

Molecular Techniques: Application to the Analysis of Microbial Communities Colonising Art Works and to the Monitoring of Changes.

Case Study: Wall Paintings of the Castle of Herberstein

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1. State of the art

1.1 Introduction

Microorganisms can be responsible for the destruction of cultural heritage, together with several environmental conditions, ageing and the chemical structure of substrate. There is a number of reviews giving a comprehensive picture of the role of microorganisms in the degradation of art objects, such as paintings, stone, wood, paper, masonry, leather, parchment, glass and metal (Bock and Sand, 1993; Cifferi, 1999; Griffin *et al.*, 1991).

Restoration efforts do not always obtain the expected result, and sometimes they even accelerate the deterioration process. Restoration works should take in account biodecay as an integral part of the global deterioration process. Therefore, an inventory of the existing microorganisms associated with the damage of the selected objects of art is a prerequisite to be included in any restoration project.

When microbiologists and restorers are confronted with a request to investigate the presence and role of microorganisms on a defaced art work, they have to deal with a substrate on which, often, microbial colonisation has taken place for years. The microbial flora is usually the result of successive colonisation by different microorganisms. The alterations suffered by the substrates are the result of modifications of their chemical composition, to which the microorganisms themselves may contribute partially. Furthermore, the substrate only represents the situation at the present moment, and not a time elapsed picture of the development of the microbial communities existing and evolving during the life of the object of art. Restorers and microbiologist are usually asked to give a diagnosis and to provide the information necessary to design corrective interventions in a short period of time. Therefore, it would be necessary to characterise the microbial flora present on art works before any alteration becomes evident. By determining which microorganisms are colonising an object at time “zero”, it would then be possible to make a reasonable assumption about how the microbial community will develop. In this context, it would be possible to suggest the nature and the mode of treatment for stopping microbial colonisation before the damage becomes irreversible. These studies should start with a descriptive analysis, cataloguing which organisms are found on which substrate. This is a necessary starting point for any restoration treatment. However, it is also important to understand the mechanisms responsible for the microbial attack. To that purpose, it will be necessary to follow-up the variations in the microbial populations when environmental conditions change. This research would allow to establish a protocol for “monitoring” the rate of microbial colonisation of an art object, the changes in the microbial population as a function of the substrate composition, environmental conditions and, eventually restoration.

1.1.1 The need of culture-dependent techniques

Traditionally, microbiology research carried out in the field of Biodeterioration was mainly based on classical cultivation methods. Culture-based approaches, while extremely useful for understanding the physiological potential of isolated organisms, do not necessarily provide a

comprehensive information on the composition of microbial communities. The results obtained by culture-dependent techniques covered only those few organisms that could be cultivated. Due to this well documented disparity between cultivable and in situ diversity, it is often difficult to assess the significance of cultured members in microbial communities. Several studies have employed culture-independent techniques to show that cultivated microorganisms from diverse environments often may represent very minor components of the microbial community as a whole. It is generally accepted that cultivation methods recover less than 1% of the total microorganisms present in environmental samples (Amann *et al.*, 1995; Ward *et al.*, 1990), therefore, microbial investigations based only on cultivation strategies can not be regarded as reliable in terms of reflecting the microbial diversity present in art samples. In addition, extensive cultivation strategies require more sample material than could be obtained from art objects.

However, the use of conventional culture techniques and the developing of new culture media are encouraged due to the advantages of having pure isolates to perform physiological and metabolic studies. Most of the unculturable bacteria fall into one of the following categories:

- a) Obligately symbiotic and parasitic organisms thriving under host-provide conditions but failing to grow in/or on bacteriological media.
- b) known species for which the applied cultivation technique are just not suitable or which have entered a non-culturable state.
- c) unknown species that have never been cultivated before for lack of suitable methods.

Using both 16S rDNA phylogenetic analysis and enrichment culture techniques, it is possible to characterise the microbial diversity and culture characteristics of the isolated microorganisms in different environments, allowing a more complete picture. The phylogenetic information obtained by using molecular techniques about the identity of the members of a bacterial community can be a very useful tool for the specific design of appropriate culture media.

