Unraveling the microbiotas and key genetic contexts identified on stone heritage using illumina and nanopore sequencing platforms

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ABSTRACT

The application of metagenomics uncovering stone-dwelling microbiotas and its functional capabilities are of great significance for early warning of stone monuments deterioration and screening of antimicrobial agents. Here, by harnessing the combination of Nanopore and Illumina sequencing, we investigated the microbial community compositions and potential risks resulting from elemental nitrogen (N) and sulfur (S) metabolism and environmental resistomes. Taxonomic profiling showed that lichenized Ascomycota and Actinobacteria dominate the microbial population in the biofilm, followed by Cyanobacteria, Proteobacteria, and Acidobacteria. Certain microbial groups and gene families were responsible for the biogeochemical N/S cycling, probably contributing to the succession and expansion of stone biodeterioration. High abundant and diverse antibacterial, biocide and metal resistance genes were retrieved from assembled genome contigs, including those encoding resistance to copper (copR and copS), zinc (znuC/yebM and zraK/hydh), and quaternary ammonium compounds (gale and vcaM). Conversely, antibiotic resistance genes conferring resistance to multiple-antibiotic, aminoglycoside, and glycopeptide accounted for the relatively low percentage of total microbial metagenome. Binned genomes have further confirmed that bacterial species contained diverse antimicrobial resistance genes and mobile genetic elements, implying the possibility of horizontal gene transfer between bacterial lineages. Overall, our findings expand our knowledge of stone-dwelling microbiome and suggest appropriate treatment for stone biodeterioration.

1. Introduction

Various microbial taxa that grow in the form of biofilms on stone monuments pose a double-edged sword for cultural heritage conservation (Liu et al., 2022b). Among them, researchers are more concerned about their negative effects (Liu et al., 2020; Urzi, 2004), including discoloration, corrosion by biogenic acids, crystallization of secondary soluble salts, physical penetration, categorically called ‘biodeterioration’. Biochemical reaction is a more aggressive and invisible biodeterioration type, and this process typically involves the specifically active key microbes and biogeochemical cycles of nutrients such as nitrogen (N) and sulfur (S) cycling (Dakal and Cameotra, 2012; Ding et al., 2022; Zhang et al., 2019). A special class of microbial community members, including nitrifying bacteria (Mansch and Beck, 1998), ammonia-oxidizing bacteria/archaea (AOB/OA) (Ding et al., 2020, 2021), sulfur-oxidizing bacteria/fungi (Kusumi et al., 2011; Xu et al., 2018), and sulfate-reducing bacteria (Cappitelli et al., 2006) which can colonize stone surface and oxidize the N/S compounds to biogenic acids (nitrous acid, nitric acid, sulfuric acid, and sulfenic acid), eventually causing mineral constituents dissolution and stone structure destruction. In the N cycling, AOA and AOB oxidize NH₃ into nitrite and nitrate by nitrifiers through a two-step or a complete nitrification process (Daims et al., 2015; Stein and Klotz, 2016). In the S cycling, sulfur-oxidizing bacteria such as Sulfovorum and Thiobacillus attack stone materials by driving the S cycling from elemental S and S compounds to extremely corrosive sulfuric and sulfurous acids (Koch and Dahl, 2018), subsequently resulting in the formation of hydrated/dehydrate sulfate salts or black
and brown sulfated crust over the stone (Wang and Liu, 2021).

Prior researches based on rRNA gene next-generation sequencing revealed a high diversity of stone-dwelling microbiome (Meng et al., 2020; Ortega-Morales et al., 2019), and the key microbial genera can participate in a series of complex biogeochemical processes including elemental N and S cycling (Liu et al., 2018). Although these results allow describing microbial communities in detail and provide insights towards metagenome functional content (Louca et al., 2016), the amplification of ribosomal sequences does not provide direct evidence on any functional genes, and therefore the microbial functional capabilities remain uncertain. High-throughput sequencing-based metagenomics is a DNA sequencing-based technology that allow deep investigations of microbial communities and enable direct access to the entire genomic content and information (Quince et al., 2017), providing more specific biogeochemical descriptions. Information on functional genes delivers the microbial communities and enable direct access to the entire genomic content and information (Quince et al., 2017), providing more specific biogeochemical descriptions. Information on functional genes delivers the microbial communities and enable direct access to the entire genomic content and information (Quince et al., 2017), providing more specific biogeochemical descriptions.

