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The community distribution of bacteria and fungi on ancient wall paintings of the Mogao Grottoes

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In this study, we compared the microbial communities colonising ancient cave wall paintings of the Mogao Grottoes exhibiting signs of biodeterioration. Ten samples were collected from five different caves built during different time periods and analysed using culture-independent and culture-dependent methods. The clone library results revealed high microbial diversity, including the bacterial groups *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Cyanobacteria*, *Bacteroidetes*, *Gemmatimonadetes*, *Planctomycetes*, and *Chloroflexi* and the fungal groups *Euascomycetes*, *Dothideomycetes*, *Eurotiomycetes*, *Sordariomycetes*, *Saccharomycetes*, *Plectomycetes*, *Pezizomycetes*, *Zygomycota*, and *Basidiomycota*. The bacterial community structures differed among the samples, with no consistent temporal or spatial trends. However, the fungal community diversity index correlated with the building time of the caves independent of environmental factors (e.g., temperature or relative humidity). The enrichment cultures revealed that many culturable strains were highly resistant to various stresses and thus may be responsible for the damage to cave paintings in the Mogao Grottoes.

Biodeterioration is often observed on items of cultural heritage, historic artefacts and monuments, and even for modern materials, buildings, museums and private collections[1–5]. Microorganisms can colonise the surfaces of a wide range of materials and niche types in ecosystems; thus, biodeterioration is observed ubiquitously. The biodeterioration of precious and culturally significant items is undesirable and a public concern. Many studies have demonstrated extensive growth of various microorganisms and resultant stains and spots on painting surfaces[6,7]. Some novel microbial species have been associated with the deterioration of rock paintings using culture-dependent methods, and high microbial diversity has been observed in different environments based on rRNA gene-PCR amplification and sequencing methods[8,9]. Although distinguishing the functional groups of microorganisms within the microbial communities of different environments is difficult, a common, core microbial group has been associated with biodeterioration in caves with similar climate conditions[10].

The Mogao Grottoes are located 25 km southeast of Dunhuang City in Gansu Province, China. Dunhuang City is a northwest oasis city located in the western Hexi Corridor and was an important strategic location on the ancient Silk Road connecting Europe and Asia. The caves of the Mogao Grottoes were built from the Northern Wei (386-534 AD) to the Mongolian-ruled Yuan Dynasty (1276-1368 AD) over a period of approximately 1,000 years, with more than 700 caves built and nearly 45,000 square meters of mural paintings completed. A total of 452 caves have survived the damage inflicted by nature and humans. The Mogao Grottoes are a famous world cultural heritage site known for its numerous caves, mural areas, and documents and its long time span. The Mogao Grottoes were added to the World Heritage List in 1987 as the only site that satisfied all six criteria for inclusion; a site must meet at least one requirement for inclusion on the World Heritage List[11].

The Mogao Grottoes are inland and are surrounded by desert, including the Gobi desert of Mongolia to the northeast and the Taklimakan desert to the northwest. It has an arid continental climate with an average annual temperature of 10.9°C, average annual relative humidity of 28.5%, yearly rainfall of 39.9 mm, and mean evaporation of 2,490 mm. These dry climate conditions limit the proliferation of most microorganisms and, consequently, many of the organic materials used in the mural paintings have resisted significant damage or deterioration. Although the remote location and arid climate ensured that the Mogao Grottoes remained, visible

discoloration and damage have accumulated over a long period of time. Large areas of the mural paintings exhibit visible signs of decay due to salt precipitation and subsequent flaking, powdering or delamination. Damage due to pigmentation and discoloration induced or caused by microbial contamination, colonisation and growth is also prevalent (Figure S1). Several methods of desalination to control salt damage of mural paintings have been evaluated with promising results and are now widely used. Although the microbial biodeterioration of Dunhuang mural paintings has been studied, efficient, effective, safe, reliable measures have not been developed[12]. Biocides were once frequently used to combat biodeterioration but have since been shown to be effective for only a short period of time[5,13]. Research is now focused on analysing the characteristics of microbial community composition and structure related to biodeterioration. The microbial population on Dunhuang mural paintings was first characterised in the 1990s, providing primary insights into the microbes associated with mural decay. The culturable microorganisms from 51 discoloured samples from 6 ancient caves were identified, and several genera including *Aspergillus*, *Cladosporium* and *Flavobacterium*, were associated with accelerated ageing of cementing materials and the discoloration of mural paintings[14]. Subsequent experiments in which simulated mural paintings were inoculated with the isolated microbial strains revealed that microbial metabolites (e.g., pigments and oxalate) altered the crystal shape of the paints and the chemical valence state of the metallic elements in the paints. These processes played an important role in the discoloration of the mural paintings, particularly for red pigment containing red lead (lead tetroxide)[15]. Aerial microorganisms and their seasonal dynamics were recently investigated both inside and outside of the caves at the Mogao Grottoes. The influence of tourists and environmental factors were identified as the main factors contributing to biodeterioration[16,17]. In addition, microbial species with the potential to damage paintings or act as opportunistic pathogens to visitors were identified[18,19].

The objectives of the present study were to characterise and identify the microorganisms present on cave paintings with signs of damage and investigate the growth characteristics of culturable strains; to elucidate the distribution patterns of microbial communities in different caves in the context of temporal and spatial analyses of their building time and positions at the site; to assess the influence of environmental conditions on community distribution patterns; and to attempt to define the relationship between microbial distribution patterns and environmental factors.

Results

Microbial diversity of clone libraries. Information on the bacterial and fungal communities was obtained by PCR amplification and the clone library method using genomic DNA extracted from samples. A total of 10 bacterial libraries and 5 fungal libraries were constructed and analysed from the 10 samples in this study. A total of 1543 bacterial and 1771 fungal clones were analysed.

