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Identification of Bacteria in a Biodegraded Wall Painting by Denaturing Gradient Gel Electrophoresis of PCR-Amplified Gene Fragments Coding for 16S rRNA

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Medieval wall paintings are often affected by biodecay. An inventory of the existing microorganisms associated with the damage to the paintings is not yet an integral part of the restoration process. This stems from the lack of effective means for such a stocktaking. Nevertheless, fungi and bacteria cause severe damage through mechanical processes from growth into the painting and its grounding and through their metabolism. Detailed information on the bacterial colonization of ancient wall paintings is essential for the protection of the paintings. We used a molecular approach based on the detection and identification of DNA sequences encoding rRNA (rDNA) to identify bacteria present on an ancient wall painting without prior cultivation of the organisms, since it has been shown that most of these bacteria cannot be cultivated under laboratory conditions. To trace the noncultivated fraction of bacteria, total DNA from a biodegraded wall painting sample from a 13th century fresco was extracted and 194-bp fragments of the 16S rDNA were amplified with eubacterial primers. The 16S rDNA fragments of uniform length obtained from the different bacterial species were separated according to their sequence differences by denaturing gradient gel electrophoresis (DGGE). By sequencing excised and reamplified individual DNA bands, we characterized the phylogenetic affiliation of the corresponding bacteria. Using this approach, we identified members or close relatives of the genera *Halomonas*, *Clostridium*, and *Frankia*. To our knowledge, these groups of bacteria have not yet been isolated and implicated by conventional microbiological techniques as contributing to the biodegradation of wall paintings.

It is now well recognized that microorganisms can be responsible for the destruction of buildings and cultural heritages, including the decay of concrete, marble, and sandstone (7, 18, 23, 43). Hence, it is not surprising that mural paintings can also support the growth of microorganisms, which thus could contribute to the biodeterioration of the paintings and their grounding (19, 32, 40). Bacteria which, inter alia, grow on the surface of such paintings might discolor the painting not only through their own pigments but also by excreting metabolic products. Heterotrophic bacteria can use organic compounds from the paint layer as growth substrates, producing acids, which cause discoloration of the paint or change its consistency. In addition, mycelia of fungi and actinomycetes can penetrate into the painting and its grounding, resulting in the mechanical destruction of the cultural heritage (19, 50).

Current restoration efforts can conceivably have the opposite effect to restoration, particularly if these measures use substrates which support the growth of microorganisms and, consequently, accelerate the deterioration process (6). Often not sufficiently respected, microbial colonization should be taken into consideration when planning the restoration of ancient wall paintings. Therefore, an inventory of the existing microorganisms associated with the damage of the paintings is a prerequisite for including biodecay as an integral part of the restoration process. DNA-based techniques of identification of microorganisms have revealed that conventional microbiological efforts fail to isolate all microorganisms present in natural samples (46) and that only a minor fraction of all bacteria have been isolated so far (13, 48). Thus, little is known about the genetic diversity of microbial communities, their potential metabolic activities, and their role in certain degradation processes.

To obtain a better understanding of the microbial community and the potential role of these bacteria in the process of degradation of ancient wall paintings, we applied the molecular approach of PCR-amplified gene fragments coding for 16S rRNA (rDNA) separated by denaturing gradient gel electrophoresis (DGGE). By using DGGE, DNA fragments of the same length but with different base pair sequences, such as PCR fragments obtained from a mixture of target DNAs, can be separated. This method has recently been introduced into molecular microbial ecology to determine the genetic diversity of natural microbial communities and to identify the phylogenetic affiliation of the community members (27–29, 47). For this approach, it is not necessary to cultivate the bacteria before identification. We extracted DNA from the original wall-painting material 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 present in the sample. Individual bands in the electrophoresis pattern were excised from the gel and sequenced. These sequences were used to identify the phylogenetic affiliation of the bacteria corresponding to individual bands in the electrophoresis pattern.

Because of the sensitivity of the PCR, only small quantities of samples are necessary to identify different groups of bacteria present in the samples. As several PCR-derived samples can be

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electrophoresed on one gel simultaneously, this approach allows mapping of the diversity of bacteria collected from several different places on the wall painting. In addition, the 16S rDNA fragments of bacteria which could be isolated from an aliquot of the same wall-painting material by conventional microbiological techniques were sequenced and analyzed phylogenetically. Our results demonstrate that the molecular biological approach is a potential tool to investigate microorganisms in biodegradation processes.

MATERIALS AND METHODS

Sampling of wall paintings. Fresco samples from the chapel in Castle Herberstein, Styria, Austria, were obtained in collaboration with the restorer. The fresco, which dates to the 13th century, was last restored around 1950 and at present shows serious damage by fungi and other microorganisms. We focused on an area which showed a rosy cover of various intensities. The samples were taken by scraping off surface material and plaster to a depth of 3 to 5 mm. The total sample was split into two parts. One part was used for the isolation of aerobic, heterotrophic bacteria by standard cultivation techniques. The other part was used for extraction of DNA, from which the 16S rDNA fragments were amplified and separated according to their sequence variations by DGGE analysis. In addition, particles of surface material were used for scanning electron microscopy.

Electron microscopy. Particles of the fresco were mounted with Tempfix on aluminum stubs and sputter coated to a 3-nm depth with gold-palladium (80:20), using a magnetron sputter coater. Electron microscopy was performed with a Hitachi S-4100 field emission scanning electron microscope.

