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Application of metagenomic methods for selection of an optimal growth medium for bacterial diversity analysis of microbiocenoses on historical stone surfaces

Magdalena Dyda [a], Przemyslaw Decewicz [a, b], Krzysztof Romaniuk [b], Martyna Wojcieszak [c], Aleksandra Sklodowska [c], Lukasz Dziewit [b], Lukasz Drewniak [a, c], Agnieszka Laudy [d, *]

[a] Research and Development for Life Sciences Ltd., Miecznikowa 1/5a, 02-096 Warsaw, Poland
[b] University of Warsaw, Faculty of Biology, Institute of Microbiology, Department of Bacterial Genetics, Miecznikowa 1, 02-096 Warsaw, Poland
[c] University of Warsaw, Faculty of Biology, Laboratory of Environmental Pollution Analysis, Miecznikowa 1, 02-096 Warsaw, Poland
[d] Museum of King John's III Palace at Wilanow, Laboratory of Environmental Analysis, Stanislawa Kostki Potockiego 10/16, 02-958, Warsaw, Poland

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ABSTRACT

Identification of microorganisms on the surfaces of historical objects traditionally involves classical microbiology techniques. Cultivation methods allow, to a limited extent, the investigation of microbial biodiversity occurring in the environment. The aim of this study was to select microbiological media, the use of which enables cultivation of bacteria in proportions and diversity most similar to that observed *in situ* in the microbiocenoses of historical stone objects. We carried out scanning electron microscopy (SEM) imaging, quantitative analysis of microorganisms using culture-dependent methods and high-throughput sequencing of the bacterial communities taken from the surfaces of the Pergola located at the Wilanow Palace gardens in Warsaw (Poland). Total DNA was isolated directly from the collected samples and from the grown colonies of microorganisms grouped together from a particular type of microbiological agar media. Amplification of the V3-V4 region of the 16S rRNA gene for *Bacteria* was performed for each sample. Illumina MiSeq technology was used for sequencing. Metagenomic analysis of the sequences revealed that the agar Wort medium is the best choice to determine the bacterial diversity present *in situ* in the biocenoses grown on historical sandstone surfaces.

1. Introduction

Isolation and identification of microorganisms from historical objects are traditionally based on classical cultivation methods, which also enable quantitative and biochemical activity analyses of isolated strains. On the other hand, classical microbiology approaches are highly limited, as it is likely less than 1% of bacteria from environmental samples are cultivable (Giovannoni et al., 1990; Ward et al., 1990; Hugenholtz, 2002).

To address this challenge, numerous DNA-based techniques have been adapted for analyses of microbial communities. The most commonly used molecular techniques in the context of conservation and protection of cultural heritage are: construction of a clone library, genetic fingerprinting, e.g. denaturing or temperature gradient gel electrophoresis (DGGE and TGGE, respectively), amplified ribosomal RNA restriction analysis (ARDRA), terminal restriction fragment length polymorphism (T-RFLP), single strand conformation polymorphism (SSCP), or automated ribosomal intergenic spacer analysis (ARISA) (Otlewska et al., 2014).

These molecular methods were applied during microbiological analyses on different historical objects including paintings, books, parchments, textiles, sculptures, mural paintings, buildings or contamination of the air in exhibition space (Michaelsen et al., 2009; Diaz-Herraiz et al., 2013; López-Miras et al., 2013a; Piñar et al., 2013; Ettenauer et al., 2014a; Koziróg et al., 2014; Lupan et al., 2014; Otlewska et al., 2015; Pangallo et al., 2015; Piñar et al., 2015a).

* Corresponding author.
E-mail addresses: magdalena.dyda@rdls.pl (M. Dyda), decewicz@biol.uw.edu.pl, przemyslaw.decewicz@rdls.pl (P. Decewicz), romaniuk@biol.uw.edu.pl (K. Romaniuk), mciezkowska@biol.uw.edu.pl (M. Wojcieszak), asklodowska@biol.uw.edu.pl (A. Sklodowska), ldziewit@biol.uw.edu.pl (L. Dziewit), ldrewniak@biol.uw.edu.pl, lukasz.drewniak@rdls.pl (L. Drewniak), alaudy@muzeum-wilanow.pl (A. Laudy).

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The most significant examples of priceless and unique masterpieces that were subjected to microbiological analyses with the application of the above mentioned methods are: (i) the Leonardo da Vinci's self-portrait (Piñar et al., 2015b), (ii) Leonardo Da Vinci's Atlantic Codex (Tarsitani et al., 2014), (iii) Archimedes Palimpsest (Piñar et al., 2015c), (iv) Palaeolithic paintings in Lascaux Cave in France (Saiz-Jimenez et al., 2012) or (v) the Bayon temple in the historical city of Angkor Thom in Cambodia (Kusumi et al., 2013).

Amongst the techniques used for monitoring the microbiological agents that cause biodeterioration of cultural heritage objects, next-generation sequencing (NGS) is currently the most promising one. This approach enables not only the characterization of microbial diversity, but also allows for a better understanding of the functions, activities and dynamics of microbial communities in their natural environments (Zhou et al., 2015). For example, it has been successfully applied to the identification of biodeterioration agents on materials from historic buildings in the Auschwitz–Birkenau State Museum (Gutarowska et al., 2015), microbial communities on carbonate stones of the medieval church in Italy (Chimienti et al., 2016) and identification of the source animal species of parchments from the 17th and 18th centuries (Teasdale et al., 2015).

Regardless of the advantages of molecular methods, classical microbiology methods cannot be underestimated due to their availability to conservators, the minimal equipment needed and their relatively low cost. However, the most problematic aspect of these methods is the selection of microbiological media most accurate for isolation of bacterial strains, which are difficult to culture on the solid agar media commonly used in laboratories for environmental analyses.

