

Exploring the role of organotrophic microbes in geochemical cycling of cold seep sediments

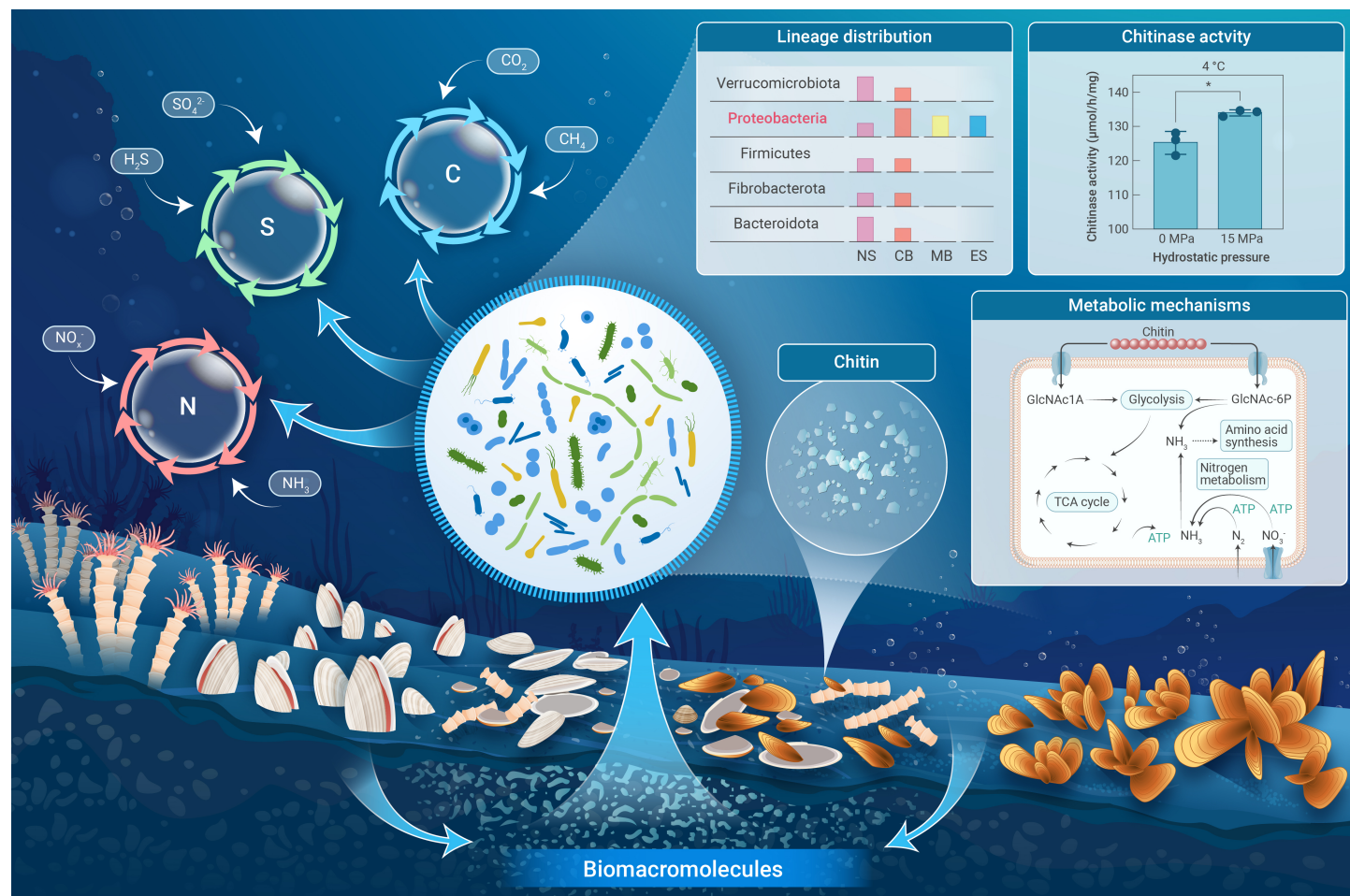
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GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- Cold seep sediment prokaryotic lineages exhibit habitat preference.
- Lineages involved in geochemical cycling of carbon, sulfur, and nitrogen can also utilize biomacromolecules.
- Chitin is characterized as one of the most abundant biomacromolecule and an enduring impact of seep activity.
- Coexistence of chitin and nitrogen metabolic pathways suggests chemoorganotroph-driven N cycling in cold seep.
- The cold seep derived chitinase exhibits increased activity under conditions of 15 MPa and 4°C.

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Owing to the high productivity of cold seep ecosystems, sediments are often enriched in organic compounds, such as biomacromolecules, which may serve as resources for local microbial communities. In this study, we investigated the sedimental microbiome of the Haima cold seep with 225 nonredundant metagenome-assembled genomes (MAGs). Among these, 68 MAGs affiliated with 12 phyla were prevalent to active or extinct cold seep habitats. They primarily harbored the key genes linked to carbon, nitrogen, and sulfur cycles. In addition, most of them potentially engaged in hydrocarbon and biomacromolecule metabolisms, suggesting an essential role of organic compounds in geochemical cycling in cold seeps. Notably, a proteobacterial lineage prevalent in active and extinct habitats, coinciding with the presence of benthic fauna, was found carrying genes for chitin and nitrogen metabolisms, indicating its significance in local chitin recycling. Further enzymatic analysis showed that the chitinase of this lineage remained structurally stable but displayed a significant enhancement in enzymatic activity to $133.95 \pm 0.89 \mu\text{mol L}^{-1} \text{h}^{-1}$ under conditions of 15 MPa and 4°C, suggesting its adaptation to deep-sea environment. Collectively, our work shed light on the contribution of organotrophs in geochemical cycling, proposing chitin as a long-term carbon and nitrogen storage in cold seeps.

INTRODUCTION

Cold seeps are areas where hydrocarbon-rich fluid, such as methane, seeps out from the seafloor. Such fluids nourish one of the richest ecosystems in deep sea, consisting of unique communities of animals and microorganisms that have adapted to extreme conditions. Cold seep ecosystems are commonly comprised of typical chemotrophic communities that were dominantly sustained by hydrocarbon-driven chemical reactions,^{1,2} highlighting the fundamental role of these organic compounds as basic resources in cold seeps. Over the past few decades, numerous studies have demonstrated that microorganisms play a key role in biogeochemical cycling in cold seeps, coupling the decomposition of organic compounds with geochemical cycling. In the anoxic sedimental environment, anaerobic oxidation of methane (AOM) averagely consumes more than 80% of the methane in the fluid,³ AOM usually couples to sulfate reduction (SR), mediated by the well-known syntrophic partners, anaerobic methanotrophic archaea (ANME) and sulfate-reducing bacteria (SRB). In recent years, researchers have found that AOM can also be coupled to complete or partial denitrification.^{4,5} Furthermore, an increasing number of studies have revealed that reduction of metals, such as iron, manganese, arsenic, and chromium, are also able to couple to AOM.^{6–8} These findings emphasize the significant role of methane in driving geochemical cycling in cold seeps. In addition to methane, many other hydrocarbons are also enriched in cold seep sediments.¹ Previous investigations have revealed that anaerobic oxidation of non-methane alkanes (e.g., ethane, butane, propane) and aromatic hydrocarbons (e.g., naphthalene) by microorganisms also can be coupled to sulfate reduction^{1,9–13} suggesting the involvement of non-methane hydrocarbons in integration of geochemical cycling in cold seeps. Therefore, hydrocarbons, particularly methane, have a profound impact on the geochemical cycles of carbon, nitrogen, sulfur, and metallic elements in cold seep sediments, whereby the organotrophic microorganisms play a crucial role in linking these cycles.

Owing to the high productivity and biomass, cold seep sediments are usually enriched for biological organic matters,^{14–16} such as proteins, nucleic acids, and polysaccharides, which are valuable resources for organisms in deep-sea habitats. These biomacromolecules predominantly consist of carbon, nitrogen, and sulfur, indicating their potential contribution in geochemical cycles. Protein degradation has been reported in cold seep, performed by Proteobacteria that interacts with nitrogen cycling.¹⁷ Additionally, a recent study identified a lineage affiliated with Tenericutes that utilizes extracellular DNA as carbon resources, thereby attributing to phosphorus as well as nitrogen and carbon geochemical cycles.¹⁸ While the significance of polysaccharides as crucial resource in terrestrial ecosystems is well-established,^{19–22} their roles in deep-sea ecosystems, especially in cold seeps, are poorly understood to date. Chitin, the second most abundant biomacromolecule on Earth after cellulose, consists of repeating units of N-acetylglucosamine (GlcNAc) monomers. It can serve as source of fuels, carbon, and nitrogen, particularly in the nitrogen-limited deep-sea environment.²³ Many benthic invertebrate species in cold seeps, such as Crustaceans and Polychaeta, produce chitin for their exoskeletons, indicating the potential enrichment of chitin in cold seep sediments. Moreover, several recent studies have identified the ubiquitous presence of chitinase encoding gene from various lineages in cold seep sediments,^{17,24,25} suggesting the participation of chitin in geochemical cycling in cold seeps. However, the mechanisms of chitin degradation and its specific role in geochemical cycling remain unclear in cold seeps to date.

