Metagenomics Revealing Molecular Profiling of Microbial Community Structure and Metabolic Capacity In Bamucuo, Tibet

Cai Wei

Shanghai Ocean University

Dan Sun

Shanghai Ocean University

Wenliang Yuan

Jiaying University

Lei Li

Fudan University

Chaoxu Dai

Shanghai Ocean University

Zuozhou Chen

Shanghai Ocean University

Xiaomin Zeng

Central South University Xiangya Public Health School

Shihang Wang

Shanghai Ocean University

Yifan Tang

Hunan Normal University School of Medicine

Shouwen Jiang

Shanghai Ocean University

Zhichao Wu

Shanghai Ocean University

Xiaoning Peng

Hunan Normal University School of Medicine

Linhua Jiang

Fudan University

Sihua peng (✉ shpeng@shou.edu.cn)

Shanghai Ocean University <https://orcid.org/0000-0001-7231-666X>

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Abstract

Background: The Qinghai-Tibet Plateau (QTP) is the highest plateau in the world, and the microorganisms there play vital ecological roles in the global biogeochemical cycle; however, detailed information on the microbial communities in QTP is still lacking.

Results: Here, we performed a landscape survey of the microorganisms in Bamucuo, Tibet, resulting in 160,212 (soil) and 135,994 (water) contigs by shotgun metagenomic methods, and generated 75 nearly complete metagenome-assembled genomes (MAGs). *Proteobacteria*, *Actinobacteria* and *Firmicutes* were found to be the three most dominant bacterial phyla, while *Euryarchaeota* was the most dominant archaeal phylum. Surprisingly, *Pandoravirus salinus* was found in the soil microbial community. The KEGG annotations showed that the genes related to metabolism accounted for 62.9% (soil) and 58.4% (water), respectively. Of the 75 MAGs, 63 were found to contain the genes related to all the six carbon fixation pathways, and the heavy metal resistance genes, the pressure response subsystem, and the secondary metabolite biosynthesis gene clusters (BGCs) were discovered to be abundant.

Conclusions: We concluded that the microorganisms in Bamucuo fix carbon mainly through the 3-hydroxypropionic bi-cycle pathway; this study, for the first time, characterized the microbial community composition and metabolic capacity in QTP high-altitude areas (with an altitude of 4,555 meters), confirmed that QTP is a huge and valuable resource bank in which more new non-resistant antibiotics and many other bioactive substances could be developed, and provided the expanded information for further microbial community studies in QTP.

Background

The Qinghai-Tibet Plateau (QTP) is the highest plateau on the earth and is known as the "third Pole" of the world, which is one of the most important water resources in East Asia and plays a vital role in regulating global climate change [1, 2]. Over the past 50 years, QTP has been warming at more than twice the global average [2], and the increase of temperature leads to the increase of soil carbon emissions. QTP is generally a carbon sink, but in the near future, warming may have a significant impact on the net ecosystem carbon balance of the ecosystem, which will gradually weaken the function of QTP carbon sink [3, 4]. There are many microorganisms in QTP, which is one of the most important life forms in the extreme environments. Through various metabolic pathways, the microorganisms form the basis of global carbon, nitrogen and various nutrient cycles [5, 6], and affect the global climate change to a certain extent, especially to the emission of the greenhouse gases (CO₂, CH₄, N₂O) [7–9]. However, up to now, the diversity information and metabolic potential of the microbial communities in QTP are still lacking. To address this issue, we initiated this research by using metagenomics to increase the understanding of the interactions between the microorganisms and the environments.

With the development of sequencing technology, it is now possible to obtain information from unculturable microorganisms through metagenomics [10]. In recent years, the related studies have mainly

focused on the permafrost and the Qaidam Basin, which is located at an altitude of 3,000 meters or below.

The permafrost in QTP is one of the most sensitive areas for climate warming. Due to its unique and fragile characteristics, the diversity of the microbial communities has become the focus of most research [11–14]. Han et al. revealed the structure and diversity of Keke Salt Lake microbial community [15]. Xing's research on the soil microbial community in Qaidam Basin showed that the environmental factors are the main driving force for the formation of bacterial community structure [16]. However, in QTP with an altitude of more than 4,000 meters, there are few environmental microbiology studies, which are mainly based on 16S Ribosomal RNA Gene Sequencing (16S) rather than shotgun metagenomic sequencing [11, 13–17]. Compared with shotgun metagenomic sequencing, 16S has obvious deficiencies: 1) it cannot fully detect the abundance of the microbial community; 2) it can only assign microorganisms to the genus level, but not to specific species; and 3) it only sequence some fragments of the certain regions of the bacterial genome, and the whole microbial genome information cannot be obtained [18, 19].

Shotgun metagenomic sequencing improves the accuracy of species detection, not only providing a stronger and more reliable assessment of microbial diversity, but also providing valuable information on the metabolic potential of microbial communities [20–23]. The draft genomes reconstructed from the datasets can be used to understand the potential mechanisms of microbial adaptation to the extreme environments and their relationship with climate change [24, 25].

Bamucuo, not far from its sister lake Namcuo, is located in QTP, with an altitude of 4,555 meters. The lake is mainly fed by surface runoff surrounding. Here we used shotgun metagenomics to investigate the microbial community in Bamucuo. To the best of our knowledge, this study is the first shotgun metagenomics study of the environmental microbial community at an altitude of more than 4,000 meters in QTP.

Materials And Methods

The overall analysis process in this study is shown in Fig. 1.

Sampling site and sample collection

The soil and water samples involved in this study were collected at Bamucuo (31°34'N, 90°6'E), at an altitude of 4,555 meters, Bango County, Nagqu Prefecture, Tibet, China, on June 20, 2017. We collected 30L water sample at depth of 0.5m through a filter membrane with a pore diameter of 0.22μm, and the water temperature was 13°C when sampling. The ambient temperature was 19°C. The soil sample was collected from 0.2m below the surface, and then stored in ice boxes for bringing back to the laboratory. For simplicity, in this article, we refer to these two examples as s05-02 (soil) and s05-03 (water).

