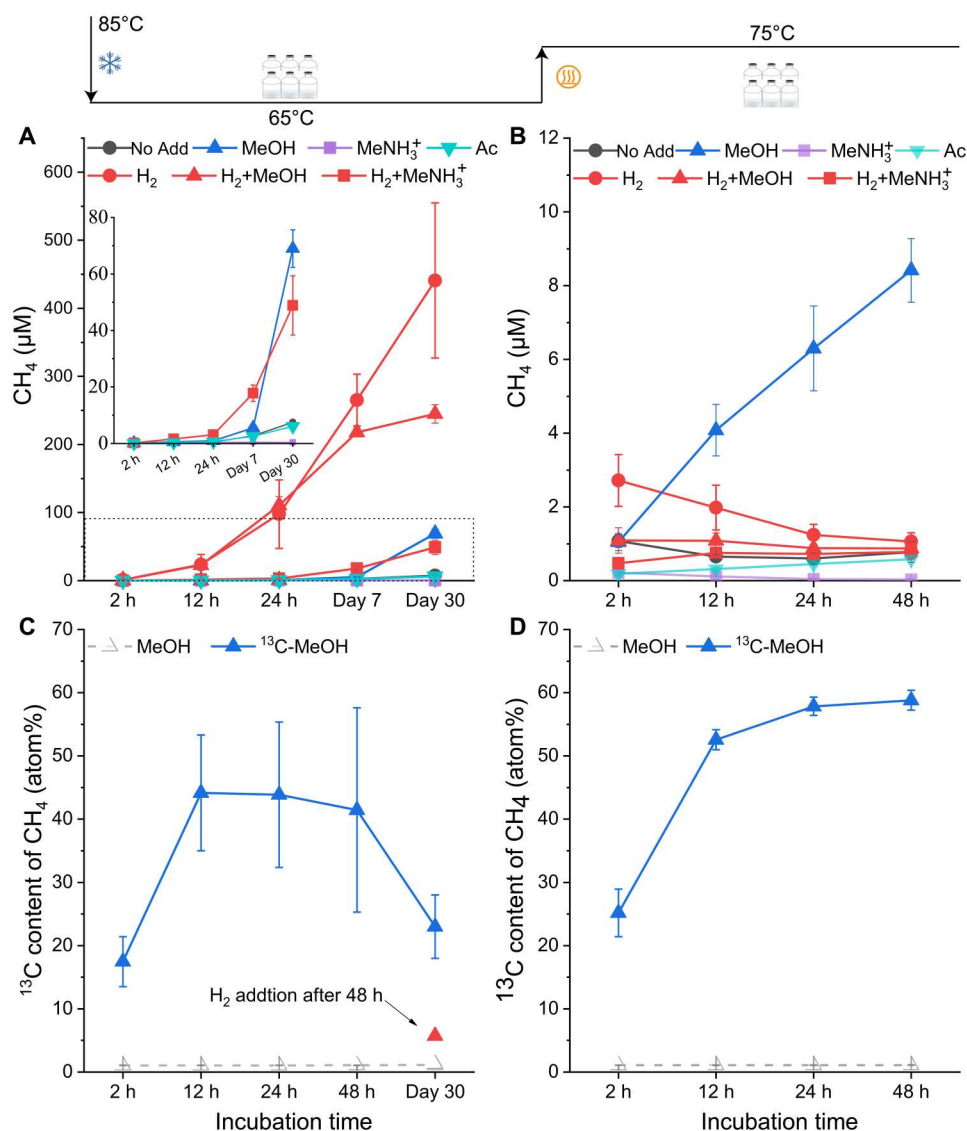


**Fig. 2. Dynamics of methanogenesis in microcosm incubations [“GuMingQuan Pool” hot spring (GMQP)] coupled experimental cooling/heating and the addition of different methanogenic substrates.** (A and C) Sediment and water were first stabilized at 65°C for 30 days in microcosms; then, microcosms were amended with substrates and incubated at 65°C for 30 days. (B and D) The temperature of the microcosms was then changed to 75°C and incubated for 30 days; last, the microcosms were reamended with substrate and incubated at 75°C for 48 hours. The vial headspace was replaced by flushing with  $\text{N}_2$  before substrate amendments. The inset in (A) summarizes dynamics of methane formation in microcosms of No Add, MeOH,  $\text{MeNH}_3^+$ , and  $\text{H}_2 + \text{MeNH}_3^+$  (in the dotted box). SD reflects results from three microcosms ( $n = 3$ ). No Add, no addition; MeOH, methanol; Ac, acetate;  $\text{MeNH}_3^+$ , methylamine. For incubation details, see fig. S6; for  $\text{CO}_2$  dynamics in microcosm incubations, see fig. S13.

$\text{H}_2$  alone. Following the 30-day 65° and 75°C incubations, spikes of  $^{13}\text{C}$ -MeOH into microcosms at the beginning of the 48-hour incubations showed that labeled  $\text{CH}_4$  was produced after only 2 hours of incubation (Fig. 2C). Subsequent  $\text{H}_2$  injection at 48 hours substantially decreased the final  $^{13}\text{C}$  content of headspace  $\text{CH}_4$  (26% versus 6%), further confirming the preference of hydrogenotrophic methanogenesis over methylotrophic methanogenesis at 65°C (Fig. 2C). The slight decrease of  $^{13}\text{C}$  content of the  $\text{CH}_4$  in the  $^{13}\text{C}$ -MeOH treatment at day 30 without  $\text{H}_2$  injection could be attributed to unlabeled  $\text{CH}_4$  produced from preexisting unlabeled DIC via hydrogenotrophic pathway. While methane was predominantly formed from  $\text{H}_2/\text{CO}_2$  at 65°C (Fig. 2A), methylotrophic methanogenesis

predominated when microcosms were incubated at 75°C (Fig. 2B). Furthermore, after heating to 75°C, an  $\text{H}_2$  respike failed to revive  $\text{CH}_4$  production, while a MeOH respike rapidly stimulated methanogenesis after 48 hours, which was ~11 times greater than a control without added substrate (Fig. 2B). Isotopic analysis confirmed that ~60% of the  $\text{CH}_4$  was produced from MeOH in  $^{13}\text{C}$ -MeOH-amended microcosms, providing further evidence of active methylotrophic methanogenesis at 75°C (Fig. 2B). As in JZ-3, methanogenesis was more sensitive to the shift to the higher temperature than  $\text{CO}_2$  production, as shown by the overall lower  $\text{CH}_4:\text{CO}_2$  ratio at 75°C relative to 65°C. Together, these results reveal that both methanogenic activity and the predominant

pathway used by methanogens in the GMQP microcosms were sensitive to temperature changes.

Because methanogenesis assays were made at the level of the whole community, observed variations in methanogenesis rates and shifts in dominant pathways could be attributed to microbial “community-level responses” (27, 31), which encompass acclimation (physiological responses of individuals), adaptation (genetic changes within species), and ecological responses (for example, altered community structure). For JZ-3, because methanogenesis assays were made at similar in situ temperature ranges (figs. S4 and S5), the underlying microbial community should be capable of adjusting appropriately to temperature changes. For GMQP, while preincubation at lower than in situ temperatures stimulated methanogenesis, thus allowing the study of community-level responses of methanogenesis (fig. S6), incubations may have caused unintentional temperature acclimation/adaptation to 65°C. However, during our experiments, we observed no loss of *mcr*-containing community diversity, but there were changes in their relative abundances and gene expression patterns (explained in the following sections). Therefore, we believe that stabilization at 65°C allowed the widest range of *mcr*-containing archaea to be maintained, which allowed us to probe the effects of changes in temperature or substrate after the microbial community has reached a steady state.

The observed shift in methanogenic pathways in GMQP microcosms was in accordance with thermodynamic inferences at high temperatures (fig. S7). While the energy available from hydrogenotrophic methanogenesis decreases rapidly when the temperature increases from 50° to 100°C, high temperature favors methylotrophic methanogenesis. Normalization of available energy to transferred electrons indicates that methanogen in GMQP and JZ-3 hot springs live close to the thermodynamic edge (fig. S7B). Because maintenance energy is high for thermophiles, subtle variations in available energy induced by temperature changes could exert huge impacts on the underlying metabolic processes. Nevertheless, our observation based on successive incubation may not fully correspond to temperature-dependent trends for methane production in situ owing to potential microbial acclimation/adaption; they will, however, provide critical information on how temperature increase affects community-level responses of methanogenesis in geothermal habitats.

Last, we calculated short-term methanogenesis rates of GMQP and JZ-3 from the incubations discussed above (Fig. 3A). Empirical methanogenesis rates were normalized to sediment dry mass, which allowed direct comparison of our rates in high-temperature geothermal springs with those of well-documented low-temperature terrestrial ecosystems, to better understand the relevance of the rates measured. The maximum short-term methanogenesis rate in GMQP, which represents an instantaneous response of methanogens to substrate spike, decreased from  $34.0 \pm 3.9$  ( $\text{H}_2$  spike) at 65°C  $\mu\text{g C g}^{-1}$  sediment day $^{-1}$  to  $1.8 \pm 0.3$   $\mu\text{g C g}^{-1}$  sediment day $^{-1}$  at 75°C (MeOH spike). Similarly, the short-term methanogenesis rate of JZ-3 (without exogenous substrate) decreased from  $76.0 \pm 23.4$  to  $9.1 \pm 5.3$   $\mu\text{g C g}^{-1}$  sediment day $^{-1}$ . These rates fall within the range found for previous incubations (4.0° to 50°C) conducted with soils/sediments from various low-temperature terrestrial ecosystems such as wetland and paddy soils (up to 43  $\mu\text{g C g}^{-1}$  sediment day $^{-1}$ ; fig. S8 and data file S1), indicating that high-

temperature geothermal ecosystems could have methanogenic potentials comparable with low-temperature anoxic ecosystems.

### Community dynamics and metabolic reconstruction

Microcosms with a higher relative abundance of *mcr*-containing archaea in the microbial community also had higher short-term methanogenesis rates (Fig. 3, A and B). Concurrent with the substantial decrease in short-term methanogenesis rates when the incubation temperature increased from 65° to 75°C, the relative abundances of *mcr*-containing archaea also decreased from 1.72 to 0.3% for GMQP and from 5.19 to 3.22% for JZ-3.