Bacterial diversity was represented by the phyla Firmicutes, Alpha, Beta, and Gamma Proteobacteria, Actinobacteria, Acidobacteria, Cyanobacteria, Bacteroidetes, Gemmatimonadetes, Planctomycetes and Chloroflexi (Figure S2). Firmicutes, represented by members of Bacillaceae and Clostridiaceae, was the major bacterial phylum and accounted for 54.0% of the entire community. The phylum Proteobacteria accounted for the second largest number of sequences detected from all samples, with Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria representing 10.5%, 4.0% and 15.3%, respectively. Firmicutes and Proteobacteria collectively accounted for 83.8% of all samples; the remaining seven bacterial phyla only accounted for 16.2%, with 6.0% Actinobacteria, 4.9% Acidobacteria, 2.9% Cyanobacteria, 1.7% Bacteroidetes, 0.3% Gemmatimonadetes, 0.3% Planctomycetes, and 0.2% Chloroflexi (Figure 1).

Three fungal phyla (*Ascomycota*, *Zygomycota* and *Basidiomycota*) were distinguished from the 1771 clones (Figure S3). *Ascomycota* was the dominant phylum, representing 88.4% of clones, including members of *Euascomycetes* (*Alternaria* and *Westerdykella*), *Dothideomycetes* (*Cladosporium* and *Leptosphaerulina*), *Eurotiomycetes* (*Aspergillus* and *Eurotium*), *Sordariomycetes* (*Chaetomiaceae*, *Stachybotrys*, and *Fusarium*), *Saccharomycetes* (*Candida*), *Plectomycetes* (*Penicillium*), and *Pezizomycetes* (*Tricharina*). Two additional fungal phyla contributed 11.7% of the total clones, with *Zygomycota* represented by *Mucoraceae* and *Basidiomycota* by *Filobasidiaceae* (Figure 2).

Microbial diversity of culturable microbes. Enrichment cultures cultivated for 15–20 days under different pH values, temperatures and salinity were spread on agar plates for isolation, purification and identification. The bacterial communities exhibited substantial differences under different culture conditions, but only two fungal genera were detected in the enrichment cultures: *Penicillium* spp. and *Cladosporium* spp.

More bacterial strains were isolated at 37°C than at 15°C based on colony forming unit counts and OTU numbers (Figure 3). *Bacillus* and *Paenibacillus* dominated the bacterial communities at both 37°C and 15°C, with relative proportions of 63.6% and 75.3%, respectively. In addition, 16 genera accounted for 36.4% of the strains isolated at 37°C, and 10 genera accounted for 24.7% of the strains isolated at 15°C. *Pseudomonas*, *Microbacterium*, *Planomicrobium*, *Sphingomonas*, *Brevundimonas*, *Arthrobacter*, *Acinetobacter* and *Roseateles* each represented more than 1% of the bacterial isolates obtained at 37°C. By contrast, *Planomicrobium*, *Sphingomonas*, *Microbacterium*, *Cupriavidus* and *Arthrobacter* each represented more than 1% of the bacterial isolates obtained at 15°C (Table S1).

The pH of the culture had an obvious effect on bacterial growth, with some strains exhibiting relatively broader pH ranges (Table S2). *Bacillus* grew at pH values ranging from 7 to 11 and accounted for a

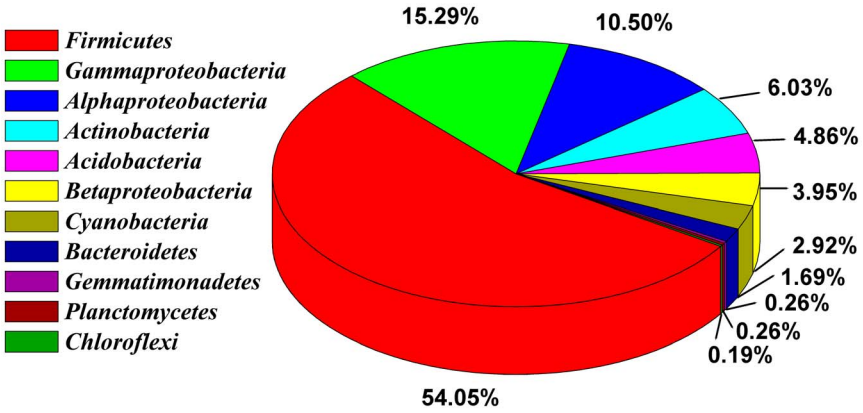

Figure 1 | Bacterial phyla detected in the samples from different caves of the Mogao Grottoes.

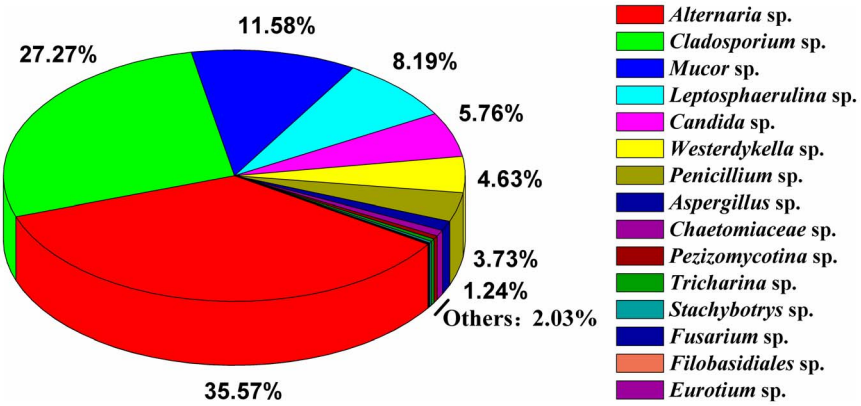

Figure 2 | **Main fungal taxa detected in the samples from different caves of the Mogao Grottoes.**

higher proportion of bacterial communities under different pH conditions, from 54.8% at pH 11 to 71.4% at pH 10. The growth characteristics of *Arthrobacter*, *Pseudomonas* and *Brevundimonas* were similar to those of *Bacillus*, and growth of these genera was detected in the same pH range. *Sphingomonas* spp. was detected at pH 7–9, while *Microbacterium* and *Micrococcus* grew at pH 7–10. *Acinetobacter*, *Roseateles*, *Psychrobacter* and *Kocuria* were only observed at pH 7 and 10, pH 7 and 11, pH 7, and pH 11, respectively.