Numerous microbial taxa, such as Planctomycete and Chloroflexi, are abundant in cold seep sediments.^{26–28} Although their roles in carbon, nitrogen, and sulfur cycling are well-established in many marine environments globally,^{5,29–31} their specific contributions to cold seep geochemical processes remain largely unexplored. These functional 'dark matters' strongly suggest the presence of additional taxa and mechanisms involved in geochemical cycling that are yet to be fully explored in cold seep environments. In this study, we provide new insights into the biogeochemical cycles in cold seep sediments through metagenome-assembled genomes (MAGs) collected from five different habitats within the Haima cold seep, South China Sea. Additionally, we have identified distributional prevalence of chitin-utilizing lineages and clarified mechanisms of chitin degradation and nitrogen metabolism in a specific lineage using genetic and enzymatic approaches, highlighting the crucial role of chitin as a carbon and nitrogen source in chemoorganotroph-driven geochemical cycling of cold seep environments.

MATERIALS AND METHODS

Sampling sites and metagenomic datasets for Haima cold seep sediments

A total of 32 sediment samples were collected with pushcores by the Haima remote operated vehicle (ROV) from five diverse habitats of the Haima cold seep (16.9°N, 110.4°E, [Figure S1](#)) that have been described in our previous studies.^{26,32} Briefly, benthic faunal community was not found in the non-seep site (NS, 1393 m). The site inhabiting Vesicomidae clams (CB, 1362 m) is usually considered to exhibit a sulfide-rich environment. Other epibenthic species, such as Echinodermata, Polychaeta, and Crustacea, were rare in site CB. Mussels usually live in methane-rich environments, composing mussel beds on the muddy sediments (MB, 1383 m), or attaching to the carbonate

rocks of methane venting sites individually (MV, 1422 m). In both MB and MV site, a large population of Crustacea, Polychaeta, and Echinodermata species were living together with the mussels. In the extinct seep site (ES, 1353 m), remains of dead benthic communities were found, such as the shells of clam and tubes of tubeworm. Our previous study has examined environmental factors, revealing significant variations in methane, sulfate, ammonium, nitrite, silicate, and phosphate levels across these five habitats.³² Briefly, methane concentrations were found evidently higher in MB and MV sites than the CB site. Sulfate and calcium concentrations exhibited dramatic declines in depth profiles of MB and MV sites, also indicating stronger methane seepage intensity here than in sites CB and NS.

The pushcores were sliced into 3 cm per sample. Total DNA was extracted from 2 g sediments using the TGuide S96 Magnetic Soil /Stool DNA Kit (Tiangen Biotech (Beijing) Co., Ltd.). Metagenomic data were generated with Illumina Novaseq 6000 platform.^{26,32} We obtained 233 Gbp raw data from a total of 32 samples and deposited them into the NCBI Sequence Read Archive under the BioProject number PRJNA846871 and PRJNA847028 (Table S1).

Metagenomic data preprocessing, assembly, and binning

Raw reads were inspected by FastQC (v0.11.9) to evaluate the general sequence quality and PCR artifacts. The raw reads were filtered, and low-quality reads, N-containing reads, and adapters were removed using Trimmomatic (v0.39). The clean data were assembled for each sample using the metaSPAdes pipeline (v3.14.1) with default parameters (k-mer = 21, 33, 55).³³ Open reading frames (ORFs) were predicted for each contig using Prodigal (v2.6.3).³⁴ Contigs from each sample were binned independently to generate metagenome-assembled genomes (MAGs) using the metabat2 tool in MetaWRAP pipeline (v1.3.2) with default parameters. Then high-quality draft genomes were extracted from the above generated MAGs using bin refinement module, and further bin reassembly module was applied to improve the quality of MAGs by enhancing the completeness and reducing the contamination. Only MAGs with completeness $\geq 50\%$ and contamination $\leq 10\%$ were retained for further analysis. MAGs were taxonomically assigned using the classify_wf module of GTDB-Tk (v1.4.0), and phylogenetic analysis was conducted with the GTDB-Tk infer module based on the set of 120 bacterial specific marker genes or 53 archaeal specific marker genes from GTDB.³⁵ The phylogenetic tree was visualized in iTOL v6 (<https://itol.embl.de/>). To calculate the relative abundance, the MAGs were dereplicated using dRep (v3.3.0) with an ANI $\geq 95\%$ threshold,³⁶ and retained 225 species-level representative genomes. Then these MAGs were merged to build an index file and the clean reads from each sample were mapped to the index file using Bowtie2 (v2.4.2).³⁷ Then, an in-house python script was used to calculate the relative abundance of MAGs and normalized to RPKM (reads per kilobase per million).³⁸

Phylogenetic analysis of chitinase gene

Amino acid sequences of *chiA* gene were aligned using the MUSCLE algorithm.³⁹ Maximum likelihood trees were constructed using the Tamura-Nei model with all sites, bootstrapped with 500 replicates by MegaX.⁴⁰ Visualization and modification of the produced tree were conducted via Interactive tree of life (iTOL v6).

Measurement of cellulose and chitin content in sediment

For each sample, 20 g sediment was freeze-dried. Cellulose content was measured based on the anthrone-sulfuric acid colorimetric assay and calculated with the equation (1).⁴¹ Briefly, a freeze-dried sample was weighted (W, g) and then hydrolyzed using sulfuric acid, and diluted to 1.25 mL (V, mL). The mixture was further reacted with anthrone reagent and then measured using a spectrophotometer at absorbance of 620 nm. Cellulose content (mg g⁻¹) of each sample was calculated using the glucose concentration calculated with calibration curve (Gc, mg mL⁻¹) and the corresponding sample weight (W, g).

$$\text{Cellulose content (mg g}^{-1}\text{)} = \frac{Gc \text{ (mg mL}^{-1}\text{)} \times V \text{ (mL)}}{W \text{ (g)}} \quad (1)$$

Chitin is insoluble in water, dilute acids and alkalis, organic solvents. However, treatment of chitin with concentrated alkali at high temperatures

(160°C) can cause deacetylation and form chitosan that is soluble in dilute acids, making it possible for quantification of chitin. In this study, chitin content was calculated with the equation (2). Briefly, freeze-dried sample was weighted (W2, g), and then heated with saturated NaOH solution in 160 °C for 20 min. After that, insoluble precipitation was rinsed with dilute water and dehydrated with 95% and 100% ethanol, and then weighted (W1, mg). M_{GlcNAc} , molecular weight of N-acetylglucosamine; M_{GlcN} , molecular weight of Glucosamine.

$$\text{Chitin content (mg g}^{-1}\text{)} = \frac{W1 \text{ (mg)} \times M_{\text{GlcN}}}{W2 \text{ (g)} \times M_{\text{GlcNAc}}} \quad (2)$$

Protein production and purification.

The GH18 chitinase coding gene (residues 21–417) of MAG IA_PS_003.bin.2, a candidate supposed to utilize chitin produced by cold seep fauna, was synthesized and subcloned into pET22b (Novagen, Madison, WI, USA) by seamless cloning (Vazyme, Nanjing, China). The recombinant plasmid pET22b-_{IA32}Chi641 was transformed into *Escherichia coli* strain BL21 (DE3) (Novagen) for protein expression. Cells were grown in Luria-Bertani medium at 37°C with shaking (180 rpm) until they reached an optical density of 0.6 at 600 nm. Cells were treated with 0.5 mM isopropyl β -D-1-thiogalactopyranoside to induce chitinase expression, and the temperature was adjusted to 16°C for 16 h. Cells were subsequently harvested by centrifugation at 6,000 g, 4°C for 10 min. The harvested cell pellets were resuspended in binding buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 5 mM imidazole) and lysed by sonication using 1-s pulse/1-s pause cycles. Cell debris was pelleted by centrifugation at 10,000 $\times$ g, 4°C for 30 min and the supernatant was loaded onto Ni-nitrilotriacetic acid (NTA) resin (Qiagen, Hilden, Germany). After extensive washing with wash buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 20 mM imidazole), the (his)₆-tagged protein was eluted using elution buffer (20 mM Tris-HCl pH 8.0, 200 mM imidazole). The eluted protein fraction was loaded onto a DEAE FF column (GE Healthcare, Chicago, IL, USA) and eluted using a linear gradient of 0 M to 1.0 M NaCl in 20 mM Tris-HCl pH 8.0 over 10 column volumes. Fractions were monitored by absorbance at 280 nm and the protein peak fractions were pooled and concentrated using an Amicon-Ultra-15 device (10,000-molecular-weight-cutoff, Merck-Millipore Co., Darmstadt, Germany). After concentrating, aliquots were flash frozen in liquid nitrogen and stored at -80°C. Protein purity was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and protein concentration was determined by absorbance at 280 nm using the molar extinction coefficient calculated from the amino acid sequence composition (<https://web.expasy.org/protparam/>).