A comparison of *mcr*-containing archaea in the field survey with microcosm incubations (Fig. 1A) shows an increase relative abundance in the microcosms of members of the *Archaeoglobales* and known euryarchaeotal methanogens at the expense of *Ca. Nezharchaeota* (Fig. 3B and fig. S10). For GMQP, *Archaeoglobales* (1.10%) replaced *Ca. Nezharchaeota* as the predominant *mcr*-containing archaea, followed by *Methanothermobacter* (0.36%), after stabilization at 65°C. The *Archaeoglobales* MAG JZ\_75\_SW\_bin\_109 accounted for 0.21% of the community and 50% of *mcr*-containing archaea in the MeOH treatments at 75°C. Similarly, incubations of JZ-3 microcosms at 65°C increased the abundance of known euryarchaeotal methanogens and *Archaeoglobales* JZ\_75\_SW\_bin\_109. These traditional euryarchaeotal methanogens decreased in relative abundance when the temperature was raised to 75°C (Fig. 3B and fig. S10). Hydrogenotrophic *Methanothermobacter* dominated in  $\text{H}_2$  plus MeOH incubations (2.79%; fig. S10), which was the only exception to the overall decrease in euryarchaeotal methanogen MAGs. However, hydrogenotrophic methanogenesis was severely limited at 75°C (Fig. 2B). Changes in temperature and/or substrate also caused variations in whole community structures (fig. S9). The in situ community inhabiting GMQP consisted mainly of *Aquificae*, *Ca. Bipolaricaulota*, *Thermotogae*, and Archaea (mainly *Crenarchaeota*), while *Ca. Bipolaricaulota* and *Thermodesulfobacteria* dominated JZ-3. Stabilization at 65°C only had a minor impact on the relative abundance of Archaea in GMQP but substantially increased the abundance of *Deinococcus-Thermus*. A notable increase of Euryarchaeota in JZ-3 was observed in microcosms relative to the in situ community. Further temperature increase to 75°C decreased the relative abundance of archaea, particularly *Crenarchaeota*, in both springs.

Inference of potential metabolisms from the *mcr*-containing archaeal MAGs (genomic characteristics, data file S2) revealed that these MAGs contained metabolic capacities for each of the four known methanogenic pathway schema: hydrogenotrophic, acetoclastic, methylotrophic, and  $\text{H}_2$ -dependent methylotrophic methanogenesis (Fig. 4 and data file S3) in GMQP and JZ-3 communities combined. Known hydrogenotrophic methanogens belonged to the genera *Methanothermobacter* and *Methanolinea*; also present was the obligate acetoclastic methanogen *Methanoxthrix* (Fig. 4) (9). In the nontraditional *mcr*-containing archaea, the presence of a complete archaeal Wood-Ljungdahl (WL)  $\text{CO}_2$  reduction pathway and all subunits of tetrahydromethanopterin S-methyltransferase (*mtrABCDEFGHI*) in one *Ca. Nezharchaeota* and all *Archaeoglobales* MAGs demonstrated the capability for hydrogenotrophic methanogenesis. The presence of *mtaABC* genes in the *Archaeoglobales* MAG suggested the capability for methylotrophic methanogenesis from methanol. The *Ca. Verstraetearchaeota* MAG also harbored *mtaABC* genes, but the lack of the WL pathway and

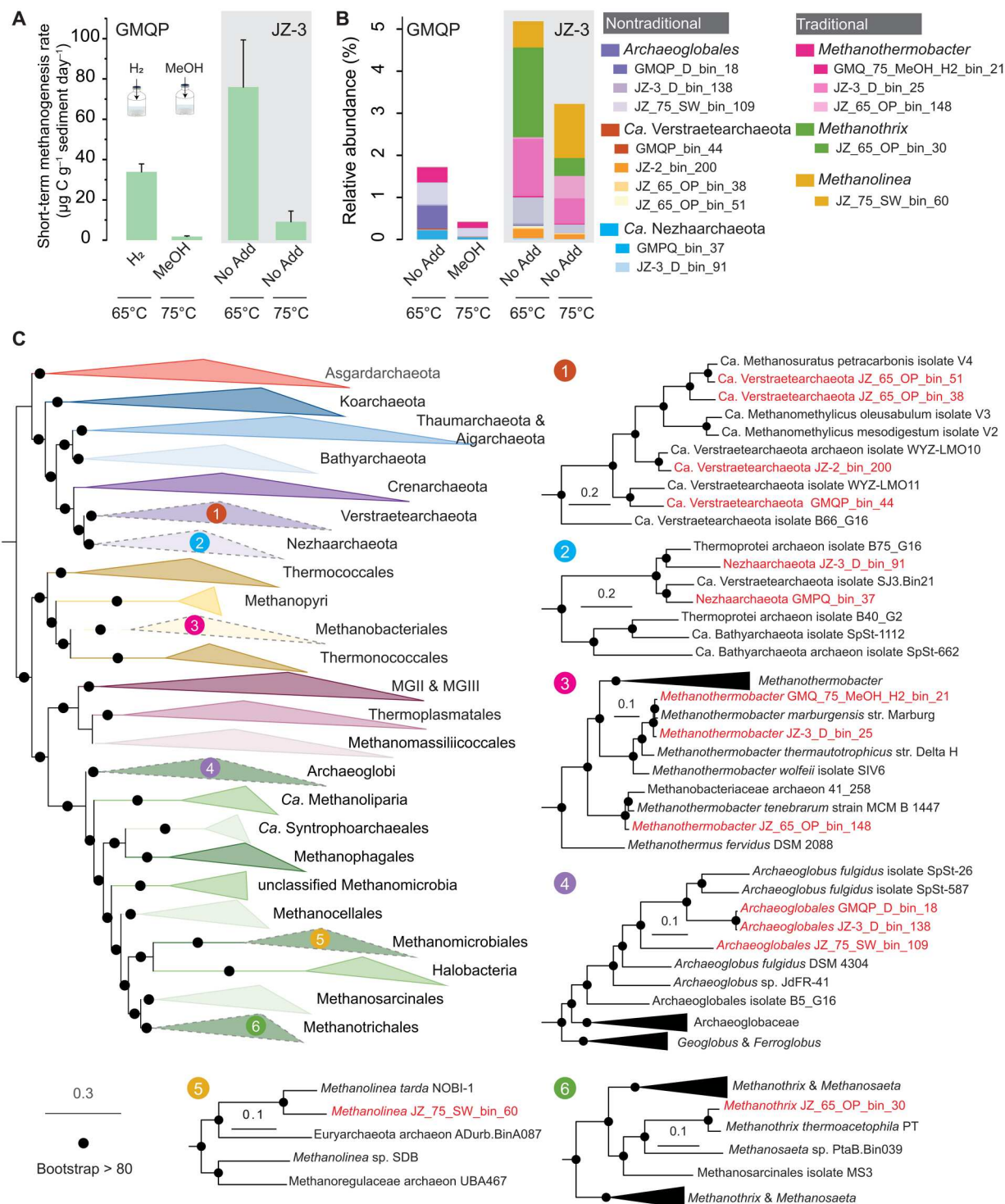


**Fig. 3. Reconstruction of *mcr*-containing archaea from microcosm experiments.** Methanogenesis rates (A), community dynamics (B), and phylogeny (C) of reconstructed *mcr*-containing metagenome-assembled genomes (MAGs) from “GuMingQuan Pool” hot spring (GMQP) and JinZe-3 hot spring (JZ-3). No Add, no addition; MeOH, microcosms that received methanol. The short-term methanogenesis rate was calculated on the basis of the sediment mass, incubation time, gas accumulation, and headspace volume following the methods of Chen *et al.* (27). The numbers in circles (C) denote the different lineages containing *mcr* genes in MAGs assembled from data in the present study. The expanded phylogenies at the right side and below display the detailed topology within each group. Items labeled in red are MAGs reconstructed in the present study. Maximum-likelihood tree was inferred from a concatenated set of 122 proteins using IQ-TREE (67) with 1000 ultrafast bootstrapping iterations. Bootstrap confidences >80% are shown on each node. For comparison of short-term methanogenesis rate with previous incubations (4.0 to 50°C) conducted with soil/sediment from various low-temperature terrestrial ecosystems, see fig. S8. For dynamics of overall microbial community, see fig. S9.

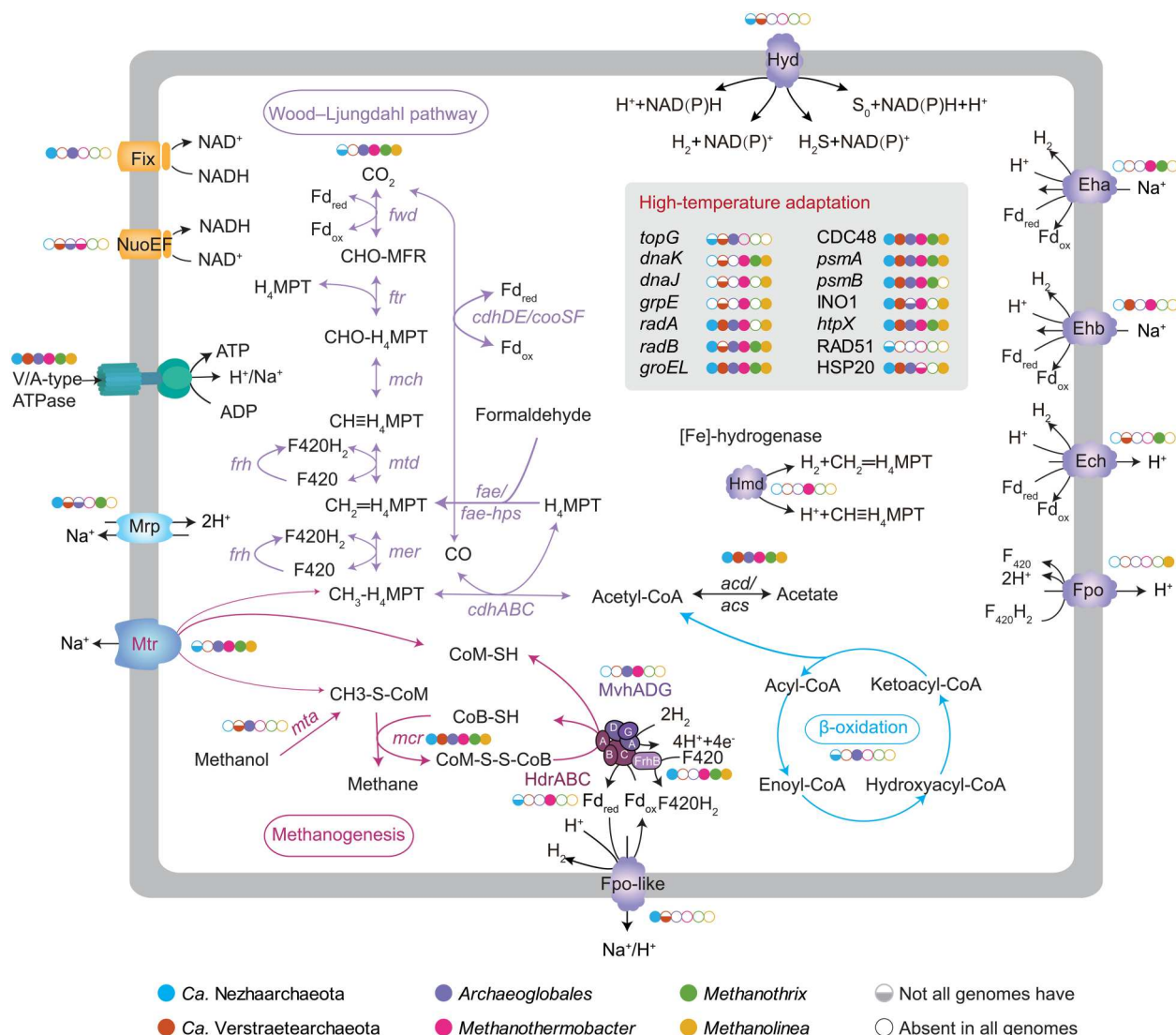


**Fig. 4. Overview of metabolic potential of *mcr*-containing metagenome-assembled genomes (MAGs) from “GuMingQuan Pool” (GMQP) and JinZe-3 (JZ-3) hot springs.** The pathways and genes detected are grouped into carbon, hydrogen, sulfur, and other metabolic pathways including sugar and amino acid utilization, energy conservation, and various transporters for each MAG grouping. Colors indicate separate metabolism modules. Detailed gene copy information associated with the above-mentioned pathways is presented in data file S3.