DNA extraction and sequencing

According to the manufacturer's instructions, genomic DNA was extracted using OMEGA's DNA extraction kit (<http://www.omegabiotek.com.cn/>, Guangzhou, China) and some modifications were made in the experimental procedure. The purity and concentration of the extracted genomic DNA were measured on NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, United States), and the quality of the extracted genomic DNA was detected by 1% agarose gel electrophoresis. The total genomic DNA was stored in a -20 °C refrigerator. Then, the genomic DNA was sent to Shanghai Meiji Biomedical Technology Co., LTD for sequencing. The length of the library was 300bp, and Hiseq 4000 high-throughput sequencer was used for sequencing.

Metagenomic assembly and gene abundance analysis

The raw reads were processed using fastp v. 0.19.5 [26] for removing adapter sequences at the 3' and 5' terminal, and the reads with the length less than 50 bp after shearing, the reads with an average quality value (Phred value) less than 20, and the reads containing N bases were all removed. FastQC v. 0.11.9 [27] was used to perform the sequence quality controlling. The assembly module of MetaWRAP v. 1.2.1 [28] was then used for assembling, which include the assembly software MEGAHIT v 1.1.3 [29], setting the parameters as follows: minimum contig length of 1000 bp, and k-mer sizes of 21, 29, 39, 59, 79, 99, 119 and 141, respectively. BMap v. 38.87 (<https://sourceforge.net/projects/bbmap/>) was used to map clean reads to each contig for calculating the coverage information. Prokka v. 1.14.6 [30] was used to annotate the assembled sequence, with all the parameters to be the default values. Salmon v. 1.3.0 [31] was then used to estimate the genetic abundance for each sample.

Species classification and functional annotation

We integrated the annotation results obtained by Kraken2 v. 2.1.1 [32] and Bracken v. 2.6.0 [33], viewing the results of the species annotation by Krona v. 2.7.1 [34].

Using Diamond v. 2.0.5 [35] as a aligner, and blastp v. 2.5.0+ [36] alignments ($e\text{-value} \leq 1E\text{-}5$) were performed against Non-Redundant Protein Sequence Database (NCBI-nr), Carbohydrate-Active Enzyme Database (CAZy) [37], Pathogen-Host Interactions Database (PHI) [38], Virulence Factors Database (VFDB) [39], and Biocide and Metal Resistance Genes Database (BacMet) [40], respectively. Rgi v. 5.1.1, based on Comprehensive Antibiotic Resistance Database (CARD) [41], was employed to predict the resistomes. The Kyoto Encyclopedia of Genes and Genomes (KEGG) [42] annotation was performed using KAAS online analysis website [43]. Clusters of Orthologous Groups of Proteins (COG) [44] and Gene Ontology (GO) [45–47] functional annotation were performed using emapper v. 2.0.1 [48] based on the eggNOG orthology data [49], and then Web Gene Ontology Annotation Plot (WEGO v. 2.0) [50] was used to classify the GO functions.

Metagenomic binning

All the data of the two samples were binned to generate the draft genomes with the binning module in MetaWRAP [28], in which three modules (MetaBAT2 [51], MaxBin2 [52], and CONCOCT [53]) were selected to perform the binning operation separately to obtain three different binning results, setting the minimum

contig length to be 1500. CheckM v 1.0.12 [54] was employed to calculate the completeness and contamination [55, 56]. Then, bin_refinement module in MetaWRAP was used to obtain the bins with the completeness of more than 70% and the contamination of less than 10% (parameter: -c 70 -x 10), and these bins were called metagenome-assembled genomes (MAGs).

By using the MetaWRAP pipeline [28], the clean reads of the each sample were mapped to the assembled MAGs by using the quant_bins module; the relative abundance of all the MAGs was estimated; a preliminary gene annotation for each MAG was obtained by using the annotate_bins module; the distribution of the contigs in each bin was visualized by the blobology module.

Taxonomic classification and phylogenetic analysis

GTDB-tk v. 1.3.0 [57] “classify” workflow was used to conduct species classification and to calculate the ANI values of the MAGs based on Genome Taxonomy Database (GTDB) [58, 59]. For phylogenetic analysis, we first searched the GTDB database to find the corresponding reference genomes which are closest to each MAG (Table S1), and then used GTDB-tk [57] “align” method to perform multiple sequence alignments of the marker genes in the MAGs and their reference genomes. After importing the above alignment results, the “infer” method (FastTree v. 2.1.10) [60] was used to construct a phylogenetic tree under the Whelan-And-Goldman (WAG) model, with the parameters to be set to “default”. Finally, the annotation information was refined using ITOL v. 5.7 [61].

Metabolism analysis

The carbon degradation enzymes were identified using dbCAN [62]. We examined the KEGG [42] biochemical maps for the six known carbon fixation pathways (reductive acetyl-CoA, 3-hydroxypropionate/4-hydroxybutyrate cycle, 3-hydroxypropionate bi-cycle, dicarboxylate/4-hydroxybutyrate cycle, reductive tricarboxylic acid cycle, and calvin cycle [55, 63]) and identified the genes encoding the enzymes involved in methanogenesis and methane oxidation; the genes related to nitrogen and sulfur metabolism were investigated (Table S2). The percentage of the number of the annotated genes to the total number of the genes is used to characterize the completeness of the pathway.

Microbial stress response and heavy metal resistance analyses

Diamond [35] was used to perform the alignments for all the MAGs (e-value < 1E-5) against BacMet Database [40]. Then, the 75 MAGs were submitted to Rapid Annotation using Subsystem Technology (RAST v. 2.0) [64] to obtain the functional annotations of the SEED subsystem [65, 66].

BGCs and ARGs identification

The MAGs were submitted to the Anti-SMASH v. 5.2.0 [67, 68] web version to screen the secondary metabolite biosynthetic gene clusters (BGCs) of these draft genomes, with the model set to 'strict'. Rgi [41] was used to predict the resistomes for the MAGs.

Result

Quality controlling and metagenome assembling

Shotgun sequencing produced 29,623,109,936 bp and 24,284,563,524 bp of unassembled raw bases from the soil (s05-02) and water (s05-03) samples (Table 1), respectively. In total, 194,202,138 (98.99%) and 159,527,496 (99.19%) reads passed the quality control in the soil and water sample dataset, respectively (Table 1).

