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Innovative metagenomic approaches for detection of microbial communities involved in biodeteriorattion of cultural heritage

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Abstract. Microorganisms can colonize any known environment and modify it due to their extremely wide and versatile metabolism. From the microorganisms point of view, cultural heritage (CH) objects represent a series of very heterogeneous habitats (from historical stones to paints or archaeological wood) where they can grow as well as in natural habitats. Aesthetical and/or structural modifications of CH materials due to microbial growth and activities are referred as biodeterioration. In most cases biodeterioration is not due to a single species of microorganisms, but is the results of the activities of a composite microbial community at some stage of its development on the artwork. The knowledge of microbial community composition and its metabolic potential is essential to individuate the microorganisms mainly responsible for deterioration and take the appropriate measures to control their growth. Microbial communities can be investigated by two different methods: culture-dependent (based on the cultivation of microorganisms) and culture-independent (based on the analysis of diagnostic molecules, mainly nucleic acids). Even if culture-dependent methods allow to study the physiology and metabolism of the isolated microorganisms, they provide limited information on the diversity of complex microbial communities since the vast majority of microorganisms (approximately 99% for environmental samples) are not yet culturable. Microbiology applied to Conservation is a relatively young branch of environmental microbiology. For many years methods used to detect microorganisms inhabiting CH objects have been culture-dependent while molecular techniques developed from microbial ecology have been adopting only in recent years. Here we discuss the potential application of innovative metagenomics approaches, to investigate microbial communities on CH. Metagenomics approaches can be divided in targeted and untargeted methods. The targeted methods amplify and sequence marker regions of the ribosomal DNA to obtain a genus level picture of the microorganisms present in different substrates. The untargeted approaches can provide strain level information of the genes and the biochemical functions present in a microbial community. This review emphasizes the importance of applying metagenomics based, culture-independent techniques, in combination with the culture-dependent ones, to assess the biodiversity of microorganisms colonizing CH objects and take proper actions for their conservation.

1. Introduction

Microorganisms can colonize any known environment and modify it due to their extremely wide and versatile metabolism. They play a key role in almost all the biogeochemical processes involved in transformation of organic as well inorganic matter. If such transformations occur in materials made

and/or used by human beings, they are very often considered as negative. Biodeterioration can be defined as "any undesirable change in a material brought about by the vital activities of organisms" [1].

From the microorganisms point of view, cultural heritage (CH) objects represent a series of very heterogeneous habitats (from historical stones to paints or archaeological wood) where they can grow as well as in natural habitats. Bacteria, archaea, fungi, algae and lichens are the microorganisms commonly causing problems in the conservation of CH because of their biodeteriorative potential since they inhabit and penetrate into the materials. Even if microbes are invisible, they cause not only visible aesthetical damages (such as patinas or discoloration), but also severe structural modifications of CH materials such as fractures and material loss, due to acid corrosion, enzymatic degradation and mechanical attack [2]. This occurs on (and in) all types of cultural artefacts (organic or inorganic materials, historic or modern ones, art objects and historical buildings, paintings, costumes, ceramics, mummies, books and manuscripts, human remains and burial-related materials) and in indoor (public museums, private art collections, churches, caves and catacombs) as well as in outdoor environments (stone monuments, architectural surfaces).

For a long time abiotic chemical and physical processes were believed the dominant factors of decay of CH materials. Only in recent decades the role of microorganisms in causing deterioration of CH has been recognized, also due to the increase of knowledge about the ecological role of microorganisms in natural environments, and since the late 1980s significant effort was applied to ascertaining the biodiversity in the component materials of works of art [2].
Microbiological investigation is then a younger scientific discipline applied to Conservation than the more traditional chemical, physical and geological disciplines and now working alongside them.
It is now generally agreed that the variety of biodeterioration phenomena observed on CH materials are determined by chemical, physical and biological factors, and their interactions, linked to the nature of the material itself and to the environmental conditions of the object exposure and keeping (Ecology of biodeterioration).

