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Variations in the bacterial community compositions at different sites in the tomb of Emperor Yang of the Sui Dynasty

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ABSTRACT

To fully understand the bacterial processes in tomb environments, it is necessary to investigate the details of the bacterial communities present under such oligotrophic conditions. Here, high-throughput sequencing based on partial 16S rRNA gene sequences was used to fully evaluate the bacterial communities at different sites in the tomb of Emperor Yang of the Sui Dynasty. We also aimed to identify the soil factors that were significant related to bacterial diversity and community composition. The results showed the presence of a broad taxonomic diversity that included nine major phyla. Actinobacteria, Firmicutes and Proteobacteria dominated the bacterial profiles in all tomb soil samples. However, significant differences between deposited soils (DS) and covering soils (CSA, CSB and CSC) were revealed by chemistry-based principal component analysis (PCA), the number of OTUs, and the Chao 1 and Shannon indexes. At the family level, hierarchically clustered heatmap and LefSe analyses showed differences in the bacterial community compositions at different sampling sites. Notably, CSA contained significant populations of Nocardioidaceae, Pseudonocardiaceae and Streptomycetaceae, which are often reported to be associated with biodeterioration in cave environments. Further, the most abundant group (>10%) in all soil samples was Streptococcaceae, whose abundance decreased from 34.66% to 13.43% with increasing soil depth. The results of redundancy analysis (RDA) and the Monte Carlo permutation test indicated that soil pH and Cu and Mn levels were significantly related to the bacterial communities in this tomb. This research offers new insight into bacterial communities in cave environments and also provides important information for the protection of this historically important tomb.

1. Introduction

Subterranean cave environments, including catacombs and tombs, are dark, with nearly constant temperature and high relative humidity (Groth and Saiz-Jimenez, 1999). Due to the lack of sunlight, primary production via photosynthesis is absolutely impossible. The limited nutrients and oxygen come from the surface via sinkholes, underground hydrology and drip waters (Barton and Jurado, 2007), but the supplies of these resources are unpredictable. Microorganisms, particularly bacteria, inhabit these extreme environments. The aphotic and oligotrophic environment restricts the development of microbial communities and only allows for the survival of species that are adapted to such conditions. Recently, some studies regarding microbial communities in caves have been reported (Stomeo et al., 2008; Krakova et al., 2015), primarily because microorganisms affect cultural heritage, such as rock art paintings (Portillo et al., 2009). However, a comprehensive and detailed understanding of microbial diversity and community composition in subterranean caves is still lacking.

Previous surveys have shown that subterranean caves contain highly diverse populations of bacteria. In particular, it was emphasized that Actinobacteria was the dominant group in the studied caves (Groth et al., 1999; Saarela et al., 2004). Krakova et al. (2015) investigated the white biofilm colonization in catacombs of St. Callixtus in Rome, showing that Actinobacteria was the most abundant phylum in both samples. Similar studies involving

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white and brown biofilms in caves and catacombs demonstrated the significant presence of Actinobacteria (Stomeo et al., 2008; Diaz-Herraiz et al., 2013; Vasanthakumar et al., 2013). In addition to Actinobacteria, Firmicutes and Proteobacteria have also been routinely found in subterranean caves (Wu et al., 2015; Krakova et al., 2015; Vasanthakumar et al., 2013). Although the bacterial diversity and community structure in caves were surveyed in all of those studies, to our knowledge, little is known about the relationships between environmental factors and the distributions of the bacterial communities in the tomb environments.

Previous studies have shown that bacteria in caves are versatile and also participate in capturing CO_2, fixing N_2, biodeterioration, and bioprecipitation (Laiz et al., 2009; Desai et al., 2013; Krakova et al., 2015; Sanchez-Moral et al., 2004). To better understand the roles and relative abundances of these functional bacteria in the cave environment, it is necessary to investigate the details of the bacterial communities occurring under such oligotrophic conditions. However, previous surveys generally used DNA/RNA-based clone libraries and denaturing gradient gel electrophoresis (DGGE) to study the bacterial community in cave environments (Schabereiter-Gurtner et al., 2002; Krakova et al., 2015). Compared with these conventional techniques, high-throughput sequencing (e.g., Illumina MiSeq sequencing) can produce more sequence information (Song et al., 2013) and provide further insight into the rare bacteria in these communities.

The tomb of Emperor Yang of the Sui Dynasty (CE 569–618) was discovered accidentally during late 2013. In the view of the important historical value, it is necessary to protect this valuable cultural heritage. Although the conservation of monuments is a multifaceted aspect, such as the effect of lights, number of visitor and humidity (Bourges et al., 2014; Russell and MacLean, 2008), bacterial communities in the caves also have diverse impacts. Bacteria usually cause deterioration (De Leo et al., 2012) and are even pathogenic and opportunistic in cave environments (Valme et al., 2010). Therefore, monitoring the microbial community and environmental parameters in the cave, in some degree, will help us to understand the biological processes in such environments. However, little is known regarding the differences among bacterial communities at the different sites in the tomb of Emperor Yang of the Sui Dynasty, as well as regarding the responses of these bacterial communities to environmental variation. Therefore, the first objective of this study was to identify the bacterial communities in the tomb. Second, we aimed to identify which factors drive bacterial richness, diversity and community composition. Finally, the main bacterial communities that might be associated with cave biogeochemical cycles in this tomb were discussed.