After assembling, a total of 160,212 (soil) and 135,994 (water) contigs were generated (Table 1). For the two samples, the longest contigs were 833,193 bp and 286,311 bp, GC content were 60.18% and 51.33%, and N50 length were 2,289 bp and 3,841 bp, respectively.

After Prokka [30] annotation, 351,242 genes were obtained from the soil sample and 390,943 genes from the water sample (Table 2).

Metagenome-assembled genomes (MAGs)

The CheckM [54] analysis showed that 75 MAGs were reconstructed with the completeness > 70% and contamination < 10%, including 18 from the soil sample and 57 from the water sample (Fig. 2a and Fig. 2b). A total of 20 MAGs met the high-quality standard with the completeness > 95% and contamination < 5%, accounting for 26.67% of all the MAGs. Table S3 lists the statistics of the 75 bins including the completeness, contamination, GC value, N50, genomic size, and GTDB [58, 59] species classification. The original reconstructed MAGs have been reassembled by using `reassemble_bins` module in MetaWRAP [28] and their genomic integrities have been significantly improved (Fig. 2c and Fig. 2d).

Microbial community composition analysis

The microbial community composition of the soil sample was visualized by Krona [34] (Fig. 3). The results showed that bacteria accounting for 99.5% were dominant, with archaea accounting for 0.3% and viruses accounting for 0.08%. The three most abundant phyla in bacteria were *Proteobacteria* (54.0%), *Actinobacteria* (18.6%) and *Firmicutes* (9.8%), and another 29 phyla were identified (Table S4a), such as *Bacteroidetes* (8.9%), *Gemmatimonadetes* (3.3%), *Planctomycetes* (1.3%), and *Acidobacteria* (1.1%). For the most abundant phylum, *Proteobacteria*, 112 families (Table S4b) were identified, in which *Sphingomonadaceae* (34.9%) was the most abundant, followed by *Bradyrhizobiaceae* (7.1%), *Methylobacteriaceae* (6.3%), etc. At the genus level of the *Proteobacteria*, 408 genera were identified (Table S4c), in which *Sphingomonas* (22.9%) had the highest abundance, followed by *Sphingobium* (4.6%), *Methylobacterium* (4.4%), etc. Among the *Actinobacteria* (Table S4d), we identified 46 families, in which *Streptomycetaceae* (22.7%) was the most abundant, and at the genus level of the *Actinobacteria*, 141 genera were identified, in which *Streptomyces* (21.5%), *Conexibacter* (7.5%) and *Micromonospora* (5.6%) were the three most abundant genera (Table S4e). A total of 2,400 bacterial species were identified

(Table S5), including *Bacillus cereus* (5.1%), *Hymenobacter sedentarius* (4.5%), *Gemmatirosa kalamazoonesis* (3.0%), *Sphingomonas indica* (2.3%), *Lactococcus piscium* (1.6%), etc.

In the soil sample, *Euryarchaeota* (81.9%) accounted for the largest proportion in the archaea (Table S6a), and *Thaumarchaeota* (14.7%) was the second most dominant phylum, followed by *Crenarchaeota* (1.7%). Table S6b, c, d, e, and f list the detailed information for the archaea at the class, order, family, genus, and species level, respectively.

Eighteen species of viruses were identified in the soil sample (Table S7). Surprisingly, *Pandoravirus salinus* (15.3%), a giant virus, was the most abundant, followed by *Bacillus virus 250* (11.5%), *Mycobacterium phage Jobu08* (7.7%), etc.

In the water sample (Fig. 4), the bacteria accounted for the majority (99.1%). Only a small portion was archaea (0.4%), and the rest was viruses (0.5%). At the phylum level, *Proteobacteria* (54.4%), *Bacteroidetes* (17.9%) and *Actinobacteria* (15.1%) were the most abundant phyla, and another 30 phyla were identified (Table S8a). Among the *Proteobacteria*, 124 families (Table S8b) were identified, including three most abundant families: *Rhodobacteraceae* (42.6%), *Comamonadaceae* (14.8%) and *Pseudomonadaceae* (3.8%). Among the 464 genera identified in the *Proteobacteria* (Table S8c), *Yoonia* (22.4%) was the most abundant, followed by *Limnohabitans* (6.8%) and *Pseudomonas* (3.7%). For the *Actinobacteria*, 46 families (Table S8d) were identified, of which *Microbacteriaceae* (23.3%) was the most abundant, and 150 genera (Table S8e) were identified, of which *Streptomyces* (13.9%), *Corynebacterium* (5.6%) and *Pontimonas* (5.1%) accounted for the largest proportion. A total of 2,714 bacterial species (Table S9) were identified, in which *Yoonia vestfoldensis* (12.2%), *Belliella baltica* (5.3%), *Limnohabitans* sp. 63ED37-2 (3.6%), *Aquiflexum balticum* (1.8%) and *Cyanobium* sp. NIES-981 (1.2%) were dominant.

Among the archaea in the water sample, *Euryarchaeota* (98.8%) accounted for the largest proportion (Table S10). Table S10b, c, d, e, and f list the detailed information for the archaea at the class, order, family, genus, and species level, respectively.

In the water sample, a total of 52 species of viruses were identified (Table S11), in which the most abundant was *Chrysochromulina ericina virus* (12.7%), followed by *Phaeocystis globosa virus* (8.8%) and *Synechococcus phage S-PM2* (5.9%).

Phylogenetic analysis

A phylogenetic analysis for the 75 MAGs was performed. Initially, using CheckM [54] to classify the MAGs, 38 of the 75 MAGs (50.67% of the datasets) were not assigned to a specific taxonomic name below a domain. Subsequently, we further refined the classification of all the MAGs by using GTDB-tk [57], and bin5 from the soil sample with the 98.06% average nucleotide identities (ANIs) was even successfully classified to the species level. Of the 75 MAGs, 28 (37.33%) lacked a genus-level match, and only one (bin13 from the water sample) lacked a family-level match (Table S3).

The phylogenetic tree constructed with the MAGs (Fig. 5) showed that the 75 MAGs could be taxonomically assigned to 10 phyla, including main members: *Proteobacteria* (16 MAGs, five from the soil sample and 11 from the water sample), *Bacteroidota* (22 MAGs, two from the soil sample and the remaining 20 from the water sample), *Actinobacteriota* (17 MAGs, three from the soil sample and 14 from the water sample), etc. The microbial community composition of the soil and water sample was roughly similar, which may be due to the fact that sampling points of the two samples were very close. However, at the phylum level and below, the species composition of the two samples gradually showed differences.

