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Analysis of bacterial communities on historical glass by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA

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Abstract

The present study describes the analysis of bacterial communities on historical window glass by denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA fragments. So far, only a few studies have been published in which the microflora and the corrosion mechanisms of glass surfaces have been investigated. Some microorganisms, especially fungi, have been isolated from different glass samples in the past. However, our results demonstrate that bacterial communities on biodeteriorated glass surfaces are much more complex than previously believed. In addition, bacteria were identified, which have never been isolated from glass samples before. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

The destruction of organic materials by microorganisms is a well-known phenomenon. This may lead to the deterioration of paper, leather, paintings and other materials used for objects of art and cultural heritages. Also inorganic materials, including concrete, marble and sandstone are subjected to deterioration by microorganisms (Urzì et al., 1991; Bock and Sand, 1993; Ariño and Saiz-Jimenez, 1996). The first report of biodeterioration of glass dates back to the beginning of this century (Mellor, 1924). But until now only a few studies have been published concerning the microflora on and the corrosion mechanisms of glassy surfaces (Krumbein et al., 1991; Krumbein et al., 1993; Drewello and Weissmann, 1997; Drewello, 1998). Although a variety of microbes, especially fungi, have been isolated from different glass samples (Nagamuttu, 1967; Kerner-Gang and Schneider, 1969; Kaiser et al., 1996; Drewello, 1998), it can be assumed that microorganisms growing on glass must be highly specialized so as to allow for sufficient nutrition for their metabolism. This may lead to difficulties in the detection of such microorganisms: (1) in the presence of faster growing but supposedly for the deterioration process not so relevant microorganisms, relevant but slowly growing bacteria and fungi may not be detected under laboratory conditions. (2) Cultivation strategies can target and reflect only a portion of existing bacteria in an environment, since the exact conditions cannot be imitated under labora-

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tory conditions; this may lead to the investigation of microorganisms, which do not fully reflect the actual population at the site.

A few years ago, DNA-based identification techniques of microorganisms were introduced to investigate the microbial diversity in different environmental habitats (Giovannoni et al., 1990; Ward et al., 1990; Muyzer et al., 1993; Wawer and Muyzer, 1995). The use of such methods demonstrated that conventional microbiological efforts fail to isolate all microorganisms present in natural samples, and that only a minor fraction of all bacteria have been isolated so far.

DNA- and RNA-based identification techniques have been applied for investigating the biodeterioration of medieval wall paintings by microbial communities (Rölleke et al., 1996, 1998). The present paper focusses on the investigation of bacterial communities on historical window glass. The glass panel from which the samples have been taken is part of a north window of the German Protestant chapel in Stockkämpen. The window dates back to about 1870 and has recently been restored. The present study has been carried out in the context of such restoration.

We applied the molecular approach of PCR-amplified 16S rDNA fragments separated by denaturing gradient gel electrophoresis (DGGE) to analyze the microbial community and the potential role of these bacteria in the deterioration process of the window glass. Using DGGE, DNA fragments of the same length but with different basepair sequences, such as PCR fragments obtained from a mixture of target DNAs, can be separated (Myers et al., 1987; Sheffield et al., 1989; Muyzer et al., 1998). Instead applying cultivation strategies, we extracted DNA from the glass samples and amplified the 16S rDNA by PCR. We used DGGE to separate these fragments. The resulting DGGE electrophoresis pattern contained 16S rDNA fragments derived from bacterial species. The obtained DGGE pattern reflects the bacterial diversity in the sample. As one DGGE band is originated from one taxon, a complex pattern would indicate a high diversity in the original sample, while only a few bands in the pattern would suggest a bacterial community with a low bacterial diversity. The comparison of DGGE patterns from different samples gives us information about similarities and differences in their community structures. Individual bands in the electrophoresis pattern can be excised from the gel and sequenced. These sequences can be used to identify the phylogenetic affiliation of the bacteria corresponding to individual bands in the electrophoresis pattern.

When restoring cultural heritage, one should take its microbial colonization into consideration so as to avoid the possibility that, after only a short period of time, microorganisms will have destroyed the restored object. Monitoring of the growth of microorganisms is also essential after completion of restoration, since restoration efforts can do more harm than good if, due to unawareness or ignorance, measures are applied that support the growth of microorganisms and accelerate the deterioration process (Bianchi et al., 1980). Efforts to eliminate microorganisms which contribute to the deterioration process will be ineffective without a better understanding of the microbial diversity present in the sample. For example, it has been recognized that biocides may provide nutrition for the growth of microorganisms when used on the object, while pure cultures of the same microorganisms would not survive (Tayler and May, 1994). Therefore, in situ tests need to be applied that allow for an appropriate monitoring of existing microorganisms and a reliable evaluation of the success of restoration efforts that aim at sustainable conservation of the cultural heritage, including historical glass. The experimental approach applied in this study necessitates using only a tiny sample of material. The approach can be used to compare microbial communities from different locations or for monitoring, as described above.

