Cultivation and characterization of a novel clade of deep-sea Chloroflexi: providing a glimpse of the phylum Chloroflexi involved in sulfur cycling

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Abstract

Chloroflexi bacteria are abundant and globally distributed in various unexplored biospheres on Earth. However, only few Chloroflexi members have been cultivated, hampering further understanding of this important group. In the current study, we firstly clarify the high abundance of the phylum Chloroflexi in deep-sea sediments via the operational taxonomic units analysis. We further successfully isolate a novel Chloroflexi strain ZRK33 from cold seep sediments by using an enrichment medium constantly supplemented with rifampicin. Phylogenetic analyses based on 16S rRNA gene, genome, RpoB and EF-tu proteins indicate that strain ZRK33 represents a novel class, and the class is designated as Sulfochloroflexia because whole set of genes encoding key enzymes responsible for assimilatory sulfate reduction are identified in the genome of strain ZRK33. Indeed, assimilation of sulfate or thiosulfate by strain ZRK33 evidently benefits its growth and morphogenesis. Proteomic results suggest that metabolization of sulfate or thiosulfate significantly promotes the transport and degradation of various macromolecules and thereby stimulating the energy production. Notably, the putative genes associated with assimilatory and dissimilatory sulfate reduction ubiquitously distribute in the metagenome-assembled genomes of 27 Chloroflexi members derived from deep-sea sediments, strongly suggesting that Chloroflexi bacteria play undocumented key roles in deep-sea sulfur cycling.

Introduction

Deep marine subsurface is one of the least-understood habitats on Earth and is estimated to contain up to 3×10^{29} microbial cells, which is equivalent to the combined microbial biomass of the oceanic water column and terrestrial soil [1]. The prokaryotic biomass in deep marine subsurface sediments exceeds 10^5 microbial cells/cm^3 even at depths of nearly to 1,000 m below the seafloor [2,3]. These microorganisms are the primary drivers of elemental cycles within deep marine subsurface sediments and play key roles in the recycling of biogeochemical nutrients to the water column [4]. Members of the phylum Chloroflexi widely distributed in various environments with high abundance, for example, in some marine subsurface sediments the number of Chloroflexi bacteria is shown to be closely equivalent to other total bacterial counts [3,5-8], strongly suggesting that the phylum Chloroflexi is an essential group to maintain the population equilibrium of marine subsurface ecosystems [9-12].

The phylum Chloroflexi, formerly called the 'green nonsulfur bacteria', is a remarkably diverse, deeply branching lineage in the domain Bacteria [13]. Currently, the phylum Chloroflexi is divided phylogenetically into nine classes, including Chloroflexia [14], Anaerolineae [15], Caldilineae [15], Ktedonobacteria [16], Thermomicrobia [17], Dehalococcoidia [18], Tepidiformia [19], Thermoflexia [20] and Ardenticatenia [21]. Concomitant with the expansion of the phylum Chloroflexi by cultivation, studies utilizing cultivation-independent techniques have revealed a

63 remarkable diversity of as-yet uncultivated microorganisms affiliated with the phylum
64 Chloroflexi [22], indicating immeasurable novel lineages of Chloroflexi existing in
65 nature. Despite the Chloroflexi bacteria being among the first widespread microbial
66 lineages discovered in deep-sea environments we still lack cultured representatives
67 (especially those with relative fast growing rate) for this group and their detailed
68 physiological, genetic and ecological properties are currently almost completely
69 obscure [13,23,24]. For example, until now, only basic physiological characteristics
70 of two cultured strains of Chloroflexi with extremely slow growth rate (doubling time
71 from 1.5 days to 19 days) from the deep-sea sediments are available [10,23], and their
72 central metabolisms and contributions to biogeochemical processes including sulfur
73 cycling are largely unknown.

74 The cycling of sulfur is one of Earth's major biogeochemical processes and is
75 closely related to the energy metabolism of microorganisms living in the cold seep
76 and hydrothermal vents [25-27]. Importantly, the coupling of sulfate/sulfite reduction
77 to oxidation of H_2, small chain fatty acids, or other carbon compounds limits the
78 availability of these substrates to other organisms like methanogens and alters the
79 energetics via syntrophic interactions, and thereby impacting the methane production
80 [25]. Given the importance of sulfur cycling in the deep biospheres, it is vital that we
81 understand which organisms can carry out the reactions and the pathways involved
82 [27]. Based on metagenomic sequencing results, some SAR202 members of the
83 phylum of Chloroflexi are predicted to be sulfite-oxidizers, making them as potential

key players in the sulfur cycle at the deep marine environment [28]; based on single-cell genomic sequencing results, some members of Dehalococcoidia class within the phylum Chloroflexi are demonstrated to possess diverse genes encoding dissimilatory sulfite reductase [4], suggesting that Dehalococcoidia bacteria could drive sulfite reduction and respire oxidized sulfur compounds. Together, some of the members of Chloroflexi are believed to play a previously unrecognized role in sulfur cycling, which needs to be verified with cultured representatives of Chloroflexi isolated from deep-sea environments.

In this study, we checked the abundance of Chloroflexi existing in both deep-sea cold seep and hydrothermal vents. Using an enrichment medium continuously supplemented with rifampicin pressure, we have successfully isolated a novel member of Chloroflexi, strain ZRK33, from the deep marine subsurface sediments collected from a typical cold seep in the South China Sea (1,146 m water depth). Strain ZRK33 is further to shown to be a representative of a novel class of the phylum Chloroflexi, designated as Sulfochloroflexia given that strain ZRK33 is demonstrated to assimilate sulfate and thiosulfate. Lastly, the broad distribution of diverse genes encoding key enzymes driving both sulfur assimilatory and dissimilatory reduction in the metagenome-assembled genomes from deep-sea sediments is detailed analyzed.

Materials and methods

Sampling and operational taxonomic units (OTUs) analysis

104 The deep-sea sediment samples were collected by *RV KEXUE* from a typical cold
105 seep in the South China Sea (E 119°17'07.322", N 22°06'58.598") at a depth of
106 approximately 1,146 m and two hydrothermal vent fields in the Okinawa Trough (E
107 126°53'50.247", N 27°47'11.096"; E 124°22'24.86", N 25°15'47.438") in July of 2018
108 as described previously [26]. In order to understand the abundance of Chloroflexi
109 phylum in the deep-sea sediments, we selected eight sedimentary samples (six cold
110 seep samples including RPC, ZC1, ZC2, ZC3, ZC4 and ZC5 at depth intervals of 0-10,
111 30-50, 90-110, 150-170, 210-230 and 230-250 cm, respectively; two hydrothermal
112 vents samples including H1 and H2 at depth intervals of 0-20 cm) for OTUs
113 sequencing performed by Novogene (Tianjin, China). Briefly, total DNAs from these
114 samples were extracted by the CTAB/SDS method [29] and diluted to 1 ng/μL with
115 sterile water and used for PCR template. 16S rRNA genes of distinct regions (16S
116 V3/V4) were amplified using specific primers (341F: 5'-
117 CCTAYGGGRBGCASCAG and 806R: 5'- GGACTACNNGGGTATCTAAT). The
118 PCR products were purified with a Qiagen Gel Extraction Kit (Qiagen, Germany) for
119 libraries construction. Sequencing libraries were generated using TruSeq® DNA
120 PCR-Free Sample Preparation Kit (Illumina, USA) following the manufacturer's
121 instructions. The library quality was assessed on the Qubit@ 2.0 Fluorometer
122 (Thermo Scientific, USA) and Agilent Bioanalyzer 2100 system. The library was
123 sequenced on an Illumina NovaSeq platform and 250 bp paired-end reads were
124 generated. Paired-end reads were merged using FLASH (V1.2.7,

http://ccb.jhu.edu/software/FLASH/) [30], which was designed to merge paired-end reads when at least some of the reads overlap with those generated from the opposite end of the same DNA fragments, and the splicing sequences were called raw tags. Quality filtering on the raw tags was performed under specific filtering conditions to obtain the high-quality clean tags [31] according to the QIIME (V1.9.1, http://qiime.org/scripts/split_libraries_fastq.html) quality controlled process. The tags were compared with the reference database (Silva database, https://www.arb-silva.de/) using UCHIME algorithm (UCHIME Algorithm, http://www.drive5.com/usearch/manual/uchime_algo.html) [32] to detect chimera sequences, and then the chimera sequences were removed [33]. Sequence analyses were performed by Uparse software (Uparse v7.0.1001, http://drive5.com/uparse/) [34]. Sequences with ≥97% similarity were assigned to the same OTUs. The representative sequence for each OTU was screened for further annotation. For each representative sequence, the Silva Database (http://www.arb-silva.de/) [35] was used based on Mothur algorithm to annotate taxonomic information.

