RESEARCH NOTE

Genetic fingerprint of microorganisms associated with the deterioration of an historical tuff monument in Italy

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Introduction

Monuments, works of art, and other cultural heritage are affected by microbial colonization that can, together with physical and chemical factors, cause serious structural and aesthetic damage. This is because cultural artifacts provide an inviting range of elements which microorganisms use in their metabolism through biosolubilization, e.g., elements such as calcium, aluminum, silicon, iron and potassium. Microorganisms can induce unsightly discolouration of building material and frescoes, formation of pigmented biofilms, biomineralization and degradation of organic binders leading to structural damage (Herrera *et al*. 2004).

Microbial solubilization of materials involves the production of organic and inorganic acids by metabolic activity and is one of the leading biogeochemical mechanisms of rock decay. The bioreceptivity of a stone depends on its structure and chemical composition, air pollutants, moisture, and the varied elemental compositions of the stones provide a suitable environment for the microorganisms to develop. Moreover, phototrophic microorganisms may grow on the stone surface or may penetrate some millimetres into the rock pore system. These organisms can potentially contribute to the breakdown of rock crystalline structures. With time, the developing microorganisms cause the deterioration of the stones on which they reside by secreting enzymes and activating other metabolic activities by providing a suitable medium for their growth on the stone pores and surfaces (Dornieden *et al*. 2000; Warscheid and Braams 2000).

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Strategies aimed at preserving monuments are rapidly adapting molecular techniques to identify microorganisms. Molecular techniques provide the actual proportion of species in the microbial populations flourishing on deteriorated monuments (Gurtner *et al*. 2000; Gonzalez and Saiz-Jimenez 2005), since only few species (those resistant to desiccation) can be retrieved by culture techniques. This is a necessary starting point for any contemporary restoration treatment. However, it is also important to have a better understanding of the mechanisms responsible for the microbial attack to formulate more effective treatments. The detection of bacteria is mainly based on the PCR-amplification of sequences of the small subunit 16S ribosomal RNA (rRNA) genes (Gurtner *et al*. 2000). The molecular identification of fungi to species level has been based mostly on the use of variable ribosomal DNA (rDNA) internally transcribed spacer (ITS) regions (Anderson *et al*. 2003). Several molecular methods to obtain the microbial community fingerprints, such as DGGE, RFLP and SSCP, have been proposed in the survey of the dominant microbial population, including uncultivated and inactive microorganisms (Gurtner *et al*. 2000; Gonzalez and Saiz-Jimenez 2005).

Palazzo De Francesco in Campania, Italy, is an important historical building of the eighteenth century made of tuff as a building stone. Tuff (from the Italian 'tufo') is a type of rock consisting of consolidated volcanic ash ejected from vents during volcanic eruption. Tuff is common in Italy, and has been used from Roman to modern times for the construction of many buildings and bridges. Neapolitan yellow tuff is a product of centuries of volcanic activity and is formed from a volcanic ash called pozzolana. It is an especially fine, sandy, volcanic ash originally discovered and excavated in Italy around the Vesuvius volcano and Campi Flegrei,

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i.e., around the modern day cities of Naples and Pozzuoli. Pozzolana is decomposed tuff that is used as a form of architectural cement. The eighteenth century 'Palazzo De Francesco' is but one example where tuff has been used in monument construction. It forms the base of the outer walls which is covered over by a baroque plaster. These walls have a massive rosy discolouration affecting the most superficial layer along with a patina which almost covered the whole area associated with the detached plaster layer. The aim of the present work was to characterize the microflora implicated in biodegradation and discoloration of outer walls of the historical De Francesco building using molecular genetic approaches. The investigations were carried out by molecular means based on the amplification of 16S and ITS rDNA fragments, clone library construction and phylogenetic sequence analysis. Microorganisms were identified by comparison of sequences with those of known bacteria and fungi listed in the Blast database (Altschul *et al*. 1990). Using this approach, we aimed to provide a relationship between bacterial contamination of the site and the deterioration of the masonry. To our knowledge, this is the first report concerning the genetic fingerprint of microorganisms associated with the deterioration of an historical tuff monument. The understanding achieved from molecular identification of the microorganisms involved in biodeterioration can be used to formulate strategies for protecting and conserving tuff historical monuments for the benefit of future generations.

Materials and methods

DNA extraction

Samples were taken from a rosy discolored plaster (often associated with detachment) that still covers parts of the outer walls of the building by scraping off surface material together with underlying material. Genomic DNA was extracted directly from the rosy discoloured plaster according to Schabereiter-Gurtner *et al*. (2001). This protocol extracts DNA from the samples derived from the monument, and these samples were later amplified using PCR.