2. Methods

2.1. Sample collection

Stone-dwelling biofilms were obtained from Leshan and Feilaifeng stone heritages, which are important components of Leshan Giant Buddha (https://whc.unesco.org/en/list/779) and West Lake (https://whc.unesco.org/en/list/1334) UNESCO World Cultural Heritage sites in China, respectively. Epilithic biofilms were collected from five outdoor exposed stone monuments, two of which belong to the Leshan Giant Buddha with signs of green (YS) and black (YR) biofilms colonization, and the other three belong to Feilaifeng with signs of lichen (ML and LG) and microalgae (RB) colonization (Fig. S1). Four samples from each site were collected and mixed, and all the mixed samples were transported to lab under low temperature and then immediately frozen and stored at −40 °C before DNA extraction.

2.2. DNA extraction and Illumina sequencing

All samples were sent to Biomarker Technology Co., Ltd, (Beijing, China) for metagenomic and diversity studies. DNeasy PowerSoil Pro Kit was used to extract total DNA according to the manufacturer’s protocols, and the obtained DNA concentration and purity was quantified with Qubit 3.0 Fluorometer and Nanodrop 2000 spectrophotometer, respectively. Metagenomic sequencing was performed on the Illumina NovaSeq 6000 platform (150 bp paired-end reads). For each Illumina dataset, clean reads were obtained after the filtration of raw tags using fastp and elimination host contamination by bowtie 2 (v2.2.4). Clean reads were assembled using MEGAHIT (v1.1.2) to remove contigs shorter than 300 bp, and QUAST (v2.3) was performed to evaluate the assembly data. Gene prediction was performed by MetaGeneMark (v3.26, http://exon.gatech.edu/meta/mghmmp_cgi) with default parameters (parameters -A -D -f G) (Zhu et al., 2010). Redundancy was removed by Mmseq2 (V12-113e3, https://github.com/soedinglab/mmseqs2) when genes share greater than 90% overlap and greater than 95% identity. The data were deposited into the NCBI SRA database under accession number PRJNA949285.

2.3. Nanopore library preparation and sequencing

The Nanopore library preparation was carried out using the PromethION platform (ONT, Oxford, UK) with the OGT 1D ligation sequencing kit (SQK-LSK109). Sequencing was performed on the PromethION platform using R9 flow cells (FLO-PR0002) for 72 h. MinKNOW software was used to collect raw sequencing data, and fast 5 files were subsequently basecalled to FASTQ format using Guppy (v4.2.2) with the recommended parameter settings, where adapters and low-quality or short-length reads (~2000 bp) were filtered and trimmed. NanoStat (De Coster et al., 2018) was carried out to assess the quality of the final clean long reads, and the preprocessed clean reads were assembled using Flye (v2.8.3, https://github.com/fenderglass/Flye). ucsm (https://github.com/isovic/racon) and Pilon (https://github.com/broadinstitute/pilon) were used to correct errors in Nanopore long-read assemblies with Illumina short reads (Vaser et al., 2017; Walker et al., 2014). QUAST (v2.3) was performed to evaluate the quality of the final assembly data. Redundancy was removed by cd-hit (Version 4.6.6, http://www.bioinfomartcs.org/cd-hit/) when genes
sharing greater than 90% overlap and greater than 95% identity. All the base-called Nanopore datasets were deposited into the NCBI SRA data base (PRJNA949288).

2.4. Taxonomy profiling, functional annotation, and binning

Each non-redundant gene was taxonomically annotated against the NR databases of NCBI at the cutoff of 10^{-5} E-value for classification of microbial community at different taxonomic levels (Buchfink et al., 2015). The annotation was performed against a curated integrative database (NCycDB, https://github.com/qichao1984/NCyc) (Tu et al., 2019) to obtain the gene corresponding to the N cycling. Functional database of the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2004) was used to annotate the functional genes corresponding to the biogeochemical S cycling. Antibacterial biocide and metal resistance genes were identified by a BLAST search against BacMet database (Pal et al., 2014). ARGs were identified by running RGI against Comprehensive Antibiotic Resistance Database (CARD) at the cutoff of 10^{-7} E-value, and those of ARGs-related mobile genetic elements were subjected to a blast search against a combined database from the ACLAME database (plasmids, prophages and viruses, http://aclame.ub.ac.be) and ISfinder (inserting sequences, https://www-is.biotoul.fr) (He et al., 2022).