Metagenomic sequencing, assembly, binning and annotation

To understand the common characteristics of Chloroflexi in deep-sea environments, four cold seep sediment samples (zhu, C1, C2 and C4) and two hydrothermal vents sediment samples (H1 and H2) were selected for metagenomic analysis in BGI (BGI, China). Briefly, total DNAs from these samples (20 g each) were extracted using the Qiagen DNeasy® PowerSoil® Pro Kit (Qiagen, Hilden, Germany) and the integrity of

DNA was evaluated by gel electrophoresis. Then 0.5 μg DNA of each sample was used library construction. The library was prepared with an amplification step for each sample. And then DNAs were cleaved into 50~800 bp fragments by the Covaris E220 ultrasonicator (Covaris, Brighton, UK) and some fragments between 150~250 bp were selected using AMPure XP beads (Agencourt, USA) and repaired using T4 DNA polymerase (Enzymatics, USA). All next-generation sequencing (NGS) was performed on the BGISEQ-500 platform (BGI, Qingdao, China) and generated 100 bp paired-end raw reads. Quality control was performed by SOAPnuke (v1.5.6) (setting: -l 20 -q 0.2 -n 0.05 -Q 2 -d -c 0 -5 0 -7 1) [36] and the clean data were assembled using MEGAHIT (v1.1.3) (setting:--min-count 2 --k-min 33 --k-max 83 --k-step 10) [37]. Assemblies of these samples were automatically binned using Maxbin2 [38], metaBAT2 [39] and Concoct [40]. MetaWRAP [41] was used to purify and organize data to generate the final bins. Finally, the completeness and contamination of metagenome-assembled genomes (MAGs) were assessed by the checkM (v1.0.18) [42]. These obtained MAGs were subsequently annotated by searching these predicted genes against KEGG (Release 87.0), NR (20180814), Swissprot (release-2017_07) and COG (update-2018_08) databases. Additionally, we utilized a custom hmmer as well as the Pfam and TIGRFAM databases to search for genes associated with sulfur metabolism using hmmsearch (e-value cut-off of 1e-20) [43].

Enrichment and cultivation of deep-sea Chloroflexi bacteria

To enrich the Chloroflexi bacteria, deep-sea sediment samples were cultured at 28 ℃ for one month in an anaerobic enrichment medium (containing 1.0 g/L NH_4Cl, 1.0 g/L $NaHCO_3$, 1.0 g/L CH_3COONa, 0.5 g/L KH_2PO_4, 0.2 g/L $MgSO_4{\cdot}7H_2O$, 1.0 g/L yeast extract, 1.0 g/L peptone, 0.7 g/L cysteine hydrochloride, 500 μL/L 0.1 % (w/v) resazurin, pH 7.0) with 50 μg/mL rifampicin. This medium was prepared anaerobically as previously described and named ORG in this study [44]. A 50 μL enrichment culture was spread on the Hungate tubes containing ORG broth supplemented with 15 g/L agar after 10,000 times dilution. These Hungate tubes were anaerobically incubated at 28 ℃ for 7 days. Individual colonies were respectively picked using sterilized bamboo sticks and then cultured in the ORG broth. Strain ZRK33 was isolated and purified by repeated use the Hungate roll-tube methods for several rounds until it was considered to be axenic. The purity of strain ZRK33 was confirmed by transmission electron microscopy (TEM) and repeated partial sequencing of the 16S rRNA gene. Strain ZRK33 was preserved at -80 ℃ in ORG broth supplemented with 20% (v/v) glycerol.

TEM observation

To observe the morphological characteristics of strain ZRK33, the cell suspension of fresh culture was collected at 5,000 ×*g* for 10 min and washed with Milli-Q water, and then taken by immersing copper grids coated with a carbon film for 10 min. Thereafter, the copper grids were washed for 10 min in Milli-Q water and dried for 20 min at room temperature [45]. Ultrathin-section electron microscopic observation was

187 performed as described previously [46-48]. The sample was firstly preserved in 2.5%
188 (v/v) glutaraldehyde for 8 h at 4 °C, washed three times with phosphate buffer saline
189 (PBS) and then dehydrated in ethanol solutions of 30%, 50%, 70%, 90% and 100%
190 for 10 min each time. Finally, the sample was embedded in a plastic resin. Ultrathin
191 sections (50~70 nm) of cells were prepared with an ultramicrotome (Leica EM UC7,
192 Gemany), stained with uranyl acetate and lead citrate. All of these samples were
193 examined using TEM (HT7700, Hitachi, Japan) with a JEOL JEM 12000 EX
194 (equipped with a field emission gun) at 100 kV.

195 **Genome sequencing and genomic analysis**

196 Genomic DNAs of strain ZRK33 were extracted from 2 L cells that cultured for 7
197 days at 28 ℃. The DNA library was prepared using the Ligation Sequencing Kit
198 (SQK-LSK109, UK), and sequenced using a FLO-MIN106 vR9.4 flow-cell for 48 h
199 on MinKNOWN software v1.4.2 (Oxford Nanopore Technologies, UK).
200 Whole-genome sequence determinations of strain ZRK33 were carried out with the
201 Oxford Nanopore MinION (Oxford, UK) and Illumina MiSeq sequencing platform
202 (San Diego, CA). A hybrid approach was utilized for genome assembly using reads
203 from both platforms. Base-calling was performed using Albacore software v2.1.10
204 (Oxford Nanopore Technologies, UK). Nanopore reads were processed using
205 protocols toolkit for quality control and downstream analysis [49]. Filtered reads were
206 assembled using Canu version 1.8 [50] with the default parameters for Nanopore data.

Finally, the genome was assembled into a single contig and was manually circularized by deleting an overlapping end.

The genome relatedness values were calculated by multiple approaches: Average Nucleotide Identity (ANI) based on the MUMMER ultra-rapid aligning tool (ANIm), ANI based on the BLASTN algorithm (ANIb), the tetranucleotide signatures (Tetra), and *in silico* DNA-DNA similarity. ANIm, ANIb and Tetra frequencies were calculated using JSpecies WS (http://jspecies.ribohost.com/jspeciesws/) [51]. The recommended species criterion cut-offs were used: 95% for the ANIb and ANIm and 0.99 for the Tetra signature. The *in silico* DNA-DNA similarity values were calculated by the Genome-to-Genome Distance Calculator (GGDC) (http://ggdc.dsmz.de/) [52]. The *is*DDH results were based on the recommended formula 2, which is independent of genome size.

Phylogenetic analysis

The full-length 16S rRNA gene sequence (1,489 bp) of strain ZRK33 was extracted from the genome, which had been deposited in the GenBank database (accession number MN817941), and other related taxa used for phylogenetic analysis were obtained from NCBI (www.ncbi.nlm.nih.gov/). The genome tree was constructed from a concatenated alignment of 37 protein-coding genes [53] that extracted from each genome by Phylosift (v1.0.1) [54], all of which were in a single copy and universally distributed in both archaea and bacteria (Supplementary Table S1). The genomes used to construct the genome tree included both draft and finished genomes

from the NCBI databases. The RpoB and EF-tu tree was constructed by using RpoB or EF-tu protein sequences, which were identified from 49 genomes using the hidden markov models (HMMs) TIGR02029 and TIGR00485 from TIGRfams (http://www.jcvi.org/cgi-bin/tigrfams/index.cgi), respectively. Phylogenetic trees were constructed by using W-IQ-TREE web server (http://iqtree.cibiv.univie.ac.at) [55] with LG+F+I+G4 model. The online tool Interactive Tree of Life (iTOL v5) [56,57] was used for editing trees.

Growth assays of strain ZRK33

Growth assays were performed at atmospheric pressure. Briefly, 15 mL fresh strain ZRK33 culture was inoculated in 2 L Hungate bottles containing 1.5 L ORG broth supplemented with 20 mM Na_2SO_4, 200 mM Na_2SO_4, 20 mM $Na_2S_2O_3$, 200 mM $Na_2S_2O_3$, 1 mM Na_2SO_3 and 1 mM Na_2S, respectively. Each condition had three replicates. These Hungate bottles were then anaerobically incubated at 28 ℃ for 12 d. Bacterial growth status was monitored by measuring the OD_{600} value every 12 h until cell growth reached the stationary phase. For the morphological observation of strain ZRK33, we took 20 μL culture that cultivated for 12 d, which was then checked and recorded under an inverted microscope (NIKON TS100, Tokyo, Japan) equipped with a digital camera. For the determination of the dynamics of the concentrations of Na_2SO_4 and $Na_2S_2O_3$ in the culture, we selected three cultivation time points at 5 d, 8 d and 12 d, respectively, and each condition had three replicates. The supernatant was collected at 12,000 *g* for 10 min and diluted 80 times, and the concentrations of SO_4^{2-}

and $S_2O_3^{2-}$ in the diluted supernatant were respectively measured by the ion chromatograph (ECO IC, Herisau, Switzerland) with an chromatographic column (Metrosep A Supp5). The column was eluted with mobile phase A (3.2 mmol/L Na_2CO_3) and mobile phase B (1.0 mmol/L $NaHCO_3$) at 25 ℃.

Proteomic analysis

Proteomic analysis was performed by PTMBiolabs (Hangzhou, China). Briefly, strain ZRK33 was respectively cultivated in the ORG broth (set as the control group and indicated as "C"), ORG broth supplemented with 200 mM Na_2SO_4 (set as the experimental group and indicated as "S") and 200 mM $Na_2S_2O_3$ (set as the experimental group and indicated as "T") for 8 d at 28 ℃. Then the cells were collected and sonicated three times on ice using a high intensity ultrasonic processor in lysis buffer (8 M urea, 1% Protease Inhibitor Cocktail). The remaining debris was removed by centrifugation at 12,000 *g* at 4 ℃ for 10 min. Finally, the supernatant was collected and the protein concentration was determined with a BCA kit (Solarbio, China) according to the instructions. The detailed protocols of proteomics sequencing technology were described in the Supplementary information. The heat map was made by HemI 1.0 based on the KEGG enrichment results.

