

Response of methanogenesis to the local environment along a 2.0-km deep Quaternary Saline Lacustrine Source Rocks

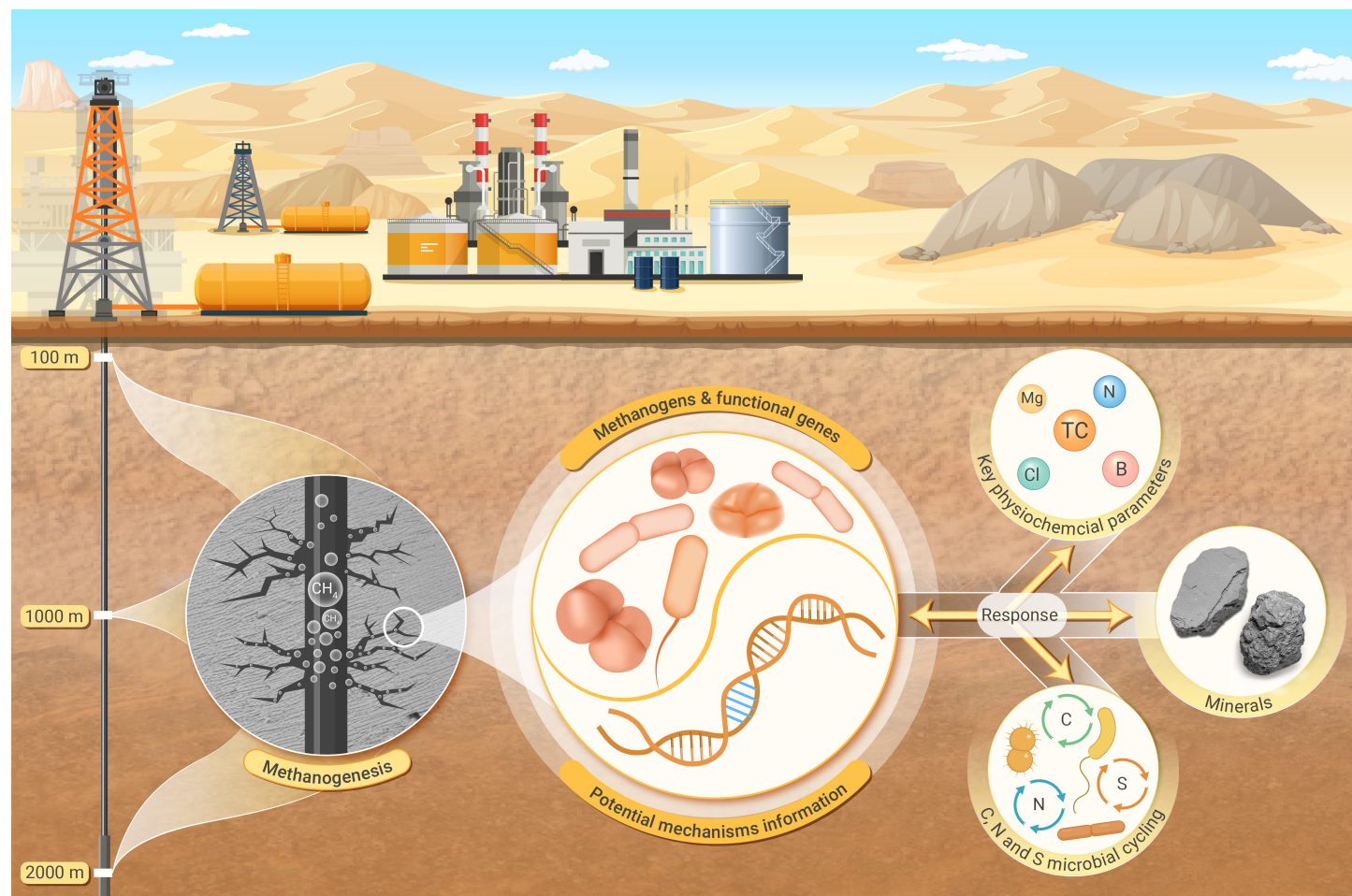
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GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- The dominant methanogens were *Methanosarcina* and *Methanohalophilus*.
- Methanogens abundance matched that of methane metabolism genes abundance.
- The only detected pathway was acetoclastic methanogenesis.
- Lithology might serve as an indicator of methanogenic potential.
- Methanogenesis was auxiliary impacted by microbial C, N and S cycling.

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Biogenic gas in deep subsurface has gained significant attention as a clean and efficient energy source. Due to the challenges associated with sample extraction, knowledge regarding the methanogenesis mechanism in deep biogenic gas fields remains limited. Herein, metagenomic sequencing was employed to investigate the microbial communities, functional genes, metabolic pathways, and their potential determinants in rock samples collected from depths of 100, 1000, and 2000 meters within Quaternary biogenic gas fields in the Qaidam Basin. The analysis revealed substantial variation in the methanogenesis-associated microbiome across different depths. Key physicochemical parameters influencing microbial community composition and the relative abundance of methanogenesis-related functional genes included total carbon, carbon-to-nitrogen ratio, chlorine, boron, magnesium, and mineral composition. The dominant methanogens identified were *Methanosarcina* and *Methanohalophilus*, with their relative abundance correlating with the distribution of methane metabolism genes. Notably, only genes associated with acetate utilization methanogenic metabolic pathway were detected. Interestingly, significant increases in dolomite concentration at 1000 m and calcite at 2000 m were linked to variations in the abundance of methanogenesis-related functional genes, suggesting that lithological characteristics may serve as an indicator of methanogenic potential. Additionally, the study identified connections between carbon, nitrogen, and sulfur microbial cycling pathways and the abundance of methanogenesis-related functional genes, highlighting the auxiliary role of microbial-mediated carbon, nitrogen, and sulfur cycling in methanogenesis. These findings provide direct evidence of methanogenesis processes and their determinants, offering valuable insights into Quaternary biogenic gas production.

INTRODUCTION

Fossil fuels are associated with significant environmental and economic costs, prompting growing interest in the exploitation of biogenic gas.¹ Compared to fossil fuels, biogenic gas, composed primarily of methane is a clean-burning and efficient energy source, offering considerable potential for reducing air pollution and greenhouse gas emissions.² Current estimates indicate that biogenic gas reserves amount to approximately $15.5 \times 10^{12} \text{ m}^3$, constituting over 20% of the total natural gas on the earth.³ Furthermore, shale gas represents an abundant resource,⁴ with reserves reaching approximately $7,299 \times 10^{12} \text{ m}^3$. Over the past decades, research on biogenic gas has expanded, focusing on its resource potential,⁵ accumulation mechanism⁶ and reservoir characteristics.⁷ Biogenic methane is predominantly generated through anaerobic biodegradation of organic matter via microbial metabolism,⁸ underscoring the importance of investigating the distribution of microbiota and microbial metabolism involved in biogenic gas formation.

Deep subsurface microbial communities are recognized as widespread, exhibiting complex physiological and phylogenetic diversity.⁸ The advent of culture-independent techniques such as low and high throughput sequencing has facilitated direct exploration of these communities.⁹ Wang et al.¹⁰ used 16S rRNA-based clone library to study microbiota in rock samples collected from a depth of 1033.77 meters, identifying 23 distinct microbial sequences, including 11 Proteobacteria and 9 Bacillus species. Zhang et al.¹¹ employed 16S rRNA sequences analysis to characterize the microbial

community in ultra-high pressure rocks from the Chinese Mainland Scientific Drilling Project, finding Proteobacteria to be predominant. Daly et al.¹² reconstructed 31 unique genomes coupled with metabolite data from fluid samples in the Marcellus and Utica shales, revealing the important role of *Frackibacter*, *Halanaerobium*, *Methanohalophilus*, and *Methanolobus* in deep subsurface methane production. Nyyssonen et al.¹³ used metagenomics to investigate microbial diversity in a 2,516-meter-deep drill hole in the continental crystalline crust in Outokumpu, Finland, identifying bacteria Proteobacteria, Firmicutes, and Tenericutes and archaea *Methanobacterium*, and *Methanobacteriaceae* as key contributors to methane production. Despite these advances, comprehensive information on *in situ* microbial community and functional genes in deep subsurface environments remains limited due to the challenges of obtaining rock samples from such depths.

The Quaternary system of the Qaidam Basin, the world's largest biogenic gas reservoir, holds more than 320 billion cubic meters of methane-rich biogas (> 98%), derived primarily from the Quaternary saline lacustrine source rocks.¹⁴ Previous studies have proposed two mechanisms for methane generation in the Sanhu area of the Qaidam Basin: acetic acid fermentation and carbon dioxide deoxygenation.¹⁵ Chen et al.¹⁶ analyzed the geochemical parameters from 44 biogenic methane wells and concluded that carbon dioxide deoxygenation is the main biogas production mode in this region. Similarly, Zhang et al.¹⁴ examined the chemical compositions and carbon isotopes of 21 gas samples from the Sebei NO. 1 and NO. 2 gas fields, detecting evidence of biogenic gas formation through carbon dioxide reduction. While these studies have explored the chemical composition of the biogenic gases and the depositional conditions of Quaternary rocks in the Qaidam Basin, the characterization of *in situ* microbial communities and their functional roles in the deep rocks has not been well studied.

In this study, we investigated bacterial and methanogenic community composition and methanogenic metabolic pathways in the shallow, middle, and deep depths within a 2-km profile of Quaternary saline lacustrine source rocks in the Qaidam Basin using metagenomic analysis. Environmental parameters shaping the microbiota's structure and functions were also assessed. This study elucidates the fundamental mechanism of methanogenesis in the deep subsurface and underscores the important role of microbial activity in the Quaternary ecosystem of the Qaidam Basin. To the best of our knowledge, this is the first report addressing the microbial mechanism of methanogenesis in deep rock samples from this region.

MATERIALS AND METHODS

Rock samples

The sampling point of Sebei 1 well is in the saddle area of Sebei 1 and 2 gas fields within the Sanhu Depression area of the eastern Qaidam Basin, with an elevation of 2,716.44 meters. Rock core samples were collected using a PVC-sealed drilling strategy between 31 August and 14 September 2004, utilizing DBS30 drilling machines (Honghua Group, China). This sealing strategy effectively protected the core samples from potential contamination by drilling fluid. Following extraction, the core samples were well sealed and transported on 15 September 2004 to the Drilling Core Samples Storage Center of Qinghai Petroleum Corporation, China, where they were preserved at room temperature (ranging from -5°C to 25°C), alongside other cores from Qaidam Basin dating back to 1957 (Figure S1).