Salinity exerted a strong selection pressure on the growth of microorganisms, and few strains were detected in the high-salinity

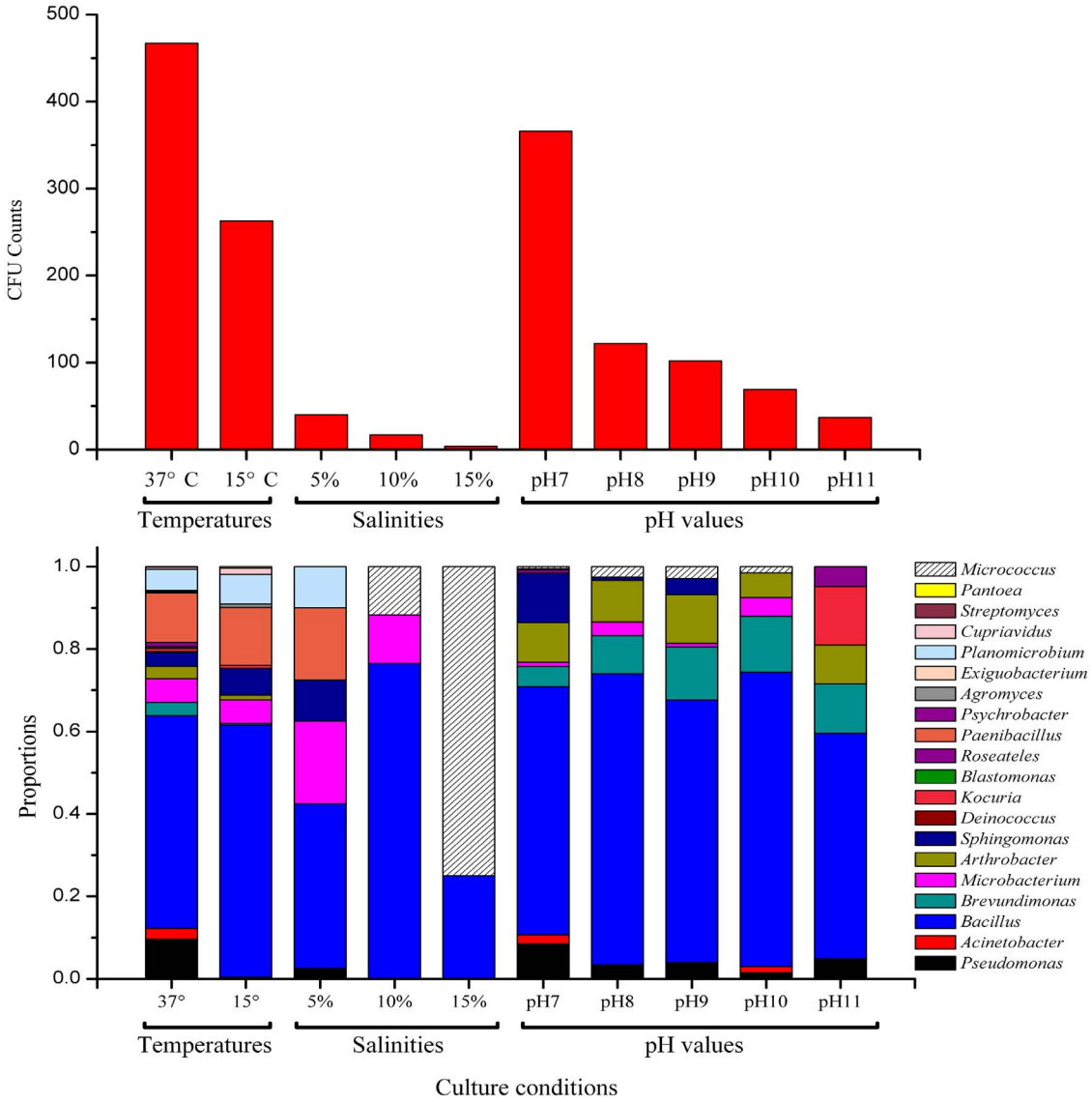

Figure 3 | **Culturable bacterial communities under different temperature, pH and salinity conditions.**

Table 1 | Description of the sampling sites and related observations in the Mogao Grottoes in this study

Samples	Floor	Position	Time	Clone library	Description
TM, CB	3	Right side of west wall	Northern Wei Dynasty	Only bacteria: TMB, CBB	TM was fragments collected from cracks; CB was sampled from darkened paintings with visible spots.
RM, RN, BN	2	West wall	Western Wei Dynasty	Bacteria: RMB, RNB, BNB Fungi: RMF	RM and RN were sampled from paintings with dense, small spots; sample RM was obtained at a higher position than RN. BN without spots was used as a control.
FM, FN	1	South wall	Tang Dynasty	Bacteria: FMB, FNB Fungi: FNF	FM and FN were sampled from paintings with a zonal distribution of spots; sample FM was obtained at a higher position than FN.
FMH, FNH	1	North wall	Tang Dynasty	Bacteria: FMHB, FNHB Fungi: FMHF, FNHF	FMH and FNH were sampled on paintings with a zonal distribution of spots; sample FMH was obtained at a higher position than FNH.
CY	3	Left side of east wall	Yuan Dynasty	CYB, CYF	CY was sampled on darkened paintings in which the primary picture is indistinct.

cultures (Table S3). *Bacillus* was the only bacterial genus detected at salinities of 1–15%, while *Micrococcus* was observed at salinities of 10%–15%, indicating a halophilic nature. The growth of *Paenibacillus*, *Sphingomonas*, *Planomicrobium* and *Pseudomonas* was detected at salinities of up to 5%, while *Microbacterium* could tolerate salinity up to 10%.

Temporal and spatial distribution of the microbial communities. The samples were divided into different groups according to the building times of the caves and sampling positions. A total of 4 samples were collected from the ground floor, 3 from the second floor, and 3 from the third floor of the Mogao Grottoes (Table 1).

Firmicutes and *Proteobacteria* including *Bacillus*, *Planococcus*, *Clostridium*, *Aerococcus*, *Paenibacillus*, *Shigella* and *Azospirillum* were dominant in all samples from all three floors, accounting for 52.1% and 23.6% of all clones from the ground floor, 49.0% and 27.2% of all clones from the second floor, and 55.1% and 26.2% of all clones from the third floor, respectively. *Cyanobacteria* was found in all samples from all three floors and accounted for a small fraction of total strains (1.2% to 5.6%), while *Acidobacteria* was only detected on the ground floor (5.0%) and third floor (7.3%). *Betaproteobacteria* was also present in all samples from all three floors at a proportion of 1.6–6.1%, while *Actinobacteria* had a higher coverage of 3.2–6.2%. *Flavobacteria* was also detected on the ground floor (2.4%) and third floor (1.2%) but not the second floor. A small fraction of *Gemmatimonadetes* ranging from 0.2% to 0.4% was detected in all samples from the three floors. *Planctomycetes* was detected on the ground floor (2.9%) and second floor (1.8%), but *Chloroflexi* was only detected on the ground floor (0.5%) (Figure 4).