Metagenomic functional annotation

For the two samples, a total of 232,679 and 294,919 genes were annotated against nr database, 104,704 and 117,467 genes against KEGG [42] database, and 45,638 and 59,921 genes against GO [46] database, respectively. More annotation results are shown in Table 2.

The COG [44] functional annotation results were visualized using ggplot2 (Fig. 6a). Except for function unknown (12.8%), the three most abundant categories in the soil sample were replication, recombination and repair (4.7%), translation, ribosomal structure and biogenesis (3.9%), and transcription (3.9%). In the water sample, except for function unknown (11.0%), translation, ribosomal structure and biogenesis (4.3%) was the most abundant, followed by replication, recombination and repair (3.6%), amino acid transport and metabolism (3.5%), etc.

WEGO [50] was used to show the three main categories (cellular component, molecular function and biological process) and the first two levels of the GO terms, with bar charts showing the number of the genes annotated. In the soil sample (Fig. 6b), the dominant category was biological process (35.5%), followed by molecular function (33.0%) and cellular component (31.5%). We found that among the biological processes, the top three GO terms were cellular process (33,656), metabolic process (32,600) and response to stimulus (8,678); among the molecular functions, the top three were catalytic activity (27,005), binding (17,692) and structural molecule activity (3,089); and among the cellular components, the top three were cell (33,716), cell part (33,716) and membrane (14,342). Whereas in the water sample (Fig. 6b), biological process (35.4%) was the dominant category, followed by molecular function (32.7%) and cellular component (31.9%). The top three GO terms were as follows: cellular process (45,113), metabolic process (43,200) and response to stimulus (12,611) among the biological processes; catalytic activity (35,860), binding (24,853) and structural molecule activity (4,247) among the molecular functions; and cell (45,101), cell part (45,101) and membrane (19,524) among the cellular components.

ggplot2 was used to display the annotation information at the first two levels of KEGG [42], with the bar graphs representing the number of the annotated genes for each metabolic pathway. For the soil sample (Fig. 6c), the genes related to metabolism (about 62.9% of the datasets) were the most abundant, and the rest were genetic information processing (9.7%), environmental information processing (9.4%), human diseases (7.3%), cellular processes (6.8%), and organismal systems (3.9%). Further analysis on the metabolism showed that the most abundant was carbohydrate metabolism (23.6%), followed by amino

acid metabolism (20.5%), metabolism of cofactors and vitamins (10.7%), energy metabolism (10.1%), etc.

Similarly, for the water sample (Fig. 6c), the most abundant metabolic function module was metabolism (58.4%), followed by human diseases (9.7%), environmental information processing (8.5%), cellular processes (6.4%), organismal systems (6.3%), and genetic information processing (6.2%). In metabolism, carbohydrate metabolism (24.5%) was the most abundant, followed by amino acid metabolism (20.7%), metabolism of cofactors and vitamins (10.6%), energy metabolism (9.7%), etc.

Metabolic potential analyses based on the MAGs

Carbon degradation: The 75 MAGs contained abundant hydrolases from the 101 glycoside hydrolases (GH) family (Table S12), among which the MAGs containing GH13 accounted for 77.3% (58 out of 75), and the MAGs containing GH3 and GH23 accounted for 76.0% (57 out of 75), respectively. The enzymes of interest included alpha-amylase, beta-amylase, glucoamylase, alpha-glucosidase, isoamylase and pullulanase (involved in starch degradation); xylanase, beta-mannanase, alpha-L-arabinofuranosidase, alpha-D-glucuronidase and beta-xylosidase (involved in hemicellulose degradation); endo-1,4-glucanase, exo- β -1,4-glucanases and β -1, 4-glucosidases (involved in cellulose degradation); and other carbohydrate degradation enzymes.

Carbon fixation: Carbon fixation capacity was examined for each of the 75 MAGs (Fig. 7). Almost all the MAGs had the genes encoding the enzymes involved in the 3-hydroxypropionate bi-cycle, reductive tricarboxylic acid cycle, and calvin cycle pathways (related enzymes are listed in Table S2). The two MAGs (bin2 and bin18 in the soil sample) possessed more than 50% complete 3-hydroxypropionate bi-cycle pathway. In the results, 27 MAGs (not listed) had one or more genes encoding the key enzymes, 2-oxoglutarate: ferredoxin oxidoreductase, pyruvate: ferredoxin oxidoreductase, and ATP-citrate lyase, of the reductive tricarboxylic acid cycle pathway. Although the calvin cycle pathway was relatively complete based on the KEGG modules, the key enzyme, ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO), was only identified in the two MAGs (bin9 in the soil sample and bin32 in the water sample), and another key enzyme of this pathway, phosphoribulokinase (PRK), was identified in the two MAGs (bin15 and bin44 in the water sample). Further, except for the two MAGs (bin9 in the soil sample and bin15 in the water sample), other MAGs had the genes encoding the enzymes involved in the reductive acetyl-CoA pathway, and most of the MAGs had the key enzyme, acetyl-CoA synthase, of this pathway. Except for bin12 in the water sample, 74 MAGs had the genes related to the dicarboxylate/4-hydroxybutyrate cycle pathway; the genes encoding the enzymes that related to the 3-hydroxypropionate/4-hydroxybutylate cycle pathway existed in 64 MAGs.

Methane metabolism: As we know, microbial methanogenesis and methane oxidation play essential roles in the biogeochemical cycles of the earth system. We found that, except bin54 in the water sample, almost all the MAGs contained genes related to the methanogenesis pathway, however, this pathway was incomplete. It can be seen from Fig. 7 that bin42 in the water sample possessed 17% complete methanogenesis pathway. The key enzyme of the methanogenesis pathway, methyl coenzyme M

reductase (MCR), was found in 17 MAGs (not listed). Furthermore, no enzyme related to the methane oxidation pathway was identified in all the MAGs in the two samples (Fig. 7).