For genome binning, MetaBat2 and Maxbin2 were used to bin probable genomes from the combination of Nanopore and Illumina sequencing reads using default parameters. DAS tool (https://github.com/cmkgs/das_tool) was used to aggregate genomes from each treatment, and genome bins were evaluated by CheckM. In general, genomes with completeness >80% and contamination <10% were selected for downstream analyses. All high-quality bins were dereplicated using dRep (Olm et al., 2017) and repetitive sequence were predicted by RepeatMasker (http://www.repeatmasker.org) against Repbase database. The closest taxonomic annotation of dereplicated high-quality bins were conducted by GTDB-Tk (https://github.com/Ecogenomics/GTDDBTk/).

3. Results

3.1. Nanopore and Illumina sequencing read statistics

The combination of Nanopore and Illumina sequencing greatly improved the quality of the metagenomic contigs for the five microbial samples. In detail, a total of on average 4.6 × 10^7 Illumina-based reads and 1.6 × 10^6 Nanopore-based reads (with a max length of 304,680 bp and an average length of 8510 bp) per sample assembled on average 10,207 contigs totaling 3.1 × 10^9 bp (with a largest length of 6.1 × 10^6 bp). By contrast, using only Illumina reads and Nanopore reads led to an average of 542,843 and 9390 contigs, respectively. Of note, longer length of total assembled sequences is observed as compared to the assemblies obtained using Illumina reads alone. The combination of Nanopore and Illumina sequencing had an average N50 of 137,765 bp (range from 101,937 bp to 215,568 bp), much longer than those contigs assembled using Illumina short reads (Tables S1 and S2).

3.2. Composition of microbiota associated with stone deterioration

A total of 2.3 × 10^8 high quality reads were obtained from 5 samples with a range of 4.1 × 10^8 to 5.3 × 10^8 reads per samples. These sequences were assigned to bacteria, fungi, and archaea, accounting for an average of 47.2%, 19.2%, and 0.1%, respectively. Fungi are generally more abundant in samples LG, ML, and YS, accounting for 34%, 32.3%, and 26.5%, respectively. Bacteria accounted for 20.5%–89.1% of the total microbiome, with the highest abundance in sample RB and the lowest in sample LG (Fig. 2a). Ascomycota and Actinobacteria were the two dominating phyla, accounting for an average of 18.3% and 14.7%, respectively (Fig. 2b). In sample RB, it was found that Cyanobacteria
(49.8%) was also predominant phylum. Ascomycota was the predominant fungal phylum, which account for 0.84% of total abundance. In samples LG, ML, and YS, *Umbilicaria* was the predominant genus with the relative abundance of 7.8%, 8.8%, and 5.2% respectively (Fig. 2c), constitute a major component of lichen. The other dominated bacteria were grouped into 7 genera, namely *Sphingomonas*, *Pseudonocardia*, *Actinomycetospora*, *Rubrobacter*, *Nocardioides*, *Leptolyngbya*, and *Nostoc*. *Leptolyngbya*, *Nostoc*, and *Cyanothece* were the most abundant cyanobacteria in sample RB, accounting for 5.3%, 3.2%, and 2.3% respectively, which constitute the main component of green biofilm. At the species level, lichen-forming fungus *Umbilicaria* was mainly annotated as *Umbilicaria pustulat*, and *Actinomycetospora* was mainly annotated as *Actinomycetospora cinnamomea* (Fig. 2d). *Microcoleus* sp. PCC 7113, *Leptolyngbya* sp. IPPAS B-1204, *Leptolyngbya frigida*, and *Pleurocapsa minor* are the predominant cyanobacteria species in sample RB. The combination of Nanopore and Illumina sequencing enables generation of long sequence read lengths, allowing better classification at the genus and species levels (Matsuo et al., 2021). At the genus level (Fig. S2a), samples YR and RB were dominated with *Escherichia*, with relative abundance of 11.6% and 9.1%, respectively. Streptomyces was recognized in all five samples, with relative abundance ranging from 3.1% to 5.0%. In sample RB, *Nostoc* was identified with relative abundance of 3.9%, which is close to 3.2% from Illumina sequenced reads. At the species level (Fig. S2b), the most abundant species classified in samples YR and RB was *Escherichia coli* belonging to the phylum Proteobacteria. *Chroococcidiopsis thermals* was recognized in sample RB with relative abundance of 1.3%.