In the present study, a metagenomics approach was used to select microbiological media to enable the most accurate determination of the bacterial diversity present *in situ* in microbiocenoses observed on historical stone objects.

2. Material and methods

2.1. Sampling sites

Samples were obtained from different sites at the historical Northern Pergola placed in the Museum of King John's III Palace at Wilanow in Warsaw (Poland). The Pergola was built between the courtyard and garden in 1852 within the extension of the northern wing of the Palace. The Pergola is made of sandstone with iron gates and fencing spans, and has not been renovated since the 19th century. Lack of conservation treatments has resulted in visible symptoms of biodeterioration on its sandstone surfaces, i.e. discoloration, spotting and visible signs of lichen and mold growth. Sampling of microbiocenoses on the Pergola's surfaces was performed in autumn 2014 on its northern side. Analyses were performed for 12 samples that displayed different morphology and deterioration symptoms. Each sampling site was documented by color photography (Fig. 1).

Warsaw (Poland) is located at 52°9′N, 20°57′E in the mild/cool summer subtype of the warm, humid continental climate zone with rather cool summers and long, cold winters (Rubel and Kottek, 2010). The average daily temperature in the city in 2014 ranged between −2.2 °C and 18.3 °C and the mean year temperature was 9.1 °C. Temperatures often reached 28 °C in the summer and −8 °C in the winter. The yearly rainfall average was 600–700 mm (Ustrnul et al., 2014).

2.2. Scanning electron microscopic observations of biocenoses samples

Biofilm samples for microscopic images were carefully collected from 12 sampling sites on the Northern Pergola's surfaces (Fig. 1) of approx. 2 cm^2. Samples were scraped with sterile scalpels to a depth of 1 mm, and immediately placed in sterile laboratory tubes. The preparations were fixed in formaldehyde vapor desiccator for 3 weeks in the presence of silica gel desiccant. Prior to observation, the samples were sputtered with gold. Preparations were viewed in a scanning electron microscope LEO 1430VP (LEO Electron Microscopy).

2.3. Quantitative analysis of microbiological community in biocenoses – classical microbiology approach

The microbial colonization of the Northern Pergola was assessed by the count plate method. Swabs were taken from different sampling sites on the Pergola's surfaces (Fig. 1) from areas of 50 cm^2. The sterile sampling swabs were immersed in 2 mL of sterile saline (0.85% NaCl) in the laboratory tubes, shaken, and spread (0.1 mL) onto agar media for bacteria or fungi. Agar media were selected according to the standards of microbiological analyses in the Museum of King John's III Palace at Wilanow. Media dedicated to yeast and fungi were also used as, during preliminary microbiological analyses of the Pergola's surface growth, a large number of various bacterial colonies on these agar media were also observed. All plates with agar media were manufactured by BTL (Poland): (i) nutrient agar (peptone – 8 g l^{-1}, beef extract - 2 g l^{-1}, NaCl - 5 g l^{-1}, agar–15 g l^{-1}); (ii) Blickfeldt medium (yeast extract - 2,5 g l^{-1}, peptone - 10 g l^{-1}, glucose–10 g l^{-1}, lactose - 10 g l^{-1}, $CaCO_3$–5 g l^{-1}, agar - 20 g l^{-1}); (iii) Frazier medium (peptone – 5 g l^{-1}, beef extract - 2 g l^{-1}, NaCl - 4 g l^{-1}, gelatin - 40 g l^{-1}, yeast extract - 2 g l^{-1}, agar - 20 g l^{-1}); (iv) Czapek-Dox agar ($NaNO_3$ -3 g l^{-1}, $MgSO_4$ x $7H_2O$ – 0.5 g l^{-1}, K_2HPO_4 - 1 g l^{-1}, KCl – 0.5 g l^{-1}, $FeSO_4$ x $7H_2O$ – 0.01 g l^{-1}, saccharose - 30 g l^{-1}, agar 15 g l^{-1}) and (v) Wort agar (malt extract – 15 g l^{-1}, pepton K – 1 g l^{-1}, maltose – 12.5 g l^{-1}, glucose – 2.5 g l^{-1}, K_2HPO_4 – 1.0 g l^{-1}, NH_4Cl – 1.0 g l^{-1}, agar – 20 g l^{-1}). The samples on agar plates with various microbiological media were incubated at room temperature (22–26 °C) for 10 days. The results were given as the number of Colony Forming Units on 100 cm^2 of analyzed surfaces (CFU/100 cm^2). All tests were performed in triplicate.

2.4. Total DNA isolation and purification

Biofilm samples were carefully collected from 12 sampling sites on the Northern Pergola's surfaces (Fig. 1) of approx. 9 cm^2. For comparison of bacterial diversity grown on different microbiological media, 0.1 g of each of the collected samples was suspended in saline (0.85% NaCl) and spread on each of microbiological media listed above. After 10 days of incubation at room temperature, colonies grown on each of the microbiological media were flooded with sterile saline (5 mL) and the microbial suspension from all 12 samples grown on particular medium were pulled together (finally 5 pooled samples from 5 microbiological media were obtained), transferred to sterile laboratory tubes and used for the isolation of total DNA. Total DNA was also isolated and purified from the 0.1 g of each of the 12 environmental samples (as a control, reflecting bacterial diversity *in situ*). Total DNA isolation was performed using Power Soil® DNA Isolation Kit (Mo Bio Laboratories Inc). Then, obtained DNA was additionally purified using Wizard® DNA Clean-Up System (Promega).

2.5. Amplicon preparation

For the PCR amplicon preparation, the following primer pairs were used: 16S_V3-F: 5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG 3′ and 16S_V4-R: 5′

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