IDENTIFICATION OF BACTERIAL TAXA IN ARCHAEOLOGICAL WATERLOGGED WOOD

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1. Introduction

The conservation of archaeological waterlogged artifacts presents manifold problems due to the vulnerability of the materials which are affected, often synergistically, by physical, chemical and biological factors, particularly as a result of fungal and bacterial colonization [1 - 2].

In characterizing the state of conservation of archaeological waterlogged wood, studies mainly focalize on micro-morphological analysis, using Optical and Scanning Electron Microscopy [3 - 4].

In addition, information about the environment where the artifacts are found (pH, temperature, water salinity, sediment characteristics) is required, as it, too, has contributed to their abiotic and biotic deterioration [2].

It is of fundamental importance to detect and identify any morphological alterations caused by biological colonization, so as to distinguish degradation processes that have occurred during the time spent in the underwater site from those that have occurred during maintenance and storage in museum deposits or exhibition spaces.

The action of some microorganisms leads to important changes in the anatomical structure, physical properties and chemical composition of wood, with implications for the stability of the archaeological finds. As regards bacterial colonization, waterlogged wooden finds provide an ideal habitat for many communities, but the identification of the species is mainly carried out through in vitro culture or by evaluating the micromorphological alteration of the wooden structure [5 - 8].

The main colonizing bacteria in waterlogged wood are, erosion bacteria (EB), which are tolerant of low oxygen concentrations, and tunneling bacteria (TB). SEM analyses revealed their erosion effects manifest as deep channels, parallel to micro-fibrils in the cell wall [4] and loss of lingo-cellulosic structure. Tunneling bacteria are able to alter middle lamella elements, rich in lignin, deteriorating tracheids, fibers and vessels, before reaching the lumen of the cell wall [2, 8 – 9].

When a find is recovered from the seabed and brought into an aerobic environment, with fluctuating humidity, iron sulfides start to oxidize, producing iron sulfate minerals,

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such as melanterite, rozenite, jarosite. Moreover, the presence of sulfur oxidation can induce formation of yellow, orange and white mineral aggregates or pyrite [10 – 12].

In anaerobic conditions, organic and cellulose residuals produced by erosion bacteria (EB) activity induce development of sulfur-reducing bacteria (SRB), able to produce hydrogen sulfide (H_2S). The HS- ions react with lignin-rich parts of the wood to form organic sulfur compounds (mainly thiols) or produce, together with iron ions that come from the corrosion of metal substrates, particles of iron sulfides deposited in wood cavities [10].

Thus, microbial degradation of wood resting within marine sediments in low oxygen conditions is usually accompanied by accumulation of sulfur and iron compounds [12 – 15].

In the last few decades, the use of molecular techniques has allowed microbial species to be detected and identified in archaeological finds too, based on the analysis of specific sequences of microbial genomic DNA, extracted directly from wood samples, corresponding to the regions 16S and ITS - Internal Transcribed Sequence rRNA [13 – 15].

In this study, SEM analysis and molecular investigations have revealed specific structural alterations and cells in wood attributable to bacterial colonization, as well as the presence of framboidal pyrite (FeS_2), also found in clusters.

Molecular analysis based on in vitro amplification (Polymerase Chain Reaction) of 16S and ITS rRNA sequences [16 -18], allowed the identification of microbial consortia with ligninolytic and cellulosolytic activity (*Cellulomonas, Bacillus, Pseudomonas, Sphingomonas and Xanthomonas genera*), in addition to iron-oxydizing and sulfate-reducing bacteria (*Marinobacter spp., Desulforudis audaxviator*), otherwise undetectable with traditional *in vitro* culture methods.

2. Material and methods

2.1. Sampling

Wood samples were collected by sterile scalpel and loops, from blackish–brown areas (Figure 1). The samples (w1, w2, w3, w4, w5) were utilized for SEM analysis and for microbial genomic DNA extraction, obtained directly from the wooden fragments or the single colonies isolated on Nutrient agar (Difco).

Figure 1. Wooden finds sampled (w1-w5)

2.2. Microscopic analysis

Morphology of wooden sections was investigated by Scanning Electron Microscope (Leica, Cambridge Leo-400), coating the samples with gold micro-particles (13 nm), by *Agar-Auto-Sputter-Coater* (B7341).

2.3. DNA extraction and bacterial identification

Bacterial genomic DNA was obtained from single colonies isolated on Nutrient Agar, by means of rapid lysis in 1X TE (10mM Tris-HCl, pH 7.5/ 1mM EDTA) at 94°C for 3 min. using either the Stool mini Kit (Quiagen) or genomic DNA Purification kit (Fermentas), modified as follows: incubation at 65°C for 4 hours in presence of 5 mg/ml Proteinase K (Invitrogen).