On 14 May 2019, rock core samples were retrieved from the storage center

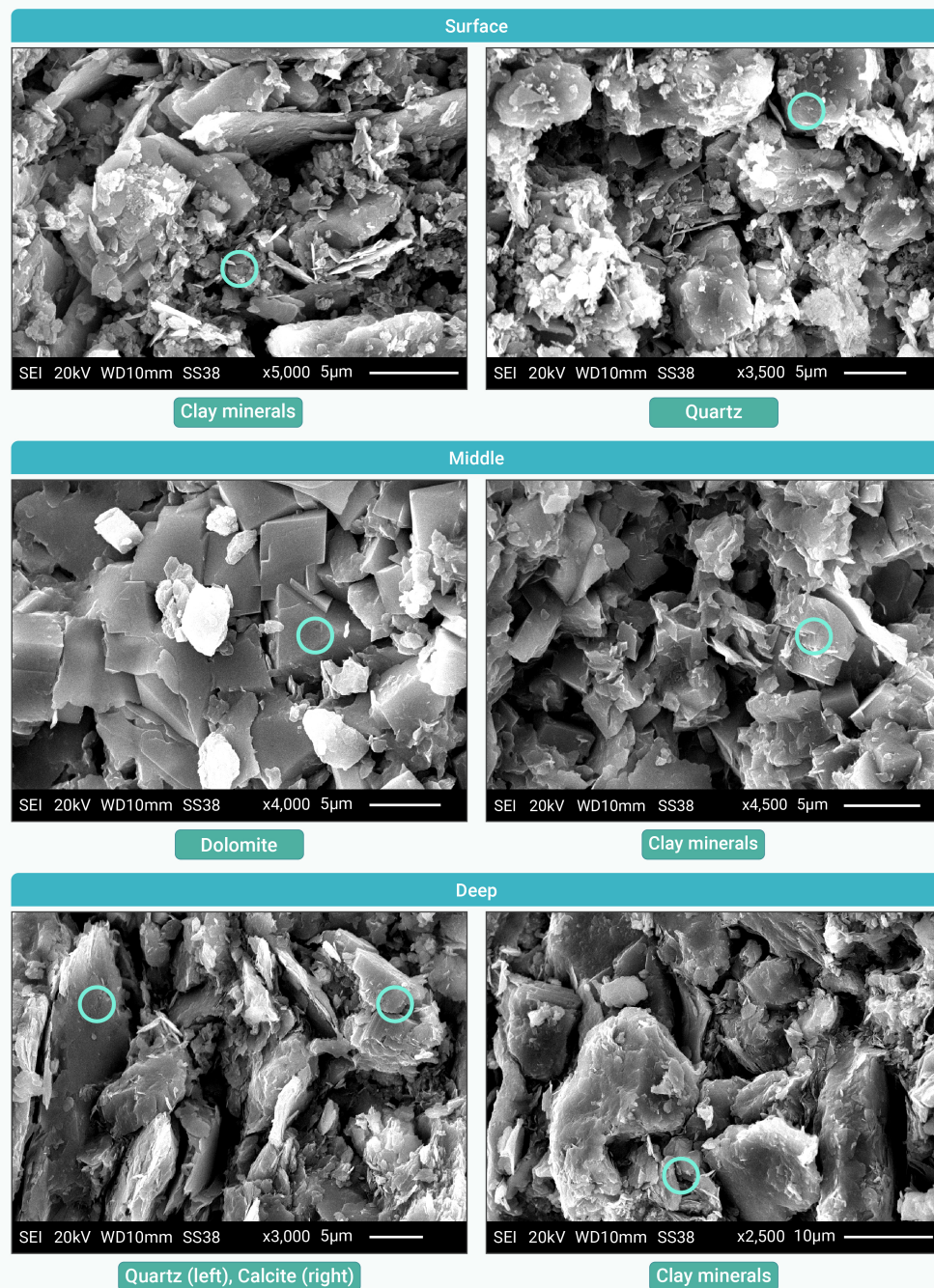


Figure 1. Scanning electron microscopy images of the minerals with the highest proportion at three depths. The green circles indicate the observation points.

Fe, and Mg) were directly quantified using an X-ray diffractometer (Bruker D8 advance, Germany). Total carbon (TC), total organic carbon (TOC), total nitrogen (TN), total phosphorus (TP), and total potassium (TK) were determined according to the standards analysis of soil.¹⁶ Briefly, TC and TOC were analyzed using the sulphochromic oxidation method. TN was measured according to Kjeldahl method. TP was determined using the molybdenum blue spectrophotometric method. TK was analyzed by hydrofluoric acid digestion method.

Metagenome DNA extraction and shotgun sequencing

DNA was extracted from rock samples using the Mag-Bind Soil DNA Kit (M5635-02) (Omega Bio-Tek, Norcross, GA, USA). Extracted DNA concentrations were quantified with a NanoDrop2000. Extracted microbial DNA was then processed to construct metagenome shotgun sequencing libraries with 400-bp insert sizes by using Illumina TruSeq Nano DNA LT Library Preparation Kit (Illumina, San Diego, CA, USA). Each library was sequenced with the Illumina NovaSeq platform (Illumina, USA) with PE150 strategy at Personal Biotechnology Co., Ltd. (Shanghai, China).

Sequence analysis

Cutadapt (v1.2.1)¹⁷ was used to identify and strip the 3' end of the sequence (match length with connector sequence > 10 bp, base mismatch rate < 20%). The sliding window method was used to screen the sequence quality (length > 50 bp, no fuzzy base), the window size > 5 bp, moving from the first base position at the 5' end and truncating the sequence from the first window with an average quality value < Q20. Quality-filtered reads were de novo assembled to construct the metagenome for each sample by IDBA-UD (Iterative De Bruijn graph Assembler for sequencing data with highly Uneven Depth).¹⁸ MetaGeneMark ([http://](http://exon.gatech.edu/GeneMark/metagenome)

exon.gatech.edu/GeneMark/metagenome)¹⁹ was used to predict all coding regions (CDS) of metagenomic scaffolds > 300 bp.

Cluster Database at High Identity with Tolerance²⁰ was used to cluster CDS sequences of all samples. Soap coverage (<http://soap.genomics.org.cn/>) was used to estimate gene abundance in each sample based on the number of aligned reads. The lowest common ancestor taxonomy of the nonredundant genes was obtained by aligning them against the NCBI-NT database using BLASTN (e value < 0.001). Similarly, the functional profiles of the nonredundant genes were obtained by annotation using Gene Ontology (<http://www.geneontology.org/>), Kyoto Encyclopedia of Genes and Genomes (KEGG, <http://www.genome.jp/kegg/>), EggNOG (http://eggnog.embl.de/version_4.0.beta/, v4.0) and Carbohydrate-Active enZymes (CAZy, <http://www.cazy.org/>) databases by using DIAMOND²¹ alignment algorithm.

Metagenomic binning

The metagenomic binning process begins with filtering the metagenomic contigs based on length. Following this, binning assembly is performed using MetaBAT (version 2.12.1), CONCOCT (version 0.5.0), and Maxbin2 (version

for analysis, representing shallow (S; 100m), middle (M; 1000m), and deep (D; 2000m) layers (Figure S1). These samples were sealed in sterile sampling bags and transported back to the laboratory at 4°C within 12 hours. To minimize contamination, sample preparation began immediately upon arrival at the laboratory. The outer surfaces of the rock samples were first disinfected with 96% ethanol. Approximately 0.9-1 cm of the superficial outer layer was removed using a sterile chisel blade. The exposed fresh cores were then chipped and ground into fine powder using sterile chisel blade and pestle (Figures S2 & S3). All the treatments were operated under a laminar flow hood to maintain sterile conditions.

Rock sample physiochemical parameters analysis

The morphology of rock samples was observed by scanning electron microscope (JEOL JSM-6610LV, Japan) under GB/T 18295-2001 instruction. Element content at target points within the rocks was determined by an X-max energy disperse spectrometer (OXFORD, UK) following GB/T 17359-2012 guidelines, and the mineral composition was subsequently analyzed. In compliance with SY/T5163-2018 standard, measurable elements (Cl, B, Ca,



Figure 2. Physiochemical parameters (A), mineral content (B), and clay mineral content (C) profiles across depths Values are given as the mean $\pm$ SD derived from three replicate measurements. Superscript letters are used to distinguish sample groups that were significantly different (adjusted $p < 0.05$); values with superscript letters differing from letters assigned to other values designate a significant difference between sample groups. Significance was determined using nonparametric Kruskal–Wallis tests, in conjunction with Nemenyi test. Qtz: quartz, Dol: dolomite, TCCM: total content of clay minerals, Pl: plagioclase, Cal: calcite, K: kaolin, C: chlorite, I: illite, S: montmorillonite, CM: clay minerals.

Table 1. Sequences reads information (mean $\pm$ SD).

Depth	S	M	D
Total	8410530.33 $\pm$ 840098.23	7277003 $\pm$ 80190.92	7667020.67 $\pm$ 1119141.65
Bacteria	6106967.33 $\pm$ 617571.29	5458020 $\pm$ 77001.58	5612947.33 $\pm$ 758247.92
Archaea	72.60% $\pm$ 0.21%	75.01% $\pm$ 1.71%	73.29% $\pm$ 1.09%
Eukaryota	74.67 $\pm$ 14.15	71.33 $\pm$ 10.97	69 $\pm$ 10.39
Viruses	0.0009% $\pm$ 0.0002%	0.0010% $\pm$ 0.0001%	0.0009% $\pm$ 0.0002%
Unclassified	172706.67 $\pm$ 17163.48	135816 $\pm$ 12327.80	154335.67 $\pm$ 28600.49
	2.05% $\pm$ 0.07%	1.87% $\pm$ 0.15%	2.01% $\pm$ 0.11%
	142174.67 $\pm$ 14317.69	112931.33 $\pm$ 7475.23	125608.67 $\pm$ 22899.08
	1.69% $\pm$ 0.01%	1.55% $\pm$ 0.09%	1.63% $\pm$ 0.06%
	1988607 $\pm$ 192470.86	1570164.33 $\pm$ 121379.80	1774060 $\pm$ 314163.65
	23.65% $\pm$ 0.14%	21.57% $\pm$ 1.47%	23.07% $\pm$ 0.94%

2.2.5). The bins produced by these tools are then integrated using DAS Tool (version 1.1.0), which refines and merges bins across platforms to generate a consolidated set. These refined bins are further purified using RefineM (Version 0.0.24). Quality assessment of the bins is conducted using CheckM (Version 1.0.12), where only bins with completeness $\geq 50\%$ and contamination $< 10\%$ are retained. Subsequently, deduplication and clustering are carried out using dRep (Version 3.4.2). Species annotation for the bins is performed using GTDB-TK (Version 2.3.0).

Statistical analysis

Mantel tests and Spearman correlations were performed to reveal correlations between microbial community composition, species abundance (quantified as Reads Per Kilobase of exon model per Million mapped reads, RPKM), functional genes abundance (Transcripts Per Kilobase of exon model per Million mapped reads, TPM), physiochemical parameters, and minerals composition (R packages: vegan v2.6-4 and linkET v0.0.7.2). Functional gene

abundance was normalized, and variations in abundance were compared (R packages: circlize v0.4.15 and ComplexHeatmap v2.13.3). Unpaired T-test was used to compare the statistical difference in parameters (relative abundance of bacteria, archaea, genes, and CAZy modules) across depths (IBM SPSS Statistics 27).