### 3.3. Occurrence of biogeochemical N cycling

In particular, we focused on the key genes related to N metabolism, which was the predominant subcategory in the energy metabolism and are very important in maintaining the stability of stone monuments. We found a total abundance of 330,030 sequences with 66 nitrogen cycling gene families (Tu et al., 2019), involving in the processes of denitrification (11.6%), assimilatory nitrate reduction (11.9%), dissimilatory nitrate reduction (12.7%), nitrogen fixation (2.6%), nitrification (0.16%), and anammox (0.02%), organic N degradation and synthesis (65.2%) and hydroxylamine reduction (Fig. 3). Five gene families involved in N fixation, including *anfG*, *nifD*, *nifH*, *nifK*, and *nifW*, were abundant in sample RB, which can be attributed to the high-abundance Cyanobacteria (such as *Nostoc* and *Cyanothece*). Archaeal and bacterial *amoA* and *amoB* genes responsible for nitrification were detected in all five samples, potentially contributing to the deterioration of stone materials by production of nitrate and nitric acid (Ding et al., 2022). The abundance of functional genes for denitrification showed significant differences among the five samples, ranging from 4819 sequences in sample RB to 11,235 sequences in sample LG. For assimilatory nitrate reduction pathway, a total of 39,279 sequences attributed to six gene families (*nasA*, *nasB*, *narB*, *narC*, *NR*, and *nirA*) were collected, responsible for nitrate reduction to nitrite and nitrite reduction to ammonia. Sample LG was dominated with genes *NR* and *nasA*, which respectively encode assimilatory nitrate reductase electron transfer subunit and nitrate reductase (NAD(P)H). Gene families including *nirB*, *nirD*, *nrfA*, *nrfB*, and *nrfD* are responsible for nitrite reduction to ammonia, and these genes were mainly detected in sample YR, followed by samples LG and ML. A small number of annotated sequences responsible for hydrazine production (gene families *hzsA*, *hzsB*, and *hzsC*) and hydrazine oxidation into nitrogen (gene families *hzo*) were retrieved, suggesting that anammox may occur on these deteriorated stone. Moreover, the functional gene *hcp* playing a role in hydroxylamine reduction was recruited across all 5 samples.
3.4. Occurrence of biogeochemical S cycling

The functional annotation of sequences showed the presence of gene families and pathways involved in assimilatory sulfate reduction (32.7%), dissimilatory sulfate reduction and oxidation (7.3%), SOX system (8.5%), sulfide cycling (1.2%), sulfur assimilation (16.7%), sulfur mineralization (12.0%), and sulfur uptake (21.6%) in the microbiome (Fig. 4). A total of 8 gene families (cysC, cysD, cysH, cysJ, cysN, cysNC, and sir) with 27,443 representative sequences were recruited for assimilatory sulfate reduction pathway, responsible for the transformation of adenosine 5′-phosphosulphate (APS) via phosphoadenosine 5′-phosphosulphate (PAPS) and sulphite to sulfide (Grein et al., 2013). However, only 892 to 1672 annotated sequences in all five samples were involved in the conversion between sulfate and APS. Six gene families for SOX systems, including soxA, soxB, soxC, soxX, soxY, and soxZ, were also detected in all the metagenomes and responsible for thiosulphate oxidation to sulfate. Genes fccA and sqp were recruited for sulfide cycling and accounted for only a small amount of representative sequences involved in S cycling pathways. Especially gene fccA encoded cytochrome subunit of sulfide dehydrogenase responsible for the transformation of sulfide to sulfur. In sample RB, a total of 3214 representative sequences were involved in the transformation of organic S to sulfite, higher than other samples. Furthermore, sample RB was dominated with gene families responsible for sulfur uptake, having 8447 associated sequences. A range of microbial genera involved in S cycling were retrieved in the microbiome, such as SOX systems mediated by Bradyrhizobium, Mesorhizobium, and Paracoccus, sulfur reduction and oxidation by Vibrio.

