Identification of microorganisms on stone and mural paintings using molecular methods

Diplomarbeit

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Overview

Abbreviations

A	Adenin
Ap	Ampicilin
APS	Ammoniumpersulfat
bp	Basepairs
BSA	Bovin Serum Albumin
C	Cytosin
℃	Degree Celsius
cDNA	copy DNA
cm	Centimeter
dATP	desoxy Adenin
dCTP	desoxy Cytosin
ddH_2O	double destilled water
ddNTPs	Didesoxy nucleotide
DGGE	Denaturing Gradient Gel Electrophoresis
dGTP	desoxy Guanin
dH_2O	destilled water
DMSO	Dimethylsulfoxid
DNA	Desoxyribonucleinacid
dNTP	Desoxy nucleotide
dsDNA	double stranded DNA
DSM	German Collection of Microorganisms
dTTP	desoxy Thymin
EDTA	Ethylenediaminetetraacetic acid
EMBL	European Molecular Biology Laboratory
G	Guanin
IPTG	Isopropyl-beta-D-thiogalactopyranoside
ITS	Internally transcribed Spacer
ITS-HHP	Internally Transcribed Spacer- Homoduplex Heteroduplex Polymorphism
JCM	Japanese Collection of Microorganisms
KM	Samples taken from the `Kindermuseum`
kV	Kilovolt
MDE	Mutation Detection Enhancement
mg	Milligramm
min	Minute
ml	Millilitre
µl	Microlitre
mm	Millimeter
mM	Millimolar
PAA	Polyacrylamid

PCR	Polymerase Chain Reaction
pmol	Picomol
RAPD-PCR	Random Amplified Polymorphic DNA PCR
rDNA	ribosomal DNA
RDP-II	Ribosomal Database Project-II
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleid acid
rRNA	ribosomal RNA
S	Samples taken from the Berglzimmer
ssDNA	single stranded DNA
T	Thymin
TAE	Trisacetic-EDTA
TBE	Tris Borat EDTA
Tc	Tetracylin
TE	Tris-EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
Temp	Temperature
U	Units
V	Volt
VK	Samples taken from the Chapel of St.Virgil
vol	Volume
W	Watt
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

Summary

A wide number of investigations have shown that microorganisms play, in addition to chemical and physical processes, a major role in degradation of objects of art, such as paintings, stone-works, wood, paper, masonry, leather, parchment, glass and metal (Bock and Sand, 1993; Cifferi, 1999; Griffin *et al*., 1991; Kowalik 1980; Montegut *et al*., 1991).

A great variety of organisms colonizing stones and mural paintings including bacteria, archaea, fungi, algae, lichens, mosses and plants have been reported (Rölleke *et al*., 1996; 1998; Schabereiter-Gurtner, 2000; Schabereiter-Gurtner *et al*., 2001a, b, c, Heyrmann *et al*., 1999; Piñar *et al*., 2001a,b,c; Saiz-Jimenez, 2002). These organisms are involved in biodeteriorative processes and may cause severe damage to objects of art. Therefore, it is important for means of prevention and restoration of our Cultural Heritage to know which microorganisms are colonising valuable substrates, where they originate from, to guest their potential deteriorative effects and at last but not least to investigate which microorganisms may even protect Cultural Heritage.

Nowadays, it is known that by using classical cultivation methods far less than 1% of all existing bacteria can be cultivated from environmental samples (Amann *et al*., 1995). Therefore, culture-independent methods have been shown to be a useful tool for the screening of microbial communities on objects of art (Schabereiter-Gurtner *et al*., 2001a). The methodology used in this study includes a molecular approach using a special DNA extraction protocol for small sample amounts, PCR directed to specific molecular markers and the fingerprinting technique Denaturing Gradient Gel Electrophresis (DGGE). Samples subjected to investigation in this study have been taken from mural paintings located in the `Berglzimmer` and `Kindermuseum` at the Palace of Schönbrunn (Vienna), as well as from salt efflorescences covering the stone work of the medieval Chapel of St.Virgil (Vienna). Samples were screened for the presence of bacterial, fungal and archaeal communities. Several members of bacteria mainly of the group of *Gamma-Proteobacteria* could be detected, as well as several moderately halophilic bacteria belonging to different genera. Furthermore, archaeal species belonging to the halophilic group were detected in samples taken from the stone work of the Chapel of St.Virgil. Fungal species of the *Ascomycota* and *Basidiomycota* could be identified colonising the samples taken from the mural paintings of the palace of Schönbrunn. The results presented in this Diploma work

are giving hints for the origin of bacteria, fungi and archaea colonising valuable objects of art as well as suggesting biodeteriorative effects caused by these microorganisms on such objects.

In addition, this work includes the detailed description of ten halophilic, Gram-positive, spore forming bacterial strains which grouped in three potentially new species closely related to members of the genus *Halobacillus*. Phenotypical features as well as a detailed molecular typing of the newly isolated bacterial strains is presented here.

1. Introduction

1.1. Mural painting- Substratum for growth and colonization scenario

In general growth of diverse organisms on mural paintings is supported by humidity, slight alkaline pH values and the presence of organic and inorganic nutrient sources (Weirich, 1988). In mural paintings, pigments are suspended in water or oil, often binders such as casein and milk are used, and they are supplied on the damp lime plaster (Ciferri, 1999). The calcium carbonate formed on contact with air consolidates the pigments. Thus frescos contain mainly inorganic components.

While chemoautotrophic nitrifiers and thiobacilli obtain their energy for growth by oxidizing ammonium and reducing sulphur-compounds respectively, heterotrophic bacteria and fungi depend on organically bound carbon (Karpovich-Tate and Rebrikova, 1991; Lazar, 1971; Petushkova and Lyalikova, 1986; Saiz-Jimenez and Samson, 1981).

Thus primary colonization of photoautotrophic *Cyanobacteria*, chemolithoautotrophic nitrifying bacteria and sulphuric acid producing thiobacilli represent the first supply of organic matter. The death and lysis of such bacteria promotes the secondary settlement of heterotrophic bacteria and fungal populations (Ortega-Calvo *et al*., 1991). Further nutrients for heterotrophic bacteria and fungi are available from dust layers which accumulate over the painting. These dust layers are also capable of retaining moisture which helps the growth of bacteria and fungal spores and even attract fungal spores (Tilak *et al*., 1970; Tilak, 1991). Metabolites of autotrophic bacteria, dripping water, animal faeces (mosquitoes, bats, etc.) and, last but not least, the organic compounds in the paint layers themselves, including casein and the bonding agents linseed oil, egg and gum (Weirich, 1988), promote the microbial settlement.

This leads to changes in the microflora within time and a successive colonisation takes place. A scenario of this settlement is shown in Figure 1 (Täubel, 2001). Considering this fact, it is obvious that taking samples from mural paintings at defined points in time is only a snapshoot of a gradual changing biocoenosis. Therefore, it is necessary to monitor the microbial growth along the time in case of restoration before and after the treatment.

Previous studies based on culture-dependent techniques showed that the flora present on frescos 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*) (Arino *et al*., 1996; Ortega-Calvo *et al*., 1993).

1.2. Stone monuments

Early workers suggested that the presence of living microorganisms on stone increased its susceptibility to damage through their water-binding capacity. It has recently been reported that the mineralogy, porosity, surface roughness and capacity to collect water and organic materials control its bioreceptivity and tendency to biodeterioration (Krumbein and Gorbushina, 1995). In addition, environmental factors as temperature, light, pH and relative humidity affect the number and type of colonising species (Tiano *et al*., 1995) and hence the progress of colonisation. Biofilms on decayed stones consist on mixed microbial populations embedded in a polymeric matrix. Fungi, algae, filamentous and unicellular bacteria are found in these complex communities. A variety of bacteria, have been isolated from different stone samples, confirming the dominance of Gram-positive organisms, especially spore-forming bacteria such as *Bacillus* and filamentous actinomycetes (Tayler and May, 1991). In addition there are several works confirming the field observations of extreme tolerance of bacteria on stone monuments to high salt levels (May *et al*., 1999). The addition of NaCl to a basic heterotrophic medium has been used to assess the presence of halotolerant and moderate halophilic bacteria. However, salt addition had an inhibitory effect on the recovery of bacteria. Cultivation strategies, especially in the case of this “extreme” conditions, reflect only small portion of the microbes present on stone monuments.

Using molecular methods, a number of bacteria isolated from stone monuments have been genotypically characterised through RFLP of the 16S rDNA analyses (ARDRA) (Daffonchio *et al*., 1999), and recently the sequencing of SSU (18S RDNA) (Sterflinger *et al*., 1999) and LSU genes (interspaces ITS1 and ITS2) (De Hoog et al., 1999) have been applied to phylogenetic studies and to describe the taxonomic position of fungi.

1.3. Moisture and limiting factor water activity

Water activity is probably one of the most important limiting factors in organic materials. The water activity determines the water availability for the germination of microbial spores. Many fungal and bacterial species start their development depending on the available moisture on an object`s surface. The risk of microbial contamination of a material can be evaluated by the water activity number (a_W).

It has been reported (Caneva *et al*., 1991) that most microorganisms can grow in an a_W range of 0.6-0.98. Bacteria require an a_W above 0.95, while fungi need a lower a_W (commonly 0.70-0.85). Xerophilic fungi even grow at 0.60 a_W .

Sources of humidity are dust layers that accumulate over the surface of the paintings and can retain moisture. Hygroscopic salts, atmospheric condensation, dripping water and even the exhaled air of visitors bring in moisture.

1.4. Microniches on mural paintings and stone-works

Building stone and wall paintings contain a variety of hydroscopic salts, including carbonates, chlorides, nitrates, sulfates, etc, dispersed within the porous material or locally concentrated. These salts are solubilised and migrate with the water in and out of the stone. Due to changes in physical parameters, the so called salt efflorescences appear on the surfaces providing extremely saline environments and building niches for settlement of halophilic microorganisms. Halite, and other hygroscopic salts, can retain considerable quantities of moisture when entrapped in the building materials and thus favouring the bacterial growth. In turn these microorganisms can mediate the crystallization process. These crystallizations can produce destructive effects. Some salts may crystallize or recrystallize to different hydrates, which occupy a larger space and exert additional pressure producing cracking, powdering and flaking (Saiz-Jimenez and Laiz, 2000).

Halophilic bacteria and archaea were thought to be found only in extreme environments like salt lakes or black smokers (Vreeland et al, 1998; Rothschild and Mancinelli 2001; Sass *et al*., 2001; Litchfield and Gillevet 2002; Oren 2002). However, it has been shown that mural paintings and stones are a common habitat for extremely salt tolerant and moderate halophilic (Saiz-Jimenez and Laiz, 2000) bacteria and archaea (Piñar *et al*, 2001a; 2001b).

In a previous study (Piñar *et al*., 2001a, b) carried out on samples taken from the medieval wall paintings at the Catherine chapel in the Castle of Herberstein (Styria, Austria) several sequences phylogenetically affiliated with members of the halophilic

group of *Archaea* were detected by molecular methods, including PCR using archaeal-specific primers and DGGE analysis. The sequences were closely related to the species *Halococcus morrhuae*. Furthermore, several strains of moderately halophilic bacteria, phylogenetically affiliated with the genus *Halobacillus,* were identified, cultivated and monitorized by molecular techniques (Piñar *et al*, 2001c). DNA-DNA reassociation experiments proved that the isolated strains belonged to a hitherto unknown new species within this genus (Piñar *et al*, 2001c). In this Diploma work, members of the genus *Halobacillus* could anew be isolated from samples of another medieval building, the chapel of St.Virgil (Vienna, Austria) which gives an evidence for frequency of this genus in Cultural Assets.

1.5. Damage due to microorganisms

Microbial induced deterioration processes can cause different kinds of alterations such as discolouration of materials, the formation of crusts on surfaces and the loss of material, which leads to structural damage.

First of all, microorganisms can cause severe problems due to the excretion of aggressive metabolic products such as organic or inorganic acids. Secondly, material components can be used as substrates for microbial metabolism. Exoenzymes such as cellulases, proteases, tannases, beta-glucosidase produced by fungi and bacteria are responsible for hydrolysis of cellulose and proteins. Further examples are mechanical processes caused by swelling and shrinking of slime or mycelia penetrating inside plaster of the painting resulting in loss of cohesion and detachment of the paint layer (Rölleke, 1996; Schabereitner-Gurtner, 2000).

Discolorations are caused by excretion of pigments and microbiologically induced reduction/oxidation-processes (Krumbein and Petersen, 1990). Degradation of the bindings is followed by reduction of stability. Oxidation of ammonium and reduced sulphur compounds results in acidification of the environment. Sulphuric acid and the variety of organic acids produced by the heterotrophs cause solubilization of calcareous binding material and chelation of metal ions (Bock and Sand, 1993).

Furthermore, the risk of formation of indoor bioaerosols in residential and public buildings (offices, schools, museums, etc) have attract interest in the last years due to socio-economic as well as sanitary reasons (Górny and Dutkiewicz, 2002). Thus it is important in aspects of health and maintenance of Cultural Heritage to analyse the complete microflora to further develop biocides for harmful microorganisms and methods for restoration of damaged objects of art.

Table 1 gives examples for microbial activity connected with damage symptoms observed in mural paintings and stone surfaces.

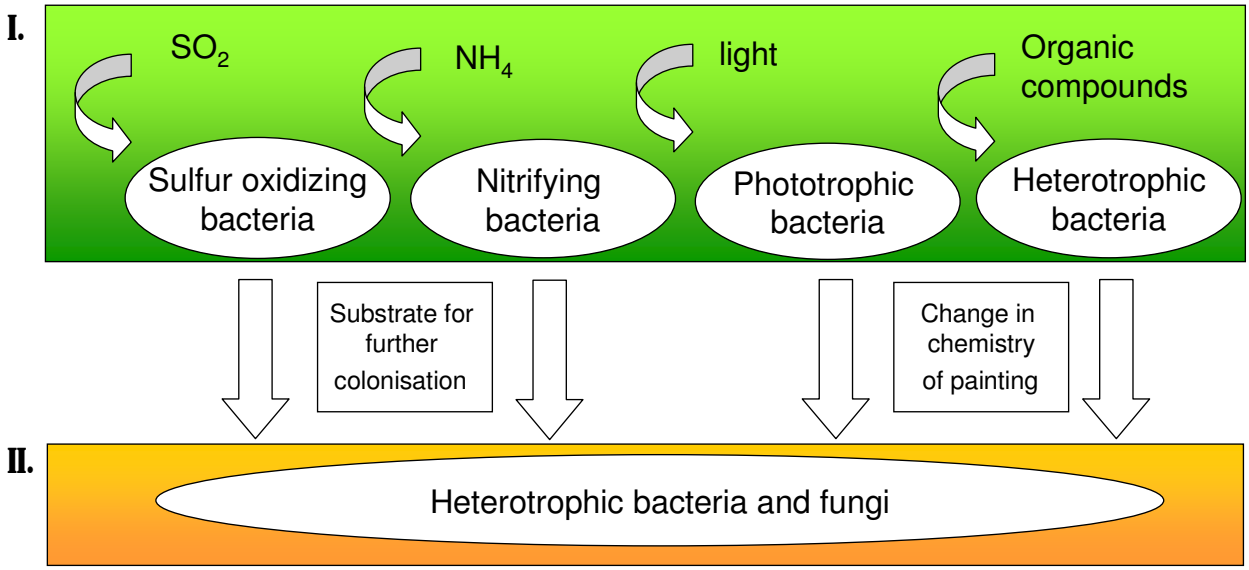


Figure 1 Schematic overview showing successive colonization of paintings: I. primary colonization. II. Successive settlement of different groups of microorganisms

Damage	Microbial activity
Green, brown, red, grey surface layers (patinas)	Pigments produced by microorganisms (intra-or extracellular) as chlorophyll and carotinoides
Dislodging of painting layers	Use of organic binders as nutrient- and energy source
Acid attack of carbonates	Acid produced by fungi or bacteria
Discolouration of pigments	Redox-reactions induced by fungi and bacteria
Loss of paint layer	Growth of microorganisms deep into the plaster
Hydrolysis of synthetic polymers	Chemoorganotrophic activity of bacteria and fungi

Table 1 Examples for microbial damage on mural paintings (Rölleke, 1996)

1.6. Methods for analysis of microrgansims on Cultural Assets

A great deal of research has been done in this field mainly by using cultivation-dependent methods. However, by using culture-dependent methods, only a very small proportion of microorganisms can be obtained. Far less than 1% of organisms in the environment can be cultivated by standard cultivation techniques (Pace, 1996). Recent studies have shown that culture-dependent techniques yielded mainly spore-forming bacteria suggesting that plating leads to an overestimation of the number of spore-forming bacteria (Laiz *et al.*, 2003). In addition, extensive cultivation strategies require far more sample material than could be obtained from objects of art. 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) Obligate 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 techniques are just not suitable or which have entered a non-culturable state.
c) Unknown species that have never been cultivated before due to the lack of suitable methods.

Using both, culture-independent as well as enrichment culture techniques, it is possible to characterise the microbial diversity and culture characteristics of the microbial flora present in environmental samples, allowing a more complete picture. The phylogenetic information obtained by using molecular techniques about the identity of the bacteria desirable to be cultivated can be a very useful tool for the specific design of appropriate culture media.

1.6.1. Molecular Methods for the detection of microorganisms

In recent years the use of novel molecular methods for culture-independent analysis has been developed and established for mural paintings and stones (Muyzer *et al.*, 1993; Schabereiter-Gurtner *et al.*, 2001). By using molecular techniques it is possible to detect a wider variety of microbial species than by using culture-dependent methods. This picture probably demonstrate the actual proportion of species present in art samples (Laiz *et al.*, 2003).

Most of the experiments which have been carried out in this field so far are based on ribosomal sequences, which are used as phlyogenetic markers (Woese, 1987). The

ribosomal sequences are present in all organisms and they contain variable and highly conserved regions which allow distinguishing 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 phylogenetic identification.

One of these molecular approaches is the PCR amplification of 16SrRNA genes followed by Denaturing Gradient Gel Electrophoresis (DGGE) and in parallel the construction of clone libraries containing PCR fragments of the ribosomal gene and screening of the clones with DGGE (Schabereiter-Gurtner *et al*., 2001). 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 cultivation (see Figure 2).

By using molecular techniques, 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). The identified sequences were belonging to genera *Actinobispora, Amycolata, Asiosporangium, Promicromonospora, Pseudonocardia, Rubrobacter, Streptomonospora, Saccharopolyspora, Sphaerobacter, Thermocrismum, Aquaspirillum, Chromohalobacter, Erythrobacter, Porphyrobacter, Salmonella*, members of the *Cytophagales* (Gurtner *et al*., 2000; Schabereiter-Gurtner *et al*. 2001b) as well as members of the halophilic group of Archaea (Piñar *et al*., 2001a, 2001b; Rölleke *et al*., 1998). To our knowledge, all these genera have not been previously reported on wall paintings by standard cultivation methods.

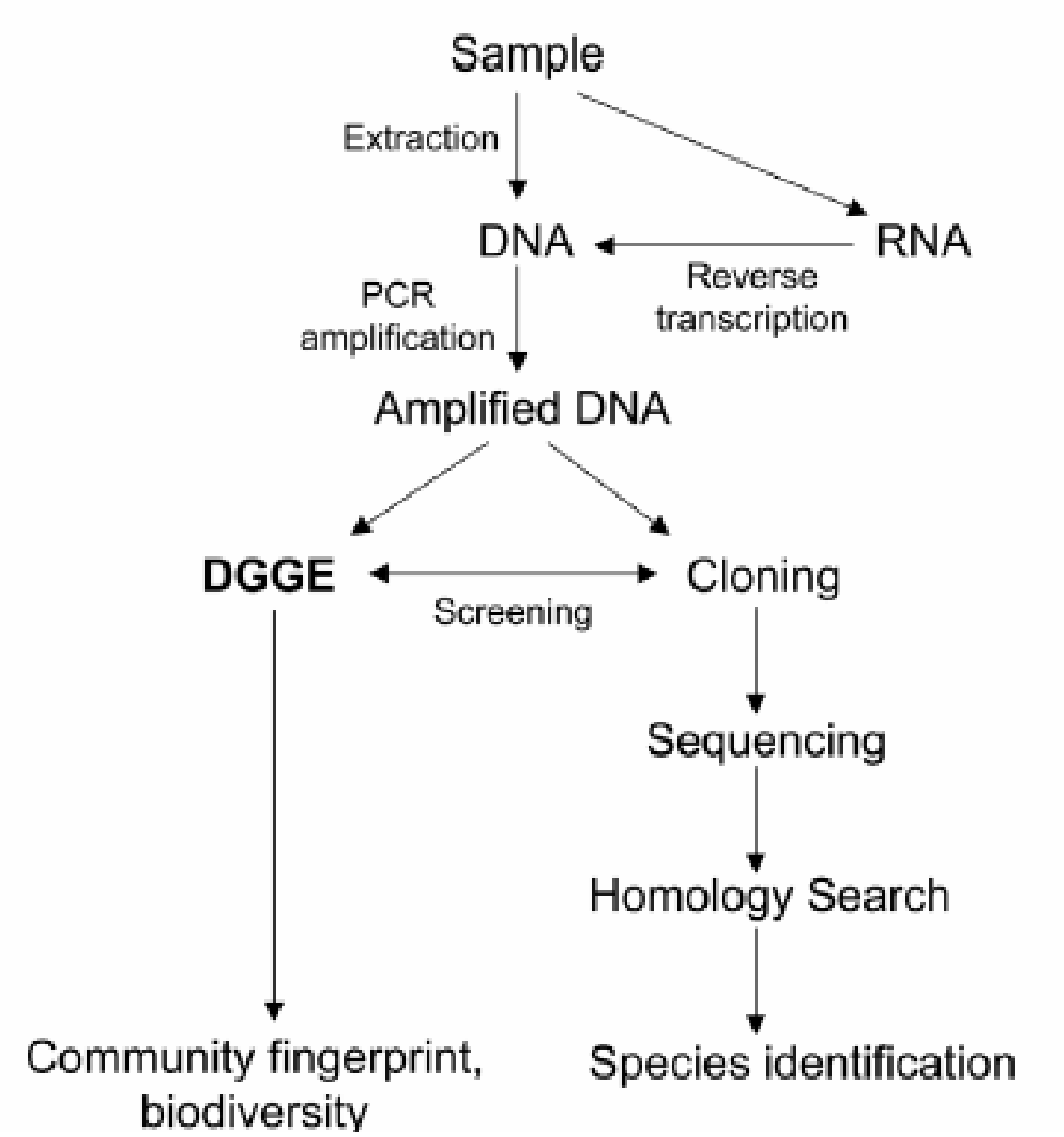


Figure 2 The molecular analysis of microbial diversity in cultural heritage studies. DGGE is required for generating community fingerprints or biodiversity analysis and can be essential for screening through the clones of a DNA library of PCR amplified 16S rDNA sequences (Gonzalez and Saiz-Jimenez, 2004).

1.6.2. Molecular markers

In the early 60ies molecular methods based on analysis of DNA were developed (Rosseló-Mora *et al*., 2001) Investigations were then based on the 5SrRNA molecule (ca. 120 Nukleotide) (Amann *et al*., 1995).