1.1.2 Advantages of molecular techniques over culture-dependent techniques for the study of microbial communities.

New DNA based techniques have been developed, which allow the identification of single bacterial species in sample material without the cultivation of the organisms (Amann *et al.*, 1995; Ludwig *et al.*, 1994; Muyzer *et al.*, 1993; Ward *et al.*, 1990). Most of the experiments which have been carried out in this field so far are based on ribosomal sequences, which are used as phylogenetic markers (Woese, 1987). The ribosomal sequences are present in all organisms and they contain variable and highly conserved regions which allow to distinguish between organisms on all phylogenetic levels. In addition, a lot of data exist in the databases (Maidak *et al.*, 1999), which can be used to compare the DNA-sequences of unknown microorganisms and allow a phylogenetic identification.

To identify bacteria in sample material, ribosomal sequences are analysed by transcribing ribosomal RNA into cDNA, which can then be cloned (Ward *et al.*, 1990). Alternatively, extracted DNA can be used as a template to amplify ribosomal gene fragments with primers for universal sequences by PCR (Polymerase Chain Reaction). The PCR amplified fragments can be cloned as well. The result of both strategies is a clone library, containing ribosomal sequences as inserts. By sequencing individual inserts and comparing the obtained sequences with sequences present in databases, it is possible to identify the phylogenetic position of the corresponding bacteria without their cultivation.

An alternative to this approach is the Denaturing Gradient Gel Electrophoresis (DGGE) of PCR-amplified gene fragments coding for rRNA (Muyzer *et al.*, 1993). This technique allows the separation of partial 16S rDNA amplified fragments of identical length but different sequence due to their different melting behaviour in a gel system containing a gradient of denaturants. As a result, a band pattern is obtained, which reflects the complexity of the microbial community. The

reliability of the technique is very high, all species present in the community that are over 1% of the total population can be detected by DGGE analysis. This percent is much higher than cultivation. By excising individual DGGE bands from the gel and reamplifying the DNA, it is possible to get sequence information of single community members (Muyzer *et al.*, 1993; Muyzer and Smalla, 1998). However, phylogenetic analyses of sequences obtained directly from DGGE patterns are often difficult. Sequence information obtained by direct sequencing of manually excised bands does not always allow reliable phylogenetic analyses due to the short sequence length (200-500 bp). Furthermore, co-migration of several different 16S rDNA sequences, which have the same melting behaviour and therefore the same position in the gel, leads to overlapping DGGE bands which cannot be sequenced directly (Röllerke *et al.*, 1996).

1.1.3 Differential and complementary characteristics of culture-dependent and -independent techniques.

- a) The use of culture-dependent and -independent techniques have important differential characteristics but they are simultaneously complementary:
- b) The amount of sample needed to study microbial communities by using molecular techniques is smaller than that needed for cultivation efforts. This fact is a big advantage in some specific studied environments, as it is the case for objects of art.
- c) Molecular techniques allow the identification of the cultivable and non-cultivable fraction of microorganisms present in one sample. By cultivation techniques it is possible to identify only those microorganisms able to grow in the selective media used.

Cultivation techniques are able to discriminate living and dead microorganisms, while molecular techniques do not.

Taking all together, culture-dependent and -independent techniques are neither contradictory nor excluding, they are complementary.

Table 1 shows an overview of the complementary characteristics.

1.2 Activities performed at the university of Vienna, Austria.

The University of Vienna is pioneer in the application of molecular methods for the study of microbial communities colonising art objects. The results obtained from such activities revealed the presence of microorganisms which had never been identified in these environments before. By applying these methods, Röllerke *et al.* (1996) demonstrated their potential to investigate biodeterioration processes and suggested that such techniques should be integrated as a part in restoration strategies. Hence, the application of molecular strategies to study biodiversity has to undergo permanent improvement to overcome any inherent limitations.

The University of Vienna has been mainly working on the development, optimisation and application of culture-independent techniques for the study of the microbial communities present on art objects. One of the major challenges when art objects are investigated by molecular methods is the extraction of DNA, because only small quantities of sample material can be taken without damaging the object. In addition, samples from such materials often contains PCR inhibitors such as salts, pigments, exopolysaccharides, humic acids and other unknown substances. We have developed a DNA extraction protocol adapted for samples taken from art objects (Schabereiter-Gurtner *et al.*, 2001a). Using this protocol it was possible to extract PCR-amplifiable DNA from many different materials, such as stone, paint layer and glass, derived from a variety of art pieces. The DNA extracts were used to amplify 16S ribosomal fragments which were subsequently analysed by denaturing gradient gel electrophoresis (DGGE).

