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Distribution and diversity of bacteria and fungi colonizing ancient Buddhist statues analyzed by high-throughput sequencing

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ABSTRACT

The Klippe statues in Hangzhou, China, are a large number of exquisite Buddhist statues which date back to the Yuan dynasty (1276–1368 AD) and are considered to be one of the best representations of bas-reliefs on precipices. The site was added to the World Heritage List in 2011 because of its important role in the study of ancient Chinese Buddhist culture and the unique craftsmanship of its engravings. However, biodeterioration has not only caused severe aesthetical damage to the statues but also altered their material structure and thermo-hygric properties. In this study, the microbial communities colonizing the stone statues were characterized through a combination of high-throughput sequencing and culture-dependent techniques. Four samples were collected from four different environmental conditions with signs of deterioration, and their diversity and composition were analyzed by means of bioinformatics software and diversity indices. In addition, the ability of the isolates to degrade synthetic materials commonly present in the artworks was tested. While the diversity of the microorganisms varied with conditions, a large proportion of the isolates had the ability to degrade protective materials.

1. Introduction

Much of the world's cultural heritage consists of stone monuments, such as sculptures, historic buildings and murals, which have suffered severe and irreversible degradation and deterioration by microorganisms. The transformation of stone into sand and soil is an essential process that recycles complex inorganic matter and sustains life on Earth (Scheerer et al., 2009). However, these processes also cause aesthetic and structural damage to stone monuments. The most common mechanisms of microbial biodeterioration involve biofilms which may cause discoloration or physical damage through the release of inorganic and organic acids and osmolytes (Danin and Caneva, 1990; Saiz-Jimenez, 1995; Mansch and Beck, 1998; Gaylarde and Gaylarde, 2004; Kemmling et al., 2004; Salinas-Nolasco et al., 2004). Extracellular polymeric substances (EPS) from surface biofilms cause discoloration and are widely present in many tomb murals and sculptures spread all over the world (Ortega-Morales et al., 2001; Perry et al., 2004; Diaz-Herraiz et al., 2013; Ma et al., 2015). Some workers have suggested that, in general, microbial colonization, whether under wet or dry conditions, initially involves a large proportion of phototrophic microorganisms, represented by different colors of Cyanobacteria and algae (Tomaselli et al., 2000). Although the contribution of algae and Cyanobacteria to biodegradation and biodeterioration has not been thoroughly researched, the accumulation of photosynthetic biomass and inorganic nutrients leads to subsequent colonization of the surface by heterotrophic microbial communities (Ortega-Morales et al., 2001). Cyanobacteria have been recognized as important pioneer organisms on the exposed surfaces of historic stone monuments (Grant, 1982). Published data on the distribution of phototrophic microorganisms do not show a distinct relationship between microbial taxa and stone substrate composition, but climate conditions have been shown to be an important influencing factor (Tiano et al., 1995; Tomaselli et al., 2000; Gaylarde and Gaylarde, 2005). Studies of the microbial communities on historic stone monuments should therefore take into account a variety of factors, including the local climate and the interactions of microbial groups.

The Klippe statues, a group of ancient Chinese stone Buddha

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statues built over a period of approximately 500 years from the Five Dynasties (907–960 AD) to the Mongolian-ruled Yuan Dynasty (1276–1368 AD), are located in Hangzhou city in Zhejiang province. A total of about 380 Buddha statues have survived. Of the preserved Buddhist statues, 61% were built during the Song Dynasty and are distributed among Qinglin and Yuru caves, another 100 during the Yuan Dynasty and 10 during the Five Dynasties period. Thanks to their flowing lines and exquisite sculpting, the Klippe statues occupy a significant position in the history of Chinese sculpture. They were added to the World Heritage List in 2011 because of their unique craftsmanship and importance in the study of ancient Chinese Buddhist culture.

Like most ancient stone monuments, the Klippe statues are composed of limestone with a calcareous matrix. The growth of microbial biofilms promotes the accumulation of metabolites, including a range of anionic sugar molecules and organic acids, which can lead to leaching or chelating of calcium from limestone surfaces once it has been solubilized from the stone matrix (Perry et al., 2004). To manage damage to the Klippe, equipment was installed for real-time monitoring of changes in the environment and the statues. However, the complicated relationship between the environment, the statues and the microbial communities means there are still no effective measures to control the deterioration. Synthetic polymers commonly used to protect and conserve artwork may be the best option, since biocidal treatments may have a negative impact on the stone monuments.

The goals of our study were to use high-throughput sequencing to identify and characterize the microbial communities on the surfaces of Klippe statues showing signs of damage, to illuminate the distribution patterns of different microbial communities in the four sampling sites, and to understand the biodeterioration mechanisms of microbial communities on the statues. Using traditional culture-dependent methods, we isolated different strains of bacteria and fungi and assessed their ability to degrade the synthetic materials used to protect and reinforce the stone monuments. Our findings will help provide effective protection and restoration schemes.