*mtrABCDEFGHIH* suggested that it is an obligate  $H_2$ -dependent methylotrophic methanogen (14). No genes encoding for any conventional terminal respiratory reductases or multiheme c-type cytochromes (MHCs) were identified in *Archaeoglobales* or *Ca. Nezaarchaeota* MAGs, which suggested that these two nontraditional lineages are not likely to perform anaerobic methane oxidation like known anaerobic methanotrophic archaea (32, 33). While all MAGs encoded the capacity to synthesize diverse molecular chaperones, including heat-shock proteins (HtpX and Hsp20) and DNA repair enzymes (RadAB), only nontraditional *mcr*-containing archaea harbored genes encoding reverse gyrase (*topG*), a key determinant of a hyperthermophilic lifestyle at or above 85°C (34).

We further constructed phylogenetic trees of key diagnostic protein sequences for methane metabolism (*McrABG*) and methanol utilization (*MtaABC*) (figs. S11 and S12). Phylogenetic

inference revealed that *Mcr* sequences from *Archaeoglobales*, *Ca. Nezaarchaeota*, and *Ca. Verstraetearchaeota* are closely related (fig. S11), although their host taxa are diverse (Fig. 3C). Therefore, we deduce that *mcrABG* genes were horizontally transferred to *Archaeoglobales* from TACK lineages. According to the phylogenetic inference (fig. S12), no explicit evidence supports the idea that *mtaABC* genes in *Archaeoglobales* were acquired horizontally. The deep branching of the MTA complex within *Archaeoglobales* and TACK suggests that methanol utilization may represent an ancient feature in Archaea, particularly in TACK, which evolved even earlier than certain traditional methanogens.

#### Gene expression patterns under methanogenic conditions

We used metatranscriptomics to reveal gene expression patterns related to methane metabolism and energy conservation from

microcosm communities with high CH<sub>4</sub> production (Fig. 5 and data file S4). A stringent criterion was applied to define active methane producers based on the expression of all three *mcr* subunits (*mcrABG*) from a single MAG. This implicated nontraditional *mcr*-containing archaea from the *Archaeoglobales* and *Ca. Nezhaarchaeota* and the traditional euryarchaeotal methanogens *Methanothermobacter* and *Methanothrix* as active members in microcosm cultures (Fig. 5 and data file S4).

Transcripts of the *mcrABG* genes from *Archaeoglobales* JZ\_75\_SW\_bin\_109 were identified in all microcosms that vigorously produced CH<sub>4</sub> (Fig. 5). In particular, this MAG was the only *mcr*-containing archaeon that expressed *mcrABG* genes at 75°C in both JZ-3 native microcosms without added substrates and the GMQP MeOH-amended microcosm in which active CH<sub>4</sub> production from MeOH was demonstrated (Fig. 2). The *mcrABG* transcripts of another *Archaeoglobales* MAG, JZ-3\_D\_bin\_138, were also present in JZ-3 in microcosm incubations at 65°C. In parallel, genes related to the WL pathway and other core methanogenesis genes (*mtrA-H*; *mtaABC*) were expressed by this *Archaeoglobales* MAG.

Despite a low relative abundance in incubations (Fig. 3B), *Ca. Nezhaarchaeota* JZ-3\_D\_bin\_91 from JZ-3 also produced

*mcrABGCD* transcripts at 65°C (Fig. 5C and data file S4). Unlike the other *Ca. Nezhaarchaeota* MAG, GMQP\_bin\_37, most genes involved in the WL pathway were not detected, suggesting that functional differentiation may have occurred and further led to the acquisition/loss of autotrophic capacity in certain lineages. Although *Ca. Nezhaarchaeota* GMQP\_bin\_37 harbors the complete WL pathway, most genes associated with the WL pathway exhibited either low or no expression. Yet, lack of the *mta* complex in this lineage precludes the utilization of methanol for methanogenesis, and gene expression patterns related to methanogenesis in *Ca. Nezhaarchaeota* remain to be investigated. However, the possibility that the incomplete genome (91.9%) might obscure gene expression patterns cannot be ruled out (data file S2). Instead of the cytoplasmic F<sub>420</sub>-nonreducing hydrogenase encoded by *mvhADG*, both *Ca. Nezhaarchaeota* and *Ca. Verstraetearchaeota* MAGs encoded homologs of F<sub>420</sub>-methanophenazine oxidoreductase (*fpo*) (35), which could form an Fpo-like complex to reoxidize reduced ferredoxin. The Fpo-like complex was highly similar to the membrane-bound NADH (reduced form of nicotinamide adenine dinucleotide)-ubiquinone oxidoreductase (Nuo) complex with respect to the composition and amino acid sequence identity. This complex is similar to those in *Methanomassiliicoccales* (36) and

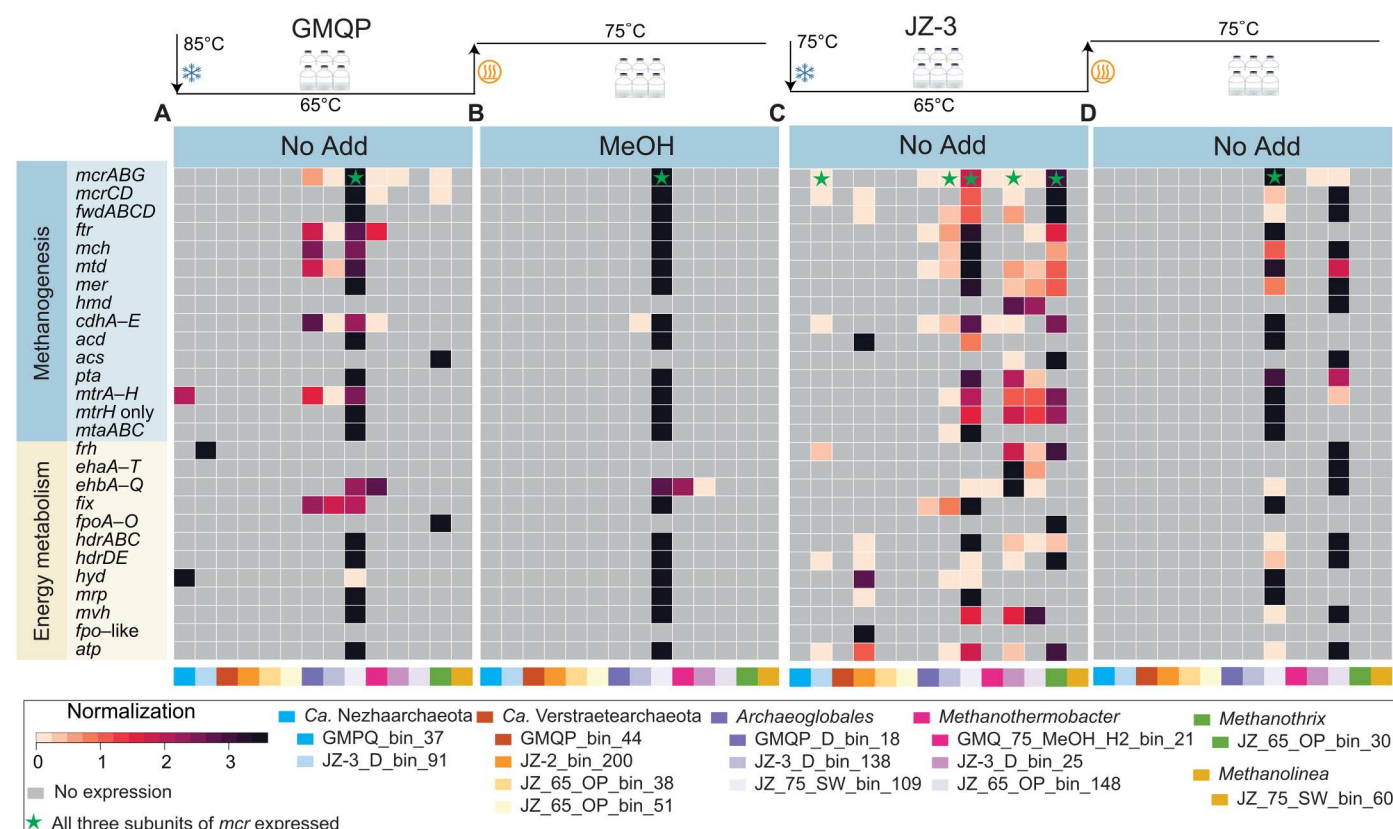


**Fig. 5. Gene expression patterns related to methanogenesis and energy metabolism from microcosm incubations forming notable quantities of CH<sub>4</sub>.** (A) Transcripts mapped to MAGs in the GMQP microcosms at the end of the 30-day stabilization at 65°C. (B) Transcripts mapped to MAGs in the GMQP microcosms at 75°C with MeOH amendment. (C) Transcripts mapped to MAGs in the JZ-3 microcosms at end of the 30-day stabilization at 65°C without substrate amendments. (D) Transcripts mapped to MAGs in the JZ-3 microcosms at 75°C without substrate amendments. No Add, no addition; MeOH, methanol; the green star indicates the expression of all three subunits of *mcrABG* in the respective MAGs. Items shaded with tan colors represent genes with transcript mapping, and gray items denote genes with no transcript mapping. Gene expression data were normalized across taxa, and z scores were generated using "scale" program in R v4.0.3 with parameters: center = F, scale = T. Detailed gene expression values are recorded in data file S4.

*Methanotherix thermophila* (37), which lack the subunits required for binding NADH (NuoEFG) and F<sub>420</sub> (FpoFO). This result confirms the preference of ferredoxin as an electron donor. Expressed *mcrABG* and *mtrABCDEFGH* genes from the traditional hydrogenotrophic *Methanothermobacter* and acetoclastic methanogen *Methanotherix* were also detected in JZ-3 microcosm at 65°C. However, heating JZ-3 microcosms from 65° to 75°C decreased the expression of *mcrABG* from both *Methanothermobacter* and *Methanotherix*.

Last, gene expression patterns related to methane metabolisms and energy conservation were explored from two GMQP microcosm communities with low CH<sub>4</sub> production at 75°C (i.e., No Add and H<sub>2</sub>). *Archaeoglobales* JZ\_75\_SW\_bin\_109 was the only *mcr*-containing archaeon that expressed *mcrABG* genes in both microcosms, further strengthening the role of this lineage as a major active methane producer at 75°C (fig. S14). Furthermore, MeOH-utilizing *mtaABC* genes had much higher expression levels than that of genes associated with the WL pathway (data file S4). This pattern suggested that the methylotrophic pathway in *Archaeoglobales* JZ\_75\_SW\_bin\_109 might be favored at 75°C, consistent with our experimental observations (Fig. 2) and thermodynamic inferences (fig. S7).