Molecular approaches based on ribosomal nucleotide sequences opened new perspectives for microbial taxonomy and ecology (Olsen *et al*., 1986; Amann *et al*., 1995).

Due to sequence similarities cultivable and non-cultivable microorganisms could be detected, discriminated and phlyogenetically characterized.

Nowadays the small subunit of the ribosoms, the 16S rRNA of prokaryotes and the 18SrRNA of eukaryotes are analysed. Recent studies refered to the Internally Transcribed Spacer (ITS) (Peterson, 1996) region to be used as molecular marker within fungi. The non-coding ITS region, consisting of ITS1, the 5.8S ribosomal DNA (rDNA), and ITS2, as target sequence for amplification, should produce a highly sensitive assay, because of its high copy number in fungal genome as part of tandemly repeated nuclear rDNA (Jasalavich *et al*., 2000).

All of the regions mentioned above showed to be good phylogenetic markers for several reasons:

1) They can be found in all organisms,
2) Due to the central function in gene expression the forces of selection are not very strong
3) There is no horizontal gene transfer
4) there are highly conserved regions
5) Variable regions for discrimination of different microbial taxa, even to species level (Ludwig *et al*., 1994).

Ribosomal sequences are not enough for definition of a species. Organisms which show in DNA-DNA hybridisation tests more than 70% reassociation of the whole genom belong to the same species. (Wayne *et al*., 1987). Empiric data showed that microorganisms with 16SrRNA similarities under 97% always have DNA-DNA reassociation rates under 60% and therefore belong to different species (Stakebrandt, 1994).

1.6.3. PCR amplification

One of the major problems when dealing with analysis of art objects is the usually small amount of sample material. The small amount of material together with the usual high concentration of impurities in samples make sometime difficult to perform reliable molecular analysis. A solution to overcome this problem is the so-called nested-PCR. Therefore, two different primer sets are used: the first set to increase the concentration of the template and the second one to amplify the desired DNA fragment in a second round of PCR. The binding sites for the second primer pair are within the PCR fragment of the first round (nested) (Gonzalez, 2003) (see Figure 3A). For further fingerprint DGGE analysis one primer of the second round of PCR has the GC clamp at its 5`end.

1.6.4. DGGE-Denaturing gradient gel electrophoresis (DGGE)

Denaturing Gradient Gel Electrophoresis (DGGE) is an appropriate tool for analysing biodiversity in environmental samples as well as for the screening of clone libraries. DGGE uses a chemical gradient formed with formamide and urea in a polyacrylamide gel. Through this chemical gradient, the double stranded DNA melts depending on their GC content (three hydrogen bonds between GC, thus stronger than AT pairing with two H-bonds) (see Figure 3 B). As each bacterial species has its characteristic ribosomal DNA sequence, theoretically, each renders a different electrophoretic position in the denaturing gel. The melting behaviour is stabilized using a GC-rich tail (about 40b) at the 5`side of one primer (Muyzer *et al.*, 1993). Temperature and Volt are stable during the run. Bands can be either cut out of the gel, sequenced and then compared with known sequences in different data bases (e.g.: EMBL) or, for more reliable information, clone libraries of selected samples are constructed and different clones are selected with DGGE for sequencing.

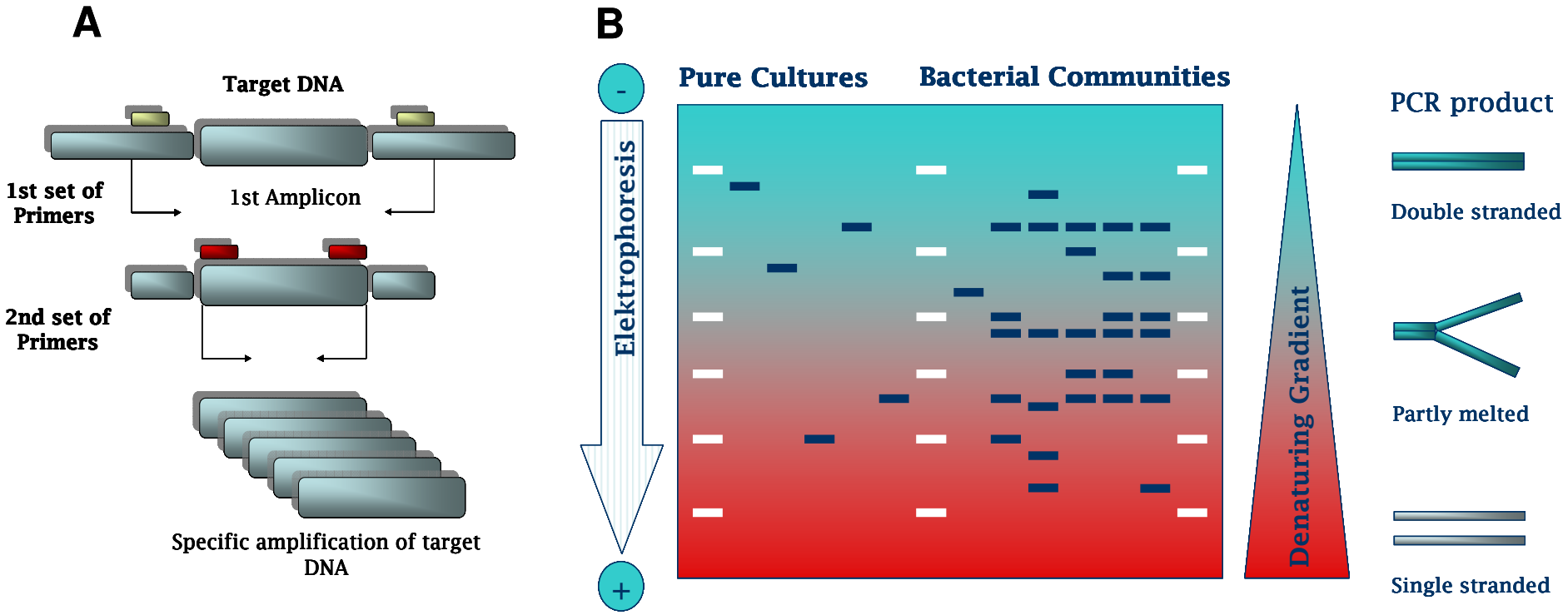


Figure 3 A Nested PCR: Two sets of primers are used to generate specific PCR products of the target DNA in high amounts. The second primer set binds within the first one.

B Denaturing Gradient Gel Electrophoresis schematic overview. DGGE uses a gradient of urea and formamid in a polyacrylamid gel. Through this gradient the double stranded PCR products melt depending on their GC content

1.6.5. Molecular methods- A critical view

There are some critical factors about this approach using DNA extraction, PCR and DGGE.

1. DNA extraction. The extraction of the DNA is a limiting factor as small cells, gram-positive bacteria or spores are hard to lyse. Furthermore PCR inhibitors as humic acids, salts, metals, pigments and other unknown substances are common in samples taken from objects of art. This problems could be partially overcome by a newly developed DNA extraction protocol for small sample material (Schabereitner-Gurtner *et al*., 2000)
2. Broad range nucleic acid amplification. Sometimes primer binding sites in normally conserved regions can be degenerated and may lead to selective amplification of defined organisms (Polz and Cavanaugh, 1998). Different numbers of operons lead to variations in copy numbers and may lead to an overestimation of the bacterial community (Ward *et al*., 1992; Farrelly *et al*., 1995). Copy numbers also depend on the G+C content of the amplified sequences. Sequences with lower G+C content are probably better denatured during PCR than those with higher G+C levels and are therefore preferentially amplified (Reysenbach *et al*., 1992). By using broad range 16S rDNA amplification and DGGE- fingerprinting theoretically only organisms more than 1% of the whole population are detectable (Muyzer *et al*., 1993; Murray *et al*., 1996)
3. Preferential cloning of defined PCR products can lead to further shifts (Rainey *et al*., 1994)
4. Sequence differences between operons or between strains can be up to 5% (interoperon) or 16% (interstrain). This leads to complex DGGE patterns and as a consequence to an overestimation of the complexity of the community. (Amann *et al*., 1995; Clayton *et al*., 1995; Ninet *et al*., 1996).
5. Overlapping of bands may occur when different sequences have the same melting behaviour. This fact leads to an underestimation of the complexity of the bacterial community (Schabereitner-Gurtner et al., 2000).
6. Chimeric molecules may be built during PCR amplification and mimic 16S rDNA of an organism which does not actually exist (Liesack *et al*.1991; Wang and Wang, 1997)

7. Identification is always as good as the existing database. Most sequences from environmental samples show low sequence homologies to known bacteria.
8. The extreme sensitivity of nucleic acid detection holds the danger of contamination. Therefore special treatments when handling solutions, PCR products and master mix are necessary (Schabereitner-Gurtner *et al*., 2000).

1.7. Characterisation of new bacterial species

In a study done by Piñar and coworkers (2001) several moderately halophilic gram positive, spore forming bacteria were isolated by conventional enrichment cultures from ancient wall paintings and building materials in the Castle of Herberstein. 16S rDNA sequencing gave high score similarities with *Halobacillus litoralis*. DNA-DNA reassociation experiments identified the isolates as a population of hitherto unknown new *Halobacillus* species. They were brought in connection with rosy discolouration and biodeterioration of the binding material of the paintings as they showed strong casein hydrolysis activity. In the frame of this Diploma work, members of the genus *Halobacillus* could anew be isolated from samples of another medieval building the chapel of St.Virgil (Vienna, Austria) which gives an evidence for frequency of this genus in cultural assets. No further classification was done so far. In the present study a detailed characterization of all isolated bacterial strains was performed.

Usually to describe a new genus or species the approach to bacterial taxonomy should be polyphasic. The first step is phenotypic grouping of strains by morphological, biochemical and any other characteristics of interest, followed by checking the molecular and genetic characteristics. Table 2 shows categories and characters applied in bacterial systematics (Busse, H.-J. *et al*., 1996).

For molecular characterizations the most common methods are 16S rDNA sequencing and Random amplified Polymorphic DNA- Polymerase Chain Reaction (RAPD-PCR). In this study four different methods were used for the molecular typing of the newly isolated strains: Denaturing Gradient Gel Electrophoresis (DGGE), Random amplified Polymorphic DNA (RAPD-PCR), 16S rDNA sequencing and Intergenic transcribed spacer homoduplex-heteroduplex PCR (ITS-HHP). Figure 5 shows an overview of the principles and differences between the four typing methods. For DGGE analysis and 16S rDNA sequencing see description above.

For RAPD PCR short (~10b) arbitrary primers that bind with low stringency are used. PCR products are resolved electrophoretically on agarose gels to yield DNA fingerprints which differ according to the degree of relatedness (Power, 1996).

Genera of *Bacillus*, *Brevibacillus*, *Geobacillus* and *Paenibacillus* show high variety in their Intergenic Transcribed Spacer (ITS) regions between the 16S and the 23SrDNA. Using specific primers targeting these regions leads to formation of Hetero- and Homoduplexes depending on whether there is a tRNA gene located in the operon or not (Daffonchio *et al*., 2003).

A

Categories and characters applied in bacterial systematics

Categories	Examples
Cultural	Colony morphology
	Color of colonies
	Fruiting bodies
	Mycelia
Morphological	Cell morphology
	Cell size
	Motility
	Flagellation type
	Reserve materials
	Gram stain
	Acid-fast stain
Physiological	Temperature range
	pH range
	Salinity tolerance
Biochemical	*Carbon source utilization*
	Oxidation of carbohydrates
	Fermentation of carbohydrates
	Enzyme profile
Inhibitory tests	Selective media
	Antibiotics
	Dyes
Serological	Agglutination
	Immundiffusion
Chemotaxonomic	*Fatty acids*
	Polar lipids
	Mycolic acids
	Lipopolysaccharide composition
	PAGE of lipopolysaccharide
	Cell wall diaminoacids
	Cell wall aminoacid composition
	Whole cell sugars
	Cell wall sugars
	Cellular pigments
	Quinone system
	Polyamine content
	Whole cell protein PAGE
Genotypic	*DNA base ratio (G+C-content)*
	Random amplified polymorphic DNA (RAPD)
	Restriction fragment length polymorphism (RFLP)
	Pulsed field gel electrophoresis (PFGE) of DNA fragments
	DNA probes
Phylogenetic	*DNA:DNA hybridization*
	DNA:rRNA hybridization
	16S rRNA sequence
	23S rRNA sequence
	Sequence of the β-subunit of ATP-synthase
	GroEL (Chaperonin) sequence

B

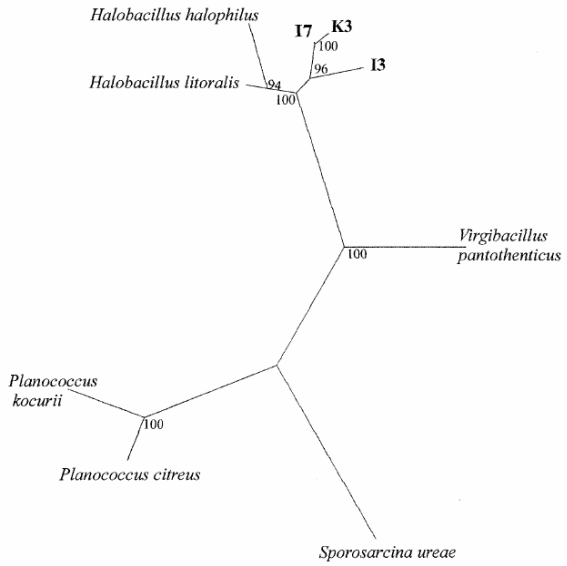


Figure 4 A Categories and characters applied in bacterial systematics (Busse et al., 1996) **B** Unrooted phylogenetic tree showing the relationship of isolates K3-1, I3, and I7 to related genera based on a comparison of 16S rDNA sequences. 16S rDNA sequences used in this study for phylogenetic analysis are as follows: isolate K3-1, AJ291752; isolate I3, AJ291753; isolate I7, AJ291754; Sporosarcina ureae DSM 2281T, AF202057; Halobacillus litoralis SL-4T, X94558; Halobacillus halophilus NCIMB 2269T, X62174; Virgibacillus pantothenticus IAM 11061T, D16275; Planococcus citreus NCIMB 1493T, X62172; and Planococcus kocurii NCIMB 629T, X62173. The tree was derived from a distance matrix based on a selection of 16S rRNA sequences of moderately halophilic and nonhalophilic gram-positive bacteria with low DNA G_C contents. Scale bar, 1% estimated sequence divergence (Piñar et al., 2001).

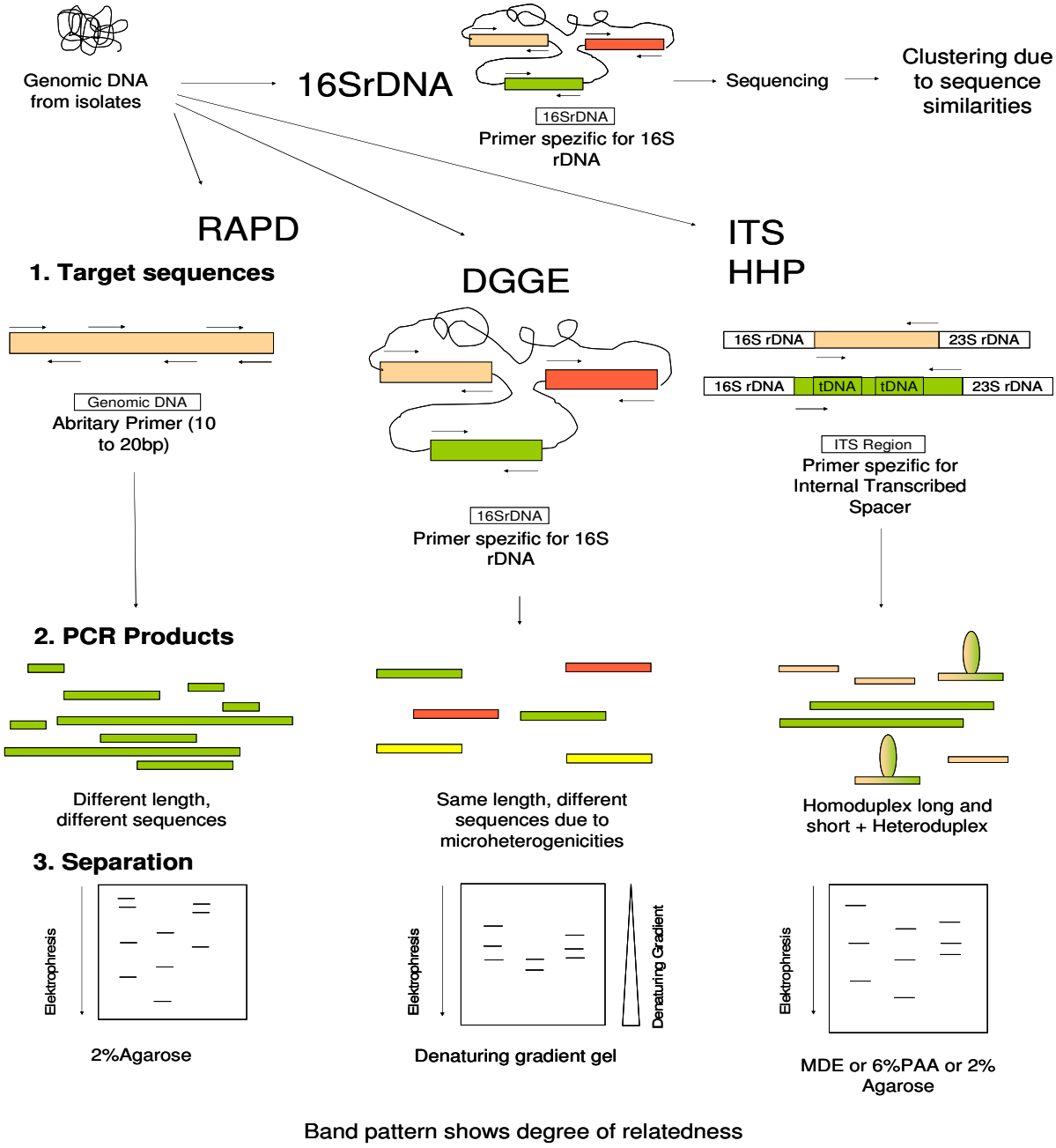


Figure 5 Schematic overview showing four different molecular methods for typing of bacterial species.

1.8. Aim of the work

Since microbial activity have been shown to play an undeniable role in damaging mural paintings and stone-works, the aim of this Diploma work was to determine which microorganisms are colonizing such substrates (a mural painting and a stone-work) and to guest were they originate from and their potential biodeteriorative effects. Locations selected for our investigation were the mural paintings placed in the "Berglzimmer" at the Palace of Schönbrunn (Vienna) built in the 18th century and the stone work of the medieval Chapel of St.Virgil (Vienna). Both locations are under controlled climate conditions.

In this work we have identified the microbial communities present in art samples with culture-independent methods. By using this information, we have tried to make a statement on the biodeteriorative potential of the detected species, where these microorgansims may originate from, and if they may emit harmful substances. Therefore, all samples taken were analysed for the presence of fungi, bacteria and archaea by using direct DNA extraction followed by PCR amplification using specific primers directed against each of these three domains. Visualisation of the microbial communities was performed by the fingerprinting technique Denaturing Gradient Gel Electrophoresis (DGGE). In parallel, clone libraries were constructed in order to obtain reliable phylogenetic information of independent 16S rDNA sequences of archaea and bacteria, as well as the ITS1 (Internally transcribed spacer) region of fungi.

The second part of this study included the characterization of new bacterial species. Several bacterial strains isolated from salt efflorescences in the Chapel of St.Virgil, Vienna, showing the highest phylogenetic similarity with members of the genus *Halobacillus,* were compared with strains previously isolated from the castle of Herberstein, which were also belonging to the *Halobacillus* genus. A polyphasic approach using phenotypical and genotypical methods to identify the new species has been carried out with all strains.

2. Material and Methods

2.1. Sampling

2.1.1. Palace of Schönbrunn

The present report describes the investigations on samples taken from partially damaged mural paintings of the “Berglzimmer” situated in the Palace of Schönbrunn (Vienna, Austria). These rooms are situated in the lower part of the castle of Schönbrunn facing the garden. In the years 1769 to 1778 these rooms were decorated with extensive paintings showing exotic scenery. Restoration was done in 1891 but as time going on paintings showed cumulative decay as black biofilms, cracks and discoloration spread. Figure 6 shows pictures taken from damaged places.

In January 2004 several samples were taken from the wall paintings by scraping off surface material to a depth of 1 to 3mm with a sterile scalpel. Samples KM1-1, KM1-2, KM2-1, KM2-2 and KM3-1 were taken from the “Kindermuseum”. Samples S1-1, S1-2, S2, S3 and S4 were taken in the eastern part of the building. Table 2 and Figure 6 show the location of sampling.

Figure 6 Pictures showing the location of sampling at the Berglzimmer in the Palace of Schönbrunn. See table two for details

Sample	Origin
KM 1-1	Black biofilm on the wall **(A)**
KM 1-2	Surface of the painting showing a flower **(G)**
KM 2-1	Surface of the painting showing a fruit **(B)**
KM 2-2	Surface of the wall, behind the fireplace over the vent, showing black biofilm **(C)**
KM3-1	Black colonies colonizing the wall behind the fireplace **(D)**
S 1-1	Surface of the painting showing green leaves, behind the fireplace **(I)**
S 1-2	Surface of the wall showing white-discoloured spots **(F)**
S 2-1	Surface of the painting showing green leaves, behind the fireplace **(E)**
S 3	from a detached area of the painting behind the fireplace **(J)**
S 4	surface of the painting showing a fruit **(H)**

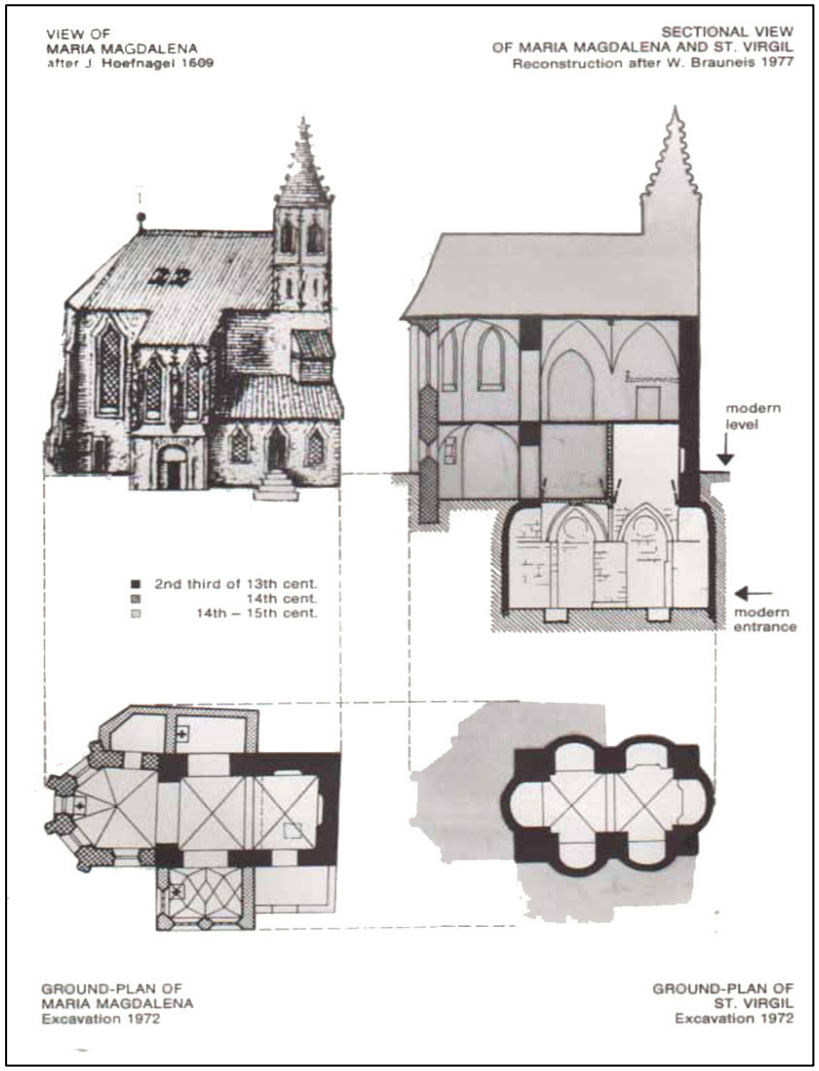
Table 2 Origin of samples taken from Berglzimmer

2.1.2. Chapel of St.Virgil

The St. Virgil Chapel, (Vienna, Austria) dates back from the beginning of the 14 century. It is located under the ruins of St. Mary Magdaleine Chapel. The St. Virgil Chapel was originally created as a tomb. After the Chapel of St. Mary Magdaleine was destroyed in 1781 by fire and demolished, the underground room was abandoned. The chapel was rediscovered in an excavation in 1972 (Figure 7A). The rectangular room is 10.5 m long and 6 m wide. The mortar and rubble walls are 1.5 m thick, containing 6 recesses with pointed arches, of which one was removed from where the present entrance is situated. They are decorated with large, red crosses painted onto white plaster (Figure 7B). One sample was taken from this mural painting (VK5). The whole chapel is covered by salt efflorescences visible by naked eye. A sample (VK3) was taken directly from a salt efflorescence (Figure 7C). An addition sample was taken from detached wall material collected on paper placed on the floor (VK1).