As mentioned above, DGGE analysis are most successful with DNA fragments of short length (200-500 bp) and consequently, the phylogenetic identification of microorganisms is often limited by the poor sequence information obtained by direct sequencing of excised and reamplified DGGE

bands. To overcome this problem and to identify microorganisms on the basis of longer 16S rDNA fragments, our working group applied an alternative strategy to the direct sequencing of DGGE bands. In parallel to DGGE analysis, a clone library containing larger fragments of the ribosomal gene was constructed (Ward *et al.*, 1990) and clones were screened by DGGE. Clone libraries allow the inclusion of the entire 16S rDNA sequence in the phylogenetic analyses of microorganisms, obtaining a more reliable identification of microbes. This strategy combines the advantage of the original cloning method of the entire 16S rDNA with the advantage of DGGE, which allows both the screening for different clones and the analysis of the structure of the bacterial community in one gel.

This approach has been successfully applied to investigate the microbial communities consisting on bacteria (Gurtner *et al.*, 2000; Piñar *et al.*, 2001c; Rölleke *et al.*, 1996, 2000; Schabereiter-Gurtner *et al.*, 2001a, 2001b), archaea (Piñar *et al.*, 2001a, 2001b, 2001d; Rölleke *et al.*, 1998) and fungi (Schabereiter-Gurtner *et al.*, 2001c) present on three different mural paintings located at the Catherine chapel of Castle Herberstein (Styria, Austria), which dates back to the 14th century; the Necropolis of Carmona (Andalusia, Spain), from the first and second centuries A.D.; and the St. Martin's church in Greene (Niedersachsen, Germany) from the 16th century. This investigation was a part of the European Union funded project "Novel molecular tools for the analysis of unknown microbial communities of mural paintings and their implementation into the conservation/restoration practice" (ENV4-CT98-0705), in collaboration with the CSIC in Sevilla (Spain), the University of Oldenburg (Germany) and the University of Gent (Belgium) as well as with the restoration company "Ochsenfarth" Paderborn (Germany).

The University of Vienna has collaborated with the Institute of glass and ceramics, at the University of Nuremberg (Germany). In this project, the microbial community (bacteria and fungi) present on historical glass at the church of Stockkämpfen (Germany), which dates back to 1870 (Rölleke *et al.*, 1999; Schabereiter-Gurtner *et al.*, 2001c) was investigated.

An additional collaboration with the CSIC in Sevilla (Spain) allowed the study of the microbial communities colonising the prehistoric caves of Altamira, Llonin, La Garma and Tito Bustillo, in the north of Spain, (Schabereiter-Gurtner, 2000, Schabereiter-Gurtner *et al.*, 2001d; 2001e; 2001f).

The University of Vienna is also involved at the moment in a European Concerted Action, EVK4-CT-1999-2001, whose acronym is COALITION: "A concerted action on molecular microbiology as an innovative conservation strategy for indoor and outdoor cultural assets". This project is funded by the European Commission. The project combines the major lab groups working in this field existing in Europe, being involved the CSIC in Seville (Spain), the CSIC in Madrid (Spain), the company Genalysis® in Luckenwalde (Germany), the University of Oldenburg (Germany), the University of Gent (Belgium), the University of Helsinki (Finland), the University of Florence (Italy), and the University of Messina (Italy).

2. The molecular approach to study microbial communities

2.1 Analyses of naturally occurring rRNA and rDNA

The starting point for the molecular approach and related procedures is the extraction of nucleic acids of sufficient quality to permit activity of the enzymes used in subsequent procedures, as Polymerase Chain Reaction (PCR). This is not a trivial matter and it has been one of the main challenges in our working group as discussed above.

There are two strategies based on rRNA and rDNA to identify bacteria in sample material. The first approach is based on the recovery of rRNA that is transcribed into cDNA, cloned and sequenced (Ward *et al.*, 1990). The alternative approach is based on the recovery of high molecular weight DNA directly from sample material, followed by the amplification of rDNA by PCR (Polymerase Chain Reaction), cloning and sequencing. The result of both strategies is a clone library, containing ribosomal sequences as inserts.

