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The microbial community dwelling on a biodeteriorated 16th century painting

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ABSTRACT

The microbial community dwelling on the damaged parts of a 16th century painting displayed in the medieval church of San Giacomo Maggiore (Bologna-Italy), was evaluated through both culture-dependent and molecular methods. Among the fungal and bacterial isolates, strains belonging to *Penicillium* and *Bacillus* genera, respectively, were present. Growth on a standard restoring organic glue paste was assessed for both genera, while degradation of lignocellulosic materials was only ascribed to the fungal fraction of the microbial consortium. Through 16S rRNA gene library screening and Terminal-Restriction Fragment Length Polymorphism (T-RFLP) analysis, a description of the actual bacterial diversity developed upon the biodeterioration process was obtained. This analysis revealed, along with the isolated bacilli, the presence of a complex community, which also included several heterotrophic bacterial genera. According to this study, the observed biodeterioration phenomenon was due to the biodegradability of the lining materials with the occurrence of environmental conditions that caused the switch from a dormant to a metabolically active state of the heterotrophic consortium.

1. Introduction

Biodeterioration is a relevant issue in the conservation of cultural heritage as works of art and monuments represent a widely diversified group of ecological niches for various organisms. Depending on the chemical nature of the material, chemo-organotrophic and/or chemo-/photo-lithotrophic microbial metabolisms may develop, thus giving rise to both aesthetic and structural damage of the piece or pieces. For this reason, a more complete knowledge of biodeterioration phenomena and microbial diversity may provide useful indications and suggestions on the best restorative and preserving strategies.

During the last decade, most of the studies on the characterization of microbial communities associated with biodeterioration phenomena have been focused on damaged stone-monuments, mural paintings, and frescoes (Ciferri, 1999; Gorbushina et al., 2004; González and Saiz-Jiménez, 2005; Urzì et al., 2008; Scheerer et al., 2009). Nevertheless, removable paintings on canvas or wood-panels may also provide a wide range of micro-habitats suitable for microbial growth. Microbial growth may indeed occur both in the support material (cellulose, canvas, wood, parchment, silk, and wool), in the materials used to adhere the paintings to the support (animal or plant glues), as well as in the organic binders in which pigments are emulsified (oils, waxes, polysaccharides) (Ciferri, 1999). However, only a few studies have been completed that describe biodeterioration cases involving removable paintings on canvas or wood-panels. Examples of such studies include the bacterial and fungal populations colonizing easel paintings artificially exposed to soil and biofilm formation on the pictorial layer (Wazny and Rudniewski, 1972; O'Neil, 1986; Santos et al., 2009). Microbial colonization has also been studied on so-called "mock paintings", i.e. control painted canvas prepared employing the traditional materials utilized for paint works and inoculated with bacterial and fungal strains (Seves et al., 1996), also subjected to artificial aging (Seves et al., 2000). Thus, until now few efforts have been devoted to the elucidation of the microbial diversity occurring on actual biodeteriorated removable paintings.

Among the different materials that are present on removable paintings, organic glue pastes used to coat the back of paintings with linen canvas (an operation usually referred to as "re-lining") may represent a rich nutrient source for microbial growth. Utilization of such substrates by microbes may lead directly to structural damages, even if the painted surface appears unaffected (Caneva et al., 1994). Albeit the high biodegradability of the materials employed in re-lining operations of antique paintings (linen canvas, animal glues mixed with flour), such phenomena are quite rare, but may nonetheless occur under certain environmental conditions, e.g. high relative humidity, that favours the growth of

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airborne microorganisms otherwise occurring in the artwork in a dormant metabolic state.

Until now, the degradation of lining canvas and glue pastes has been examined from a chemical point of view (Shepard et al., 2000; Filippidis et al., 2009), where little attention has been paid to the microbiological aspects possibly associated with the degradation process. Particularly in recent years the most commonly used molecular fingerprinting techniques applied to biodeteriorated artworks were restricted to Denaturing Gradient Gel Electrophoresis (DGGE) (Rölleke et al., 2000; Möhlenhoff et al., 2001; González and Saiz-Jiménez, 2004, 2005). On the other hand, Terminal-Restriction Fragment Length Polymorphism (T-RFLP) has been shown to be a reliable and reproducible method to investigate microbial environmental communities (Smalla et al., 2007; Hartmann and Widmer, 2008; Capodicasa et al., 2009).

Here, the T-RFLP technique was applied for the first time to dissect the microbial community of a biodeteriorated piece of art in order to verify the potential use of T-RFLP as a diagnostic tool for biodeteriorated artwork. This latter was a 16th century panel painting that was showing in the medieval church of San Giacomo Maggiore (Bologna-Italy). This painting was completely detached from the wooden frame on which it was fixed, due to the widespread degradation of the lining glue paste and canvas, which were applied in the course of a restoration intervention in 1983.

Growth tests on the cultivable bacteria and fungi were also performed to elucidate their possible role in the occurred damage. Further, the physical interaction between the damaged canvas fibres and bacterial cells was investigated by fluorescent microscopy.

The results of this work represent an interesting case report on the dynamics of the biodeterioration phenomena caused by the microbial degradation of the materials typically used to restore ancient paintings.