Enzyme assay.

Chitinase activity was monitored at 40°C in 20 mM phosphate buffer, pH 7.0, containing *p*-nitrophenyl N, N'-diacetyl- β -D-chitobiose, *p*Np-diNAG (Sigma-Aldrich, St. Louis, MO, USA) as substrate in a final volume of 0.1 mL. The reactions were initiated by the addition of the purified enzyme and interrupted after convenient time intervals by heating at 90°C for 10 min. To mimic the environment where chitin operates in the deep sea, the ambient temperature and pressure were precisely regulated. A sealed pouch enclosing 1000 μ L of the enzyme reaction mixture was inserted into a pressure chamber, with the internal pressure of the chamber stabilized at 15 MPa. Subsequent to an 18-hour incubation in a 4°C water bath, the reaction was quenched to assess the product content. The amount of product, *p*-nitrophenol, is determined by reading the absorbance at 405 nm and using a molar extinction coefficient of 1725 M⁻¹cm⁻¹, using an EnSight™ Multimode microplate reader (PerkinElmer Inc., Spokane, Washington, USA). The kinetic parameters K_m and k_{cat} were determined by fitting the initial rates to Michaelis-Menten equation $v_0 = k_{cat}[E]_0[S]_0/(K_m + [S]_0)$, where v_0 is the initial velocity, $[E]_0$ is the initial enzyme concentration, and $[S]_0$ is the initial substrate concentration.

Crystallization, X-ray diffraction, and structure determination.

Crystallization trials were performed via the sitting drop method by mixing 0.4 μ L purified proteins (10 mg mL⁻¹) with 0.4 μ L reservoir solution and allowing the drop to equilibrate at 20°C in 96-well protein crystallization plates (Viola, Osaka, Japan). The reservoir solution for the crystallization of

^{IA32}Chi641 was 0.15 M potassium bromide, and 30% (w/v) polyethylene glycol monomethyl ether 2,000. Crystals were flash frozen in a cryoprotectant comprising mother liquor supplemented with 22% ethylene glycol and X-ray data were collected on a Rigaku Oxford XtaLAB Pro diffractometer equipped with Cu K α radiation ($\lambda = 1.54184 \text{ \AA}$) at 100 K. Diffraction data were indexed, integrated, and scaled using the Rigaku CrysAlisPro software system. The ^{IA32}Chi641 structure was determined by molecular replacement. Iterative refinement cycles were performed using PHENIX.REFINE and COOT. Data collection and refinement statistics are shown in Table S4. All structures were depicted using PyMOL (v.2.3; Schrödinger, LLC., New York, NY, USA). The atomic coordinates and structure factors for ^{IA32}Chi641 were deposited in the Protein Data Bank under accession number 8XBD.

RESULTS

Phylogeny and spatial distribution of the MAGs in different habitats

We assembled metagenomic data sequenced from 32 sediment samples obtained from five habitats of Haima cold seep (Figure S1), inclusive of non-seep (NS), clam bed (CB), mussel bed (MB), mussel bed overlying a methane vent (MV), and extinct seep (ES). After binning, 467 metagenome-assembled draft genomes (MAGs) were recovered (>50% completeness and <10% contamination). Classification of MAGs via Genome Taxonomy Database (GTDB)⁴² showed 225 nonredundant prokaryotic species affiliated to 21 phyla, including 3 archaeal and 18 bacterial phyla (Figure 1A & Table S2). The genome size of the 225 representative MAGs ranged from 0.5 to 5.6 Mbp.

We examined the relative abundance of all 225 MAGs in the different habitats within the Haima cold seep. Non-metric multidimensional scaling (NMDS) analysis based on MAG abundances demonstrated obvious clusters of samples from different habitats (stress = 0.05, Figure 1B). In addition, the separation of clusters suggested potential niche preference that associated with taxonomy. The MAGs affiliated to Campylobacterota, Chloroflexota, Desulfobacterota, Gemmatimonadota, Halobacteriota, Methyloirabilota, Planctomycetota, Proteobacteria, and Thermoplasmatota were prevalent to the mussel (MB and MV) and extinct seep (ES) habitats (Figure S2). While the ones affiliated to Acidobacteriota, Actinobacteriota, Bacteroidota, Cyanobacteria, Elusimicrobiota, Fibrobacterota, Firmicutes, Patescibacteria, Spirochaetota, Thermoproteota, and Verrucomicrobiota were prevalent in non-seep (NS) or clam (CB) habitats (Figure S3).

Metabolic potential of carbon fixation, and nitrogen, sulfur geochemical cycling

Carbon fixation. Lineages prevalent to MB, MV, and ES were predominantly carrying the key genes of carbon fixation pathways (Figures 2A, S2 & S4). Members in Desulfobacterota (12/12) and Chloroflexota (4/4) harbored at least one key gene for Wood-Ljungdahl (WL) pathway (Figures 2A & S4). Members in Methyloirabilota (2/2), Thermoplasmatota (3/3), and Proteobacteria (8/20) harbored the key genes in Calvin-Benson-Bassham (CBB) cycle (Figures 2A & S4). But only 2 MAGs affiliated with Halobacteriota and 1 with Campylobacterota were carrying the genes for reductive tricarboxylic acid (rTCA) cycle (Figures 2A & S4). On the other hand, the MAGs prevalent in NS and CB were seldom carrying the genes for carbon fixation. Only a group of Firmicutes (14/68) were found to carry the key genes for WL pathway (Figures 2A, S3 & S5).

Nitrogen cycling. Members in Campylobacterota (6/6), Gemmatimonadota (3/3), Acidobacteriota (1/1), and Desulfobacterota (6/12) harbored the key genes for dissimilatory nitrate reduction to ammonia (DNRA) pathway (Figures 2B & S4). While lineages affiliated to Methyloirabilota (1/2), Planctomycetota (2/3), and Proteobacteria (6/20) carried the key gene for nitrate reduction via denitrification pathway. Some lineages from Desulfobacterota (3/12), Halobacteriota (3/6), and Thermoplasmatota (3/3) carried a set of nitrogenase molybdenum-iron protein encoding genes, suggesting their potential in nitrogen fixation (Figures 2B & S4). On the other hand, inhabitants in NS and CB were less partitioning in nitrogen geochemical cycling. Only a few species from Bacteroidota (3/53), Firmicutes (9/68), and Spirochaetota (6/22) harbored the key genes for denitrification and nitrogen fixation (Figures 2B, S3 & S5).

Sulfur cycling. Similarly, the lineages involved in sulfur cycling were also

prevalent to MB, MV, and ES (Figures 2C, S2, and S5). A majority members of Desulfobacterota (12/12), Planctomycetota (3/3), and Proteobacteria (7/20) were carrying the genes in dissimilatory sulfate reduction (DSR) pathway (Figures 2C and S4), which was essential for the sulfate reduction or sulfur oxidation cascades.^{43,44} Lineages affiliated to Campylobacterota (6/6), Chloroflexota (3/4), Planctomycetota (2/3), and Proteobacteria (7/20) harbored the genes for sulfide oxidation (*sqr* and *fccB*). Interestingly, several Proteobacteria (6/20) harbored a set of genes for oxidative reactions of sulfur compounds, such as thiosulfate and sulfite (Figures 2C and S3), indicative of their essential role in sulfate production. While only a few MAGs were found to carry the key genes for DSR and sulfur oxidation cascades in NS and CB (Figures 2C, S3 & S5).

Functional profiles in utilization of organic compounds

Hydrocarbon degradation. Most of the lineages (48/63) inhabited MB, MV, and ES were potentially able to utilize hydrocarbons (Figures 3A, S2 & S4). It is unsurprising that the well-known anaerobic methanotrophs (ANMEs), affiliated to Halobacteriota (previously classified as Euryarchaeota), harbored the genes of reverse methanogenesis pathway. Also, species from Thermoplasmatota were found to carry the methyl-coenzyme M reductase (MCR) encoding genes, suggesting their potential *rt*-chain alkane degradation. Members from Proteobacteria (11/20) possessed the genes (*ass* and *alkM*) responsible for long-chain alkane degradation. Additionally, members from Acidobacteriota (1/1), Desulfobacterota (9/12), Gemmatimonadota (2/2), Planctomycetota (3/3), Proteobacteria (8/20), and Thermoplasmatota (2/3) possessed the *paaH* and/or *atoB* gene, suggesting that they were potentially able to utilize aromatic hydrocarbons (Figures 3A & S4). While for the NS and CB lineages, Firmicutes (22/68) and Bacteroidota (6/53) harbored the key genes for aromatic hydrocarbon degradation. Verrucomicrobiota (8/12) were likely able to utilize long-chain alkanes. None of the MAGs prevalent in NS or CB had the potential to utilize short-chain alkane or methane (Figures 3A, S3 & S5).