2.2 The PCR-clone-sequence approach

The extracted DNA is subjected to PCR amplification using “universal” primers or primers designed to amplify rRNA genes from particular group of organisms. The broad-range amplification of 16S rDNA genes with universal 16S rDNA primers allows the unselective detection of unexpected or hitherto unknown bacteria in medical and environmental samples. The use of specific primers for a specific group of microorganisms can be both highly specific and sensitive.

The PCR products can then be cloned by overhanging 3' deoxyadenosine residues and blunt-end ligation procedures, or by using commercially available kits for the cloning of PCR products.

2.3 Sequencing of specific clones

Automated DNA sequencing systems have greatly facilitated the rapid screening and analysis of large gene libraries. Initial screening of rRNA gene-containing clones by different methods such as restriction fragments length polymorphism (RFLP) analysis of purified plasmid DNA or insert DNA obtained by colony PCR for the presence of near identical sequences, can greatly reduce the number of clones that require complete sequencing. However, RFLP is of limited use for demonstrating the presence of specific phylogenetic groups and is a time-consuming method.

By sequencing individual clones and comparing the obtained sequences with sequences present in databases, it is possible to identify the phylogenetic position of the corresponding bacteria without their cultivation.

2.4 Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing Gradient Gel Electrophoresis (DGGE) is a method by which fragments of partial 16S rDNA amplified fragments of identical length but different sequence can be resolved electrophoretically due to their different melting behaviour in a gel system containing a gradient of denaturants. This method has been applied to the analysis of 16S rRNA genes from environmental samples (Muyzer *et al.*, 1993). As a result, a band pattern is obtained, which reflects the complexity of the microbial community. By excising individual DGGE bands from the gel and reamplifying the DNA, it is possible to get sequence information of single community members (Muyzer *et al.*, 1993; Muyzer and Smalla, 1998). DGGE is relatively rapid to perform, and many samples can be run simultaneously. The method is, therefore, particularly useful when examining time series and population dynamics. Once the identity of an organism associated with any particular band has been determined, fluctuations in individual components of a microbial population, due to environmental perturbations, can be rapidly assessed. DGGE represents a powerful tool for monitoring microbial communities.

2.5 Whole-cell hybridisation

This approach is Fluorescent *In Situ* Hybridisation (FISH). End-labeled oligonucleotides are sufficiently sensitive to allow the specific detection of individual microbial cells directly in sample materials. Fluorescent rRNA-targeted oligonucleotide probes confer fluorescent stain specifically to cells of a phylogenetically coherent group on various taxonomic levels from species up to the kingdom level. They can be applied to samples without prior cultivation and determine the cell morphology and identity of microorganisms, their abundance and the spatial distribution *in situ* (Amann *et al.* 1995). Cells showing specific hybridisation with the fluorochrome-labelled probe can be identified and enumerated. There are also some limitations associated with the technique. These can be divided in four main categories: cell permeability problems, target site accessibility, target site specificity and sensitivity.

Figure 1 shows the flow diagram of the various steps for the study of the structure of microbial communities by the combination of culture-independent and –dependent techniques.

3. CASE STUDY: The wall paintings at the Castle of Herberstein, Styria, Austria.

3.1 Biodeterioration of wall paintings.

It is a well established knowledge that wall paintings can be attacked and destroyed by microorganisms (Ciferri, 1999). Wall paintings provide a variety of ecological niches, allowing primary colonisation by photoautotrophic and chemolithoautotrophic bacteria as well as secondary growth by heterotrophic bacteria. Nutrients for heterotrophic bacteria are available from metabolites of autotrophic bacteria, from airborne organic contamination and dripping water, from animal faeces and, last but not least, from organic compounds in the paint layers themselves. Concerning wall paintings, pigments are often suspended in water or oil, mostly together with organic binders such as casein, egg yolk and milk before application on the damp lime plaster. Organic substances may also be applied later, during restoration campaigns.