3.5. Occurrence of antibacterial biocide and metal resistance genes, and ARGs

BLAST analysis against BacMet database further revealed stone metagenome contained a broad spectrum of antibacterial biocide and metal resistance genes. A total of 482 gene families were recruited for biocides, metals, and multi-compound metals biocides resistance. Compared to other four samples, a large number of representative sequences were detected in sample RB, accounting for 35.8% of total metagenomic reads related to antibacterial biocide and metal resistance. Gene families including fabI, ygaA, wpc, and nrsS were the top 3 most dominant genes, showing phenolic compounds, multi-metal (tungsten and molybdenum), and nickel resistance, respectively. Twelve gene families for copper (Cu) resistance were recruited, including copR, copS, corR, ctpV, cdpR, copB, corS, copA, actP, hmrR, supT/ygiE, and fpvA.
Among these, copBRS, corRS, ctpV, and crdR only showed resistance to Cu, and copA, actP, hmrR, supT/ygiE, and fpvA showed resistance to Cu and other metals or biocides. Gene families conferring resistance to zinc (Zn) and iron (Fe) were commonly detected across five samples, indicating that epilithic biofilms have the ability to resist Zn and Fe oxide nanoparticles. Some gene families (such as galE, vcaM, cpxR, and mdeA) were involved in resistance against quaternary ammonium compounds such as benzylkonium chloride (BAC), tetraphenylphosphonium (TPP), and cetyltrimethylammonium bromide (CTAB). The resistance genes of antibacterial biocide were also identified, including those encoding resistance to triclosan, chlorhexidine, tetrachlorosalicylanilide, acriflavine, and hydrogen peroxide.

We found a total abundance of 15,709 ARGs with 608 types, belong to 29 different antibiotic classes across five samples. Multiple resistance genes (57.8%) were the predominant resistance type in all five samples, followed by aminoglycoside (8.2%), glycopeptide (6.5%), tetracycline (6.1%), peptide (4.1%), phenicol (2.6%), carbapenem (2.3%), and macrolide (2.1%) (Fig. 6a). Sample LG has high abundance of ARG subtypes encoding resistance to aminoglycoside, carbapenem, macrolide, peptide, and phenicol as compared with other four samples. The abundance of top 30 ARG subtypes accounted for 51.7% of the total subtypes. The resistance gene adeF is the most abundant types of efflux pump genes encoding resistance tetracycline and fluoroquinolone (Fig. 6b). Abundance variations of ARG ermA of antibiotic target alteration were observed in different samples, such as sample ML had a high percentage of ermA (up to 72%) of total ermA subtype. Genes OXA-357, mdsB, LRA-17, and efrB encoding multi-drug resistance such as cephalosporin, penam, carbapenem, cephamycin, and fluoroquinolone, were abundant in sample YR. The ARGs-carrying microbes were identified using the assembled contigs. Among the total annotated ARGs-carrying microbial species, Actinobacteria is the most abundant bacterial phyla to impart resistance, accounting for 38.7%, followed by Proteobacteria (33.5%), Cyanobacteria (7.5%), and Acidobacteria (6.0%) (Fig. S3). Actinobacteria has high abundance of dominant ARG types of rifamycin and tetracycline, accounting for 25.4% and 24.9%, respectively. The most abundant ARG types of multidrug resistance were present in phyla Proteobacteria and Acidobacteria.

By use of the combination of Nanopore and Illumina sequencing datasets, binning resulted in formation of 10 high-quality binned genomes. Four of which had an N50 of over 3 Mb, and others had N50 value ranging from 141 kb to 971 kb. Among the high-quality binned genomes, nine reached family identification level, including Pseudonocardiaceae, Roseiflexaceae, Gloeobacteraceae, Rhodocyclaceae, Geminicoccaceae (Fig. 7), Acetobacteraceae, and Pyrinomonadaceae,
only one was annotated as *Hydrococcus*. Four binned genomes annotated as Rhodocyclaceae (bin 87), Roseiflexaceae, and Pseudonocardiaceae possess at least two ARGs, and *Hydrococcus* (*mphG*), *Gloeobacter* (*adeF*), Rhodocyclaceae (bin 2, *adeF*), Geminicoccaceae (*adeF*), and Acetobacteraceae (LRA-9) were identified to carry only one ARG. A series of plasmids, phages, integrons, and transposons were identified on the chromosomes, most probably indicating horizontal gene transfer (HGT) events of ARGs between different species. Moreover, genes involved in metal (i.e., Cu, Zn, and Ag) and quaternary ammonium compounds resistance, and N/S metabolism were also detected in binned genomes.