16S rDNA and ITS clone libraries

To obtain sequence information of individual members of the bacterial and fungal communities, the DNA extracted from the rosy discoloured plaster was amplified by PCR. Ribosomal fragments 16S and ITS were amplified and the respective rDNA clone libraries constructed. A clone library containing 800-bp 16S rDNA fragments was constructed by cloning the PCR product amplified with primers SSU1 and SSU2 (Berschick 1997). A clone library containing fragments of about 300 bp in size, specific for the internal transcribed spacer region 1 (ITS1) located between 18S and 5.8S rDNA, was constructed by cloning the PCR product amplified with primers ITS1 and ITS2 (White *et al*. 1990).

The purified PCR products for bacteria and fungi, respectively, were ligated and cloned into the pGEM-T Vector System (Promega, Mannheim, Germany) following the protocol of the manufacturer. The cloned DNA fragments were reamplified from 30 clones for each library with the SP6 and T7 primers (Qiagen, Hilden, Germany) matching the flanking regions of the pGEM-T polylinker. Amplicons were screened by amplified ribosomal DNA restriction analysis (ARDRA) with endonucleases according to Imperi *et al*. (2007) and Hunt *et al*. (2004). Clones showing identical restriction profile with both enzymes were grouped; the distinct taxonomic units were defined by 10 bacterial species and four fungal species. These were represented in the 30 clones for each library, which were sequenced.

Sequencing of 16S rDNA and ITS inserts and phylogenetic analysis

For sequencing of clone inserts, PCR product generated with primers SP6 and T7 was purified with a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced using an ABI DNA Sequencer model 373 (Applied Biosystems, Carlsbad, USA) and the Taq DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems). Sequences were deposited in the GenBank database (accession numbers are provided in figure 1; table 1).

Nucleotide sequence similarities were determined by using BLAST, version 2.0 (National Center for Biotechnology Information databases). Phylogenetic analysis was performed using MEGA version 4.0 (Tamura *et al*. 2007) after multiple alignment of data by Clustal W 1.8 (Thompson *et al*. 1994). Distance matrix and neighbour-joining methods were applied for tree construction.

Results and discussion

The sample DNA was isolated directly from areas of the monument that suffered deterioration (discolouration), associated with detachment of the plaster layer. The microbial population in the samples was identified by constructing 16S ribosomal and ITS clone libraries. Clone libraries allow the inclusion of the 16S and ITS rDNA sequences, providing a more reliable phylogenetic identification of microorganisms.

The molecular approach used in this study led to the identification of 10 bacterial species and four fungal species, as shown in table 1 and figure1. Large varieties of heterotrophic bacteria seem to be the first colonizers of moist frescos and building materials, and thus represent the first supply of organic matter (Karpovich-Tate and Rebrikova 1991). They excrete organic acids that have a biocorrosive action, thus contributing to the discolouration of the painted surface. The bacterial groups isolated in this work demonstrated phylogenetic relationships with *Bacillus* strains and indicated the presence of species previously isolated in other studies from mural paintings and monuments (Gorbushina *et al*. 2004; Saarela *et al*. 2004). In fact, *Bacillus* sp. are also the most frequent isolates from monuments affected by salt efflorescence (Saiz-Jimenez and Laiz 2000). Strains belonging to *Bacillus megaterium* and *Bacillus* (cadmium resistant) were also isolated here. These reflect

