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TITLE: Development of a Rapid Indicator of Biodeterioration of Historic Stone

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1. Executive Summary

Biodeterioration plays an important role in the degradation of stone in historic buildings, monuments, and archeological sites. Microbial deterioration occurs through the action of organic and inorganic acids produced by biofilms. Detection of microbial deterioration of culturally important stone objects is difficult. The use of microbiological indicators of environmental conditions is common (e.g., *E. coli* is a key indicator of fecal contamination of water). The objective of this project was to compare the microbial community on deteriorated and undeteriorated stone. The microbial community of both deteriorated and undeteriorated locations was dominated by Cyanobacteria. In undeteriorated locations the dominant organism was *Anabaena cylindrica*. In deteriorated locations, the dominant organism was *Chroococcidiopsis* sp. Differences between the communities suggest that microbial indicators could provide a simple and rapid means for early detection of stone biodeterioration.
2. Introduction

Biodeterioration plays an important role in the degradation of stone in historic buildings, monuments, and archeological sites (e.g., Saiz-Jimenez 1999). A wide range of microorganisms, such as Bacteria (Sand and Bock 1991), Archaea (Rölleke et al. 1998), cyanobacteria and algae (Tomaselli et al. 2000), fungi (Gorbushina et al. 1993), and lichens (Garcia-Rowe and Saiz-Jimenez 1991) have been implicated in the degradation of stone. Additionally, stone objects may support unique communities of microorganisms (e.g., alkaliphiles, halophiles and endoliths) that function in the biodeterioration process (Saiz-Jimenez and Laiz 2000).

Microorganisms that colonize stone surfaces form biofilms. Biofilms are collections of bacterial cells on surfaces that are maintained by electrostatic forces and adhering exopolymers. Formation of biofilms begins with the initial adhesion of microorganisms to a surface. Division of attached cells produces microcolonies containing large amounts of exopolymer separated by sparse areas relatively devoid of growth. Production of exopolymer and other exudates is stimulated in response to cellular density by cell-cell signaling. The exopolymer matrix is composed mainly of polysaccharides, and serves a variety of functions such as providing protection from desiccation, radiation, erosion, and disinfectants, as well as storage of organic carbon and nutrients (Costerton et al. 1995, Flemming and Wingender 2001). Additionally, the exopolymer matrix limits the rate of diffusion within microcolonies, resulting in the formation of microenvironments due to gradients in pH, O₂, nutrients, and organic carbon (Rittman et al. 1999).

Microbial biodeterioration of stone is widely thought to occur through the action of organic and inorganic acids produced as metabolic by-products of biofilms (Gu et al. 2000). We have observed that bacteria isolated from biofilms at the Maya site of Ek Balam, Yucatan, Mexico, are capable of producing stone dissolving exudates (Perry et al. 2003). However, interactions between microorganisms and stone are complex and not all organic acids produced by microorganisms cause immediate dissolution of stone. For example, oxalic acid may have a protective role due to the formation of calcium oxalate on stone surfaces (Di Bonaventura et al. 1999).

Adding to the complexity of interactions between microorganisms and stone, we have found that biofilm exopolymers may play an important role in stone deterioration (Perry et al. 2003). Bacterial exopolymers are large macromolecules consisting of varied sugar molecules exhibiting functional groups (e.g., acidic carbonyls, Ford et al. 1991) capable of binding cations (Smidsrød and Haug 1965). Negatively charged carboxylic and hydroxyl groups of exopolymeric materials, such as alginic acid, may deteriorate stone through the formation of complexes with the mineral surface (Perry et al. 2004).