Microbial-induced deterioration processes cause structural as well as aesthetic damage to wall paintings. The formation of pigmented biofilms, biomineralisation, the dissolution of metals by acids and chelating agents, the degradation of organic binders and consolidants, and the degradation and discoloration of pigments are some of the damaging phenomena triggered by microbial growth (Bock and Sand, 1993; Ciferri, 1999; Guglielminetti *et al.*, 1994; Karpovich-Tate and Rebricova, 1990; Petushkova and Lyalikova, 1986; Saiz-Jimenez and Samson, 1981; Sorlini *et al.*, 1987). The flora present on frescoes are fungi (predominant species of *Penicillium*, *Aspergillus*, *Cladosporium* and *Engyodontium*) (Guglielminetti *et al.*, 1994; Jeffries, P. 1986), bacteria (predominant species of *Bacillus*, *Arthrobacter*, *Micrococcus*, *Streptomyces* and *Pseudomonas*) (Altenburger *et al.*, 1996; Heyrman *et al.*, 1999) cyanobacteria and eukaryotic algae (species of *Nostoc*, *Lyngbya* and *Chlorophyceae*) (Ariño *et al.*, 1996; Ortega-Calvo *et al.*, 1993).

3.2 Molecular techniques applied to study the microbial communities associated to the wall paintings of Castle Herberstein.

3.2.1 Location

The location of the case study here presented is the Catherine Chapel of the Castle Herberstein (Styria), Austria. This chapel is decorated with a collection of frescoes of the 14th century. The frescoes were covered with plaster around 1580 probably due to a profane use of the room for several hundred years. This plaster layer was removed in 1942 and during this year and in November 1949 restoration works were carried out. Today the paintings show a remarkable microbial growth including detachment in many areas, as well as serious fading due to salt efflorescence formation and microbial attack. It is remarkable that the microbial community likely developed on the walls only in the last 50 years, after removal of the plaster covering the paintings and cleaning. This colonisation was favoured by the restoration process (addition of casein in paintings and mortars), the use of the chapel as storage room and the possible contamination from the stored goods. The site is subjected at irregular intervals to frequent exposures of humidity.

Seven samples, H1 to H7 (20-50 mg each) were taken from the walls of the chapel. Three different aliquots were taken in replicate from sampling areas delimited in squares of around 20 cm². Sample H1 was taken from a zone with a biofilm with brownish stripes below the chancel's east wall's window. Sample H2 was taken from the paint layer material together with a black biofilm on the chancel's vault. Sample H3 was taken from a black biofilm from an area about 40 cm below sample H1. Sample H4 was taken from a green biofilm on the chancel's north wall. Sample H5 was taken from a brownish-black biofilm located on one of the side walls of the painting. Sample H6 was obtained from a zone of the painting with an intense rosy discoloration on the chancel's north wall. Sample H7 was taken from a rosy cover together with plaster on the east wall.

3.2.2 Microbial communities colonising the wall paintings of Castle Herberstein.

The seven samples (H1-H7) taken from different areas of the wall paintings from the Chapel of Castle Herberstein were analysed by using the methodological approach described above. A surprising number of DNA sequences from bacteria were obtained and identified as members of microbial communities colonising wall paintings, (Gurtner *et al.*, 2000; Rölleke *et al.*, 1996; Schabereiter-Gurtner *et al.*, 2001b). The identified sequences were belonging to genera *Actinobispora*, *Amycolata*, *Asiosporangium*, *Promicromonospora*, *Pseudonocardia*, *Rubrobacter*, *Streptomonospora*, *Saccharopolyspora*, *Sphaerobacter*, *Thermocrisum*, *Aquaspirillum*, *Chromohalobacter*, *Erythrobacter*, *Porphyrobacter*, *Salmonella*, members of the *Cytophagales*. To our knowledge, all these genera have not been previously reported on wall paintings by standard cultivation techniques.

The most surprising results were those concerning the detection and identification of members of the halophilic group of *Archaea* (Piñar *et al.*, 2001a; 2001b; 2001d; Rölleke *et al.*, 1998). Until a few years ago, the domain *Archaea* was considered as inhabitants of hostile environments (Tindal, 1992). *Archaea* were excluded in previous molecular investigations by using only eubacterial specific primers, and overlooked in cultivation experiments due to the use of unsuitable culture media with inadequate salt concentration, and/or the non-culturability of this group of microorganisms under laboratory conditions.

Recent molecular studies have shown that *Archaea* are also present in non-extreme environments, including marine ecosystems, estuaries, freshwater, petroleum reservoirs, in association with marine invertebrates, in the digestive tracts of fishes and in terrestrial ecosystems. A largely neglected terrestrial environment with high salt concentrations is found in deteriorated monuments. The presence of extremely salt tolerant and moderate halophilic bacteria has been demonstrated in monuments (Krumbein *et al.*, 1991; Saiz-Jimenez and Laiz, 2000), where salt efflorescences on the surfaces is a common phenomenon as a result of changing physical parameters. Practically all porous materials subjected to rainwater and rising damp contain soluble salts, dispersed within the porous materials or locally concentrated in some areas. These salts are solubilized and migrate with the water through the stone followed by the drying out of the solution at the exposed surface. This process results in the formation of deposits of hygroscopic salts in the surface, commonly know as efflorescences.