In most cases biodeterioration is not due to a single species of microorganisms, but is the results of the activities of a composite microbial community at some stage of its development on the artwork. The knowledge of microbial community composition inhabiting the CH objects and its metabolic potential is essential to identify the microorganisms and mechanisms mainly responsible for deterioration and take the appropriate measures to control their growth, and has become an important research field both for conservators and for microbiologists [3].

Microbial techniques for investigating microbial communities can be divided into culture-dependent and culture-independent methods. Culture-dependent methods are based on the cultivation of microbial strains, thus allowing to study the physiology and metabolism of the isolated microorganisms. The drawback is that they provide limited information on the diversity of complex microbial communities. In fact it has been estimated that the vast majority of environmental microorganisms occurring in a viable state (approximately 99%) cannot be cultured according to standard procedures [4]. This is partly due to the lack of appropriate methods to grow environmental microorganisms, in particular those belonging to unknown or poorly known species or the anaerobes.

Moreover, very often culture conditions favor fast-growing opportunistic species and hinder the development of microorganisms playing a significant role in the environment. Microbiology applied to Conservation is a relatively young branch of environmental microbiology and has adopted many techniques developed from other sciences, in particular microbial ecology and clinical microbiology, to detect microorganisms. For many years methods used to investigate microorganisms inhabiting CH objects have been culture-dependent, often associated to Microscopy. This has allowed Microbiology to achieve important results in demonstrating the deteriorative role of microorganisms. Nevertheless, the limitation of traditional cultivation techniques and the value of culture-independent methods to assess the biodiversity of complex microbial communities were recognized by microbiologists working on CH at the beginning of the current century [5].

Culture-independent methods are mainly based on DNA level analyses to assess microbial community structure, function and dynamics regardless of the requirement to cultivate microorganisms.

The application of these techniques to CH has been reviewed in detail in [6] and in [7]. The most common techniques used so far have been genetic fingerprinting analyses such as DGGE/TGGE, ARDRA, t-RFLP, SSCP, ARISA, despite of interest their limitation is to fail to assign a taxonomic identification of the different members of a given microbial community. The most promising molecular techniques are those based PCR amplification of specific target genes, mainly rRNA genes, that serve as evolutionary clock and allow taxonomic assignment of the sequences obtained from a microbial community. Initially these techniques were applied to single colonies or libraries from environmental samples, following cloning of the single PCR products, yet in recent years, the application to microbial communities of next generation sequencing techniques has revolutionized microbiology, enabling massive sequencing of DNA samples, independently of cultivation and cloning. This field has been named metagenomics, the term refers to a study of genetic material isolated directly from an environmental sample, whereby it is possible to analyze all of the microorganisms from the sample, including the non-culturable ones [7]. Many studies on microbial communities carried out by sequencing of DNA libraries, carried out over the first decade of our century can be considered as metagenomic studies. Nevertheless, the depth of the investigation is being now enormously enhanced by the fast pace advancement of high throughput next-generation sequencing (NGS) technologies, which allow in-depth sequencing and data analysis of various types of environmental samples [3]. The NGS based methods are increasingly being employed to successfully characterize microbial community composition and functional diversity in different environments, from soil to human gut [8]. In the field of CH these methodologies are being adopted from few years and only few publications are available in the literature.

We will describe these state-of-the-art methodologies in Section 2 and review very recent studies that used them in assessing microbial diversity in CH objects in Section 3.

2. Metagenomics approaches and tools

Metagenomics offers a powerful lens for microbial microorganisms directly in their natural environments bypassing the need for isolation and cultivation of individual species. Before the coming of Next Generation Sequencing (NGS), microorganisms analysis were only based on cultured studied and sequencing with Sanger method [9]. The consequent advance offered by NGS is the ability to produce an enormous volume of data rapidly and cheaply. The ability to sequence the whole genome of many related organisms has allowed large-scale comparative and evolutionary studies that were unimaginable just few years ago [10]. NGS, and in particular Pyrosequencing analysis, "allows to provide cost-effective, time-consuming steps, rapid and highly parallel sequencing of large numbers of fragments from complex samples of DNA or even transcriptomes" [11].