4. Discussion

Stone-dwelling microbial biofilms pose a potential threat to historical stone monuments and its surrounding environment. A varied group of microbes are also included in the biofilms, such as phototrophs and chemolithotrophs, forming a stable ecosystem and metabolically cooperative network to drive the biogeochemical cycles, AMR genes horizontal transfer, and stone biodeterioration. Within the mature epilithic
biofilms, the outermost layer of phototrophs assimilate CO$_2$ into organic compounds and then provide necessary nutrients and suitable niche for subsequent colonizers (Liu et al., 2020). Certain microbial groups such as chemolithotrophic bacteria and archaea, usually located in the middle layer of the epilithic biofilms, likely contribute to the succession and expansion of stone biodeterioration and enhance other microbial growth (He et al., 2022). The heterotrophic bacteria, actinobacteria, and fungi can serve as the primary colonizer when sufficient organic matter is available (Nuhoglu et al., 2006), inducing a significant change in the chemical and physiochemical natures of the substratum materials by pigmentation, penetration, and mineralization (Gu and Katayama, 2021; Sterflinger, 2010).

Next-generation sequencing (NGS) has revolutionized the profiling of complex stone-dwelling microbiotas and functional genes and pathways involved in biogeochemical cycles and antimicrobial resistance (Ding et al., 2022; Schröer et al., 2020). However, using the reads generated by NGS technologies led to much shorter contigs and fail to span most repetitive sequences. Third-generation Nanopore sequencing technologies generate long reads, allowing significant improvements in diverse applications such as real-time sequencing, de novo genome assembly, and structural variation detection (Chen et al., 2022). Meanwhile, hybrid assembly using Nanopore and Illumina reads increases assembly quality and enables analysis of high-complexity ecosystems (Liu et al., 2021). For instance, in our large-scale sequencing of
stone-dwelling microbiome the average contig N50 value of metagenomic assemblies was 137.8 kb, much higher than those by only using illumina reads. *Sphingomonas, Pseudonocardia, Rubrobacter,* and *Leptolyngbya* were the most dominant genera and were frequently detected in stone monuments (Jroundi et al., 2020; Skipper et al., 2022). Fungal or lichen induced encrustation and biomineralization could serve a dual role as protective coatings to shield the surfaces from direct rainfall impacts and also as biodeteriogens to accelerate aging of stone substratum (Liu et al., 2022b). Samples LG, ML, and YS were collected from deterioration areas with visual signs of lichen colonization, and the most abundant fungi are Ascomycota. The representative lichenized fungal genera are *Umbilicaria,* with the main species being *Umbilicaria pusulata* (7.3%). Another lichenized fungus *Cladonia uncialis* was only detected in samples LG, ML, and YS, and has been found to display better antibacterial activity (Studzinska-Sroka et al., 2019). *Leptolyngbya* is the most abundant Cyanobacteria in sample RB, and this phototrophic microbe precipitated calcite while forming biofilms (Leiser et al., 2021). Compared to Illumina-based sequencing, the combination of Nanopore and Illumina sequencing provides the most comprehensive taxonomic classification at the species level (Klain et al., 2023). Genus *Escherichia* was also detected in samples YR and RB with relative abundance >9% and *Escherichia coli* was the dominant species. High relative abundance of Genus *Nostoc* was mainly present in sample RB, contributing to the green biofilm formation and contributing to the stone deterioration.