2. Material and methods

2.1. Sampling

Small amounts of test material were taken by mechanical scraping off the organic layer from the solid glass surface. The biolayer originates from a historical glass panel from a north window of the church of Stockkämpen, Germany, which dates back to 1870. The sampling was carried out in collaboration with the restorer of the window.

2.2. Chemical analysis of the glass and surface layers

The glass was characterized by analysing a polished sample using X-ray fluorescence analysis (Siemens SRS 3000). In order to analyse the surface coatings, a cross-section of the glass was examined using a scanning electron microscope fitted with energy and wavelength dispersive X-ray analysis (Cambridge Instruments Stereoscan 250 SK3). For the characterization of organic compounds (cold settings) and corrosion products a Fourier-transformed infra-red spectrometer was used (Perkin-Elmer FT-IR-Microscope, Spectra 2000). Crystalline precipitates were additionally determined by FT-IR and X-ray diffraction (Siemens D500).

2.3. DNA extraction from the glass

In order to obtain bacterial DNA from the surface material of glass, a small amount (5–10 mg) of sample material was dissolved in H_2O and subjected to three to five freeze–thawing cycles to lyse the bacterial cells. After centrifugation, the supernatant was used directly as template DNA in the PCR reaction.

2.4. PCR amplification of 16S rDNA gene fragments

Primers complementary to conserved regions were used to amplify 194 basepairs of the 16S rDNA corresponding to nucleotide positions 341–534 in the *E. coli* sequence (Muyzer et al., 1993). As a forward primer we used a eubacterial specific sequence: (5′-CCTACGGGAGGCAGCAG-3′). This primer contains at its 5′ end a 40-base GC-clamp, (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGG-GCACGGGGGGC-3′) to stabilize the melting behavior of the DNA fragments (Sheffield et al., 1989). The universal consensus sequence (5′-ATTACCGCGGCTGCTGG-3′) was used as a reverse primer. A 'touch-down PCR' (Don et al., 1991) with annealing temperature changing from 63 to 55°C over 16 cycles was used to avoid non-specific primer annealing. PCR was carried out as described previously (Rölleke et al., 1996), with a total of 30 cycles and 1 unit of *Taq* polymerase (Boehringer) per 50 μl PCR reaction. A small amount of all PCR products of the amplified 16S rDNA was first analyzed by electrophoresis in 2% (w/v) agarose gels, before DGGE analysis.

2.5. Analysis of PCR products by DGGE

For the mixture of PCR fragments obtained by direct amplification of the DNA extracts, we used 200 μl PCR product, which were precipitated and resuspended in 20 μl H_2O. For the separation of the different total 16S rDNA fragments, DGGE analysis was performed as described previously (Muyzer et al., 1993; Rölleke et al., 1996). The gel electrophoresis was performed in 0.5 × TAE (20 mM Tris-acetate, pH 7.8), 8% (w/v) acrylamide with a linear gradient from 25 to 55% of urea and formamide. All gels were run at 60°C, 200 V and for 225 min. After completion of electrophoresis, the gels were stained in an ethidium bromide (0.5 μg/ml) solution and documented with a Pharmacia documentation system. Main bands were excised from the gel and the DNA was eluted as described elsewhere (Rölleke et al., 1996).

2.6. Cloning of excised DGGE bands

Some of the excised DGGE-bands could not be sequenced directly because of the sequences present in the background. These bands were cloned, to facilitate later sequence analyses. Reamplified excised bands were ligated into a *Xcm*I restriction site of a pks-derived plasmid. *E. coli* XL 1 Blue (Tet^r) was used as host strain for transformation (Sambrook et al., 1989). Before sequencing of the cloned fragments an aliquot was used to verify that the correct fragment was obtained, by checking the position in a DGGE.