RESULTS

Morphology and environmental parameters

The morphology of representative rock samples at three depths is shown in Figure 1, with their lithology characterized by element content (Table S1). At depths S and D, clay minerals were observed surrounding rigid grains such as quartz and calcite, with numerous pores within the clay minerals. In contrast, at depth D, the presence of glassy, diamond-shaped flaky minerals identified as dolomites was notable. The increased abundance of dolomite at this depth corresponded to a reduction in pore space relative to depths S and M. This decrease in porosity at depth D may be linked to the compaction and

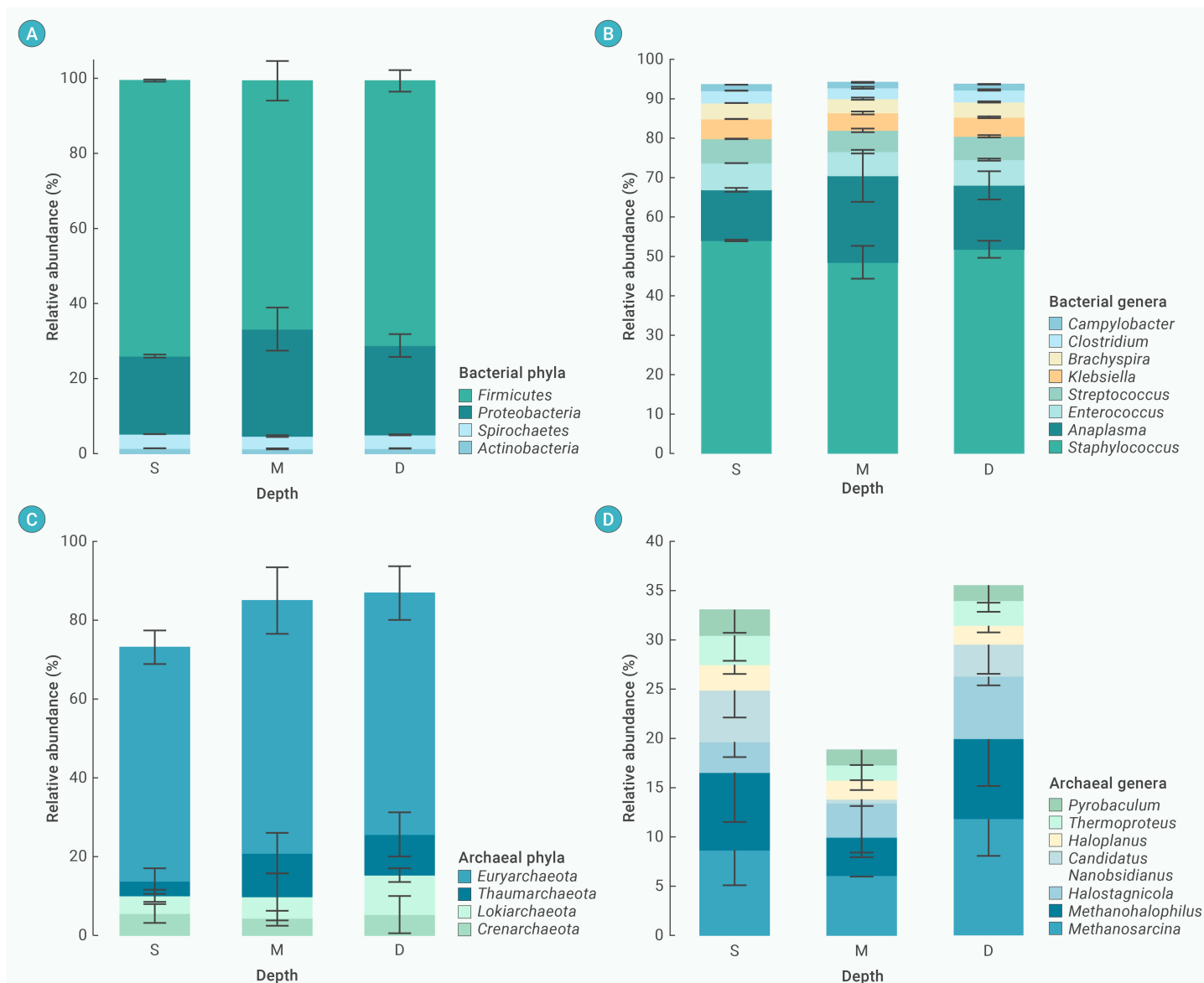


Figure 3. Relative abundance of top four bacterial phyla (mean $\pm$ standard error) (A), top eight bacterial genera (mean $\pm$ SD) (B), top four archaeal phyla (mean $\pm$ SD) (C), and top seven archaeal genera (mean-SD) (D)

cementation effects associated with dolomite formation, influencing both the rock's microstructure and its potential as a reservoir for microbial activity and methane storage.

The measured physiochemical parameters, including TN, TP, TC, TOC, carbon/nitrogen (C/N) ratio, Cl, B, Ca, Fe, Mg, and TK showed significant differences across depths ($p < 0.05$) (Figure 2A). The TN and TK content significantly decreased from depth S (0.57 ± 0.05 and 11.13 ± 0.06 g/kg, respectively; mean $\pm$ standard error (SD)) to depth M (0.40 ± 0.00 and 11.13 ± 0.06 g/kg, respectively) and then significantly increased at depth D (1.57 ± 0.03 and 12.74 ± 0.04 g/kg, respectively) ($p < 0.05$). Although depth D had a higher percentage of TOC than that at depth S or depth M ($3.49 \pm 0.00\%$, $0.40 \pm 0.00\%$, and $1.78 \pm 0.00\%$, respectively), the TC and the C/N ratio at depth M was significantly higher than those at depth S or depth D ($p < 0.05$) (100.67 ± 0.19 , 30.57 ± 0.03 , and 43.03 ± 0.14 g/kg, respectively, for TC, and 4.48 ± 0.018 , 0.22 ± 0.04 , and 0.96 ± 0.040 , respectively, for T/C). The TP and Fe content significantly increased from depth S to depth M and then further significantly increased at depth D ($p < 0.05$) (0.21 ± 0.00 , 0.27 ± 0.00 , and 0.33 ± 0.00 g/kg, respectively, for TP, and 12.42 ± 0.05 , 14.50 ± 0.27 , and 19.40 ± 0.13 g/kg, respectively for Fe). The B and Cl content significantly decreased from depth S to depth M and then significantly increased at depth D ($p < 0.05$) (0.04 ± 0.00 , 0.03 ± 0.00 , and 0.04 ± 0.00 g/kg, respectively for B, and $24,952.00 \pm 0.00$, $8,334.00 \pm 0.00$, $12,617.00 \pm 0.00$ ppm, respec-

tively for Cl).

The Ca and Mg content significantly increased from depth S to depth M and then significantly decreased at depth D ($p < 0.05$) (22.59 ± 0.55 , 70.91 ± 1.12 , and 20.31 ± 0.68 g/kg, respectively, for Ca, and 5.35 ± 0.31 , 31.30 ± 1.20 , and 3.31 ± 0.17 g/kg, respectively, for Mg).

Quartz (Qtz) (S: 23.2%, M: 10.0%, and D: 34.7%), dolomite (Dol) (S: 21.8%, and M: 63.8%), clay minerals (CM) (S: 29.6%, M: 17.5%, and D: 23.2%) plagioclase (Pl) (S: 10.1%, M: 3.0%, and D: 15.6%), and calcite (Cal) (S: 0.3%, M: 0%, and D: 18.6%) were the predominant minerals in the rock samples at the three depths (Figure 2B) and the CMs were mainly composed of illite (S: 22%, M: 33%, and D: 29%) and illite/smectite mixed layers (S: 65%, M: 54%, and D: 50%; mixed layer ratio at depth S: 8, M: 5, and D:10) (Figure 2C).

Microbial community composition

The relative 16S rRNA gene-based sequence abundance of bacteria was close at depth S, depth M, and depth D ($72.60\% \pm 0.21\%$, $75.01\% \pm 1.71\%$, and $73.29\% \pm 1.09\%$, respectively) (Table 1). The bacterial community at the three depths was mostly represented by the phylum Firmicutes (S: $73.42\% \pm 0.30\%$, M: $66.16\% \pm 5.28\%$, and D: $70.52\% \pm 2.86\%$), followed by Proteobacteria (S: $20.78\% \pm 0.40\%$, M: $28.52\% \pm 5.73\%$, and D: $23.79\% \pm 3.04\%$), Spirochaetes (S: $3.79\% \pm 0.01\%$, M: $3.39\% \pm 0.24\%$, and D: $3.63\% \pm 0.13\%$) and Actinobacteria (S: $1.42\% \pm 0.02\%$, M: $1.28\% \pm 0.12\%$, and D: $1.38\% \pm 0.05\%$)