2. Material and methods

2.1. Artwork description and sampling

The object of this work was the oil painting on wood panel "*Madonna col Bambino in gloria fra S. Caterina, il B. Riniero e S. Lucia*" painted in 1598 by Denys Calvaert and displayed in the medieval (13th century) church of San Giacomo Maggiore in Bologna (Italy). The painting was placed in a niche of the inner wall at a distance of 2 m from the church pavement. The relative humidity and temperature were measured by means of a Thermo-Hygro Sensor (THGR122NX, Oregon Scientific, USA).

No alterations were detectable on the painted layer, while a wide damaged area was visible on the peripheral areas of its back, namely: the degradation of the lining canvas and glue paste used in a re-lining operation carried out in 1983. This caused the sudden detachment of the painting from its wooden support during an ordinary cleaning intervention. Samples of the residual canvas were removed with a sterile scalpel from three areas on the back of the painting: along the damaged zone, along the wooden support on which the panel was fixed, as well as from the central zone of the painting back where no visible damage was observed.

2.2. Fluorescent microscopy

Samples of the lining canvas were stained for 30 min with 1 μg ml^{-1} Hoechst 33324 dye (Hoechst, Frankfurt am Main, Germany), washed with physiological solution (0.9% w/v NaCl) and observed using a coverslip. Bacterial cells were visualized by immunofluorescent analysis using a digital imaging system, comprising an inverted epifluorescence microscopy (Eclipse Ti-U, Nikon, Japan) and Omega filter pinkel set XF66-1 triband (Omega Optical Inc., Brattleboro, VT, USA). Images were captured with a back-illuminated Photometrics Cascade CCD camera system (Roper Scientific, Tucson, AZ, USA) and acquisition/analysis software Metamorph (Universal Imaging Corp., Downingtown, PA, USA). Cell morphology was visualized using a 60 × 1.4 oil immersion objective.

Beside samples taken from the damaged parts of the lining canvas, microscope observation was carried out on negative (sterile) and positive (inoculated) controls. A sterile control was prepared by autoclaving (20 min, 121 °C) some pieces of canvas samples and by detaching the dead cells through sonication (30 min in physiological solution). A positive control was obtained by incubating overnight sterile canvas samples with a cell suspension of one of the isolated *Bacillus* sp. (see below).

2.3. Isolation and characterization of microorganisms

About 500 mg of the canvas samples were washed with 10 ml of physiological solution by shaking for 30 min at 150 rpm and 30 °C. Serial dilutions were then plated on TSA medium for bacterial growth and malt extract agar 3% with peptone 0.5% and chloramphenicol 0.0002% for fungal growth and incubated for 15 days at 30 °C.

For the phylogenetic identification of the isolated bacteria, a portion of the 16S rRNA gene was sequenced. DNA was extracted as follows: 1.5 ml of cell suspension were centrifuged 5 min at 16,000 g, washed in TE, lysed with 30 mg ml^{-1} lysozime and 20 mg ml^{-1} proteinase K, and deterged with SDS 10%; cell debris were extracted with 1 volume of phenol–chloroform and successively with 1 volume of chloroform–isoamilic alcohol; DNA precipitation was carried out with 0.7 volume of iso-propanol and pellet washed with 200 μl of cold 70% ethanol. After re-suspension of the dried pellets, DNA was visualized for quantification on 0.7% agarose gel in TAE. The 16S rRNA gene amplification was performed from about 50 ng of genomic DNA with bacterial universal primers 27F and 1525R (Lane, 1991). The reaction mixture contained template DNA, 5 μl of 10× reaction buffer, 1.5 mM $MgCl_2$, 200 μM (each) deoxy nucleotide triphosphate (dNTP), 400 nM (each) primer and 3 U of *Taq* DNA polymerase. DNA amplification was performed in a thermocycler (Biometra, Göttingen, Germany) with the following profile: initial denaturation for 5 min at 95 °C; 30 cycles of denaturation (45 s at 95 °C), annealing (45 s at 55 °C) and extension (45 s at 72 °C); and finally an extension at 72 °C for 8 min. The success and yield of each amplification reaction were determined by electrophoresis on 0.7% agarose gel in TAE buffer with 5 μl aliquots of PCR products.

For fungal DNA extraction, an UltraClean microbial DNA isolation kit (MoBio Laboratories, Dianova, Hamburg, Germany) was used with minor modifications (Loncaric et al., 2008). 18S rRNA gene was amplified by means of the EukA/EukB primer set (Díez et al., 2001) with the following steps: initial denaturation at 94 °C for 3 min, 30 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 1 min, extension at 72 °C for 3 min, and a final extension at 72 °C for 5 min (Díez et al., 2001).

Purification of the 16S and 18S rRNA gene amplicons was carried out by means of the QIAquick PCR Purification Kit (QIAGEN, Chatsworth, CA, USA). Nucleotide sequences were determined through the Sanger method by means of an ABI 3730XL sequencer (Applied Biosystems, USA) and analysed by means of the BLAST software.

Phylogenetic clustering of the bacterial strains isolated from the non-degraded linen canvas was performed through ARDRA (Amplified Ribosomal DNA Restriction Analysis) by comparing them with the characterized strains from the degraded linen. About 50 ng of the 16S rRNA gene, amplified and purified as previously