The fungal composition of the samples from different floors was less diverse than that of the bacterial communities, but greater variation was observed among the different floors. The three predominant fungal genera in the samples from the ground floor were *Cladosporium*, *Alternaria* and *Leptosphaerulina*, which represented 90.0% of the fungal communities in these floor samples; an additional six genera contributed only 10.0%: *Penicillium*, *Candida*, *Pezizomycotina* sp., *Westerdykella*, *Stachybotrys* and *Fusarium*. The three predominant fungal genera in the samples from the second floor were *Alternaria* (63.27%), *Westerdykella* (16.1%) and *Candida* (15.0%), which represented 94.5% of the fungal population. An additional two genera, *Cladosporium* and *Penicillium*, accounted for 5.3% and 0.2%, respectively, of the population on the second floor. *Mucor* was the main fungal genus in the third-floor samples, accounting for 67.9%. Ten additional genera represented 32.1%: *Aspergillus* (7.3%), *Cladosporium* (6.3%), *Alternaria* (6.0%), *Chaetomiaceae* (4.6%), *Penicillium* (4.0%), *Tricharina* (2.0%), *Pezizomycotina* (0.7%), *Candida* (0.7%), *Filobasidiales* (0.3%) and *Eurotium* (0.3%) (Figure 4).

The caves sampled in the Mogao Grottoes were built during different time periods. The ten samples used in this study represented four time periods. Two samples were collected from caves built during the Northern Wei Dynasty (386-557 AD), three from the Western Wei Dynasty (535-556 AD), four from the Tang Dynasty (618-907 AD), and one from the Yuan Dynasty (1271-1368 AD) (Table 1).

Firmicutes and *Proteobacteria* were the dominant members in all samples from all four periods, with coverages of 61.2% and 19.5% in samples from the Northern Wei Dynasty, 49.0% and 30.0% in samples from the Western Wei Dynasty, 52.1% and 29.7% from the Tang Dynasty, and 36.6% and 51.2% from the Yuan Dynasty. The distribution of *Firmicutes* exhibited a decreasing trend from the earlier Northern Wei Dynasty to the later Yuan Dynasty, while *Proteobacteria* exhibited an increasing trend. *Betaproteobacteria* were detected in all samples from the four periods with the exception of the earliest, the Northern Wei Dynasty. *Actinobacteria*, including *Blastococcus*, *Arthrobacter*, *Propionibacterium*, and *Kocuria*, and *Cyanobacteria,* appeared in all samples from the four periods and contributed proportions of 3.2–8.0% and 1.2–5.6%, respectively. *Flavobacteria* were detected in samples from the Tang Dynasty (2.4%) and Yuan Dynasty (3.2%), while *Acidobacteria* were detected in all samples from the Northern Wei Dynasty (6.7%) and Tang Dynasty (7.3%). The diversity of the bacterial communities was higher in all samples from the Western Wei Dynasty than in those from the other three periods, with more unique groups, including *Virgibacillus* (9.5%), *Desemzia* (5.1%), *Aerococcus* (2.8%), *Ochrobactrum* (2.8%), *Kocuria* (1.8%), *Renibacterium* (1.5%), *Streptomyces* (1.3%), *Carnobacterium* (1.0%), *Paracoccus* (0.8%), *Tumebacillus* (0.5%), *Oceanobacillus* (0.5%), *Variovorax* (0.5%), *Roseomonas* (0.5%), *Agrococcus* (0.3%), *Devosia* (0.3%), *Thermoactinomycetaceae* (0.3%), *Trabulsiella* (0.3%), and *Nocardiopsis* (0.3%) (Figure 4).

Fungal communities were extracted from the samples associated with the Western Wei Dynasty, Tang Dynasty and Yuan Dynasty but not the Northern Wei Dynasty. Only 5 fungal genera were detected in samples from the Western Wei Dynasty, while 9 and 11 genera were detected in those from the Tang Dynasty and Yuan Dynasty, respectively. The fungal communities in samples from the Western Wei Dynasty comprised *Alternaria* (63.3%), *Westerdykella* (16.1%), *Candida* (15.1%), *Cladosporium* (5.3%) and *Penicillium* (0.2%). Three main fungal genera contributed 90.0% of the sequences in the samples from the Tang Dynasty, including *Cladosporium* (44.0%), *Alternaria* (31.4%) and *Leptosphaerulina* (14.5%), while the other six genera collectively contributed 10.0% of the sequences, including *Penicillium* (5.3%), *Candida* (2.9%), unidentified *Pezizomycotina* sp. (0.6%), *Westerdykella* (0.6%), *Stachybotrys* (0.4%), and *Fusarium* (0.2%). The most prevalent fungal genus in the Yuan Dynasty samples was *Mucor* (67.9%); *Aspergillus* (7.3%), *Chaetomiaceae* (4.6%), *Tricharina* (2.0%), *Filobasidiales* (0.3%), and *Eurotium* (0.3%) were also detected in these samples (Figure 4).