In the frame of a project started by Dr. Busse and Prof. Lubitz, from the University of Vienna, Austria, an artificial wall painting (Figure 8) was created in the year 1998 by members of the masterschool for restoration and conservation of the Academy of Building Arts in Vienna, Austria (Klose, 2001). The artificial wall painting was done in fresco technique using five different pigments and 80 different investigation areas. Defined areas were inoculated with a combination of different bacterial strains (cocktail α, β, γ) (Table 4) formally isolated from damaged medieval wall paintings. This artificial mural painting has been exposed in the Chapel of St.Virgil. After two months of exposure, three different biocides (Metatin101, Metatin 58-10 and Metatin 58-10/101) used commonly in restoration, were applied by brushing. (for details see the Diploma Thesis of Klose, 2001). In the present work, three samples (VK6, VK7, VK9) were taken from different areas of this artificial wall painting (see Table 3).

A



B

C

Figure 7 A Picture showing the location and ground plan of St.Virgil; **B** Red wheeled cross painted onto white plaster; **C** Salt efflorescence on the stone work of the chapel

Table 3 Origin of samples taken from artificial wall painting

Quadrant	Sample	Treatment
Iα	VK6 Sample taken with scalpel over a green colored area with red spots	Cocktail α, Biocide Metatin 101
IIIØ	Vk7 a piece of the whole wall painting: painting + support material was taken	No cocktail , Biocide Metatin 58-10
IIIβ	VK9 Sample taken with scalpel from a hole produced in a former sampling procedure. Area with white pigmentation.	Cocktail β, Biocide Metatin 58-10

Table 4 Phylogenetic affiliation of inoculated strains

Cocktail	Strain	Length [bp]	Highest 16SrDNA similarity	Similarity [%]
Cocktail α	D-1, 1a	1212	*Agrococcus citreus*	100
	D7	1430	*Micrococcus luteus*	98.7
	D3	680	*Bacillus licheniformis*	93.6
	D-4, 2	1239	*Clavibacter michiganensis*	92.7
	D-2, 4	1336	*Moraxella osloensis*	97.5
Cocktail β	5-2	726	*Bacillus pumilis*	96.7
	4-0	941	*Micrococcus luteus*	96.0
	1a-1b	950	*Agromyces ramosum*	97.9
	3a-1	517	*Cellulomonas*	95.0-95.7

Figure 8 Artificial wall painting in the Chapel of St.Virgil

2.2. DNA extraction and purification

2.2.1. Fast DNA Spin kit protocol

DNA was extracted directly from wall painting and stone-work samples by using the Fast DNA SPIN kit for soil (Bio 101). The protocol of the manufacturer was modified as follows. Powder obtained from wall painting samples were placed in the MULTIMIX2 tissue tube. 978 µl sodium phosphate buffer and 122 µl of the MT buffer (Bio 101) were added. The mixture was then bead beaten two times for 1 min with one intervening minute on ice and centrifuged at 14000xg for 2min. The PPS reagent and the binding matrix suspension (Bio 101) were applied to the supernatant following the instructions of the manufacturer. The resulting suspension was transferred to a spin filter and centrifuged at 14000 x g for 1min. DNA was washed twice with 500µl of the SEWS-M solution (Bio 101) and eluted from the binding matrix with 160 µl DES (Dnase/Pyrogene free water).

The extracted DNA was further purified with the QIAamp Viral RNA mini kit (Quiagen) and finally eluted from the silica column with 100 µl ddH_2O.

2.2.2. DNA Extraction Protocol for objects of art (Schabereiter-Gurtner *et al.*, 2001a)

DNA Extraction of microorgansims from samples of art was used as follow:

Solutions:

Buffer 1:
150 mM Na_2EDTA (15 ml of 0.5M stock/50ml)
225 mM NaCl (0.66 g/50ml)
pH 8.5

Buffer 2:
100mM Na_2 EDTA (10ml of 0.5M stock/50ml)
400mM Tris-HCl (2.42g/50ml)
400mM Na_2phosphat buffer [pH 8.0] (20ml of 1 M stock/50ml)
5.55 M NaCl (16.21 g/50ml)
4% CTAB [hexadecylmethylammonium bromide] (2g/50ml)
pH 8.0

Protocol:

1. Add approximately 100 mg of sample to 100 µl buffer 1
2. Add 45 µl lysozyme (stock: 50mg/ml) to get a total vol. of 150 µl. Mix with vortex.
3. Incubate at 37°C 30min with agitation.
4. Add 3 µl of 25% SDS and 3µl proteinase K (stock: 20mg/ml).
5. Incubate at 37 1h with agitation.

6. Add 50 µl of buffer 2 and 9 µl of 25 %SDS (as buffer 2 is of a gelatinous consistency, it must be heated up to 65°C to be pippeted). (Alternatively, use 100µl of CTAB/NaCl).
7. Incubate at 65°C 1h with agitation.
8. 3 cycles of freezing (-80°C) and thawing (65°C), vortex after each cycle (to release DNA from the cells)
9. Centrifuge at 14000xg 5min.
10. Transfer supernatant to a clean Eppendorf tube.
11. Add 200 µl chloroform-isoamyl alcohol (24:1, vol.:vo.l) to remove the CTAB/protein/polysaccharide complex. A white interface forms.
12. Centrifuge at 14000xg 5min
13. Transfer the supernatant to a clean Eppendorf tube.
14. The DNA in the supernatant is purified with the QIAamp viral RNA mini kit.

2.2.3. Purification of DNA using the QIAamp viral RNA mini kit

1. Pippet sample into 1.5 ml microfuge tube (approx. 140µl)
2. Add 560 µl buffer AVL containing carrier RNA to the sample. Mix with vortex.
3. Incubate at RT (15-35°C) 10min.
4. Add 560 µl of ethanol (96-100%) to the sample. Mix with vortex.
5. Place a QIAamp spin column in a 2ml collection tube.
6. Apply 630 µl of the mixture from step 4 to the QIAamp spin column.
7. Centrifuge at 6000xg (8000rpm) 1min.
8. Place QIAamp spin column in a new 2ml collection tube.
9. Repeat step 6+7
10. Wash with 500µl of buffer AW1. Centrifuge at 6000xg (8000rpm) 1min.
11. Place QIAamp spin column in a new 2ml collection tube.
12. Wash again with 500µl of buffer AW2. Centrifuge at 6000xg (8000 rpm) 1min.
13. Centrifuge again at full speed for a further 2min.
14. Place QIAamp spin column in a clean 1.5ml microfuge tube.
15. Elute the DNA with 100µl of ultra-pure water preheated to 80°C.
16. Centrifuge at 6000xg (8000 rpm) for 1min.
17. Repeat steps 15+16 three times to have three different eluents.

2.3. PCR Amplification

2.3.1. PCR amplification of eubacterial 16S rDNA fragments

For all reactions 2x PCR Master Mix from Promega [50 units/ml of TaqDNA Polymerase supplied in a proprietary reaction buffer (pH 8.5), 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP, 3mM MgCl2] was diluted to 1x and 12.5 pmol of each primer were added. Primer sequences used in this study are shown in Table 2.

PCR amplification for genetic fingerprinting by DGGE was carried out with primer 341f specific for eubacterial 16S rDNA and 907r a universal primer for 16SrDNA. Afterwards, this step was followed by a semi-nested PCR performed with primers 341fGC and 518r. The forward primer possessed a 40-base GC clamp at its 5`end which stabilizes the melting behaviour of DNA fragments in DGGE analysis (Muyzer

et al., 1993). For the first round of PCR 25µl volume and 2µl template were used. For nested PCR 100µl volumes separated on two tubes to 50µl and 3.5µl template to each tube were applied.

PCR were performed in a Robocycler with the following thermocycling program:5 min denaturation at 95°C, followed by 30 cycles each consisting of 1 min denaturation at 95°C, 1 min annealing at 55°C and 1 min extention at 72°C. Five minutes at 72°C were used as final extension step.

Ten µl of each PCR product were run on a 2% agarose gel for ~ 30 min at 200V, stained with ethidium bromide and visualized by a UVP documentation system.

2.3.2. PCR amplification of archaeal 16SrDNA

PCR was carried out as described above using the specific archaeal primers ARC344 (forward) (Raskin *et al*., 1994) and ARC915 (reverse) (Raskin *et al*., 1994) using the following conditions: 5 min denaturation at 95°C, followed by 40 cycles consisting of 1 min denaturation at 95°C, 1 min primer annealing at 60°C and 1 min extension at 72°C, with a final extension step af 72°C for 5 min (Piñar *et al*., 2001).

The 2nd round was done with universal primer 518r carrying the GC clamp (Muyzer *et al*., 1993) and the archaea-specific primer ARC344. The same thermocycling program was used as described above.

2.3.3. PCR amplification of ITS1 Region (Fungi)

PCR was carried out as described above using primers ITS1 and ITS2 specific for the internal transcribed spacer region 1 located between the 18S and 5.8SrDNA (White *et al*., 1990). For 2nd round of PCR the ITS1 primer had a GC clamp on its 5`end. The thermocycling program for the first round was as followed: 5min at 95°C, followed by 35 cycles 1min at 95°C, 1min at 62°C, 1min at 72°C and a final extension step 5 min at 72°C. For PCR with the GC primer annealing temperature was set at 60°C (Michaelsen *et al*., 2005).

2.4. (DGGE)- Denaturing Gradient Gel Electrophoresis

For genetic fingerprinting 100 µl of semi-nested PCR products were precipitated in 1 ml 96% ethanol (–20^{o}C overnight), resuspended in 15µl ddH_2O water (Sigma) and separated by DGGE analysis.

Gels for the screening of bacterial communities were run in 0.5xTAE buffer [20mM Tris, 10mM acetate, 0.5mM Na_2EDTA; pH 7.8] for 3.5 hours at 200V and 60°C in a D GENE System (BioRad, Munich, Germany) (Muyzer, *et al*; 1993). A denaturing

gradient from 30 to 55% of urea and formamide in an 8% (w/v) polyacrylamide gel (BioRad, Munich, Germany) was used.

For the screening of archaeal communities chemical gradients ranged in between 25 to 55% of urea and formamide. Gels were run during 3 hours at 200V and 60°C (Piñar *et al*., 2001).

For the screening of fungal communities gels contained 30 to 50% of chemical denaturants and were run for 14 hours at 100V and 60°C (Michaelsen *et al.,* 2005).

After completion of electrophoresis, gels were stained in an ethidium bromide solution and documented with an UVP documentation system.

DGGE solutions

Two stock solutions of acrylamide containing 0 and 80% of denaturants were used for pouring the gels (Table 3). By mixing the two solutions in proper proportions all desired concentrations of high-acryl and low-acryl can be made.

	1.5 ml	TAE (50x)
0 % urea and formamide	30 ml	40% Acrylamide (Biorad)
	120 ml	H20
	Σ=150 ml	
	1,5 ml	TAE (50x)
	30 ml	40 % Acrylamide
80% urea and formamide	30 ml	H_2O
	48 ml	Formamide deionised (Roth)
	50,4 g	urea
	Σ=150 ml	

Table 5 Stock solutions for DGGE

Example:

Separating zone:

For a gradient 30-55%, 10 ml of the 30% solution are mixed with 10ml of 55% solution. The polymerisation is started with 50µl APS (10%) and 7µl TEMED (in 10 ml).

Stacking zone:

8ml of 0% solution, + 30µl APS (10%) + 4µl TEMED

2.5. Elution of Bands from Polyacrylamide Gels

1. Complete electrophoresis, staining of the gel and gel documentation.
2. Excise intensely stained bands out of the gel (put in one eppendorf tube).
3. Incubate o/n at 37℃ in 500 µl elution buffer (0.3 M NaCl; 3mM EDTA; 30mM Tris (pH7.6)) to elute the DNA.
4. Centrifuge the tubes and transfer supernatant to a clean tube.
5. Supernatant is precipitated in 1ml ethanol.
6. Centrifuge, remove ethanol and dry DNA.
7. DNA is resuspended in 15-20 µl ultra pure water.
8. DNA is used as template for a new PCR with primers for DGGE (341f/518r)
9. Run samples in DGGE to prove that is only one band!
10. DNA is used as template for a new PCR for sequencing.

2.6. Construction of clone libraries

For the construction of clone libraries for each sample 2 x 3.5µl DNA templates were amplified in 2 x 50µl volumes using the primers 341f and 907r for Eubacteria, ITS1 and ITS2 for fungi and ARC344 (forward) and ARC915 (reverse) for Archaea. The PCR products were pooled and purified using the QIAquick PCR Purification Kit Protocol (qiagen, Hilden, Germany) and eluated with 30µl ddH_2O water (Sigma).

5.5µl of purified PCR product were ligated into the pGEM –T easy Vector system (Promega, Vienna, Austria) following the instructions of the manufacturer. The ligation products were transformed into *E.coli* XL Blue^{Tc}, which allows the identification of recombinants (white colonies) on an indicator LB medium containing ampicilline (100µg/ml), tetracycline (10µg/ml), X-Gal (5 bromr-4-cloro-3-indolyl-ß-D-galactorpyranoside; 0.1 mM) and IPTG (isopropyl-ß-D-thiogalactopyranoside: 0.2 mM) (Sambrook et al., 1989).

2.6.1. Ligation protocol

1. 100µl PCR product, visualise 7-8µl in an agarose gel electrophoresis
2. PCR product is purified using the PCR purification kit from QIAGENE and resuspended in 20-25 µl ultrapure water.
3. Cloning using the kit from Promega (PGEM system):
   7.5 µl Buffer
   1 µl vector
   1 µl ligase
   5.5 µl PCR product previously purified
   15 µl total volume-incubate o/n at 4°C

2.6.2. Transformation protocol

1. Transformation of competent cells of *E.coli* XLBlue^{Tc}
2. Transfer 10 µl of the ligation to 100 µl competent cells in ice for 1h
3. Heat-shock: 90 seconds at 42 °C, and immediately 5 additional min. on ice.
4. Add 500 µl LB media on the competent cells and incubate at 37 °C 1h.

5. Centrifugate at low speed (5000 rpm)
6. Discard the supernatant, reducing until 150 µl (additionally until 200 µl and share in two plates each with 100 µl).
7. Plate 150 µl (or 100 µl in two plates) on LB plates with Ap, Tc, IPTG, X-gal and incubate o/n at 37 °C.
8. Next day visualisation of the colonies.
9. Transfer each white colony to a clean Eppendorf tube containing 40µl TE buffer
10. 3 cycles of freeze-thawing to lyse the cells, and use directly 3 µl as template for PCR in a 25 µl reaction.
11. PCR is performed using primers T7 and SP6 which are present in the vector (annealing temperatute 46 °C for 35 cycles), visualise PCR products in 2% agarose gels.
12. Nested PCR using primers for DGGE analysis (341fGC/518r)
13. Screening of clones in DGGE.

Plates for transformation	End concent.	Stock concent.
LB media		
Ap	100 µg/ml	100 mg/ml
Tc	10 µg/m1	10 mg/ml
X-Gal	0.1mM	2% (dilute 1000 folder)
IPTG	0.2mM	1M (80 µl in 400 ml)

2.7. Screening of clones by DGGE and PCR

About 50 clones from each clone library are harvested and resuspended in 50µl TE buffer [10mM TrisHCl, 1mM EDTA; pH 8.0] followed by three steps of freeze and thawing.

Screening of the clone libraries by PCR and DGGE was performed as described by Schabereiter-Gurtner and coworkers (2001) as follows: To amplify the inserts the vector specific primers SP6 and T7 were utilized. For PCR the following conditions were used: 5 min at 95°C denaturation followed by 35 cycles each consisting of 1 min at 95°C, 40 sec at 46°C and 1 min at 72°C. Three minutes were used as final extension step.

A nested PCR for DGGE analysis was carried out with the primer pair 341fGC and 518r. PCR was performed as described above in 25µl volumes. Ten µl of each PCR product were run on a 2% agarose gel for ~ 30 min at 200V, stained with ethidium bromide and visualized via an UVP documentation system (BioRad quantity one).

The rest was used for DGGE analysis under the same conditions as described above. Clones showing different motility behaviour in DGGE analysis were selected for sequencing.

2.8. Sequencing and identification

100µl of PCR products amplified with primers SP6 and T7 were purified with a QIAquick PCR Purification Kit (qiagen, Hilden, Germany). Sequencing was done by VBC (Vienna Bio Centre, Austria) and the obtained sequences were compared with know sequences in the EMBL database using the search tool FASTA (Pearson, 1994).

2.9. Characterisation of new Halobacillus species- Polyphasic Approach

Usually to describe a new genus or species the approach to bacterial taxonomy should be polyphasic. The first step is phenotypic grouping of strains by morphological, biochemical and any other characteristics of interest, followed by checking the molecular and genetic characteristics.

For categories of characters used in classification see Table 6.

Category	Examples
Cultural	Colonial morphology, pigmentation
Morphological	Cell shape, staining reactions, motility
Physiological	Growth temperatures, anaerobic growth
Biochemical	Acid from carbohydrates, nitrate reduction
Nutritional	Organic acids as sole carbon and energy sources, vitamin requirements
Chemotaxonomic	Amino acids in interpeptide brides of cell wall, types of lipids in membranes
Inhibitory tests	Sensitivities to antibiotics, dye tolerance
Serological	Agglutination by antisera to reference strains
Genomic	Mol%GC in DNA, DNA-DNA reassociation

Table 6 Categories of characters used in classification

2.9.1. Enrichment and isolation.

Conventional enrichment cultures were performed in 300 ml Erlenmeyer flasks containing 30 ml M2 medium (Tomlinson and Hochstein 1976) as well as Maintenance Medium (MM) (Spring et al. 1996). To avoid fungal growth, media were supplemented with 50 µg ml^{-1} cycloheximide (Sigma). All enrichments were incubated aerobically at room temperature (22 ^{o}C ± 3 ^{o}C) and at 37 ^{o}C in a water-bath by shaking using magnetic stirring bars. During a total period of 1 to 3 weeks,

once a week aliquots of 100 µl enrichments were plated onto four different solid media: M2 medium (20 % wt/vol NaCl), M2A medium (20 % wt/vol NaCl) (Denner et al. 1994), Halococci medium (25 % wt/vol NaCl) and LB medium (Atlas 1995). All media were incubated aerobically at room temperature and at 37 °C.

2.9.2. Bacterial strains.

Strains K3-1, I3, I7, I3R and I3A were isolated from the chapel of castle of Herberstein, Styria (Austria). Five additional strains, named S3, S4, S20, S21 and S22, were isolated from the stonework of the chapel of St.Virgil ,Vienna (Austria). All isolates were stored at −70 °C in MM broth containing 20 % (wt/vol) glycerol as cryoprotectant. Bacterial inocula for all experiments were taken from cryopreserves, streaked onto MM plates and cultivated at 37 °C for 48 h. Reference type strains of *Halobacillus* used in this study were: *H. karajensis* DSM 14948^T, *H. halophilus* DSM 2266^T, *H. litoralis* DSM 10405^T, *H. trueperi* DSM 10404^T, *H. locisalis* DSM 16468^T and *H. salinus* JCM 11546^T (DSM-German Collection of Microorgansims; JCM-Japanese Collection of Microorganisms).

2.9.3. Growth requirements

Bacterial strains were grown and maintained in a complex medium (MM) containing (gl^{-1}): NaCl, 100; $MgSO_4.7H_2O$, 5; casein peptone, 5; yeast extract, 3 (as recommended by Spring *et al.*; 1996).

Growth at different NaCl concentrations (0, 1, 5, 10, 15, 20, 25, 30% w/v) was determined on plates containing M_2A medium (l^{-1} : yeast extract, 1g; Tris, 12g; NH_4Cl, 1g; SL-6, 1ml; Sol-1, 25ml. SL-6 l-1: $BrCl_2x6H_2O$, 0.042 g; H_3BO_3, 0.027 g; $MnCl_2x4H_2O$, 0.01 g; $Na_2Si_3O_7xH_2O$, 0.005 g; NaF, 0.003 g; NH_4NO_3, 0.002 g; NH_4Cl, 0.5 g. Sol-1 l^{-1}: $MgCL_2x7H_2O$, 400 g; KCl, 40 g; $CaCl_2x2H_2O$, 4 g) (Denner *et al.*, 1994). All media were incubated aerobically at 37°C.

2.9.4. Morphological characteristics

Determination of colony shape, margin, elevation, surface appearance, opacity, texture, pigmentation and colony size was performed by microscopical observation in on a Leitz Diaplan (Germany) phase contrast microscope.

2.9.5. Phenotypic characterization.

2.9.5.1. Motility

To identify unknown bacteria it is often necessary to determine whether or not the microorganism is motile. There are several ways to performe this determination. The simplest way is to take a loop full of a pure culture of the desired microorganism and examine it with a phase contrast microscope (wet amount). Other possibilities are the hanging drop method or culture methods (e.g.0,3% Agar; SIM Agar).