Isolation of bacteria. Sample material from the wall painting was suspended in saline containing 0.001% (vol/vol) Tween 80, shaken for 1 h to remove the cells from the particles (41), and transferred to agar plates in dilutions from 10^{-1} to 10^{-5}. The following media were used: (i) nutrient broth (2), (ii) glycerol-arginine (9), and (iii) casein minimal medium (K_2HPO_4, 0.6 g/liter; $Na_2HPO_4 \cdot 0.5$ g/liter; $MgSO_4 \cdot 7H_2O$, 0.05 g/liter; $MgCl_2 \cdot 7H_2O$, 0.1 g/liter; KNO_3, 0.2 g/liter; $FeCl_3 \cdot 6H_2O$, 0.01 g/liter; $CaCl_2$, 0.1 g/liter; yeast extract, 0.4 g/liter; casein, 0.8 g/liter; and 15 g of agar per liter for solid media [pH 7.0]). The plates were incubated at 22°C for 8 weeks under aerobic conditions. The number of CFU was determined every 3 days. Isolation of well-defined single colonies resulted in the isolation of five different bacterial species as pure cultures. Colonies were selected initially according to their color and their differences in shape and identified by 16S rDNA sequencing.

DNA extraction. Genomic DNA from the isolated bacteria was obtained by chloroform-phenol extraction and ethanol precipitation (36). To obtain bacterial DNA from wall painting material, a small amount (ca. 50 mg) of sample material was dissolved in TE buffer (10 mM Tris HCl, 1 mM EDTA [pH 8]) and subjected to three freeze-thaw cycles to lyse the bacterial cells. After centrifugation, 5 μl of the supernatant was used directly as template DNA in the PCR. Different protocols for DNA extraction, including microwave heating, freeze-thawing, lysis solutions, and combinations of these methods, were tested. However, only the protocol described above proved successful in isolating DNA from small quantities of wall painting material.

PCR amplification of 16S rDNA fragments. Enzymatic amplification (35) of the 16S rDNA (24, 27) was performed on DNA extracted from the bacterial isolates and from the original wall-painting material. Primers complementary to conserved regions were used to amplify a 194-bp fragment of the 16S rDNA corresponding to nucleotides 341 to 534 in the *Escherichia coli* sequence (27). The nucleotide sequence of the forward primer, which is specific for eubacteria (5′-CCTACGGGAGGCAGCAG-3′), contains at its 5′ end a 40-base GC clamp (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3′) to stabilize the melting behavior of the DNA fragments (39). The universal consensus sequence (5′-ATTACCGCGGCTGCTGG-3′) was used as a reverse primer. The PCR was performed with a Trio-Thermoblock (Biometra). The PCR mixture, containing 50 to 100 ng of genomic DNA of the bacterial isolates or 5 μl of DNA preparations from wall-painting samples, 25 pmol of each primer, 200 μM each deoxyribonucleoside triphosphate, and 5 μl of 10× PCR buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl [pH 8.3]), was made up to 50 μl with sterile water (Sigma Chemical Co., Ltd.), transferred to 0.5-ml tubes, and overlaid with 3 drops of mineral oil (Merck). The samples were first incubated at 94°C for 5 min to denature the DNA and then cooled to 80°C, at which temperature 1 U of *Taq* DNA polymerase (Boehringer) was added. To increase the specificity of the amplification and to reduce the formation of spurious byproducts, a "touchdown" PCR (8, 27) was performed. A touchdown PCR is a PCR in which the annealing temperature is set 10°C above the expected annealing temperature (65°C) and decreased by 1°C every second cycle until a touchdown of 55°C, at which temperature six additional cycles were carried out. Denaturing was carried out at 94°C for 1 min, primer annealing was performed at the appropriate temperature for 1 min, and primer extension was performed at 72°C for 3 min. All PCR products (10-μl volumes) were analyzed by electrophoresis in 2% (wt/vol) agarose gels (36) before DGGE analysis was performed.

Analysis of PCR products by DGGE. PCR products (25 μl) obtained from genomic DNA of pure culture were used for separation in denaturing gradient gels. To analyze the mixture of PCR fragments obtained by amplification of the DNA extracted from wall-painting material, we pooled 300 μl of PCR products, which were precipitated to reduce the volume, resuspended in 15 μl of TE buffer, and loaded onto the gel.

DGGE was performed as previously described (27, 30) with 8% (wt/vol) acrylamide gels (in 0.5× TAE: 20 mM Tris acetate [pH 7.8], 10 mM sodium acetate, 0.5 mM disodium EDTA) containing a linear chemical gradient ranging from 25 to 55% denaturant. Gels were made with 8% (wt/vol) acrylamide stock solutions (acrylamide–*N*,*N*-methylene-bisacrylamide, 37:1) containing 0 and 100% denaturant (7 M urea and 40% [vol/vol] formamide, deionized with AG501-X8 mixed-bed resin [Bio-Rad Laboratories, Inc.]). The gels were run for 225 min at 60°C and 200 V.

After completion of electrophoresis, the gels were stained in an ethidium bromide solution (0.5 μg/ml) and documented with a Polaroid system with a UV transilluminator. Intensely stained bands were excised from the gel and incubated overnight at 37°C in a solution containing 0.3 M NaCl, 3 mM EDTA, and 30 mM Tris (pH 7.6) to elute the DNA. The acrylamide was removed by centrifugation, and the DNA in the supernatant was precipitated with ethanol and resuspended in TE buffer.

The DNA obtained from the excised bands was reamplified with the same primer pair, and the PCR products were checked by DGGE to verify that distinct bands were eluted.