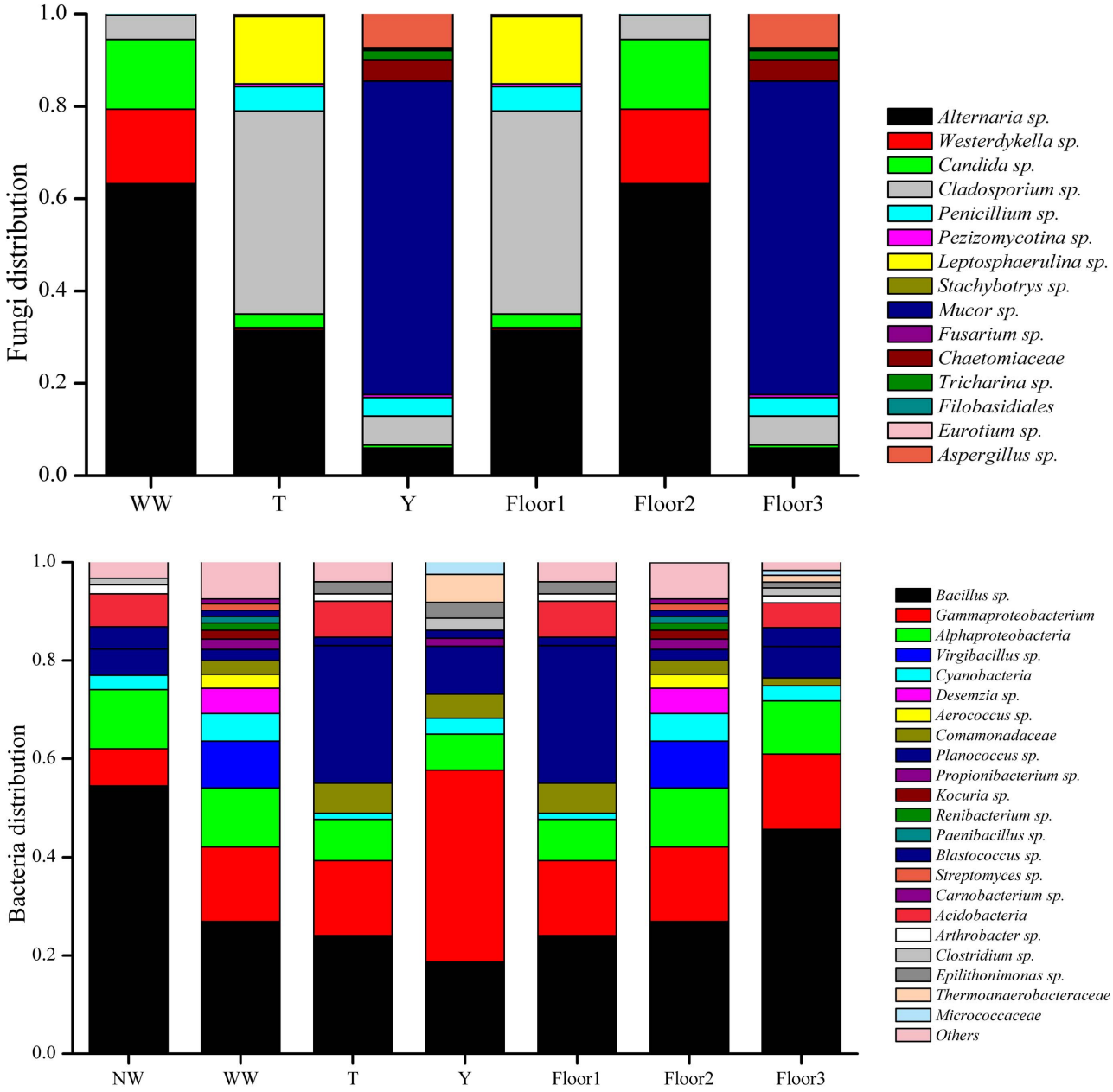

Figure 4 | Distribution patterns of fungi and bacteria from caves built at different times and specific locations in the caves investigated. The cave positions increase in altitude from floor 1 on the Ground Level to floor 3 on the highest level. NW stands for Northern Wei Dynasty (386-557 AD), WW for Western Wei Dynasty (535-556 AD), T for Tang Dynasty (618-907 AD), and Y for Yuan Dynasty (1271-1368 AD).

Influence of environment factors on the microbial community distribution. The microbial communities in the samples were assembled and assessed using statistical analysis methods; the results of principal component analysis (PCA) are shown in Figure 5. The structures of the ten bacterial communities were similar, with the exception of the control sample BNB, which was more diverse. However, five fungal communities differed distinctly from each other. There were greater differences between the control sample BNB and the other samples, primarily due to differences in the OTUs *Kocuria*, *Paenibacillus*, *Tumebacillus*, *Streptomyces*, and *Desemzia*. Although the distribution characteristics of the microbial communities differed among samples collected from different floors/levels and different time periods, no correlation between microbial community features and location could be identified. Only indistinct correlations were observed between fungal communities and floor location ($r = 0.869, p = 0.056$). The culturable bacterial communities were also subjected to PCA of the cultivation conditions (Figure 6). The structures of the bacterial communities were similar when cultured at different pH values but differed distinctly when cultured at 15°C and 37°C. The original community data and environmental characteristics were also subjected to correlation analysis (Table 2). There were three sets of community data, including the bacterial communities from the clone libraries, the bacterial communities from enrichment cultures, and the fungal communities from the clone libraries. Among the environmental characteristics of the caves investigated (e.g., temperature, relative humidity, floor location and building time), Pearson correlation analysis revealed significant correlations only between fungal communities and the time periods when the caves were built. The fungal diversity index decreased as the age of the cave paintings increased (Table 2).