2.9.5.2. Gram staining

Gram staining is the most important staining procedure in microbiology. It is used to differentiate between gram-positive organisms and gram-negative organisms. Hence, it is a differential stain.

Gram-positive cells take up the crystal violet, which is then fixed in the cell with the iodine mordant. This forms a crystal-violet iodine complex that remains in the cell even after decolorizing. It is thought that this happens because the cell walls of gram-positive organisms include a thick layer of protein-sugar complexes called peptidoglycans. This layer makes up 60-90% of the gram-positive cell wall. Decolorizing the cell causes this thick cell wall to dehydrate and shrink which closes the pores in the cell wall and prevents the stain from exiting the cell. At the end of the gram staining procedure, gram-positive cells will be stained a purplish-blue color.

Gram-negative cells also take up crystal violet, and the iodine forms a crystal violet-iodine complex in the cells as it did in the gram-positive cells. However, the cell walls of gram-negative organisms do not retain this complex when decolorized. Peptidoglycans are present in the cell walls of gram-negative organisms, but they only comprise 10-20% of the cell wall. Gram-negative cells also have an outer layer which gram positive organisms do not have; this layer is made up of lipids, polysaccharides, and proteins. Exposing gram negative cells to the decolorizer dissolves the lipids in the cell walls, which allows the crystal violet-iodine complex to leach out of the cells.

<u>Test procedure:</u>

1. Perform a bacterial smear.
2. Saturate the smear with crystal violet for 1 minute.
3. Rinse the slide gently with iodine.
4. Saturate the smear with iodine for 1 minute.

5. Rinse the slide gently with water.
6. Decolorize with alcohol for 3-5 seconds **ONLY**; if you leave the decolorizer on too long, it will bleach the crystal violet out of a gram positive cell!
7. Rinse the slide gently with water.
8. Counterstain with safranin for 1 minute.
9. Rinse the slide gently with water.
10. Carefully blot the slide dry with bibulous paper.
11. Observe the slide under the microscope.

    Gram positive bacteria will stain purple.

    Gram negative bacteria will stain red/pink.

2.9.5.3. Endospore staining

The endospore stain is a differential stain used to visualize bacterial endospores. Endospores are formed by a few genera of bacteria, such as *Bacillus*. By forming spores, bacteria can survive in hostile conditions. Spores are resistant to heat, desiccation, chemicals and radiation. Bacteria can form endospores in approximately 6 to 8 hours after being exposed to adverse conditions. The normally growing cell that forms the endospore is called a vegetative cell. Spores are metabolically inactive and dehydrated. They can remain viable for thousands of years. When spores are exposed to favorable conditions, they can germinate into a vegetative cell within 90 minutes.

Endospores can form within different areas of the vegetative cell. They can be central, subterminal, or terminal. Central endospores are located within the middle of the vegetative cell. Terminal endospores are located at the end of the vegetative cell. Subterminal endospores are located between the middle and the end of the cell. Endospores can also be larger or smaller in diameter than the vegetative cell. Those that are larger in diameter will produce an area of “swelling” in the vegetative cell. These endospore characteristics are consistent within the spore-forming species and can be used to identify the organism.

Because of their tough protein coats made of keratin, spores are highly resistant to normal staining procedures. The primary stain in the endospore stain procedure, malachite green, is driven into the cells with heat. Since malachite green is water-soluble and does not adhere well to the cell, and since the vegetative cells have been disrupted by heat, the malachite green rinses easily from the vegetative cells, allowing them to readily take up the counterstain (Benson, 1969).

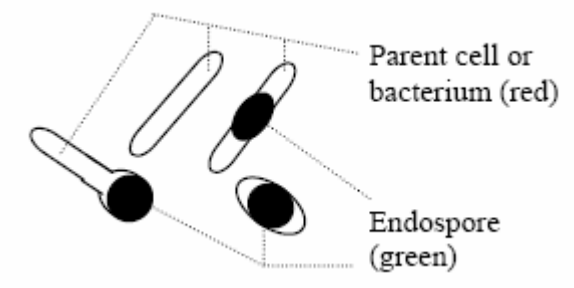


Figure 9 Position of endospores in parental cells

Test procedure:

1. Perform a bacterial smear of *your isolates*
2. Cover smear with 5% malachite green.
3. Steam on heater for 10 minutes. Add stain if stain boils off. Then let slide cool.
4. Rinse the slide gently with water for 30 seconds
5. Counterstain with safranin for about 20 seconds.
6. Rinse the slide gently with water.
7. Carefully blot the slide dry with bibulous paper.
8. Observe the slide under the microscope under oil immersion.

Endospores will stain green. Parent cells will stain red.

2.9.6. Biochemical tests

For morphological and physiological studies 10 % (w/v) NaCl was added to each media.

2.9.6.1. SIM AGAR

Sulphur reduction, motility and indole production was checked on SIM agar (Oxoid). Motility was also tested on plates containing MM and 0.3% agar. SIM medium is a differential medium that combines three test for the determination of three different parameters, which are represented by the three letters in the name: Sulphur Reduction, Indole Production and Motility. The sulphur reduction test is useful in differentiating enteric organisms. The indole test is a component of the IMViC series of tests, which is used for differentiating the *Enterobacteriaceae*. The motility test is useful for testing a wide variety of organisms. As a whole, the SIM test is primarily useful for differentiating *Salmonella* and *Shigella*. SIM medium contains nutrients, iron, and sodium thiosulfate. One of the nutrients is peptone, which contains amino

acids, including tryptophan. If an organism can reduce sulphur to hydrogen sulphide, the hydrogen sulphide will combine with the iron to form ferric sulphide, which renders a black precipitate. If there is any blackening of the medium, it indicates the reduction of sulphur producing a positive result. The sulphur and motility test results should be determined before performing the indole test. Some bacteria possess the ability to produce the enzyme tryptophanase, which hydrolyzes tryptophan. The end products of this hydrolyzation are indole, pyruvic acid, and ammonia, by way of deamination. The Kovac's reagent added to the SIM medium to test for indole contains hydrochloric acid, p-dimethylaminobenzaldehyde (DMABA), and n-amyl alcohol. DMABA reacts with indole to produce a red quinoidal compound. If the reagent turns red, the indole test is positive.

2.9.6.2. Oxidase production

Oxidase-activity was tested using commercialised test strips (Bactident oxidase; Merck).

The oxidase test identifies organisms that produce the enzyme cytochrome-oxidase. Cytochrome-oxidase participates in the electron transport chain by transferring electrons from a donor molecule to oxygen. The oxidase reagent contains a chromogenic reducing agent, which is a compound that changes colour when it becomes oxidized. If the test organism produces cytochrome oxidase, the oxidase reagent will turn blue or purple within 15 seconds.

Test procedure: Pick a single colony from each isolate and put it on an oxidase test strip (Merck).

2.9.6.3. Catalase production

The catalase production was determined as described by Smibert and Krieg (1994).

The catalase test identifies organisms which produce the catalase enzyme; this enzyme converts hydrogen peroxide to water and oxygen gas. Catalases can utilise hydrogen peroxide (H_2O_2) both as an electron acceptor and an electron donor (`catalatic' activity) yielding molecular oxygen (O_2) and water in the disproportionation reaction:

$H_2O_2 + H_2O_2 \rightarrow 2\ H_2O + O_2$

This enzyme helps protect bacterial cells against hydrogen peroxide. Hydrogen peroxide is a highly-reactive compound which damages cell components. It is sometimes formed when the electron transport chain is used to produce energy. This

test is particularly useful in differentiating staphylococci and micrococci, which are catalase-positive, from *streptococci* and *enterococci*, which are catalase-negative.
Test procedure: When a catalase-positive organism is exposed to hydrogen peroxide, the 3% hydrogen peroxide will bubble. Put a few drops of hydrogen peroxide on the organism.

2.9.6.4. Nitrate reduction
Nitrate reduction was determined as described by Smibert and Krieg (1994).

Nitrate broth is used to determine the ability of an organism to reduce nitrate (NO_3) to nitrite (NO_2) using the enzyme nitrate reductase. It also tests the ability of organisms to perform nitrification on nitrate and nitrite to produce molecular nitrogen. Nitrate broth contains nutrients and potassium nitrate as a source of nitrate.

Test procedure: After incubating the nitrate broth, add a dropperful of sulfanilic acid (Nit1) and α-naphthylamine (Nit2). If the organism has reduced nitrate to nitrite, the nitrites in the medium will form nitrous acid. When sulfanilic acid is added, it will react with the nitrous acid to produce diazotized sulfanilic acid. This reacts with the α-naphthylamine to form a red-coloured compound. Therefore, if the medium turns red after the addition of the nitrate reagents, it is considered a positive result for nitrate reduction.Negative confirmation: confirmed by adding a pinch of zinc dust to the tube and shaking vigorously. If the tube becomes red, the test is confirmed as being negative. Zinc causes this reaction by reducing nitrate to nitrite; the newly formed nitrite reacts with the reagents to produce the red colour.

2.9.6.5. Starch hydrolysis
Starch hydrolysis was determined as described by Smibert and Krieg (1994).

Starch agar is a differential medium that tests the ability of an organism to produce certain exoenzymes, including α-amylase and oligo-1,6-glucosidase, that hydrolyze starch. Starch molecules are too large to enter the bacterial cell, so some bacteria secrete exoenzymes to degrade starch into subunits that can then be utilized by the organism. Starch agar is a simple nutritive medium with starch added. Since no colour change occurs in the medium when organisms hydrolyze starch, add iodine to the plate after incubation. Iodine turns blue, purple, or black (depending on the concentration of iodine) in the presence of starch. A clearing around the bacterial growth indicates that the organism has hydrolyzed starch.

Test procedure: Plate isolates on MM+ 5gl^{-1} Starch. Incubate plates at 37℃ for ~48h. Pour a few drops of gram iodine over the growth.

2.9.6.6. Casein hydrolysis

Casein hydrolysis was determined as described by Smibert and Krieg (1994).

Media with casein agar are used to test the ability of an organism to produce an exoenzyme, called casease that hydrolyzes casein. Casein forms an opaque suspension in milk that makes the milk appear white. Casease allows the organisms that produce it to break down casein into smaller polypeptides, peptides, and amino acids that can cross the cell membrane and be utilized by the organism. When casein is broken down into these component molecules, it is no longer white. If an organism can break down casein, a clear halo will appear around the areas where the organism has grown.

Test procedure: Plate isolates on Agar with 8gl^{-1} milk powder. Incubate plates at 37℃ for ~48h.

2.9.6.7. Tween80 hydrolysis

Hydrolysis of Tween80 was determined as described by Friedrich Burkhardt (1992).

Tween80 (Polysorbat 80) is an oleic acid ester thus it is a test for lipase. For testing of hydrolysis of Tween80 CaCl2 is added to the media. If the test strain is able to hydrolyze Tween the hydrolysis products (fatty acids) interact with the CaCl2 and opaque haloes can be observed due to granulation.

Test procedure: Plate isolates on MM + Tween80. Incubate plates at 37℃ for ~ 1 week.

2.9.6.8. DNAse Agar

DNase agar is a differential medium that tests the ability of an organism to produce an exoenzyme, called deoxyribonuclease or DNase that hydrolyzes DNA. DNase agar contains nutrients for the bacteria, DNA, and methyl green as an indicator. Methyl green is a cation which binds to the negatively-charged DNA. Deoxyribonuclease allows the organisms that produce it to break down DNA into smaller fragments. When the DNA is broken down, it no longer binds to the methyl green, and a clear halo will appear around the areas where the DNase-producing DNAse production was determined on DNAse agar (Oxoid) plus 0.05gl^{-1} Methylgreen.

2.9.6.9. Other biochemical tests

KOH-lysis test and conventional biochemical tests (i.e. oxidative-fermentative metabolism of glucose [O/F test], citrate utilization) were carried out as described by Smibert and Krieg (1994).

Utilization of various carbohydrates and the production of acid were determined on phenolred agar as described by Atlas (2004).

For Gelatine hydrolyse 12% (w/v) Gelatine was added to medium maintenance MM (Bast, 2001).

Enzymatic activities were tested using API Zym test strips (biomerieux) following the instructions of the producer. Therefore cell mass was resuspended in 10% saline to reach a McFarland standard of 5. The strips were incubated for 4.5 hours at 37°C.

2.9.7. Molecular characterisation of bacterial strains

2.9.7.1. DNA extraction from pure bacterial strains

Genomic DNA from pure bacterial strains was extracted according to the protocol provided by Ausubel et al. (1991) as followed: Isolates were grown on plates containing medium maintainance (MM) at 37°C for 48 hours. From these cell cultures one loop of each strain was resuspended in 540µl TE (10x TE: 100mM Tris (hydromethyl) aminomethan; 10mM EDTA; pH 8) and 50µl Lysozym (stock solution: 50mg/ml) and incubated for 20 min at 37°C. For break down of the cell walls and membranes 11.5µl 25% SDS (endconcentration: 0.5%) and 6µl proteinase K (endconcentration: 200µg/ml) were added and the mixture was left for 1 hour at 37°C. To prevent the formation of a CTAB-DNA complex 100µl 5M NaCl were given to ever tube. Afterward 80µl CTAB/NaCl (10% CTAB, 0.7 M NaCl) solution was admixed followed by 10 min incubation at 65°C. To remove the CTAB/Protein/Polysaccharide complex that had formed an equal volume of chloroform/isoamylalcohol (24:1) was applied and well mixed. A centrifugation step (4-5min at 13.000rpm) separated the two phases. The supernatant containing the DNA was transferred in a new eppendorf and the same procedure was repeated with Phenol/Chloroform/Isoamylalcohol (25:24:1). Again the supernatant was transferred into a new eppendorf and precipitated in 1ml 96% Ethanol overnight at -20°C. After centrifugation (10min, 13.000rpm) the resulting pellet was resuspended in 100µl ultrapure water (Sigma).

2.9.7.2. PCR analysis

For PCR analysis, 2x PCR Master Mix (Promega) [50 units/ml of TaqDNA Polymerase supplied in a proprietary reaction buffer (pH 8.5), 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP, 3mM $MgCl_2$] was diluted to 1x and 12.5 pmol of each primer were added. PCR was carried out in 25µl volumes and 2.5µl template was added.

Two different PCR reactions were carried out to amplify the eubacterial 16S rDNA fragments. For DGGE analysis, PCR was performed as described in paragraph 2.3.1.

For sequencing analyses, ~1500 base pairs 16S rDNA fragments were amplified using the forward primer 27f and the reverse primer 1492r (Lane 1991). PCR reactions were performed using a Robocycler (Stratagene). PCR conditions: 5 min denaturation (95 ^{o}C), followed by 30 cycles consisting of 1 min denaturation (95 ^{o}C), 1 min primer annealing (55 ^{o}C) and 2 min primer extension (72 ^{o}C), with a final extension step of 72 ^{o}C for 5 min.

2.9.7.3. Denaturing Gradient Gel Electrophoresis (DGGE) analysis

16S rDNA was amplified from each isolate for the detection of a possible microheterogeneity in rRNA encoding genes.

DGGE was done as previously described for the screening of eubacterial in paragraph 2. 4. using a lineal chemical gradient ranging from 25 to 60 % (100 % denaturant contains 7 M urea and 40 % vol./vol. formamide).

2.9.7.4. 16S rDNA sequencing and phylogenetic analyses.

The individual 16S rDNA fragments (~1400 bp) were purified with the QIAquick PCR Purification Kit (Qiagen) and sequenced as described previously (Schabereiter-Gurtner *et al*., 2001). The obtained sequences were compared with known 16S rDNA sequences of prokaryotes contained in the Ribosomal Database Project (RDP) (Maidak *et al*,. 1999) and the EMBL database. Pairwise evolutionary similarities and distances were computed by using the phylogeny inference package (PHYLIP) (Cole *et al*., 2003) of the online analysis of the RDP-II. A total of 1262 bases were compared.

Initial sequence data base search was performed by the SIMILARITY-RANK tool of the RDP and the FASTA search option (Pearson, 1994).

2.9.7.5. Random Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR)

For RAPD PCR short (~10b) arbitrary primers that bind with low stringency are used. PCR products are resolved electrophoretically on agarose gels to yield DNA fingerprints which differ according to the degree of relatedness.

PCR was performed with 10 different primers (see table 7). Five of them are 10 bp in length and the other half ≥17 nt. They also differ in their GC content which affects the annealing behaviour. With the short primers lesser bands are obtained because they have fewer possibilities to bind. As shown in in Figure 10 primer extension is only possible when (b) perfect annealing or (c) annealing at the 3`end occurs. For longer primers an additional alternative is the formation of a hairpin structure or a loop (d). Thus more bands are gained using longer oligonucleotides.

To the 1x diluted Mastermix (Promega) primer concentrations from 3 to 15nM were applied. PCR was carried out in 25µl volumes and 2.5µl template was added.

When using 10-nt primers PCR was performed under the following thermocycling conditions: 4 cycles of [94°C, 5min; 36°C, 5min; and 72°C, 5min], 30 cycles of [94°C, 1min; 36°C, 1min; and 72°C, 2min] followed by 10min at 72°C (Williams et al. 1990).

When primer oligos ≥17nt were used, the cycling program was 4 cycles of [94°C, 5min; 40°C, 5 min; and 72°C, 5min; low stringency amplification], 30 cycles of [94°C, 1min; 55°C, 1min; and 72°C, 2 min; high stringency amplification] and a final elongation step for 10min at 72°C (Welsh and McClelland 1990).

The whole reaction batches were run on a 2% (wt/vol) agarose gel at 160 V for 2 hours, stained with ethidium bromide and visualized with an UVP documentation system.

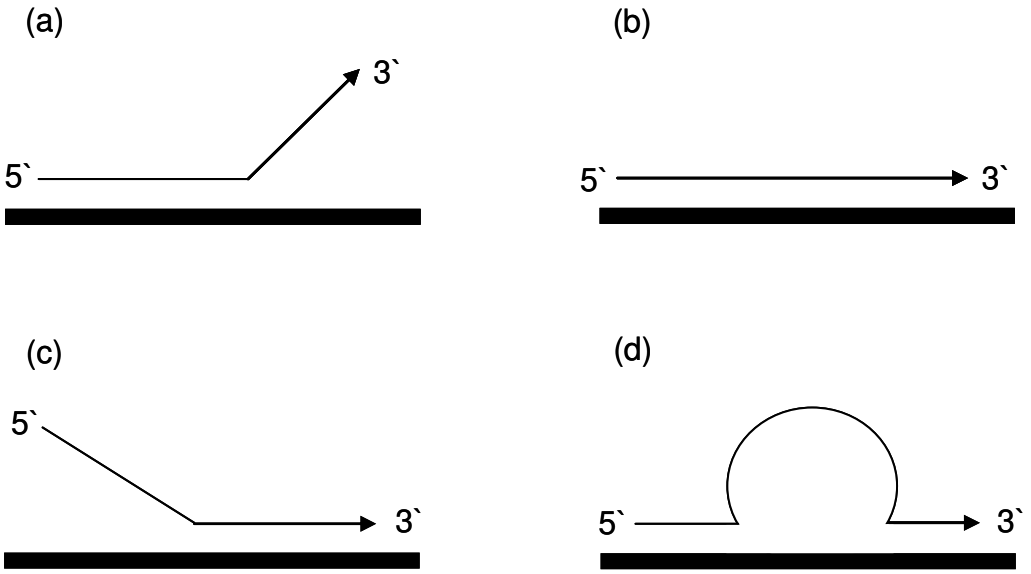


Figure 10 Possible primer-template interactions in DNA amplification (a) No annealing at the 3`end, no extension possible; (b) perfect annealing; (c) no annealing at the 5`end though extension is possible; (d) formation of a hairpin or loop, allowing extension (Power, 1996)

Primer	Sequence	%G+C
10 nt primers		
1254	CCGCAGCCAA	70
1283	GCGATCCCCA	70
1247	AAGAGCCCGT	60
1281	AACGCGCAAC	60
1290	GTGGATGCGA	60
≥ 17 nt primers		
D14216	NNNAACAGCTATGACCATG	~44
D11344	AGTGAATTCGCGGTGAGATGCCA	52
D8635	GAGCGGCCAAAGGGAGCAGAC	67
D9355	CCGGATCCGTGATGCGGTGCG	71
D14307	GGTTGGGTGAGAATTGCACG	55

Table 7 Primers used for RAPD analysis. 10nt primers: 1254, 1283, 1247, 1281, 1290; ≥ 17 nt primers: D14216, D11344, D8635, D9355, D14307

2.9.7.6. Intergenic transcribed spacer homoduplex heteroduplex PCR (ITS-HHP)

Genera of *Bacillus*, *Brevibacillus*, *Geobacillus* and *Paenibacillus* show high variety in their Intergenic Transcribed Spacer (ITS) regions between the 16S and the 23SrDNA. Using specific primers targeting these regions leads to formation of Hetero- and Homoduplexes depending on whether there is a tRNA gene located in the operon or not (See Figure 11, Daffonchio *et al*., 2003).

For PCR 12.5 pmol of each primer S-D-Bact-1494-a-S-20 (5`-GTC GTA ACA AGG TAG CCG TA-3`) and L-D-Bact-0035-a-A-15 (5`-CAA GGC ATC CAC CGT-3`) (Daffonchio *et al*., 2003) were used. PCR was carried out in 50µl volumes to which 3.5µl template were added. PCR conditions: 4 min denaturation at 94°C, then 35 cycles [94°C, 1min; 55°C, 7min; and 72°C, 2 min] with a final extension step at 72°C for 7 min (Daffonchio *et al*., 1998).

The PCR products were run on three different kinds of gels: first, 25µl PCR product were run at 160 V for 2 hours on a 2% (wt/vol) agarose gel; second, 6% (wt/vol) acrylamide gels and mutation detection enhancement (MDE) gels were used for a better separation. For reducing band broadening 10% (vol/vol) glycerol was added to the 6% (wt/vol) acrylamide gels (Kumeda and Asao 1996). For the same reason urea was added to the MDE gel matrix as recommended by the manufacturer (Cambrex, USA). 15µl of ITS-HHP PCR products were loaded on the polyacrylamide and MDE gels and electrophoresed in 1x TBE buffer (5x TBE /l: 54g Tris; 27.5g Borat, 20 ml 0.5M EDTA; pH 8.0) for 7 hours at 100 V using a D GENE System (BioRad, Munich, Germany).