Sequencing of 16S rDNA fragments. DNA extracted from the bacterial isolates and DNA eluted from the excised DGGE bands were reamplified with the same forward primer (5′-CCTACGGGAGGCAGCAG-3′) including an additional sequence extension (T3; 5′-AAAATTAACCCTCACTAAAG-3′) at its 5′ end and the reverse primer (5′-ATTACCGCGGCTGCTGG-3′) with the additional sequence (M13r; 5′-AAATTCACACAGGAAACAG-3′) at its 5′ end to facilitate DNA sequence analysis. The newly obtained PCR fragments were purified (28) and sequenced directly with a LI-COR model 4000 L automatic sequencing system. This system is based on the detection of infrared fluorophore-labeled DNA molecules by a scanning infrared fluorescence microscope during electrophoresis (25). The sequencing reaction was carried out by cycle sequencing with the SequiTherm system (Epicentre). The PCR product (80 fmol) was sequenced with 2 pmol of fluorescently labeled T3 or M13r primer and 5 U of SequiTherm thermostable DNA polymerase. Both strands of each PCR product were sequenced.

Identification of bacteria by comparative sequence analysis. The sequences obtained from the isolated bacteria and the DGGE bands were sent to the Ribosomal Database Project (RDP [21]) and to the EMBL nucleotide sequence database for comparison. The SIMILARITY_RANK tool of the RDP and the FASTA search option (31) for the EMBL database were used to search for close evolutionary relatives. Prealigned sequences of representatives of several groups were obtained from the RDP and used for identification of similarities to our sequences. Sequence alignments were performed with the sequence alignment editor SEQAPP (12). Distance matrices were calculated with DNADIST as implemented in the software package PHYLIP (version 3.5) developed by Felsenstein (10), using the Jukes-Cantor model, which assumes independent change at all sites with equal probability (15). Phylogenetic trees were constructed from the evolutionary distances, calculated with the algorithm of Fitch and Margoliash (11) as implemented in the program FITCH in the software package PHYLIP (version 3.5) and checked by bootstrap resampling (100 replicates) using the program SEQBOOT. In this calculation, nucleotides of the sequence data set are deleted and replaced to produce random variations typical of the variation to be obtained by introducing new sequences. The sequence data set is varied and analyzed multiple times. The bootstrap numbers indicate the percentage of resampling which supports a specific branching pattern. A bootstrap value of 100 indicates that a branching pattern was confirmed in all resamplings, whereas a bootstrap value of 50 indicates that the branching pattern was reproduced only in 50% of the resamplings.

Nucleotide sequence accession numbers. The sequences obtained in this study are available at the EMBL database under the accession numbers X95940 (HB1-1), X95941 (HB1-2), X95942 (HB1-4), X95943 (HB1-5), X95944 (HB1-3), X95945 (band A2), X95946 (band A3), X95947 (band A6), X95948 (band A7), and X95949 (band A8).

RESULTS

Electron microscopy of wall-painting material. The research object is a fresco in the apsis in the chapel of Castle Herberstein, which dates from the 13th century (Fig. 1). Electron microscopy of a wall-painting sample, which was taken from the surface, revealed a dense microbial colonization of the material (Fig. 2). The dominant colonizing microorganisms showed filamentous morphologies ranging in length from 4 to 15 μm. The filaments not only were attached to the material but also grew within the interstices of the material, occasionally forming tuberous growths. Figure 2 suggests that the growth of

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FIG. 1. Portion (approximately 2 by 3 m) of the fresco in the chapel of Castle Herberstein, showing the wall painting on the ceiling. The wall painting is continued on the sidewalls. Samples for the present study were taken from one of the sidewalls. This painting shows parts of the Christian hagiography and dates to the 13th century. It was last restored around 1950 and is now highly degraded by microorganisms.

these microorganisms resulted in a loosening of the consistency of the ground material.

Isolation of bacteria from wall-painting samples. We were able to isolate five separate strains of heterotrophic bacteria. With the exception of strain HB1-3 (<10^3 CFU), the isolates were obtained in quantities ranging from 10^3 to 10^4 CFU/g of material. HB1-1 and HB1-5 were grown on nutrient broth and casein minimal medium, while HB1-2 was grown on nutrient broth. HB1-3 and HB1-4 grew on casein minimal medium. No colonies could be isolated from glycerol-arginine plates, which were used to grow actinomycetes (9), because they were overgrown by fungi. Colony pigmentation among the purified bacterial strains varied; isolate HB1-1 showed an intense yellow color, HB1-2 was nonpigmented, HB1-3 was white, HB1-4 was yellowish, and isolate HB1-5 produced a red pigmentation.

DGGE analysis of 16S rDNA PCR products. Figure 3 shows a DGGE analysis pattern of PCR products obtained after amplification of two DNA aliquots isolated from a wall-painting sample (lanes 5 and 6) and from the five purified bacterial strains, which were isolated from the same samples (lanes 1 to 4 and 7 to 11). The equal-sized 16S rDNA fragments were separated according to their sequences to study the microbial

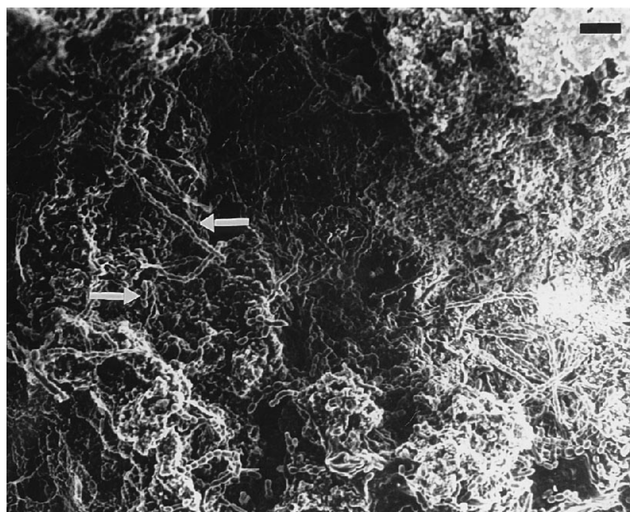

FIG. 2. Electron micrograph of surface material of the fresco sample. The material shows a dense microbial colonization. Filamentous microorganisms which are attached to the material are indicated by arrows. Bar in the upper right corner, 3.34 μm.