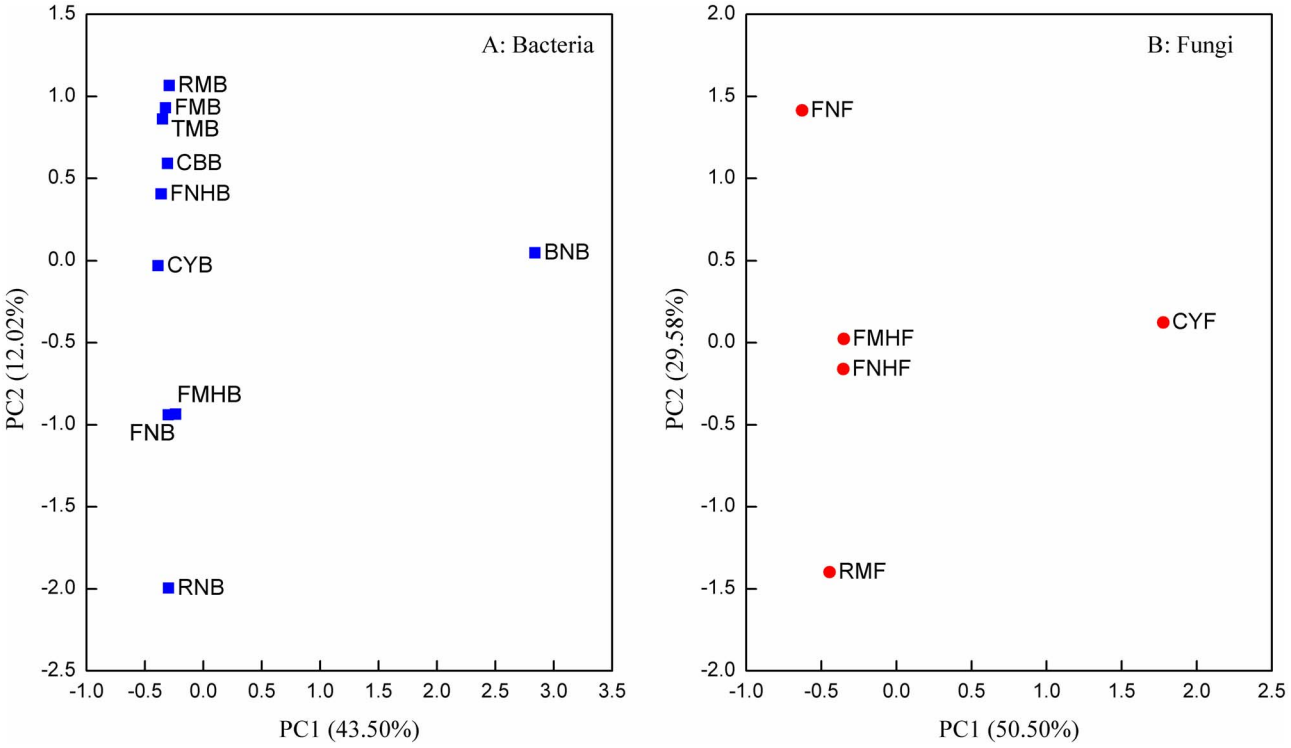

Figure 5 | Principal component analyses of the bacterial and fungal communities from different caves in the Mogao Grottoes. RMB stands for the bacterial community from sample RM and RMF for the fungal community from sample RM.

Discussion

Microorganisms play an important role in the biodeterioration of objects of cultural and historical significance, but their detailed biochemical and ecophysiological functions and roles remain unclear. Although many studies have reported that a high diversity of microorganisms participate in the biodeterioration process, no consistent conclusion has been achieved[20,21]. Bacteria, fungi and archaea have been mentioned in many reports, as have lichens and insects[22–24]. The characteristics of previously studied microbial communities were often related to the environmental conditions, with similar ecosystems (e.g., environmental parameters, geochemistry, and the availability and nature of organic matter) harbouring similar microbial compositions[25]. The results of these studies vary substantially depending on the methods employed; this variability makes the study of biodeterioration of cultural relics complex and challenging. Culture-independent methods are often considered more conveni-

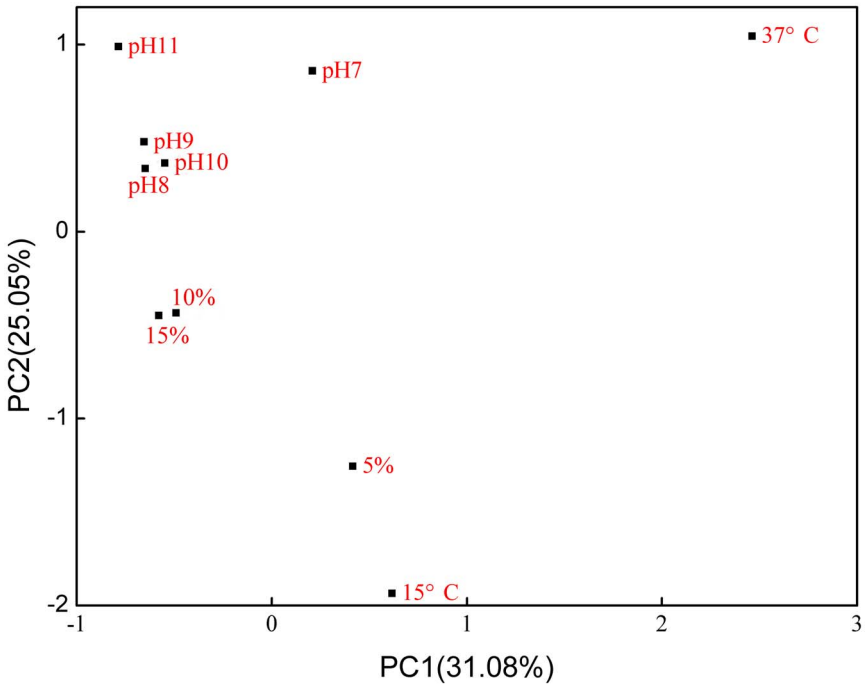

Figure 6 | Principal component analyses of culturable bacterial communities under different cultivation conditions.

Table 2 | The α-diversity indices of different communities and environmental characteristics determined by Pearson correlation analysis

α-Diversity Shannon_H Simpson_1-D	Temperature (°C)	RH (%)	Floor	Time
Clone-bacteria	0.082	0.265	0.237	−0.071
	−0.058	0.126	0.210	−0.062
Clone-fungi	−0.106	0.470	−0.170	0.721*
	−0.108	0.513	−0.278	0.651*
Culture-bacteria	−0.606	−0.607	−0.321	0.426
	−0.833	−0.832	−0.838	−0.100

*Correlation is significant when $p < 0.05$ (two-tailed).

ent and informative than cultivation-based methods, which only enable the detection of 1–5% of the total microbial community[20]. However, increased attention has been devoted to cultivation analysis because the isolated strains can be used to further investigate the mechanisms and processes associated with specific microorganisms.