## DISCUSSION

This study demonstrates the importance of nontraditional *mcr*-containing archaea in mediating biological methanogenesis in volcanogenic geothermal springs. The <sup>13</sup>C-MeOH-amended methanogenic assays revealed active methylotrophic methanogenesis in GMQP spring from 65° to 85°C (Figs. 1C and 2, C and D). The enrichment of headspace <sup>13</sup>C-CH<sub>4</sub> was detectable within 2 hours of incubation, showing an instant response of methylotrophic methanogens to substrate addition. Community-level genomic reconstruction revealed that only two nontraditional lineages in *mcr*-containing communities, i.e., *Archaeoglobales* and *Ca. Verstraetearchaeota*, encoded the capacity for methylotrophic methanogenesis from MeOH (Fig. 4 and data file S3). The involvement of traditional methanogens in this pathway could be ruled out because all of them lack *mtaABC* genes necessary for methanol utilization (Fig. 4 and data file S3). The participation of *Archaeoglobales* in methylotrophic methanogenesis was further corroborated by the high expression of key methylotrophic methanogenesis-related genes in MeOH-amended microcosms (Fig. 5B and data file S4). Within *Archaeoglobales*, JZ\_75\_SW\_bin\_109 stood out in GMQP spring because it expressed key genes for H<sub>2</sub>/CO<sub>2</sub> and MeOH methanogenesis in both 65° and 75°C incubations. Likewise, in these 65° and 75°C microcosm incubations, the predominant methanogenic pathways were shown to be hydrogenotrophic and methylotrophic, respectively (Fig. 2, A and B). Moreover, the involvement of *Archaeoglobales* in methanogenesis was not an isolated phenomenon because similar gene expression patterns were also found in JZ-3 spring under in situ-like conditions (Fig. 5, C and D). These results indicate the potential coexistence of active hydrogenotrophic and methylotrophic pathways in *Archaeoglobales*, which could confer a selective advantage in adapting to changing environmental conditions such as variations in temperature and substrate energetics. Could our collective observations be alternatively explained by methane production from other organisms that do not contain recognizable *mcr* complexes? We argue that they cannot, because the

extreme physicochemical conditions studied here are not conducive to any of these other known methane-producing organisms (38–40). Ultimately, our experiments do not rule out other pathways for methanogenesis in geothermal environments, but we believe that the evidence presented here firmly establish methanogenesis by a member of the *Archaeoglobales* (JZ\_75\_SW\_bin\_109) and potentially other archaea as well.

A comparison of 5-year field surveys to microcosm incubations showed much higher proportions of *Ca. Nezhaarchaeota* (Fig. 3B). A parsimonious explanation for this disparity would be that *Ca. Nezhaarchaeota* may benefit from the continuous supply of resources or removal of products in situ, conditions that were not replicated during in vitro incubations. In contrast, traditional euryarchaeotal methanogens appear not to be fully adapted in GMQP to in situ temperatures of 85° to 90°C, reflected by the lack of genes encoding reverse gyrase (*topG* in Fig. 4 and data file S3), and correspondingly hydrogenotrophic methanogenesis by traditional methanogens was severely inhibited at 75°C (Figs. 2B and 5). The abundant DIC (>10 mM; mainly HCO<sub>3</sub><sup>−</sup>) and the deficiency of other typical terminal electron acceptors (e.g., sulfate, nitrate, and Fe<sup>3+</sup>) in these geothermal springs (30) (table S2) render anaerobic methane oxidation thermodynamically unfavorable, and therefore, this process is unlikely in our experiments. Correspondingly, we could not identify any terminal reductases or MHCs in genomes of *Archaeoglobales* or *Ca. Nezhaarchaeota*. Combined with the expression of *mcrABG* under methanogenic conditions (Fig. 5C) and the predominance of *Ca. Nezhaarchaeota* among native *mcr*-containing communities (Fig. 1A), we reason that *Ca. Nezhaarchaeota* might be the key participant mediating the observed in situ hydrogenotrophic methanogenesis in GMQP (Fig. 1C). Furthermore, based on high relatedness between *Mcr* sequences from *Archaeoglobales*, *Ca. Nezhaarchaeota*, and *Ca. Verstraetearchaeota* (fig. S11), which reflects functional similarity of the respective archaeal lineages (4, 13), we propose that these nontraditional *mcr*-containing archaea are the origin of methanogenesis in these springs. Our results therefore also support the hypothesis that the last common ancestor of Euryarchaeota and TACK archaea could have been a methanogen (41, 42).

The observation of functional methanogenesis in *mcr*-containing *Archaeoglobales* promises to resolve an ongoing debate over its evolutionary transition from methanogenesis to a sulfate-reducing lifestyle by gaining bacterial dissimilatory sulfite reductase genes (*dsr*) (9, 43). However, the *mcr*-containing *Archaeoglobales* MAGs from our alkaline springs in Tengchong show no co-occurrence of *mcr* and *dsr* genes. We suggest that the previously reported *Archaeoglobales* MAGs from sulfidic Yellowstone hot springs (15) that contain both *mcr* and *dsr* genes may represent an intermediate stage in the transition from methanogenesis to sulfate reduction. A similar evolutionary adaptation also likely occurred in *Ca. Korarchaeota* MAGs that harbor both *mcr* and *dsr* genes (18). By presenting a benchmark where methanogenic lifestyles in *mcr*-containing *Archaeoglobales* are shown, the methodologies used in Tengchong hot springs presented here could be applied to other geothermal systems to expand and understand the evolutionary relationship between methanogenesis and sulfate reduction.

On early Earth, which was anoxic, more volcanically active (22, 23), and had a higher surface temperature (>60°C) than today (26), the carbon cycle is widely believed to have been dominated by methanogenesis (1, 25). Previous early Earth ecosystem-planetary

models were often grounded on the assumption that hydrogenotrophic and acetoclastic methanogenesis predominated in primitive CH<sub>4</sub>-cycling ecosystems and was constrained to our understanding of traditional euryarchaeal methanogens (1, 44). Our results indicate that primitive ecosystems may have been dominated by diverse *mcr*-containing archaea performing hydrogenotrophic and methylotrophic methanogenesis (Figs. 2 and 3), in line with recent evolutionary inference (41, 42). Moreover, those primitive, high-temperature ecosystems could have had methanogenic potentials comparable to contemporary low-temperature anoxic ecosystems (fig. S8 and data file S1). In sufficient numbers, these systems would have had global consequences and affected the climate on early Earth.

While traditional euryarchaeotal methanogens populate nearly every conceivable anoxic habitat on contemporary Earth (9), it is currently unclear how widely distributed nontraditional *mcr*-containing archaea are. However, recent metagenomic studies suggest that these nontraditional lineages are not limited to hydrothermal settings (9, 14, 19). This study provides a view into the bulk methanogenic activities and underlying active members by coupling in situ and ex situ incubations, isotope tracers, metagenomics and metatranscriptomics, and biogeochemistry. A full understanding of the ecophysiology of those nontraditional *mcr*-containing archaea would further require targeted enrichment/cultivation strategies coupled with fluorescence in situ hybridization–nano secondary ion mass spectrometry. On a larger scale, how the coexistence or competition of traditional euryarchaeal and nontraditional methanogens in diverse ecosystems affects the biogeochemical carbon cycle and contributions of nontraditional methanogens to global CH<sub>4</sub> emissions remains to be investigated. Geothermal springs represent early Earth analogs harboring a vast repository of microbial dark matter (45) that will continue to inspire multidisciplinary research about the evolution of life on Earth.

## MATERIALS AND METHODS

### Location and geochemical characterization of the study site

Two terrestrial geothermal springs, GMQP (98°26'10"E, 24°57'3'N) and JZ-3 (98°27'36"E, 25°26'28"N), are located in the Tengchong volcanogenic geothermal area (Yunnan, China), which belongs to the Yunnan-Sichuan-Tibet Geothermal Province, a part of the Mediterranean-Himalayas geothermal belt. GMQP (in situ temperature: 85° to 90°C; pH: 9.4) is formed by the outflow of geothermal water originating from a boiling reservoir fluid, while JZ-3 (in situ temperature: 65° to 75°C; pH: 8.9) is likely formed primarily via conductive cooling of a reservoir fluid or its mixing with low-temperature shallow groundwaters. GMQP and JZ-3 are typical of the high-temperature geothermal springs located in the Rehai and Diantan areas. These systems are the largest and second largest hydrothermal areas in Tengchong, respectively (46). The geological and geothermal characteristics of Rehai and Diantan have been reported previously by Guo and Wang (46) and are summarized in table S1.

### Sample collection and processing

The microbial communities from GMQP and JZ-3 springs were monitored using metagenomic analyses of sediment samples collected twice yearly from 2017 to 2021. These sediments were collected anaerobically, flash-frozen on-site in liquid nitrogen, and

transferred to the laboratory before being stored at –80°C, and total genomic DNA was extracted using previously described methods (4, 47). For laboratory-based microcosm experiments, sediment and geothermal water were sampled aseptically from the GMQP and JZ-3 springs in 2021. The collected biomass was stored without headspace in amber-colored glass vials (sealed with a gas-tight polytetrafluoroethylene (PTFE) gasket and screw cap) and transported to the laboratory for microcosm setup at ambient temperature.

### In situ methanogenesis assays

At the second 2021 sampling, a static respiration chamber was used to estimate the in situ CH<sub>4</sub> flux in the GMQP spring (in situ temperature: 85°C) (fig. S1). In situ experiments were not conducted in JZ-3 due to the water depth not allowing deployment of the chamber. The chamber was made in-house and consisted of a polyvinyl chloride housing with a vent tube for pressure equilibration between accumulated gas and the external atmosphere, a three-way gas sampling valve, and an internal fan to homogenize accumulated gas before sample collection (fig. S1C).

The active pathways underlying methanogenesis in the hot springs were identified using microcosm-based enrichments amended with either <sup>13</sup>C-labeled NaHCO<sub>3</sub> or methanol (MeOH) and compared to unlabeled H<sub>2</sub>, NaHCO<sub>3</sub>, or MeOH. These anaerobic microcosm assays were prepared by suspending ~5 g of fresh sediment in autoclaved serum bottles (100 ml) with 20 ml of geothermal water under an N<sub>2</sub> headspace. Vials were then capped with butyl rubber septa and immediately returned to the GMQP spring for incubation. A total of six treatments, each with four replicates, were incubated in the GMQP spring and consisted of (i) control without amendment (No Add), (ii) H<sub>2</sub> (~6%, 5 ml of H<sub>2</sub> into 80 ml of N<sub>2</sub>-headspace), (iii) H<sub>2</sub> plus 10 mM NaHCO<sub>3</sub>, (iv) H<sub>2</sub> plus 10 mM <sup>13</sup>C-labeled NaHCO<sub>3</sub>, (v) 10 mM MeOH, and (vi) 10 mM <sup>13</sup>C-labeled MeOH. MeOH was used as a representative substrate for methylotrophic methanogenesis as it is the most abundant oxygenated volatile organic compound in the atmosphere (48) and is a common substrate of anaerobic metabolism (49). Moreover, MeOH may be derived from endogenous synthesis via the mineral-catalyzed fixation of CO<sub>2</sub> with H<sub>2</sub> under alkaline hydrothermal conditions (50). Acetate was not used as the presently known upper-temperature limit for acetoclastic methanogenesis is ~70°C (51).

