International Biodeterioration & Biodegradation 134 (2018) 1–6

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

Mycobiota of silk-faced ancient Mogao Grottoes manuscripts belonging to the Stein collection in the British library

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ARTICLE INFO

Keywords:
The Stein collection
Manuscripts
Conservation science
Silking
Scanning Electron Microscopy (SEM)
Energy dispersive X-ray spectroscopy (EDAX)
Mycobiota
Culture dependent methods
Amplicon sequencing analyses
Conidia
Cellulolytic activity

ABSTRACT

Silking, a conservation technique which involved gluing silk gauze over the face of a manuscript was popular in the mid-20th Century, especially for treating early Chinese documents. The method is now little used, and the question as to whether silking interventions should be reversed is controversial, given the high economic cost of active intervention, and there are few scientific studies as to the long-term consequences of the technique. Silk-facing materials from documents of the Stein collection were analysed using scanning electron microscopy coupled with energy dispersive X-ray spectroscopy. The mycobiota diversity was unravelled through the combination of culture dependent methods and amplicon sequencing analyses. The SEM micrographs showed smooth regular nodules of *ca.* 3–5 μm diameter on both silk threads and glue paste. This morphology differs from the irregular and the crystalline morphologies of glue paste and inorganic crystallites, respectively, but it is consistent with that of small-sized conidia (asexual spores of fungi) or yeasts. Glue paste demonstrated three fungal strains: *Aspergillus tubingensis*, *Penicillium crustosum* and *Chrysonilia sitophila* which display cellulolytic activity, except for the last one. Amplicon sequencing revealed that silk threads and glue paste host distinct mycobiota. Here, we preliminary show that the silking method may be affecting the overall integrity of the silk-faced manuscripts, principally due to contamination with cellulolytic fungal strains. Unless the silk facing is removed, irreversible damage to the documents is highly probable.

1. Introduction

The conservation and preservation of ancient manuscripts is an area of huge social, historical, religious, and cultural significance, and yet one which has attracted little scientific study. In the field of conservation, there has been a *volte-face* in acceptable techniques with the new guiding principle being minimalist intervention and reversibility (The Institute of Conservation, 2015). In the field of analysis, there have been tremendous advances in the past decades (Mazzoli et al., 2018; Sawoszczuk et al., 2018). It is critical that these “two cultures”, of classical historical contexts and analytical science, are brought together to the advantage of preserving our culture (Cappitelli et al., 2010; Sterflinger and Pinzari, 2012).

The preservation of ancient manuscripts - invaluable information carriers - in modern libraries and archives currently benefits from advanced environmental control systems that efficiently block the impact of numerous exogenous factors like acidity, heat, UV light, humidity, oxygen and pollutants (Cappitelli et al., 2010; Sterflinger and Pinzari, 2012). However, other influential issues during historical conservation treatments perhaps are easily neglected, which could cause unforeseen detrimental effect.

Restoration of historical manuscripts and paper documents can be traced back to China, almost 2000 years ago - the birth of well-known techniques including mounting, remounting, backing, lining, *etc*. The first evidence of such techniques appearing in the Western world dates back to 1837 in the United States of America, and to 1858 in Europe (Marwick, 1964). The silking technique was first applied in the 1940s, and consists of the use of fresh silk gauze as an ideal solution to

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https://doi.org/10.1016/j.ibiod.2018.07.010
Received 21 December 2017; Received in revised form 15 July 2018; Accepted 20 July 2018

strengthen the manuscript pages (Marwick, 1964). This technique has been formerly used, extensively, to preserve numerous manuscripts in a wide range of institutions. In particular, it constituted the major conservation effort for thousands of manuscripts belonging to the Stein collection in the British Library in the 1960s-1970s. However, scientific studies on silk-faced manuscripts are lacking, especially on the detrimental impacts. This constitutes a serious omission, because water, starch paste and animal glue paste, which were often used, might increase the manuscripts' ability to be colonised by living organisms upon silking. Such potential vulnerabilities through the decades might have opened the door for microbial colonisation, mainly fungi (Cappitelli et al., 2010; Sterflinger and Pinzari, 2012). Moreover, microbial colonisation can provoke serious damage/degradation of the affected manuscripts (Cappitelli et al., 2010; Sterflinger and Pinzari, 2012).

The silk facing procedure is, of course, no longer used in an era defined by minimal intervention. However, the question remains “how diverse is the community now colonising the silk-facing materials?” This study aims at evaluating the presence of fungal contamination on ancient Chinese manuscripts from the Mogao Grottoes that have been subjected to the silking conservation technique and are currently requiring further conservation (Fig. 1A) and weight arguments on whether silk should or not be removed. The data obtained provide sufficient evidence of both the physical damage and the fungal contamination of the ancient Chinese silk-faced manuscript selected for study.

2. Materials and methods

2.1. Samples

The manuscripts originate from Dunhuang, dated from the 5th to early 11th Centuries, discovered by Yuanlu Wang in 1900 (Wang and Perkins, 2008). They were sealed in Cave 17, known as the Library Cave, in the Mogao Grottoes, where closely packed layers of heaped bundles of scrolls were discovered, along with textiles, such as banners, as well as figurines of Buddha (damaged) and other Buddhist artefacts. Mogao Grottoes enclose important cultural heritage and have been listed officially as UNESCO World Heritage Site in 1987 (Wu et al., 2017). The manuscripts, many of which reside in the British Library, are referred to as the Stein Collection (Wang and Perkins, 2008).