The clone library results in this study revealed a rich diversity of microbial communities on wall paintings. Some of the identified microorganisms have been reported previously at other sites. *Firmicutes* and *Proteobacteria* (particularly *Gammaproteobacteria*) were the most predominant bacterial taxa found on the paintings. *Firmicutes* is often associated with earthy and drought environments, while *Proteobacteria* is frequently found in subterranean environments. The bacterial phyla *Actinobacteria*, *Acidobacteria*, *Cyanobacteria*, *Gemmatimonadetes*, *Bacteroidetes*, *Planctomycetes* and *Chloroflexi* are also frequently detected in caves and subterranean environments[2,26]. Autotrophic microflora such as *Cyanobacteria* have been identified in many sites under conditions of high humidity and illumination, high porosity and the presence of fissures and cavities, similar to the conditions at the Mogao Grottoes, which features a heterogeneous substrate[27]. Although the climate of the Mogao Grottoes is dry due to a lack of rainfall, occasional seasonal rainstorms often inflict serious damage and losses to some caves. This brief increase in available moisture may promote the growth of dormant microorganisms and accumulate considerable organic matter for the further growth of microflora.

Due to their ability to form hyphae, fungi may colonise and damage precious historical objects such as books, mural paintings and architectural surfaces. Hyphae penetrate deeply into materials and release extracellular enzymes, resulting in aesthetic spoiling and mechanical attack due to material loss, pigment contamination, acid corrosion, and enzymatic degradation[28–30]. Among fungi, *Alternaria*, *Cladosporium* and *Mucor* are major plant pathogens, and their spores are wind-dispersed and often extremely abundant in outdoor air[18]. *Candida* is the most frequently found fungal genus in the order *Saccharomycetales*, while *Penicillium* and *Aspergillus* are often associated with aerobic micro-environments. Fungi can also seriously threaten the health of conservationists and visitors due to the potential for allergic reactions, mycotoxin production, and the risk of systemic infections in humans[31]. *Aspergillus niger* and *Aspergillus flavus* have been proposed to be the causative agents of the 'curse of the pharaoh', a lung infection or systemic mycosis (Aspergillosis) that has been implicated in the deaths of several archaeologists[32].

The predominant spore-forming bacteria detected in this study have frequently been detected on other historical articles, such as wall paintings, rock paintings, book papers, and oil paintings[3,33–36]. Moreover, many members of these genera, including *Bacillus* spp., can form endospores, permitting survival under extreme conditions for a long period of time[1,37]. Many of the microorganisms identified in this study are biodeteriogens that cause spots-disease, such as *Aspergillus* and *Pseudomonas*, and produce organic acids in the environment[38,39]. The occurrence of fungi such as *Alternaria* and *Aspergillus* and bacteria such as *Bacillus* and *Pseudomonas* in the air at heritage sites has been reported repeatedly[19,40]. The present study also revealed that the microbial communities on the wall paintings did not differ from those in other caves in the Mogao Grottoes, and no unique genus with a vital contribution to the biodeterioration of wall paintings was identified.

The microbial community structures observed in this study varied depending on the detection method. The culture-dependent method enabled the detection of spore-forming bacteria, while the culture-independent method favoured the detection of the predominant groups/members of the microflora[41]. *Bacillus* spp. accounted for 31.67% of all sequences but 51.60% of species detected using enrichment cultures. This discrepancy is likely attributed to the reduced susceptibility of spores to DNA extraction. In contrast to the results of Lopez-Miras *et al.*[42], we observed that spore-forming bacteria on wall paintings in the Mogao Grottoes were partially active and may represent crucial members of the community. Two genera, *Deinococcus* and *Psychrobacter*, were only detected in enrichment cultures. Many of the isolates were resistant to low temperature, high salinity and high pH. These results may reflect the conditions on the wall paintings, which are characterised by low temperature, high salinity and dryness[43]. Consistent with a previous study of oil paintings[44], the most predominant fungal members belonged to the phylum *Ascomycota*, as determined by both culture-dependent and culture-independent methods. These may due to the similar organic materials contained in both paintings. All fungal isolates in the enriched cultures belonged to the genera *Penicillium* and *Cladosporium*, which were also detected in the clone libraries using culture-independent methods; no other genera were detected. Both of these genera are known biodeteriogens responsible for the biodeterioration of complex polymeric materials[45]. The enrichment cultivation results revealed that most strains belonged to *Penicillium*, whereas the molecular-based results revealed a dominance of *Alternaria*. This discrepancy is most likely due to low DNA extraction from *Penicillium* spores; fungal spores are likely dominant on the wall paintings. The enrichment conditions may also have contributed to this discrepancy by facilitating the rapid germination and enrichment of spores of *Cladosporium* and *Penicillium* over other fungi, resulting in the quick establishment of these two genera.

Some genera in this study have been reported previously on other heritage sites and paintings. For example, *Arthrobacter* and *Acinetobacter* have been shown to contribute to the biodeterioration of various art works[46–48]. Other genera, including *Bacillus*, *Kocuria*, and *Penicillium*, have been identified in various environments, including soil, water, buildings and aerosols[49–52]. These genera are known for their cosmopolitan and widespread distribution and are important components of the environment. Thus, the genera colonising wall paintings may have comprehensive origins, either as indigenous species in painting materials or foreign species transported by airflow[53]. The biodeterioration of wall paintings suggests that small fractions of the detected microbial community may grow and cause damage. These microbes may have the ability to survive under harsh climate conditions using the extremely limited organic matter available or have special metabolic capabilities[54]. The enrichment culture results in this study demonstrated that some strains had

high levels of resistance to various stresses, including low temperature, high pH and high salinity. These strains may be able to grow on wall paintings and damage paintings due to their specialised biochemical functions and capabilities.

The microbial community structures differed among the samples, and the distribution patterns of the various microorganisms were temporally or spatially irregular among the investigated caves. The alpha-diversity of microbial communities in this study was estimated and characterised as a function of environmental parameters via correlation analysis. However, only the building time of the caves was positively correlated with the diversity index of the fungal community, and this correlation relationship did not exhibit a positive association with the temperature and relative humidity of the caves. Differences in building techniques and materials may affect the characteristics of wall paintings as substrates for microbial growth. In addition, the fungi were frequently present in the form of dormant spores, from which genomic DNA is more difficult to extract. The bacteria community from sample BNB exhibited higher diversity than all other communities, suggesting that diversity is lost as biodeterioration progresses and those specialised members of the community are responsible for the biodeterioration of wall paintings.