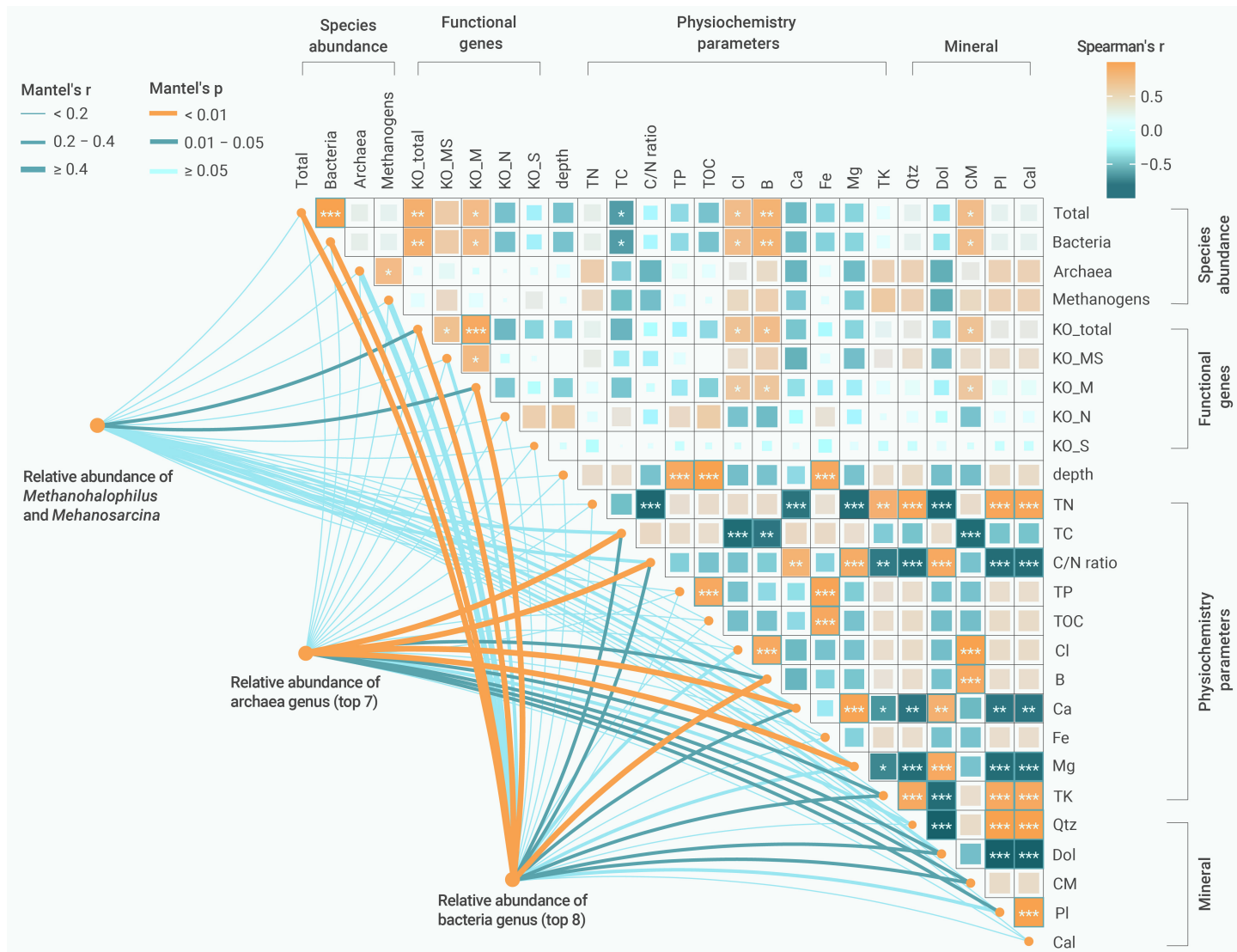


Figure 4. Correlation between microbial community composition, species abundance, functional genes abundance, the concentration of physiochemical parameters, and mineral (**p < 0.01, 0.001 < **p < 0.01, 0.01 < *p < 0.05). Abbreviation: KO_MS: total abundance of functional genes for methanogenic substrate metabolism, KO_M: total abundance of functional genes for methane metabolism, KO_N: total abundance of functional genes for nitrogen metabolism, KO_S: total abundance of functional genes for sulfur metabolism, and CM: clay minerals.

(Figure 3A). At the genus level, the eight largest genera (*Staphylococcus*, *Anaplasma*, *Enterococcus*, *Streptococcus*, *Klebsiella*, *Brachyspira*, *Clostridium*, and *Campylobacter*) (total relative abundance > 1%) accounted for > 93% of the total bacterial community at the three depths. The relative abundance of the genus *Staphylococcus*, which occupied the highest proportion, decreased from depth S to depth M and then increased at depth D (54.04% ± 0.22%, 48.50% ± 4.16%, and 51.80% ± 2.18%, respectively). The relative abundance of the genus *Anaplasma* increased from depth S to depth M and then decreased at depth D (12.85% ± 0.50%, 21.93% ± 6.60%, and 16.23% ± 3.59%, respectively) (Figure 3B).

The relative 16S rRNA gene-based sequence abundance (metagenome derived) of archaea was < 0.001% at all three depths (Table 1). The archaeal community at the three depths was mostly represented by phylum Euryarchaeota (S: 58.19% ± 4.94%, M: 63.18% ± 9.21%, and D: 61.28% ± 6.87%), followed by Thaumarchaeota (S: 3.64% ± 3.18%, M: 10.92% ± 5.03%, and D: 10.30% ± 5.63%), Lokiarchaeota (S: 4.38% ± 1.41%, M: 5.25% ± 5.74%, and D: 10.03% ± 1.79%), and Crenarchaeota (S: 5.49% ± 2.37%, M: 4.29% ± 1.76%, and D: 5.31% ± 4.80%) (Figure 3C). At the genus level, the seven largest genera (*Methanosarcina*, *Methanohalophilus*, *Halostagnicola*, *Candidatus Nanobsidianus*, *Haloplanus*, *Thermoproteus*, and *Pyrobaculum*) (total relative abundance > 1%) accounted for 33.06% ± 3.26%, 18.84% ± 7.13%, and 35.56% ± 6.30% at depth S, M, and D, respectively. The relative abundance of the *Methanosarcina*, *Methanohalophilus*, *Candidatus Nanobsidianus*, and

Pyrobaculum genera decreased from depth S (8.66% ± 3.57%, 7.87% ± 5.02%, 5.25% ± 2.76%, and 2.61% ± 2.35% respectively) to depth M (6.09% ± 0.12%, 3.87% ± 2.01%, 0.40% ± 0.69%, and 1.54% ± 1.54% respectively), and then increased at depth D (11.81% ± 3.75%, 8.12% ± 4.76%, 3.25% ± 2.97%, and 1.61% ± 1.77%, respectively). The relative abundance of the *Halostagnicola* genus at depth D (6.34% ± 0.89%) was higher than that at depth S or M (3.11% ± 1.53%, and 3.47% ± 5.02%, respectively). The relative abundance of the *Haloplanus* and *Thermoproteus* genera at depth S was higher than that at depth M and D (2.59% ± 0.95%, 1.94% ± 1.00%, and 1.92% ± 0.68% respectively, and 2.96% ± 2.56%, 1.54% ± 1.54%, and 2.50% ± 1.10%, respectively) (Figure 3D).

To identify species, we reconstructed metagenome-assembled genomes (MAGs) using metagenomics data. After quality filtering and dereplication of the resulting bins, only five low-quality bins were obtained (Table S2). Due to their low completeness (50–54%) and high levels of contamination (7–9%), the reliability of both taxonomic and functional annotations was considered insufficient for further analysis. This may be attributed to insufficient sequencing depth, which is often a limitation when working with subsurface rock samples where microbial density is relatively low.

The link between microbial community composition and physiochemical parameters

The correlation between microbial community composition (dominant

methanogens, archaea, and bacteria), species abundance, functional genes (Table S3) abundance, physiochemical parameters, and mineral content was analyzed (Mantel test) (Figure 4). Significant correlations were mainly detected between species abundance and functional genes, and between physiochemical parameters and mineral content. The results suggested that the concentration of TC, C/N ratio, B, Ca, Mg, TK, and mineral content, including Dol, CM, and Pl, was significant (Mantel's $p < 0.05$) factors influencing the composition of the dominant archaeal genus, and in addition to Mg and Pl, were significant (Mantel's $p < 0.05$) factors influencing the composition of the dominant bacterial genus. The functional genes of methanogenic substrate metabolism, methanogenic functional genes, and the relative abundance of dominant methanogens were significantly ($p < 0.05$) correlated with the total abundance of genes and methanogenic functional genes. The total abundance of methanogenic functional genes (Figure 6B & Table S3) was also significantly (Spearman's $p < 0.05$) correlated with the Cl, B, and CM concentrations.

Metabolism profiles along depths

The microbial communities' functional gene content was summarized as the CAZy families (Table S4). At depth S, the relative abundance of functional genes matching CAZy reference sequences was higher than that at depth M (Figure 5). This included a near-significant increase in auxiliary activities (AA; T-test, $p < 0.1$) and a significant increase in glycosyl transferases genes (T-test, $p < 0.05$). While the average relative proportion of functional gene matching CAZy reference sequences was highest at depth D, the differences between depth D and depths S and M did not achieve statistical significance (T-test and Wilcoxon two-sided rank sum test).

A significant (T-test, $p < 0.05$) shift in mean abundance of total KO terms (Table S3) between depth S (417351.09 ± 39648.45 TPM) and depth M (334019.26 ± 26590.81 TPM) was found and included a significant (T-test, $p < 0.05$) decrease in functions involved in methanogenic substrate metabolism and methane metabolism from depth S (909.70 ± 84.91 TPM and 831.72 ± 84.36 TPM, respectively) to depth M (741.13 ± 45.71 TPM and 645.49 ± 32.99 TPM, respectively). A significant (excluding specific values at depth D, $p < 0.05$) increase in functions involved in methanogenic substrate metabolism and methane metabolism from depth M to depth D (1039.64 ± 97.96 TPM and 834.25 ± 23.67 TPM, respectively) was also found (Figures 6A-B). Substrate metabolism included glycolysis (S: 837.97 ± 87.92 TPM, M: 659.79 ± 38.11 TPM, and D: 746.45 ± 131.86 TPM) and microbial synthesis of acetate (S: 51.99 ± 3.92 TPM, M: 60.48 ± 10.95 TPM, and D: 131.13 ± 115.04 TPM), carbon dioxide (S: 19.73 ± 7.45 TPM, M: 17.40 ± 3.48 TPM, and D: 31.83 ± 17.48 TPM), and methanol and methylamine (M: 3.47 ± 3.36 TPM, and D: 5.64 ± 8.92 TPM). This evaluation was primarily based on KEGG pathway ko01200 corresponding to carbon metabolism and pathway map00680 corresponding to methane metabolism. Among the four methanogenesis modules (M00567, M00356, M00357, and M00563), only part of the functional genes in the KEGG module M00357 corresponding to methanogenesis from acetate were detected, while key functional genes of methanogenesis metabolism, including gene K00399 (*mcrA*, methyl-coenzyme M reductase alpha subunit), were not detected.²² A near-significant ($p < 0.1$) decrease in the mean abundance of functional genes in the KEGG module M00357 that were detected between depth S (11.92 ± 0.97 TPM) and M (9.13 ± 1.87 TPM) and a significant (excluding specific values at depth D, $p < 0.05$) increase between depth M and D (22.58 ± 3.59 TPM) were found (Figure 6B). Accordingly, the acetoclastic methanogenesis functional genes including K00625, K13788 (phosphate acetyltransferase), K00925 (acetate kinase), K01895 (acetyl-CoA synthetase), 2-Phosphosulfolactate phosphatase (ComB), coenzyme M (CoM) (K05979) and coenzyme B (CoB) (K16792, K16793) have been detected. For nitrogen metabolism, the functional genes of nitrate reduction (*narG*, *narH*, and *narI*) (S: 12.25 ± 2.36 TPM, M: 11.97 ± 5.21 TPM, and D: 24.61 ± 25.60 TPM), denitrification (M00529: *nirS*) (S: 2.00 ± 1.28 TPM, M: 1.40 ± 0.31 TPM, and D: 2.37 ± 0.82 TPM), and nitrate/nitrite transporter (NRT) (S: 7.08 ± 3.30 TPM, M: 9.12 ± 1.96 TPM, and D: 12.68 ± 6.53 TPM) at the three depths, assimilatory nitrate reduction (ANR) (M00531: *nasC*) at depth D (D2: 2), dissimilatory nitrate reduction (DNR) (M00530: *nirB*, *nirD*) (M: 2.04 ± 0.075 TPM and D: 8.14 ± 8.00 TPM) and nitrate/nitrite transport system substrate-binding protein (*nrtA*, *nrtB*, and *nirC*) (M: 1.78 ± 1.07 TPM and D: 3.59 ± 2.45 TPM) at depth M and D, and nitrogen fixation (M00175: *nifH*) at depth M (M2: 2.73) were detected (Figure 6C). A metabolic pathway