Data availability

The raw amplicon sequencing data have been deposited to NCBI Short Read Archive (accession numbers: PRJNA675395 and PRJNA688815). The BioProject accession number of metagenome-assembled genomes (MAGs) of Chloroflexi bacteria used in

this study is PRJNA667788. The full-length 16S rRNA gene sequence of *S. methaneseepsis* ZRK33 has been deposited at GenBank under the accession number MN817941. The complete genome sequence of *S. methaneseepsis* ZRK33 has been deposited at GenBank under the accession number CP051151. The mass spectrometry proteomics data have been deposited to the Proteome Xchange Consortium with the dataset identifier PXD023380.

Results

Chloroflexi bacteria possess high abundance in the deep-sea environments

To gain preliminary insights of Chloroflexi bacteria existing in the deep-sea environments, OTUs sequencing was firstly performed to detect the abundance of the phylum Chloroflexi present in the cold seep sediments at depth intervals of 0-10 cm (sample RPC), 30-50 cm (sample ZC1), 90-110 cm (sample ZC2), 150-170 cm (sample ZC3), 210-230 cm (sample ZC4), 230-250 cm (sample ZC5) and hydrothermal vents sediments at depth intervals of 0-20 cm from the surface to the deep layer in two different sampling sites (samples H1 and H2). As previously reported [2,6,7], the Chloroflexi group was both the second most abundant phylum in cold seep and hydrothermal vents sediments, suggesting Chloroflexi was dominant in these deep-sea regions (Figs. 1A and 1B). The proportion of Chloroflexi respectively accounted for 6.15%, 10.92%, 5.04%, 8.67% and 13.67% of the whole bacterial domain at the phylum level in samples RPC, ZC1, ZC2, H1 and H2 (Figs. 1A and 1B).

Moreover, the ratio of the phylum Chloroflexi to the whole bacteria domain is much higher in the top layer than that in the bottom one, providing a preliminary hint of distribution of Chloroflexi bacteria in the deep-sea sediments. To obtain further insights into the deep-sea Chloroflexi bacteria, we analyzed the abundance of Chloroflexi members at the class level and found that Dehalococcoidia and Anaerolineae were the top two classes in the cold seep (Fig. 1C) and hydrothermal vents sediments (Fig. 1D). In particular, Dehalococcoidia class bacteria are an absolute dominant population in 7 of 8 samples, strongly suggesting the importance of this lineage in deep-sea environments.

Cultivation and morphology of a novel Chloroflexi bacterium isolated from the deep-sea cold seep

To culture novel isolates belonging to the phylum Chloroflexi from deep-sea environments, we improved the enrichment method by using a specific medium that constantly supplemented with 50 μg/mL rifampicin, given that many members of Chloroflexi were reported to tolerate rifampicin [13,23] while most of other bacteria are sensitive to this antibiotics. Using this strategy, we anaerobically enriched the deep-sea sediment samples at 28 ℃ for one month. Thereafter, the enriched samples were plated on solid medium in Hungate tubes, and individual colonies with distinct morphology were picked and cultured (Fig. 2A). Excitingly, some of the cultured colonies were identified as Chloroflexi bacteria based on their 16S rRNA sequences. Among them, strain ZRK33 possessed a fast growth rate and was chosen for further

311 study. Under TEM observation, the cells of strain ZRK33 were filamentous, generally
312 more than 20 μm long and 0.5-0.6 μm wide, and had no flagellum (Figs. 2B and 2C).
313 Ultrathin sections of whole cells of strain ZRK33 revealed a cytoplasmic membrane
314 surrounded by a cell wall surface layer (Figs. 2D and 2E). The strain did not possess a
315 clearly visible sheath-like structure (Fig. 2D) as shown in the *Pelolinea submarina*
316 strain MO-CFX1^{T}, a typical Chloroflexi bacterium belonging to the class
317 Anaerolineae [23]. Based on the 16S rRNA sequence of strain ZRK33, a sequence
318 similarity calculation using the NCBI server indicated that the closest relatives of
319 strain ZRK33 were *Anaerolinea thermophila* UNI-1 (82.75%) (class Anaerolineae),
320 *Ornatilinea apprima* P3M-1 (82.32%) (class Anaerolineae), *Thermomarinilinea*
321 *lacunifontana* SW7 (82.42%) (class Anaerolineae) and *Caldilinea aerophila* DSM
322 14535 (81.87%) (class Cadilineae). Recently, taxonomic thresholds based on 16S
323 rRNA gene sequence identity values were suggested [58]: for classes, the proposed
324 thresholds for median and minimum sequence identity values were 86.35% and
325 80.38%, respectively. Based on these criteria, we propose that strain ZRK33 might be
326 a representative of a novel class-level Chloroflexi.

327 **Genomic characteristics and phylogenetic analysis of strain ZRK33**

328 To understand more characteristics of strain ZRK33, its whole genome was sequenced
329 and analyzed. The genome size of strain ZRK33 was 5,631,885 bp with a DNA G+C
330 content of 52.76% (Fig. 3A and Supplementary Table S2). Annotation of the genome
331 of strain ZRK33 revealed it consisted of 4,885 predicted genes that included 55 RNA

genes (6 rRNA genes, 46 tRNA genes and 3 other ncRNAs). When exploring the detailed genomic composition of strain ZRK33, we found that various genes encoding key enzymes responsible for sulfur metabolism existing in the genome of ZRK33 (Fig. 3B). And these enzymes are thought to involve in assimilatory sulfate reduction, strongly indicating that strain ZRK33 might be a representative of a novel clade that driving deep-sea sulfur cycling.

To further clarify the phylogenetic position of strain ZRK33, the genome relatedness values were calculated by the average nucleotide identity (ANI), *in silico* DNA-DNA similarity (*is*DDH) and the tetranucleotide signatures (Tetra), against six genomes (strain ZRK33, and five strains MO-CFX2, MO-CFX1, UNI-1, IMO-1 and P3M-1 belonging to class Anaerolineae) (Supplementary Table S3). The average nucleotide identities (ANIb) of ZRK33 with strains MO-CFX2, MO-CFX1, UNI-1, IMO-1 and P3M-1 were 64.81%, 63.06%, 63.42%, 63.41% and 63.29%, respectively. The average nucleotide identities (ANIm) of ZRK33 with MO-CFX2, MO-CFX1, UNI-1, IMO-1 and P3M-1 were 85.21%, 82.63%, 83.42%, 83.15% and 83.23%, respectively. The tetra values of ZRK33 with MO-CFX2, MO-CFX1, UNI-1, IMO-1 and P3M-1 were 0.48145, 0.67572, 0.64677, 0.65234 and 0.65126. Based on digital DNA-DNA hybridization employing the Genome-to-Genome Distance Calculator GGDC, the in silico DDH estimates for ZRK33 with MO-CFX2, MO-CFX1, UNI-1, IMO-1 and P3M-1 were 23.30%, 24.20%, 20.40%, 21.60% and 23.80%, respectively. These results together demonstrated the genome of strain

ZRK33 to be obviously below established 'cut-off' values (ANIb: 95%, ANIm: 95%, isDDH: 70%, Tetra: 0.99) for defining bacterial species, suggesting strain ZRK33 represents a novel taxon within the phylum Chloroflexi as currently defined.

To further confirm the taxonomic status of strain ZRK33, we performed the phylogenetic analyses with 16S rRNA genes from all cultured Chloroflexi representatives, some uncultured SAR202 representatives and other uncultured Chloroflexi bacteria. The maximum likelihood tree of 16S rRNA placed strain ZRK33 as a sister of the strain MO-CFX2, which together formed a distinct cluster separating from other classes of the phylum Chloroflexi (Fig. 4). Furthermore, the genome tree also placed the novel clade as a sister of the Anaerolineae class belonging to the phylum Chloroflexi (Supplementary Figure S1). The phylogenetic analysis of strain ZRK33 using the beta subunit of RNA polymerase (RpoB), which also showed that the novel clade formed a separate branch from the Anaerolineae class (Supplementary Figure S2). More importantly, the broader phylogeny of elongation factor Tu (EF-Tu) supported the placement of the novel clade within the phylum Chloroflexi (Supplementary Figure S3). Based on phylogenetic, genomic and phenotypic characteristics, we proposed that strain ZRK33 together with strain MO-CFX2 (previously classified as a representative of a novel order of class Anaerolineae) were classified as the type strains of a new class of the phylum Chloroflexi. Given the broad distribution of genes associated with sulfur metabolism in the genome of strain ZRK33 (Fig. 3B) and its significant potential involved in sulfur cycling, we propose

374 Sulfochloroflexia classis nov., Sulfochloroflexales ord. nov., Sulfochloroflexaceae
375 fam. nov. and *Sulfochloroflexus methaneseepsis* gen. nov.

376 **Description of *Sulfochloroflexus* gen. nov. and *Sulfochloroflexus methaneseepsis***
377 **sp. nov.**

378 *Sulfochloroflexus* (Sul.fo'ch.lo.ro.fle.xus. N.L. fem. pl. n. Sulfo, sulfur; N.L. masc.
379 *chloroflexus* a bacterial genus; N.L. masc. n. *Sulfochloroflexus*, *chloroflexus* sulfur
380 loving. Facultatively anaerobic, mesophilic, neutrophilic and moderately halophilic
381 (Supplementary Table S2). Cells are non-motile. Gram-staining reaction is negative.
382 The phylogenetic position is in the family Sulfochloroflexaceae, order
383 Sulfochloroflexales, class Sulfochloroflexia of the phylum Chloroflexi. The type
384 species is *Sulfochloroflexus methaneseepsis*.