Biomacromolecule utilization. We accessed the metabolic potential of the MAGs in biomacromolecule degradation, including DNA, protein, and saccharide. Firstly, many lineages were carrying the nucleases in cold seep sediments. For example, the MB, MV, and ES prevalent Campylobacterota (6/6) ubiquitously harbored the gene encoding for 3'-5' exonuclease, while Chloroflexota (3/4), Desulfobacterota (9/12), Planctomycetota (3/3), and Proteobacteria (8/20) usually carried the *nuc* gene encoding for micrococcal nuclease (Figures 3B, S2 & S4). The NS and CB prevalent Bacteroidota (43/53) and Spirochaetota (17/22) usually carried the encoding genes for extracellular DNA degradation protein (*Eddb*), CRISPR-associated exonuclease (*cas4*), and 5'-3' exonuclease (*exoA*, Figures 3B, S3 & S5). Additionally, lineages affiliated to Chloroflexota (2/4), Desulfobacterota (8/12), Firmicutes (50/68), and Spirochaetota (18/22) were found to possess the potential to transport nucleoside, suggesting that microbes living in different habitats in cold seep might benefit from DNA degradation and metabolisms (Figures 3C & S4-S5). Secondly, most MAGs harbored at least one of the amino acid degradation-associated genes (Figures 3B & S4-S5). Considering the potential of peptide transportation, Chloroflexota (4/4) and Desulfobacterota (10/12) possessed the key genes for oligopeptide and/or dipeptide ATP-binding cassette (ABC) transporter. Planctomycetota (2/3) and Proteobacteria (8/20) prevalent to MB, MV, and ES (Figure S2), as well as Firmicutes (51/68) and Spirochaetota (22/22) prevalent to NS and CB (Figure S3), carried the key genes for oligopeptide ABC transporter, but they did not possess the dipeptide ABC transporter (Figures 3C & S4-S5). Therefore, they might get involved in exocellular protein degradation and acquire resources from the related processes. Thirdly, among the MAGs from MB, MV, and ES, Proteobacteria carried a set of genes for saccharide metabolisms, inclusive of polysaccharide, starch, and chitin. Taken together with the multiple sugar ABC transporter, rather than the ones for sugar, Proteobacteria might mediate the degradation of polysaccharide into smaller fragments and further metabolize intracellularly (Figures 3B-C, S2 & S4). On the other hand, members of Bacteroidota, Firmicutes, and Spirochaetota might be responsible for degradation for polysaccharide in NS and CB (Figures 3B-C, S2 & S5).

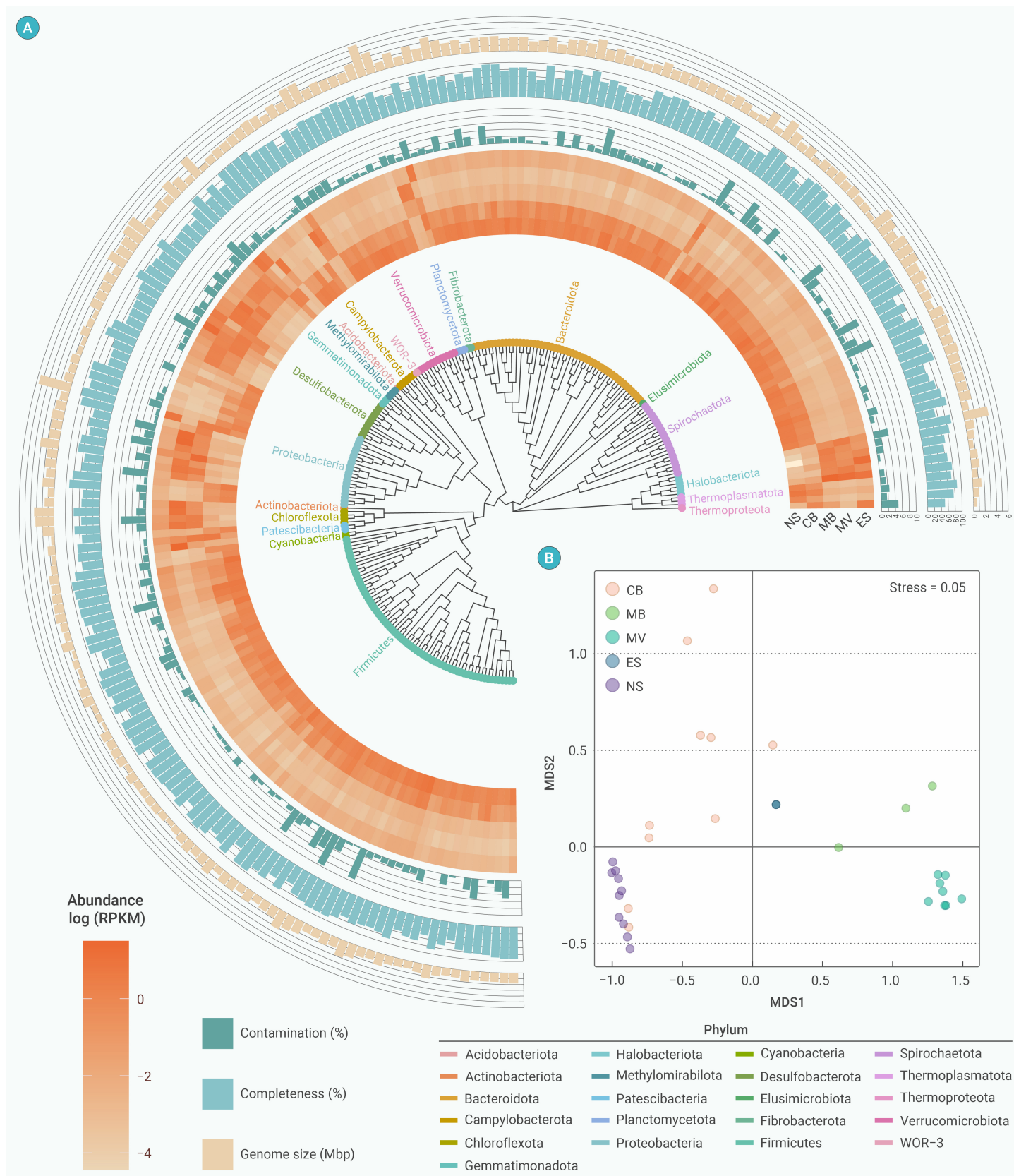


Figure 1. Phylogeny and niche prevalence of MAGs derived from sediments of Haima cold seep (A) A total of 225 nonredundant prokaryotic MAGs were retrieved and phylogenetically affiliated to 18 bacterial and 3 archaeal phyla, respectively. Phylogenetic affiliation of each MAG was indicated by the tip color. The heatmap clearly showed the distribution of the nonredundant MAGs. (B) Non-metric multidimensional scaling (NMDS) analysis based on MAG abundances suggested niche prevalence of MAGs in cold seep sediments. Stress = 0.05.

Participation of chitin degradation in carbon and nitrogen cycling in cold seep sediment

To identify the potential contribution of chitin to geochemical cycling in the

cold seep sediments, we first evaluated the content of chitin in sediment. It was averagely 7.41 mg g^{-1} in dried sediment but did not show significant differences between the habitats (One-way ANOVA, $P = 0.9303$). In addition,