Gas headspace from the respiration chamber and microcosm assays were sampled at 24 and 48 hours and stored in pre-evacuated gas-sampling bags (Delin, LB-301A) before analysis. Short incubation times were used to prevent methanogen adaptation to substrate spikes in microcosms and provided estimates of an instant response of the native community (27). The potential for microbial substrate adaptation from the in situ microcosms was investigated by transfer of enrichment cultures to the laboratory incubators at 85°C for 30 days to mimic the thermal conditions of the GMQP spring. The methane quantity in the headspace was sampled on days 10 and 30.

### Laboratory microcosm incubations

Further anaerobic microcosm incubations using a combination of fresh sediments and water samples from GMQP and JZ-3 were prepared aseptically under N<sub>2</sub> headspace inside a laboratory anoxic glovebox. GMQP sediments (dry weight: ~3.0 g) were mainly composed of sand (main metal contents, table S4), while JZ-3 sediments (dry weight: ~0.31 g) were composed of dark green, slippery

streamer material (photo, fig. S3). Geothermal water (20 ml in each microcosm) was filtered (0.2  $\mu\text{m}$ ) and  $\text{N}_2$ -purged (30 min) to prevent oxygen contamination as described in a previous study (52).

Methanogenesis was stimulated in the GMQP microcosm incubations over a stepwise temperature gradient from 65° to 85°C by amending samples with several methanogenic precursors summarized in fig. S6. In summary, two multiple-stage incubation programs were used to cultivate methanogens. The first experimental program consisted of materials being prestabilized at 65°C for 30 days, and then, methanogenic substrates were amended and incubated at this temperature to perform methanogenic assays for an additional 30 days. Subsequently, the assays were heated to 75°C for 30 days, after which the substrates were reamended and incubated at 75°C for an additional 48 hours. The second experimental program consisted of materials being prestabilized at 85°C for 30 days, and then, methanogenic substrates were amended at this temperature and incubated to perform methanogenic assays for an additional 30 days. Prestabilization helps to minimize the perturbations during sediment/water sampling and transport and the concomitant influence on microbial communities before methanogenesis assays. In addition, the stabilization period allows microbial communities to adjust during temperature transition (e.g., 65°C/75°C), facilitating the study of community-level responses after steady conditions have been reached. Subsequently, the assays were cooled to 75°C for 30 days, after which the substrates were reamended and incubated at 75°C for an additional 48 hours. The temperatures of 65°, 75°, and 85°C were selected on the basis of our previous genome-based inferences that most extant *mcr*-containing genomes from terrestrial geothermal springs thrived optimally under temperatures ranging from ~60° to 83°C (4). Incubations contained  $\text{H}_2$  at ~6% of headspace and exploited indigenous DIC in geothermal water, as this reflects physiological levels found in the Tengchong volcanogenic geothermal areas that varied between 0.32 and 5.15% (53). This  $\text{H}_2$  level also avoids classical cultivation/enrichment methods that use high-energy yielding 80:20  $\text{H}_2/\text{CO}_2$ , which is ecologically irrelevant (54), especially in terrestrial hot springs. In total, seven incubation treatments each with three replicates were used: (i) No Add without amendment, (ii)  $\text{H}_2$  (~6%), (iii) 10 mM MeOH, (iv)  $\text{H}_2$  + 10 mM MeOH, (v) 10 mM methylamine ( $\text{MeNH}_3^+$ ), (vi)  $\text{H}_2$  + 10 mM methylamine ( $\text{MeNH}_3^+$ ), and (vii) 10 mM sodium acetate (acetate). Both the instantaneous and long-term responses to substrate addition were monitored in 48-hour and 30-day respiration assays with the  $\text{CH}_4$  and  $\text{CO}_2$  measured during the processes. To distinguish whether  $\text{CH}_4$  production was derived directly via methylotrophic methanogenesis or indirectly from MeOH syntrophy (first oxidizing MeOH to  $\text{H}_2$  and  $\text{CO}_2$ ), short-term respiration assays with 10 mM  $^{13}\text{C}$ -labeled MeOH at 65° and 75°C along incubations were performed. The indigenous carbonate/bicarbonate buffering system guaranteed stable pH values of ~9.4 over the whole experiment (table S5). Before the addition of substrates, the vials were flushed with high-purity  $\text{N}_2$  gas (30 min) to minimize the potential inhibitory effects of high  $\text{CH}_4$  concentrations on methanogenesis as previously described (27).

For JZ-3 with in situ temperatures ranging from 65 to 75°C, only the aforementioned first incubation program was used (see fig. S4 for details). No exogenous substrate was provided, given that vigorous  $\text{CH}_4$  production was found under in situ temperature ranges in our laboratory microcosm incubations.

## Geochemical analytical techniques

Headspace  $\text{CH}_4$  and  $\text{CO}_2$  concentrations along with their  $^{13}\text{C}$  contents were measured at several time intervals with cavity ring-down spectroscopy (Picarro G2201-i, USA) using a discrete small-volume gas sample module (Picarro Small Sample Isotope Module, SSIM Ao314, USA) (27). The short-term  $\text{CH}_4$  respiration rate was calculated on the basis of the soil mass (dry weight), incubation time (24 hours), gas accumulation, and headspace volume (80 ml) following the methods of Chen *et al.* (27).

For hydrochemical properties, geothermal waters were collected and analyzed using standard methods (46, 55). Nonstable chemical parameters (temperature, pH, redox potential, and free sulfide) were measured immediately on-site using portable HACH meters. All water samples were filtered through a 0.45- $\mu\text{m}$  membrane on-site, transferred on ice, kept anoxic in the dark, and stored at 4°C before analysis. The contents of dissolved organic carbon (DOC), DIC, and dissolved total nitrogen were analyzed by Total Organic Carbon Analyzer (Shimadzu TOC-L). Concentrations of anions, that is, nitrate ( $\text{NO}_3^-$ ), sulfate ( $\text{SO}_4^{2-}$ ), phosphate ( $\text{PO}_4^{3-}$ ), and chloride ( $\text{Cl}^-$ ), were analyzed in unacidified samples by ion chromatography (930 Compact IC with Metrosep A Supp 17-150/4.0 column, Switzerland).

## Thermodynamic calculations

The thermodynamic basis for the observed temperature dependency of methanogenesis in GMQP materials was calculated on the basis of temperatures from 50° to 100°C for the free energy available. Reactions for (i) hydrogenotrophic methanogenesis from  $\text{H}_2/\text{HCO}_3^-$ , (ii) methylotrophic methanogenesis from MeOH, and (iii)  $\text{H}_2$ -dependent methylotrophic methanogenesis from MeOH are given in table S6 [ $\Delta G^\circ$  from Whitman *et al.* (56)]. Available energy is computed with Geochemist's Workbench and the Lawrence Livermore National Laboratory (LLNL) thermodynamic database by taking pH at 9.6, 3 to 6%  $\text{H}_2$ , 10 mM methanol, 11.25 mM bicarbonate, and 10  $\mu\text{M}$  methane from aqueous chemistry and microcosm incubation setups.

## Metagenomic and metatranscriptomic analyses

Community genomic DNA and RNA were extracted using commercial kits (ALFA-SEQ Advanced Soil DNA Kit and MOBIO RNA PowerSoil Total RNA Isolation Kit, respectively) according to the manufacturer's instructions. DNA extract concentrations and constructed libraries were assessed on the Qubit 4.0 Fluorometer and the Qsep400 High-Throughput Nucleic Acid Protein Analysis System. Ribosomal RNA transcripts were removed from the total RNA pool using an ALFA-SEQ rRNA Depletion Kit. Whole mRNA-sequencing libraries were generated using the NEB Next Ultra Nondirectional RNA Library Prep Kit for Illumina (New England Biolabs, MA, USA). Metagenomic and metatranscriptomic sequencing were conducted by applying Illumina HiSeq 4000 instruments at Guangdong Magigene Biotechnology Co. Ltd. (Guangzhou, China), generating, on average, 30-giga base pairs (Gbp) raw sequence data per sample. For metagenomics sequencing data, raw sequence reads were preprocessed using an in-house Perl script (v1.0.0 last accessed at 15 April 2023; <https://github.com/hzhengsh/qualityControl>) (57). The quality-filtered reads were assembled individually for each sample using SPAdes v3.9.0 (--meta -k 21,33,55,77,99,127) (58). Genomic bins were obtained for each assembly with MetaBAT v2.12.1 (59) using tetranucleotide

frequency and sequence depth parameters. Bins were merged if the combination resulted in a notable increase in completeness with no or minimal increase in contamination score. Genome completeness and contamination were evaluated using CheckM v1.0.12 (60). Scaffolds with abnormal sequence depth and discordant positions in the Emergent self-organizing map (ESOM) map (61) were manually eliminated. To enhance the quality of genomic bins, sequences belonging to each genome bin were recruited by mapping clean reads to preliminary bins using BBmap v35.85 (<http://sourceforge.net/projects/bbmap/>) and reassembled allowing correction using SPAdes v3.9.0 (--care -k 21,33,55,77,99,127). Last, 14 high-quality MAGs that contained the methyl-coenzyme reductase subunits A, B, and G (*mcrABG*) genes were further analyzed. Putative protein-coding genes (CDSs) for each MAG were predicted using Prodigal v2.6.3 (-single) (62). Functional annotation was conducted by comparing all CDSs against databases, including National Center for Biotechnology Information–non redundant (NCBI-nr), and archaeal Clusters of Orthologous Genes (arCOG), using Diamond v2.0.11.149 ( $E$  value  $<1 \times 10^{-5}$ ) (63). KEGG Orthology (KO)-based functional annotation was conducted using KofamScan v1.3 ([https://github.com/takaram/kofam\\_scan](https://github.com/takaram/kofam_scan)).

For metatranscriptomic data, raw reads were quality-controlled using Sickel v1.33 (-t sanger --quiet -l 50; <https://github.com/najoshi/sickle>). The generated clean reads were mapped onto the aforementioned *mcr*-containing MAGs using Bowtie v2.4.4 (-p 15 -D 20 -R 3 -N 1 -L 24 -i S,1,0.75) (64). Cufflinks v2.2.1 (65) analysis was performed to calculate the abundances of all transcripts and test the differential gene expression. Briefly, the “cufflinks,” “cuffmerge,” and “cuffdiff” commands within the Cufflinks package were sequentially performed with the default parameters.

## Phylogenetic tree reconstruction Phylogenomic tree

A phylogenomic tree of the 14 *mcr*-containing MAGs was constructed using a multiple sequence alignment of 122 archaeal-specific conserved marker genes using GTDB-Tk v1.6.0 (66). These concatenated sequence alignments were used as input to reconstruct the phylogenomic tree using IQ-TREE v1.6.12 (67) with the best model of LG + F + R10.