Genomic DNA molecules were utilized as a template in the PCRs (Polymerase Chain Reaction) using 16S-rDNA primers specific for Pseudomonas, Cellulomonas and Bacillus genera, or universal primers for the ITS (16S-23S, Internal Transcribed Spacer) rDNA region [19]. The PCR reaction mixture consisted of: genomic bacterial DNA (lysis solution 5 µl or DNA 40 ng); 1X Reaction Buffer; 10 µM forward primer; 10 µM reverse primer; 2 µM dNTP mix; 2 mM MgCl2; 5 U/µl Taq DNA polymerase (Invitrogen). The PCR reactions are performed by the following amplification profiles: 1 initial cycle of denaturation at 95°C for 5 min; 30 cycles as follows: denaturation at 94°C for 1 min, annealing at 50°C (16S)–58°C (ITS) for 1 min, extension at 72°C for 2 min. A final extension step (72°C for 7 min) was added to ensure that all PCR products are full-length. DNA fragments were analyzed on 2% agarose gel in 1X TAE (50X TAE = 40 mM Trizma base, 20 mM acetic acid, 1 mM EDTA, pH 8.4), in 1X SYBR-safe DNA gel stain (Invitrogen).

The nucleotide composition of the PCR products was determined by Eurofins MWG Operon Sequencing Service [20] and the homology research was undertaken by BLAST analyzer [21].

3. Results

3.1. Microscopy analyses

SEM analyses of radial and transversal sections indicated middle lamella and cell wall alteration (Figure 2 B). Structural deterioration of the wood is shown in Figure 3 A-B, where the cell walls appear more distorted and fractured, due also to the mechanical action of crystalline aggregates inside the structure (Figure 3 C). The presence of pyrite framboids, single or clustered, is evident in Figure 4 A-B-C. Erosion effects, attributable to bacteria activity, are shown in Figure 5 A-B.

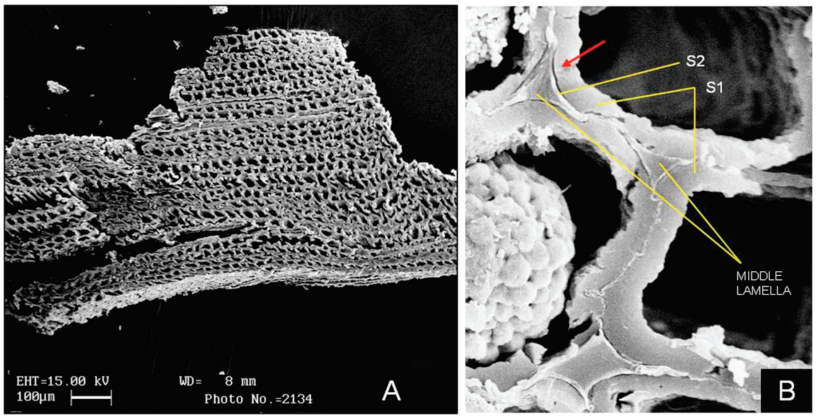

Figure 2. SEM micrographies: A) Transverse view; B) xilema degradation, red arrows indicate detachment of secondary cell walls S2 from middle lamella

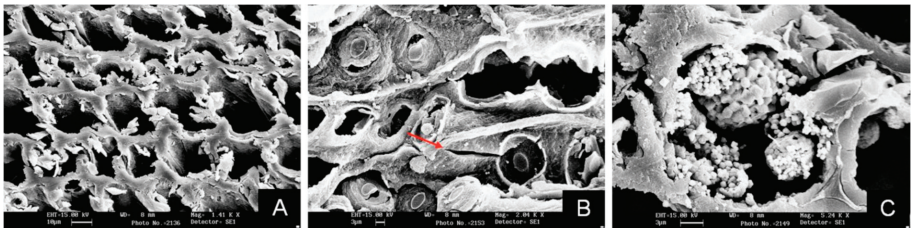

Figure 3. SEM micrographies: A) transverse section: the structure appear distorted for loss of mechanical resistance; B) radial section: cracks and fractures (red arrow) in cell walls; C) transverse view: the presence of crystalline aggregates induce breaking in cell wall

Figure 4. SEM micrographies: single (A) or clustered (B) Pyrite framboids; diatoms (C)

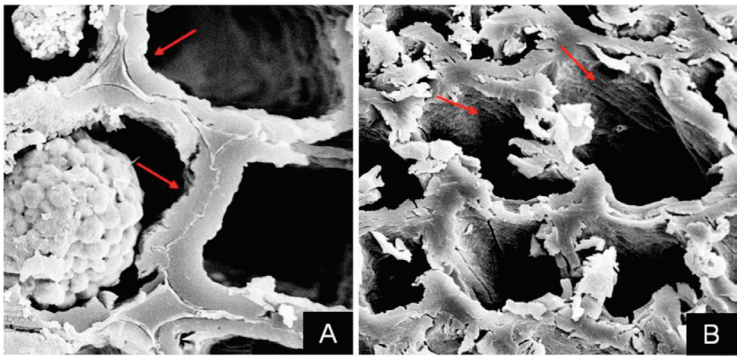

Figure 5. SEM micrographies: A- B) Cell walls erosion decay, probably induced by bacteria (red arrows)

3.2. DNA extraction and bacterial identification

PCR reaction products were analyzed by electrophoresis on 2% agarose gel, the sequencing and sequences analysis using the BLAST platform allowed cellulosolytic bacteria belonging to Cellulomonas, Bacillus, Pseudomonas, to be identified in almost all the samples analyzed.