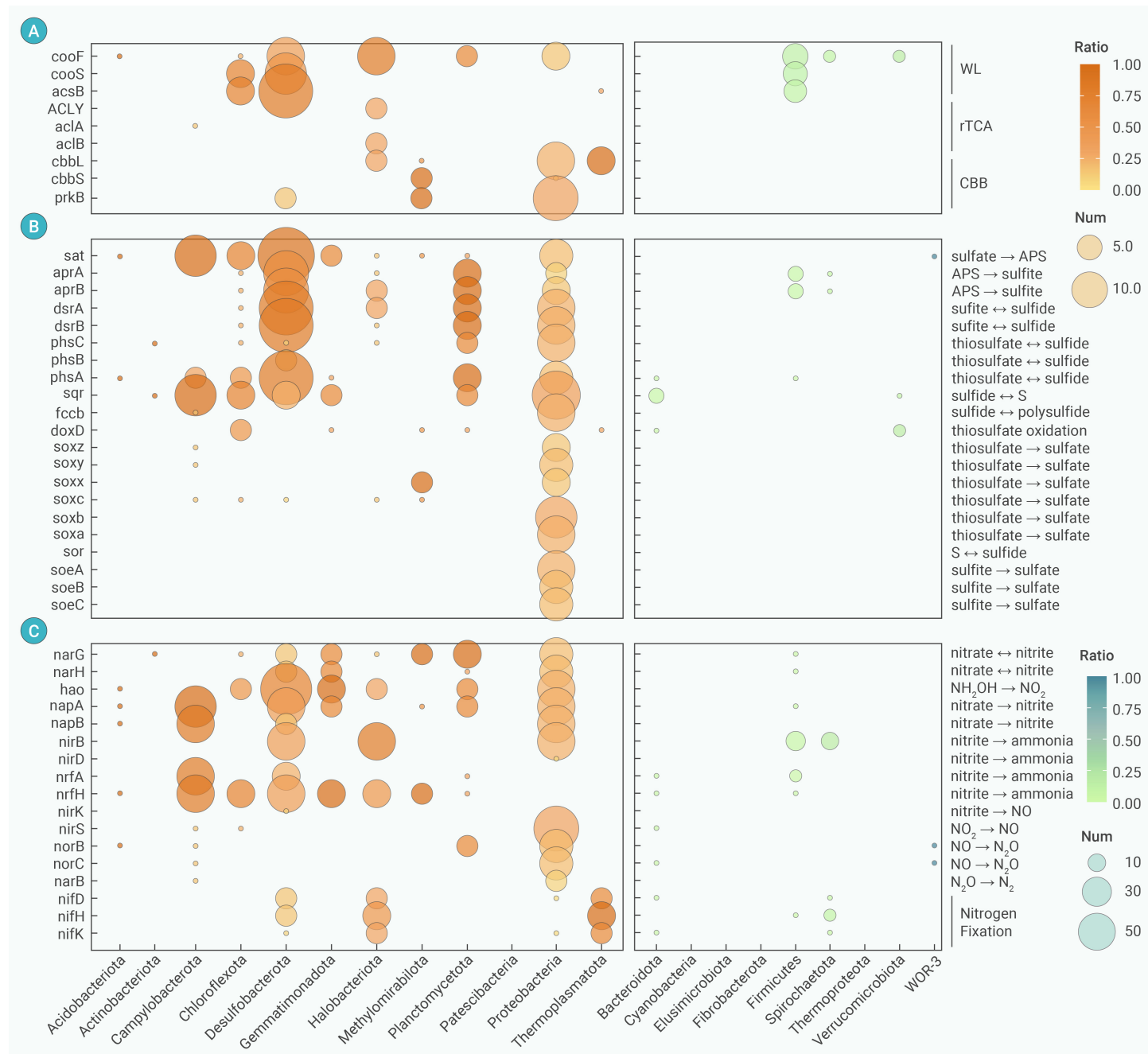


Figure 2. Metabolic potentials of MAGs in C, N, S geochemical cycling Key genes involved in carbon fixation (A), sulfur cycling (B), and nitrogen cycling (C) were prevalent in the phyla inhabited in MB and MV (left panel). The ratio and number of each gene in corresponding phylum are indicated by color and size of the circle, respectively.

chitin contents were close to the cellulose contents (Figure S6). The correlation of *chiA* gene abundance with ammonium (Spearman, $P = 0.04$, $|R| = 0.39$) and nitrite (Spearman, $P = 0.09$, $|R| = 0.33$), as well as the correlation of chitin content with nitrite (Spearman, $P = 0.01$, $|R| = 0.51$) and nitrate (Spearman, $P = 0.04$, $|R| = 0.37$) further suggested the potential contribution of chitin recycling to nitrogen cycling in cold seeps (Figure 4A). Furthermore, we investigated the MAGs and identified 14 lineages carrying the *chiA* gene, most of which (13/14) were affiliated to Verrucomicrobiota, Bacteroidia, Fibrobacteriota, and Firmicutes, and prevalent to NS and CB. However, the MAG IA_PS_003.bin.2, phylogenetically affiliated with *Klebsiella*, was prevalent to the CB, MB, and ES (Figure 4B) and highly parallel to the occurrence of benthic communities, supposed to utilize the chitin produced by the cold seep fauna. Phylogenetic analysis of amino acid sequences also showed that the ChiA of IA_PS_003.bin.2 clustered to the *Klebsiella* ChiA sequences (Figure 4C).

In addition to chitinase, IA_PS_003.bin.2 encodes a series of enzymes for

hydrolytic degradation of chitin to fructose-6-phosphate (Fru-6P). Most of genes in this pathway were clustered in series, including *chiQ* and *chiP* for chitinase, *nagE* for N-acetylglucosamine-specific IIC component, *nagA* for N-acetylglucosamine-6-phosphate deacetylase, and *nagB* for glucosamine-6-phosphate deaminase, suggesting an operon structure. However, the *chiA* gene was not included in this structure (Figure 5A, Table S3). Interestingly, a pathway for oxidative chitin degradation was also identified in this genome, including the gene encoding lytic polysaccharide monooxygenases (LPMO) and the *ong* gene cluster (Figure 5B, Table S3), suggesting that this microbe might utilize chitin via two different pathways. Additionally, chitin might be assimilated for biosynthesis in this microbe (Figure 5C). Besides, IA_PS_003.bin.2 harbored the genes (*narG/H/I*, *nirB/D*) for DNRA cascades, as well as the nitrogenase molybdenum-iron protein encoding genes (*nifD/K/H*), suggesting that it has the potential to mediate nitrate reduction and nitrogen fixation. Furthermore, the genes encoding nitrate/nitrite ATP-binding cassette (ABC) transporter were also present, suggesting its poten-

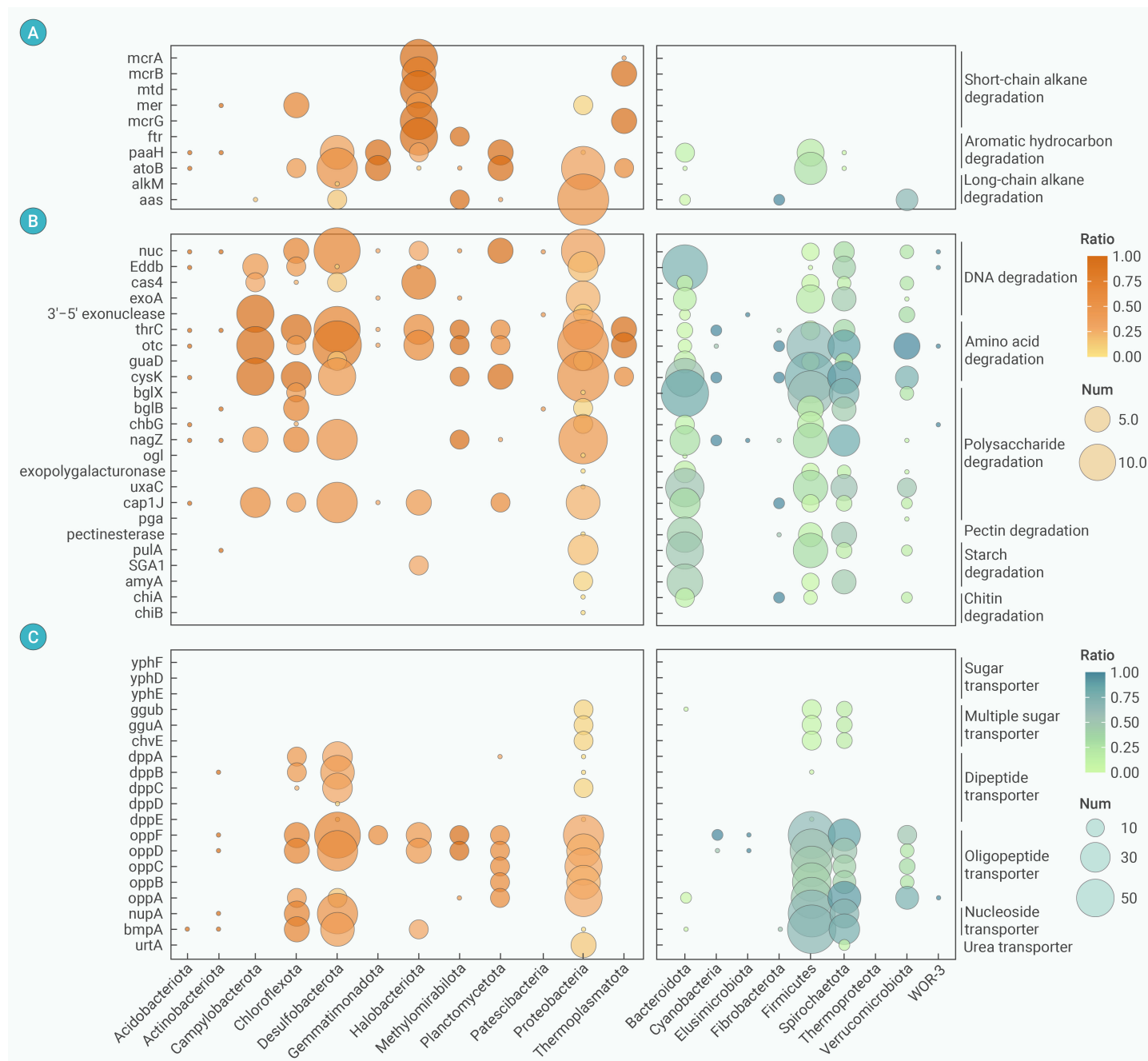


Figure 3. Functional profiles of MAGs in organic compound utilization Key genes for degradation of hydrocarbons (A), biomacromolecules (B), and corresponding ABC transporters (C) were present in both groups of MAGs. The ratio and number of each gene in corresponding phylum are indicated by color and size of the circle, respectively.

tial to utilize nitrate and/or nitrite (Figure 5C).