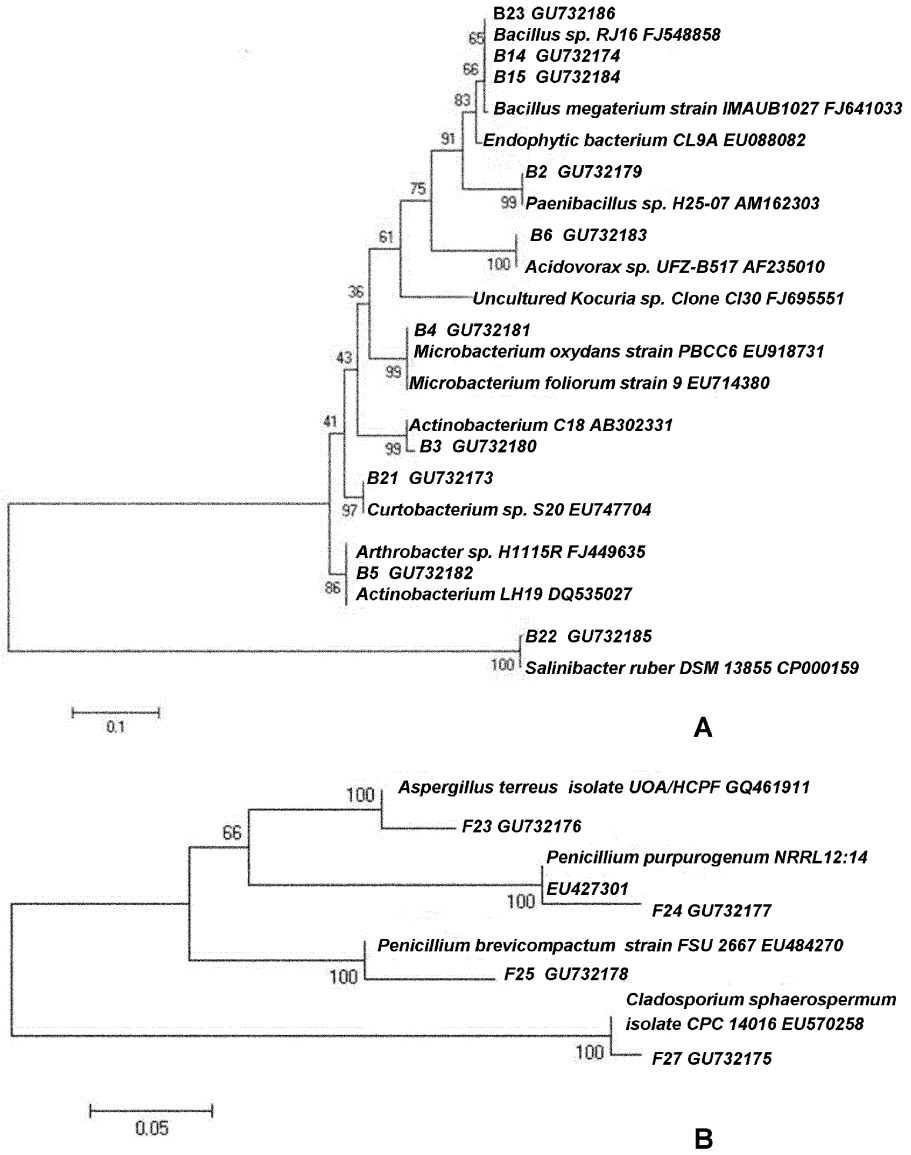

Figure 1. Phylogenetic tree of (A) bacteria, and (B) fungal strains developing on the stone work of Palazzo De Francesco and their nearest neighbours derived from maximum parsimony analysis. The identities of strains correspond to the identities given in the databases and accession numbers are given in parentheses after the taxonomic assignment. The phylogram was calculated from the divergence in partial sequences of 16S rDNA (bacteria) and internal transcribed spacer region 1 (ITS1) located between the 18S and 5.8S rDNA (fungi). Numbers given below the branches are frequencies (expressed as percentages) with which a branch appeared in 1000-bootstrap replicates. Branch lengths are proportional to nucleotide differences as indicated by numbers on branches. Consistency index = 0.878, retention index = 0.980, homeoplasy index = 0.122.

the adaptive opportunities of these isolates that can occupy this specific niche due to their osmotic adaptation. In deteriorated monuments, salt efflorescence on the surfaces is the result of changing physical parameters; this phenomenon creates micro-environments with high salt concentrations, facilitating the growth of extremely salt tolerant and moderately halophilic bacteria (Saiz-Jimenez and Laiz 2000). Here, we found for the first time on a historical monument, three clones showing 99% similarity with *Salinibacter ruber*, which has been found repeatedly in significant numbers in climax saltern crystallizer communities.

Salinibacter is a bacterium representing a convergence among archea and hyperhalophilic bacteria, displaying many remarkable similarities to haloarchaea, like a high salt concentration requirement in both cases, (Mongodin *et al*. 2005). Like haloarchaea, *Salinibacter* contains a high proportion

Table 1. Phylogenetic affinities of partial 16S rRNA bacterial and ITS rRNA fungal coding sequences detected in the stone work of 'Palazzo De Francesco'.