2.7. Sequencing of 16S rDNA gene fragments

The DNA eluted from the excised DGGE bands was reamplified using the same forward primer (5′-CCTACGGGAGGCAGCAG-3′) including an additional sequence extension (T3; 5′-AAAAT-TAACCCTCACTAAAG -3′) at its 5′ end and the reverse primer (5′-ATTACCGCGGCTGCTGG-3′) with the additional sequence (M13r; 5′-AAAT-

TCACACAGGAAACAG-3′) at its 5′ end to facilitate later sequence analyses. For the cloned fragments, the plasmid sequences T3 and the T7 were used as primers to amplify a 400-bp fragment containing the 200 bp of the16S rDNA. The newly obtained PCR fragments were purified with the Quiagen gel extraction Kit and sequenced directly using a LI-COR model 4000 L automatic sequencing system (Middendorf et al., 1992). The sequencing reaction was carried out by cycle sequencing using the Sequitherm (Epicentre) system. The sequencing was carried out in both directions.

2.8. Identification of bacteria by comparative sequence analysis

All sequences were sent for comparison to the Ribosomal Database Project (Larsen et al., 1993) and to the EMBL nucleotide sequence database. The SIMILARITY_RANK tool of the RDP and the FASTA search option (Pearson, 1990) for the EMBL database were used to search for close evolutionary relatives.

2.9. Nucleotide sequence accession numbers

The sequences obtained in this study are available at the EMBL database under the following accession numbers: AJ009891 (GII21K), AJ009892 (GII18K), AJ009893 (GII20K), AJ009894 (GII.4), AJ009895 (GCII.1), AJ009896 (GII10).

3. Results and discussion

The present paper describes the investigation of bacterial communities on a 19th century soda-lime silicate glass panel from the German Protestant chapel in Stockkämpen. The glass panel, from which the samples have been taken, is part of the northern window of the chapel. The window dates back to about 1870 and has recently been restored. Fig. 1 shows a detail of the window, before restoration, with microbial-induced deterioration. The samples have been taken from one of the outer panels (see marking).

The glass consists of typical alkaline–earth–alkaline silicate glass of the late 19th century. Identification marks for this type of glass are a high concentration of network forming SiO_2 (~70%, w/w), the addition of network stabilizing CaO (10–12%, w/w), and relatively low concentrations of network forming Na_2O and K_2O (up to 14–18%, w/w). Minor components are the oxides of aluminium, manganese and iron. The brownish color is due to the remarkable raised manganese concentration. For aesthetic reasons the panels have been covered by a layer of lead silicate glass which contains a mixture of coloring pigments (metal oxides of Pb, Mn, Fe, Co, Cu, Zn). On the interior, the layer has a thickness of about 20 μm, on the exterior it is below 5 μm.

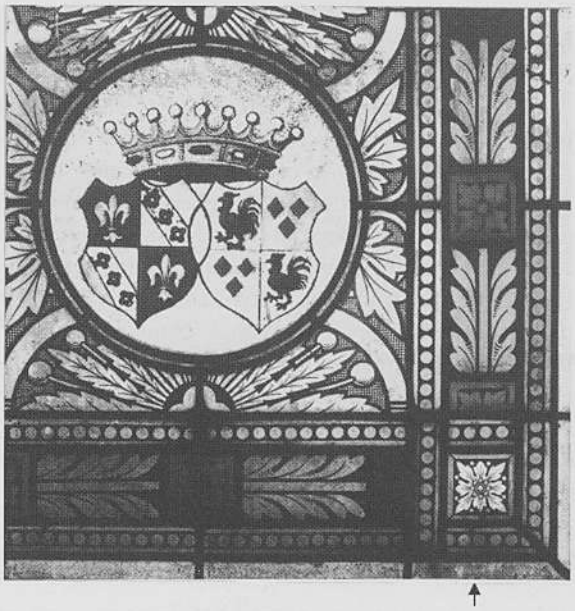

Fig. 1. Detail (58 × 57 cm) of a northern historical window, from a German Protestant chapel in Stockkämpen. The glass panel, which has been investigated in this study, was taken from the bottom left corner of the window (see marking).

The bulk glass shows a high chemical stability and appears to be homogeneous without any phase separation. On the contrary, the layer on the interior is melted incompletely, full of bubbles and partly corroded. In the surface region, potassium, calcium, and lead and the transition metals iron and manganese are significantly enriched. Crystalline corrosion products have been precipitated inside the bubbles and on the surface. They consist of the sulfates of lead, potassium and calcium (anglesite,

gypsum, syngenite). On the exterior the glass is in good condition and without any corrosion symptoms, except for the depletion of K, Ca, and Pb out of the layer of lead silicate glass. The leading has been attacked by sulfuric acid, inducing the deposition of anglesite beneath the metal frame.