385 *Sulfochloroflexus methaneseepsis* (me.th.ane'seep.sis. L. gen. pl. n.
386 *methaneseepsis* of the deep-sea methane seeps). Cells are generally more than 20 μm
387 long and 0.5-0.6 μm wide, filamentous, facultatively anaerobic and have no flagellum.
388 From the sole carbon source utilization test, growth is stimulated by arabinose,
389 fructose, glucose, galactose, mannose, ribose, fumarate, pyruvate and peptone.
390 Growing at pH values of 6.0-8.0 (optimum, pH 7.0). The temperature range for
391 growth is 28-32 ℃ with an optimum at 28 ℃. Growth occurs at NaCl concentrations
392 between 0.0-5.0% with optimum growth at 3.0% NaCl. Containing significant
393 proportions (>10 %) of the cellular fatty acids $C_{16:0}$, $C_{15:0}$2-OH, $C_{17:1}\omega$6c and $C_{18:1}\omega$7c.

394 The type strain, ZRK33^T, was isolated from the sediment of deep-sea cold seep, P.R.
395 China. The DNA G+C content of the type strain is 52.76%.

396 The detailed descriptions of other levels of Sulfochloroflexia were shown in the
397 Supplementary information.

398 **_S. methaneseepsis_ ZRK33 assimilates sulfate and thiosulfate for growth**

399 Given that strain ZRK33 had a complete set genes of assimilatory sulfate reduction
400 and it was isolated from the deep-sea cold seep where is rich of different
401 sulfur-containing compounds [25,26], thus, we tested the effects of different
402 sulfur-containing inorganic substances (including Na_2SO_4, Na_2SO_3, $Na_2S_2O_3$, Na_2S)
403 on the growth of *S. methaneseepsis* ZRK33. The results showed that the supplement
404 of high concentration (200 mM) of Na_2SO_4 and $Na_2S_2O_3$ could significantly promote
405 the growth of strain ZRK33 (Figs. 5A and B). While low concentration of Na_2SO_4 and
406 $Na_2S_2O_3$ (20 mM) had no evident effects on the growth of strain ZRK33
407 (Supplementary Fig. S4), indicating this bacterium is only sensitive to high
408 concentrations of Na_2SO_4 and $Na_2S_2O_3$. Meanwhile, it is noting that the
409 concentrations of Na_2SO_4 and $Na_2S_2O_3$ were respectively decreased from 200 mM to
410 120 mM and 140 mM along with the growth of strain ZRK33 for 12 d, suggesting that
411 strain ZRK33 could effectively metabolize Na_2SO_4 and $Na_2S_2O_3$ (Figs. 5A and B).
412 Moreover, the average length of filamentous cells of strain ZRK33 became apparently
413 longer in the medium supplemented with 200 mM Na_2SO_4 (Fig. 5D) or $Na_2S_2O_3$ (Fig.
414 5E) than that in the control group (Fig. 5C), strongly suggesting that ZRK33 could

assimilate Na_2SO_4 and $Na_2S_2O_3$ and thereby generating extra energy for growth. In comparison, the supplement of very low concentration (1 mM) of Na_2SO_3 and Na_2S inhibited the growth of strain ZRK33 (Supplementary Fig. S4), indicating that SO_3^{2-} and S^{2-} were harmful sulfur-containing compounds against the growth of strain ZRK33.

Proteomic analyses of sulfur metabolism in *S. methaneseepsis* ZRK33

To better describe the sulfur metabolism of *S. methaneseepsis* ZRK33, we performed the proteomic analysis of strain ZRK33 that cultured in the medium amended with or without Na_2SO_4/$Na_2S_2O_3$ to explore the underlying mechanism of growth promotion, given that ZRK33 could effectively assimilate Na_2SO_4/$Na_2S_2O_3$ for its growth. The results showed that the expression of sulfurtransferase (TST), sulfatase-like hydrolase and cysteine desulfurase-like protein were obviously up-regulation compared with the control group, which were associated with sulfur metabolism (Fig. 6A). In particular, TST is a key enzyme catalyzing $S_2O_3^{2-}$ to SO_3^{2-} and thereby joining into sulfur assimilation (Fig. 3B), and it was significantly up-regulated in the presence of high concentrations of Na_2SO_4/$Na_2S_2O_3$, especially $Na_2S_2O_3$. Surprisingly, the expressions of other proteins associated with assimilatory sulfate reduction were not significantly up-regulated in experimental groups, partly due to the single sampling time point that might miss the exact time to detect the up-regulation of key proteins associated with sulfur metabolism. Alternatively, the expressions of many proteins associated with organic matter metabolisms toward energy production were evidently up-regulated,

436 including amino acids and sugar ABC transporters (Fig. 6B),
437 saccharides/peptides/amino acids degradation (Fig. 6C), and energy production (Fig.
438 6D). Correspondingly, the expressions of almost all genes involved in EMP glycolysis
439 were also significantly up-regulated (Supplementary Figures S5). Thus, we speculated
440 that metabolization of sulfate and thiosulfate by strain ZRK33 may accelerate the
441 hydrolysis and uptake of saccharides and other organic matter and thereby
442 synthesizing energy to promote the growth [48]. Combining the results of catalyzing
443 of sulfate and thiosulfate to other formations (Figs. 5C and 5D), we believe that strain
444 ZRK33 possesses a capability to assimilate inorganic sulfur-containing compounds
445 (e.g. sulfate and thiosulfate) that ubiquitously existing in the deep-sea environments
446 and thereby contributing to the deep-sea sulfur cycling to some extent.

447 Based on the combination of proteomic, genomic and physiological
448 characteristics, we propose a model towards central metabolic traits of strain ZRK33
449 (Fig. 7). In this model, central metabolisms including EMP glycolysis, oxidative
450 pentose phosphate pathway, TCA cycle (tricarboxylic acid cycle), assimilatory sulfate
451 reduction, urea cycle and electron transport system are shown. All the above items are
452 closely related to the energy production in strain ZRK33. Briefly, strain ZRK33
453 contains a number of genes related to ABC transporters of amino acids, peptides and
454 sugar, which could transport these organic matters into the cell to participate in EMP
455 glycolysis and oxidative pentose phosphate pathway. These processes eventually
456 drive the formation of pyruvate and acetyl-CoA, which enter the TCA cycle to

457 produce energy for the growth of strain ZRK33. Of note, sulfate and thiosulfate could
458 be converted to cysteine and thereby entering the pyruvate synthesis pathway through
459 the assimilatory sulfate reduction, which might promote the saccharides degradation
460 and utilization via some unknown mechanisms. Moreover, strain ZRK33 could fix
461 nitrogen and carbon dioxide to involve in the urea cycle, and corresponding
462 metabolites can join into the TCA cycle for energy generation. Meanwhile, the F-type
463 ATP synthase, cytochrome bd ubiquinol oxidase and H^+-transporting NADH:
464 Quinone oxidoreductase required for energy production are also present in the
465 genome of strain ZRK33. Overall, ***S.*** *methaneseepsis* ZRK33 is a representative of a
466 novel clade of the phylum Chloroflexi that possessing diverse metabolic pathways for
467 energy production, providing an evidence that Chloroflexi members are a group of
468 high-abundance bacteria ubiquitously distributed in different environments.

469 **Wide distribution of assimilatory and dissimilatory sulfate reduction pathways in**
470 **the deep-sea Chloroflexi bacteria**

471 To evaluate the contribution of Chloroflexi bacteria to the deep-sea sulfur cycling, we
472 further analyzed the distribution of genes encoding key enzymes responsible for both
473 assimilatory (Fig. 8A) and dissimilatory (Fig. 8B) sulfate reduction in 27
474 metagenome-assembled genomes (MAGs) of Chloroflexi bacteria derived from both
475 deep-sea cold seep and hydrothermal vents sediments. Through a thorough analysis of
476 27 MAGs, we found that diverse genes encoding key enzymes in charge of
477 assimilatory and dissimilatory sulfate reduction, including adenylyl-sulfate kinase

(CysC), 3', 5'-bisphosphate nucleotidase (CysQ), sulfate adenylyltransferase (CysN), anaerobic sulfite reductase (AsrA, AsrB and AsrC) and dissimilatory sulfite reductase (DsrA and DsrB), were widely distributed in both cold seep and hydrothermal vents derived MAGs (Fig. 8C). Of note, genes encoding AsrA and AsrB were present in most MAGs, however, genes encoding DsrA and DsrB only broadly existed in the hydrothermal vents-derived MAGs (Fig. 8C). DsrA and DsrB are typical symbols of microbes mediating dissimilatory sulfate reduction [4]. Therefore, we propose dissimilatory sulfate reduction might be often adopted by the members of Chloroflexi in the hydrothermal vents. Nonetheless, Chloroflexi bacteria should be important participants in sulfur cycling in the deep-sea environments, given their high abundance in both cold seep and hydrothermal vents.

Discussion

Microorganisms in the deep marine subsurface sediments represent a large unexplored biosphere, exploring and resolving their metabolisms are essential to understand the global biogeochemical cycles [59-61]. Despite the global importance of these microorganisms, deep-sea sediments are among the least understood environments, partly due to the difficulty of sampling as well as the complexity of inhabiting communities [59]. With this, the majority of deep-sea microbial diversity remains uncultured, hampering a more thorough understanding of their unique biology [3,59,62]. One of the striking characteristics of these uncultured lineages is that most of them are dominant population, for example, the large proportion of uncultured

499 microbes was estimated to make up more than 75% of sediment genera [63].
500 Therefore, it is crucial to increase our capability for bringing microorganisms from
501 the environment into culture [60], which will advance our understanding of their
502 global biogeochemical cycles [48]. Among the uncultured majorities, the phylum
503 Chloroflexi is ubiquitous and often abundant in sediments, soils and wastewater
504 treatment systems, as well as in deep-sea extreme environments [64].