population of the wall painting. Each band in the electrophoresis pattern presumably originates from one bacterial species present in the original material. Theoretically, bands at the same position in the electrophoresis pattern contain DNA fragments with identical sequences (42). However, this should be confirmed by sequencing the bands. Furthermore, overlapping of fragments with different sequences cannot be excluded in DGGE separation patterns derived from complex bacterial populations. The DGGE pattern of different 16S rDNA fragment amplified from wall-painting DNA (lanes 5 and 6)

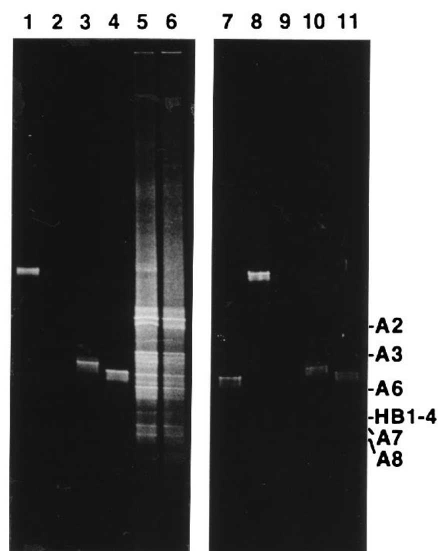

FIG. 3. Ethidium bromide-stained DGGE separation pattern of bacterial DNA fragment coding for the 16S rRNA. Lanes 1 to 4 and 7 to 11 show the fragments obtained from bacterial isolates HB1-1 (lane 7), HB1-2 (lanes 1 and 8), HB1-3 (lanes 4 and 11), HB1-4 (lanes 2 and 9), and HB1-5 (lanes 3 and 10). (Lanes 2 and 9, representing isolate HB1-4, contained too little DNA. A weak band was visible only in the original gel. The position of the band is marked.). Lanes 5 and 6 show the reproduced band patterns of the 16S rRNA genes which were amplified directly from DNA extracted from wall-painting material. Bands A2, A3, A6, A7, and A8 were excised and sequenced.

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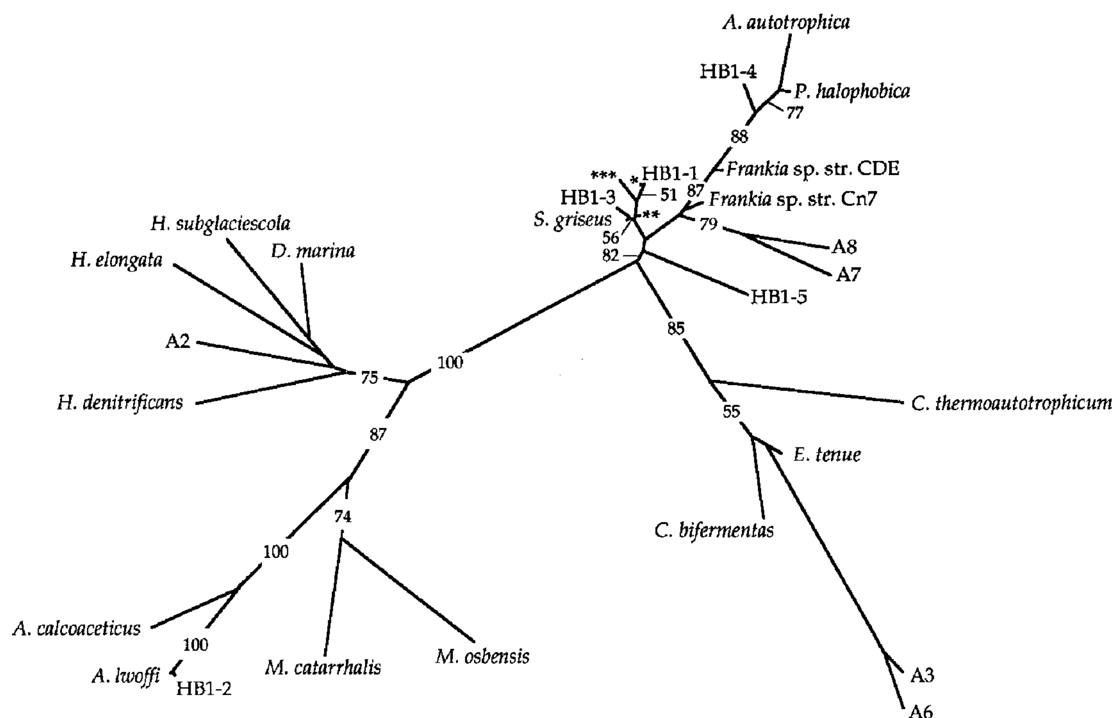

FIG. 4. Unrooted distance tree of nucleotide-sequencing data based on 16S rDNA sequences showing the affiliation of bacterial isolates (HB1-1, HB1-2, HB1-3, HB1-4, and HB1-5) and molecular isolates obtained by sequencing individual DGGE bands (A2, A3, A6, A7, and A8) obtained in this study. Sequences used in preparing this tree were derived from the RDP (11). The numbers on the branches indicate the percentages of bootstrap replications that support the notion that the group descends from that branch. Symbols: *, *Arthrobacter globiformis*; **, *Streptomyces ambofaciens*; ***, *Micrococcus luteus*.

showed 15 distinguishable bands which were retrieved from bacteria in the wall painting. The background smear may contain more bands, which, however, are not clearly distinguishable. When comparing the DGGE pattern originating from wall-painting material with the pattern obtained from control material taken from a suitable area without paint, we observed absent or missing bands in the control material; e.g., band A2 is missing (data not shown). This indicates that the corresponding bacteria were absent in the control material. The DGGE patterns of the isolated bacteria showed only one clearly separated band (Fig. 3). The band of isolate HB1-1 (lane 7) had the same position as one of the bands in the DGGE pattern of 16S rDNA fragments amplified from wall-painting DNA. However, because of overlapping of amplified PCR products, we could not confirm that the sequence of the bacterial isolate was identical to a sequence from the DGGE pattern.