Strain number	Closest identified phylogenetic relatives	GenBank accession no.	Identity (%)
Bacteria			
B2	*Paenibacillus* sp. *H25-07 (AM162303)*	*GU732179*	99
B3	*Actinobacterium C18 (AB302331)*	*GU732180*	99
B4	*M. oxydans* strain *PBCC6 (EU918731)*	*GU732181*	99
B5	*Arthrobacter* sp. *H1115R (FJ449635)*	*GU732182*	86
B6	*Acidovorax* sp. *UFZ-B517 (AF235010)*	*GU732183*	98
B14	*E. bacterium CL9A (EU088082)*	*GU732174*	99
B15	*B. megaterium* strain *IMAUB1027 (FJ641033)*	*GU732184*	99
B21	*Curtobacterium* sp. *S20 (EU747704)*	*GU732173*	97
B22	*Salinibacter rubber (DQ915829)*	*GU732185*	99
B23	*Bacillus* sp. *RJ16 (FJ548858)*	*GU732186*	99
Fungi			
F23	*A. terreus (GQ461911)*	*GU732176*	97
F24	*P. purpurogenum (EU427301)*	*GU732177*	98
F25	*P. brevicompactum (EU484270)*	*GU732178*	95
F27	*C. sphaerospermum (EU570258)*	*GU732175*	98

of carotenoids in its membrane, producing red colonies that probably contributed to the rosy discolouration of the mansory. Members belonging to *Acidovorax, Kocuria, Curtobacterium, Actinobacterium*, widely present in hostile environments, were also isolated here (figure1A).

In our study, we observed that discolouration was sometimes associated with detachment of the paint layer and/or to the development of efflorescence or a patina. We isolated from these sampling points some heterotrophic filamentous microbes, like fungi belonging to the genera *Cladosporium, Aspergillus* and *Penicillium*, which contributed to the mechanical destruction of wall paintings due to mycelia production (figure1B; table 1). *Penicillium* sp., *Aspergillus* sp. and *Cladosporium* sp. are found on substrates that varies widely in paint chemistry, such as paint breakdowns on historic buildings and artifacts, and also vary between exposed and hidden surfaces. These fungi are able to penetrate into the rock material by hyphal growth and bio-corrosive activity, owing to secretion of organic acids and by oxidation of mineral-forming cations, such as iron and manganese. Fungi are able to interact with organic compounds, minerals and metals through biochemical and biomechanical processes, making them suitable as biological agents of rock and building stone. Their corrosive actions also include discolouration of stone surfaces due to the excretion of pigments (Warscheid and Braams 2000).

Growth of fungi on buildings results in damage to the structure and can have adverse health effects on humans too. Historic buildings are often unused and not heated and this, in turn, may be a cause of overcooling and a trigger for the production of fungal toxins. Restoration work such as replacing old plaster, or painting the walls, usually increases the humidity of the materials (Flaninngan 1992). We identified here *Cladosporium sphaerospermum*, which has a worldwide distribution and is very common on wet building material such as soil painted walls; its spores can elicit allergic reactions in susceptible individuals. It is reported that *C. sphaerospermum* has been involved in human corneal ulcer, skin lesions and infection of nails.

Moreover, from some of the sampling points, we isolated fungi belonging to the species *Aspergillus terreus* and *Penicillium brevicompactum* associated with the different species of *Bacillus*. It is been reported that the death and lyses of such bacteria would promote the growth of fungi (Garg *et al*. 1995). Ciferri (1999) in his researches on contaminated painted canvases reported that cellulolytic and proteolytic activities of *Aspergillus niger* stimulated the survival and growth of *B. pumilus*.

To our knowledge, the molecular strategy presented here, is the first report concerning the genetic fingerprint of microorganisms associated with the deterioration of an historical tuff monument. However, our data show several similarities with results derived from analysis performed on other monuments made with different materials and located in different geographic locations. These results prove the widespread distribution of hyperhalophilic bacteria found in hostile environments. Moreover, we found a trophic interrelationship made by fungi and bacteria in the microbial communities. Finally, we have shown that molecular characterization of microorganisms from biofilms enables, in great detail, more practical characterization of their deteriorative potential that would be useful for development of new biocontrol methods. Beside, the restoration and conservation of old constructions may help to protect human health from the toxic effect of mould fungi growing on building materials.

We conclude that genetic fingerprints in microbial investigations are very important branch of good conservation practice of antique buildings which includes close collabo-

ration between art and science, and can involve effects on human health too.

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References

Altschul S. F., Gish W., Miller W., Myers E. W. and Lipman D. J. 1990 Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410.

Anderson I. C., Campbell C. D. and Prosser J. I. 2003 Potential bias of fungal 18S rDNA and internal transcribed spacer polymerase chain reaction primers for estimating fungal biodiversity in soil. *Appl. Environ. Microbiol*. **5**, 36–47.