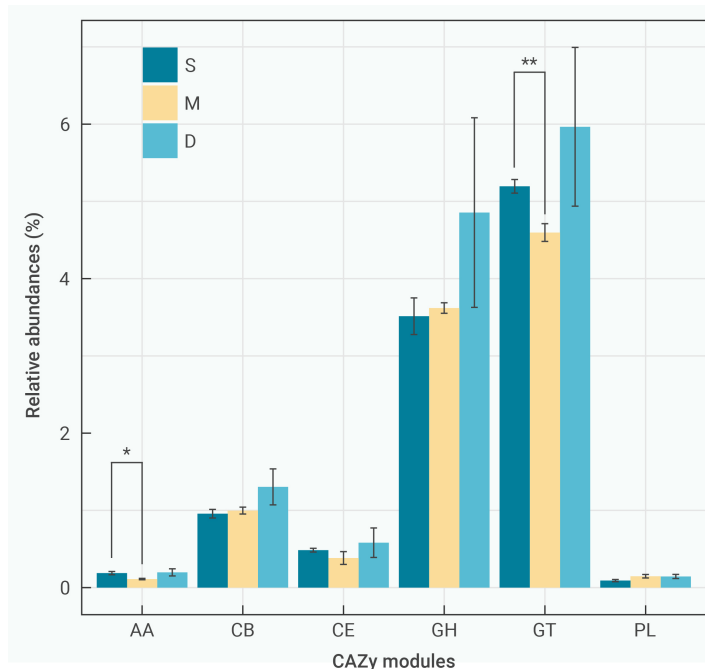


Figure 5. Relative abundance of CAZy modules, including auxiliary activities (AA), carbohydrate binding (CB), carbohydrate esterases (CE), glycoside hydrolases (GH), glycosyl transferases (GT), and polysaccharide lyases (PL) (* $p < 0.1$, ** $p < 0.05$).

of assimilation sulfate reduction, which was incomplete due to the undetected functional genes encoding bifunctional enzyme CysN/CysC and 3'-phosphoadenosine 5'-phosphosulfate synthase (PAPSS), was found at the three depths (S: 9.78 ± 5.26 TPM, M: 11.53 ± 5.10 TPM, and D: 11.52 ± 9.48 TPM) (Figure 6D).

DISCUSSIONS

The Sanhu area of the Qaidam Basin contains the vast majority of biogenetic gas, with an estimated volume of $1,591,012 \text{ m}^3$. Previous studies have examined the chemical composition and carbon isotope of gas samples, identifying acetate fermentation¹⁵ and carbon dioxide deoxidation¹⁴ as the primary methanogenic pathways. However, the microbial community composition and the genetic evidence supporting methanogenic pathways have not been well explored.

Methanogens and methane metabolism associated functional genes

In this study, we observed depth-related variation in the relative abundance of methanogens, particularly *Methanosarcina* and *Methanohalophilus*, which dominate the methanogens in rock samples. The abundance of these methanogens was significantly (Mantel's $p < 0.05$) correlated with methanogenic functional genes (Figure 4), underscoring their importance in methane production. Both *Methanosarcina* and *Methanohalophilus* belong to the *Methanosarcinaceae* family, which includes many halophilic or highly salt-tolerant members such as *Methanohalophilus*, all of which exhibit methylotrophic metabolism.²³ *Methanosarcina* is capable of producing methane through a mixed fermentation pathway, utilizing acetate and H_2/CO_2 .²⁴ The predominant species, *Methanosarcina acetivorans* (relative abundance > 91%), is a well-characterized acetotrophic methanogens.²⁵ It converts acetate to acetyl-CoA using one ATP molecule and subsequently to methane through a reaction catalyzed by acetate kinase (AckA) and phosphate acetyltransferase (Pta). This pathway is more energy-efficient than that of *Methanosaeta* (not detected in this study), which uses two ATP molecules for a similar conversion through acetyl-CoA synthetase (ACSS1_2).²⁶ Due to its high affinity for acetate and lower energy consumption, *Methanosarcina* is better suited to nutrient scarce environments,²⁷ such as deep rocks.

Consistently, *Methanosarcina* exhibited relatively higher abundance at depths S and D, aligning with the distribution of functional genes involved in acetate-dependent methanogenesis, including K00625, K13788 (phosphate acetyltransferase), K00925 (acetate kinase), and K01895 (acetyl-CoA

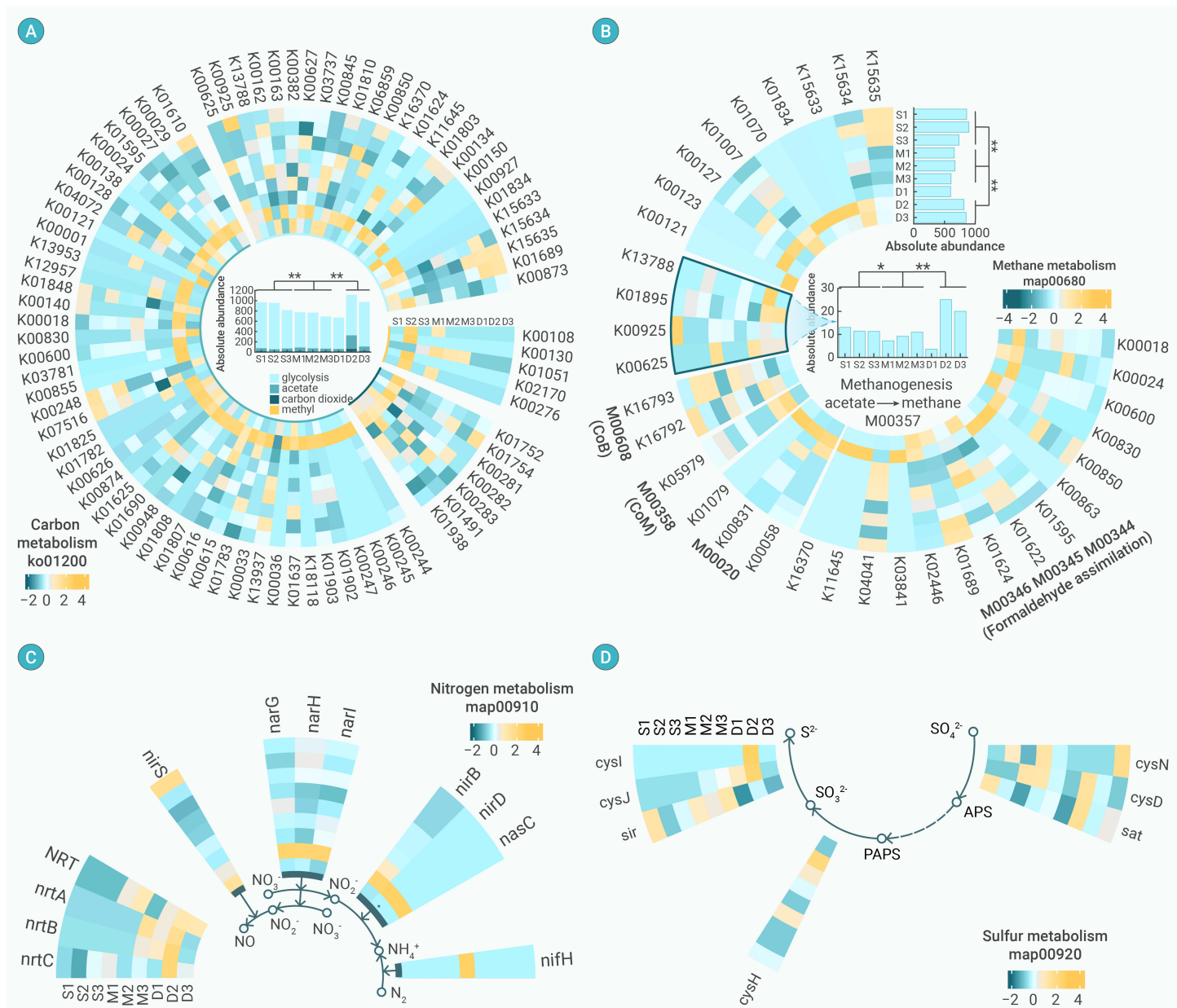


Figure 6. Heatmaps showing the gene abundances for methanogenic substrate metabolism (A), methane metabolism (B), nitrogen metabolism (C), and sulfur metabolism (D). The values for each gene were normalized by the mean relative abundance of all nine rock metagenomes. Bars correspond to the absolute abundance of functional genes for methanogenic substrate metabolism, methane metabolism and methanogenic from acetate, respectively (*p < 0.1, **p < 0.05).

synthetase). This relative enrichment of *Methanosarcina* and its functional genes supports the occurrence of acetate-utilizing methanogenesis in deep rock. Zhang et al.¹⁴ also observed *Methanosarcina* in core samples at a depth of 1264 meters east of Tajin Lake in the Sanhu area of Qaidam Basin and concluded the existence of acetate-utilizing methanogenesis pathways in the area.

Methanohalophilus is a prevalent methanogen in source rock across various unconventional reservoirs and serves as a keystone species in these ecosystems.¹² At present, the genus *Methanohalophilus* comprises a single identified species, *Methanohalophilus* WGI-DM, a dimethylamine-utilizing methanogens previously detected in fracturing fluids from Oklahoma wells²⁸ and the current study. This microorganism relies on glycine betaine (GB) production and uptake as a halotolerance mechanism, a trait encoded in its genome by a glycine-metabolic pathway.¹² However, only the GB synthesis gene specific to the choline pathway (Figure 6A) was detected in our samples, probably due to the low abundance of *Methanohalophilus*.

Previous studies have shown that *Methanohalophilus* species thrive in environments with NaCl concentrations ranging from 1.5% to 20%, with optimal growth at 6% to 15%.^{23,29} In contrast, the Cl⁻ concentrations in this study

ranged from 0.83% to 2.50%, which is lower than the microorganism's preferred salinity range. Despite this, *Methanohalophilus* had a higher average relative abundance at depths S and D, corresponding to relatively higher salinity levels in these layers. The relative enrichment suggests that *Methanohalophilus* plays a significant role in methylotrophic methanogenesis in deep subsurface rocks.