This field of study is constantly evolving and its application can be divided mainly in two different approaches, targeted and untargeted metagenomics. Targeted metagenomics, the most common, sequences in parallel and massively target genes, serving as evolutionary clocks. The evolutionary clock of election is ribosomal RNA (rRNA). The success of this biomarker is based on the enormous database of rRNA gene sequences (more than 200,000) that have been collected for reconstructing the universal Tree of Life and that is exponentially increasing both thanks to targeted and untargeted seqeucning methods [12]. All organisms have rRNAs similar enough to each other that they can be recognized as the "same molecule" but different enough that the differences are a good measure of evolutionary distance.

The 16S rRNA gene has become an accepted standard in studies of Bacteria and Archea, in which the discrimination of different species occurs when two organisms have less than 97% identity of this gene [13]. 16S gene consists of about 1500 pairs of base [10], composed of conserved regions and nine short hypervariable regions, numbered from V1 to V9 (fig.1.5) that characterize the bacterial taxonomy. From literature emerges, that the nine-hypervariable regions show different capacities in the identification of bacteria [14]. In order to obtain a meaningful microbial identification, genus-or species-level classification is important. Using 16S rRNA gene sequence data, genera and species are typically distinguished at levels of 95% and 97% pairwise sequence identities, respectively [15]. The HMP Consortium, basing on NGS data, found out that 454-generated data describe microbial community

better than those generated with 3730 Sanger sequencing and determined that the statistically significant most accurate window region was the V3-V5, with the V6-V9 giving the worse results (Group Jumpstart Consortium Human Microbiome Project Data Generation Working Group, 2012 and [16]). In the case of metagenomics analysis of fungi, the region analysed was the ITS1 region. The ribosomes of eukaryotic organisms are composed of two structural subunits, 60S and 40S. The 60S subunit contain three different types of rRNA fragments: 28S, 5.8S and 5S rRNA. The 40S has a single 18S RNA fragment. The genes coding for the different fragments of rRNA are repeated genes, constituting the nucleolus organize region (NOR). Within the chromosome are clustered into structural units separated by non-coding spacer sequences called NTS (non-transcribed spacer) and IGS (intergenic spacer). Each unit consists of a structural and external transcribed spacer (ETS), located at the 3 'end of the 18S gene, two internal transcribed spacers (ITS), situated respectively: the ITS1 between the gene 18S and 5.8 S gene, the ITS2 between the 5.8S 3 'end and the 28S gene and 5S gene is separated from the 28S gene from another non-coding spacer sequence. Currently the ITS1 region, such V3-V5 region of the 16S, is a hypervariable regions, allowing identification of fungi at the species or genus level, and in the case of a metagenomic analysis, to understand the composition of the fungal community present in a particular habitat [17].

From a technological point of view, initially the metagenomics revolution was led by, Roche/454 pyrosequencing Life Sciences (Roche), used for the identification of ribosomal RNA and for the investigation of entire genomes. Pyrosequencing generates a large number of rDNA sequence tags by amplifying select variable regions within 16S or ITS rRNA gene that are currently regarded as the most versatile phylogenetic markers.

In parallel to pyrosequencing Illumina has recently developed a number of instruments enabling both targeted and untargeted sequencing of 16s or ITS hypervariable regions. The chemistry and chip design are different from Pyrosequencing, yet the potentials are greater, since the throughput and costs are exponentially decreasing.

Illumina is also the technology of choice for untargeted massive sequencing [18]. Untargeted metagenomics is significantly more expensive than targeted, and is based on generating large amount of short reads on the entire DNA community. Assignment of sequence to an OTU is a challenge dependent on the number of sequenced reference genomes available, thus this approaches are viable for the human metagenome, but are quite challenging for environmental samples. Yet the big advantage of these technologies is to provide unique insight on strain level variation [19] and on the microbial functions present [20].