2. Materials and methods

2.1. Soil sampling and physicochemical analysis

The tomb was discovered in Yangzhou, which was naturally closed for thousands of years and accidentally discovered at the end of 2013. A stone epitaph found in the western side of the brick-lined tomb was inscribed with the title "Tomb Epitaph of the late Emperor Yang of Sui", indicating that the tomb was that of Emperor Yang. The footprint of the tomb is 4.98 × 5.88 m in dimension. The top of the tomb had been damaged by buildings subsequently built on top of it, and the surface soil was moved away. During the burial, approximately 10 cm of soil was deposited in the tomb. In June 2014, soil samples were collected from various sites in this tomb. The deposited soils (DS) in the bottom of the tomb were collected. In addition, covering soils (CSA, CSB and CSC) that were on top of the tomb gate were also collected every 30 cm. A sketch map is shown in Fig. 1. Three replicate soil samples were collected at each

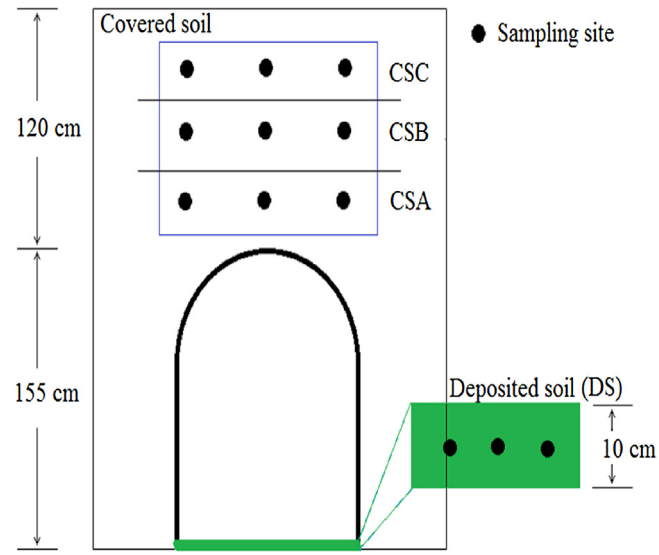

Fig. 1. A simple sketch map indicates the sampling locations in the tomb of Emperor Yang of Sui. Deposited soil in the tomb is indicated by the green color at the bottom, and the covered soils are divided into three sections, CSA, CSB and CSC. The black dots mean the sampling site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

site, and all samples were taken back to the laboratory immediately. Soil subsamples were stored at 4 °C for biological analysis or at −80 °C for molecular analysis, and the remaining soils were air-dried in the laboratory over several weeks for further analysis.

Soil pH was measured with a pH meter (PHS-3CT, Shanghai) using a soil extract solution made at a ratio of 1:2.5 soil:distilled water (w/v). Soil total organic carbon (TOC) and total nitrogen (TN) were determined according to previously reported methods (Lu, 1999). In brief, soil available potassium (K) was extracted using 2 M HNO_3 and analyzed by flame photometry (FP640, INASA, China). Soil available phosphorus (P) was extracted using 0.5 M $NaHCO_3$ and analyzed using the molybdenum blue method (Bao, 2005). Soil available Fe, Mn, Zn, Al and Cu were extracted using 0.05 M DTPA (diethylenetriaminepentaacetic acid) and analyzed using an inductively coupled plasma optical emission spectrometry (ICP-OES, Optima 8000). The chemistry-based principal component analysis (PCA) was conducted using Canoco software for Windows (version 4.5).

2.2. DNA isolation, PCR conditions, multiplexing and sequencing

Community DNA was extracted from 500 mg of each soil sample using the FastDNA® 24 instrument (MP Biomedicals) according to the manufacturer's instructions. DNA concentration was determined using the NanoDrop-2000 (Thermo Scientific, USA). DNA samples were amplified using the bacterial primers 519F (5′-CAG CMG CCG CGG TAA NWC-3′)/907R (5′-CCG TCA ATT CMT TTR AGT T-3′) according to the literature (Caporaso et al., 2012; Shehab et al., 2013). For Illumina sequencing, a 5-bp barcode sequence was added to the 5′ end of each primer. The PCRs were carried out in a total volume of 50 μl, containing 5 μl of 10× buffer with 15 mM $MgCl_2$, 4 μl of dNTPs (2.5 mM), 1 μl of each primer (5 μM), 0.5 μl of Taq DNA polymerase (5 U) and 50 ng of DNA. The following cycling parameters were used: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 45 s, annealing at 58 °C for 45 s, extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. Triplicate reaction mixtures for each soil sample were then mixed. The PCR products were purified using a PCR Clean Up Kit (Amresco). The amplicons from each triplicate sample were subjected to Illumina MiSeq sequencing. All the sequences used in this