Some key functional gene associated with acetoclastic methanogenic metabolism in deep rocks have been detected, including K00625, K13788 (phosphate acetyltransferase), K00925 (acetate kinase), K01895 (acetyl-CoA synthetase), 2-Phosphosulfolactate phosphatase (ComB), coenzyme M (CoM) (K05979) and coenzyme B (CoB) (K16792, K16793). Among them, 2-Phosphosulfolactate phosphatase (ComB), an acid phosphatase involved in the biosynthesis of CoM,³⁰ was detected, which serves as the terminal methyl carrier in methanogenesis. Although homologs of ComB have been identified in bacteria and algae,³¹ its detection here does not conclusively prove methylotrophic methanogenesis. Nonetheless, the detection of methanogen homoaconitase subunits, which participate in the reduction of methyl-coenzyme M to methane,³² provides direct evidence for methanogenic metabolism in the deep subsurface. Methane metabolism at depth D may benefit from

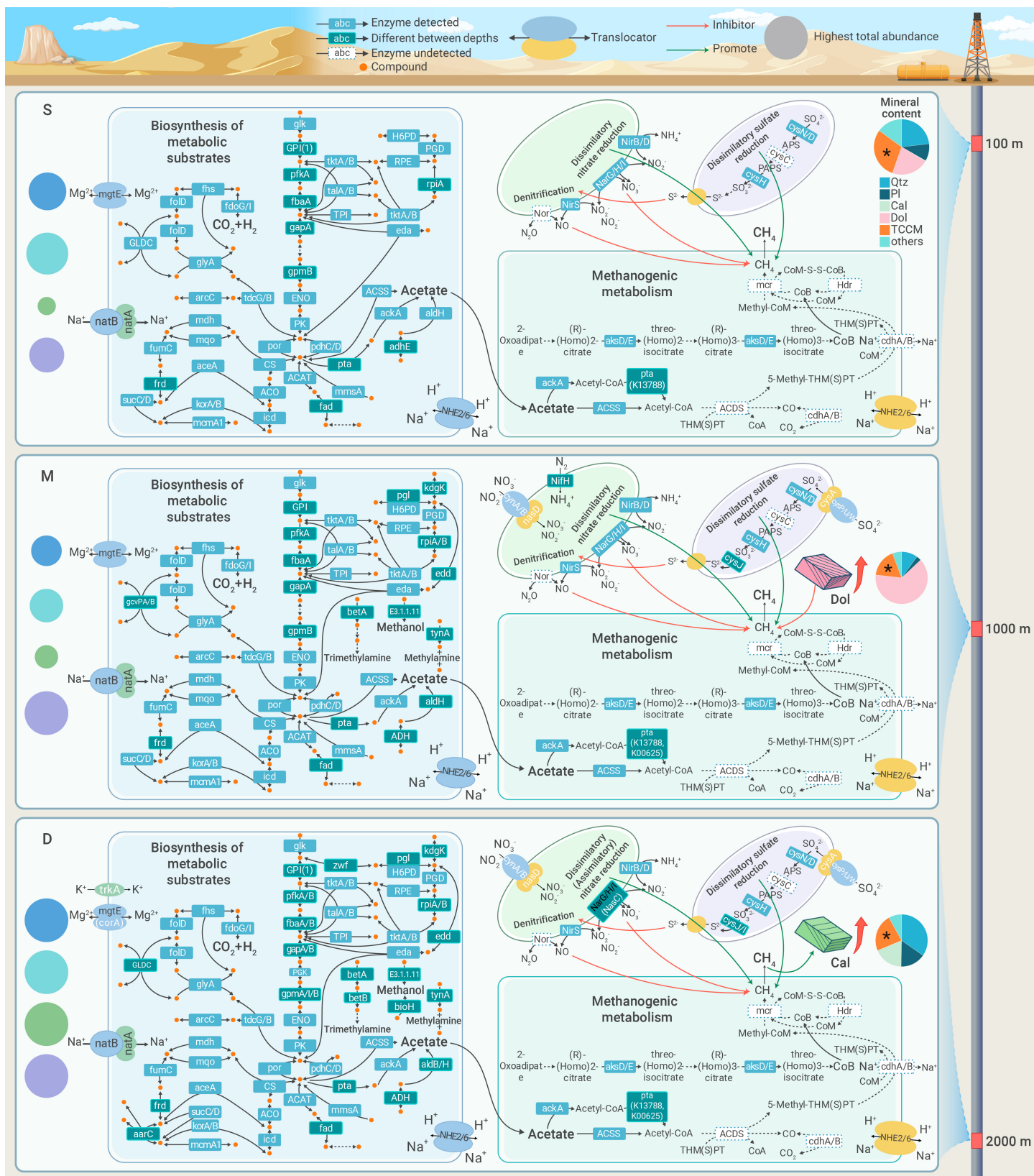


Figure 7. Methanogenesis-related metabolic pathways and potential determinants across depths.

increased substrate support, as indicated by the higher abundance of functional genes associated with the production of substrates such as acetate, carbon dioxide, methanol, and methylamine. However, no direct evidence of carbon dioxide-dependent methanogenesis pathways was detected except for that of substrate metabolism functional genes.

Other microbiota, such as *Haloplanus* and *Thermoproteus* also contributed

to the metabolic potential of the subsurface ecosystem. For instance, *Haloplanus natans*, the sole species in the genus *Haloplanus*, was found at all depths. Known for its nitrate-reducing capabilities but inability to ferment sugar,³³ *Haloplanus* likely plays a role in nitrogen cycling within the sampled environment. Similarly, *Thermoproteus uzoniensis*, thermophilic anaerobes isolated from hot springs and soil of the Uzon caldera (SW of

Kamchatkapen)³⁴ and capable of acetate production, was abundant at depths S and D. Its distribution aligns with the presence of functional genes for acetate-dependent methanogenesis (Figure 6B), suggesting that *Thermoproteus* may contribute to methanogenic metabolism by supplying acetate.

Limited pioneer works have speculated on the methanogenesis mechanism of biogenic methane generation in deep rock and shale. Daly et al.¹² detected the genus *Methanohalophilus* in input and output fluids over a year following hydraulic fracturing, demonstrating that methylamine cycles facilitated by microbial populations play a crucial role in methanogenesis within the deep biosphere. Nyssönen et al.¹³ identified multiple methanogenesis metabolic mechanisms in drill hole water, including methylotrophic and acetoclastic methanogenesis at all depths, as well as autotrophic methanogenesis from CO₂ and H₂ at 2300 meters. However, these works inferred methanogenesis pathways primarily by analyzing fluids associated with rock and shale drilling, rather than the rock and/or shale themselves. In this study, the dominant mitogens *Methanosarcina* and *Methanohalophilus* and acetoclastic methanogenesis pathways were detected by direct detection of *in situ* rock samples. A combination of the results of the aforementioned studies and this work suggests that multiple methanogenesis might contribute to methane production in deep rock. This conclusion aligns partially with prior chemical and isotopic analyses.^{14,15} This might be due to the following two reasons. First, a lack of comparative studies in the area constrains the ability to generalize methanogenic pathways. Second, the difficulty in obtaining and analyzing *in situ* rock samples has hindered the direct investigation of microbial communities and functional genes.

While this study directly analyzed rock samples, the long interval between sample collection and laboratory detection posed challenges to assessing functional gene expression and enzyme activity. These limitations underscore the need for future *in situ* research with improved sampling methods to better understand the microbial and metabolic dynamics driving methanogenesis in deep subsurface environments.

Link of microbiota and key physiochemical parameters and lithology

Decoupling between microbial community characteristics and rock sampling depth has been observed previously. Nyssönen et al.¹³ reported that microbial diversity was unrelated to sampling depth, and similar patterns were observed in this study. However, significant ($p < 0.05$) correlations were observed between the content of minerals profiles and the depths (Figure 4). For example, at depth M, the enrichment of dolomite (Dol), which is rich in Ca and Mg,³⁵ corresponded to a significant increase in the Ca and Mg abundance. There were notable (Mantel's $p < 0.01$) correlations between the concentrations of Ca and Mg and the dominant archaeal composition (Figure 4). Importantly, Dol has been shown to inhibit methane production in rumen fermentation,³⁶ which may explain the reduced abundance of methanogens and functional genes of methane metabolism at depth M. This inhabitation likely results from the increased concentrations of Ca and Mg caused by Dol enrichment.

Mari et al.¹³ also reported a significant ($p < 0.05$) correlation between the microbial community composition and concentrations of B, Ca, and Mg in the Outokumpu deep subsurface. Boron, as a nonmetal micronutrient for microorganisms,³⁷ plays an important role in microbial function, though its specific influence on methane metabolism in deep rock remains unclear. In this study, significant correlations were identified between B content and the abundance of functional genes of methane metabolism, suggesting that further exploration of the Boron's effect on methane metabolism in deep rocks could yield valuable insights.

Interactions between bacteria and CMs were also evident. CMs provide critical nutrients and habitat for the microbe, while microbial metabolism promotes the formation and transformation of CMs.³⁸ Meanwhile, CMs rich in illite/smectite mixed layers exhibit high adsorption capacity for methane.³⁹ Consistent with these observations, CMs content showed a significant ($p < 0.05$) positive correlation with bacterial species abundance and microbial metabolism, particularly methane metabolism (Figure 4).

Other minerals, such as calcite and quartz, can also be associated with methanogenesis in deep rocks. Hein et al.⁴⁰ proposed that carbon produced by the anaerobic oxidation of methane is a major source of Cal in deep mud-volcano cores. In deep rock formations, methane is stored via physisorption.⁴¹ In shales with low porosity, high content of Qtz and CMs increases the brittleness and promotes fracture development, thereby

enhancing methane storage capacity. This study observed a significant increase in the abundance of Cal, Qtz, and CM at depth D, which corresponds to the higher abundance of methanogens and methanogenic functional genes at this depth.

Overall, the interaction between microbial metabolism and mineral content in deep rocks shapes the environmental differences across depths, which in turn influences the abundance, activity, and function of methanogenic communities. These findings highlight the importance of mineralogical factors in determining the microbial dynamics and methane production potential of deep subsurface environments.

Link of microbiota and functional genes

Bacterial communities and the abundance of methanogenic functional genes were found to be significantly (Mantel's $p < 0.01$, Spearman's $p < 0.05$) correlated (Figure 4), indicating the integral role of bacteria in supporting methanogenesis. Various bacterial taxa contribute to the degradation of complex organic compounds into simple substrates that methanogens can utilize.⁴² For example, members of Firmicutes (e.g., *Staphylococcus*, *Enterococcus*, *Streptococcus*, and *Clostridium*) are known cellulose-degraders that hydrolyze cellulose to sugars.⁴³ Members of Proteobacteria (e.g., *Anaplasma*, *Klebsiella*, and *Campylobacter*) participate in hydrolysis⁴⁴ and play a significant role in methane-metabolizing microbial community. Previous studies on anaerobic methanogenesis have demonstrated a shift in bacterial dominance from Firmicutes to Proteobacteria as the system transitions from the acidification stage to the methanogenesis stage.^{45,46} Consistent with this, our study observed depth-related variations in bacterial community composition, with a notable decrease in the relative abundance of Firmicutes and an increase in Proteobacteria at depth M (Figure 3A). The abundance of functional genes involved in the production of methanogenic substrates (Figure 6A) was significantly lower at depth M than at depth S or D (Figure 6B), corresponding to the significantly lower abundance of methanogenic functional genes at depth M. Accordingly, the observed decrease in methanogenesis-related activity at depth M may be explained by the reduced availability of substrates such as acetate. In sum, these findings indicate that bacteria in deep subsurface rocks serve as primary suppliers of substrates for methanogenic metabolism. Their abundance, community composition, and metabolic activity directly influence the efficiency of methanogenesis and methane production.