## *mcrABG* and *mtaABC* gene trees

Amino acid sequences derived from *mcrABG* and *mtaABC* genes (when present) were extracted from reconstructed MAGs generated in this study. The *McrABG* sequences were added to a reference dataset of *McrABG* sequences derived from isolates and MAGs from a previous study (4). *MtaABC* sequences were added to a reference database generated by searching against the National Center for Biotechnology Information (NCBI) RefSeq database (downloaded on 16 August 2021) using Diamond ( $E$  value:  $1 \times 10^{-5}$ ). Further *Archaeoglobales* and *Ca. Verstraetearchaeota* *MtaABC* sequences were added by using those from the current study to recruit more diversity into the reference database. For the *MtaABC* sequences, Basic Local Alignment Search Tool (BLAST) hits were further searched against the Kyoto Encyclopedia of Genes and Genomes database and were kept if the annotated KOs were K14080, K04480, and K14081. Other *mtaA*, *mtaB*, and *mtaC* genes were eliminated if they did not occur within six consecutive genes of the same scaffold. After collating concatenated sequences for each operon, amino acids were aligned and trimmed using MUSCLE v3.8.31 (68) and Trimal v1.4.rev22 (69). This resulted

in 165 and 471 sequences with 1337 and 1102 amino acids in length for phylogenetic inference. IQ-TREE was used to build the phylogenies, and the best models for *mcrABG* and *mtaABC* were LG + F + R6 and LG + F + R6, respectively.

## Statistical analyses and data availability

All statistical analyses were performed in the R statistical computing environment. Significant differences were obtained by two-way/one-way analysis of variance (ANOVA) with means compared using Duncan's multiple range test at the level of  $P < 0.05$  using the function “aov.” The 14 near-complete archaeal genomes were submitted to the NCBI database under the ProjectIDs PRJNA544494 and PRJNA955688, and Whole genome sequencing (WGS) accessions JARXPE010000000 (*Ca. Verstraetearchaeota* archaeon JZ-2\_bin\_200), JARXPF010000000 (*Ca. Verstraetearchaeota* archaeon GMQP\_bin\_44), JARXPG010000000 (*Ca. Nezharchaeota* archaeon GMPQ\_bin\_37), JASFYD010000000 (*Archaeoglobales* archaeon GMQP\_D\_bin\_18), JASFYE010000000 (*Methanothermobacter* sp. GMQ\_75\_MeOH\_H2\_bin\_21), JASFYF010000000 (*Ca. Nezharchaeota* archaeon JZ-3\_D\_bin\_91), JASFYG010000000 (*Methanothermobacter* sp. JZ-3\_D\_bin\_25), JASFYH010000000 (*Archaeoglobales* archaeon JZ-3\_D\_bin\_138), JASFYI010000000 (*Methanolinea* sp. JZ\_75\_SW\_bin\_60), JASFYJ010000000 (*Archaeoglobales* archaeon JZ\_75\_SW\_bin\_109), JASFYK010000000 (*Ca. Verstraetearchaeota* archaeon JZ\_65\_OP\_bin\_51), JASFYL010000000 (*Ca. Verstraetearchaeota* archaeon JZ\_65\_OP\_bin\_38), JASFYM010000000 (*Methanothermobacter* sp. JZ\_65\_OP\_bin\_30), and JASFYN010000000 (*Methanothermobacter* sp. JZ\_65\_OP\_bin\_148). Supplementary data, including phylogenies of *mcrABG* and *mtaABC* in newick format, are available in FigShare repository at <https://figshare.com/s/fb3c5dfae25e1213a532>.

## Supplementary Materials

This PDF file includes:

Figs. S1 to S14

Tables S1 to S6

Legends for supplementary data S1 to S4

References

Other Supplementary Material for this manuscript includes the following:

Supplementary data S1 to S4

[View/request a protocol for this paper from Bio-protocol.](#)

## REFERENCES AND NOTES

- B. Sauterey, B. Charnay, A. Affholder, S. Mazevet, R. Ferrière, Co-evolution of primitive methane-cycling ecosystems and early Earth's atmosphere and climate. *Nat. Commun.* **11**, 2705 (2020).
- G. Yvon-Durocher, A. P. Allen, D. Bastviken, R. Conrad, C. Gudas, A. St-Pierre, N. Thanh-Duc, P. A. del Giorgio, Methane fluxes show consistent temperature dependence across microbial to ecosystem scales. *Nature* **507**, 488–491 (2014).
- H. Mißbach, J.-P. Duda, A. M. van den Kerkhof, V. Lüders, A. Pack, J. Reitner, V. Thiel, Ingredients for microbial life preserved in 3.5 billion-year-old fluid inclusions. *Nat. Commun.* **12**, 1101 (2021).
- Z.-S. Hua, Y.-L. Wang, P. N. Evans, Y.-N. Qu, K. M. Goh, Y.-Z. Rao, Y.-L. Qi, Y.-X. Li, M.-J. Huang, J.-Y. Jiao, Y.-T. Chen, Y.-P. Mao, W.-S. Shu, W. Hozzein, B. P. Hedlund, G. W. Tyson, T. Zhang,