Active GH18 family chitinase from IA_PS_003.bin.2 in cold seep environment

The mature GH18 family glycosidase $_{IA32}Chi641$ (Figure 6A), encoded by the *chiA* gene of IA_PS_003.bin.2, was expressed in *E. coli* and the recombinant protein was purified by the combination of affinity and ion exchange chromatography (Figure S7). $_{IA32}Chi641$ can effectively degrade chitin oligosaccharides into lower polymerization oligosaccharides (Figure S8). The optimum temperature for the enzyme is 40 °C, and the activity of chitinase can still be detected below 10 °C; the optimum pH of $_{IA32}Chi641$ is 7.0, and the activity decreases significantly when the pH is less than 5 or more than 9 (Figures 6B–C). Under standard conditions (40 °C and pH=7.0), the catalytic efficiency constant (k_{cat}/K_m) of the enzyme for pNP-Chi2 was $50.91 \pm 5.05 \text{ mM}^{-1}\text{s}^{-1}$, which is comparable to the activity of chitinase derived from

Pyrococcus furiosus.⁴⁵ In addition, it is interesting to note that the specific activity of $_{IA32}Chi641$ under 4 °C increased from $125.20 \pm 3.36 \mu\text{mol L}^{-1} \text{h}^{-1}$ to $133.95 \pm 0.89 \mu\text{mol L}^{-1} \text{h}^{-1}$ while hydrostatic pressure elevated from 0 MPa to 15 MPa (Figure 6D). These results indicate that $_{IA32}Chi641$ is a chitinase that can be active in deep-sea cold seep environments.

The crystal structure of $_{IA32}Chi641$ was solved at 2.4 Å resolution with space group / 121 (Table S4). The structure consists of two molecules in the asymmetric unit, while PISA analysis indicated that dimer is unstable in the solution state. $_{IA32}Chi641$ contains a canonical (β/α)₈ TIM-barrel structure within which the active residues (Tyr28, Asp149, Asp151, and Asp153) deduced from the structural alignment are observed. These residues perform the hydrolysis of chitin *via* a substrate-assisted retention mechanism.⁴⁶ In addition to the catalytic domain, an $\alpha\beta$ insertion domain is present at the carboxyl terminus, indicating that $_{IA32}Chi641$ is a member of subgroup A chitinases (Figure 6E). $_{IA32}Chi641$ was further superimposed with the homolo-

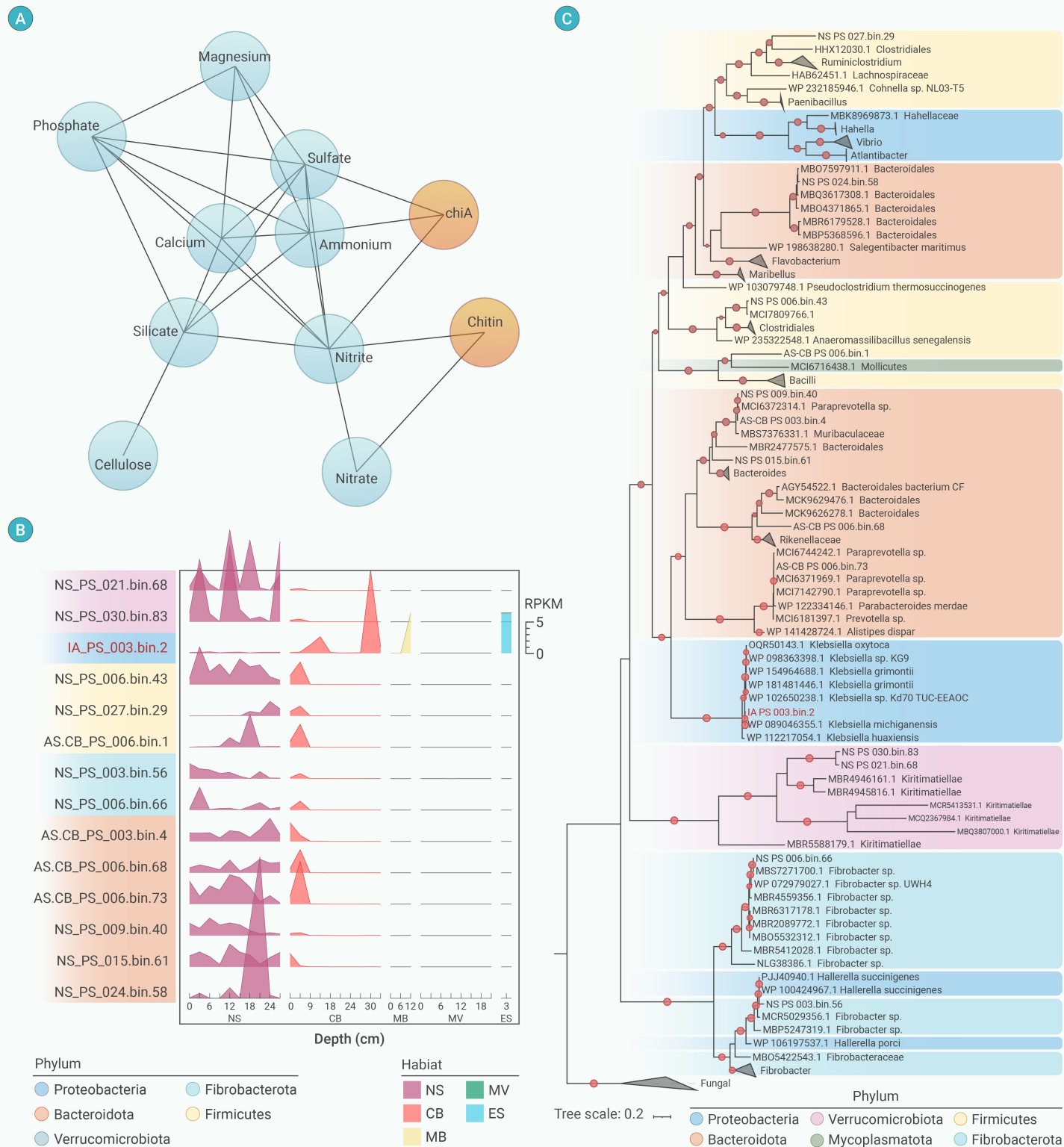


Figure 4. Potential contribution of chitin to carbon and nitrogen cycling in Haima cold seep (A) Content of chitin and relative abundance of *chiA* gene were correlated with nitrate, nitrite, and ammonium (Spearman, $|R| > 0.3$), suggesting the potential of chitin as a nitrogen supplier in Haima cold seep. (B) Distribution of the *chiA* gene carrying MAGs. The occurrence of IA_PS_003.bin.2 is highly parallel to that of faunal aggregates. Y axis illustrates the RPKM of each MAG, and x axis stands for the samples from different habitats (NS, CB, MB, MV, and ES) and depths (cm). (C) Maximum-likelihood phylogenetic trees of detected *chiA* sequences based on alignments of amino-acid sequences of *chiA* genes. Bootstrap values of >70% are indicated with red circles. NS, non-seep habitat; CB, clam bed habitat; MB, mussel bed habitat; MV, mussel habitat with methane vent; ES, extinct seep habitat.

gous complexed structures with chitobiose and allosamidin to identify the chitin-binding residues at the positive and negative subsites that facilitate the catalysis (Figure 6F). Most of the residues in the substrate pocket form π - π stacking interactions with the substrate, suggesting that such interactions are essential for chitin substrate recognition and catalysis.