The future of next generation sequencing technologies is now the ability to obtain long reads longer than 10kb, using single molecule sequencing approaches, that do not require amplification, allowing entire assembly of small genomes or organelles [21].

The fast pace at which the NGS is evolving promises to further reduce the costs and expand the potential, nevertheless the decision on the best technology to be used really depends on a careful definition of the biological question [22]. The main challenge being the bioinformatics development and the ability to extract meaningful information from the large datasets generated.

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3. Recent studies on CH biodeterioration based on Metagenomics and NGS Methods

Authors [28] characterized algal and fungal epilithic communities living on the surface of stone heritage structures in Belfast, UK, by using molecular techniques, namely TRFLP and high-throughput 454 pyrosequencing, to investigate the relationship between eukaryotic community composition and a variety of substrate characteristics. They found that community structure varied in association with major differences in stone geochemistry (i.e. limestone vs. sandstone). By 454 pyrosequencing a number of algal species that have not been associated with building stone were identified. However, algal communities were found relatively homogeneous and largely unaffected by minor differences in sandstone type or by morphological features such as surface profile or surface texture. In contrast, fungal communities were much richer and more spatially heterogeneous than the algae. According to the

authors, the relative simplicity of epilithic algal communities, coupled with their spatial invariance, should make these assemblages amenable to efforts to model the impacts of future climate change (e.g. through the use of manipulative experiments).

Authors in [23] investigated the diversity and abundance of microbial communities thriving in degraded mural paintings, selected for representing different environmental conditions in Portugal, by combining culture-dependent methods and culture independent methods, such as denaturing gradient gel electrophoresis (DGGE) and pyrosequencing. The coupled use of culture-dependent methods and DGGE did not give enough information to investigate the diversity and abundance of microorganisms present in wall paintings. Pyrosequencing, used for the first time for this kind of materials, allowed the identification of a large number of microorganisms, confirming some already identified by the cultivation-dependent methods (such as fungi of the genera Penicillium and Cladosporium), but also providing a great contribution in the identification of several genera and species not previously identified in these artworks. The results obtained on several mural painting samples showed a strong relationship between the most deteriorated areas of the paintings and higher microbial contamination. According to the authors, the application of pyrosequencing provided an important and exhaustive description about the microbial population that develops on murals paintings and allowed to expand the knowledge about them, giving a detailed overview of contaminants which was not possible with the other techniques.

Authors in [8] studied the phylogenetic diversity and activated metabolic pathways of microbial communities colonizing construction materials, such as wood, brick, mortar, and stone in historic buildings located in the former Auschwitz II–Birkenau concentration and extermination camp in Oświęcim, Poland. For this purpose molecular, microscopic and chemical methods were combined. Selected specimens were examined using Field Emission Scanning Electron Microscopy (FESEM), metabolomics analysis by ultra-high performance liquid chromatography (UPLC) coupled to high-resolution mass spectrometry (HRMS) and high-throughput Illumina sequencing. UPLC/HRMS- based metabolomics was used for the first time in the study of biodeterioration of heritage materials. DNA sequences documented the presence of 15 bacterial phyla representing 99 genera and nine fungal classes represented by 113 genera, confirming NGSM as a superior tool for interrogating the diversity of the microbial populations associated with heritage materials compared with culturing methods and clone library approach (previously used by the same authors on historic building materials). Most of the identified sequences were characteristic of organisms implicated in deterioration of wood and brick. Metabolomic data allowed the detection of numerous active metabolic pathways present in heritage materials, including those regulating the production of primary and secondary metabolites. The study demonstrated that a combination of FESEM imaging with metabolomics and genomic techniques allows to link the phylogenetic information and metabolic profiles of microbial communities, to shed new light on biodeterioration processes and to develop novel conservation strategies.