- W.-J. Li, Insights into the ecological roles and evolution of methyl-coenzyme M reductase-containing hot spring Archaea. *Nat. Commun.* **10**, 4574 (2019).
5. Y. Ueno, K. Yamada, N. Yoshida, S. Maruyama, Y. Isozaki, Evidence from fluid inclusions for microbial methanogenesis in the early Archaean era. *Nature* **440**, 516–519 (2006).
  6. R. Mei, M. Kaneko, H. Imachi, M. K. Nobu, The origin and evolution of methanogenesis and Archaea are intertwined. *PNAS Nexus* **2**, pgad023 (2023).
  7. A.-L. Reysenbach, E. Shock, Merging genomes with geochemistry in hydrothermal ecosystems. *Science* **296**, 1077–1082 (2002).
  8. W. Martin, J. Baross, D. Kelley, M. J. Russell, Hydrothermal vents and the origin of life. *Nat. Rev. Microbiol.* **6**, 805–814 (2008).
  9. P. N. Evans, J. A. Boyd, A. O. Leu, B. J. Woodcroft, D. H. Parks, P. Hugenholtz, G. W. Tyson, An evolving view of methane metabolism in the Archaea. *Nat. Rev. Microbiol.* **17**, 219–232 (2019).
  10. P. Prondzinsky, S. Toyoda, S. E. McGlynn, The methanogen core and pangenome: Conservation and variability across biology's growth temperature extremes. *DNA Res.* **30**, dsac048 (2023).
  11. P. S. Garcia, S. Gribaldo, G. Borrel, Diversity and evolution of methane-related pathways in Archaea. *Ann. Rev. Microbiol.* **76**, 727–755 (2022).
  12. Y. Liu, W. B. Whitman, Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. *Ann. N. Y. Acad. Sci.* **1125**, 171–189 (2008).
  13. G. Borrel, P. S. Adam, L. J. McKay, L.-X. Chen, I. N. Sierra-García, C. M. K. Sieber, Q. Letourneur, A. Ghazlane, G. L. Andersen, W.-J. Li, S. J. Hallam, G. Muyzer, V. M. de Oliveira, W. P. Inskeep, J. F. Banfield, S. Gribaldo, Wide diversity of methane and short-chain alkane metabolisms in uncultured archaea. *Nat. Microbiol.* **4**, 603–613 (2019).
  14. I. Vanwonterghem, P. N. Evans, D. H. Parks, P. D. Jensen, B. J. Woodcroft, P. Hugenholtz, G. W. Tyson, Methylophilic methanogenesis discovered in the archaeal phylum Verstraetearchaeota. *Nat. Microbiol.* **1**, 16170 (2016).
  15. Y. Wang, G. Wegener, J. Hou, F. Wang, X. Xiao, Expanding anaerobic alkane metabolism in the domain of Archaea. *Nat. Microbiol.* **4**, 595–602 (2019).
  16. Y.-F. Liu, J. Chen, L. S. Zaramela, L.-Y. Wang, S. M. Mbadinga, Z.-W. Hou, X.-L. Wu, J.-D. Gu, K. Zengler, B.-Z. Mu, Genomic and transcriptomic evidence supports methane metabolism in *Archaeoglobi*. *mSystems* **5**, e00651–e00619 (2020).
  17. B. A. Berghuis, F. B. Yu, F. Schulz, P. C. Blainey, T. Woyke, S. R. Quake, Hydrogenotrophic methanogenesis in archaeal phylum Verstraetearchaeota reveals the shared ancestry of all methanogens. *Proc. Natl. Acad. Sci. U.S.A.* **116**, 5037–5044 (2019).
  18. L. J. McKay, M. Đlakić, M. W. Fields, T. O. Delmont, A. M. Eren, Z. J. Jay, K. B. Klingensmith, D. B. Rusch, W. P. Inskeep, Co-occurring genomic capacity for anaerobic methane and dissimilatory sulfur metabolisms discovered in the Korarchaeota. *Nat. Microbiol.* **4**, 614–622 (2019).
  19. Y.-F. Ou, H.-P. Dong, S. J. McIlroy, S. A. Crowe, S. J. Hallam, P. Han, J. Kallmeyer, R. L. Simister, A. Vuillemin, A. O. Leu, Z. Liu, Y.-L. Zheng, Q.-L. Sun, M. Liu, G. W. Tyson, L.-J. Hou, Expanding the phylogenetic distribution of cytochrome b-containing methanogenic archaea sheds light on the evolution of methanogenesis. *ISME J.* **16**, 2373–2387 (2022).
  20. L. J. McKay, R. Hatzenpichler, W. P. Inskeep, M. W. Fields, Occurrence and expression of novel methyl-coenzyme M reductase gene (*mcrA*) variants in hot spring sediments. *Sci. Rep.* **7**, 7252 (2017).
  21. B. J. Baker, V. De Anda, K. W. Seitz, N. Dombrowski, A. E. Santoro, K. G. Lloyd, Diversity, ecology and evolution of Archaea. *Nat. Microbiol.* **5**, 887–900 (2020).
  22. L. R. Kump, M. E. Barley, Increased subaerial volcanism and the rise of atmospheric oxygen 2.5 billion years ago. *Nature* **448**, 1033–1036 (2007).
  23. J. C. Rosas, J. Korenaga, Archaean seafloors shallowed with age due to radiogenic heating in the mantle. *Nat. Geosci.* **14**, 51–56 (2021).
  24. A. Y. Mulkidjanian, A. Y. Bychkov, D. V. Dibrova, M. Y. Galperin, E. V. Koonin, Origin of first cells at terrestrial, anoxic geothermal fields. *Proc. Natl. Acad. Sci. U.S.A.* **109**, E821–E830 (2012).
  25. K. S. Habicht, M. Gade, B. Thamdrup, P. Berg, D. E. Canfield, Calibration of sulfate levels in the Archean ocean. *Science* **298**, 2372–2374 (2002).
  26. N. T. Arndt, E. G. Nisbet, Processes on the young Earth and the habitats of early life. *Annu. Rev. Earth Planet. Sci.* **40**, 521–549 (2012).
  27. H. Chen, T. Zhu, B. Li, C. Fang, M. Nie, The thermal response of soil microbial methanogenesis decreases in magnitude with changing temperature. *Nat. Commun.* **11**, 5733 (2020).
  28. Q. Guo, Hydrogeochemistry of high-temperature geothermal systems in China: A review. *Appl. Geochem.* **27**, 1887–1898 (2012).
  29. M. Liu, Q. Guo, X. Zhang, Y. Wang, Characteristic solutes in geothermal water from the Rehai hydrothermal system Southwestern China. *J. Earth Sci.* **26**, 140–148 (2015).
  30. B. R. Briggs, E. L. Brodie, L. M. Tom, H. Dong, H. Jiang, Q. Huang, S. Wang, W. Hou, G. Wu, L. Huang, B. P. Hedlund, C. Zhang, P. Dijkstra, B. A. Hungate, Seasonal patterns in microbial communities inhabiting the hot springs of Tengchong, Yunnan Province, China. *Environ. Microbiol.* **16**, 1579–1591 (2014).
  31. K. Karhu, M. D. Auffret, J. A. J. Dungait, D. W. Hopkins, J. I. Prosser, B. K. Singh, J.-A. Subke, P. A. Wookey, G. I. Ågren, M.-T. Sebastià, F. Gouriveau, G. Bergkvist, P. Meir, A. T. Nottingham, N. Salinas, I. P. Hartley, Temperature sensitivity of soil respiration rates enhanced by microbial community response. *Nature* **513**, 81–84 (2014).
  32. G. L. Chadwick, C. T. Skennerton, R. Laso-Pérez, A. O. Leu, D. R. Speth, H. Yu, C. Morgan-Lang, R. Hatzenpichler, D. Goudeau, R. Malmstrom, W. J. Brazelton, T. Woyke, S. J. Hallam, G. W. Tyson, G. Wegener, A. Boetius, V. J. Orphan, Comparative genomics reveals electron transfer and syntrophic mechanisms differentiating methanotrophic and methanogenic archaea. *PLOS Biol.* **20**, e3001508 (2022).
  33. S. E. McGlynn, G. L. Chadwick, C. P. Kempes, V. J. Orphan, Single cell activity reveals direct electron transfer in methanotrophic consortia. *Nature* **526**, 531–535 (2015).
  34. M. Kampmann, D. Stock, Reverse gyrase has heat-protective DNA chaperone activity independent of supercoiling. *Nucleic Acids Res.* **32**, 3537–3545 (2004).
  35. S. Bäumer, T. Ide, C. Jacobi, A. Johann, G. Gottschalk, U. Deppenmeier, The  $F_{420}H_2$  dehydrogenase from *Methanosarcina mazei* is a redox-driven proton pump closely related to NADH dehydrogenases. *J. Biol. Chem.* **275**, 17968–17973 (2000).
  36. K. Lang, J. Schuldes, A. Klingl, A. Poehlein, R. Daniel, A. Brune, M. A. Elliot, New mode of energy metabolism in the seventh order of methanogens as revealed by comparative genome analysis of "*Candidatus* Methanoplasma termitum". *Appl. Environ. Microbiol.* **81**, 1338–1352 (2015).
  37. C. Welte, U. Deppenmeier, Bioenergetics and anaerobic respiratory chains of aceticlastic methanogens. *Biochim. Biophys. Acta* **1837**, 1130–1147 (2014).
  38. P. Carini, A. E. White, E. O. Campbell, S. J. Giovannoni, Methane production by phosphate-starved SAR11 chemoheterotrophic marine bacteria. *Nat. Commun.* **5**, 4346 (2014).
  39. Y. Zheng, D. F. Harris, Z. Yu, Y. Fu, S. Poudel, R. N. Ledbetter, K. R. Fixen, Z.-Y. Yang, E. S. Boyd, M. E. Lidstrom, L. C. Seefeldt, C. S. Harwood, A pathway for biological methane production using bacterial iron-only nitrogenase. *Nat. Microbiol.* **3**, 281–286 (2018).
  40. M. Bižić, T. Klintzsch, D. Ionescu, M. Y. Hindiyyeh, M. Günthel, A. M. Muro-Pastor, W. Eckert, T. Urich, F. Keppler, H. P. Grossart, Aquatic and terrestrial cyanobacteria produce methane. *Sci. Adv.* **6**, eaax5343 (2020).
  41. P. S. Adam, G. E. Kolyfets, T. L. V. Bornemann, C. E. Vorgias, A. J. Probst, Genomic remnants of ancestral methanogenesis and hydrogenotrophy in Archaea drive anaerobic carbon cycling. *Sci. Adv.* **8**, eabm9651 (2022).
  42. Y. Wang, G. Wegener, T. A. Williams, R. Xie, J. Hou, C. Tian, Y. Zhang, F. Wang, X. Xiao, A methylophilic origin of methanogenesis and early divergence of anaerobic multicarbon alkane metabolism. *Sci. Adv.* **7**, eabj1453 (2021).
  43. N. A. Chernykh, S. Neukirchen, E. N. Frolov, F. L. Sousa, M. L. Miroshnichenko, A. Y. Merkel, N. V. Pimenov, D. Y. Sorokin, S. Ciordia, M. C. Mena, M. Ferrer, P. N. Golyshin, A. V. Lebedinsky, I. A. Cardoso Pereira, E. A. Bonch-Osmolovskaya, Dissimilatory sulfate reduction in the archaeon "*Candidatus* Vulcanisaeta moutnovskia" sheds light on the evolution of sulfur metabolism. *Nat. Microbiol.* **5**, 1428–1438 (2020).
  44. K. Ozaki, E. Tajika, P. K. Hong, Y. Nakagawa, C. T. Reinhard, Effects of primitive photosynthesis on Earth's early climate system. *Nat. Geosci.* **11**, 55–59 (2018).
  45. R. Stone, Biological dark matter exerts irresistible pull in Yunnan. *Science* **333**, 1209–1209 (2011).
  46. Q. Guo, Y. Wang, Geochemistry of hot springs in the Tengchong hydrothermal areas, Southwestern China. *J. Volcanol. Geotherm. Res.* **215–216**, 61–73 (2012).
  47. Z.-S. Hua, Y.-N. Qu, Q. Zhu, E.-M. Zhou, Y.-L. Qi, Y.-R. Yin, Y.-Z. Rao, Y. Tian, Y.-X. Li, L. Liu, C. J. Castelle, B. P. Hedlund, W.-S. Shu, R. Knight, W.-J. Li, Genomic inference of the metabolism and evolution of the archaeal phylum Aigarchaeota. *Nat. Commun.* **9**, 2832 (2018).
  48. D. J. Jacob, B. D. Field, Q. Li, D. R. Blake, J. de Gouw, C. Warneke, A. Hansel, A. Wisthaler, H. B. Singh, A. Guenther, Global budget of methanol: Constraints from atmospheric observations. *J. Geophys. Res.* **110**, 10.1029/2004JD005172, (2005).
  49. V. De Anda, L.-X. Chen, N. Dombrowski, Z.-S. Hua, H.-C. Jiang, J. F. Banfield, W.-J. Li, B. J. Baker, Brockarchaeota, a novel archaeal phylum with unique and versatile carbon cycling pathways. *Nat. Commun.* **12**, 2404 (2021).
  50. M. Preiner, K. Igarashi, K. B. Muchowska, M. Yu, S. J. Varma, K. Kleinermaans, M. K. Nobu, Y. Kamagata, H. Tüysüz, J. Moran, W. F. Martin, A hydrogen-dependent geochemical analogue of primordial carbon and energy metabolism. *Nat. Ecol. Evol.* **4**, 534–542 (2020).
  51. S. H. Zinder, Conversion of acetic acid to methane by thermophiles. *FEMS Microbiol. Rev.* **6**, 125–137 (1990).
  52. J. Wang, C. F. Kerl, P. Hu, M. Martin, T. Mu, L. Brüggewirth, G. Wu, D. Said-Pullicino, M. Romani, L. Wu, B. Planer-Friedrich, Thiolated arsenic species observed in rice paddy pore waters. *Nat. Geosci.* **13**, 282–287 (2020).
  53. Z. Shangquan, W. Huo,  $\delta D$  values of escaped  $H_2$  from hot springs at the Tengchong Rehai geothermal area and its origin. *Chin. Sci. Bull.* **47**, 148–150 (2002).