505 Indeed, our OTUs sequencing results clearly show that the abundance of the
506 phylum Chloroflexi is both the second most in the domain Bacteria that living in the
507 cold seep and hydrothermal vents sediments (Figs. 1A and 1B). Although Chloroflexi
508 bacteria are widespread on Earth, the puzzling thing is the extreme difficulty to
509 culture Chloroflexi members from various environments, leading to a poor
510 understanding with regard to their unique metabolisms that endowing them with
511 tremendous vitality. Therefore, it is an urgent need to obtain more uncultivated
512 isolates for better resolving their diversity and ecological roles, especially from the
513 deep-sea environments given their potentials to participate in sulfur cycling [4,28].
514 When we looked through the previously reported characteristics of Chloroflexi
515 isolates, one of the striking features attracted our attention: most cultured Chloroflexi
516 could tolerate a high concentration of rifampicin (50 μg/mL) [13,23]. It is known that
517 rifampicin is an effective inhibitor of DNA-dependent RNA polymerase and inhibits
518 the growth of many bacteria [23]. Therefore, in the present study, we developed an
519 effective enrichment method by keeping a constant rifampicin pressure in the

enrichment and isolation medium (Fig. 2A). Indeed, we successfully obtained a novel Chloroflexi isolate, strain ZRK33, from the cold seep samples (Fig. 2). Strikingly, strain ZRK33 possessed a very fast growth rate (4 h for doubling time) compared to other reported Chloroflexi isolates (6 h to 19 days for doubling time) (Supplementary Table S2), providing a great advantage for us to promptly perform various assays. Overall, we strongly recommend the researchers to use rifampicin as a selection pressure to enrich and culture novel isolates of Chloroflexi in the future.

Additionally, we proposed strain ZRK33 as a representative of a novel class of the phylum Chloroflexi. The reasons are as following: (1) strain ZRK33 showed only ~82% 16S rRNA gene identity with other cultured isolates, which meets the proposed thresholds for median (86.35%) and minimum (80.38%) sequence identity values to build a novel class [58]; (2) the phylogenetic analyses based on the genome (Supplementary Figure S1), beta RpoB (Supplementary Figure S2) and EF-Tu (Supplementary Figure S3) all support the classification of strain ZRK33 as the type strain of a new class; (3) strain ZRK33 is facultatively anaerobic, however, the Anaerolineae class bacteria are obligately anaerobic (Supplementary Table S2)[13], even though the novel clade shows the highest identity with the Anaerolineae class. Taken together, we propose strain ZRK33 together with *Aggregatilinea lenta* MO-CFX2^{T} to represent a novel class of Chloroflexi phylum, though strain MO-CFX2^{T} was thought to be a representative of a novel order of the class Anaerolineae [13].

Notably, we find that strain ZRK33 contains a complete set of genes associated with assimilatory sulfate reduction (Fig. 3B), providing potentials to involve into sulfur cycling. Therefore, we name this novel isolate as *Sulfochloroflexus methaneseepsis* ZRK33, which belonging to Sulfochloroflexia classis nov., Sulfochloroflexales ord. nov., Sulfochloroflexaceae fam. nov.. The cycling of sulfur is a dominant metabolism pathway for the marine subsurface microorganisms [26,65], and deep-sea Chloroflexi bacteria were predicted to respire oxidized sulfur compounds [4] and metabolize multiple organosulfur compounds [28] based on metagenomics data. However, to date, no studies based on the pure culture have verified that Chloroflexi members indeed drive sulfur cycling of deep-sea environments. Take advantage of pure cultivation of *S. methaneseepsis* ZRK33, we verified its actual involvement of both sulfate and thiosulfate assimilatory processes (Fig. 5), and the sulfur assimilatory greatly facilitates the growth and morphogenesis of ZRK33 via promoting the transport and metabolization of saccharides and other organic matter (Fig. 6). However, strain ZRK33 only responds to high concentrations of sulfate and thiosulfate (200 mM), given the high concentrations of different sulfur-containing compounds [25,26] existing in the cold seep and some microbes possessing a capability to enrich sulfur-containing compounds (such as elemental sulfur and polysulfide [26,66]), we speculate this phenomenon is possible to happen in the deep-sea cold seep sediments.

Most importantly, large portion of the genes associated with assimilatory or dissimilatory sulfate reduction are widely distributed in the Chloroflexi MAGs derived from deep-sea cold seep and hydrothermal vents (Fig. 8), which strongly suggesting Chloroflexi bacteria are key players in the sulfur cycling of deep biosphere. In combination with the reports that the other two Chloroflexi lineages (SAR202 group and Dehalococcoidia class) possessing potentials to drive sulfur metabolizations, it is reasonable to affirm the phylum Chloroflexi greatly contributes to the ocean sulfur cycling. Actually, we tried to check the metabolisms of strain ZRK33 that cultured in the deep-sea cold seep as performed previously [48], unfortunately, the cells of strain ZRK33 were invaded by some unknown microbes that leading the failure of *in situ* test toward its involvement of sulfur cycling. We are improving the experimental apparatus and procedure, which will greatly benefit us to check the central metabolisms of strain ZRK33 *in situ* in the near future.

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586 Author contributions

587 RZ and CS conceived and designed the study; RZ conducted most of the experiments;
588 RL, YS and GL collected the samples from the deep-sea cold seep; RC helped to
589 analyze the metagenomes; RZ and CS lead the writing of the manuscript; all authors
590 contributed to and reviewed the manuscript.

591 Conflict of interest

592 The authors declare that there are no any competing financial interests in relation to
593 the work described.

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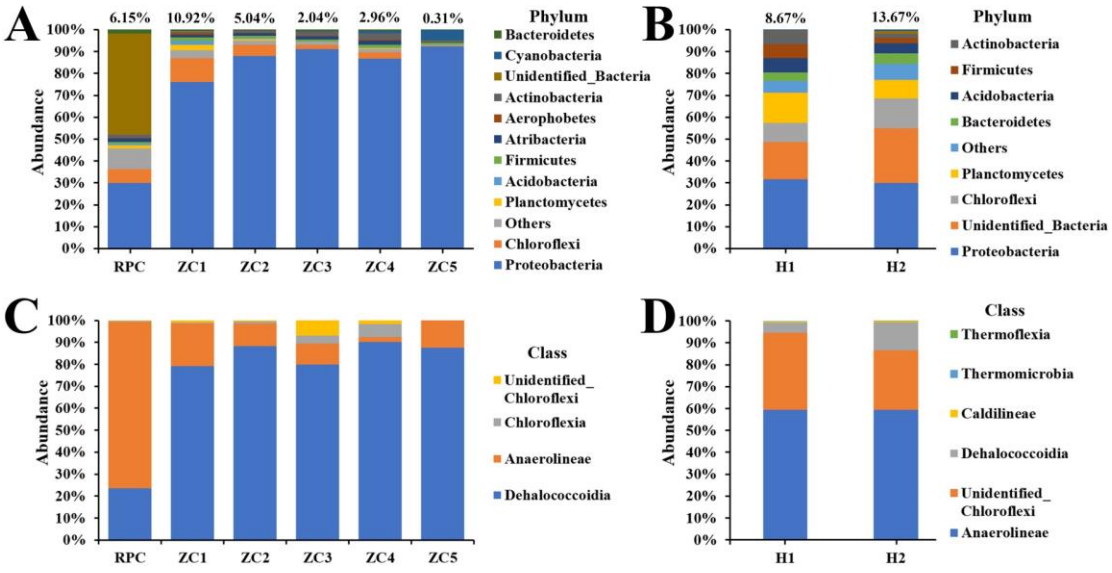
804 # Figure



805

806 **Fig. 1.** Detection of the abundance of the phylum Chloroflexi derived from the
807 deep-sea cold seep and hydrothermal vents sediments. The community structure of six
808 sampling sites in the cold seep sediments and two sampling sites in the hydrothermal
809 vents sediments as revealed by 16S rRNA gene amplicon profiling. The relative
810 abundances of operational taxonomic units (OTUs) representing different bacteria are
811 shown at the phylum level (A and B) and class level (C and D). Panels A and C
812 represent samples from the cold seep; Panels B and D represent samples from the
813 hydrothermal vents.