Berschick P. 1997 One primer pair amplifies small subunit ribosomal DNA from mitochondria, plastids and bacteria. *Biotechniques* **23**, 494–498.

Ciferri O. 1999 Microbial degradation of painting. *Appl. Environ. Microbiol*. **65**, 879–885.

Dornieden Th., Gorbushina A. A. and Krumbein W. E. 2000 Biodecay of cultural heritage as a space/time-related ecological situation—an evaluation of series of studies. *Int. Biodeterioration Biodegrad*. **6**, 261–270.

Flaninngan B. 1992 Indoor microbiological pollutants–sources, species, characterization and evaluation, chemical, microbiological, health and comfort aspects of indoor air quality–state of the art in SBS, Kluwer Acad., Amsterdam, The Netherlands.

Garg K. L., Kamal K. J. and Mishra A. K. 1995 Role of fungi in the deterioration of wall paintings. *Sci. Total Environ.* **167**, 255–271.

Gonzalez J. M. and Saiz-Jimenez C. 2005 Research review: application of molecular nucleic acid-base techniques for the study of microbial communities in monuments and artwork. *Int. Microbiol*. **8**, 189–194.

Gorbushina A. A., Heyrman J., Dornieden T., Gonzales-Delvalle M., Krumbein W. E., Laiz L. l. *et al*. 2004 Bacterial and fungal diversity and biodeterioration problems in mural painting environments of St. Martins church (Greene-Kreisen, Germany). *Int. Biodeterioration Biodegrad*. **53**, 13–24.

Gurtner C., Heyrman J., Pinar G., Lubitz W., Swings J. and Rölleke S. 2000 Comparative analyses of the bacterial diversity on two different biodeteriorated wall paintings by DGGE and 16S rDNA sequence analysis. *Int. Biodeterioration Biodegrad*. **46**, 229–239.

Herrera L. H., Arroyave C., Guiamet P., de Saravia S. G. and Videla H. 2004 Biodeterioration of peridotite and other constructional materials in a building of the Colombian Cultural Heritage. *Int. Biodeterioration Biodegrad*. **53**, 135–141.

Hunt J., Boddy L., Randerson P. F. and Rogers H. J. 2004 An evaluation of 18S rDNA approaches for the study of fungal diversity in grassland soils. *Microb. Ecol*. **47**, 385–395.

Imperi F., Caneva G., Cancellieri L., Ricci M. A., Sodo A. and Visca P. 2007 The bacterial aetiology of rosy discoloration of ancient wall paintings. *Environ. Microbiol*. **9**, 2894–2902.

Karpovich-Tate N. and Rebrikova N. L. 1991 Microbial communities on damaged frescos and building materials in the Cathedral of the Nativity of the Virgin in the Pafnutii-Borovskii Monastery, Russia. *Int. Biodeterioration Biodegrad*. **27**, 281–296.

Mongodin E. F., Nelson K. E., Daugherty S. and De Boy R. T. 2005 The genome of *Salinibacter ruber:* Convergence and gene exchange among hyperhalophilic bacteria and archaea. *Proc. Natl. Acad. Sci. USA* **102**, 18147–18152.

Saarela M., Alakomi H. L., Suihko M. L., Maunuksela L., Raaska L., Mattila-Sandholm T. *et al*. 2004 Heterotrophic microorganisms in air and biofilm from Roman catacombs, with special emphasis on actinobacteria and fungi. *Int. Biodeterioration Biodegrad*. **54**, 27–37.

Saiz-Jimenez C. and Laiz L. 2000 Occurrence of halotolerant/halophilic bacterial communities in deteriorated monuments. *Int. Biodeterior*. **46**, 319–326.

Schabereiter-Gurtner C., Pinar G., Lubitz W. and Rolleke S. 2001 An advanced molecular strategy to identify bacterial communities on art objects. *J. Microbiol. Meth*. **45**, 77–87.

Tamura K., Dudley J., Nei M. and Kumar S. 2007 *MEGA4:* Molecular evolutionary genetics analysis (mega) software version 4.0. *Mol. Biol. Evol*. **24**, 1596–1599.

Thompson J. D., Higgins D. G. and Gibson T. J. 1994 Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res*. **22**, 4673–4680.

Warscheid T. H. and Braams J. 2000 Biodeterioration of stone: a review. *Int. Biodeterioration Biodegrad*. **46**, 343–363.

White T. J., Bruns T., Lee S. and Taylor J. 1990 Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR protocols: a guide to methods and applications* (ed. M. A. Innis, D. H. Gelfand, J. J. Sninsky and T. J. White), pp. 315–322. Academic Press, NewYork, USA.

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