Biogeochemical reactions of the microbial N and S cycling are the key determinants responsible for stone deterioration (Liu et al., 2020), for instance, microbes responsible for the N cycling convert ammonia and nitrogenous oxides into inorganic nitrous and nitric acids and cause acidic erosion of stone materials (Kuyers et al., 2018). In this study, a wide range of genera capable of performing many important N transformation reactions as well as specific ammonia oxidizing genera were detected. The bacterium *Azotobacter vinelandii,* encoding three types of nitrogenases, had very low relative abundances (<0.01%) in the stone-dwelling microbiome. The genus *Cyanothecce,* contributing significantly to the N and C cycle, had a higher relative abundance in samples ML and RB. Genera of *Azotobacter, Azospirillum, Azospira, Azorhizobium,* and *Azoarcus,* representing a total of 0.3% of the microbiome, were capable of fixing dinitrogen into ammonia. The symbiotic unicellular cyanobacterium *Candidatus Atelocyanobacterium thalassa,* which lives symbiotically with haptophyte algae, showed significantly lower abundances. The *NifH* gene encoding iron protein of the nitrogenase is the most commonly used target gene for tracking the nitrogen-fixing microorganisms due to its highly conserved property (Zehr et al., 2003). It is observed that the *NifH* genes were present in all five samples, accounting for 1.56% of total N cycling genes. Ammonia oxidation is the first and typically rate-limiting step in nitrification and is performed by both AOA and AOB (Prosser and Nicol, 2008). From the microbiome dataset across all five samples, a number of known microbial ammonia oxidizers were detected, such as *Nitrososphaera viennensis,* *Nitrosospira,* and *Nitrosopumilus maritimus,* can also grow chemolithoautotrophically by oxidizing ammonia to nitrite. The genus *Nitrosira* was found to be capable of nitrite oxidation and complete ammonia oxidation (comamox), and the latter oxidize ammonia to nitrate (Kits et al., 2017). *Nitrospira* was the major ammonia oxidizers on samples LG, ML, and RB. Moreover, the specific microbial species and functional gene markers responsible for denitrification (*Rhodobacter, Nitrireductus,* and *Ochrobactrum; nirKS*) and nitrite oxidation (*Nitrospira* and *Nitrobacter; nitrA*) were detected across all samples, thus forming an essentially complete N cycling (Ding et al., 2022).

The identification of the environmental sequences in the KEGG database enabled mapping of the S cycling in the deteriorated stone monuments. High abundance of the functional genes including *cysCDHLDU,* *cysNC,* and *sir* were detected, indicating epiphytic microbial mediated assimilatory sulfate reduction pathway. This process can result in organic sulfur accumulation, and then work together with the SOX system, eventually accelerating the stone deterioration (Meng et al., 2022). Representative sequences involved in SOX system were abundant in samples YR and RB, which primarily comprised Actinobacteria, Proteobacteria, and Acidobacteria (harboring genes involved in S cycling). Three bacterial genera of *Bradyrhizobium, Mesorhizobium,* and *Para- coccus* involving sulfur oxidizing were detected in our metagenome dataset. In the dissimilatory sulfate reduction and oxidation pathway, *Desulfovibrio* with an average relative abundance of 0.06% may mediate the reduction of sulfate and produces large quantities of inorganic sulfide (H2S) (Yu et al., 2021). Gene families of *fcaC* and *apr* participated in sulphide oxidation and accounted for only a small proportion of total S cycling associated gene sequences. A total of 24,067 representative sequences participate in sulfur assimilation and sulfur mineralization pathways, responsible for linking inorganic and organic S transformation. Gene families including *saaDE, msmAB,* and *muc* were involved in the conversion of organic S compounds to sulphite, and further induce mineralization reaction taking place at the surface of stone (Wang and Liu, 2021).

The accelerating occurrence and spread of ARGs have becoming emerging environmental and global public health concerns (Tysers and Wright, 2019). In natural stone ecosystems, a wide variety of microbes live in matrix-enclosed biofilm and contribute substantially to energy metabolism and stone deterioration (Gaylarde and Little, 2022; Özdemir et al., 2020). Among them, AMR issues cannot be ignored, since the HGT of antibacterial biocide and metal resistance genes, and ARGs among different stone-dwelling microbes facilitates the survival and persistence of multidrug-resistant microbial colonizers when combating antimicrobial agents (Franco-Castillo et al., 2021; He et al., 2022). Genes encoding resistance to critically important biocide and metal were detected across five samples. For example, genes *znuC*/*yehM* encoding zinc ABC transporter ATP-binding protein *ZnuC* can tolerate high concentrations of zinc (*Hudek et al., 2009,* gene *copR* encoding DNA-binding response regulator plays a significant role in copper resistance (*Wei et al., 2009,* gene *acn* encoding aconitate hydratase *AcnA* shows resistance to Fe, gene *mdeA* encoding methionine gamma-lyase allows microbes to tolerate quaternary ammonium compounds such as BAC, cetrimide, and dequalinium (*Huang et al., 2004,*). It is worth noting that the zinc oxide, copper, and quaternary ammonium salt exhibit antimicrobial activity with potential for the treatment of stone deterioration (Franco-Castillo et al., 2021; Gallo et al., 2020). BAC is a biocide disinfectant and can promote the evolution of resistant bacteria (*Short et al., 2021,* and the emergence of bacterial resistance in Lascaux Cave might be closely related to its frequent use (*Bastian et al., 2009,*). Gene *egsS* is abundant in sample RB, which could be attributed to the higher abundance of *Escherichia coli* detected via the combination of Nanopore and Illumina sequencing (*Eguchi and Utsumi, 2014,*). *Pseudomonas aeruginosa* is an opportunistic human pathogen and contributes to multidrug resistance (*Poole and Srikumar, 2001,* with average relative abundance of 0.03%.