Sequence analysis of isolated bacteria and DGGE bands. The 16S rDNA fragments corresponding to nucleotide positions 341 to 534 in the *E. coli* sequence of the five isolated bacteria and of bands from the DGGE pattern, which were amplified from wall-painting material (Fig. 3), were sequenced.

Comparison of the 16S rDNA sequences with sequences available in the RDP and EMBL databases revealed high similarity values for four of our bacterial isolates, i.e., HB1-1, HB1-3, HB1-4, and HB1-5, with respect to sequences of different actinomycetes. The actinomycetes belong to the gram-positive bacteria and show a wide morphological diversity, including coccoid organisms, coccus-rod cycles, branching or nonbranching rods, fragmenting hyphal forms, or those which produce a highly differentiated branched mycelium. The sequence obtained from the fifth bacterial isolate, HB1-2, showed high similarity to sequences from acinetobacters. They are ubiquitous aerobic, heterotrophic organisms (4).

Sequences obtained from excised DGGE bands, which originated from bacteria that had not undergone prior cultivation, showed high similarities to sequences of bacteria from the genera *Halomonas* (band A2), *Clostridium* (bands A3 and A6), and *Frankia* (bands A7 and A8).

To obtain a more detailed identification based on the nucleotide sequencing data for our bacterial isolates and for the bacteria corresponding to the separated DGGE bands, we performed a sequence comparison with aligned 16S rDNA sequences of several representatives of different bacterial groups. Figure 4 shows a tree based on distance estimates obtained after comparison of aligned 16S rDNA sequences corresponding to nucleotide positions 341 to 534 in the *E. coli* sequence. The tree shows the affiliation of the sequences from our isolates and from the DGGE bands with sequences from known bacteria. Bootstrap analysis was used to evaluate the reliability of this tree.

Bacterial isolates HB1-1, HB1-3, HB1-4, and HB1-5 are apparently all related to the actinomycete lineage. In particular, HB1-1 is most closely related to *Arthrobacter globiformis* (bootstrap value, 51%) whereas HB1-3 clusters with *Streptomyces griseus* (bootstrap value, 56%). Bacterial isolate HB1-4 is related to members of the *Pseudonocardiaceae* (bootstrap value, 88%). Isolate HB1-5 is also related to the actinomycetes but only distantly; a close relative of this isolate could not be found. Isolate HB1-2 is affiliated with the gamma subdivision of the *Proteobacteria* and is very closely related to *Acinetobacter lwoffi* (bootstrap value, 100%).

The sequence derived from DGGE band A2, also affiliated with the gamma subdivision of the *Proteobacteria*, is related to sequences from the genus *Halomonas* (bootstrap value, 75%). Bacteria of the genus *Halomonas* are gram negative and aerobic but can also grow anaerobically in the presence of NH_3,

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when they can ferment glucose (44). They are chemoorganotrophic and extremely halotolerant.

DGGE bands A3 and A6 contained sequences which group with sequences of the genus *Clostridium* (bootstrap value, 85%). This genus encompasses over 100 species with a wide diversity in morphology, nutritional requirements, and metabolic activities. They are obligate anaerobes and may grow autotrophically or heterotrophically; they can ferment a variety of different substrates, such as polysaccharides and proteins (14).

Sequences obtained from DGGE bands A7 and A8 are related to those of species from the *Frankia* group. Members of this group are similar to most aerobic actinomycetes in that they produce a filamentous mycelium. All *Frankia* strains investigated so far are slowly growing, with generation times ranging from 1 day to several days (22). Most species can fix atmospheric nitrogen and are symbiotic with plants (22), although they may occur free in soil (3).

DISCUSSION

In the present study, bacteria on a 13th century wall painting in Castle Herberstein, Austria, were identified by molecular biological techniques, including DGGE analysis of PCR-amplified 16S rDNA fragments. The application of this approach allows the identification of bacteria without the need for prior cultivation, which is important because most bacteria in environmental samples cannot be cultivated under laboratory conditions (13, 46, 48). In addition, we used conventional microbiological methods to cultivate bacteria from wall-painting material.

The colonization of the fresco by different fungi has recently been shown by Berner et al. (5). At present, a ratio of damage caused by fungi and by bacteria cannot be estimated. The identification of bacteria aims at a better understanding of possible genera and species involved in the biodegradation processes of this example of cultural heritage and similar works of art.

Actinomycetes, such as streptomycetes, arthrobacters, and micrococci, have been isolated from ancient wall paintings in the past. They were identified on the basis of their morphological and physiological features (32, 50). Altenburger et al. (1) identified *Micrococcus* and *Agromyces* spp. from wall paintings by means of a polyphasic approach (26). Two other isolates, which could not be affiliated with known genera, were also shown to belong to the order *Actinomycetales* (1). The role of mycelium-producing actinomycetes in biodeterioration has been described by various authors (50, 51). Actinomycetes contribute to the mechanical destruction of wall paintings as a result of the production of mycelia, which grow into the painting and its grounding (50). By using conventional microbiological techniques, we could also isolate bacteria of this group. They were identified by 16S rDNA sequence analysis. Four of the five isolates are related to the gram-positive bacteria with high G+C content, i.e., the actinomycete lineage (Fig. 4).