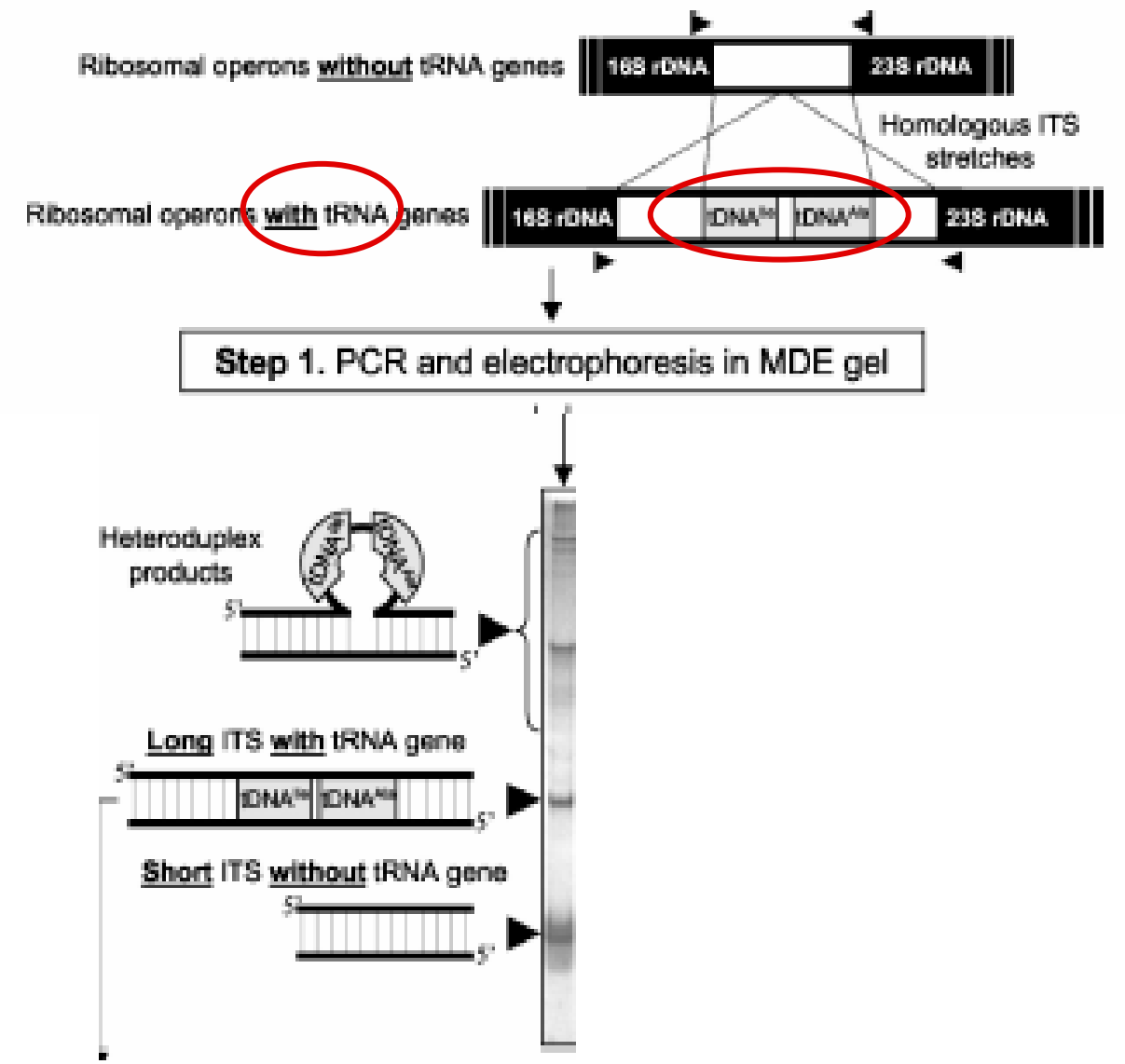


Figure 11 General principle of ITS Homoduplex Heteroduplex PCR; Daffonchio *et al.*, 2003

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4. Manuscripts

I. Screening of bacterial and fungal diversity with DGGE on mural paintings under controlled climate conditions in the Castle of Schönbrunn
II. Investigation of moderate and extreme halophilic microorganisms on mural paintings
III. Molecular characterisation of *Halobacillus* populations isolated from different medieval wall paintings and building materials
IV. *Halobacillus virgiliensis sp. nov.*, *Halobacillus styriensis sp.nov* and *Halobacillus herbersteinensis sp.nov* isolated from medieval mural paintings and stone-works in two different lcations in Austria

I. Screening of bacterial and fungal diversity with DGGE on mural paintings under controlled climate conditions in the Castle of Schönbrunn

1. Introduction

Colonization of art works by microorganisms usually depends on environmental conditions like moisture, temperature and light, as well as the chemical nature of the substratum (Saiz-Jimenez, 1993). Microorganisms are often responsible for the biodeterioration and degradation presented by art objects such as paintings, stone, wood, paper, masonry, leather, parchment, glass and metal (Bock and Sand, 1993; Cifferi, 1999; Griffin *et al.*, 1991; Kowalik 1980; Montegut *et al.*, 1991). Microbial induced deterioration processes can cause different kinds of alterations such as discolouration of materials, formation of crusts on surfaces and the loss of binding material, which leads to structural damage. First of all, microorganisms cause severe problems due to the excretion of aggressive metabolic products such as organic and inorganic acids. Secondly, material components can be used as substrates for microbial metabolism. In mural paintings, pigments are suspended in water or oil and casein and milk are often used as binding materials supplied on the damp lime plaster (Ciferri, 1999). Therefore, mural paintings contain mainly inorganic substrates able to support their colonization by autotrophic microorganisms. These autotrophic organisms establish a basis for a further colonization of mural paintings by heterotrophic microorganisms. Thus the microflora changes within time and a successive colonisation takes place. A scenario of this settlement is shown in Figure

12 (Täubel, 2001). Considering this, it is obvious that taking samples from mural paintings at defined points in time is only a snapshoot of a gradual changing biocoenosis. Therefore, it is necessary to monitor the microbial colonization of objects of art at different points of time in case of restoration before and after the treatment. Furthermore, the microorganisms present on art objects may emit bioaerosols (Piñar, 2002) or even be harmful. Thus it is important in aspects of health and maintenance of cultural heritage to analyze the complete microflora to further develop biocides for harmful microorganisms and methods for restoration of degraded objects of art.

A great deal of research in this field has been done mainly by using culture-dependent methods. However, these traditional methods allow only the detection of a very small proportion of present microorganisms. Far less than 1% of organisms in the environment can be cultivated by standard cultivation techniques (Pace, 1996). In addition, recent studies have shown that culture-dependent techniques yield mainly spore-forming bacteria suggesting that plating leads to an overestimation of the number of this kind of microorganisms (Laiz *et al*, 2003). Last but not least, extensive cultivation strategies require far more sample material than could be obtained from objects of art without damage.

In recent years the use of novel molecular methods for culture-independent analysis has been developed and established for mural paintings and stones (Schabereiter-Gurtner, *et al*. 2001). Using molecular techniques it is possible to detect more biodiversity in samples as when using culture-dependent methods and this fact probably demonstrate the actual proportion of species present in art samples (Laiz, 2003). The molecular approach used in this study was described elsewhere (Schabereiter-Gurtner *et al*., 2001). It includes the direct DNA extraction from small pieces of art samples followed by PCR amplification of 16S rRNA genes and fingerprinting by Denaturing Gradient Gel Electrophoresis (DGGE). In parallel clone libraries, containing PCR fragments of the ribosomal gene for bacteria as well as the ITS1 region for fungi, are constructed, screened by DGGE analysis and clones showing different electrophoretic motility in DGGE are sequenced.

This study describes the investigations on samples taken from partially damaged mural paintings from the Bergl-Zimmer situated in the lower part of the castle of Schönbrunn (Vienna, Austria). In the years 1769 to 1778 these rooms were decorated with extensive paintings showing exotic scenery. Restoration was done in

1891 but as time going on paintings showed cumulative decay as black biofilms, cracks and discoloration spread.

Analysis included DNA extraction from small sample amounts, the study of bacterial and fungal communities present in the samples, the construction and screening of clone libraries as well as sequencing of clones. Research evidenced that microorganism play a major role in the degradation of these paintings.

2. Material and Methods

2.1. Sampling

In January 2004 wall painting samples were taken from the Berglzimmer in the Palace of Schönbrunn, Vienna (Austria) by scraping off surface material to a depth of 1 to 3mm with a sterile scalpel. Samples KM1-1, KM1-2, KM2-1, KM2-2 and KM3-1 were taken from the "Kindermuseum". Samples S1-1, S1-2, S2, S3 and S4 were taken in the eastern part of the building. Table 8 and Figure 13 show the location of sampling.

2.2. DNA extraction and purification

DNA was extracted directly from wall painting samples by using the Fast DNA SPIN kit for soil (Bio 101). The protocol of the manufacturer was modified as follows. Powder obtained from wall painting samples were placed in the MULTIMIX2 tissue tube. 978 µl sodium phosphate buffer and 122 µl of the MT buffer (Bio 101) were added. The mixture was then bead-beaten two times for 1 min with one intervening minute on ice and centrifuged at 14000xg for 2min. The PPS reagent and the binding matrix suspension (Bio 101) were applied to the supernatant following the instructions of the manufacturer. The resulting suspension was transferred to a spin filter and centrifuged at 14000 x g for 1min. DNA was washed twice with 500µl of the SEWS-M solution (Bio 101) and eluted from the binding matrix with 160 µl DES (Dnase/Pyrogene free water).

The extracted DNA was further purified with the QIAamp Viral RNA mini kit (Quiagen) and finally eluted from the silica column with 100 µl ddH_2O.

2.3. PCR amplification of 16S rDNA fragments

For all reactions 2x PCR Master Mix from Promega [50 units/ml of TaqDNA Polymerase supplied in a proprietary reaction buffer (pH 8.5), 400µM dATP, 400µM

dGTP, 400µM dCTP, 400µM dTTP, 3mM MgCl2] was diluted to 1x and 12.5 pmol of each primer were added. Primer sequences used in this study are shown in Table 9.
PCR amplification for genetic fingerprinting for bacteria was carried out with primer 341f specific for eubacterial 16SrDNA and 907r a universal primer for 16SrDNA. This step was followed by a semi-nested PCR performed with primers 341fGC and 518r. The forward primer possessed a 40-base GC clamp at its 5`end which stabilizes the melting behaviour of DNA fragments in DGGE analysis (Muyzer *et al*., 1993). For the first round of PCR 25µl volume and 2µl template were used. For nested PCR 100µl volumes separated on two tubes to 50µl and 3.5µl template to each tube were applied.
PCR were performed in a Robocycler with the following thermocycling program:5 min denaturation at 95°C, followed by 30 cycles each consisting of 1 min denaturation at 95°C, 1 min annealing at 55°C and 1 min extension at 72°C. Five minutes at 72°C were used as final extension step.
Ten µl of each PCR product were run on a 2% agarose gel for ~ 30 min at 200V, stained with ethidium bromide and visualized by a UVP documentation system.

2.4. PCR amplification of ITS1 Region (Fungi)

PCR was carried out as described above using primers ITS1 and ITS2 specific for the internal transcribed spacer region 1 located between the 18S and 5.8SrDNA (White *et al*; 1990). For 2nd round of PCR the ITS1 primer had a GC clamp on its 5`end. The thermocycling program for the first round was as followed: 5min at 95°C, followed by 35 cycles 1min at 95°C, 1min at 62°C, 1min at 72°C and a final extension step 5 min at 72°C. For PCR with the GC primer annealing temperature was set at 60°C.

2.5. DGGE- Denaturing Gradient Gel Electrophoresis

For genetic fingerprinting 100µl of semi-nested PCR products were precipitated in 1 ml 96% ethanol (–20^{o}C overnight), resuspended in 15µl ddH_2O water (Sigma) and separated by DGGE .
Gels screening bacterial communities and clone libraries were run in 0.5xTAE buffer [20mM Tris, 10mM acetate, 0.5mM Na_2EDTA; pH 7.8] for 3.5 hours at 200V and 60°C in a D GENE System (BioRad, Munich, Germany) (Muyzer, *et al*; 1993). A denaturing gradient from 30 to 55% of urea and formamide in an 8% (w/v) polyacrylamide gel (BioRad, Munich, Germany) was used.

Fungal communities and clone libraries were screened on gels containing 30 to 50% of denaturants for 14 hours at 100V and 60°C (Michaelsen *et al*., 2005).
After completion of electrophoresis, gels were stained in an ethidium bromide solution and documented with an UVP documentation system.

2.6. Construction of clone libraries

For the construction of clone libraries for each sample 2 x 3.5µl DNA templates were amplified in 2 x 50µl volumes using the primers 341f and 907r for bacteria and ITS1 and ITS2 for fungi. The PCR products were pooled and purified using the QIAquick PCR Purification Kit Protocol (Qiagen, Hilden, Germany) and resuspended in 30µl ddH_2O water (Sigma).
5.5µl of purified PCR product were ligated into the pGEM –T easy Vector system (Promega, Vienna, Austria) following the instructions of the manufacturer. The ligation products were transformed into *E.coli* XL Blue^{Tc}, which allows the identification of recombinants (white colonies) on an indicator LB medium containing ampicilline (100µg/ml), tetracycline (10µg/ml), X-Gal (5 bromr-4-cloro-3-indolyl-ß-D-galactorpyranoside; 0.1 mM) and IPTG (isopropyl-ß-D-thiogalactopyranoside: 0.2 mM) (Sambrook *et al*., 1989).

2.7. Screening of clones by DGGE and PCR

Of each clone library about 50 clones were harvested and resuspended in 50µl TE buffer [10mM TrisHCl, 1mM EDTA; pH 8.0] followed by three steps of freeze and thawing.
Screening of the clone libraries by PCR and DGGE was performed as described by Schabereiter-Gurtner and coworkers (2001) as follows: To amplify the inserts the vector specific primers SP6 and T7 were utilized. For PCR the following conditions were used: 5 min at 95°C denaturation followed by 35 cycles each consisting of 1 min at 95°C, 40 sec at 46°C and 1 min at 72°C. Three minutes were used as final extension step.
A nested PCR for DGGE analysis was carried out with the primer pair 341fGC and 518r for bacterial clone libraries and ITS1GC and ITS2 for fungal clones. PCR was performed as described above in 25µl volumes. Ten µl of each PCR product were run on a 2% agarose gel for ~ 30 min at 200V, stained with ethidium bromide and visualized via an UVP documentation system (BioRad quantity one).

The rest was used for DGGE analysis under the same conditions as described above. Clones showing different motility behaviour in DGGE analysis were selected for sequencing.

2.8. Sequencing and identification

100µl of PCR products amplified with primers SP6 and T7 were purified with a QIAquick PCR Purification Kit (qiagen, Hilden, Germany). Sequencing was done by VBC (Vienna Bio Centre, Austria) and the obtained sequences were compared with know sequences in the EMBL database using the search tool FASTA (Pearson, 1994).

3. Results and Discussion

3.1. DNA extraction from mural painting samples and DGGE fingerprint analysis

This study introduces an advanced strategy for identification of the microbial flora colonising mural paintings by using molecular techniques, including a DNA extraction procedure for small sample amounts. The modified DNA extraction protocol described in the section of Methods, based on the commercial FastDNA SPIN kit for soil, has the advantage that is at least five times faster than previous methods described for DNA extraction from soil and particulate samples (Leff *et al*., 1995). Furthermore, the protocol includes an extra purification step for removing PCR inhibitors which results in very pure colourless DNA suitable for cloning and PCR amplification.

To determine the quality of the extracted DNA, PCR analysis using 16S rDNA universal eubacterial-primers were performed to amplify the extracted DNA. Crude DNAs directly extracted from all wall painting samples showed to be amplifiable after 30 amplification cycles. All samples were subsequently analysed by DGGE analysis.

In a nested PCR, 200 bp fragments spanning the hypervariable V3 region of the 16S rDNA were reamplified and genetic fingerprinting was performed by DGGE. Figure 14 shows a detail of the DGGE fingerprints derived from the bacterial communities colonizing the mural paintings from the Palace of Schönbrunn. DGGE fingerprints showed similar profiles for all samples, with four to five different predominant bands. Therefore, it can be assumed that the diversity of bacteria in these samples is rather low. As all the samples showed nearly the same band-

patterns, two samples, S1-1 and KM1-2, showing the more complex DGGE profiles were selected for the construction of clone libraries.

Genetic fingerprinting was carried out to visualise the fungal communities colonizing the mural paintings from the Palace of Schönbrunn by DGGE analysis (Figure 15). PCR was performed as mentioned in the section of Methods. DGGE fingerprints derived from fungal communities showed more discrepancies in between the different samples as those derived from bacterial communities. In general, two to six different predominant bands and some more other faint bands could be visualised. Two samples, S1-1 and KM2-1 were selected for the construction of clone libraries to obtain a detailed phylogenetic identification.

3.2. Construction of clone libraries containing 16S rDNA sequences and phylogenetic analysis

Fifty clones from clone library S1-1 as well as 34 clones from clone library KM1-2 were screened as described in the section of Methods. The screening of the clone libraries revealed that biodiversity of bacterial populations on samples was higher than expected. From clone library S1-1, 16 different clones could be obtained (see figure 16). For clone library KM1-2, 11 clones showed different migration behaviour (see figure 17). Clone libraries gave more information both in the quantity of bacteria identified as well as in the length of sequences for alignment. All clones showing different positions in DGGE were subjected to sequencing. The phylogenetic affiliations obtained for clones derived from samples S1-1 and KM1-2 are shown in Table 10 and 12 respectively. Comparative sequence analysis showed percent of similarities of 94.2 % to 99.8 % in between the sequenced clones and sequences from the EMBL.

Sequences derived from sample S1-1 were phylogenetically affiliated with genera of the cultivated gamma-proteobacteria as the family *Moraxellaceae* (*Acinetobacter*, clone K16), *Pseudomonadaceae* (*Pseudomonas*, clone K3) and *Enterobacteriaceae* (*Enterobacter*, clone K5, K12 and K38 and *E. coli*, clone K20; *Pantoea* clone K11, K18 and K37). Some sequences affiliated with the cultivated actinobacteria as the family *Brevibacteriaceae* (*Brevibacterium*, clone K2), *Micrococcaceae* (*Arthrobacter*, clone K19) and *Propionobacteriaceae* (*Propionibacterium,* clone K39). Clone K36 was phylogenetically affiliated with a genus of the cultivated Bacillales, *Bacillaceae*, namely species of the genus *Bacillus*. Clones K7, K30 and K42 resulted from heteroduplex bands and were not sequenced.

Some clones derived from Clone library S1-1, showing different migration behaviour in DGGE screening, gave a high score similarity with the same species, as clones K5, K12 and K38 with *Enterobacter* sp. For these clones it can be assumed that they derived from different operons of the same organism showing microheterogeneity of the 16S rDNA.

Most of the sequences derived from sample KM1-2 were phylogenetically affiliated with genera of the cultivated gamma-Proteobacteria as the family *Moraxellaceae* (*Moraxella*, clone K8 and *Acinetobacter*, clone K4 and K28), *Aeromonadaceae* (*Aeromonas*, clone K19), *Pseudomonadaceae* (*Pseudomonas*, clone K32) and *Enterobacteriaceae* (*Enterobacter*, clone K10 and K26). In addition clone K5 showed the highest phylogenetic affiliation with the genera *Thermoactinomyces*, which belong to the Bacillales, family *Thermoactinomycetaceae.* Clone K9 was phylogenetically affiliated with a genus of the cultivated alpha-proteobacteria, the family *Acetobacteraceae*, namely species of the genus *Rhodophila.* Finally, clone K13 was phylogenetically affiliated with a genus of the cultivated beta-Proteobacteria, the family *Comamonadaceae*, namely species of the genus *Acidovorax*.

3.3. Construction of clone libraries containing ITS1 fungal sequences and phylogenetic analysis

Sample KM2-1 derived from the Kindermuseum and sample S1-1 from the Berglzimmer were selected for detailed phylogenetic analysis including the construction of clone libraries containing ITS1 fungal sequences. DGGE analysis were carried out for the screening of clones followed by sequencing of the inserted fragments. From Sample KM2-1, seven different clones could be obtained. Sample S1-1 showed a higher biodiversity as 10 different clones could be obtained and sequenced (Figure 18 and 19).

For phylogenetic identification, the obtained sequences were compared with sequences of known fungi listed in the EMBL nucleotide sequence database. Sequencing results for sample S1-1- and KM2-1 are shown in Table 11 and 13 respectively. Comparative sequence analysis showed percent of similarities in between 95.8 % and 100 % of fungal sequences obtained in this study with sequences from the EMBL database.

Sequences derived from sample S1-1 were phylogenetically affiliated with genera of the Ascomycota group Pezizomycotina as *Cladiosporum herbarum* (clone S17 and

S35), *Ascochyto* sp. (clone S58), *Aspergillus restrictus* (clone S68), *Eurotium amstelodami* (clone S56) and one member of the group of mitosporic Ascomycota named *Wallemia sebi* (clone S13). Also members of the Basidiomycota group homobasidiomycetes as *Coprinus cothurnatus* (clone S12), *Hyphodontia paradoxa* (=*Schizopora paradoxa*) (S18) and *Fomitopsis pinicola* (S52) could be identified.

Clones derived from sample KM2-1 gave high score similarities with the Ascomycetes *Cladiosporum sphaerospermum* (clone K7), *Eurotium amstelodami* (clone K36), *Ascochyto sp*. (clone K29), *Alternaria tenuissima* (clone K30) and one member of Homobasidiomycetes *Schizophyllum commune* (clone K27). In this sample a member of Heterobasidiomycetes called *Trichosporon mucoides* (clone K28) could be detected as well.

In both samples investigated one sequence gave high score similarities with a member of viridiplantae known as *Lycium ruthenicum,* as clone S28 of sample S1-1 and clone K6 of sample KM2-1.

4. Discussion

The objective of this work was to obtain an understanding of the microbial flora (bacteria and fungi) present on wall paintings located at the Palace of Schönbrunn which are under T^a and humidity controlled conditions, and to compare the data obtained with those obtained from former research works carried out by our working group on wall paintings located in other locations under non-climatic controlled conditions.

To analyse and compare the microbial flora associated with different samples, a molecular approach consisting on the combination of fingerprinting by DGGE (denaturing gradient gel electrophoresis) with the screening by DGGE of clone libraries containing either eubacterial 16S rDNA fragments or ITS1 fungal fragments. The screening was followed by sequencing of selected clones showing different migration behaviour in DGGE analysis. By using a strategy based on culture-independent techniques we reduced possible culturing bias by analysing rDNA sequences directly amplified from DNA isolated from wall paintings. Second, a method for extraction and purification of DNA was chosen to ensure that microorganisms from many different taxa (bacteria and fungi) would be lysed efficiently.

4.1. Bacterial colonization of mural paintings at the Palace of Schönbrunn

To obtain a detailed phylogenetic information about the bacterial flora present on the wall paintings of the Palace of Schönbrunn, two samples were selected for the construction of clone libraries (samples S1-1 and KM1-2). Phylogenetic analysis showed percent of similarities in between 99.8 % and 94.2 % with sequences contained in the EMBL nucleotide database. In general, most of the bacteria found in the observed samples belong to the subgroup of *gamma-Proteobacteria*, as well as members of the *Actinobacteria, Bacillales* and in a minor proportion to the alpha- and beta- subclass of *Proteobacteria* .

First of all, comparative sequence analysis allowed us to infer about the origin of the bacteria detected on the mural paintings at the Palace of Schönbrunn. Bacteria may derive from plants or pollen as many sequences obtained in this study gave high score similarities with bacteria isolated from plants, like *Moraxella osloensis, Enterobacter sp., Enterobacter cloace, Enterobacter agglomerans,* and *Pantoea endophytica.* Bacteria may also derive from animal faeces like flies, as clone 4 derived from KM1-2 sample, gave high scores similarity with *Acinetobacter junii* isolated from wild culex quinquefasciatus mosquito midgut (Pidiyar, 2004). This author was able to isolate also members of the genera *Bacillus, Pseudomonas, Aeromonas* and *Panteoa*. All this genera could be detected in the samples analysed in our study as well.