DISCUSSION

In the past decades, geochemical cycling has been one of the hottest topics in cold seep research. While some key cascades are well clarified, there remain unknown links in the complete cycles, suggesting the existence of undiscovered taxa and mechanisms involved. Our study unveiled involve-

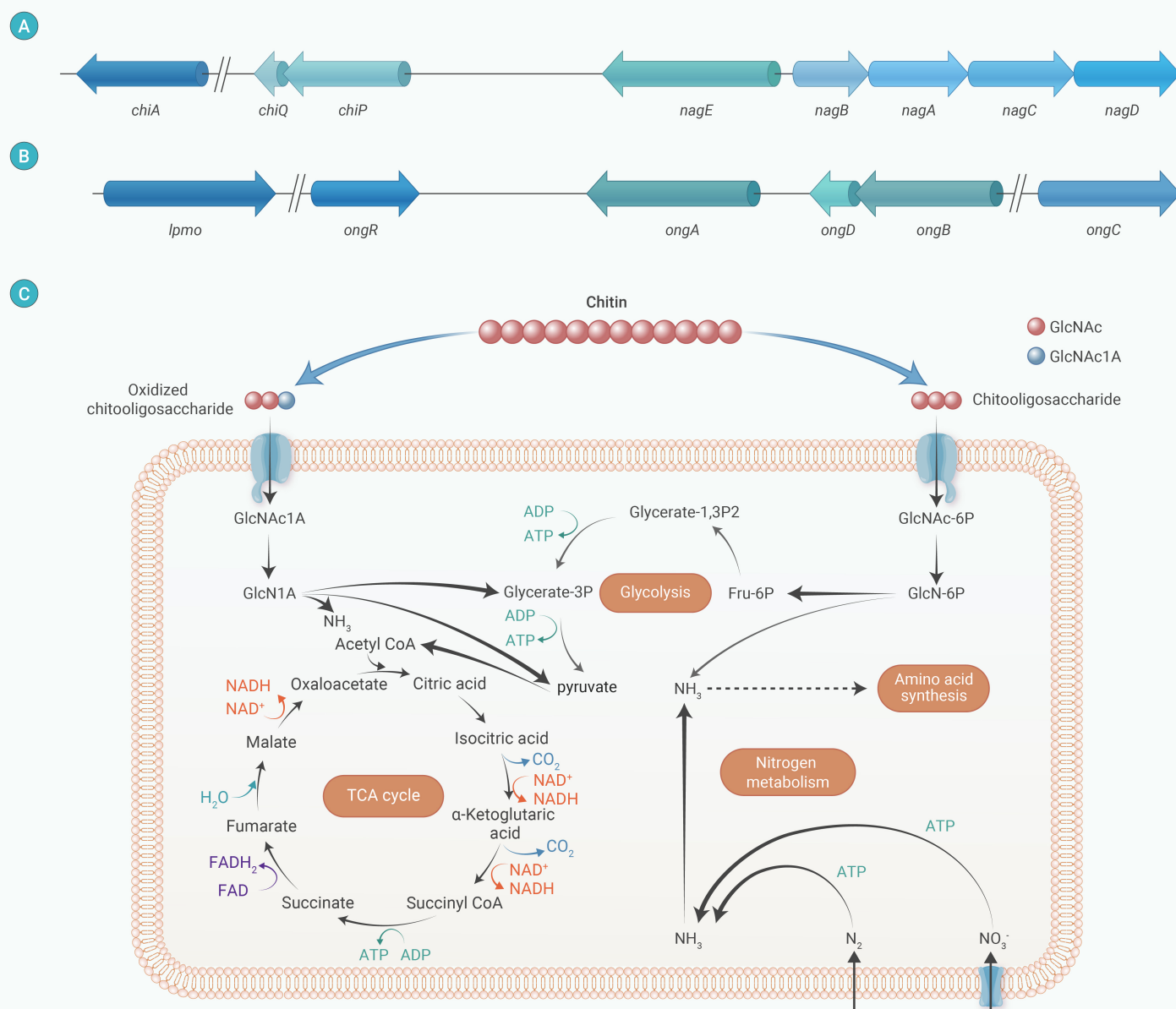


Figure 5. Genomic context of chitin degrading genes and schematic overview of pathways for chitin degradation (A) Concatenated genes for the classical hydrolytic chitin utilization pathway in IA_PS_003.bin.2. (B) Genes in the oxidative chitin degradation pathway were partially concatenated. (C) IA_PS_003.bin.2 possesses both pathways for chitin degradation that can produce ammonium. Furthermore, it also harbored genes for nitrogen fixation, DNRA, and ABC transporter for nitrate.

ment of microbial lineages potentially linking carbon, sulfur, and nitrogen geochemical cycling in cold seep sedimental environments. For instance, Planctomycetota and Chloroflexota, the well-established members involved in sulfur and nitrogen cycles in other environments,^{31,47,48} were identified as potential contributors to coupling sulfur and nitrogen cycling in cold seep sediments. Moreover, functional redundancy was ubiquitous within the sedimental microbiome of the Haima cold seep. Planctomycetota and Chloroflexota, complementing the roles of Proteobacteria and Desulfobacterota as previously observed^{2,49}, exhibited high functional redundancy in sulfate reduction within sediments of the Haima cold seep. Functional redundancy plays a pivotal role in sustaining ecological resilience and stability within ecosystems⁵⁰, ensuring that essential biogeochemical processes continue even in the face of environmental disturbances. Meanwhile, many lineages affiliated to Planctomycetota, Chloroflexota, Proteobacteria also showed high functional diversity that they might conduct carbon, sulfur, and nitrogen within one cell, facilitating the occupation of multiple niches in such extreme environments. These lineages are also potential contributors to ecosystem stability and resilience by performing various ecological tasks.⁵¹ Therefore, our study provides a comprehensive overview of functional structure of the

microbiome in cold seep sediments, suggesting the significance of microorganisms in ecological succession and resilience in deep-sea cold seeps.

Carbon metabolism is usually coupled with other geochemical cycles in cold seep environments. The well-known cooperation between SRB and ANME is a typical example of coupling of carbon and sulfur cycling.⁵² Methanoperedenaceae (formerly referred as ANME-2d) can assimilate methane for further biosynthesis processes by coupling it to nitrogen or metal element cycling, solely or with their partners^{4,5,53-55}. Additionally, SRBs are also found to assimilate inorganic and organic carbon sources simultaneously in this study and couple carbon, sulfur, and nitrogen cycling. This finding is consistent with the previous studies that they can employ multiple pathways for assimilation of inorganic carbon and a wide range of organic compounds, inclusive of carbohydrates, organic acids (i.e. formate, lactate, acetate, propionate, and butyrate), alcohols (methanol and ethanol), alkane, and aromatic hydrocarbons (i.e. benzene, toluene, ethylbenzene, and xylene) by coupling with sulfate reduction.⁵⁶⁻⁵⁸ An increasing number of prokaryotic lineages have been proposed to utilize hydrocarbons in couple with the oxidation or reduction of nitrogen or/and sulfur compounds,^{1,59} making hydrocarbons a keystone for geochemical cycling in cold seeps. In addition to