The fifth isolate is an acinetobacter, closely related to *Acinetobacter lwoffi*. Although previously not identified on wall paintings, acinetobacters are ubiquitous aerobic, heterotrophic organisms. The kind of damage that acinetobacters can do to wall paintings has not yet been clarified; however, since they can live on a wide variety of compounds, including aliphatic alcohols, some amino acids, decarboxylic and fatty acids, unbranched hydrocarbons, and sugars (4, 16), they may also use organic compounds, which are usually present in the paint layer of ancient wall paintings (33, 49), as a carbon source.

Using DGGE analysis of amplified 16S rDNA fragments from wall painting DNA, we identified sequences with high similarity to bacteria which have not yet been isolated on ancient wall painting by conventional microbiological means. One rDNA sequence showed a high similarity to sequences of species from the genus *Halomonas*, which are extremely salt tolerant and grow in media or niches with salt concentrations ranging from 0 to 32% (44). This halotolerance suggests that they may also grow on salt efflorescence areas on wall paintings. While the microbial colonization of salt efflorescence areas on stone and mural paintings by halotolerant bacteria, i.e., *Brevibacterium linens*-type species, has already been shown (20, 38), *Halomonas* spp. have not yet been identified on wall paintings. During the planning of restorative procedures, the microbial colonization by halophilic and halotolerant bacteria on salt efflorescence has more or less been ignored (19). In this context, we consider that the ability of *Halomonas* species to ferment organic compounds, a process which includes the production of organic acids (44), indicates a potential for biodecay.

In addition, we have identified two sequences, A3 and A6, that are related to members of the gram-positive group with a low G+C content, especially clostridia. Clostridia are obligatory anaerobic bacteria and are nutritionally versatile. While clostridia have been identified on stone (37), their presence on and potential role in the destruction process of ancient wall paintings have not been taken into consideration so far. Clostridia can ferment a variety of different substrates, such as polysaccharides and proteins. Therefore, it is not surprising to find these bacteria in ancient wall paintings, since ancient paints were often made from natural products, including egg, oil, and gum (33, 49). As a result of their growth on these substrates, they can produce a variety of organic acids and alcohols, which may lead to discolorations, loss of paint material, and loosening of the material consistency of the painting.

Finally, we sequenced two DGGE bands, A7 and A8, which group with sequences of the genus *Frankia. Frankia* species, like other actinomycetes, build mycelia, which may grow into the painting. The mechanical destructive capability of actinomycetes has been described above. As *Frankia* species grow extremely slowly, they are difficult to isolate in the presence of other, faster-growing bacteria. As a result, they are likely not to be taken into consideration as contributors to the biodegradation of wall paintings.

DGGE has proven to be a useful means of detecting bacteria on wall paintings. As mentioned above, we identified five sequences representing different species which have not previously been isolated from wall paintings. They deserve better attention from restorers of medieval wall paintings, since the metabolism of these bacteria suggests that they play a major role in the destruction of this art. In fact, we believe that these findings are the tip of the iceberg and that many more bacteria which may cause severe damage to ancient wall paintings will be identified in the future. Figure 3 demonstrates many more bands in the pattern originating from the wall-painting material. We assume that sequencing more bands proves the presence of a whole range of bacteria in the sample. This assumption is also based on the fact that in only few cases were directly amplified 16S rRNA sequences from an environmental sample identical to 16S rRNA sequences of cultivated species (17, 29). This is generally interpreted as an indication of a wide microbial diversity, which supports the theory that some bacteria are isolated by selective enrichment while the vast majority remain undetected.

Detection of bacteria by DGGE analysis of PCR-amplified 16S rDNA fragments contributes to a better understanding of the actual bacterial species composition. Our results suggest

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that the cultivated organisms may not necessarily be the predominant species in the sample. For example, we could easily isolate acinetobacters at high CFU, but they were not represented as a strong band in the DGGE pattern. The overestimation of acinetobacters in environmental samples as a result of cultivation-dependent population shifts was shown in the past. Wagner et al. (45) showed by in situ hybridization that the number of acinetobacters in activated sludge samples was overestimated.

We are well aware that the extraction of DNA from wall-painting material, as the first step, is decisive, because potential errors are multiplied in further experiments. The applied freeze-thawing method did not necessarily ensure that all bacteria in the sample were lysed. Eventually, this leads to an underestimation of the bacterial species present in such samples. However, taking into consideration the cultural value of the painting, which restricted the quantities of our samples, detailed experiments which would have taken eventual lysing problems into account were not possible. Test experiments, in which different protocols for DNA extraction were evaluated, showed that freeze-thawing worked best for the extraction of DNA from small quantities. Furthermore, our results show that gram-positive bacteria can also be detected.

The analysis of short sequences might not give a reliable phylogenetic inference. Therefore, we used the constructed tree only for identification purposes. For real phylogenetic analysis, we would need more information on the DNA sequences. This could be obtained by DGGE analysis of larger fragments (28, 29).

In principle, DGGE analysis of 16S rDNA fragments does not distinguish whether the template DNA originates from active or dead bacteria or from free DNA (34). Consequently, in future investigations, we will consider using RNA instead of DNA as the starting material for our analysis. It has recently been shown that the use of RNA favors the identification of more active bacterial populations (42).