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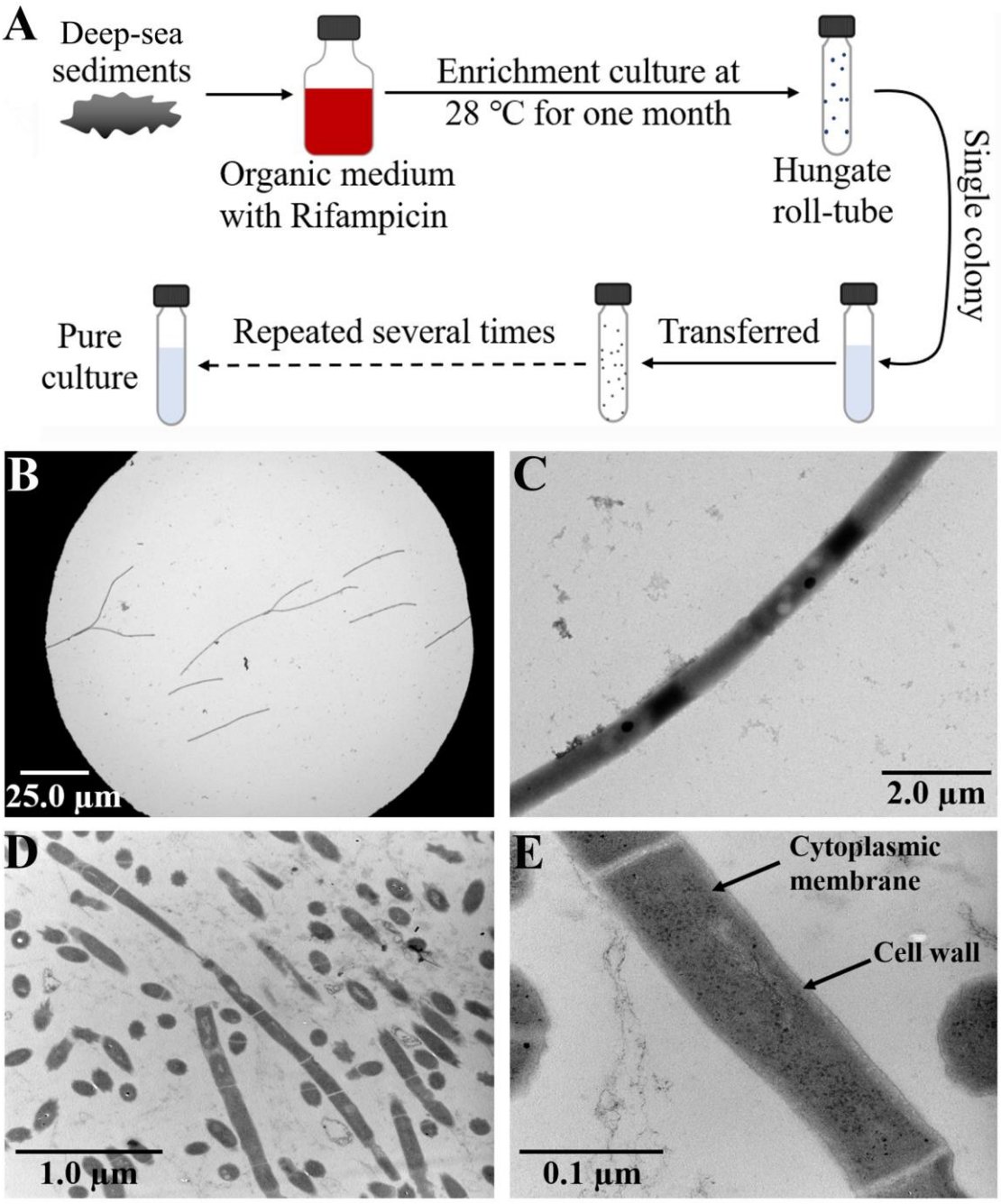


819

820 **Fig. 2.** Rifampicin resistance-driven enrichment and isolation strategy of Chloroflexi
821 bacteria. (A) Diagrammatic scheme of enrichment and isolation of Chloroflexi
822 bacteria. (B, C) TEM observation of strain ZRK33. (D, E) TEM observation of the
823 ultrathin sections of strain ZRK33.

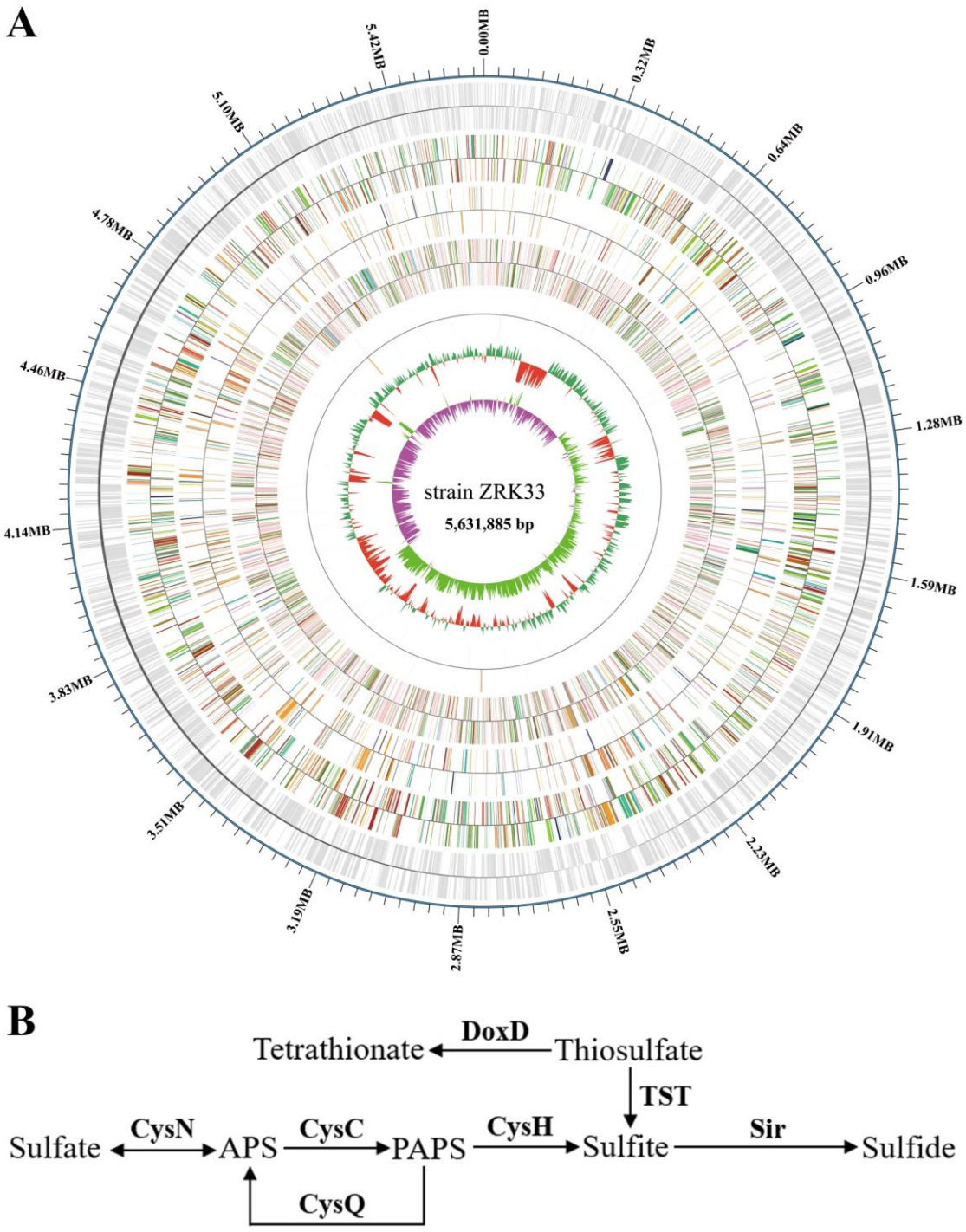


Fig. 3. Genomic analysis of strain ZRK33. (A) Circular diagram of the genome of strain ZRK33. Rings indicate, from outside to the center: a genome-wide marker with a scale of 320 kb; forward strand genes, colored by COG category; reverse strand genes, colored by COG category; gene function annotation (COG, KEGG, GO, NR, CAZy, TCDB); RNA genes (tRNAs blue, rRNAs purple); GC content; GC skew. (B)

Proposed assimilatory sulfate reduction pathway identified in the genome of strain ZRK33. Abbreviations: CysN, sulfate adenylyltransferase; Sat, sulfate adenylyltransferase; CysC, adenylyl-sulfate kinase; CysQ, 3', 5'-bisphosphate nucleotidase; CysH, phosphoadenosine phosphosulfate reductase; Sir, sulfite reductase; TST, thiosulfate/3-mercaptopyruvate sulfurtransferase; DoxD, thiosulfate dehydrogenase (quinone) large subunit; APS, adenosine 5’-phosphosulfate; PAPS, 3’-phosphoadenosine-5’-phosphosulfate (3’-phosphoadenylylsulfate).