DGGE analyses of PCR-amplified DNA with *Archaea*-specific primers were carried out in combination with the construction of clone libraries to identify members of the archaeal community without prior cultivation of the organisms. In addition, Fluorescent *In Situ* Hybridization (FISH) analyses were carried out to obtain more information about the distribution and abundance of bacteria and *Archaea* on wall painting samples. PCR products using *Archaea*-primers were obtained from 2 out of the 7 samples from Herberstein (H1 and H6 samples). The presence of halophilic *Archaea* in the Chapel of Castle Herberstein agrees with the mineralogical analyses of the samples, which show a salt composition of about 10% halite in the whole sample, although local concentrations or deposits can increase the figure. This concentration of halite is growth-limiting for a number of bacteria, but could promote the successful colonisation of halophilic *Archaea* in the salt efflorescences microniches.

DGGE analyses were performed with the two positive samples to get information on the diversity and complexity of the species composition. The DGGE band patterns obtained from the original sample H1 and H6 of Castle Herberstein contained 5 and 3 main individual bands, respectively. Each band represented an *Archaea*-taxon in the sample material.

In parallel to DGGE analysis of 16S rDNA archaeal-fragments, cloning of environmental PCR products was carried out in order to avoid problems such as the excision of overlapping bands from the band patterns. A total number of 60 positive clones for sample H1 and 37 clones for sample H6 were screened by DGGE. Sequence analyses of selected clones were performed to obtain information on the identity of the corresponding organisms, as well as to verify that the amplified fragments were derived from *Archaea*. Three sequences obtained from sample H1 from

Herberstein Castle bore close similarity to extremely halophilic *Archaea*, sharing similarity values ranging in between 93.55% and , 91.15% with the 16S rDNA sequence of the haloarchaeon str. PW5.4. (McGenity *et al.*, 2000). In addition, two other sequences grouped with another extremely halophilic archaeon, sharing similarity values ranging in between 98.13% and 94.37% with the 16S rDNA sequence of *Halococcus morrhuae*. The sequences obtained from sample H6 grouped with those of extremely halophilic *Archaea*, corresponding with close relatives of the genus *Halococcus* (similarity values in between 91.00 and 94.03%).

Monitoring of variations in the composition of the archaeal communities over a time-interval between samplings was performed by DGGE analysis. A second sampling in H1 and H6 areas were carried out at an interval of 14 months. Based on the number and positions of DGGE fragments, the archaeal community present in the sample H1 was stable over this time interval as the band patterns obtained after 14 months were identical. However, for sample H6 a significant change was observed with time. The band pattern obtained most recently showed the loss of one band in the higher position of the run which was already identified as a sequence belonging to the *Halococcus* genus.

In addition, Fluorescent *In Situ* Hybridization (FISH) analysis were carried out to obtain more information about the distribution and abundance of bacteria and *Archaea* on wall painting samples.

To this end, the samples were first checked for autofluorescence before hybridization. All the samples showed a high green autofluorescence, therefore, probes labelled with Cy3 or Cy5 fluorochromes were used for further experiments. Eubacterial cells were visualized after hybridization of wall painting samples with probe EUB338 labeled with Cy5. No signal was detected without the use of the specific dye, indicating no false results due to autofluorescence using this fluorochrome. However, by using the described conditions it was difficult to get a clear hybridization signal using the *Archaea*-probe ARC915 labeled with Cy5. This could be due to:

- 1) Lysis of *Archaea* cells during the fixation and hybridization procedure due to the low amount of NaCl in the standard fixation and hybridization solutions.
- 2) Low number of *Archaea* cells in the samples and impossibility to visualize them.
- 3) Low fluorescence intensity after *in situ* hybridization, due either to low accessibility of the target molecule in *Archaea* or to low growth rate of the cells giving rise to low concentration of rRNA.