Future activities will focus on three aspects: (i) sequencing of more bands from the DGGE pattern to explore the microbial diversity in more detail, (ii) attempting to isolate bacteria related to DGGE sequences, such as anaerobic and halotolerant bacteria, and (iii) determining the abundance of particular bacterial groups which have been found either by microbial isolation or by DGGE analysis. For this purpose, we plan to use in situ hybridization with available fluorescence-labeled group-specific probes (45) for bacteria which have been identified on wall paintings.

We hope that an integrated approach of molecular and microbiological techniques will lead to a better understanding of the composition of bacterial species on ancient wall paintings and to new ways to restore and conserve this cultural heritage.

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REFERENCES

1. **Altenburger, P., P. Kämpfer, A. Makristhathis, W. Lubitz, and H.-J. Busse.** Classification of bacterial strains from a medieval wall painting. J. Biotechnol., in press.
2. **American Public Health Association.** 1980. Standard methods for the examination of water and wastewater, 1st ed. American Public Health Association, Inc., Washington, D.C.
3. **Baker, D., and D. O'Keefe.** 1984. A modified sucrose fractionation procedure for the isolation of *Frankiae* from actinorhizal root nodules and soil samples. Plant Soil **78:**23–28.
4. **Baumann, P.** 1968. Isolation of *Acinetobacter* from soil and water. J. Bacteriol. **96:**39–42.
5. **Berner, M., G. Wanner, and W. Lubitz.** Unpublished data.
6. **Bianchi, A., M. A. Favali, N. Barbieri, and M. Bassi.** 1980. The use of fungicides on moldcovered frescoes in San Eusebio in Pavia. Int. Biodeterior. Bull. **16:**45–51.
7. **Bock, E., and W. Sand.** 1993. The microbiology of masonry biodeterioration. J. Appl. Bacteriol. **74:**503–514.
8. **Don, R. H., P. T. Cox, B. J. Wainwright, K. Baker, and J. S. Mattick.** 1991. "Touchdown" PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res. **19:**4008.
9. **El-Nakeeb, M. A., and H. A. Lechevalier.** 1963. Selective isolation of aerobic actinomycetes. Appl. Microbiol. **11:**75–77.
10. **Felsenstein, J.** 1993. PHYLIP (Phylogeny Inference Package) version 3.5c. Department of Genetics, University of Washington, Seattle.
11. **Fitch, W. M., and E. Margoliash.** 1967. Construction of phylogenetic trees: a method based on mutation distances as estimated from cytochrome c sequences is of general applicability. Science **155:**279–284.
12. **Gilbert, D. G.** 1992. SeqApp—a bio-sequence analysis application. Indiana University, Bloomington.
13. **Giovannoni, S. J., T. B. Britschgi, C. L. Moyer, and K. G. Field.** 1990. Genetic diversity in Sargasso Sea bacterioplankton. Nature (London) **345:**60–63.
14. **Hippe, H., J. R. Andreesen, and G. Gottschalk.** 1992. The genus *Clostridium*—nonmedical, p. 1800–1866. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The prokaryotes, vol. 2. Springer-Verlag, New York.
15. **Jukes, T. H., and R. R. Cantor.** 1969. Evolution of protein molecules, p. 21–132. *In* H. N. Munro (ed.), Mammalian protein metabolism. Academic Press, Inc., New York.
16. **Juni, E.** 1978. Genetics and physiology of *Acinetobacter*. Annu. Rev. Microbiol. **32:**349–371.
17. **Kane, M. D., L. K. Poulsen, and D. A. Stahl.** 1993. Monitoring the enrichment and isolation of sulfate-reducing bacteria by using oligonucleotide hybridization probes designed from environmentally derived 16S rRNA sequences. Appl. Environ. Microbiol. **59:**682–686.
18. **Krumbein, W. E.** 1968. Zur Frage der biologischen Verwitterung: Einfluß der Mikroflora auf die Bausteinverwitterung und ihre Abhängigkeit von edaphischen Faktoren. Z. Allg. Mikrobiol. **8:**107–117.
19. **Krumbein, W. E., and K. Petersen.** 1990. Mikroorganismen beschleunigen den Zerfall mittelalterlicher Wandgemälde. Wandmalereischäden Arbeitshefte Denkmalpflege Niedersachsen **8:**115–121.
20. **Krumbein, W. E., V. Schostak, and K. Petersen.** 1991. On novel halophilic and extremely halotolerant bacteria from environments near the North Sea coast and the Steinhuder Meer. Kiel. Meersesforsch. Sonderh. **8:**173–177.
21. **Larsen, N., G. J. Olsen, B. L. Maidak, M. J. McCaughey, R. Overbeek, T. J. Macke, T. L. Marsh, and C. R. Woese.** 1993. The ribosomal database project. Nucleic Acids Res. **21:**3021–3023.
22. **Lechevalier, M. P.** 1994. Taxonomy of the genus *Frankia*. Int. J. Syst. Bacteriol. **44:**1–8.
23. **Lyalikova, N. N., and Y. P. Petushkova.** 1991. Role of microorganisms in the weathering of minerals in building stone of historical buildings. Geomicrobiol. J. **9:**91–101.
24. **Medlin, L., H. J. Ellwood, S. Stickel, and M. L. Sogin.** 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA coding regions. Gene **71:**491–499.
25. **Middendorf, L. R., J. C. Bruce, R. C. Bruce, R. D. Eckles, D. L. Grone, S. C. Roemer, G. D. Sloniker, D. L. Steffens, S. L. Sutter, J. A. Brumbaugh, and G. Patonay.** 1992. Continuous, on-line DNA sequencing using a versatile infrared laser scanner/electrophoresis apparatus. Electrophoresis **13:**487–494.
26. **Murray, R. G. E., D. J. Brenner, R. R. Colwell, P. De Vos, M. Goodfellow, P. A. D. Grimont, N. Pfennig, E. Stackebrandt, and G. A. Zavarzin.** 1990. Report of the ad hoc committee on approaches to taxonomy within the *Proteobacteria*. Int. J. Syst. Bacteriol. **40:**213–215.
27. **Muyzer, G., E. C. de Waal, and A. G. Uitterlinden.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59:**695–700.
28. **Muyzer, G., S. Hottenträger, A. Teske, and C. Wawer.** 1996. Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA—a new molecular approach to analyze the genetic diversity of mixed microbial communities, p. 3.4.4.1–3.4.4.22. *In* A. D. L. Akkermans, J. D. van Elsas, and F. J. de Bruijn (ed.), Molecular microbial ecology manual, 2nd ed. Kluwer Academic Publishers, Dordrecht, The Netherlands.
29. **Muyzer, G., A. Teske, C. O. Wirsen, and H. W. Jannasch.** 1995. Phylogenetic