Secondly, comparative analysis of the sequences obtained in this study with sequences contained in the database allowed us to infer about the biodeteriorative capabilities of the bacteria identified on the wall painting samples. Sequences reveal high similarities with cultivated bacteria showing potential degradation capacities, what could be of advantage for the microbial flora inhabiting the altered wall paintings. As Lazar and Dumitru (1973) reported studying mural paintings in a Romanian monastery, the genera *Bacillus*, *Arthrobacter* and *Pseudomonas* have been proved to produce salt efflorescences and were even considered to cause the blanching phenomenon of frescoes (Saiz-Jimenez and Laiz, 2000). Furthermore, strains of genus *Acinetobacter* have shown to precipitate aragonite (Laiz *et al.,* 1999). These crystallizations produce destructive effects. Salts may crystallize or recrystallize to different hydrates, which occupy a larger space and exert additional pressure producing cracking, powdering and flaking of substrates. Halite, and other hygroscopic salts, can retain considerable quantities of moisture when entrapped in

the building material and thus favouring the bacterial growth. In turn these microorganisms can mediate the crystallization process (Saiz-Jimenez and Laiz, 2000). In addition, other sequences, as *Moraxella* sp., *Enterobacter* sp. and *Pantoea* sp., all of them showing heterotrophic ambitions were detected in this study. It can be assumed that the bioflora is in secondary stage of colonisation.

Finally and surprisingly, clone K5 derived from KM1-2 sample showed high scores similarity with *Thermoactinomyces sacchari,* a bacterium formally isolated from air samples. This bacteria is involved in hypersensitivity pneumonitis (HP) and therefore, potentially harmful for humans. Studies showed that HP can be caused by, amongst other substances, a subset of 4 thermophilic mycelial bacteria: *Saccharopolyspora rectivirgula, Saccharomonospora viridis, Thermoactinomyces sacchari*, and *Thermoactinomyces vulgaris* (Harvey *et al*, 2001). This data suggest the importance to control the microbial flora present in public buildings subjected to visitors.

Comparative analysis in between the bacterial sequences obtained in this study and formal studies performed by our working group on mural paintings showed common results. This is the case of species belonging to the actinobacteria group as *Arthrobacter*, *Brevibacterium* and *Propionibacterium*. The biodeteriorative potential of this group of bacteria includes the excretion of aggressive metabolism products such as organic and inorganic acids and the mechanical destruction of the paint layer by penetration of the mycelium deeply into the plaster of the paintings (Scharbereiter-Gurtner *et al*., 2001). The occurrence of members of the genera *Bacillus*, *Brevibacterium*, *Arthrobacter* and *Pseudomonas* has already been demonstrated on mural paintings before by our working group (Necropolis of Carmona, Herberstein Castle, Greene Church) (Gurtner, et al, 2000). Furthermore, clone K36 derived from sample S1-1 gave a similarity of 98.2 % with *Bacillus pumilus* sp. already isolated from biodeteriorated wall paintings of the Servilia Tomb in the Necropilis of Carmona, Spain (Heyrmann *et al*., 1999) In these locations, wall paintings were under non-controlled climatic conditions what suggest that these bacteria are common inhabitants of this kind of substrates independently of the climatic preservation of the objects.

4.2. Fungal colonization of mural paintings at the Palace of Schönbrunn

Relative humidity, temperature and availability of nutrients are the three most important factors for mould growth over any type of material. According to Rebricova (1991, 1993), the formation of different microbial communities on the surface of wall paintings depends upon the painting techniques as well as the climatic conditions. Fungi find optimal growth conditions in environments where relative humidity is over 65%. By mural paintings those needs may be fulfilled by atmospheric humidity. In many cases the dampness in the walls is responsible for meeting the humidity requirements. According to Strzelczyk (1981), the spores of most fungal forms are capable of utilizing condensation moisture. Under conditions of relative humidity (75-95%), the spores germinate into mycelium. If humidity conditions remain unchanged, developing hyphae produce further spores within 48-72 hours, dominating the surface of the painting.

A factor which plays a vital role in the development of fungi is the dispersal of fungal spores. Mostly it is through air. However, many times the spores may reach the surface through the persons visiting the wall painting sites (Emoto and Emoto, 1974; Agrawal *et al*., 1988, 1991). In some instances, the materials used for the conservation treatment are the source of fungal growth (Sorlini *et al*., 1982, 1987; Sampo and Mosca, 1989; Hammer and Lux, 1990; Karpovich-Tate and Rebricova, 1991).

Samples taken from the palace of Schönbrunn were investigated for the presence of fungal communities colonising their mural paintings. As the rooms in Schönbrunn are all under controlled climate conditions fungal growth should be no problem. Nevertheless fungal colonization could be observed in all samples as showed by DGGE analysis. Two samples, S1-1 and KM2-1, were subjected to cloning and sequencing analysis showing that species belonging to both the *Ascomycetes* as well as the *Basydiomycetes* could be detected.

4.2.1. Ascomycetes

Strains of species of *Alternaria* and *Cladiosporium,* both of them identified on the mural paintings of the Palace of Schönbrunn, are the most common genera isolated from stone and mural paintings in indoor environments with standard cultivation methods. Saprophytic species of the genus *Cladiosporium* are among the most abundant fungi isolated in saltern samples. They grow optimally on media with low water activity due to high salt or sugar concentrations, seem to propagate

vegetatively in the salterns, and are thus halotolerant/osmotolerant species rather than simple flush-through occurrences. *Cladiosporum herbarum* was frequently isolated from hypersaline waters, dead and dying plant material, air, soil, food, fruit, cereals, paint and textiles (Ventosa, 2004).

There are some fungal species like *Aureobasidium pullulans* that are unable to grow in the absence of other fungal forms like *Alternaria* and *Cladiosporium*. (Winters *et al*.,1976) *Alternaria* aids in the initial colonisation of these fungi due to hydrolysis of hydroxyl ethyl cellulose present within the paint film. Thus growth of these moulds indicates further colonization of other species.

In temperate climates, fungi like *Aureobasidium pullulans*, *Cladosporium herbarum* and species of *Alternaria*, *Penicillium* and *Aspergillus* have been found to be common on outdoor paint surfaces. (Garg *et al*., 1995)

Saiz-Jimenez and Samson (1981) suggested that saline deposits may provide a suitable habitate for fungi, especially for species like *Cladiosporum sphaerospermum*.

Wallemia is an important genus of xerophilic food-borne fungi. *Wallemia sebi,* which was identified on sample S1-1, seems to have a natural ecological niche in salterns. This species has a minimal water activity for growth of 0.69-0.75 and has been isolated from dry food and soil.

Members of *Eurotium* and *Aspergillus* , detected in both samples, have also been found to be actively growing in low water activity environments. (Ventosa, 2004)

As all of these fungi show active growth in environments with low water activity they should be considered not only airborne. That means they grow on the surface of the mural paintings and into the plaster and are metabolically active which may lead to biodeterioration of art work. The mycelia grow on the surface and into the plaster of the paintings which may lead to dislodging of various layers, powdering and formations of cracks. In addition the development of fruiting bodies leads to further damage.

Metabolic activity and the associated production of organic acids results in solubilization of cations or chelation with metal ions present in the pigments of the paintings. Many fungal species produce exoenzymes that hydrolyse components of the painting material. *Cladiosporium herbarum*, for example, produces protease, amylase and acetic acid. (Valentin, 2003). In addition, some strains are black pigmented, what produces patinas.

For the aspects of health the production of mycotoxins and cell debris that cause allergies are a common problem connected with fungal growth. *C. herbarum* is known to produce mycotoxins and is a potential pathogenic species for humans. The genera *Alternaria, Aspergillus* and *Wallemia* are potential toxin producing fungi commonly found in microbially damaged nonliving materials (Saiz-Jimenez, 2003). Different strains of the same species may produce different toxins, several toxins or none at all. Thus it is difficult to say if the fungal strains present on the subjected mural paintings produce toxins or not. Actually there are only few reports on toxins produced under indoor environment.

4.2.2. Basidiomyctes

To our knowledge there is hardly any report of *Basidiomycetes* on mural paintings or stone. Therefore these results were rather surprising on the first sight. The *Basidiomycetes* found in the samples all form rather big fruiting bodies and are known to grow on rotten woods (Shah, 2000). Member of the genus *Corprinus* are living as saprophytes on humus, waste, wood and rest of plants. Most of them prefer eutrophic environments (Heinrich Dörfelt and Gottfried Jetschke, 2001).

They need a humid environment for outgrowth of spores. Therefore the mural paintings in the Kindermuseum and the Berglzimmer seem to offer not the right place for these fungi to grow as the rooms are under T^a and humidity controlled conditions. So it can be assumed that the DNA isolated from these samples was obtained not from growing material but from spores. As the rooms are indoor environment the question is where this spore originate from.

To our knowledge the air condition leads to an old tunnel in the woods. No filter has been installed. Therefore the surrounding air full of fungal spores, pollen, small organic and inorganic compounds and various microorganisms is transported via air condition into the rooms. Here the small particles are spread all over the rooms and accumulate on the walls and paintings. Even if these microorgansims or spores are not able to grow there they are input of organic material and serve as nutrient sources for adapted microorganisms.

Taking into consideration the small amount of sample taken from the paintings it is obvious that there has to be a big amount of spores in the room.

Also one member of the group of yeasts namely *Trichosporon mucoides* was detected in Sample KM2-1. Osmotolerant yeasts frequently occur on the phylloplane of plants (Inacio *et al*., 2002), in soil and water (van Uden *et al*., 1968). In literature it

is reported with human infections e.g. meningitis, skin lesions, pubic white piedra (AIDS) and others (Butinar *et al*., 2005). In a study by Butinar and co-workers (2005) investigating yeast diversity in eight different hypersaline habitats worldwide, *T.mucoides* was one of the species who showed the largest geographical distribution. This study also suggest that *T.mucoides* could be indigenous and probably represents a member of the stable core of microorganisms in hypersaline environments. Thus *T.mucoides* is believed to be able to grow in environments showing low water activities such as mural paintings. As osmotolerant yeasts showed to occur on phylloplanes of plants it can be assumed that one way of bringing them in is through the air condition.

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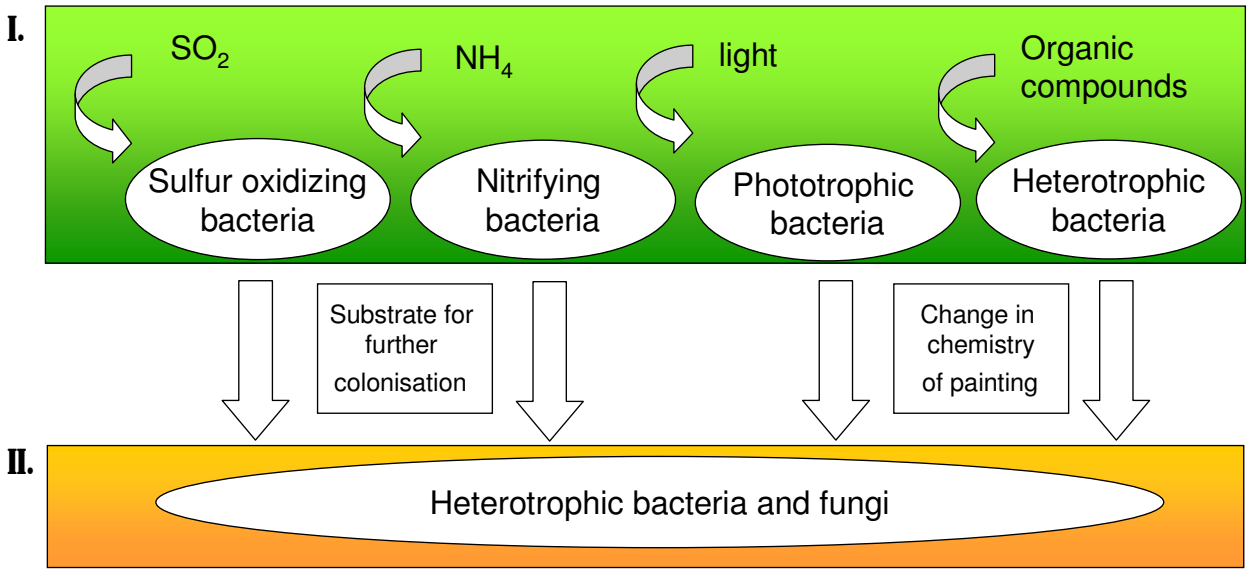

Figure 12 Diagram showing a scenario of the settlement of autotrophic organisms establishing a basis for further growth of the heterotrophic microbial flora (Täubel, 2001).

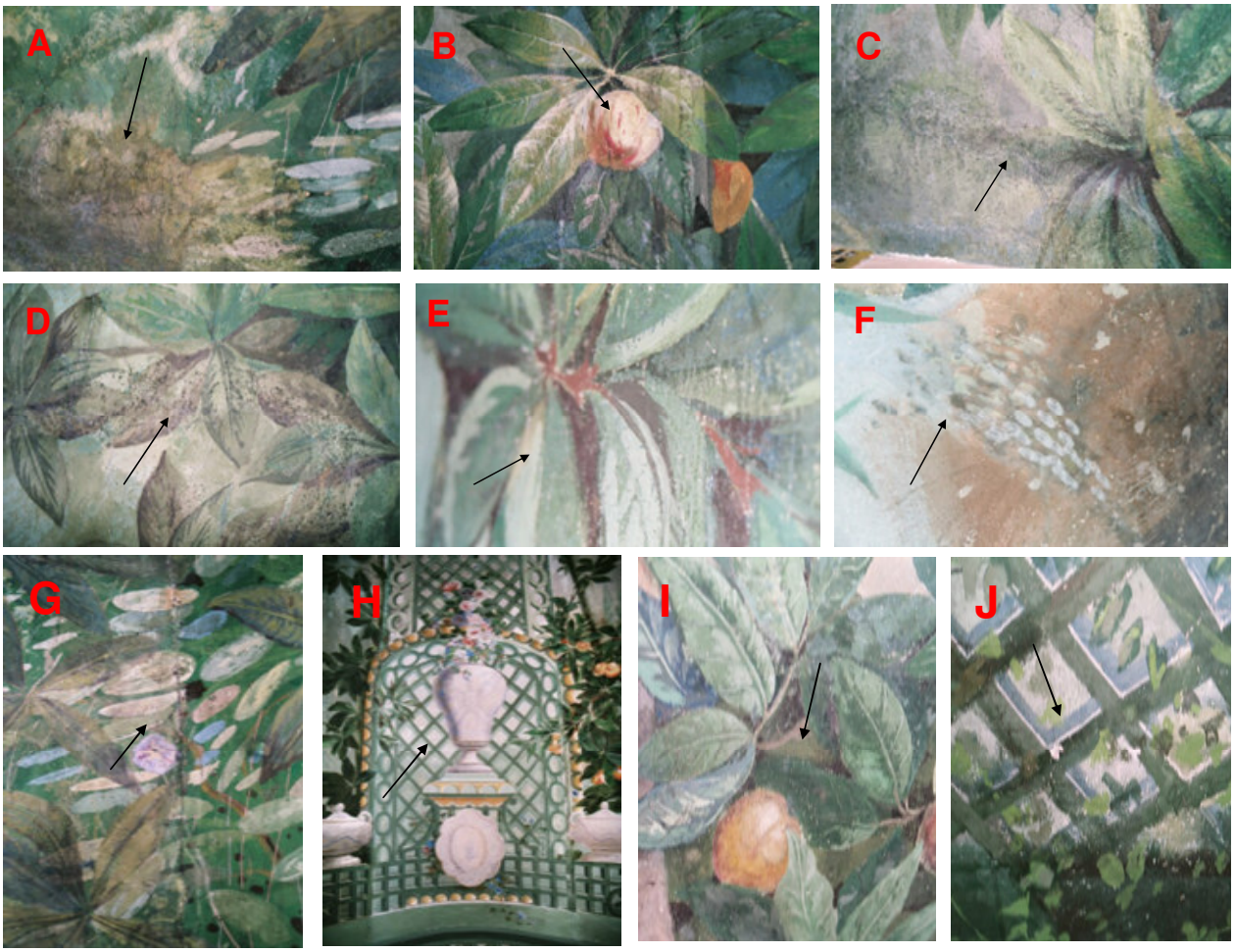

Figure 13 Pictures showing the location of sampling at the Berglzimmer in the Palace of Schönbrunn

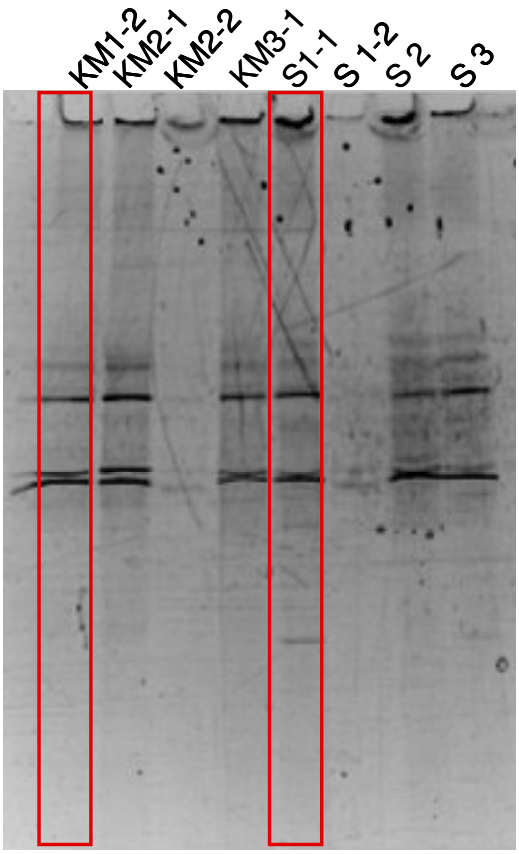

Figure 14 Detail of the ethidium-bromide-stained 16S rDNA DGGE community fingerprints of samples derived from mural paintings from the Palace of Schönbrunn. Lane 1: sample KM1-2, lane 2: sample KM2-1, lane 3: sample KM2-2, lane 4: sample KM3-1, lane 5: sample S1-1, lane 6: sample S1-2, lane 7: sample S2 and lane 8: sample S3. (shown denaturing gradient ranges from 30 to 55%).

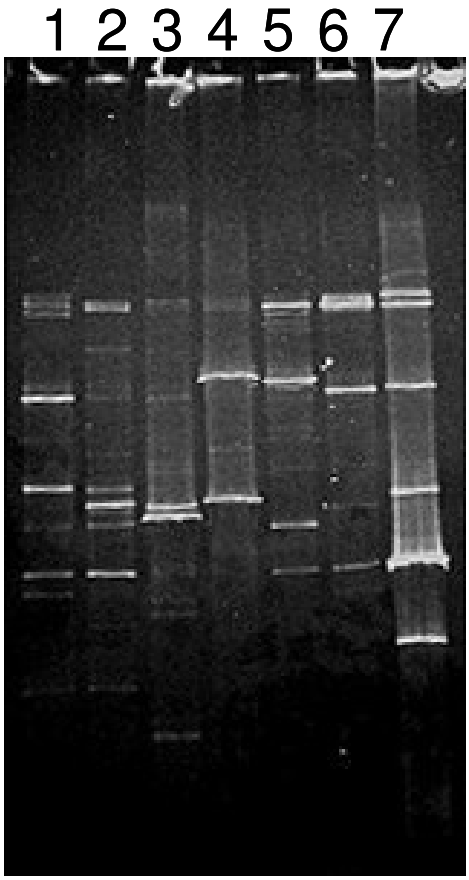

Figure 15 Detail of the ethidium-bromide-stained ITS1 DGGE community fingerprints of samples derived from mural paintings from the Palace of Schönbrunn: Lane 1: S1-1, lane 2: S2, lane 3: S3, lane 4: S4, lane 5: KM2-1, lane 6: KM3-1, lane 7: ITS1 Marker (band 1-6 from top to bottom: Band 1 *Alternaria alternate*; Band 2 *Chaetonium globosum*; Band 3 *Cladiosporum cladosporoides*; Band 4 *Eurotium chevalieri* and *Aspergillus hollandicus*; Band 5 *Aspergillus tereus*; Band 6 *Aspergillus versicolor*

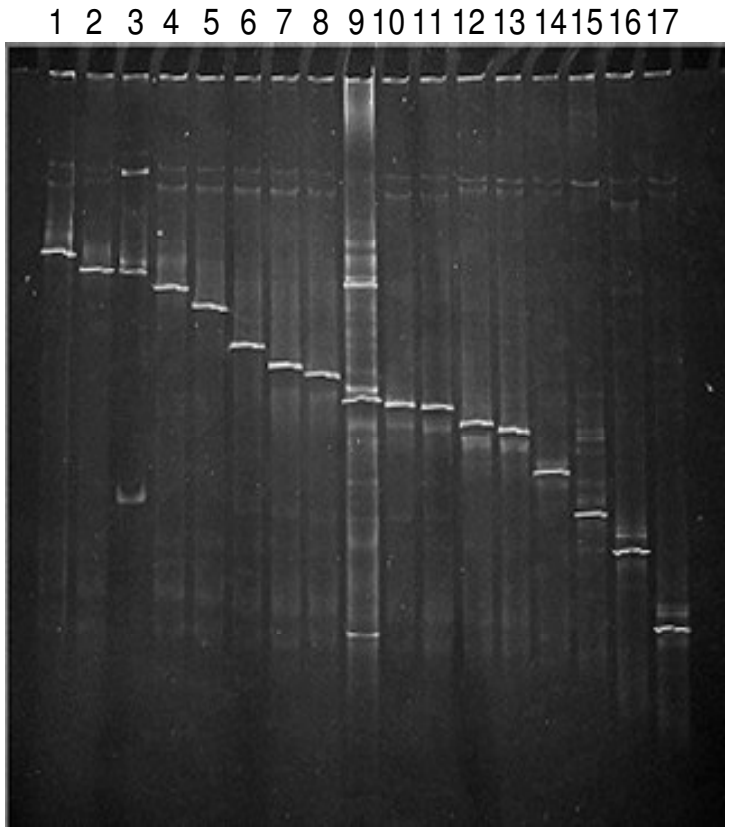

Figure 16 Details (from 30-55% of the denaturing gradient) of the ethidium bromide stained 16S rDNA DGGE fingerprint derived from clones obtained from sample S1-1. Lane 1: clone K3, lane 2: clone K42, lane 3: clone K7, lane 4: clone K19, lane 5: clone K16, lane 6: clone K20, lane 7: clone K12, lane 8: clone K36, lane 9: Community fingerprint of sample S1-1, lane 10: clone K5, lane11: clone K38, lane 12: clone K11, lane 13: clone K18, Lane 14: clone K37, lane 15: clone K30, lane 16: clone K2 and line 17: clone K39.