Nitrogen metabolism: As shown in Fig. 7, the genes related to the assimilation nitrate reduction pathway were identified in eight MAGs, while 13 MAGs had the genes encoding the enzymes related to the dissimilation nitrate reduction pathway. Only one MAG had the gene encoding the key enzyme, respiratory nitrate reductase, of the dissimilation nitrate reduction pathway. Besides, the genes related to the nitrogen fixation pathway were identified in three MAGs (Fig. 7), while the nitrogenase genes (*nif*, *vnf* and *anf*) were not found. Four MAGs (Fig. 7) had the genes related to the denitrification pathway, while no MAG had the genes related to the nitrification pathway. There were only two MAGs which had the genes encoding the key enzyme, nitrite reductase, of the denitrification pathway.

Sulfur metabolism: For sulfur metabolism, 52 MAGs (Fig. 7) had the genes related to the assimilatory sulfate reduction pathway, while eight MAGs had the genes related to the dissimilatory sulfate reduction pathway and 12 MAGs had the genes related to the thiosulfate oxidation by SOX complex pathway. Among the 12 MAGs, the marker gene, *soxB*, used to analyze the thiosulfate oxidation by SOX complex pathway was found in the ten MAGs (bin1 in the soil sample and bin1, bin2, bin35, bin41, bin44, bin46, bin51, bin54, bin55 in the water sample).

Stress response and metal resistance analyses

We investigated the subsystems of all the MAGs, with the results showing that more than 19 types of the subsystem [65] categories were identified, including the three most abundant categories: carbohydrates, amino acids and derivatives, and protein metabolism (see Table S13 for more information). All the MAGs contained respiration, stress response, and virulence, disease and defense categories. However, there were only four MAGs (one *Planctomycetota* MAG and three *Proteobacteria* MAGs) containing photosynthesis category. All RAST [64] annotations showed that the highest subsystem coverage was 33.0%, and the lowest was 12.0%.

Both the samples contained many stress response subsystems (Table S14), of which the detoxification subsystems were found in all the MAGs, the oxidative stress subsystems were found in the abundant MAGs except for the two *Actinobacteriota* MAGs, and the osmotic stress subsystems were found in 54 MAGs. Besides, we found that two MAGs (one *Bacteroidota* MAG and one *Proteobacteria* MAG) in the water sample had proteins associated with cold shock (*CspA* family of proteins). These proteins are induced when the temperature drops, allowing cells to adapt to lower temperature [69].

The annotation for heavy metal resistance genes revealed diverse resistance genes that confer resistance to 19 heavy metals such as silver, copper, iron, nickel, zinc, and antimony, including *actP*, *copA*, *copB*, *silP*, *acn*, *ctpC*, *ziaA*, etc (related genes are listed in the Table S15). The results showed that more than 1,064 heavy metal resistance genes were identified in all the MAGs (Fig. 8), among which the heavy metal resistance genes conferring resistance to multi-metals were abundant. Furthermore, the genes conferring resistance to antimony, arsenic, molybdenum and tungsten have been identified in all MAGs.

Biosynthetic gene cluster (BGC) identification

By using Anti-SMASH [67], a total of 270 putative BGCs clusters of secondary metabolites were identified in the 75 MAGs, revealing that the two samples contained 25 types of BGCs such as terpene, T3PKS, NRPS, lanthipeptide, and bacteriocin (Table S16); there were 96 terpenes (35.56%) categories, 40 NRPS (14.81%) categories, 35 T3PKS (12.97%) categories, and 27 bacteriocin (10.00%) categories. We found that most NRPSs existed in the *Acidobacteriota* MAGs, for example, 25 of 40 NRPSs existed in the *Acidobacteriota* MAGs. Furthermore, terpenes were found in 61 of all the MAGs. Similarly, T3PKSs were found in many MAGs in the two samples, NRPSs were found in the *Acidobacteriota* MAGs and the *Bacteroidota* MAGs, and PKSs were found in the *Bacteroidota* MAGs and the *Actinobacteria* MAGs. Eleven bacteriocins were found in the *Cyanobacteria* MAG. Interestingly, some of the BGCs identified in this study were highly similar to known gene clusters, while the others were not closely related to any known gene clusters.

Antibiotics resistance gene (ARG) annotation

ARGs were found in 15 of the 75 MAGs, including *adeF*, *rpsL* (mycobacterium tuberculosis *rpsL* mutations conferring resistance to streptomycin), etc. Among these 15 MAGs, *adeF* existed in all the soil sample MAGs, and *ampS* existed in bin10. In the water sample, besides *adeF* and *ampS*, *rpsL* accounted for the majority (5 out of 8). The detailed ARGs annotation information is shown in Fig. 9.

Discussion

Microbial community structure

Extreme environment is an ideal model to illustrate the adaptability of microorganisms [70]. To the best of our knowledge, our study, for the first time, reveals the composition of microbial communities in the surface water and nearshore soil in Bamucuo, Tibet. We found that the dominant bacterial phyla of the soil and water microbial communities were *Proteobacteria*, *Actinobacteria* and *Firmicutes* (Fig. 3 and Fig. 4), which were also found to be dominant phyla in other areas in QTP such as Qaidam Basin [16] and Qinghai Lake [71]. As the dominant bacteria we found in this study, *Proteobacteria* plays an essential role in various energy metabolism in QTP [16, 72], and at the class level of the *Proteobacteria*, *Alphaproteobacteria* was the most prevalent in Bamucuo microbial community, with the most common genus belonging to *Sphingomonas* (22.4%) in the soil microbial community (Table S4c) and *Yoonia* (22.9%) in the water microbial community (Table S8c); however, a study on Lake Meyghan reported that *Gammaproteobacteria* is the most abundant class of *Proteobacteria* [73]. We believed that this is a unique feature of the microbial composition in Bamucuo because of the differences in environmental conditions (such as altitude). *Actinobacteria* is the main source of new antibiotics and bioactive molecular candidates [74]. However, many of the existing antibiotics used by human have developed drug resistance [75]. Therefore, the discovery of many *Actinobacteria* in the water and soil microbial communities in Bamucuo may become important natural resources to develop new antibiotics.

Firmicutes, one of the abundant phyla in Bamucuo, can also be found in desert [76] and deep sea sediment [77] due to their spores, so our study confirmed that *Firmicutes* could adapt to various extreme environments including QTP.