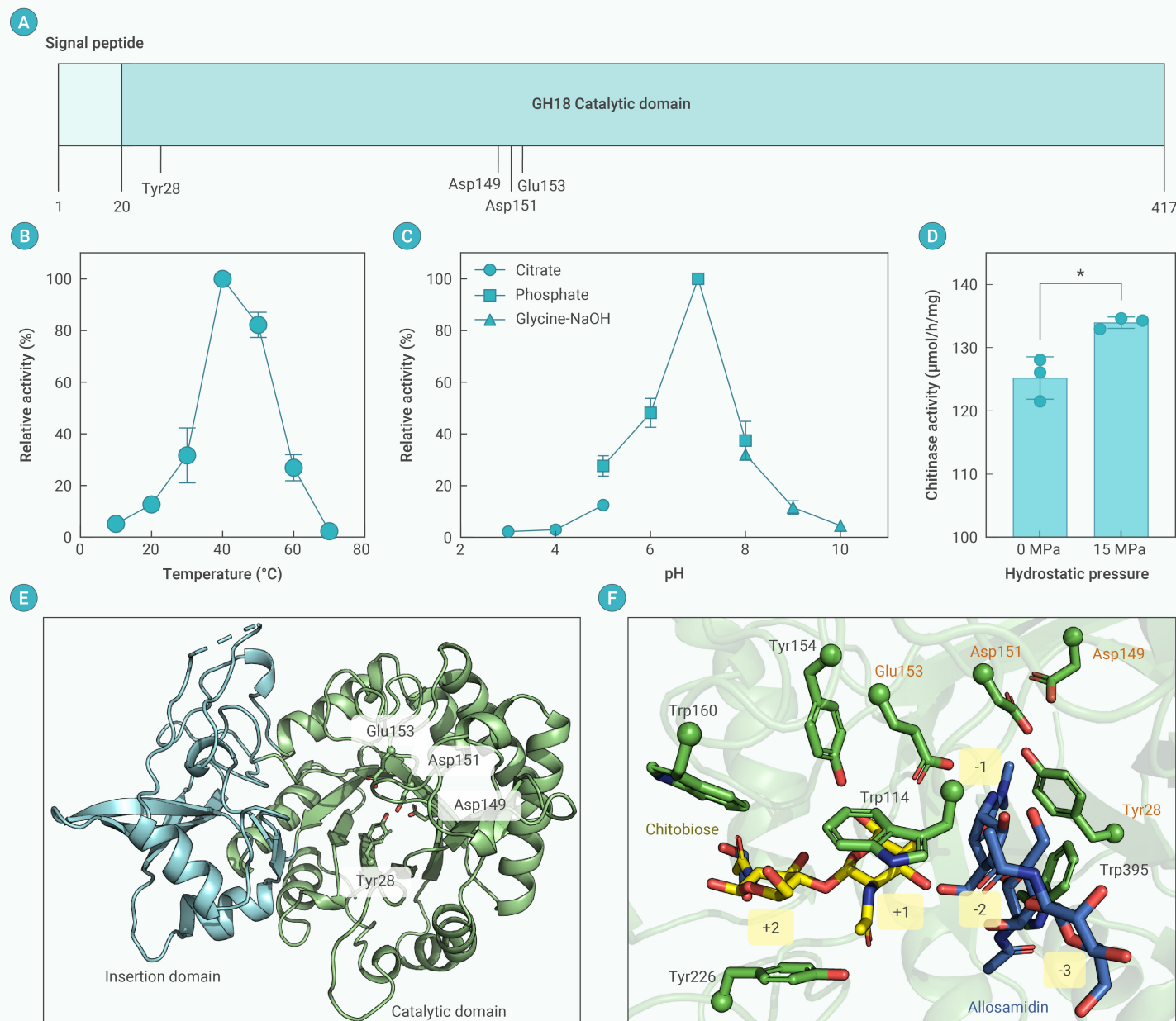


Figure 6. Catalytic and structural properties of GH18 chitinase *IA32Chi641* (A) Organization of the functional units of *IA32Chi641*. The enzyme contains a signal peptide (residues 1-20) and a GH18 catalytic domain. Temperature (B) and pH (C) dependency of chitinase activity. (D) Effect of hydrostatic pressure on enzyme activity. All experiments were carried out in a 4°C water bath for 18 h. Hydrostatic pressure was applied using a pressure tank. *, $P < 0.05$. (E) Ribbon presentation of the overall structure of *IA32Chi641*. The $\alpha\beta$ insertion domain is colored in wathet blue. The catalytic residues Tyr28, Asp149, Asp151, and Asp153 are indicated as green sticks. (F) Superimposed structure of *IA32Chi641* and chitinase from *Serratia proteamaculans* 568 in complexed with chitobiose at the positive subsites (amino acid identity 77.6%, PDB code: 6f8n) and inhibitor allosamidin at negative sites (PDB code: 6hm1). The presumed substrate binding residues of *IA32Chi641* are indicated as green sticks. Data are represented as mean $\pm$ SD.

hydrocarbons, biomacromolecules containing carbon, nitrogen, sulfate, phosphate, and metallic elements are also essential resources for deep-sea microbial communities and may contribute to geochemical cycling.⁶⁰ Recent studies have demonstrated the importance of proteins and nucleic acids as fuels and resources for surface or subsurface sedimental microbiomes and their contribution to geochemical cycling of nitrogen and phosphate.^{17,18,59} Our study further revealed that taxa driving nitrogen and sulfur geochemical cycling usually possessed the potential for utilizing biomacromolecules, suggesting the significance of organotrophs and biomacromolecules in the deep-sea geochemical cycles. While our data have identified numerous MAGs containing genes associated with classical carbon fixation pathways in the Haima cold seep, it is essential to note that the reverse Wood-Ljungdahl (WL) pathway, which shares many enzymes with the WL pathway,⁹ is also involved in the oxidation of long-chain hydrocarbon degradation. This suggests that MAGs harboring genes related to the WL pathway are not warranted to be autotrophs. Taken together, the significance of organotrophs,

especially those utilizing biomacromolecules, in geochemical cycling in cold seep sediments may have been previously underestimated.

Chitin stands out as the most abundant renewable polymer in marine environments. It emerges as valuable carbon (approximately 47% by weight) and nitrogen (approximately 6.9% by weight) source for the deep-sea ecosystems, being produced by marine organisms ubiquitously.⁶¹ Typically, crustaceans, encompassing the planktonic and benthic members, are major contributors to marine chitin production. Given the unique community of cold seep ecosystem, polychaetas, such as the tubeworms, emerge as another important chitin contributor in cold seep environments,⁶² indicating differing chitin sources between non-seep and seepage-associated environments.^{53,64} It is worth noting that chitin content might be underestimated, especially in the active and extinct habitats, due to the exclusion of large fragments like tubeworm relics and bivalve shells by measuring sedimental samples. These relics can be preserved in deep-sea environments for thousands of years,⁶⁵ suggesting the significance of chitin as long-term carbon and nitrogen

source for the cold seep affected habitats. Intriguingly, MAGs harboring the *chiA* gene showed distinct niche prevalence. The only prevalent MAG in active and extinct habitats, IA_PS_003.bin.2, might potentially utilize chitin synthesized by the cold seep fauna, making it a key model for studying chitin recycling in seepage-influenced environments. *Klebsiella* is a ubiquitous clade in environment and aquatic animals, inclusive of the marine bivalves.^{66,67} Members of this clade usually possess a set of genes responsible for chitin degradation, making them a key link for chitin recycling in various environments.⁶⁸ However, their functionality in deep-seep cold seep ecology has never been reported before. Our enzymatic results have demonstrated the involvement of IA_PS_003.bin.2 in chitin recycling, highlighting the significance of *Klebsiella* in carbon and nitrogen cycling in deep-sea environments. The increase of activity in the deep-sea environment (4°C and 15 MPa) strongly indicates the modification for adaptation to low temperature and high pressure. In addition, IA_PS_003.bin.2 is also found to harbor a set of genes for the novel oxidative chitin degradation pathway. Although the LPMO appeared to be inactive or with extremely low activity in this study (Figure S8), they are believed to play a key role in the initial degradation of marine chitin particles and promote its hydrolysis by chitinase.^{68,69} In addition, we are delighted to find that IA_PS_003.bin.2 carries the key genes for nitrogen fixation and DNRA, providing genetic evidence for the potential chemoorganotroph-driven nitrogen cycling in cold seep environments. Given the long-term reservation of chitin relies in cold seeps, our findings emphasized the enduring impact of the seepage activities to deep-sea ecosystems.

By the way, cellulose, the most abundant polymer on Earth, comprising of glucose units, was also found abundant in sediments of Haima cold seep, in suggestive of a significant carbon sink. Since little is known about its production by the local communities, cellulose in the Haima cold seep was supposed to be transported from terrigenous input. Unfortunately, key genes essential for cellulose degradation, such as the encoding genes of 1,4-beta-cellobiosidase and beta-glucosidase, were absent in the MAGs obtained in this study. Considering its high abundance, it could be worth elucidating the mechanisms of cellulose recycling to refine carbon cycling in deep-sea cold seeps in the future.

CONCLUSION

In summary, we presented a comprehensive view of the biogeochemical cycling of carbon, nitrogen, and sulfur in the sediments of the Haima cold seep, highlighting the significance of biomacromolecules and organotrophs in the geochemical cycling of cold seeps. Given the abundance of chitin in cold seep environments and cooccurrence of chitin degradation and nitrogen metabolism pathways within a specific lineage, chitinase activity under deep-sea conditions provided compelling evidence of chemoorganotroph-driven nitrogen cycling, emphasizing the enduring influence of biomacromolecule recycling to deep-sea cold seep ecology.

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AUTHOR CONTRIBUTIONS

Y.C., J.L. and S.Z. conceived the original concept. Y.C., Q.L. and L.L. collected the samples. Y.C., X.H. and J.Z. performed metagenomic data analyses. J.Y. conducted the enzymatic analysis. The manuscript was written by Y.C. and J.Y., and supervised by J.L. and S.Z. All authors contributed to the manuscript and approved the final version.

DECLARATION OF INTERESTS

The authors declare no competing interests.

DATA AND CODE AVAILABILITY

The metagenomic sequencing data have been deposited at the NCBI Sequence Read Archive under the BioProject number PRJNA846871 and PRJNA847028. The atomic coordinates and structure factors for _{1A32}Chi641 were deposited at the Protein Data Bank under accession number 8XBD.

SUPPLEMENTAL INFORMATION

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