The bacterial ARG profiles showed that more aminoglycoside and glycopeptide resistance genes, primarily composed of AAC-group and van-group respectively, were retrieved from the genomic data than those resistant to tetracycline and peptide. The abundances of ARGs conferring resistance to phenicol, carbapenem, macrolide, sulfonamide, lincosamide, and cephapycin were slightly higher in samples LG and ML than those in other three samples. Tetracycline resistance genes are the most commonly observed ARGs, and an array of tet genes (i.e. *tetA, tetG,* and *tetH*) contribute to tetracycline resistance by encoding efflux pumps proteins and ribosomal protection proteins (*Peng et al., 2021,* Roberts, 1996). ARGs can be categorized into two major groups based on their HGT potential, i.e., the intercellularly mobile group and the chromosomal group, and the former could be transferred between bacterial cells (*Che et al., 2019,*). A variety of ARGs such as *tet(E), sul 4, cmaA,* and *mhpN* were detected in the mobile group, indicating that stone-dwelling biofilms might be considered as a reservoir of resistome and mobile. Based on the metagenomics data obtained, bacterial antibiotic resistance issues within stone settings do not seem to pose a major threat to stone artifacts and its surrounding environment, since ARG sequences...
were found within stone biofilms at a much lower frequency than in hospital (Casenaz et al., 2022), wastewater treatment plant (Wu et al., 2022), and livestock farms (Li et al., 2022). On the contrary, more attention should be paid to the effect of antibacterial biocide and metal nanoparticles treatments on the development and spread of bacterial resistance. Binned genomes have further confirmed that bacterial species contained at least one AMR gene, for example, genes copA, mtrR, gall, and mdtB in the binned Geminicoccaceae, genes mdtB, eogS, merA, and copA in binned Hydromonas. These diverse AMR genes and mobile genetic elements (e.g., prophages, plasmids, and insertion sequences) carried by active microbes may enhance their adaptive resistance to a variety of traditional and novel antimicrobials commonly used on stone monuments.

5. Conclusions

This study constitutes the detailed description of stone-dwelling microbiotas and key genetic contexts by the combination of Nanopore and Illumina sequencing. Taxonomic profiling showed that high diversity and abundant Ascomycota and Actinobacteria distributed in epilithic biofilms, serving a significant role in stone deterioration. A great variety of microbes and gene families were responsible for the biogeochemical N and S cycling, indicating the potential to participate in the stone destruction by converting N/S compounds into inorganic acids. Compared to antibiotic resistance, genes conferring resistance to antibacterial biocides and metals should receive more attention since they account for a relatively large percentage of the total microbial metagenome and may mediate microbes escape from the antimicrobial agents commonly used in stone monuments. High-quality genome assemblies further uncover the putative HGT event of AMR genes which drives the evolution of microbes and enhances its adaption to the extreme environmental conditions. Our study offers a broader perspective on the stone microbiome and proposes the combination of Nanopore and Illumina sequencing as an alternative to investigate genetic contexts in stone heritage samples.

CRediT authorship contribution statement

Qi Li: Experiment designing and operation, Data analysis, Writing original draft, Conceptualization, Funding acquisition, and Review. Chao Wu: Investigation and revision. Jintao He: Data analysis. Bingjian Zhang: Review and editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibiod.2023.105688.

References

Gu, J.-D., Katayama, Y., 2021. Microbiota and biochemical processes involved in biogeochemical N and S cycling, indicating the potential to participate in the stone destruction by converting N/S compounds into inorganic acids. Compared to antibiotic resistance, genes conferring resistance to antibacterial biocides and metals should receive more attention since they account for a relatively large percentage of the total microbial metagenome and may mediate microbes escape from the antimicrobial agents commonly used in stone monuments. High-quality genome assemblies further uncover the putative HGT event of AMR genes which drives the evolution of microbes and enhances its adaption to the extreme environmental conditions. Our study offers a broader perspective on the stone microbiome and proposes the combination of Nanopore and Illumina sequencing as an alternative to investigate genetic contexts in stone heritage samples.

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