Furthermore, we confirmed that *Euryarchaeota* is the most representative archaea in the Bamucuo microbial community in QTP, and similar observation in Lonar soda Lake in India was reported by Chakraborty et al. [78], suggesting that the phylum *Euryarchaeota* can adapt to the most extreme environmental conditions. However, *Thaumarchaeota* and *Crenarchaeota* were only identified in the soil sample in Bamucuo, suggesting *Thaumarchaeota* and *Crenarchaeota* might be more suitable for survival in the soil.

Philippe et al. reported that *Pandoravirus*, the largest known virus to date, was isolated from the sediments of the estuary of central Chile and the freshwater pond near Melbourne (Australia) [79]. However, in our research, *Pandoravirus salinus* was found in the nearshore soil, and this finding proved that the habitat of *Pandoravirus* may not only be in water.

In phylogenetic analysis (Fig. 5), we observed that in the same habitat, the species diversity of water microbial community is greater than that of soil microbial community. In summary, as a special extreme environment, the microbial community in Bamucuo has different characteristics from other extreme environments such as deep sea and desert.

Carbon metabolism

Microorganisms play very important roles in carbon fixation [80]. We found that the majority of the microbial community possessed relatively complete carbon fixation pathways (Fig. 7), suggesting that the microbial community in Bamucuo obtains energy through carbon fixation by autotrophic bacteria. Although many different carbon fixation pathways were found in both the samples, the 3-hydroxypropionate bi-cycle (9.5%-52.4%), calvin cycle (11.8%-52.9%) and reductive tricarboxylic acid (2.3%-37.2%) pathways were the most complete (Fig. 7). Among them, the most represented carbon fixation pathway in the 75 MAGs was the 3-hydroxypropionate bi-cycle pathway, which is generally rarely found in most environments because of its high energy cost [81]. Interestingly, the abundant genes related to the 3-hydroxypropionate bi-cycle pathway were found in all the MAGs, and the key enzymes, acetyl-CoA carboxylase and propionyl-CoA carboxylase, of this pathway were present in most MAGs, suggesting that the 3-hydroxypropionate bi-cycle pathway may be dominant under extreme environmental conditions, such as low oxygen, low pressure, low temperature and high salinity. The calvin cycle pathway is the main CO₂ fixation pathway, which is proved to be widely present in green plants and many autotrophic bacteria in most environments, such as trench [82]. However, even if the calvin cycle pathway was high complete, its key enzymes were lacking in our results. Compared with the calvin cycle pathway, the reductive tricarboxylic acid pathway has lower energy consumption, which was found in certain autotrophic mesophilic bacteria and archaea [81]. Our results showed that 27 MAGs had the key enzymes in the reductive tricarboxylic acid pathway, indicating that the microorganisms in Bamucuo may prefer to use this pathway instead of the calvin cycle pathway. In addition to the most

common three carbon fixation pathways mentioned above, the three less common pathways were the reductive acetyl-CoA (0–30.0%), dicarboxylate/4-hydroxybutyrate (0–39.1%), and 3-hydroxypropionate/4-hydroxybutyrate cycle (0–27.8%) pathways. The reductive acetyl-CoA pathway, which requires strict anoxic conditions, plays a role in both psychrophiles and hyperthermophiles [81]. At least 73 MAGs had the key enzyme, acetyl-CoA synthase, of the reductive acetyl-CoA pathway (Fig. 7), but the completeness of this pathway is low. The reductive acetyl-CoA pathway was found to be the most abundant carbon fixation pathway in deep biosphere [55, 83], however, our results suggested that the microorganisms in Bamucuo may not use this pathway. The 3-hydroxypropionate/4-hydroxybutyrate cycle and the dicarboxylate/4-hydroxybutyrate cycle pathways existed in thermophilic archaea, and the 3-hydroxypropionate/4-hydroxybutyrate cycle seems to be the dominant pathway in anoxic marine zones [84], which is different from our results.

Therefore, the 3-hydroxypropionate bi-cycle pathway is the preferred methods for microorganisms to fix inorganic carbon into organic carbon in Bamucuo.

Methane metabolism

In methane metabolism, CO_2 is converted to CH_4 . CH_4 is the second largest greenhouse gas, accounting for about 17% of global warming impact [85], while CO_2 accounting for about 60% [86]. The results showed that the most representative features of our samples are the low completeness of the methanogenesis pathway and the unpredicted methane oxidation pathway (Fig. 7). In the methanogenesis pathway of microbial communities, the methane was generated through the key enzyme, methyl-coenzyme M reductase (MCR), which was identified both in the KEGG [42] annotation results of the MAGs and the metagenomic data, whereas the study on Pangong Lake reported the absence of *MCR* [87]. In the contigs of the two samples, the existence of methanogens, such as *Methanobacterium*, *Methanosarcina* and *Methanocaldococcus*, suggested that methanogens can survive in the hypoxic environment in Bamucuo and are crucial in the methane production.

Methane oxidation is an important process to slow down CH_4 emission [88], which is performed by CH_4 oxidizing bacteria (methanotrophs). We found that methanotrophs, such as *Methylomonas*, *Methylochromium*, and *Methylococcus*, existed in the two samples. However, in the KEGG [42] annotation results, the key functional genes for methane oxidation were not found, which may be due to insufficient sequencing depth.

Nitrogen cycle

Because the MAGs possessed the incomplete nitrogen metabolism pathways, the nitrogen metabolism might rarely occur in energy metabolism in Bamucuo, Tibet. Nitrogen loss in QTP is mainly related to denitrification [89]. In our study, there are few genes related to the denitrification pathway. However, denitrifying bacteria, such as *Pseudomonas* and *Bacillus*, were identified in the metagenomic data of both the samples, indicating that denitrification with nitrate as the terminal electron acceptor is a rare but indispensable pathway to remove nitrogen in Bamucuo. Nitrification is a key process in biogeochemical

nitrogen cycling, which oxidizes ammonia to nitrate via nitrite [90]. In our result, nitrosifier (such as *Nitrosomonas* and *Nitrosococcus*) and nitrifying bacteria (such as *Nitrobacter* and *Nitrospina*) were low abundant, and the nitrification pathway was not identified, suggesting that nitrification in the surface water and soil microbial communities was relatively rare. However, Rasigraf et al. reported that nitrification and denitrification are the main processes in the Bothnian Sea sediment [91], which is contradictory to our results. It was previously reported that *Proteobacteria* plays an important role in the nitrogen cycle [92], and our results also confirm that the nitrogen fixation pathway has only been found in the *Proteobacteria* MAGs. In addition, the assimilatory nitrate reduction pathway and the dissimilatory nitrate reduction pathway were more abundant than other pathways of nitrogen cycle, indicating that these two pathways are relatively common in the microbial communities in Bamucuo, which is similar to the results reported by Rathour et al. [87].