Specifically for sample w3, in Figure 6A fragments of 220 bp, 750 bp, 680 bp (PCR reactions using ITS rDNA primers) correspond to Desulforudis audaxviator; Marinobacter spp. (sulfate - reducing and iron - oxidizing) and Xanthomonas spp. (ligninolytic) respectively. The presence of Cellulomonas, Bacillus and Pseudomonas genera was detected using 16S rDNA primers, whose amplification products were 201 bp, 181 bp and 338 bp in length respectively (Figure 6B). In addition, mono and polysaccharides hydrolyzing bacteria, belonging to Sphingomonas genera, were revealed in sample w2.

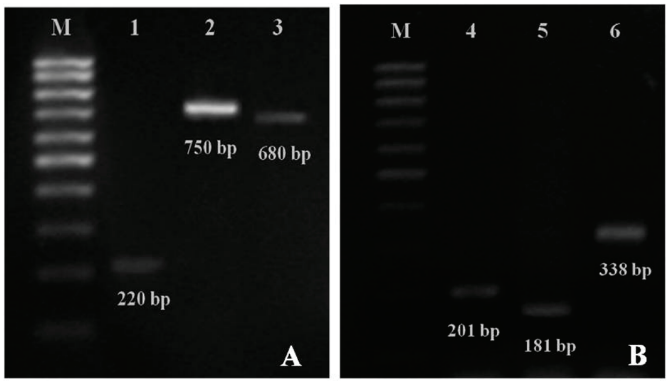

Figure 6. Gel electrophoresis on 2% agarose gel of w3 sample PCR reactions: A) lanes 1-3), in vitro amplification by universal ITS 16-23S rDNA primers; B) lanes 4-6, in vitro amplification by 16S-rDNA specific primers. M= DNA marker Sharpmass (Euroclone)

Table 1. Bacterial identification in five samples (w) through 16S and ITS amplification. Bacteria revealed in w3 are marked in red

DNA (BACTERIAL COLONIES)						DNA (WOODEN FRAGMENTS)									
	ITS – PCR					ITS - PCR					16S - PCR				
	w1	w2	w3	w4	w5	w1	w2	w3	w4	w5	w1	w2	w3	w4	w5
Pseudomonas spp.	+	+	+	+							+	+	+	+	
Cellulomonas spp.	+	+			+						+	+	+		
Bacillus spp.	+	+		+									+	+	+
Xanthomonas spp.			+												
Desulforudis audaxviator						+		+							
Sphingomonas spp.							+								
Marinobacter spp.								+							

4. Conclusions

The diagnostic tests performed on objects of historical and artistic interest provide information essential for assessing the state of preservation and in particular for identifying microbial colonization related to processes of biodeterioration. Although not always easy to implement, the identification of the components of a microbial consortium is of considerable importance in the implementation of operations compatible with both the artifact / specimen, and with the operator, as well as with the environment; this allows microbial growth to be eliminated or counteracted in a targeted manner. In the present study protocols were used developed specifically for the characterization of bacterial taxa that colonize waterlogged archaeological wood. In particular, the study involved the use of a scanning electron microscope (SEM) to observe the structural changes in the wood, and the set up of molecular protocols, based on the analysis of microbial genomic DNA, for the detection and identification of bacterial colonization.

In particular, bacteria were identified with ligninolytic or cellulolytic activity, belonging to the genera Cellulomonas, Bacillus, Pseudomonas, and Xanthomonas (besides Sphingomonas in sample w2) and iron-oxidising bacteria or iron sulfate - reducers, respectively Marinobacter sp. and Desulforudis audaxviator, otherwise undetectable with conventional in vitro culture methods.

Molecular studies have allowed us to complete identification of the bacteria colonizing the wooden finds through the analysis of microbial genomic DNA obtained from the bacteria colonies isolated on agar medium or directly extracted from the wood samples. Combining the results of the SEM observations and molecular studies, it is possible to correlate the microbial populations with the structural alterations with a view to compiling appropriate conservative restoration protocols which respect the integrity of the archaeological find.

This microscopy and molecular biology integrated approach provides an overview on microbial colonization and related alterations, in order to define appropriate restoration protocols.

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