End of 1999 the first treatment with biocides was performed in two different discoloured areas on the wall paintings of the Chapel of Castle Herberstein. The design and application of biocides was done by restorers of the company Ochsenfarth (Paderborn, Germany). Three different biocides, i.e. C: 2% Clotrimazol; H: 2% Hergal HS21, and P: 2% Prevantol R50 (all of them in 70% Isopropanol) were applied separately in squares of 10x10 cm.

Two different monitoring samplings have already been performed after the application of the biocides. The first sampling was done in 1999 and the second in 2000. DGGE analysis were carried out to monitor the impact in the microbial communities caused by the biocides. The results obtained from this monitoring were relevant, because they were a validation of the treatments used routinely by restorers. We proved that the biocide Clotrimazol was the most appropriate for application, while biocide Prevantol could have even harmful effect causing the proliferation of new microbial species. We are expecting to continue with a long-term monitoring of these paintings.

4. Bibliography

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Table 1. Differential characteristics from molecular and cultivation techniques

	MOLECULAR TECHNIQUES	CULTIVATION TECHNIQUES
AMOUNT OF SAMPLE	Small	Moderate
IDENTIFICATION	Culturable and non- culturable microorganisms	Culturable microorganisms
DISCRIMINATION Microorganisms Death material	No	Yes

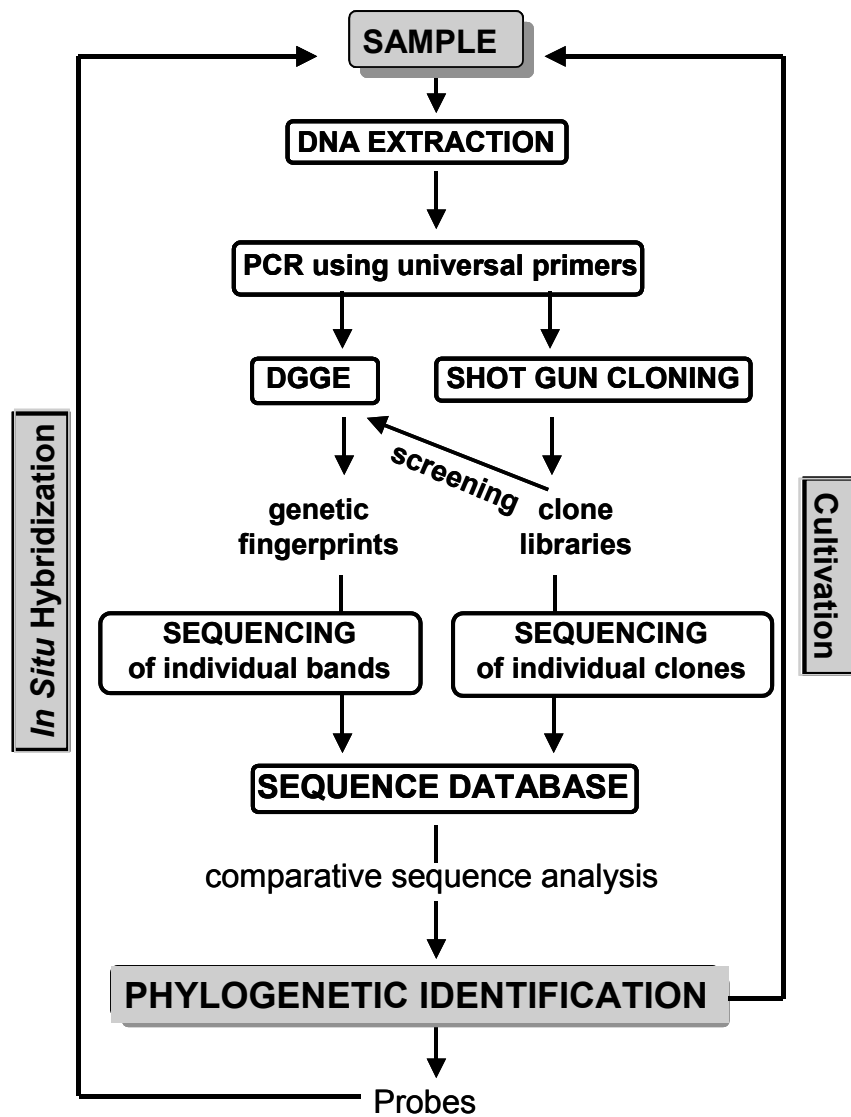


Figure 1