2. Materials and methods

2.1. Characteristics of the location and samples

The Klippe statues are located in the middle and lower reaches of the Yangtze River in China in an area surrounded by mountains (Fig. 1). The region has a subtropical monsoon climate with an average annual temperature of 17.8 °C, an average relative humidity of 70.7%, and an average yearly rainfall of 1454 mm. The temperature and high humidity are suitable for the growth of photosynthetic microorganisms and may amplify the damage caused by them.

Six samples, referred to as SN1, SN2, SN3, SN4, SN5 and SN7, were collected from five different Buddhist statues of the Klippe (Fig. 1). All of the samples had signs of microbial deterioration. They were collected by means of an aseptic graver and stored at 4 °C until further treatment and analysis in the laboratory. The samples from the five sites were each divided into three parts; one part was used for SEM observations, while the other two parts were used for isolation of microbial strains and biodiversity analysis with high-throughput sequencing. The sample SN5 and SN7 are for microbial isolation and degradation assays only because of the failure to amplify the total DNA.

2.2. High-throughput sequencing analysis

Total genomic DNA was extracted from the microbial samples using the Power Soil® DNA Isolation Kit (MO BIO Laboratories, Inc., CA, USA) according to the manufacturer's instructions. Next generation sequencing library preparations and Illumina MiSeq sequencing were conducted by GENEWIZ, Inc. (Beijing, China). DNA samples were quantified using a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) and DNA quality was checked on a 0.8% agarose gel. 5–50 ng of DNA was used to generate amplicons using a MetaVx™ Library Preparation kit (GENEWIZ, Inc., South Plainfield, NJ, USA). Prokaryotic diversity was estimated by Illumina MiSeq sequencing with a panel of proprietary primers which were designed to anneal to the relatively conserved regions bordering the V3, V4, and V5 hypervariable regions. The sequences were as follows: $F_{V3\text{-}V4}$ (5′-CCTACGGRRBGCASCAGKVRVGAAT-3′) and $R_{V3\text{-}V4}$ (5′-GGAC-TACNVGGGTWTCTAATCC-3′). $F_{V4\text{-}V5}$ (5′-GTGY-CAGCMGCCGCGGTAA-3′) and $R_{V4\text{-}V5}$ (5′-CTTGTGCGGKCCCCCGYCAATTC-3′). Eukaryotic diversity was assayed by Illumina MiSeq sequencing with a panel of proprietary primers which were designed to anneal to the relatively conserved regions bordering the ITS1 and ITS2 hypervariable regions. The sequences were as follows: F_{ITS1} (5′-ACCTGCGGARGGAT-3′) and R_{ITS1} (5′- GAGATCCRTTGYTRAA-3′). F_{ITS2} (5′-GTGAATCATCGARTC-3′) and R_{ITS2} (5′-TCCTCCGCTTATTGAT-3′). Besides the 16S and ITS target-specific sequences, the primers also contain adaptor sequences allowing uniform amplification of the library with high complexity ready for downstream NGS sequencing on Illumina Miseq.

DNA libraries were validated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), and quantified by Qubit and real time PCR (Applied Biosystems, Carlsbad, CA, USA). DNA libraries were multiplexed and loaded on an Illumina MiSeq instrument according to manufacturer's instructions (Illumina, San Diego, CA, USA). Sequencing was performed using a 2 × 250 or 2 × 300 paired-end (PE) configuration; image analysis and base calling were conducted by the MiSeq Control Software (MCS) on the MiSeq instrument. The sequences were processed and analyzed by GENEWIZ. Taxonomy analysis was carried out on the QIIME platform.

2.3. Microbial cultivation, identification and biodegradation assays

Three media were prepared in Petri dishes following standard microbiological protocols for cultivation of bacteria and fungi. The composition of the media was as follows: LB agar medium: NaCl 10 g, yeast 5 g, peptone 10 g, sterile distilled water 1000 ml; R2A agar medium: 0.5 g yeast extract, 0.5 g proteose peptone 0.5 g, casamino acids 0.5 g glucose, 0.5 g soluble starch, 0.3 g sodium pyruvate, 0.3 g K_2HPO_4, 0.05 g $MgSO_4 \cdot 7H_2O$, 3 g NaCl, 15 g agar, sterile distilled water 1000 ml, pH 7.0 (Krakova et al., 2015; Pangallo et al., 2015; Pavic et al., 2015); fungal PDA agar medium: potato 200 g, glucose 20 g, agar 20 g, sterile distilled water 1000 ml. Streptomycin was added to PDA medium for inhibiting the bacteria growth, while Nystatin was for inhibiting fungi growth. Microbial samples collected from stone statues with signs of biodeterioration were cultured using serial dilutions, spread onto plates and incubated at 30 °C for several days until the growth of microorganisms became apparent. After selection of pure colonies, bacteria and fungi were cultured on LB and PDA agar plates for subsequent characterization.

To identify the isolated bacteria and fungi, total nucleic acids from the pure cultures were extracted with the CTAB method (Stewart and Via, 1993). The DNA was subsequently used as a template for a PCR in a reaction volume of 50 μl with the bacterial and fungal universal primers 341F-907R (Teske et al., 1996) and ITS1-ITS4(T.J. White et al., 1990). The PCR reaction mix for bacteria and fungi included 5 μl PCR buffer (TIANGEN), 0.2 μM of each