Detection of biodeterioration of culturally important stone objects is inherently difficult. In some cases, detection is based on macroscopic observations of pitting or etching of stone (Wakefield and Jones 1998). When macroscopic evidence of biodeterioration is absent, laboratory experiments have been used to determine the potential for biodeterioration (Di Bonaventura et al. 1999). There is no standard technique used to
measure the potential of a community to cause biodeterioration; a variety of time consuming and inadequate methods have been used. Simple methods such as measuring mass loss of stone samples may require long incubation times before changes are detectable. Additionally, there is the potential for interference by the accumulation of microbial biomass on the stone. Scanning electron microscopy, which is used to observe surface degradation, fails to detect changes below the surface unless samples are sectioned, potentially causing severe disturbances to the sample. Methods such as nuclear magnetic resonance (Alesiani et al. 2000) and acoustic wave velocity (Papida et al. 2000) must be correlated with other measurements (e.g., mass loss). X-ray computed tomography (Bentz et al. 1995, Jacobs et al. 1995) and more recently high resolution micro-computed x-ray tomography (McNamara et al. 2002, McNamara et al. 2003) have been used to examine stone building materials and may potentially be applied to analysis of microbial biodeterioration. However, the analytic method is expensive and not easily available. Stimulation of aqueous calcium release from carbonate minerals by microorganisms has been used as evidence of biodeterioration. In a non-quantitative method, calcium carbonate has been incorporated into solid laboratory media and solublization of the mineral has been inferred from the presence of clear zones underneath bacterial colonies (Di Bonaventura et al. 1999). Calcium dissolution has been quantified in liquid media using chelator titration (Lyalikova et al. 1991, Urzi et al. 1991), ion selective electrodes (Lewis et al. 1987), and Ca$^{2+}$ binding fluorochromes (McNamara et al. 2005).

In addition to their individual limitations, each of the techniques described above requires the isolation and cultivation of microorganisms in the laboratory. Studies from many environments, including cultural heritage materials, have demonstrated that the majority of microorganisms are not culturable (Jannasch and Jones 1959, McNamara et al. 2003). Furthermore, the metabolic (and thereby biodeteriorative) activity of microorganisms growing on laboratory media may differ significantly from the activity of the same organisms in situ. Therefore, we have used non-culture based, molecular techniques to search for microbiological indicators of stone biodeterioration.

The use of microbiological indicators of environmental conditions is quite common. The best known instance is the use of *Escherichia coli* as an indicator of fecal contamination of water (Toranzos et al. 2002). Other examples of microbial indicators of fecal contamination include total coliforms, fecal streptococci and enterococi, *Clostridium perfringens*, and bacteriophages (Toranzos et al. 2002). Microorganisms have also been used as indicators of the potential for skin infections (staphylococci), trophic status of bodies of water (Rosen 1981), forensic postmortem submersion interval (Casamatta and Verb 2000), and of radioactive and chemical contamination (Lemke et al. 1997). On stone cultural heritage materials, Mitchell and Gu (2000) found larger numbers of sulfur-oxidizing and hydrocarbon-utilizing bacteria on limestone exposed to air pollution than were present at an unpolluted location, suggesting that these organisms may be indicators of deterioration due to urban air pollution.

The objective of this project was to evaluate the use of microorganisms as early indicators of stone biodeterioration. The use of microorganisms as indicators has the
potential to provide a simple and rapid detection of the early stages of stone deterioration. Samples were collected from the Woodlawn Cemetery, located in Ayer, MA. Substantial differences were found between the microbial communities on deteriorated and undeteriorated stone, indicating that microorganisms could be used as indicators of stone deterioration.
3. Materials and Methods

Samples were collected from deteriorated and undeteriorated stone at the Woodlawn Cemetery located in Ayer, MA. Samples were collected from deteriorated and undeteriorated locations of the same headstone to minimize variation in the microbial community caused by other factors (e.g., age or type of stone, exposure, prior cleaning or conservation treatments). Status of the stone (i.e., deteriorated or undeteriorated) was determined by visual examination. Stone from the undeteriorated locations was characterized by a smooth surface and sharp edges and corners. In deteriorated locations, the stone surface appeared rough and edges or corners appeared weathered or worn. Microbial growth was not visible to the naked eye at either location.

Microorganisms were removed from stone non-destructively using swabs. Swabs were immersed in a dilute detergent solution to facilitate removal of microorganisms and a 1.0 cm² area was swabbed. After sampling, swabs were placed on ice and returned to the lab.