The panel was cemented using a mixture of calcite and linseed oil. Thus, residues of the cementing and derived corrosion products have been found along with the leads (calcite, gypsum, heavy metal sulfates). An additionally applied cold setting which is rich in organic compounds could be detected on both sides of the panels.

The phenomenon of microbially influenced corrosion of glass has been investigated by conventional means previously. Environmental pollution has been supposed to be one of the causes of biodeterioration, as it offers the necessary organic material for initiating microbial growth (Krumbein et al., 1993). Conservation treatments as well as environmental conditions and glass composition are considered to be additional causes of microbial attack (Drewello, 1998). Leaching, the formation of etched traces, pitting, mineralisation, and crust formation are due to the mobilisation of mono- and divalent metals of the glass and are well-documented problems associated with the growth of microbes on glass surfaces. We assume that only little is known regarding microorganisms that play a role in the deterioration process of glass, since by conventional microbiological methods we are able to reflect only a minor fraction of microorganisms which are in fact present on historical glass.

We have investigated the bacterial growth of three different samples of one glass panel (see Fig. 1). Sample 1 was taken from a white crystalline precipitation (containing gypsum and calcite) from the outside of the chapel, while samples 2 and 3 were taken from the inside of the building. Sample 2 was taken from a black painting ('Schwarzlot') on the basis of lead silicate glass containing a mixture of coloring heavy metal oxides (Fe, Mn, Cu, Zn), and sample 3 was taken from a biogenic layer on a cold setting based on organic compounds (drying oils). Fig. 2 shows the result of a DGGE-analysis of 16S rDNA gene fragments, which we were able to amplify from DNA extracts of these three samples. Each band in such a pattern represents a different

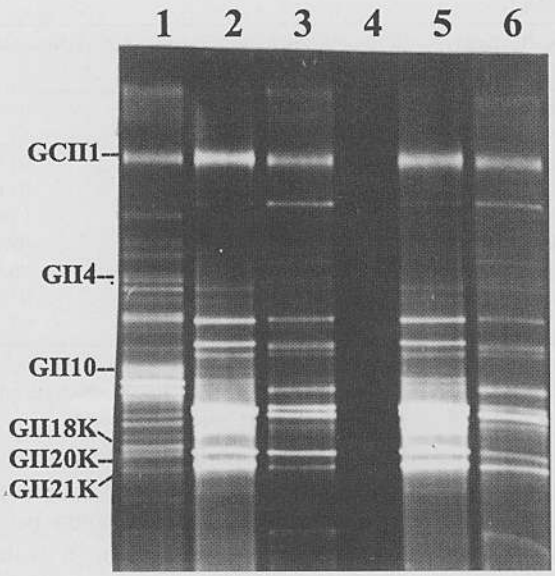

Fig. 2. Ethidium bromide-stained DGGE separation pattern of bacterial DNA fragments coding for 16S rDNA. DNA extracts from surface material of the glass panel were used as a template for the PCR amplification (lane 1, sample 1; lane 2, sample 2; lane 3, sample 3; lane 5, sample 2; lane 6, sample 3). The positions of the bands from which the sequences were obtained are marked.

bacterial taxon. Complex profiles suggest complex bacterial communities. As bands at identical positions in different lanes indicate that the sequences are identical, the same bacteria occurred in the samples. All three samples showed complex band patterns. This complexity suggests that a large variety of bacteria exist in the samples as part of a complex bacterial community. However, if we compare the patterns of the three samples (Fig. 2, lanes 1–3) with each other, considerable similarities were identified. This suggests that while the bacterial communities are complex, they are quite similar.

In order to obtain information on the phylogenetic affiliation of the bacteria corresponding to individual bands in the patterns, we excised bands from the gel and sequenced them. The results of the comparative sequence analysis are shown in Table 1. Band GCII.1 is obviously one of the most dominant DGGE bands in all samples (see Fig. 2). The bacteria, representing this sequence, are members of the order *Cytophagales* and the highest similarity in partial 16S rDNA was obtained with species of the genus *Flexibacter*. Bacteria of this group grow

Table 1

DGGE-Band	% Similarity in partial 16S rDNA with bacterial relatives	
GCII.1	*Flexibacter* sp.	89.6
GII.4	*Nitrosospira* sp.	94.3
GII.10	*Arthrobacter* sp.	91.0
	Streptomyces sp.	91.0
	Micrococcus sp.	91.0
GII18K[a]	*Frankia* sp.	99.1
GII20K	*Geodermatophilus* sp.	96.4
GII21K	*Frankia* sp.	97.3
	Geodermatophilus sp.	97.3