Sulfur cycle

The microbial sulfur cycle has been proposed as the driving force for bacterial survival [93]. The sulfur-reducing bacteria (e.g., *Desulfobacterales*, *Desulfovibrionales*, *Desulfotomaculum* and *Desulfosporosinus*) in Bamucuo, which can perform anaerobic respiration utilizing sulfate (SO_4^{2-}) as the terminal electron acceptor for reducing sulfate to hydrogen sulfide (H_2S), were found in the two samples; similar results in Pangong lake were reported by Rathour et al. [87]. The assimilative sulfate reduction pathway was originally reported in plants, but it has been shown to exist in certain bacteria, such as *Allochromatium vinosum* [94]. Our results showed that the assimilatory sulfate reduction pathway was relatively complete (Fig. 7) in Bamucuo, while the key functional gene for dissimilatory sulfate reduction, dissimilatory sulfite reductase, had not been identified, which indicated the lack of microorganisms that perform the functions of dissimilatory sulfate reduction.

The sulfur-oxidizing bacteria such as *Sulfuritalea* and *Thiobacillus* were present with a low abundance, and few genes related to the thiosulfate oxidation by SOX complex pathway were identified (Fig. 7). However, the key gene, *soxB*, related to the thiosulfate oxidation by SOX complex pathway was found in most of the MAGs which possessed this pathway, so we believed that sulfur oxidation is important in the energy metabolism of the microorganisms in Bamucuo.

Stress response and metal resistance

The subsystem annotation showed that the abundant presence of certain stress response subsystems, such as osmotic stress, oxidative stress, and cold shock (Table S14), could help the microorganisms to withstand various stress conditions in QTP such as temperature, salinity, hypoxia, and low pressure. Koo et al. reported that almost the entire cold-shock family of proteins exists in Lake Untersee in Antarctica [95], however, the cold shock pathway was rarely found in our study, it may be due to the higher temperature during sampling.

The microbial community is the main driving force for metal resistance gene distribution, and a previous study showed that these genes are found mainly in the environments contaminated with heavy metals

[96]. However, in our metagenomic data, abundant heavy metal resistance genes were identified (Fig. 8). A study supported that the pollution of heavy metals in QTP has been on the rise in recent years [97]. In addition, rainwater and runoff may cause microorganisms to accumulate in the lake, being more conducive to the enrichment of heavy metal resistance genes in the water and nearshore soil microbial communities.

A previous study on Yellow River have reported that heavy metals can induce DNA damage and oxidative stress [98]. Our study showed that the DNA repair genes (not listed), such as *recB*, *recC*, *recD*, and *recF*, were found in most of the MAGs, which is obviously the result of the microbial community in Bamucuo adapting to the environment with the abundant heavy metals.

BGCs and ARGs

Among the secondary metabolites of microorganisms, special attention is always paid to antibiotics. In recent years, some pathogens have developed "drug resistance" to certain antibiotic drugs, and the number of new antibiotics has steadily decreased [75]. Therefore, there is a urgent need to find new resources to obtain new antibiotic drugs. Obviously, QTP is the appropriate candidate because of less human activity. Many BGCs were identified in both the samples (Table S16), suggesting that there may be many novel natural product biosynthetic gene clusters in Bamucuo.

Terpenes are the largest and the most diverse natural compounds, and are widely distributed in the genomes of many plants, fungi and bacteria [99]. In our study, the content of terpenes was the highest (35.56%) (Table S16), indicating that terpenes have the potential to become a new source to develop natural products, however, until now, little is known about their ecological functions in bacteria. Non-ribosomal peptide synthases (NRPSs) and polyketide synthases (PKSs) that we pay more attention to are two families of modular mega-synthases [100], and NRPSs (14.81%) and PKSs (15.93%) were the most abundant types of BGCs except for terpenes (Table S16), which produce compounds such as antibiotics, antifungals, immunosuppressants, and iron-chelating molecules [101]. Many NRPS and PKS gene clusters were found in the *Actinobacteria* MAGs, *Acidobacteria* MAGs and *Bacteroidetes* MAGs, suggesting that besides *Actinobacteria*, *Acidobacteria* and *Bacteroidetes* may also be the potential natural product biosynthetic producers in Bamucuo. In summary, QTP can be a valuable resource bank for discovering new secondary metabolite clusters.

Antibiotic resistance gene is a new pollutant with mobility [102]. Our results showed that few ARGs were identified (Fig. 9) in the soil and water microbial communities in Bamucuo. However, studies in other regions reported that abundant ARGs have been found [78, 103]. Therefore, we inferred that Bamucuo has not yet been extensively contaminated by the ARGs. Our study is of great significance in developing new antibiotics without resistance. Interestingly, *rpsL* was only identified in the *Actinobacteriata* MAGs (Fig. 9), indicating that *Actinobacteriata* may be the source of *rpsL* in Bamucuo. Related research also supports that some ARGs found in pathogenic bacteria come from *Actinobacteriata* that produce antibiotics [104].

Conclusion

In this study, we investigated the microbial ecosystem of Bamucuo in QTP. The results showed that the water and soil in Bamucuo have unique microbial community structure and metabolic process. The presence of *Pandoravirus salinus* in the soil provides important information for further exploring this unique microorganism and discovering more new giant virus. We concluded that the 3-hydroxypropionate bi-cycle pathway is the most representative carbon fixation pathway in Bamucuo; the contribution of the microorganisms in Bamucuo to the nitrogen cycle, sulfur cycle and methane cycle is very small. Since *Actinobacteria* was one of the dominant bacteria, QTP can become a valuable resource bank for developing new and non-resistant antibiotic drugs and other bioactive substances. This study provides many draft genomes and the genome annotation information for further in-depth study of the microbial ecology in QTP. Future studies are needed to combine microbial metabolomics data and geochemical data to address the relationship between environmental factors and microbial metabolism, which will help us to clarify the impact of microbial ecological change in QTP on global warming.