In summary, high microbial diversity was detected on the wall paintings of the Mogao Grottoes using both culture-dependent and culture-independent methods. The microbial distribution characteristics were assessed as a function of the temporal and spatial patterns of the caves. The distribution patterns were not dependent on environmental parameters (e.g., temperature and relative humidity). The enrichment culture results revealed that many of the strains were highly resistant to various stresses and may inflict damage on paintings. The microbial damage observed on the wall paintings of the Mogao Grottoes was serious but did not reflect the diversity of microbes detected in this study, which suggests that most of these microorganisms are dormant or metabolically slow. Such conditions are dangerous to wall paintings because appropriate environmental conditions, particularly increased moisture, can result in a microbial outbreak[55]. These results highlight the difficulties in heritage conservation and risk monitoring works. More attention should be paid to the cautious management of microbial threaten and heritage conservation.

Methods

Sampling description. Ten samples were collected from five different caves of the Mogao Grottoes in which various degrees of damage to the wall paintings were apparent. All samples were collected as aseptically as possible. Approximately 50 mg of surface materials was collected into a sterile tube and stored at −20°C until further treatment or analysis. The sampling process was supervised by administrative staff of the Dunhuang Academy, and the sampling sites were restricted to the edges of damaged paintings. The collected samples consisted of fragments of painting layers and their substratum soil layers; these fragments could not be restored or reused. The detailed sampling sites and sample descriptions are shown in Table 1.

Enrichment cultivation. All sample materials were divided into two parts. The first part, approximately 10 mg, was inoculated into sterilised PYGV medium in a culture flask and incubated at 15°C for 15–20 days for microbial enrichment. The second part, approximately 20 mg, was used for genomic DNA extraction. The PYGV broth mimicked the nutritional conditions found in the wall paintings; the average temperature in the Mogao Grottoes is approximately 15°C. The enriched cultures were subsequently spread on LB agar plates for bacteria and PDA plates for fungi. The resulting colonies were isolated, purified and identified by PCR amplification and sequencing of the 16S or ITS gene. In general, all isolates were cultured in LB broth individually and then harvested for DNA extraction. The primers 27F/1492R for bacteria or ITS1/ITS4 for fungi was used to amplify the different isolates by PCR. The PCR products were sequenced and blasted against an online database. Each isolate was compared with known taxa.

Different culture conditions were designed to identify specific microbial isolates. The enriched cultures were inoculated on LB agar plates at two different temperatures, 15°C or 37°C. LB agar plates with different pH gradients were prepared to examine microbial growth at pH 7, 8, 9, 10 and 11. The salt tolerance of the enriched cultures was investigated by culturing in media with salinities of 5%, 10% and 15%. For each culture condition, 3–20 parallel plates were prepared and repeated at least 3 times.

DNA extraction and clone library construction. Whole genomic DNA was extracted using the Power Soil®DNA Isolation Kit (MO BIO Laboratories, Inc., CA, USA) according to the recommended procedure for all samples. The 16S rRNA genes were amplified using the primer set: 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGCTACCTTGTTACGACTT-3′)[56]. Fungal ITS regions were amplified with the primers ITS1 and ITS4[57]. The reaction mixture (25 μL) consisted of 1 unit of Taq polymerase (Tiangen Co., Beijing, China), 0.2 mM dNTPs, 2.5 μL of 10×PCR buffer, 2.5 mM $MgCl_2$, 0.2 μM each primer, and 2.5 μL (approximately 10 ng) of DNA template. The PCR amplification procedure for the 16S rRNA gene was as follows: initial denaturation at 94°C for 5 min; 30 cycles at 94°C for 40 s, annealing at 58°C for 90 s, and extension at 72°C for 90 s; and a final extension for 10 min at 72°C. The amplification procedure for fungal ITS was as follows: initial denaturation at 94°C for 5 min; 30 cycles at 94°C for 40 s, annealing at 55°C for 40 s, and extension at 72°C for 40 s; and a final extension for 10 min at 72°C. PCR products were detected by electrophoresis in 1% agarose gels and purified using a Gel Extraction Kit (Tiangen Co., Beijing, China).

The purified PCR products were used for clone library construction. Cloning was performed with the pGEM-T Vector System (Tiangen Co., Beijing, China) following the manufacturer's protocol. The ligation products were subsequently transformed into *Escherichia coli* DH5α cells for blue-white screening. Transformants were plated onto LB medium containing ampicillin (100 mg ml^{-1}), X-Gal (20 mg ml^{-1}) and IPTG (200 mg ml^{-1}). Positive clones were identified by PCR amplification with pGEM-T vector-specific primers (T7/Sp6) using the same amplification conditions used for bacterial and fungal amplification. The positive clones were first screened by double digestion (*Bsu*RI and *Csp*VI for bacteria, *Bsu*RI and *Hin*fI for fungi; MBI, Fermentas), and the clones with different patterns was used for sequencing.

The sequences obtained were analysed using the National Center for Biotechnology Information (NCBI) Blast program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The most similar sequences were extracted from the GenBank database. A phylogenetic neighbour-joining tree, including the obtained sequences and their closest relatives, was constructed using MEGA software 4.0. The sequences retrieved from this study can be accessed under EMBL (European Nucleotide Archive) accession numbers HG917219-HG917279 for bacteria and HG917280-HG917306 for fungi.

Environmental data collection. Long-term meteorological data were provided by the Dunhuang Academy. A wireless monitoring system is installed in each sampled cave. Computerised hourly data are available for statistical analysis. Environmental parameters subjected to statistical analysis included temperature (°C) and relative humidity (RH). We used the monthly average of the environmental parameter data at sampling time.

Statistical analysis. All experimental data were analysed using IBM SPSS Version 19.0 (SPSS, Standard Version). PCA was conducted using PAlaeontologicalSTatisitcs (PAST) version 2.03 (http://folk.uio.no/ohammer/past/). The relationships between the diversity index of the microbial community and environmental parameters were tested using the Pearson correlation.

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Author contributions

H.Y.F. conceived and planned the project, and Y.T.M. created the clone library and wrote the manuscript. H.Z. extracted DNA and performed sequence analyses, Y.D. cultured and tested isolates. T.T. and T.X. supervised phylogenetic analysis and performed statistical analyses. X.D.L. and F.S.W. collected samples. L.Z.A. and W.F.W. organised the sampling trips, provided access to the sampling site, and supplied historical data and climate data for the Mogao Grottoes. J.-D.G. discussed conservation methods.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

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