[a] The sequences which end with a 'K' in their labeling were obtained by cloning of the excised fragment. Bands which do not end with a 'K' in their labeling were sequenced directly.

aerobically and are microaerophilics or facultatively anaerobics. They are all organotrophs, and many are able to degrade biomolecules (proteins, chitin pectins, etc). The *Cytophagales* are found in a wide spectrum of habitats, mainly those which are rich in organic material. However, they can adapt also to low nutrient levels. It is likely, that, when growing on historical glass, these bacteria live on a biofilm, which is produced by other microorganisms.

We could identify a sequence (band GII.4) which shows the highest 16S rDNA similarity with sequences of the genus *Nitrosospira* (Table 1). This genus belongs to the lithotrophic, ammonia-oxidizing bacteria. According to their physiology they represent a distinct group. However, phylogenetically they are categorized in different subdivisions of the *Proteobacteria*. The sequence obtained in this study groups with sequences from the beta subdivision of the *Proteobacteria*. The biodeteriorative potential of this group of bacteria has been investigated in detail for building stone of historical monuments (Baumgärtner et al., 1991; Bock and Sand, 1993). As a result of the oxidation of ammonium, nitric acid is produced. This inorganic acid causes the deterioration of stone. To our knowledge, nitrifying bacteria have never been identified on glass. Nitrifying bacteria, identified in this study, which are members of or close relatives to the genus *Nitrosospira*, occurred in two out of the three sampling sides. They were present in the material taken from the white crystalline precipitation (sample 1) as well as in the material taken from a biogenic layer on an cold setting (sample 3). Nitrifying bacteria, as identified here, could play an important role in biodeterioration processes of glass surfaces, due to their ability to produce inorganic acids.

Furthermore, we found several sequences which showed the highest similarity with 16S rDNA sequences belonging to different genera of actinomycetes (see Table 1, GII.10, GII18K, GII20K, and GII21K). Like fungi, many actinomycetes are capable of producing a stable mycelium. The penetration of different materials with hyphae is associated with the ability to excrete a wide range of products, including a variety of enzymes (Williams, 1985; Weirich, 1989). Besides mechanical destruction of the grounding, actinomycetes that produce mycelia can cause different deterioration phenomena, due to their growth and metabolic products (like acids and pigments) (Urzì et al., 1991).

Actinomycetes have already been isolated from historical glass surfaces in the past. Biopitted glass samples from the Cologne Cathedral were found to be colonized by fungi and a coccoid bacteria which could be identified as *Micrococcus* sp. (Krumbein et al., 1991). We found a sequence (GII.10) showing 91% 16S rDNA similarity with sequences from the genera *Streptomyces*, *Micrococcus* and *Arthrobacter*. As demonstrated in Table 1, the sequences obtained from DGGE bands GII18K, GII20K and GII21K all represent bacteria belonging to the family *Frankiaceae*. Band GII18K shares 99.1% similarity with 16S rDNA sequences obtained from *Frankia* sp., band GII20K 96.4% similarity to sequences from *Geodermatophilus* sp., and finally band GII21K which shows 97.3% similarity with sequences from both genera.

It has been believed that bacteria belonging to *Frankiaceae* occur worldwide, but that they are soil organisms only (Baker and O'Keefe, 1984; Lechevalier, 1989). In 1996, two reports were published which showed that *Frankiaceae* can also be present in other habitats. In a study investigating the biodiversity of rockdwelling bacteria, *Geodermatophilus* sp. could be isolated from rocks and monument surfaces (Eppard et al., 1996). The application of molecular tools for the analysis of a biodeteriorated medieval wall painting in Austria demonstrated the presence of members of or close relatives to the genus *Frankia* (Rölleke et al., 1996). With this report, we have additional proof for the presence of members of the family *Frankiaceae* in

other habitats than soil. This supports the idea that, in the past, the use of selective cultivation strategies has favored the identification of those bacteria which were easy to isolate, but which were not necessarily those causing biodeterioration processes.

It becomes more and more evident that biodeterioration has to be understood as a result of the metabolism of an entire microbial community, rather than that of individual representatives of such a community. Until and unless the variety of such communities is fully known, counter measures will remain piece-meal efforts, which may even have negative effects on the preservation of historical glass.

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