Authors in [29] carried out a high-throughput investigation of the prokaryotic communities colonizing the exterior walls of the medieval church of San Leonardo di Siponto (Italy) by Illumina-based deep sequencing.

The metagenomic analysis of sequences revealed the presence of Archaea, Bacteria, and photosynthetic Eukarya. The microbial diversity was explored at qualitative and quantitative levels, evaluating the richness and the abundance of reads associated with each OTU. The results highlighted a structured community, showing low diversity, made up of extremophile organisms adapted to desiccation and UV radiation. The authors concluded that molecular tools, and in particular the easy-to-run next-generation sequencing, are powerful to perform an accurate microbiological investigation and diagnosis in order to plan better strategies for restoration and protection of historical and artistic monuments.

In [24] the microbial communities colonizing the stone monuments of biodeteriorated ancient Buddhist statues in Hangzhou, China, were identified by high-throughput Illumina sequencing (performed on a MiSeq platform) of 16S rDNA for bacteria and ITS sequences for fungi. The diversity and distribution of the microbial communities in samples collected from three different environmental conditions with signs of deterioration were analyzed by means of bioinformatics software and diversity

indices. In addition, the relationship between these communities and environmental factors, including light intensity, air humidity and concentration of NO2 and SO2 were evaluated. The authors showed a positive association between the diversity and distribution of microbial communities and the considered environmental parameters and considered their data useful for the evaluation of effective control measures.

In a following study [25], the same authors characterized the microbial (prokaryotic and eukaryotic) communities colonizing other biodeteriorated Buddhist statues with signs of deterioration from the same site through a combination of high-throughput sequencing by Illumina MiSeq and culture-dependent techniques. The latter were used to test the ability of the isolates to degrade synthetic materials used to protect and reinforce the stone monuments. They found that the diversity of the microorganisms varied with the environmental conditions where samples were collected from, while a large proportion of the isolates had the ability to degrade protective materials.

In [3], 16S rRNA amplicon NGS using the Ion Torrent™ sequencing technology platform was applied for the first time to investigate the microbiota, Bacteria and Archaea, involved in the biodeterioration of building materials, as brick and paint coating used in historical buildings in Lodz (Poland). To evaluate the sensitivity of taxon detection depending on the type of primer pairs, amplicon sequencing of the 16S rRNA gene was performed using three sets of well-known primers (universal, bacteria- and archaea-specific primers). Results showed that the Ion Torrent™ sequencing platform has an enormous potential to evaluate the microbiome of historic building materials in a fast and easy way. The brick sample displayed a total of 1178 genera belonging to 27 bacterial and 3 archaeal phyla, while the paint coating showed 145 genera belonging to 11 bacterial phyla. Nevertheless, analyses showed to be influenced by the primer choice, and the authors recommend using, in addition to the universal primers, bacteria- and archaea specific primers in order to cover a higher taxonomic diversity.

Since deteriorating monuments, especially salt weathered ones, can provide suitable habitat for the proliferation of halophilic microorganisms, a special focus was given to detect them. The analyses revealed halophilic and halotolerant bacterial sequences, represented by genera which biodegradation potential was described and discussed in the literature. Moreover, the list of halophilic microorganisms obtained in previous work from cultivation and molecular techniques, comprising mainly DGGE and cloning analysed, applied to the built cultural heritage was remarkably expanded by the implementation of next generation sequencing using the Ion PGM™ System in metagenomics studies.