Two representative manuscripts with silk-facing were selected for this study (Fig. 1A and B), namely Or.8210/S.417 and Or.8210/S.316 (*n.b.* this is the British Library registration system for manuscripts from Dunhuang in the Stein Collection, and uniquely defines a document), from which silk threads (BL1 and BL2, showing distinct yellowing of the fibres) and glue (BL3-BL6) were removed and donated by the British Library.

2.2. Surface analyses

In order to investigate the deterioration of the silk facing materials, a scanning electron microscope (SEM) coupled with energy dispersive X-ray spectroscopy (EDAX) was employed: JEOL JSM-6500F Field Emission Scanning Electron Microscope and Oxford instrument INCA X-sight 7558 (School of Mathematics and Physics at QUB). Silk samples were sputter-coated with gold, and were affixed *via* copper tape to the SEM sample holders.

2.3. Cultivable fungi isolation, identification and characterisation of cellulolytic activity

Following the identification of structures in the SEM data, of similar size and shape to fungal spores and/or yeasts, it was speculated that this may be due to fungal contamination. To isolate any cultivable fungal strains, the samples were incubated in a sterile peptone solution (2%) for three days at room temperature followed by vortex cycles, and aliquots were then directly inoculated onto Malt Extract Agar (MEA) and incubated at 27 °C. The forming colonies were monitored daily. Isolated colonies were selected for further purification by consecutive sub-culturing onto fresh MEA. Morphological characterisation (microscopy) allowed their preliminary identification. Negative controls, *i.e.* similar materials (unused paper pieces collected inside the laboratory) were considered in parallel with the study samples to discard the possibility of cross-contamination during the analysis.

DNA extractions were undertaken from the fungal isolates mycelia using a DNeasy extraction kit (Qiagen). The DNA samples were stored at −20 °C until further analysis. Amplifications of a part of the β-tubulin and calmodulin genes, and the ITS regions (including 5.8S rDNA) were done in a GeneAmp PCR system 2720 (Applied Biosystems) thermocycler using the primers Bt2a and Bt2b, CMD5 and CMD6, and V9G and LS266, respectively (Deive et al., 2011). Primer sequences are as follows: Bt2a, 5′-GGT AAC CAA ATC GGT GCT GCT TTC-3’; Bt2b, 5′-ACC CTC AGT GTA GTG ACC CTT GGC-3’; CMD5 3’ - CCG AGT ACA AGG ARG CCT TC; CMD6 - CCG ATR GAG GTC ATR ACG TGG; V9G, 5′-TTA CGT CCC TGC CCT TTG TA-3’; LS266, 5′-GCA TTC CCA AAC AAC TCG ACT-3’ (Deive et al., 2011).

The PCR products were purified using the NZY Gelpure kit (NZYTech) and then sequenced at StarSEQ (Mainz, Germany). Sequence similarity searches were performed in public databases of GenBank (http://www.ncbi.nlm.nih.gov/) with BLAST (version 2.2.30).

To assess cellulolytic activity, each strain was platted onto carboxymethylcellulose (CMC) agar (0.2% NaNO3, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl, 0.2% CMC sodium salt, 0.02% peptone, and 1.7% agar) and incubated at room temperature. At the third and tenth day of incubation, the plates were flooded with Gram's iodine (binds to CMC) for 5 min and the excess of reagent removed. The formation of a decolouration halo indicates the production of cellulases, as previously described (Kasana et al., 2008).

2.4. Next generation sequencing (NGS)

DNA was extracted from the peptone extracts of each sample (see above: Cultivable fungi isolation and identification) using a DNeasy extraction kit (Qiagen). The DNA samples were stored at −20 °C until further analysis. Amplifications of the ITS2 region were done in a GeneAmp PCR system 2720 (Applied Biosystems) thermocycler using barcoded gITS7 and ITS4 (Ihrmark et al., 2012) in three PCR reactions per sample. The PCR reactions were set as previously described (Žifčáková et al., 2016). Primer sequences are as follows: gITS7, 5′- GTG ART CAT CGA RTC TTT G-3’; ITS4, 5′- TCC TCC GCT TAT TGA TAT GC-3’. The PCR products were then tested using gel electrophoresis and finally pooled for each sample and sequenced on Illumina MiSeq. NGS analysis was performed by Gene Expression Unit at Instituto Gulbenkian de Ciência (Oeiras, Portugal).

2.5. Data processing

The amplicon sequencing data were processed using the pipeline SEED 2.0.4 (Větrovský and Baldrian, 2013). Briefiy, pair-end reads were joined using FASTQ-join (Aronesty, 2013). The ITS2 region was extracted using ITS EXTRACTOR 1.0.11 (Nilsson et al., 2010) before processing. Chimera search was done using USEARCH 8.1.1861 and deleted. Sequences were clustered using UPARSE implemented within USEARCH (Edgar, 2013) at a 97% similarity level. Consensus sequences were constructed for each cluster, and the closest hits were identified using BLASTn against GenBank. Sequences with less than 10 reads were discarded. The phylogenetic relations between the OTUs identified were estimated using Bayesian approximate branch support at PhyML 20120412, and further visualised and exported using the FigTree 1.4.2. Descriptive statistics were performed using XLSTAT 2009.1.02, and histogram analysis took into account the number of reads of each OTU at each sample, as weights. The data herein presented have been