DNA was extracted using the UltraClean Soil DNA Kit (MoBio Labs, Carlsbad, CA). The 16S rDNA was amplified using the polymerase chain reaction (PCR) as previously described (Perry et al. 2005) with primers 27f and 1492r (Lane 1991). PCR products were cloned into the pCR 2.2-TOPO vector and transformed into competent *Escherichia coli* as described in the manufacturer’s instructions (TOPO TA Cloning Kit K4500-01, Invitrogen, Carlsbad, CA).

Clone inserts were sequenced at the Dana Farber/Harvard Cancer Center High-Throughput DNA Sequencing Facility (Cambridge, MA) using a 3700 DNA Analyzer (Applied Biosystems, Foster City, CA) as described in the manufacturer’s instructions. Unaligned sequences were compared to the National Center for Biotechnology Information database using the BLAST search program to find closely related sequences (Altschul et al. 1997).
4. Results and Discussion

We analyzed over 100 clones from deteriorated and undeteriorated locations. In both locations, the microbial community was dominated by Cyanobacteria, which are also known as blue-green algae. These are relatively large, photosynthetic bacteria that are common on stone heritage materials and that have frequently been implicated as the cause of stone deterioration.

We found six different microbial taxa on stone from undeteriorated locations (Fig. 1). The most common microorganism on undeteriorated stone was *Anabaena cylindrica* (65.9% of the clones). *A. cylindrica* is a filamentous nitrogen-fixing cyanobacterium commonly found in freshwater. Other common microorganisms on undeteriorated stone were *Phormidium murrayi* (14.9% of clones) and *Chroococcidiopsis* sp. (10.6% of clones). *P. murrayi* is a filamentous Cyanobacterium that is tolerant of extreme temperatures and radiation. *Choococcidiopsis* is a unicellular Cyanobacterium that is also tolerant of harsh environments (e.g., both hot and cold deserts).

The microbial community from deteriorated locations was more diverse than that from undeteriorated locations. Nine different microbial taxa were found (Fig. 2). *Phormidium murrayi* was observed to make up approximately the same percentage of the community (16.4%) as on undeteriorated stone. *Chroococcidiopsis* sp. was the most common organism found on the deteriorated stone (61.8% of clones). *A. cylindrica* was not detected on deteriorated stone.

The differences observed between the microbial communities on deteriorated and undeteriorated stone present several possibilities for the development of a microbial indicator of deterioration. Dominance of the community by *A. cylindrica* or *Choococcidiopsis* sp. could indicate, respectively, either non-deteriorating or deteriorating communities. Another potential indicator is the difference in diversity between the two communities. Presumably, the more porous and rough surface of the deteriorated stone provides numerous microhabitats or niches in which the additional taxa can survive. The presence of consistently higher levels of microbial diversity on deteriorated stone could be an indicator of deterioration. A third possibility is that one of the less abundant taxa found on deteriorated stone (e.g., *Xylophilus ampelinus* or *Symploca atlantica*) is a good indicator of deterioration.

Additional work will be required to determine if any of these potential indicators are reliable and to determine the breadth of their applicability. Samples were collected at one location at one point in time. Previous studies have shown that while there appear to be differences in the photosynthetic microorganisms that colonize various types of stone and building materials (Tomaselli *et al*. 2000), the organisms found in tropical and temperate climates are quite similar (Crispim *et al*. 2003). Therefore, the results of our study may be valid over a range of geographic locations, but more work may be needed to characterize the communities on different materials.
Figure 1. Distribution of clones from undeteriorated samples.
Figure 2. Distribution of clones from deteriorated samples.
5. Conclusions

1. The microbial community from both undeteriorated and deteriorated stone was dominated by Cyanobacteria.
2. The microbial community on the deteriorated stone was more diverse than that on undeteriorated stone.
3. The dominant organism found on undeteriorated stone was *Anabaena cylindrica*.
4. The dominant organism found on deteriorated stone was *Chroococcidiopsis* sp.
5. Differences between the microbial communities on deteriorated and undeteriorated stone suggest that microorganisms could be used as indicators of stone deterioration.
6. References