54. B. Mukhopadhyay, E. F. Johnson, R. S. Wolfe, A novel  $p_{H_2}$  control on the expression of flagella in the hyperthermophilic strictly hydrogenotrophic methanarchaeon *Methanococcus jannaschii*. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 11522–11527 (2000).
55. Q. Guo, M. Liu, L. Luo, K. Yan, W. Guo, G. Wu, W. Yan, Y. Wang, Geochemical controls on magnesium and its speciation in various types of geothermal waters from typical felsic-rock-hosted hydrothermal systems in China. *Geothermics* **81**, 185–197 (2019).
56. W. B. Whitman, T. L. Bowen, D. R. Boone, The methanogenic bacteria, in *The Prokaryotes: Other Major Lineages of Bacteria and the Archaea*, E. Rosenberg, E. F. DeLong, S. Lory, E. Stackebrandt, F. Thompson, Eds. (Springer Berlin Heidelberg, 2014), pp. 123–163.
57. Z.-S. Hua, Y.-J. Han, L.-X. Chen, J. Liu, M. Hu, S.-J. Li, J.-L. Kuang, P. S. G. Chain, L.-N. Huang, W.-S. Shu, Ecological roles of dominant and rare prokaryotes in acid mine drainage revealed by metagenomics and metatranscriptomics. *ISME J.* **9**, 1280–1294 (2015).
58. A. Bankevich, S. Nurk, D. Antipov, A. A. Gurevich, M. Dvorkin, A. S. Kulikov, V. M. Lesin, S. I. Nikolenko, S. Pham, A. D. Prjibelski, A. V. Pyshkin, A. V. Sirotkin, N. Vyahhi, G. Tesler, M. A. Alekseyev, P. A. Pevzner, SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* **19**, 455–477 (2012).
59. D. D. Kang, J. Froula, R. Egan, Z. Wang, MetaBAT, an efficient tool for accurately reconstructing single genomes from complex microbial communities. *PeerJ* **3**, e1165 (2015).
60. D. H. Parks, M. Imelfort, C. T. Skennerton, P. Hugenholtz, G. W. Tyson, CheckM: Assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Res.* **25**, 1043–1055 (2015).
61. G. J. Dick, A. F. Andersson, B. J. Baker, S. L. Simmons, B. C. Thomas, A. P. Yelton, J. F. Banfield, Community-wide analysis of microbial genome sequence signatures. *Genome Biol.* **10**, R85 (2009).
62. D. Hyatt, G.-L. Chen, P. F. LoCascio, M. L. Land, F. W. Larimer, L. J. Hauser, Prodigal: Prokaryotic gene recognition and translation initiation site identification. *BMC Bioinformatics* **11**, 119 (2010).
63. B. Buchfink, C. Xie, D. H. Huson, Fast and sensitive protein alignment using DIAMOND. *Nat. Methods* **12**, 59–60 (2015).
64. B. Langmead, S. L. Salzberg, Fast gapped-read alignment with Bowtie 2. *Nat. Methods* **9**, 357–359 (2012).
65. C. Trapnell, B. A. Williams, G. Pertea, A. Mortazavi, G. Kwan, M. J. van Baren, S. L. Salzberg, B. J. Wold, L. Pachter, Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat. Biotechnol.* **28**, 511–515 (2010).
66. 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 (2020).
67. L.-T. Nguyen, H. A. Schmidt, A. von Haeseler, B. Q. Minh, IQ-TREE: A fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol. Biol. Evol.* **32**, 268–274 (2015).
68. R. C. Edgar, MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **32**, 1792–1797 (2004).
69. 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).
70. V. Jerman, T. Danevčič, I. Mandić-Mulec, Methane cycling in a drained wetland soil profile. *J. Soil. Sediment.* **17**, 1874–1882 (2017).
71. J. Winden, G. J. Reichart, G. J. Mcnamara, A. Benthien, J. Damsté, Temperature-induced increase in methane release from peat bogs: A mesocosm experiment. *PLOS ONE* **7**, e39614 (2012).
72. A. J. Francez, S. Gogo, N. Josselin, Distribution of potential  $CO_2$  and  $CH_4$  productions, denitrification and microbial biomass C and N in the profile of a restored peatland in Brittany (France). *Eur. J. Soil Biol.* **36**, 161–168 (2000).
73. R. Ye, Q. Jin, B. Bohannan, J. K. Keller, S. D. Bridgman, Homoacetogenesis: A potentially underappreciated carbon pathway in peatlands. *Soil Biol. Biochem.* **68**, 385–391 (2014).
74. Y. Song, C. Song, A. Hou, L. Sun, X. Wang, X. Ma, L. Jiang, C. Liu, J. Gao, Temperature, soil moisture, and microbial controls on  $CO_2$  and  $CH_4$  emissions from a permafrost peatland. *Environ. Prog. Sustain. Energy* **40**, e13693 (2021).
75. H. Zhang, E.-S. Tuittila, A. Korrensalo, A. M. Laine, S. Uljas, N. Welti, J. Kerttula, M. Maljanen, D. Elliott, T. Vesala, A. Lohila, Methane production and oxidation potentials along a fen-bog gradient from southern boreal to subarctic peatlands in Finland. *Glob. Chang. Biol.* **27**, 4449–4464 (2021).
76. R. Ye, Q. Jin, B. Bohannan, J. K. Keller, S. A. McAllister, S. D. Bridgman, pH controls over anaerobic carbon mineralization, the efficiency of methane production, and methanogenic pathways in peatlands across an ombrotrophic–minerotrophic gradient. *Soil Biol. Biochem.* **54**, 36–47 (2012).
77. S. Kumaraswamy, B. Ramakrishnan, N. Sethunathan, Methane production and oxidation in an anoxic rice soil as influenced by inorganic redox species. *J. Environ. Qual.* **30**, 2195–2201 (2001).
78. S. Mitra, D. Majumdar, R. Wassmann, Methane production and emission in surface and subsurface rice soils and their blends. *Agric. Ecosyst. Environ.* **158**, 94–102 (2012).
79. Y.-Q. Wang, M.-X. Wang, Y.-Y. Chen, C.-M. Li, Z.-F. Zhou, Microbial community structure and co-occurrence are essential for methanogenesis and its contribution to phenanthrene degradation in paddy soil. *J. Hazard. Mater.* **417**, 126086 (2021).
80. C. Hattori, A. Ueki, H. Fujii, H. Egashira, K. Ueki, Factors affecting seasonal and vertical variations in the methanogenic activity in paddy soil determined by the addition of methanogenic substrates. *Soil Sci. Plant Nutr.* **48**, 315–324 (2002).
81. N. Matthias, K. Melanie, C. Ralf, Effect of temperature change on the composition of the bacterial and archaeal community potentially involved in the turnover of acetate and propionate in methanogenic rice field soil. *FEMS Microbiol. Ecol.* **73**, 215–225 (2010).
82. D. Y. Liu, W. X. Ding, Z. J. Jia, Z. C. Cai, Relation between methanogenic archaea and methane production potential in selected natural wetland ecosystems across China. *Biogeosciences* **8**, 329–338 (2011).
83. C. Tong, W.-Q. Wang, C.-S. Zeng, R. Marrs, Methane ( $CH_4$ ) emission from a tidal marsh in the Min River estuary, southeast China. *J. Environ. Sci. Health A Tox. Hazard. Subst. Environ. Eng.* **45**, 506–516 (2010).
84. W. Wang, C. Zeng, J. Sardans, C. Wang, C. Tong, J. Peñuelas, Soil methane production, anaerobic and aerobic oxidation in porewater of wetland soils of the Minjiang river Estuarine, China. *Wetlands* **38**, 627–640 (2018).
85. A. Fuchs, E. Lyautey, B. Montuelle, P. Casper, Effects of increasing temperatures on methane concentrations and methanogenesis during experimental incubation of sediments from oligotrophic and mesotrophic lakes. *J. Geophys. Res. Biogeosci.* **121**, 1394–1406 (2016).
86. X. Zhu, Y. Yuan, X. Wei, L. Wang, C. Wang, Dissimilatory iron reduction and potential methane production in Chagan Lake wetland soils with carbon addition. *Wetlands Ecol. Manag.* **29**, 369–379 (2021).
87. Z. Yan, J. Wang, Y. Li, H. Wu, K. Zhang, L. Yan, X. Zhang, X. Kang, Waterlogging affects the mitigation of soil GHG emissions by biochar amendment in coastal wetland. *J. Soil. Sediment.* **20**, 3591–3606 (2020).
88. M.-J. Gonsalves, C. Fernandes, S. O. Fernandes, D. L. Kirchman, P. Bharathi, Effects of composition of labile organic matter on biogenic production of methane in the coastal sediments of the Arabian Sea. *Environ. Monit. Assess.* **182**, 385–395 (2011).
89. J. Xu, D. Lai, S. Neogi, Effects of land use types on  $CH_4$  and  $CO_2$  production potentials in subtropical wetland soils. *Water* **12**, 1856 (2020).
90. J. Kim, J. Lee, J. Yun, Y. Yang, H. Kang, Mechanisms of enhanced methane emission due to introduction of *Spartina anglica* and *Phragmites australis* in a temperate tidal salt marsh. *Ecol. Eng.* **153**, 105905 (2020).
91. Y. Deng, P. Liu, R. Conrad, Effect of temperature on the microbial community responsible for methane production in alkaline NamCo wetland soil. *Soil Biol. Biochem.* **132**, 69–79 (2019).
92. R. Conrad, M. Noll, P. Claus, M. Klose, W. R. Bastos, A. Enrich-Prast, Stable carbon isotope discrimination and microbiology of methane formation in tropical anoxic lake sediments. *Biogeosciences* **8**, 795–814 (2011).
93. R. Conrad, P. Claus, P. Casper, Stable isotope fractionation during the methanogenic degradation of organic matter in the sediment of an acidic bog lake, Lake Grosse Fuchskuhle. *Limnol. Oceanogr.* **55**, 1932–1942 (2010).
94. J. Wang, C. Liu, X. Gong, Y. Liu, C. Chen, Trimethylamine stimulated and dissolved organic matter inhibited methane production in sediment from the Poyang Lake, China. *Environ. Technol.* **37**, 2545–2554 (2016).
95. Y. Yang, J. Chen, T. Tong, S. Xie, Y. Liu, Influences of eutrophication on methanogenesis pathways and methanogenic microbial community structures in freshwater lakes. *Environ. Pollut.* **260**, 114106 (2020).
96. V. Mach, P. P. Chaudhary, M. Blaser, P. Claus, M. Rulik, Methane production potentials, pathways, and communities of methanogens in vertical sediment profiles of river Sitka. *Front. Microbiol.* **6**, 506 (2015).
97. G. B. Avery, C. S. Martens, Controls on the stable carbon isotopic composition of biogenic methane produced in a tidal freshwater estuarine sediment. *Geochim. et Cosmochim. Acta* **63**, 1075–1082 (1999).
98. A. Bednarek, M. Blaser, M. Rulik, Methane formation and consumption by sediments in a cross-channel profile of a small river impoundment. *J. Limnol.* **78**, 233–242 (2019).
99. X. Chen, M. Yang, J. Sun, J. Yu, L. Liu, S. Bai, F. Bai, M. Yang, Z. Chen, C. He, X. Liu, J. Liang, F. Wang, The anaerobic oxidation of methane driven by multiple electron acceptors suppresses the release of methane from the sediments of a reservoir. *J. Soil. Sediment.* **22**, 682–691 (2022).
100. S. Y. Kim, A. J. Verraart, M. Meima-Franke, P. L. E. Bodelier, Combined effects of carbon, nitrogen and phosphorus on  $CH_4$  production and denitrification in wetland sediments. *Geoderma* **259–260**, 354–361 (2015).

101. S. E. Andrews, R. Schultz, S. D. Frey, V. Bouchard, R. Varner, M. J. Ducey, Plant community structure mediates potential methane production and potential iron reduction in wetland mesocosms. *Ecosphere* **4**, art44 (2013).

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**Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. The 14 near-complete archaeal genomes were submitted to the NCBI database under the ProjectIDs PRJNA544494 and PRJNA955688 and WGS accessions JARXPE010000000 (*Ca. Verstraetearchaeota* archaeon JZ-2\_bin\_200), JARXPF010000000 (*Ca. Verstraetearchaeota* archaeon GMQP\_bin\_44), JARXPG010000000 (*Ca. Nezhaarchaeota* archaeon GMPQ\_bin\_37), JASFYD010000000 (*Archaeoglobales* archaeon GMQP\_D\_bin\_18), JASFYE010000000 (*Methanothermobacter* sp. GMQ\_75\_MeOH\_H2\_bin\_21), JASFYF010000000 (*Ca. Nezhaarchaeota* archaeon JZ-3\_D\_bin\_91), JASFYG010000000 (*Methanothermobacter* sp. JZ-3\_D\_bin\_25), JASFYH010000000 (*Archaeoglobales* archaeon JZ-3\_D\_bin\_138), JASFYI010000000 (*Methanolinea* sp. JZ\_75\_SW\_bin\_60), JASFYJ010000000 (*Archaeoglobales* archaeon JZ\_75\_SW\_bin\_109), JASFYK010000000 (*Ca. Verstraetearchaeota* archaeon JZ\_65\_OP\_bin\_51), JASFYL010000000 (*Ca. Verstraetearchaeota* archaeon JZ\_65\_OP\_bin\_38), JASFYM010000000 (*Methanothermobacter* sp. JZ\_65\_OP\_bin\_30), and JASFYN010000000 (*Methanothermobacter* sp. JZ\_65\_OP\_bin\_148). Supplementary data, including phylogenies of *mcrABG* and *mtaABC* in newick format, are available in FigShare repository at <https://figshare.com/s/fb3c5dfae25e1213a532>.

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