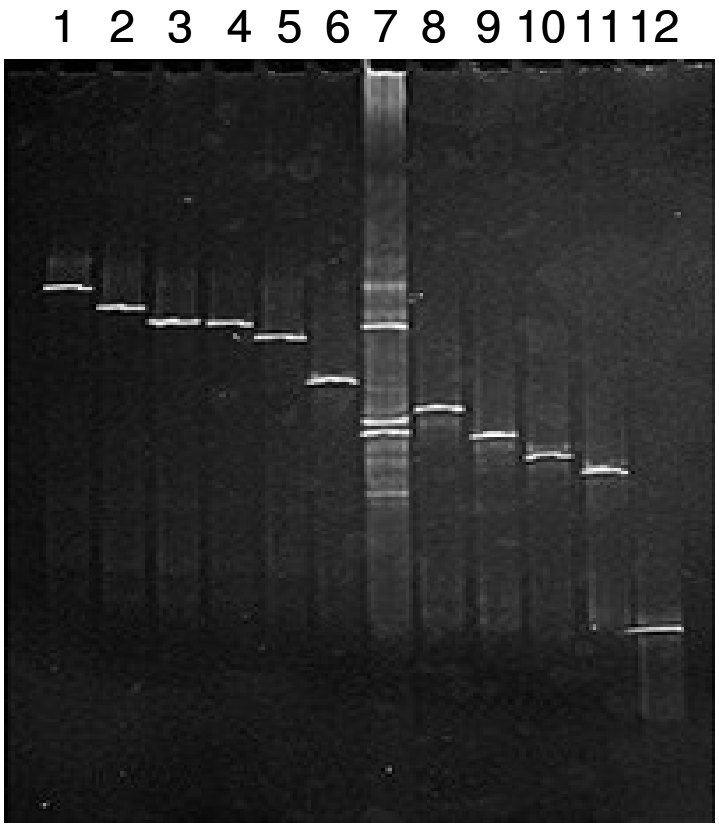

Figure 17 Details (from 30-55% of the denaturing gradient) of the ethidium bromide stained 16S rDNA DGGE fingerprint derived from clones obtained from sample KM1-1. Lane 1: clone K8, lane 2: clone K32, lane 3: clone K28, lane 4: clone K4, lane 5: clone K13, lane 6: clone K15, lane 7: Community fingerprint of sample KM1-1, lane 8: clone K10, lane 9: clone K26, lane 10: clone K5, lane11: clone K19, lane 12: clone K9.

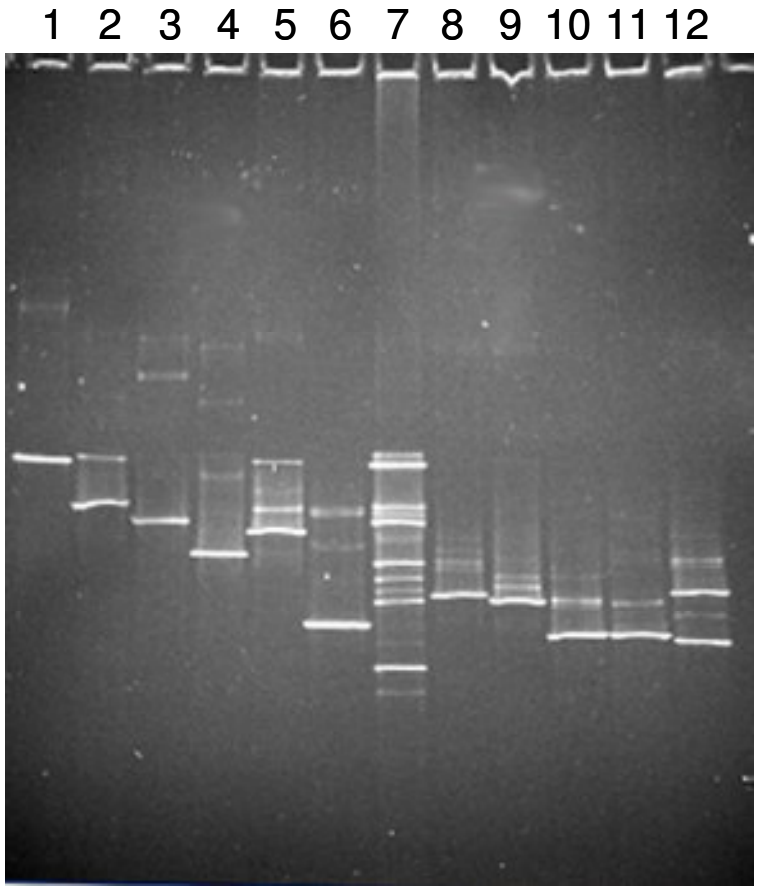

Figure 18 Details (from 30-50% of the denaturing gradient) of the ethidium bromide stained ITS1 rDNA DGGE fingerprint derived from clones obtained from sample S1-1. Lane 1: clone S13, lane 2: clone 28, lane 3: clone 17, lane 4: clone 35, lane 5: clone 52, lane 6: clone 58, lane 7 Community fingerprint of sample S1-1, lane 8: clone 68, lane 9: clone 18, lane 10: clone 56, lane 11: clone 12, lane 12: clone 75.

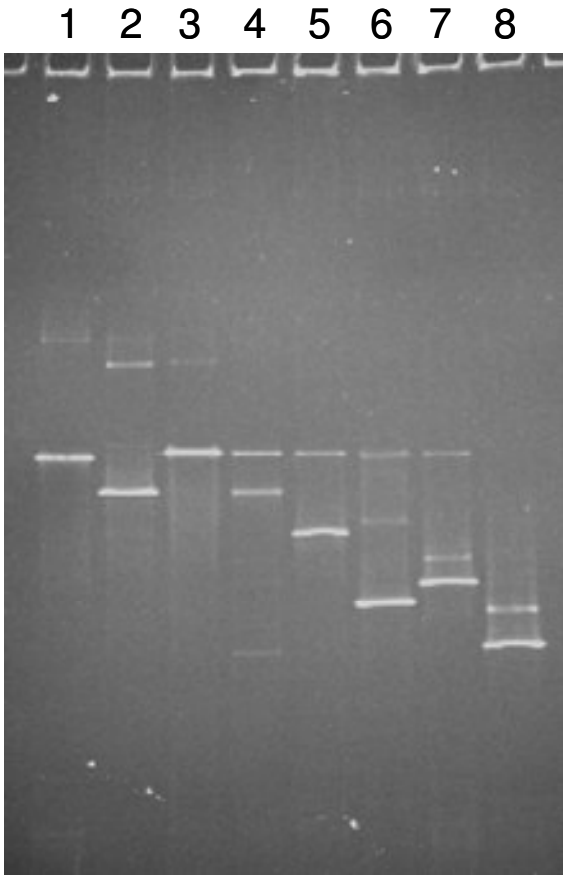

Figure 19 Details (from 30-50% of the denaturing gradient) of the ethidium bromide stained ITS1 rDNA DGGE fingerprint derived from clones obtained from sample KM2-1. Lane1: clone 28, lane 2: clone7, lane 3: clone30, lane 4: community fingerprint of sample KM2-1, lane 5: clone 6, lane 6: clone 29, lane 7: clone27, lane 8: clone36.

Sample	Origin
KM 1-1	Black biofilm on the wall **(A)**
KM 1-2	Surface of the painting showing a flower **(G)**
KM 2-1	Surface of the painting showing a fruit **(B)**
KM 2-2	Surface of the wall, behind the fireplace over the vent, showing a black biofilm **I**
KM3-1	Black colonies colonizing the wall behind the fireplace **(D)**
S 1-1	Surface of the painting showing green leaves, behind the fireplace **(I)**
S 1-2	Surface of the wall showing white-discoloured spots **(F)**
S 2-1	Surface of the painting showing green leaves, behind the fireplace **(E)**
S 3	from a detached area of the painting behind the fireplace **(J)**
S 4	surface of the painting showing a fruit **(H)**

Table 8 Origin of samples taken from the Berglzimmer in the Palace of Schönbrunn

Phylogen.group	Primer	Target region	Nucleotide sequence (5`-3`)	Reference
Eubacteria	341f	341-457	CCTACGGGAGGCAGCAG	Muyzer *et al*, 1993
Eubacteria	341fGC	341-457	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G CCT ACG GGA GGC AGC AG	Muyzer *et al*, 1993
Universal	907r	907-926	CCCCGTCAATTCATTTGAGTTT	Teske *et al*, 1996
Universal	518r	518-534	ATTACCGCGGCTGCTGG	Neefs *et al* ; 1990
CLONING	SP6		ATTTAGGTGACACTATAGAATAC	Promega
CLONING	T7		TAATACGACTCACTATAGGG	Promega
Fungi	ITS 1	ITS1	TCCGTAGGTGAACCTGCGG	White *et al*; 1990
Fungi	ITS 2	ITS1	GCTGCGTTCTTCATCGATG	White *et al*; 1990
Fungi	ITS1GC	ITS1	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG G TCCGTAGGTGAACCTGCGG	Michaelsen *et al*, 2005

Table 9 Sequences and target regions of primers used in this study

Sample S1-1	Clone no. [sequence length]		Closest identified phylogenetic relatives [EMBL accession numbers]	Similarity (%)	Accession number
Bacteria					
Gamma-Proteobacteria	K16	[635]	*Acinetobacter sp*. [AJ291842.1] IrT-R5M2-138 isolated from water samples of Monticello mill tailings.	99.3	AM161146
	K12	[635]	*Enterobacter sp* [AB114268.1]	99.6	AM161141
	K38	[630]	*Enterobacter sp*.	99.6	AM161152
	K5	[635]	*Enterobacter sp*. [AB114268.1] isolated from gramineous plants	99.8	AM161144
	K20	[633]	*Escherichia coli* [BA000007.2] enterohemorrhagic E.coli O157:H7 derived from the Sakai outbreak	99.6	AM161149
	K11	[655]	*Pantoea endophytica* [AF130956.1] Phylogenetic analysis of endophytic populations isolated from plants	99.6	AM161145
	K18	[654]	*Pantoea sp.* [AF130957.1]	99.6	AM161147
	K37	[656]	*Pantoea sp.* [AF130957.1]	99.5	AM161151
	K3	[627]	*Pseudomonas fulva* [D84015.1]	99.8	AM161143
Actinobacteria	K19	[632]	*Arthrobacter sp*. [AY641537.1]	99.3	AM161148
	K2	[617]	*Brevibacterium sp*. [AY577816.1]	99.6	AM161142
	K39	[637]	*Propionibacterium acnes* [AE017283.1]	99.6	AM161153
Bacillales	K36	[635]	*Bacillus pumilus* [AY548955.1]	98.2	AM161150

Table 10 Phylogenetic affinities of partial 16S rRNA coding sequences detected in mural paintings of the Palace of Schönbrunn. Sample S1-1.

Sample S1-1	Clone no. [sequence length]		Closest identified phylogenetic relatives [EMBL accession numbers]	Similarity (%)	Accession number
Fungi					
Ascomycota	S58	[371]	*Ascochyta sp*. CBS 110129 18 [AF520641.1] ITS sequences of wheat derived ascomycetes	99.07	AM159625
	S68	[414]	*Aspergillus restrictus* stra [AY373864.1] derived from analyzis of ten HVAC dust samples from different sources around the US	95.88	AM159626
	S35	[384]	*Cladosporium herbarum* 18S [AJ300333.1] recovered from common reed growing at Lake Constance	98.73	AM159629
	S17	[384]	*Cladosporium herbarum* ITS [AJ300333.1] colonize Phragmites australis (reed)	99.58	AM159622
	S56	[371]	*Eurotium amstelodami* strain [AY373885.1] derived from analyzis of ten HVAC dust samples from different sources around the US	99.55	AM159628
	S13	[343]	*Wallemia sebi* strain EXF-10 [AY328913.1]	99.42	AM159621
Basidiomycota	S12	[402]	*Coprinus cothurnatus* KACC49 [AF345820.1]	99.57	AM159623
	S52	[435]	*Fomitopsis pinicola* 18S rR [AJ560638.1] Fomitopsis pinicola (fr.) Karst is possibly an emergent human pathogen.	86.69	AM159630
	S18	[395]	*Schizopora paradoxa* strain [AF145571.1]	98.43	AM159627
Plants					
Viridiplantae	S28	[394]	Lycium ruthenicum 18S ribosomal DNA [AY880681.1]	99.2	AM159624

Table 11 Phylogenetic affinities of partial ITS1 sequences detected in mural paintings of the Palace of Schönbrunn. Sample S1-1.

Sample KM1-2	Clone no. [sequence length]		Closest identified phylogenetic relatives [EMBL accession numbers]	Similarity (%)	Accession number
Bacteria					
Gamma-Proteobacteria	K28	[654]	*Acinetobacter junii* [AB101444.1]	99.8	AM161163
	K4	[636]	*Acinetobacter junii* [AB101444.1] 16 S rDNA isolated from wild culex quinquefasciatus mosquito midgut.	99.5	AM161156
	K19	[653]	*Aeromonas schubertii* [X60416.1]; isolated from activated sludge	94.2	AM161155
	K26	[651]	*Enterobacter agglomerans* st [AF130960.1]) isolated from plants	99.8	AM161164
	K10	[657]	*Enterobacter sp*. [AB114268.1] 9B 2 isolated from gramineous plants.	99.8	AM161160
	K8	[654]	*Moraxella osloensis* [X95304.1] 16SrDNA isolated from activated sludge sewage treatment plants in Victoria, Australia.	99.6	AM161159
	K32	[654]	*Pseudomonas putida* [AE016774.1]	99.6	AM161157
Beta-Proteobacteria	K13	[623]	*Acidovorax sp.* [AY623930.1]	98.9	AM161161
	K15	[632]	*Acidovorax sp.* [AJ534865.1]	98.9	AM161162
Alpha-Proteobacteria	K9	[600]	*Rhodopila globiformis* [D86513.1] 16SrDNA isolated from acidic environments.	96.4	AM161158
Actinobacteria	K5	[657]	*Thermoactinomyces sacchari* [AF089890]16 SrDNA gene isolated from air samples; involved in hypersensitivity pneumonitis.	98.2	AM161154

Table 12 Phylogenetic affinities of partial 16S rRNA coding sequences detected in mural paintings of the Palace of Schönbrunn. Sample KM1-2

Sample KM2-1	Clone no. [sequence length]		Closest identified phylogenetic relatives [EMBL accession numbers]	Similarity (%)	Accession number
Fungi					
Ascomycota	K7	[487]	*Cladosporium sphaerospermum* [AJ244228.1]	99.5	AM159631
	K36	[374]	*Eurotium amstelodami* isolate [AF455536.1] Fungal biodiversity as found in nasal mucus	99.1	AM159632
	K29	[371]	*Ascochyta sp*. CBS 110129 18 [AF520641.1] ITS sequences of wheat derived ascomycetes	99.1	AM159633
	K30	[395]	*Alternaria tenuissima* [AY154712.1	98.8	AM159636
Basidiomycota	K28	[350]	*Trichosporon mucoides* [AY591346.1] Polymorphic internal transcribed spacer region 1 DNA sequences identify medically important yeasts	100.0	AM159634
	K27	[381]	*Schizophyllum commune* isolate [AY949987.1]	99.6	AM159635
Plants					
Viridiplantae	K6	[395]	*Lycium ruthenicum* 18S riboso [AY880681.1] from different geographical origins in China	98.8	AM159637

Table 13 Phylogenetic affinities of partial ITS1 sequences detected in mural paintings of the Palace of Schönbrunn. Sample Km2-1

II. Investigation of moderate and extreme halohilic microorgansims on mural paintings

1. Abstract

In the recent years mural paintings and stone works have been the subject of investigation as possible microniches of halophilic microorganisms. In the present study samples taken from an stone work (the medieval Chapel of St.Virgil, Austria) as well as from an artificial mural painting, created 7 years ago to study the dynamics of microbial communities on such substrates, were investigated for the presence of bacterial, archaeal and fungal colonization. We used a DNA extraction protocol which allowed the extraction of PCR-amplifiable DNA directly from sample materials. PCR analysis specific for the 16S rDNA (Archaea and Bacteria) and the Internal Transcribed Spacer 1 (ITS1) region (Fungi) were carried out with DNA extracts. The microbial communities were analysed by genetic fingerprinting using Denaturing Gradient Gel Electrophoresis (DGGE). For further phylogenetic analysis clone libraries were constructed from selected samples. Several moderately halophilic bacterial species could be detected as well as members of the extremely halophilic archaeal family *Halobacteriaceae*.

2. Introduction

Halophilic bacteria and archaea were thought to be found only in extreme environments like salt lakes or black smokers (Vreeland et al, 1998; Rothschild and Mancinelli 2001; Sass *et al*., 2001; Litchfield and Gillevet 2002; Oren 2002)**..** However, recently has been observed that mural paintings and stone works are a common habitat for extremely salt tolerant and moderate halophilic bacteria (see review Saiz-Jimenez and Laiz, 2000) and archaea (Rölleke *et al*, 1998; Piñar *et al*, 2001a).

Building stone and wall paintings contain a variety of hydroscopic salts, including carbonates, chlorides, nitrates, sulfates, etc, dispersed within the porous material or locally concentrated. These salts are solubilized and migrate with the water in and out of the stone. Due to changes in physical parameters, the so called salt efflorescences appear on the surface of paintings and stones and they can be considered as extremely saline environments building niches for settlement of

halophilic microorganisms. Oren (1994), studied the competition between archaea (*Halobacterium*) and halophilic bacteria (*Halomonas*), postulating that these two groups occupy different niches. Neutrophilic halophilic *Archaea (Halobacteria)* need higher salt concentrations but lower pH levels than bacteria (*Halomonas*) for growing. Nevertheless, in mural paintings the combination of moderately halophilic bacteria and neutrophilic halophilic archaea has been reported (Rölleke *et al.*,1998; Piñar *et al.*,2001a). It is assumed that in deteriorated mural paintings high variations in salinity and pH are found locally. Furthermore, the overlap of microorgansims with different growth requirements may be due to mixing of bioniches when taking samples.(Saiz-Jimenz and Laiz, 2000). This study should bring more clearance of which microorgansims are inhabiting salt efflorescences and their origin.

3. Materials and Methods

3.1. Sampling in the Chapel of St.Virgil (stone work)

The St. Virgil Chapel, (Vienna, Austria) dates back from the beginning of the 14 century. It is located under the ruins of St. Mary Magdaleine Chapel (Fig.20A). The St. Virgil Chapel was originally created as a tomb. After the Chapel of St. Mary Magdaleine was destroyed in 1781 by fire and demolished, the underground room was abandoned. The chapel was rediscovered in an excavation in 1972. The rectangular room is 10.5 m long and 6 m wide. The mortar and rubble walls are 1.5 m thick, containing 6 recesses with pointed arches, of which one was removed from where the present entrance is situated. They are decorated with large, red crosses painted onto white plaster (Fig.20B). One sample was taken from this mural painting (VK5). The whole chapel is covered by salt efflorescences visible by naked eye. A sample (VK3) (Fig.20C) was taken directly from a salt efflorescence. An addition sample was taken from detached wall material collected on paper placed on the floor (VK1).

3.2. Sampling from an artificial mural painting

In the frame of a project started by Dr. Busse and Prof. Lubitz, from the University of Vienna, Austria, an artificial wall painting was (in 1998 year) created by members of the masterschool for restoration and conservation of the Academy of Building Arts in Vienna, Austria (Klose, 2001) (Fig.21). The artificial wall painting was done in fresco technique using five different pigments and 80 different investigation

areas. Defined areas were inoculated with a combination of different bacterial strains (cocktail α, β, γ) (Table 15) formally isolated from damaged medieval wall paintings and was exposed in the Chapel of St.Virgil. After 2 months 3 different biocides (Metatin101, Metatin 58-10 and Metatin 58-10/101) used in restoration were applied with a brush. (for details see Klose, 2001). Three samples (VK6, VK7, VK9) were taken from different areas of this artificial wall painting (Table 14).

3.3. DNA extraction and purification

DNA was extracted directly from wall painting samples by using the Fast DNA SPIN kit for soil (Bio 101). The protocol of the manufacturer was modified as follows. Powder obtained from wall painting samples was placed in the MULTIMIX2 tissue tube. 978 µl sodium phosphate buffer and 122 µl of the MT buffer (Bio 101) were added. The mixture was then beadbeaten two times for 1 min with one intervening minute on ice and centrifuged at 14000xg for 2min. The PPS reagent and the binding matrix suspension (Bio 101) were applied to the supernatant following the instructions of the manufacturer. The resulting suspension was transferred to a spin filter and centrifuged at 14000 x g for 1min. DNA was washed twice with 500µl of the SEWS-M solution (Bio 101) and eluted from the binding matrix with 160 µl DES (Dnase/Pyrogene free water).

The extracted DNA was further purified with the QIAamp Viral RNA mini kit (Quiagen) and finally eluted from the silica column with 100 µl ddH_2O.

3.4. PCR Amplification

3.4.1. PCR amplification of 16S rDNA fragments (Eubacteria)

For all reactions 2x PCR Master Mix from Promega was used following the manufacturers instructions. PCR amplification for genetic fingerprinting was carried out with primer 341f (Muyzer *et al.*, 1993) specific for eubacterial 16SrDNA and 907r (Teske *et al.*, 1996) a universal primer for 16SrDNA. This step was followed by a semi-nested PCR performed with primers 341fGC (Muyzer *et al.*, 1993) and 518r (Neefs *et al.*; 1990). The forward primer possessed a 40-base GC clamp at its 5`end which stabilizes the melting behaviour of DNA fragments in DGGE analysis (Muyzer *et al.*, 1993). For the first round of PCR 25µl volume and 2µl template were used. For nested PCR 100µl volumes separated on two tubes to 50µl and 3.5µl template to each tube were applied.

PCR were performed in a Robocycler with the following thermocycling program:5 min denaturation at 95°C, followed by 30 cycles each consisting of 1 min denaturation at

95℃, 1 min annealing at 55℃ and 1 min extension at 72℃. Five minutes at 72℃ were used as final extension step. Ten µl of each PCR product were run on a 2% agarose gel for ~ 30 min at 200V, stained with ethidium bromide and visualized by a UVP documentation system.

3.4.2. PCR amplification of ITS1 Region (Fungi)

PCR was carried out as described above using primers ITS1 (White *et al*., 1990) and ITS2 (White *et al*., 1990) specific for the internal transcribed spacer region 1 (ITS) located between the 18S and 5.8SrDNA (White *et al*., 1990). For 2nd round of PCR the ITS1 primer had a GC clamp on its 5`end (Michaelsen *et al*., 2005). The thermocycling program for the first round was as followed: 5min at 95℃, followed by 35 cycles 1min at 95℃, 1min at 62℃, 1min at 72℃ and a final extension step 5 min at 72℃. For PCR with the GC primer annealing temperature was set at 60℃ (Michaelsen *et al*., 2005).

3.4.3. PCR amplification of archaeal 16SrDNA

Looking for Archaea PCR with primers ARC344 (forward) (Raskin *et al*., 1994) and ARC915 (reverse) (Raskin *et al*., 1994) specific for archaeal 16SrDNA was performed using the following conditions: 5 min denaturation at 95℃, followed by 40 cycles consisting of 1 min denaturation at 95℃, 1 min primer annealing at 60℃ and 1 min extension at 72℃, with a final extension step af 72℃ for 5 min (Piñar et al, 2001a).

The 2nd round was done with universal primer 518r carrying the GC clamp (Muyzer *et al*., 1993) and the Archaea specific primer ARC344. The same thermocycling program was used as described for amplification of 16SrDNA.