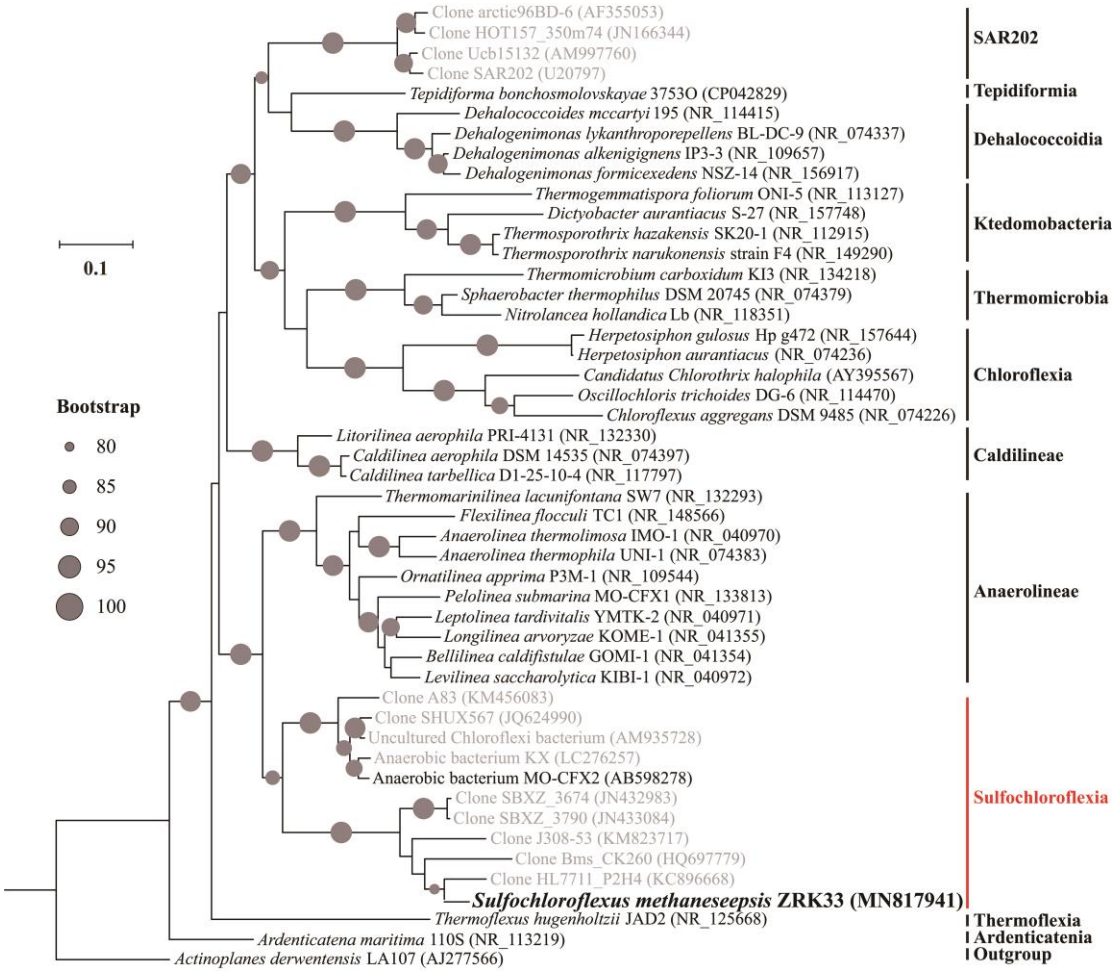
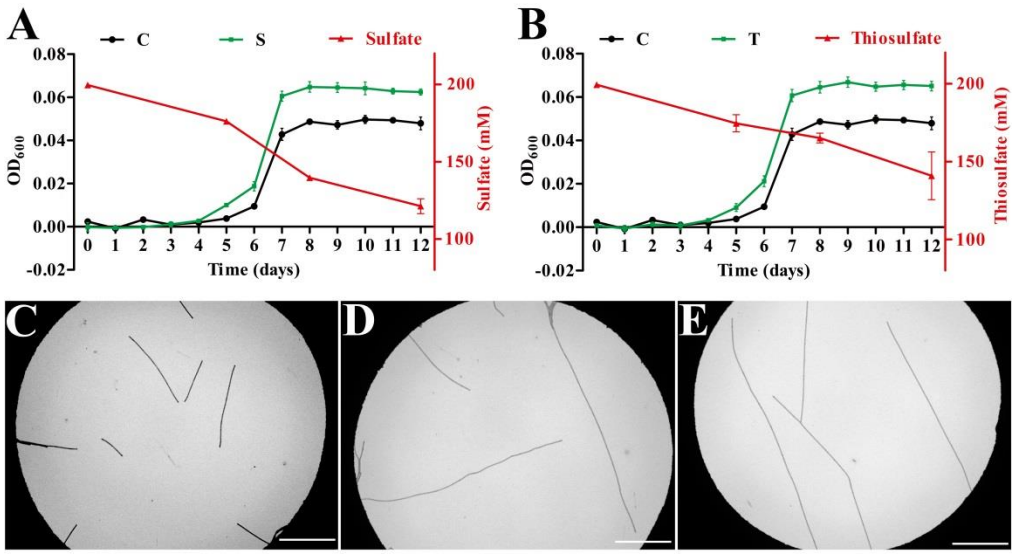


Fig. 4. Phylogenetic analysis of *Sulfochloroflexus methaneseepsis* ZRK33. Phylogenetic placement of strain ZRK33 within the phylum Chloroflexi based on almost complete 16S rRNA gene sequences. The tree is inferred and reconstructed under the maximum likelihood criterion and bootstrap values (%) > 80 are indicated at the base of each node with the black dots (expressed as percentages of 1,000 replications). Names indicated with grey color in quotation represent taxa that are not yet validly published. All sequences are labeled with their NCBI accession numbers. The 16S rRNA gene sequence of *Actinoplanes derwentensis* LA107^{T} is used as an outgroup. Bar, 0.1 substitutions per nucleotide position.



858 **Fig. 5.** Verification of sulfate and thiosulfate assimilation in *S. methaneseepsis*
859 ZRK33. (A) Growth assay and sulfate metabolization of strain ZRK33 cultured in the
860 medium supplemented without or with 200 mM Na_2SO_4. (B) Growth assay and
861 thiosulfate metabolization of strain ZRK33 cultured in the medium supplemented
862 without or with 200 mM $Na_2S_2O_3$. "C" indicates the control group, where strain
863 ZRK33 was cultured in the medium supplemented without extra Na_2SO_4 or $Na_2S_2O_3$;
864 "S" indicates the sulfate-treated group, where strain ZRK33 was cultured in the
865 medium supplemented with 200 mM Na_2SO_4; "T" indicates the thiosulfate-treated
866 group, where strain ZRK33 was cultured in the medium supplemented with 200 mM
867 $Na_2S_2O_3$. The black lines represent the growth curves of the control group; the green
868 lines represent the growth curves of experimental groups; the red lines represent the
869 variation tendency of concentrations of Na_2SO_4 or $Na_2S_2O_3$. (C) TEM observation of
870 strain ZRK33 that cultured in the ORG medium. (D) TEM observation of strain
871 ZRK33 that cultured in the ORG medium supplemented with 200 mM Na_2SO_4. (E)
872 TEM observation of strain ZRK33 that cultured in the ORG medium supplemented
873 with 200 mM $Na_2S_2O_3$. The bar is 20 μm in the panels C, D and E.