Declarations

Acknowledgements

Not applicable

Authors' contributions

S.H.P., X.N.P., and L.H.J. conceived of the study. C.W., D.S., L.L., and W.L.Y. participated in the study design. C.W., D.S., C.X.D., and S.H.W performed the wet-lab experiments. C.W., D.S., W.L.Y., Z.Z.C and Y.F.T. processed the raw data and performed the computational analyses. S.W.J. and Z.C.W designed the sampling scheme and guided the process of DNA extraction and metagenome sequencing. S.H.P and X.M.Z. guided the pipelines of all the analyses. C.W. wrote the first draft of the manuscript. S.H.P., X.N.P., and L.H.J. reviewed and revised the manuscript. All authors reviewed and approved the final version of the manuscript.

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Raw data availability

All the raw sequencing data were deposited in the NCBI Sequence Read Archive (SRA) under accession numbers SAMN17838283 (soil sample-05-02) and SAMN17838284 (water sample-05-03). The metagenomic sequence data is bundled under NCBI BioProject number PRJNA700111.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1 Summary of the sample information and the metagenomic sequencing results

	s05-02 (soil)	s05-03 (water)
Longitude	31.34	31.34
Latitude	90.60	90.60
Raw reads	196,179,536	160,824,924
Raw bases (bp)	29,623,109,936	24,284,563,524
Clean reads (%)	194,202,138(98.99%)	159,527,496 (99.19%)
Clean bases (%)	29,290,018,832(98.88%)	24,062,066,782(99.08%)
Contigs Num	160,212	135,994
Total length (bp)	356,933,003	397,377,554
Max length (bp)	833,193	286,311
N50 (bp)	2,289	3,841
GC content (%)	60.18	51.33
Average coverage (%)	31.45	45.70
Percent of reference bases covered (%)	99.90	99.92

Table 2 Summary of functional gene annotations against different databases in the two samples

Database	s05-02 (soil)	s05-03 (water)
Predicted genes	351,242	390,943
nr	232,679	294,919
KEGG	104,704	117,467
COG	211,757	222,635
GO	45,638	59,921
CAZy	52,427	51,940
VFDB	45,976	45,547
BacMet	21,273	18,305
PHI	59,516	60,782
CARD	11	12

Figures

Figure 1

Analysis flow chart in this study. MAGs: metagenome-assembled genomes; BGC: Biosynthetic gene cluster; ARG: Antibiotics resistance gene. Sup Tab 1 denotes Table S3, Sup Tab 2 denotes Table S4, etc. The detailed information of the 11 databases in the figure can be referred to the reference list.

Figure 2

The evaluations of binning and reassembled MAGs. a (s05-02, soil), and b (s05-03, water): The distribution of the contigs in each bin after refining. The abscissa represents the GC content of the contigs, and the ordinate represents the contig abundance, with one dot representing one contig. The contigs in same color belong to the same bin. c (s05-02) and d (s05-03): An evaluation of the bins after

Figure 4

The overall composition of the microorganisms in the water. The figure shows the most abundant taxa, with the remaining categories to be omitted.

Tree scale: 1

Figure 5

Phylogenetic tree of 75 reconstructed metagenome-assembled genomes (MAGs). The maximum likelihood evolutionary tree is reconstructed for all the 75 MAGs and their nearest neighbor reference genomes. The MAGs of s05-02 were represented by the red background, and the MAGs of s05-03 by the green background. High quality MAGs, namely MAGs with completeness > 95% and contamination < 5%, are marked with a star sign, and the phyla to which they belong were distinguished in outer rings in different colors.

Figure 6

Visualization of functional genes annotation (a) The relative abundance of the COG [44] functional categories of the two samples. The red bars represent s05-02 (soil), and the green bars represent s05-03 (water). (b) GO distribution analysis of the two samples. The horizontal axis indicates the GO [46] functional classifications, with the red bars representing s05-02, and the grey bars representing s05-03. All the 2-level GO terms are arranged in three main categories (cellular component, molecular function

and biological process). (c) The number of the genes annotated at the first two levels KEGG [42] pathway. S05-02 is displayed on the left and s05-03 on the right. The horizontal axes represent the number of the genes, and the vertical axes represent the metabolic pathways.

Figure 7

KEGG [42] annotation of energy metabolism for the two samples. The heatmap shows the percentage of the associated genes. The orange boxes represent carbon fixation; the red boxes represent methane

metabolism; the blue boxes represent nitrogen metabolism; the green boxes represent sulfur metabolism. Here, we investigated six carbon fixation pathways, the methanogenesis and methane oxidation pathways, nitrogen metabolism pathways, and sulfur metabolism pathways.

Figure 8

Heavy metal resistance genes identified in the two samples. The black squares represent the presence of the related genes, and the blank squares represent the absence of the related genes. V: the resistant

genes for vanadium; Mg: the resistant genes for magnesium; Hg: the resistant genes for mercury; Te: the resistant genes for tellurium; Se: the resistant genes for selenium; Ni: the resistant genes for nickel; Sb: the resistant genes for antimony; As: the resistant genes for arsenic; Mo: the resistant genes for molybdenum; W: the resistant genes for wolfram; Co: the resistant genes for cobalt; Cr: the resistant genes for chromium; Pb: the resistant genes for lead; Mn: the resistant genes for manganese; Cd: the resistant genes for cadmium; Fe: the resistant genes for iron; Ag: the resistant genes for silver; Cu: the resistant genes for copper; Zn: the resistant genes for zinc.

Figure 9

The circos of the annotation of MAGs by using CARD [41]. The ARGs information is on the left side of the peripheral ring, and the MAGs information is on the right side. The inner ring shows different MAGs and ARGs by different colors, and the scale represents the abundance information. The width of the band is proportional to the abundance of a particular ARG in each MAG.

Supplementary Files

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