Carbon cycling

The total proportion of CAZymes decreased from depth S to depth M (e.g., AA, carbohydrate esterases (CE), and glycosyl transferases (GT)). At depth D, the proportion increased, with higher levels of AA, carbohydrate binding, CE, glycoside hydrolases, and GT detected (Figure 5). Significant (Mantel's $p < 0.05$) correlations were observed between TC, C/N ratio, and dominant microbial communities (both archaea and bacteria) (Figure 4). Previous research has identified the C/N ratio as a strong predictor of the richness of bacterial carbohydrate-active enzyme genes.⁴⁷ However, in this study, no significant correlation was observed between the TC, C/N ratio, and species abundance. A significant negative correlation was found between the C/N ratio and functional genes associated with methane metabolism. These results suggest that carbon abundance, including organic carbon, is not the sole determinant of methane metabolism in deep rocks. TOC is an important parameter for assessing the hydrocarbon generation potential of shale gas reservoirs.⁴¹ Although TOC did not show a significant correlation with methanogenesis and methane metabolism functional genes (Figure 4), its significant increase with depth suggests that depth D has greater methanogenic potential. This was further supported by the correlation between the abundance of methane metabolism functional genes and methanogenic substrate metabolism, showing the need for further exploration of carbon metabolism pathways directly influencing methane production in deep rocks.

Enzymes associated with biosynthetic methanogenic metabolic substrates (Figure 6A) and their metabolic pathways increased with depth (Figure 7). Additionally, the species richness of enzymes facilitating key metabolic reactions was higher at greater depth. While the metabolic pathway at depth M was relatively intact and comparable to that at depth D, the lower total abundance of related functional genes suggests reduced metabolic potential at depth M. In contrast, depth D had a more abundant and complete metabolic

pathway for biosynthetic methanogenic substrates, providing greater material support for intensified methane metabolism at this depth.

Nitrogen cycling

Functional genes associated with nitrogen cycling pathways, including nitrate reduction (*narG*, *narH*, and *narI*), denitrification (*nirS*), ANR (*nasC*), DNR (*nirB*, *nirC*), and nitrogen fixation (*nifH*) were detected in this study (Figure 6). Among these, only *nirS* was identified among the denitrification-related genes, suggesting that denitrification pathways in deep rocks are incomplete. Denitrification is typically an anoxic process, while DNR occurs in strictly anaerobic environments.⁴⁸ This aligns with the higher abundance of DNR functional genes observed at depths M and D compared to denitrification genes, reflecting the prevailing anaerobic conditions at these depths.

The low relative abundance (< 1%) of *Pseudomonas*, the most common nitrate-reducing bacterial genus,⁴⁹ at all depths may account for the limited detection of nitrate-reducing genes. Nevertheless, the relatively complete set of nitrate reduction-related genes identified at depth D indicates that nitrate reduction metabolism is more robust at this depth. Further, the higher abundance of DNR-related genes (*nirB* and *nirD*) compared to ANR-related genes (*nasC*) suggests that DNR is the dominant nitrate reduction pathway in deep rocks. Unlike the denitrifying pathway, DNR reduces the production of nitric oxide, which are toxic intermediate that can inhibit methanogenesis⁵⁰. This could explain the observed increase in functional genes related to methanogenesis at depth D (Figure 6). The nitrogen fixation marker gene *nifH*⁵¹ was detected exclusively at depth M in extremely low abundance, indicating that nitrogen fixation is not active in deep rocks.

Sulfur cycling

Nearly all functional genes associated with assimilating sulfate reduction (ASR) pathways were detected, except those encoding PAPSS and CysN/CysC, indicating that a relatively complete ASR pathway is present in deep rocks environment. Despite this, the low abundance of ASR-related functional genes and the limited presence of sulfate-reducing bacteria suggest that the sulfur cycle operates at a low metabolic level in these environments. ASR metabolism facilitates the conversion of SO_4^{2-} to S^{2-} , which can inhibit denitrification.⁵⁰ This inhibition may promote the dominant role of DNR in nitrogen cycling at depth D. Furthermore, the expression of the ASR-related genes likely enhances DNR, indirectly reducing the inhibition of nitrate on methanogenesis metabolism.⁵⁰ The absence of functional genes encoding nitrate, nitrite, and sulfate translocators (Table S5) at depth S indicates a lower overall abundance of nitrogen and sulfur metabolism functional genes in the shallower layers. Interestingly, the nitrogen and sulfur cycles in the deep rock environment are characterized by metabolic pathways supporting methanogenesis. This interplay between nitrogen and sulfur cycling pathways and methanogenesis highlights the adaptive strategies of microbial communities in these subsurface ecosystems. The microbial composition and unique metabolic mechanisms of the deep subsurface environment within the biogas field appear to be optimized to facilitate the production of large quantities of methane.

Metabolic strategies and potential determinants

Methane metabolism in deep rocks across biological gas fields has primarily focused on the presence or absence of metabolic pathways for synthetic methanogenic substrates.^{12,13} This approach has often led to the assumption that these pathways remain consistent across depths. Additionally, functional genes related to nitrogen and sulfur cycling metabolism were largely ignored due to their low abundance or lack of significant variation between depths. In this study, we extensively analyzed enzyme composition associated with metabolic pathways at various depths (Figure 7). The results revealed that the abundance of C, N, and S metabolic pathways associated with methane metabolism increased with depth, peaking at depth D. This analysis suggests that the richness of enzyme species, and thus the completeness of metabolic pathways, directly influences the potential for methane production. For example, higher enzyme species richness at depth D correlated with the highest methane production potential, as indicated by the relative abundance of methanogen (Figure 3) and methane metabolism functional genes (Figure 6).

The analysis also demonstrated that carbon, nitrogen, and sulfur metabolic pathways are strongly tied to methanogenic metabolism in deep rock.

Despite a higher abundance of metabolic pathways at depth M compared to depth S, methane production potential was lower at depth M. This may be attributed to a significant decrease in TCCM content and an increase in Dol content in this depth, which negatively affects methanogenesis. Physico-chemical factors such as the TOC have been emphasized for their indication function for hydrocarbon generation potential in shale gas reservoirs.⁴¹ However, our findings suggest an insignificant correlation between TOC and methanogenesis (Figure 4). In contrast, a significant ($p < 0.05$) correlation was observed between the TCCM content and the abundance of methanogenic metabolism functional genes (Figure 4). This indicates that TCCM may serve as a better indicator of bioavailable organic matter in rocks across depths than TOC. Mineral composition exhibited a strong correlation with the physical and chemical factors of deep rocks, making it a reliable reference for gas exploration. In this study, the abundance of Dol, Cal, and TCCM emerged as a potential determinant of methanogenesis.

CONCLUSIONS

This study elucidates the distribution characteristics and potential determinants of microbial communities, functional genes, and metabolic pathways in rock samples from varying depths of Quaternary biogas fields in the Qaidam Basin. The findings reveal that key environmental factors influencing the distribution of microbial communities and methanogenesis-related functional genes include TC, C/N ratio, Cl, B, and Mg) and mineral composition (mainly CM and Dol). The detection of the dominant methanogen *Methanosarcina* and acetate-utilizing methanogenesis functional genes indicates the existence of an acetate-utilizing methanogenic metabolic pathway in deep rocks. These insights provide a fundamental understanding of the methanogenesis mechanism in Quaternary biogenic gas production. Additionally, the finding of the tight link between methanogenesis and microbial C, N, and S cycling offers a valuable reference for biogenic gas exploration and sustainability efforts.

This study provides direct evidence of microbial involvement in biogas production, highlighting the possibility of multiple methanogenesis pathways in the deep subsurface. However, the limitation arising from the long-term storage of rock samples must be acknowledged, as the microbial activity may not fully reflect "*in situ*" conditions. Future studies utilizing *in situ* approaches are crucial for a more accurate assessment of microbial community and functional dynamics in the deep subsurface. Results from this study and future research would contribute to a deeper understanding of methanogenesis and its role in biogas production within deep rock environments.

REFERENCES

- Wang H., Chen L., Qu Z. G., et al. (2020). Modeling of multi-scale transport phenomena in shale gas production - A critical review. *Appl. Energy* **262**:114575. DOI:10.1016/j.apenergy.2020.114575
- Yang Y., Wang L. M., Fang Y. B., et al. (2017). Integrated value of shale gas development: A comparative analysis in the United States and China. *Renew. Sust. Energ. Rev.* **76**:1465–1478. DOI:10.1016/j.rser.2016.11.174
- Singh K. P. (2014). Biogeophysical signatures of microbial natural gas accumulation. *Current Science* **107**:1880–1884.
- Castro-Alvarez F., Marsters P., Barido D. P. D., et al. (2018). Sustainability lessons from shale development in the United States for Mexico and other emerging unconventional oil and gas developers. *Renew. Sust. Energ. Rev.* **82**:1320–1332. DOI:10.1016/j.rser.2017.08.082
- Zou C. N., Yang Z., Pan S. Q., et al. (2016). Shale gas formation and occurrence in China: An overview of the current status and future potential. *Acta Geol. Sin-Engl.* **90**:1249–1283. DOI:10.1111/1755-6724.12769
- Guo T. L. (2016). Key geological issues and main controls on accumulation and enrichment of Chinese shale gas. *Pet. Explor. Dev.* **43**:349–359. DOI:10.1016/s1876-3804(16)30042-8
- Zou C. N., Dong D. Z., Wang Y. M., et al. (2015). Shale gas in China: Characteristics, challenges and prospects (I). *Pet. Explor. Dev.* **42**:753–767. DOI:10.1016/s1876-3804(15)30072-0
- Amend J. P. and Teske A. (2005). Expanding frontiers in deep subsurface microbiology. *Palaeogeogr. Palaeoclimatol. Palaeoecol.* **219**:131–155. DOI:10.1016/j.palaeo.2004.10.018
- Brazelton W. J., Nelson B. and Schrenk M. O. (2012). Metagenomic evidence for H-2 oxidation and H-2 production by serpentinite-hosted subsurface microbial communities. *Front. Microbiol.* **3**:268. DOI:10.3389/fmicb.2011.00268
- Wang Y. L., Xia Y., Dong H. L., et al. (2005). 16S rDNA analysis of bacterial community in Donghai terrestrial deep subsurface: Microbiology research of the Chinese