Authors of [26] employed and compared culture-dependent and culture-independent, using high-throughput sequencing on Illumina MiSeq platform, approaches for the first time to analyze the deteriorating microflora of book samples. It is worth noting that rRNA genes were amplified from total RNA extracted from the microflora sampled, which allows to detect metabolically active microorganisms. The comparison of results obtained from culture-dependent and culture-independent methods showed few similarities. High-throughput sequencing allowed for the detection of higher biodiversity than the culture-dependent approach, giving a deep view of the microflora contaminating different book portions. The authors compared their results with those coming from other few studies based on other culture-independent methods to analyse the bacterial communities in archival documents. Although bacteria members of the phyla Actinobacteria, Proteobacteria and Firmicutes very frequently contaminating archival objects were detected also by different approaches applied to the analysis of cellulose materials in previous works, the Illumina investigation allowed a more accurate analysis of this kind of microbial community. For fungi, Illumina results confirmed the findings on detected genera of Ascomycota, known to be plentiful in paper documents, of previous investigations and allowed to detect new taxa contaminating these materials, although their presence was in the range of 1-4%. Moreover, it evidenced the presence of members of Basidiomycota, not considered as the major fungal contaminant of archival documents, with a large diversity of this fungal group not been detected until now on archival items.

4. Conclusions and Perspectives

The high throughput NGS methods are increasingly employed to successfully characterize microbial communities occurring in different environments, from soil to human gut, since their fast-growing output per cost ratio [8, 3] and the advancements in appropriate bioinformatics tools to analyze the amount of phylogenetic data obtained. Nevertheless, these state-of-the-art methodologies are being adopted only very recently in the field of CH and only few publications are available. These studies, here reviewed, used targeted Metagenomics methods based on sequencing of partial rDNA amplicons to describe prokaryotic and eukaryotic communities colonizing deteriorated heritage materials, mainly stone structures, monuments and construction materials. The NGS sequencing platforms used were different, with the Illumina Miseq platform being the most adopted. Independently on the platform and the rDNA target region, a general agreement emerges among the authors on the indisputable advantage of using NGS methods as a powerful tool to deep analysis of microbial populations over conventional microbiological or other molecular techniques adopted in the study of biodeterioration. Moreover, these methods can be combined with a non-invasive (or micro-invasive) sampling, an essential requirement for working on historic or artistic products.

In order to get a deeper understanding of biodeterioration processes and to design effective and case-specific conservation strategies, it is of outmost importance to know not only the taxon composition of microbial communities, but also how microbial populations interact with each other and with heritage materials causing damage.

Metagenomics approaches on airborne microbial communities is also showing that climate change and pollution are changing the structure of microbial communities and their transport [27]. Thus it is expected that microbial communities involved in biodeterioration could also be affected and reflect this global change.

It is then absolutely necessary to develop appropriate methods for monitoring both composition of microbial communities and their physiological activity and combining different methods [2, 8, 26]. Monitoring methods must be optimized in order to be able to assess the function and possible community shifts, the effects of conservation treatments, climate change or biocide application [2, 26].

While cultivation methods are still widely used to investigate the physiological role of microorganisms in biodeterioration, studies attempting to investigate physiology and quantify microbial activity without cultivation in CH, i.e. studies on RNA or metabolic products, are still rare [2]. Among the studies here reviewed, only one used a metabolomic approach [8].

Metagenomics, metatranscriptomics and metabolomics technologies offer more powerful tools to understanding the activity and function of whole microbial communities and allow to generate unprecedented amount of genetic and chemical data on biodeterioration processes. These state-of-the-art technologies can be still combined to cultivation methods. NGS techniques can give an exhaustive picture of the microbial communities (richness and abundance) inhabiting CH objects allowing to identify microorganisms not identified by cultivation but probably involved in biodeterioration if detected with high percentages of reads and/or with a metabolic potential compatible with the investigated damage. These findings can direct efforts towards developing and improving cultivation strategies able to isolate selected groups of microorganisms with a potential role in deterioration. This is a challenge for next investigations in order to enhance the knowledge on cultivation of microorganisms from a specific material and their real deterioration effects [26].

Meta-omics approaches applied to the study of biodeterioration of CH are still in their infancy. Nevertheless, considering their deep detection power, the huge amount of data produced in a short time and the availability of bioinformatics tools to process them, and that the costs for molecular analysis are also going down, in the next future they should become a routine tool, also combined with implemented more conventional techniques, for monitoring structure and activity of microbial communities associated to biodeterioration.

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