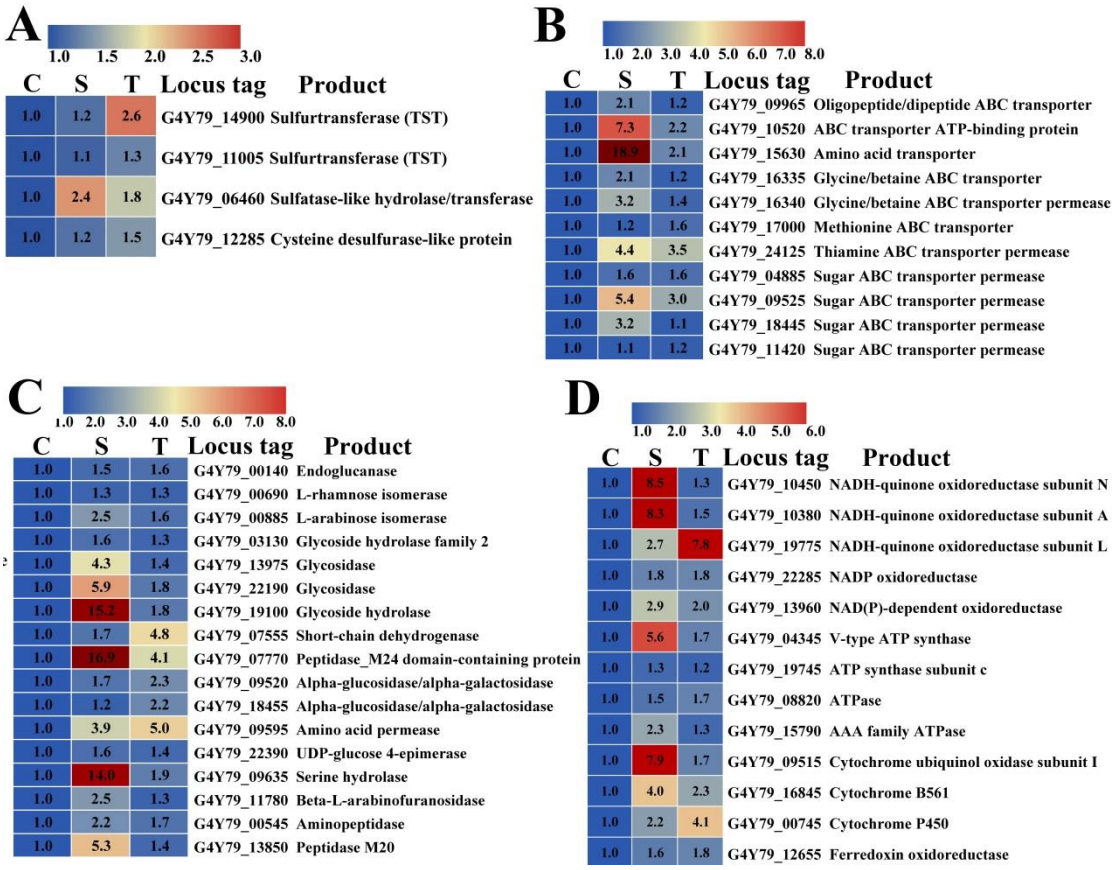
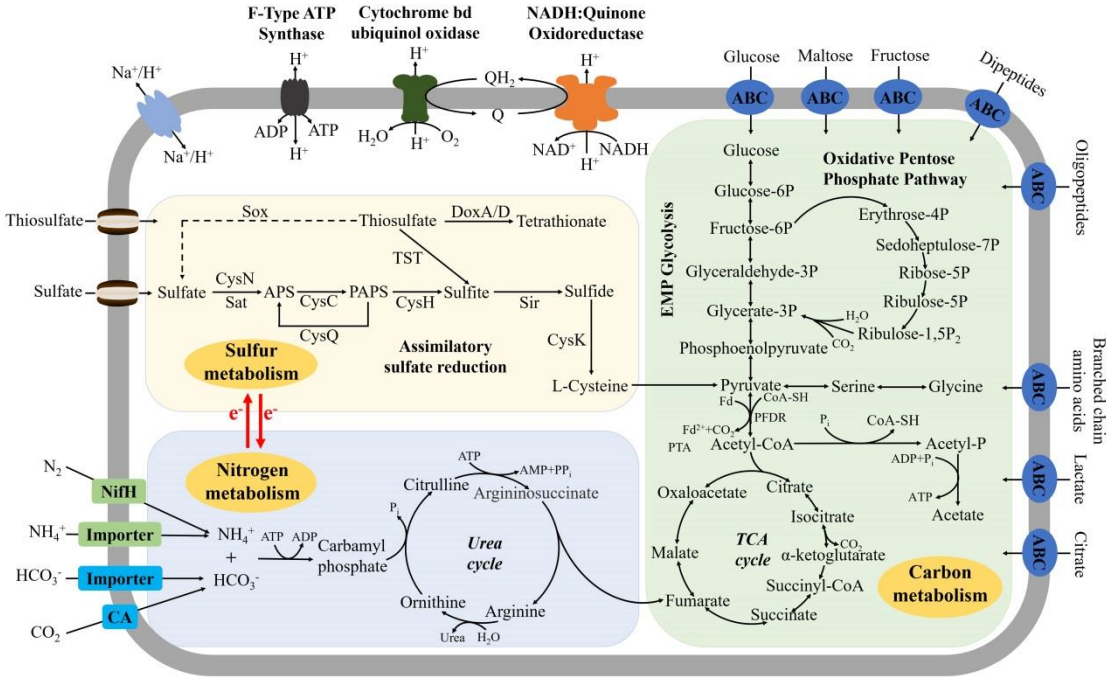
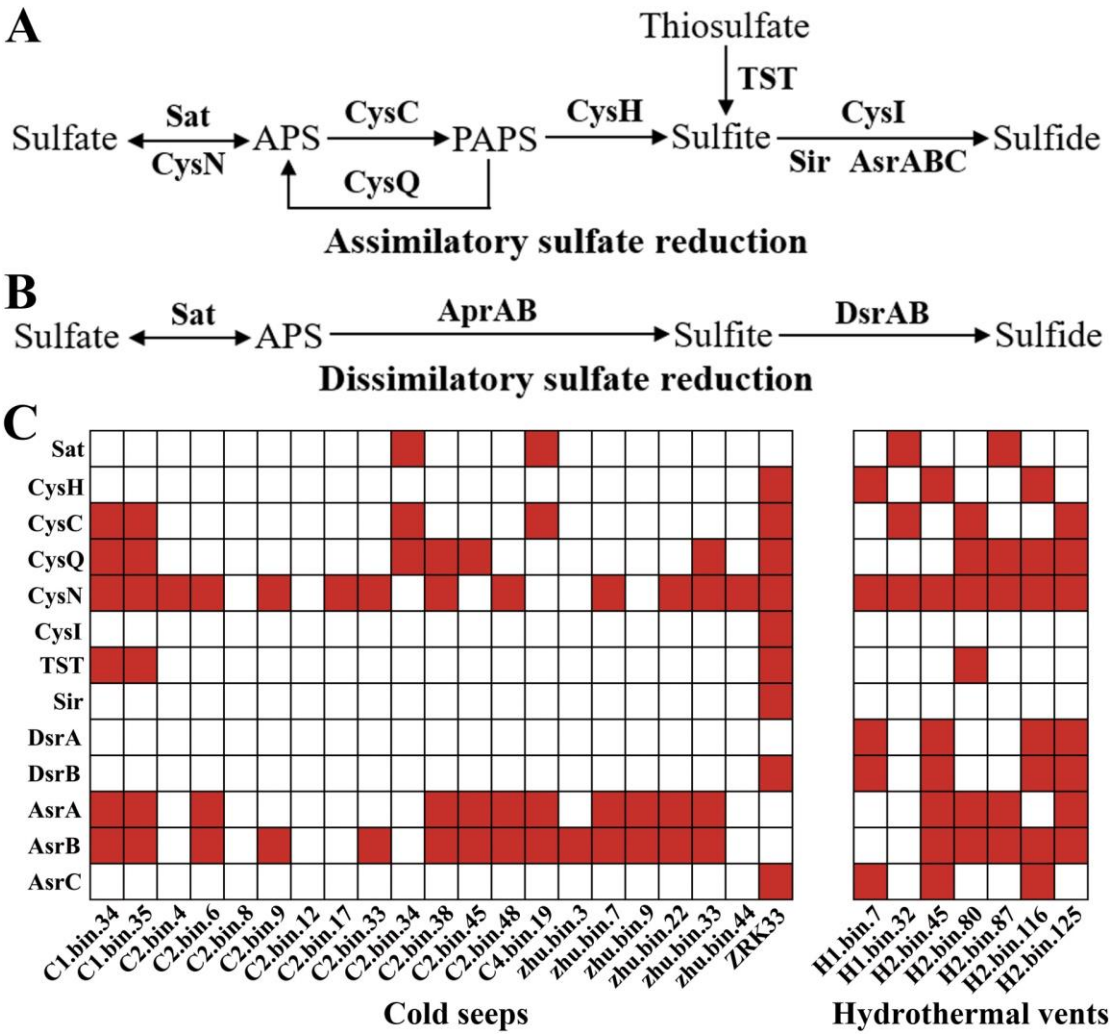


Fig. 6. Proteomic analysis of *S. methaneseepsis* ZRK33 cultured in the medium supplemented with sulfate and thiosulfate. (A) Proteomics based heat map showing all up-regulated proteins associated with sulfur metabolism. (B) Proteomics based heat map showing all up-regulated proteins associated with amino acids and sugar transporters. (C) Proteomics based heat map showing all up-regulated proteins associated with saccharides/amino acids/peptides hydrolases. (D) Proteomics based heat map showing all up-regulated proteins associated with energy production. "C" indicates the control group, where strain ZRK33 was cultured in the medium supplemented without extra Na_2SO_4 or $Na_2S_2O_3$; "S" indicates the sulfate-treated group, where strain ZRK33 was cultured in the medium supplemented with 200 mM Na_2SO_4; "T" indicates the thiosulfate-treated group, where strain ZRK33 was cultured in the medium supplemented 200 mM $Na_2S_2O_3$.



886 **Fig. 7.** Muti-omics based central metabolisms model of *S. methaneseepsis* ZRK33. In
887 this model, three central metabolic pathways (associated with carbon, sulfur and
888 nitrogen cyclings) including EMP glycolysis, oxidative pentose phosphate pathway,
889 TCA cycle, urea cycle, assimilatory sulfate reduction and some electron transport
890 systems are shown and highlighted with different colors. All the above items are
891 closely related to the energy production in *S. methaneseepsis* ZRK33. Abbreviations:
892 TCA, tricarboxylic acid cycle; Urea, urea cycle; ATP, 5'-Adenylate triphosphate;
893 ADP, adenosine diphosphate; AMP, adenosine monophosphate; CA, carbonic
894 anhydrase; NifH, nitrogenase iron protein; Q, quinone; QH_2, ubiquinone; CysN,
895 sulfate adenylyltransferase; Sat, sulfate adenylyltransferase; CysC, adenylyl-sulfate
896 kinase; CysQ, 3', 5'-bisphosphate nucleotidase; CysH, phosphoadenosine
897 phosphosulfate reductase; Sir, sulfite reductase; CysK, cysteine synthase; TST,
898 thiosulfate/3-mercaptopyruvate sulfurtransferase; DoxA, thiosulfate dehydrogenase
899 (quinone) small subunit; DoxD, thiosulfate dehydrogenase (quinone) large subunit;
900 Sox, L-cysteine S-thiosulfotransferase.



901

902 **Fig. 8.** Broad distribution of genes encoding key enzymes driving assimilatory and
903 dissimilatory sulfate reduction pathways in the metagenome-assembled genomes
904 (MAGs) of Chloroflexi bacteria derived from deep-sea cold seep and hydrothermal
905 vents sediments. (A) Typical pathway of assimilatory sulfate reduction existing in
906 bacteria. (B) Typical pathway of dissimilatory sulfate reduction existing in bacteria.
907 (C) Distribution of genes encoding key enzymes involved in assimilatory and
908 dissimilatory sulfur metabolisms in deep-sea Chloroflexi MAGs and strain ZRK33.
909 The presence of enzymes involved in the sulfur metabolic pathway is indicated by
910 using red colored rectangles. Sat, sulfate adenylyltransferase; CysN, sulfate

911 adenylyltransferase; CysC, adenylyl-sulfate kinase; CysQ, 3', 5'-bisphosphate
912 nucleotidase; CysH, phosphoadenosine phosphosulfate reductase; Sir, sulfite
913 reductase; AsrA, AsrB and AsrC, anaerobic sulfite reductases; CysI, sulfite reductase
914 (NADPH) hemoprotein beta-component; TST, thiosulfate/3-mercaptopyruvate
915 sulfurtransferase; AprA and AprB, adenylylsulfate reductase; DsrA and DsrB,
916 dissimilatory sulfite reductase.