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relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Arch. Microbiol. **164:**165–174.

30. **Myers, R. M., T. Maniatis, and L. S. Lerman.** 1987. Detection and localization of single base changes by denaturing gradient gel electrophoresis. Methods Enzymol. **155:**501–527.
31. **Pearson, W. R.** 1990. Rapid and sensitive sequence comparison with FAST and FASTA. Methods Enzymol. **183:**63–98.
32. **Petushkova, Y. P., N. N. Lyalikova, and M. N. Poglasova.** 1989. Microorganisms discovered on the frescoes of the Ferapoint Monastery. Mikrobiologiya **58:**1021–1030.
33. **Reichlin-Minoretti, A.** 1983. Bindemittelanalysen an vorindustriellen Malereien. Ph.D. thesis. ETH Zürich, Zürich.
34. **Romanowski, G., M. G. Lorenz, and W. Wackernagel.** 1991. Adsorption of plasmid DNA to mineral surfaces and protection against DNase I. Appl. Environ. Microbiol. **57:**1057–1061.
35. **Saiki, R. K., D. H. Gelfand, S. J. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with thermostable DNA polymerase. Science **239:**487–491.
36. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
37. **Schmidt, A.** 1994. Methanbildende und methanverbrauchende Bakterien in Steinen. Diploma-thesis. University of Hamburg, Hamburg, Germany.
38. **Schostak, V.** 1993. Halotolerante und moderat halophile chemoorganotrophe Bakterien auf salzbelasteten Putzen und Wandmalereien. Ph.D. thesis. Universität Oldenburg, Oldenburg, Germany.
39. **Sheffield, V. C., D. R. Cox, L. S. Lerman, and R. M. Myers.** 1989. Attachment of a 40-base pair G+C rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. Proc. Natl. Acad. Sci. USA **86:**232–236.
40. **Sorlini, C., M. Sacchi, and A. Ferrari.** 1987. Microbiological deterioration of Gambara's frescos exposed to open air in Brescia, Italy. Int. Biodeterior. **23:**167–179.
41. **Tayler, S., and E. May.** 1994. The investigation of biocides against bacteria isolated from stone and their effectiveness against in situ populations. Mater. Org. **28:**265–277.
42. **Teske, A., C. Wawer, G. Muyzer, and N. B. Ramsing.** 1996. Distribution of sulfate-reducing bacteria in a stratified fjord (Mariagerfjord, Denmark) as evaluated by most-probable-number counts and denaturing gradient gel electrophoresis of PCR-amplified ribosomal DNA fragments. Appl. Environ. Microbiol. **62:**1405–1415.
43. **Urzi, C., S. Lisi, G. Criseo, and A. Pernice.** 1991. Adhesion to and degradation of marble by a *Micrococcus* strain isolated from it. Geomicrobiol. J. **9:**81–90.
44. **Vreeland, R. H., C. D. Litchfield, E. L. Martin, and E. Elliot.** 1980. *Halomonas elongata*, a new genus and species of extremely salt-tolerant bacteria. Int. J. Syst. Bacteriol. **30:**485–495.
45. **Wagner, M., R. Erhart, W. Manz, R. Amann, H. Lemmer, D. Wedi, and K.-H. Schleifer.** 1994. Development of an rRNA-targeted oligonucleotide probe specific for the genus *Acinetobacter* and its application for in situ monitoring in activated sludge. Appl. Environ. Microbiol. **60:**792–800.
46. **Ward, D. M., R. Weller, and M. M. Bateson.** 1990. 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature (London) **345:**63–65.
47. **Wawer, C., and G. Muyzer.** 1995. Genetic diversity of *Desulfovibrio* spp. in environmental samples analyzed by denaturing gradient gel electrophoresis of [NiFe] hydrogenase gene fragments. Appl. Environ. Microbiol. **61:**2203–2210.
48. **Wayne, L. G., D. J. Brenner, R. R. Colwell, P. A. D. Grimont, O. Kandler, M. I. Krichevsky, L. H. Moore, W. E. C. Moore, R. G. E. Murray, E. Stackebrandt, M. P. Starr, and H. G. Trüper.** 1987. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int. J. Syst. Bacteriol. **37:**463–464.
49. **Weirich, G.** 1988. Wachstum von Schimmelpilzen und Bakterien auf verschiedenen Malgründen. Z. Kunsttechnol. Konservier. **2:**305–314.
50. **Weirich, G.** 1989. Investigations of the microflora of mural paintings. Mater. Org. **24:**139–159.
51. **Williams, S. T.** 1985. Streptomycetes in biodeterioration—their relevance, detection and identification. Int. Biodeterior. **21:**201–209.

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