- Continent Scientific Drilling. *Acta Petrol. Sin.* **21**:533–539. DOI:10.3321/j.issn:1000-0569.2005.02.028
11. Zhang G. X., Dong H. L., Xu Z. Q., et al. (2005). Microbial diversity in ultra-high-pressure rocks and fluids from the Chinese Continental Scientific Drilling Project in China. *Appl. Environ. Microbiol.* **71**:3213–3227. DOI:10.1128/aem.71.6.3213-3227.2005
 12. Daly R. A., Borton M. A., Wilkins M. J., et al. (2016). Microbial metabolisms in a 2.5-km-deep ecosystem created by hydraulic fracturing in shales. *Nat. Microbiol.* **1**:16146. DOI:10.1038/nmicrobiol.2016.146.
 13. Nyyssönen M., Hultman J., Ahonen L., et al. (2014). Taxonomically and functionally diverse microbial communities in deep crystalline rocks of the Fennoscandian shield. *ISME J.* **8**:126–138. DOI:10.1038/ismej.2013.125
 14. Zhang X. B., Song C. P., Qi Y. L., et al. (2005). Methanogenic pathway of the Quaternary biogenic gases in the Qaidam basin, China. *Geochem. J.* **39**:411–416. DOI:10.2343/geochemj.39.411
 15. Chen Q. L., Zhao F., Su Q., et al. (2016). Quaternary evaporites of biogenic methane gas in the eastern Qaidam Basin, China. *Carbonates Evaporites* **31**:213–218. DOI:10.1007/s13146-016-0288-3
 16. Lu R. K. (2000). Mineral total analysis of soil and colloids. Lu R. K. (ed). Analysis method of soil agricultural chemistry (China Agricultural Science and Technology Press), pp: 44–57.
 17. Martin M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet. J.* **17**:10–12. DOI:10.14806/embnet.17.1.200
 18. Peng Y., Leung H. C. M., Yiu S. M., et al. (2012). IDBA-UD: A de novo assembler for single-cell and metagenomic sequencing data with highly uneven depth. *Bioinformatics* **28**:1420–1428. DOI:10.1093/bioinformatics/bts174
 19. Zhu W. H., Lomsadze A. and Borodovsky M. (2010). Ab initio gene identification in metagenomic sequences. *Nucleic Acids Res.* **38**:e132. DOI:10.1093/nar/gkq275
 20. Fu L. M., Niu B. F., Zhu Z. W., et al. (2012). CD-HIT: Accelerated for clustering the next-generation sequencing data. *Bioinformatics* **28**:3150–3152. DOI:10.1093/bioinformatics/bts565
 21. Buchfink B., Xie C. and Huson D. H. (2015). Fast and sensitive protein alignment using DIAMOND. *Nat. Methods* **12**:59–60. DOI:10.1038/nmeth.3176
 22. Kanehisa M., Sato Y., Kawashima M., et al. (2016). KEGG as a reference resource for gene and protein annotation. *Nucleic Acids Res.* **44**:D457–D462. DOI:10.1093/nar/gkv1070
 23. Oren A. (2014). Taxonomy of halophilic Archaea: current status and future challenges. *Extremophiles* **18**:825–834. DOI:10.1007/s00792-014-0654-9
 24. Bharathi M. and Chellapandi P. (2017). Phylogenomic proximity and metabolic discrepancy of *Methanosarcina mazei* Go1 across methanosarcinal genomes. *Biosystems* **155**:20–28. DOI:10.1016/j.biosystems.2017.03.002
 25. Sowers K. R., Baron S. F. and Ferry J. G. (1984). *Methanosarcina acetivorans* sp. nov., an Acetotrophic Methane-Producing Bacterium Isolated from Marine Sediments. *Appl. Environ. Microbiol.* **47**:971–978. DOI:10.1111/j.1574-6968.1992.tb04987.x
 26. Jetten M. S. M., Stams A. J. M. and Zehnder A. J. B. (1991). Methanogenesis from acetate: a comparison of the acetate metabolism in *Methanotrix soehngenii* and *Methanosarcina* spp. *FEMS Microbiol. Rev.* **88**:181–197.
 27. R. Prathiviraj D., Berchmans S. and Paulchamy C. (2019). Analysis of modularity in proteome-wide protein interaction networks of *Methanothermobacter thermautotrophicus* strain ΔH and metal-loving bacteria. *J. Proteins Proteomics* **10**:179–190. DOI:10.1007/s42485-019-00019-5
 28. Borton M. A., Daly R. A., O'Banion B., et al. (2018). Comparative genomics and physiology of the genus *Methanohalophilus*, a prevalent methanogen in hydraulically fractured shale. *Environ. Microbiol.* **20**:4596–4611. DOI:10.1111/1462-2920.14467
 29. Guan Y., Ngugi D. K., Vinu M., et al. (2019). Comparative genomics of the genus *methanohalophilus*, including a newly isolated strain from kebrtit deep in the red sea. *Front. Microbiol.* **10**:839. DOI:10.3389/fmicb.2019.00839
 30. Graham D. E., Graupner M., Xu H. M., et al. (2001). Identification of coenzyme M biosynthetic 2-phosphosulfolactate phosphatase - A member of a new class of Mg²⁺-dependent acid phosphatases. *Eur. J. Biochem.* **268**:5176–5188. DOI:10.1046/j.0014-2956.2001.02451.x
 31. DiDonato M., Krishna S. S., Schwarzenbacher R., et al. (2006). Crystal structure of 2-phosphosulfolactate phosphatase (ComB) from *Clostridium acetobutylicum* at 2.6 angstrom resolution reveals a new fold with a novel active site. *Proteins* **65**:771–776. DOI:10.1002/prot.20978.
 32. Drevland R. M., Jia Y. H., Palmer D. R. J., et al. (2008). Methanogen Homoacetonitase Catalyzes Both Hydrolyase Reactions in Coenzyme B Biosynthesis. *J. Biol. Chem.* **283**:28888–28896. DOI:10.1074/jbc.M802159200
 33. Bardavid R. E., Mana L. and Oren A. (2007). *Haloplanus natans* gen. nov., sp. nov., an extremely halophilic, gas-vacuolate archaeon isolated from Dead Sea–Red Sea water mixtures in experimental outdoor ponds. *Int. J. Syst. Evol. Microbiol.* **57**:780–783. DOI:10.1099/ijs.0.64648-0
 34. Bonch-Osmolovskaya E. A., Miroshnichenko M. L., Kostrikina N. A., et al. (1990). *Thermoproteus uzoniensis* sp. nov., a new extremely thermophilic archaeobacterium from Kamchatka continental hot springs. *Arch. Microbiol.* **154**:556–559. DOI:10.1007/BF00248836
 35. Bonifacie M., Calmels D., Eiler J. M., et al. (2017). Calibration of the dolomite clumped isotope thermometer from 25 to 350 degrees C, and implications for a universal calibration for all (Ca, Mg, Fe) CO₃ carbonates. *Geochim. Cosmochim. Acta* **200**:255–279. DOI:10.1016/j.gca.2016.11.028
 36. Varadyova Z., Baran M., Zawadzki W., et al. (2003). Effect of dolomite, magnesium oxide (MgO) and chalk (CaCO₃) on in vitro fermentation of amorphous and crystalline cellulose and meadow hay using inoculum from sheep. *Berl. Munch. Tierarztl. Wochenschr.* **116**:50–54. DOI:10.1080/0307945031000121176
 37. Korkmaz F., Sonmez G. D., Celik P. A., et al. (2018). Toxicity and mutagenicity assessment of a boron mine drainage water. *Water Environ. J.* **32**:250–258. DOI:10.1111/wej.12321
 38. Li G. L., Zhou C. H., Fiore S., et al. (2019). Interactions between microorganisms and clay minerals: New insights and broader applications. *Appl. Clay Sci.* **177**:91–113. DOI:10.1016/j.clay.2019.04.025
 39. Ji L. M., Zhang T. W., Milliken K. L., et al. (2012). Experimental investigation of main controls to methane adsorption in clay-rich rocks. *Appl. Geochem.* **27**:2533–2545. DOI:10.1016/j.apgeochem.2012.08.027
 40. Hein J. R., Normark W. R., McIntyre B. R., et al. (2006). Methanogenic calcite, C-13-depleted bivalve shells, and gas hydrate from a mud volcano offshore southern California. *Geology* **34**:109–112. DOI:10.1130/g22098.1
 41. Rani S., Padmanabhan E. and Prusty B. K. (2019). Review of gas adsorption in shales for enhanced methane recovery and CO₂ storage. *J. Pet. Sci. Eng.* **175**:634–643. DOI:10.1016/j.petrol.2018.12.081
 42. Kato S. and Watanabe K. (2010). Ecological and Evolutionary Interactions in Syntrophic Methanogenic Consortia. *Microbes Environ.* **25**:145–151. DOI:10.1264/jsmme2.ME10122
 43. Eichorst S. A., Varanasi P., Stavila V., et al. (2013). Community dynamics of cellulose-adapted thermophilic bacterial consortia. *Environ. Microbiol.* **15**:2573–2587. DOI:10.1111/1462-2920.12159
 44. Lai Q. L. and Shao Z. Z. (2008). *Pseudomonas xiamenensis* sp. nov., a denitrifying bacterium isolated from activated sludge. *Int. J. Syst. Evol. Microbiol. Microbiology* **58**:1911–1915. DOI:10.1099/ijs.0.65459-0
 45. Ni R. J., Xu C. M., Shi X. Y., et al. (2022). Acetoclastic methanogenesis pathway stability despite the high microbial taxonomic variability in the transition from acidogenesis to methanogenesis during food waste anaerobic digestion. *J. Cleaner Prod.* **372**:133758. DOI:10.1016/j.jclepro.2022.133758
 46. Lim J. W., Ge T. S. and Tong Y. W. (2018). Monitoring of microbial communities in anaerobic digestion sludge for biogas optimisation. *Waste Manage.* **71**:334–341. DOI:10.1016/j.wasman.2017.10.007
 47. Bahram M., Hildebrand F., Forslund S. K., et al. (2018). Structure and function of the global topsoil microbiome. *Nature* **560**:233–+. DOI:10.1038/s41586-018-0386-6
 48. Burgin A. J. and Hamilton S. K. (2007). Have we overemphasized the role of denitrification in aquatic ecosystems. A review of nitrate removal pathways. *Front. Ecol. Environ.* **5**:89–96. DOI:10.1890/1540-9295(2007)5[89:Hwtro]2.0.Co;2
 49. Gao P. K., Tian H. M., Li G. Q., et al. (2015). Microbial diversity and abundance in the Xinjiang Luliang long-term water-flooding petroleum reservoir. *Microbiol. Open* **4**:332–342. DOI:10.1002/mbo3.241
 50. Wong B. T. and Lee D. J. (2011). Sulfide enhances methanogenesis in nitrate-containing methanogenic cultures. *Bioresour. Technol.* **102**:2427–2432. DOI:10.1016/j.biortech.2010.10.121
 51. Kuypers M. M. M., Marchant H. K. and Kartal B. (2018). The microbial nitrogen-cycling network. *Nat. Rev. Microbiol.* **16**:263–276. DOI:10.1038/nrmicro.2018.9

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AUTHOR CONTRIBUTIONS

S Y and LY S. conceptualized the study and provided the funding. RJ N and LY S. performed the experiments. RJ N and WQ H. performed the bioinformatics analysis. RJ N and LY S. wrote the manuscript. S Y., YH T., YF A., CW X and LY S. reviewed and edited the manuscript. All authors contributed to the manuscript and approved the final version.

DECLARATION OF INTERESTS

The authors declare no competing interests.

DATA AND MATERIALS AVAILABILITY

All raw sequences were deposited in the NCBI Sequence Read Archive under

SUPPLEMENTAL INFORMATION

It can be found online at <https://doi.org/10.59717/j.xinn-geo.2024.100125>