3.5. DGGE- Denaturing Gradient Gel Electrophoresis

For genetic fingerprinting 100µl of semi-nested PCR products were precipitated in 1 ml 96% ethanol (–20^{o}C overnight), resuspended in 15µl ddH_2O water (Sigma) and separated by DGGE .

Gels screening bacterial communities and clone libraries were run in 0.5xTAE buffer [20mM Tris, 10mM acetate, 0.5mM Na_2EDTA; pH 7.8] for 3.5 hours at 200V and 60℃ in a D GENE System (BioRad, Munich, Germany) (Muyzer, *et al*; 1993). A denaturing gradient from 30 to 55% of urea and formamide in an 8% (w/v) polyacrylamide gel (BioRad, Munich, Germany) was used.

Archaeal communities and clone libraries were screened on gels containing 25 to 55% of denaturants for 3 hours at 200V and 60°C.

After completion of electrophoresis, gels were stained in an ethidium bromide solution and documented with an UVP documentation system.

3.6. Construction of clone libraries

For the construction of clone libraries for each sample 2 x 3.5µl DNA templates were amplified in 2 x 50µl volumes using the primers 341f and 907r for Bacteria and ARC344 and ARC915 for Archaea. The PCR products were pooled and purified using the QIAquick PCR Purification Kit Protocol (Qiagen, Hilden, Germany) and resuspended in 30µl ddH_2O water (Sigma).

5.5µl of purified PCR product were ligated into the pGEM –T easy Vector system (Promega, Vienna, Austria) following the instructions of the manufacturer. The ligation products were transformed into *E.coli* XL Blue^{Tc}, which allows the identification of recombinants (white colonies) on an indicator LB medium containing ampiciline (100µg/ml), tetracycline (10µg/ml), X-Gal (5 bromr-4-cloro-3-indolyl-ß-D-galactorpyranoside; 0.1 mM) and IPTG (isopropyl-ß-D-thiogalactopyranoside: 0.2 mM) (Sambrook *et al.*, 1989).

3.7. Screening of clones by DGGE and PCR

Of each clone library about 50 clones were harvested and resuspended in 50µl TE buffer [10mM TrisHCl, 1mM EDTA; pH 8.0] followed by three steps of freeze and thawing.

Screening of the clone libraries by PCR and DGGE was performed as described by Schabereiter-Gurtner and coworkers (2001a) as follows: To amplify the inserts the vector specific primers SP6 and T7 were utilized. For PCR the following conditions were used: 5 min at 95°C denaturation followed by 35 cycles each consisting of 1 min at 95°C, 40 sec at 46°C and 1 min at 72°C. Three minutes were used as final extension step.

A nested PCR for DGGE analysis was carried out with the primer pair 341fGC and 518r for bacterial clone libraries and ARC344 and ARC915 for fungal clones. PCR was performed as described above in 25µl volumes. Ten µl of each PCR product were run on a 2% agarose gel for ~ 30 min at 200V, stained with ethidium bromide and visualized via an UVP documentation system (BioRad quantity one).

The rest was used for DGGE analysis under the same conditions as described above. Clones showing different motility behaviour in DGGE analysis were selected for sequencing.

3.8. Sequencing and identification

100µl of PCR products amplified with primers SP6 and T7 were purified with a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Sequencing was done by VBC (Vienna Bio Centre, Austria) and the obtained sequences were compared with know sequences in the EMBL database using the search tool FASTA (Pearson, 1994).

4. Results and discussion

4.1. DNA extraction

Three samples (VK1, VK3 andV5) from different areas of the walls from the Chapel of St. Virgil, Vienna, as well as three samples taken from an artificial wall painting were directly subjected to DNA extraction by using the protocol described in the section of methods. All samples rendered pure DNA able to be amplifiable by PCR analysis. Subsequently, samples were screened for the presence of bacteria, archaea and fungi. PCR analysis using eubacterial- and archaeal-specific primers showed positive results for all samples indicating the presence of these microorganisms in the samples (data non-shown). By using fungal-specific primers no amplification could be observed.

4.2. Study of microbial communities by DGGE fingerprint analysis

DGGE analysis was performed with all samples to get information on the diversity and complexity of the species composition. The DGGE profiles obtained from all samples by using eubacterial and archaeal primers are shown in Fig. 22. Fig. 22A shows the fingerprints obtained when using eubacterial primers. Samples (VK1, VK3, VK5) taken from different wall locations in the chapel of St. Virgil showed different band patterns containing about 6 main individual bands. In contrary, fingerprints obtained from samples taken from the artificial wall painting showed rather similar bands. Samples VK6 and VK7 showed exactly the same band-pattern containing four main individual bands. Even though VK6 was derived from an area inoculated with a bacterial cocktail of five different strains and different biocide were applied to both areas. The quadrant from where sample VK7 was scraped off had not been infected with bacterial cultures at all. Sample VK9 was taken from a hole produced in a former

sampling procedure and produced two additional bands in the band-pattern. The community fingerprint from sample VK3 showed a more diverse band pattern showing about 10 different bands (Fig. 23).

The same approach was carried out using archaeal-primers (Fig.22B) (Fig.24). The archaeal fingerprints showed the same similarities as those derived when analysing bacteria. Samples derived from the Chapel of St. Virgil showed more complex profiles (3-4 individual bands) different from each other and from those derived from samples taken from the artificial mural painting. Again, fingerprints derived from the artificial mural painting samples showed to be very similar. This shows correlation of the bacterial and archaeal communities present on the samples.

4.3. Construction and screening of bacterial and archaeal rDNA libraries

In parallel to DGGE analysis of 200 bp 16S rDNA bacterial and archaeal fragments, cloning of environmental PCR products containing approximately 600 bp was carried out in order to avoid excision of bands from the band patterns for obtaining phylogenetic identification. Sample VK3 (taken directly from a salt efflorescence) was chosen for this purpose. Two clone libraries were constructed, one for bacterial sequences and one for archaeal sequences. From each clone library a total of 50 clones were picked and screened with DGGE as described in the section of method. Clones differing from each other were selected for sequencing.

4.3.1. Phylogenetic affiliations of members of the bacterial community.

Sequence analyses of selected clones containing bacterial sequences (Fig. 25) were performed to obtain information on the identity of the corresponding organisms. Comparison of sequences obtained in this study with sequences of known bacteria in the EMBL database using the search tool FASTA is summarised in Table 16. For sample VK3 eleven different clones containing bacterial sequences could be obtained sharing similarities values in between 92.0 % and 100 % with sequences from the EMBL database. Most of the clones derived from sample VK3 gave high score similarities with members of the gamma-proteobacteria as *Acinetobacter junii* (clone K6), *Pantoea endophytica* (clone K10), *Enterobacter sp*. (clones K24 and K36), *Salinisphaera sp*.(clone K25) and members of actinobacteria as *Rubrobacter sp*. (clones K7 and K8) and *Thermomonospora fusca* (clones K22 and K39). One member of Firmicutes (*Bacillus sp.*; clone K13) and one of the Bacteroidetes group (*Bacteroidetes bacterium*; clone K41) were detected as well.

4.3.2. Phylogenetic affiliations of members of the archaeal community.

For sample VK3 eight different clones containing archaeal sequences could be obtained (Fig.26) sharing similarities values in between 95.2% and 99.5% with sequences from the EMBL database. Phylogenetic affiliation results are shown in Table 17. All sequenced clones gave high score similarities with members of the family *Halobacteriaceae*: *Halococcus morrhuae* (K16, K45); *Halococcus salifodinae* (K12, K46), *Halococcus dombrowskii* (K25, K23) and *Halobacterium sp.* (K22, K14).

5. Discussion

Salt efflorescences in monuments mimic the conditions found in extreme habitats favouring the growth of halophilic microorganisms. Indeed, the presence of extremely and moderate halophilic species on mural paintings and stone works has been reported in several studies (see review Saiz-Jimenez and Laiz, 2000). In this study we show that moderately halophilic bacteria and neutrophilic halophilic archaea cohabit the walls of the Chapel of St. Virgil as well as the surface of an artificial wall painting harboured inside this Chapel. Sample VK3 directly taken from a salt efflorescence on the walls of the Chapel was chosen for phylogenetic analysis. Furthermore, in a parallel study, this sample was subjected to cultivation assays using culture media containing 20 % NaCl to isolate halophilic microorganisms. Five different strains identified as new members of the *Halobacillus* genus were isolated (Ripka *et al*, 2005). No archaea could be isolated from this sample.

5.1. Detection of Moderately halophilic bacteria

Four members of moderately halophilic Bacteria could be detected and identified by using molecular methods on sample VK3: *Salinisphaera sp*, *Bacillus sp*, *Rubrobacter sp*. and *Bacteroidetes bacterium.*

Members of the genus *Rubrobacter* were previously detected in mural paintings located in two different locations, the Castle of Herberstein, Styria, Austria and the Church of St. Anna im Feld, Germany (Schabereiter-Gurtner *et al*., 2001b). The presence of *Rubrobacter sp.* on these mural paintings was associated with rosy discolouration of the masonry and lime wall paintings due to pigment production (as chlorophyll, carotin, carotenoid, melanin) as a means for protection against exposure to UV light and chemicals (Agnanstidis *et al*., 1992). Cultivation efforts from these samples render no positive results (Heyrman *et al*., 1999). Species of the genus *Rubrobacter*, as *Rubrobacter radiotolerans* and *Rubrobacter xylanophilus* showed to

be slightly halotolerant as growth at 6.0% NaCl could be determined. Both species show a temperature optimum of 60°C and resistance to desiccation. This temperature conditions are not present on mural paintings. However, one has to keep in mind that in the present study only culture independent methods were used, thus nothing is known about the physiological properties of these bacteria. Schabereiter-Gurtner and coworkers (2001b) proposed that a possible resistance to desiccation might be a selective advantage for growth of members of the genus *Rubrobacter* on masonry and wall paintings.

The genus *Salinisphaera* consists of one defined species called *Salinisphaera shabanensis* isolated from the brine–seawater interface of the Shaban Deep, northern Red Sea. (Antunes *et al*., 2003). *Salinisphaera shabanensis* is a gram-negative, monotrichous coccus that shows a remarkable physiological flexibility, as could be seen by the quite broad growth ranges for oxygen, temperature, NaCl (1% to 28% NaCl), and, to a smaller degree pH (4.0 and 8.0).

In a parallel study performed with sample VK3, cultivation assays were applied using culture media containing 20 % NaCl to isolate halophilic microorganisms. Five different strains identified as new members of the *Halobacillus* genus were isolated (Ripka *et al*., 2005). However, these microorganisms could not be detected by using molecular methods. No clone harbouring this sequence could be screened from the clone library. This fact is showing once more the disparity in between data obtained by using molecular- and cultivation-dependent methods (Laiz *et al*., 2003) indicating the need of combination of both methods for a complete overview of microbial communities in natural environments.

4.4. Extremely halophilic Archaea

In this study it was possible to identify 8 different clones showing high scores similarity with the archaeal order of the *Halobacteriales*. This order contains the extreme halophilic archaea which are highly specialized microorganisms, most of which will not grow at total salt concentrations below 2.5–3 M. When suspended in solutions containing less than 1–2 M salt, cells are irreversibly damaged, and many species will lyse (Oren, 1994). Due to the bright red, orange, or purple coloration of most representatives of the group and to the extremely high community densities at which these archaea may develop the presence of dense communities of members of the *Halobacteriales* often can be observed with the unaided eye (Oren, 2002). *Halococcus dombrowskii* and *Halococcus salifodinae* are two extremely halophilic

species isolated from pieces of dry rock salt (Denner *et al*., 1994; Stan-Lotter *et al*., 2002)**.** *Halococcus dombrowskii* shows an optimum growth in the presence of NaCl concentrations ranging from 20 to 30%. No growth of this strain occurred in media containing <15% NaCl or pH values above 9.0 or below 5.1. (Stan-Lotter *et al*., 2002). *Halococcus salifodinae* grows between pH 6.8 and 9.5. Optimal growth occurred at NaCl concentrations of 20-25%; no growth occurs at NaCl concentrations below 15% (Denner *et al*., 1994).

These strains were isolated from Austrian salt mines where the deposition of salts is thought to have occurred during the Permian period (225-280 million years ago) (Stan-Lotter *et al*., 1999, 2002; Denner *et al*., 1994). Large numbers of halophilic archaea from salt deposits in Bad Ischl (Austria) and Berchtesgaden (Germany) were isolated in this study**.** A new species of *Halococcus*, *Halococcus salifodinae* was isolated and reisolated from geographically widely separated salt deposits of similar geographical age (Stan-Lotter *et al*., 1999). Stan-Lotter and coworkers (2002) therefore suggested that these communities may be remnants of populations of halophilic archaea that inhabited the ancient hypersaline seas. However, it is difficult to assess whether the bacteria isolated were indeed trapped within the crystals at the time of the formation of the salt deposit, or these bacteria grew more recently and may have entered the salt during disturbances of the salt layer, either by natural or human activity. The archaeal communities observed in the samples taken from the stone work of the chapel itself (VK1, VK5) are similar but differ from the samples taken from the artificial wall painting (VK6,VK7,VK9) which are highly similar among each other again. This may be a hint for the origine of archaea in mural paintings. As mentioned above halophilic archaea are often found in old salt deposits (*H.salifodinae, H.dombrowskii*) and are supposed to endure over a long period of time within salt crystals. Thus they seem to be brought in through building material itself.

Halococcus morrhuae has already been detected on mural paintings before (Piñar *et al*., 2001; Rölleke *et al*., 1998) but no strains could be isolated so far from these substrates. This species was brought in connection with rosy discolouration of the paintings (Rölleke *et al*., 1998). Archaeal communities were thought to settle there due to contamination from stored goods.

4.5. *Archaea* versus *Bacteria* ?

Oren (1994) studied the competition between archaea (Halobacterium) and halophilic bacteria (Halomonas), postulating that these two groups occupy different niches. Archaea of the group of Halobacteriacae need higher salt concentrations and lower pH levels than bacteria (Halomonas). However, in this study as well as in previous works (Rölleke et al., 1998; Piñar et al., 2001a; 2001b) the combination of bacteria and archaea showing different growth requirements in mural paintings and stone works has been reported.

There are two main explanations for the simultaneous presence of archaea and bacteria with different growth requirements in natural samples. First, deteriorated mural paintings present locally high variations in salinity and pH, which could be the reason for the co-habitation of archaea and bacteria on these substrates. The second theory to explain the overlapping of microorganisms presenting different growth requirements may be the mixing of bioniches when taking samples (Saiz-Jimenz and Laiz, 2000).

In this study, the requirements as salt concentrations for growth and pH ranges of some of the detected bacteria and archaea are rather similar. Salinisphaera sp., for example, shows to be highly tolerant to salt concentrations up to 28% NaCl (w/v) as mentioned above. This species has therefore the same salt requirements as the observed Archaea. In turn, the archaeal species Halococcus dombrowskii and salifodinae are able to grow at higher pH levels than Salinisphaera sp. and thus they are better adapted to growth on the basic environment presented by mural paintings.

The similarities and correlations of DGGE bands in the community fingerprints observed in this study may be an additional evidence of this co-habitation. Surprisingly, samples VK6 and VK7 showed exactly the same band-patterns for amplified bacterial and archaeal sequences. These samples were derived from areas inoculated with different bacterial cocktails. Furthermore, different biocides were applied to both areas. Therefore, it can be assumed that either after the treatment with biocides microbial growth of the inoculated strains was inhibited and an almost homogenous bioflora developed across the painting afterwards, or that this microbial (bacterial and archaeal) communities originated from the building material itself. It is hardly possible that in all samples taken the same mixture of bioniches had occurred.

As the *Halobactericaeae* are known to be all chemoorganotrophic species and need organic compounds to survive, bacteria and archaea may perhaps interact and provide nutrients for each other to grow.
These results may give a hint for coexistence of the detected archaea and bacteria in hypersaline environments.

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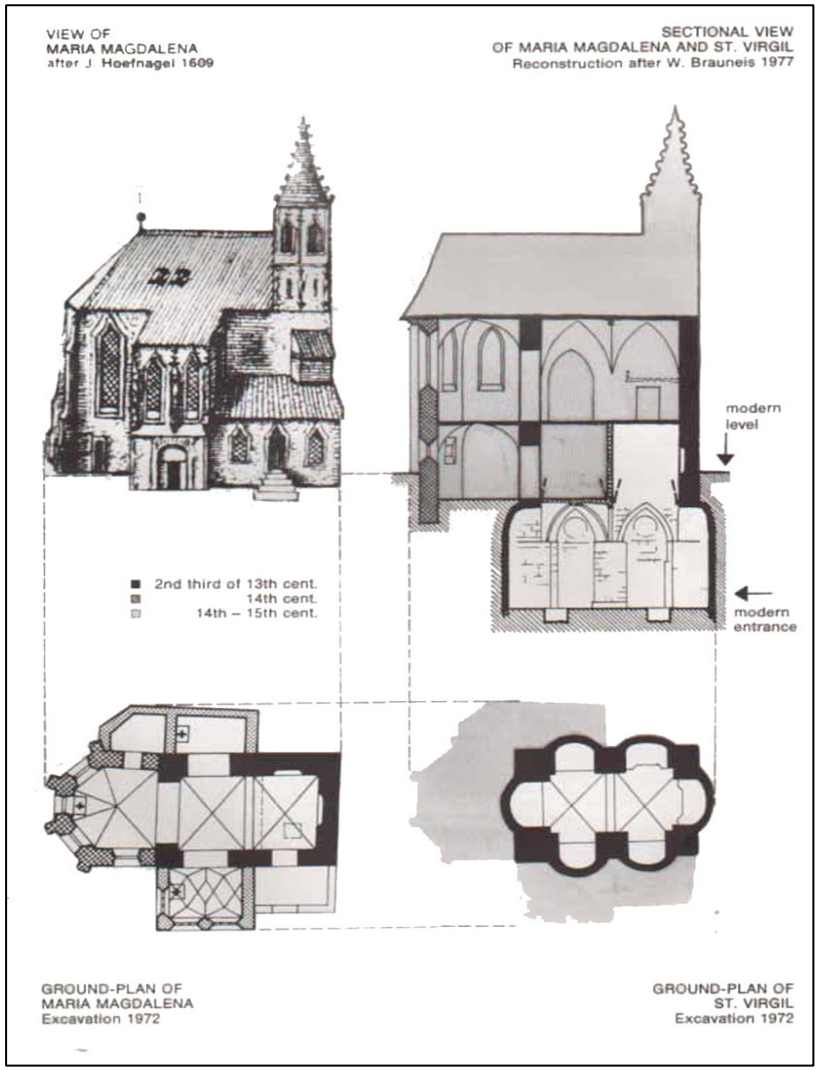
A



B

C

Figure 20 A Picture showing the location and ground plan of St.Virgil; **B** Red wheeled cross painted onto white plaster; **C** Salt efflorescence on the stone work of the chapel

Table 14 Origin of samples taken from artificial wall painting

Quadrant	Sample	Treatment
Iα	VK6 Sample taken with scalpel over a green colored area with red spots	Cocktail α, Biocide Metatin 101
IIIØ	Vk7 a piece of the whole wall painting: painting + support material was taken	No cocktail , Biocide Metatin 58-10
IIIβ	VK9 Sample taken with scalpel from a hole produced in a former sampling procedure. Area with white pigmentation.	Cocktail β, Biocide Metatin 58-10

Table 15 Phylogenetic affiliation of inoculated strains

Cocktail	Strain	Length [bp]	Highest 16SrDNA similarity	Similarity [%]
Cocktail α	D-1, 1a	1212	*Agrococcus citreus*	100
	D7	1430	*Micrococcus luteus*	98.7
	D3	680	*Bacillus licheniformis*	93.6
	D-4, 2	1239	*Clavibacter michiganensis*	92.7
	D-2, 4	1336	*Moraxella osloensis*	97.5
Cocktail β	5-2	726	*Bacillus pumilis*	96.7
	4-0	941	*Micrococcus luteus*	96.0
	1a-1b	950	*Agromyces ramosum*	97.9
	3a-1	517	*Cellulomonas*	95.0-95.7

Figure 21 Artificial wall painting in the Chapel of St.Virgil

Sample VK3	Clone no. [sequence length]		Closest identified phylogenetic relatives [EMBL accession numbers]	Similarity (%)	Accession number
Bacteria					
Gamma-Proteobacteria	K6	[657]	*Acinetobacter junii* [AB101444.1] score similarity with *Acinetobacter junii* 16SrDNA isolated from activated sludge.	99.8	AM161165
	K10	[633]	*Pantoea endophytica* [AF130956.1] isolated from plants	99.5	AM161168
	K24	[633]	*Enterobacter sp* [AB114268.1] [AY689062.1] [AY689047.1]	99.3	AM161171
	K25	[634]	*Salinisphaera sp*. ARD M17 [AB167073.1] Distribution of euryhaline halophilic microorganisms	97.8	AM161172
	K36	[627]	*Enterobacter sp* [AB114268.1] [AY689062.1] [AY689047.1]	100.0	AM161173
Actinobacteria	K7	[694]	*Rubrobacter sp* [AB166956.1] Distribution of euryhaline halophilic microorganisms	97.7	AM161166
	K8	[630]	*Rubrobacter sp*. [AB166956.1]	97.8	AM161167
	K22	[626]	*Thermomonospora fusca* [AF002264.1]	92.0	AM161170
Bacillales	K13	[656]	*Bacillus sp*. MB-11 [AF326360.1]	98.9	AM161169
Bacteroidetes group	K41	[625]	*Bacteroidetes bacterium* [AY553111.1] [AY553110.1] [AY553122.1] isolated from activated sludge; Halotolerant Aerobic Heterotrophic Bacteria from the Great Salt Plains of Oklahoma	97.4	AM161175
Acidobacteria	K39	[627]	*Bacterium Ellin*5115 [AY234532.1] Widespread and Previously Uncultured Soil Bacteria	92.0	AM161174

Table 16 Results clone library Bacteria: Phylogenetic affinities of partial 16S rRNA coding sequences detected in stone work of the Chapel of St.Virgil

Sample VK3	Clone no. [sequence length]		Closest identified phylogenetic relatives [EMBL accession numbers]	Similarity (%)	Accession number
Archaea					
Halobacteriaceae	K14	[688]	*Halobacterium sp*. NCIMB 718 [AB074300.1]	95.2	AM159641
	K22	[689]	*Halobacterium sp*. NCIMB 734 [AB074302.1] an extreme halophile in the order *Halobacteriales*	99.0	AM159640
	K25	[690]	*Halococcus dombrowskii* [AJ420376.1] *Halococcus dombrowskii sp. nov*., an archaeal isolate from a Permian alpine salt deposit	97.8	AM159642
	K23	[685]	*Halococcus dombrowskii* par [AJ420376.1]	98.4	AM159645
	K45	[687]	*Halococcus morrhuae* ribosomal RNA operon [X72588.1]	95.3	AM159643
	K16	[692]	*Halococcus morrhuae* ribosomal RNA operon [X72588.1]	96.1	AM159638
	K46	[685]	*Halococcus salifodinae* 16S [AJ131458.1]	97.7	AM159644
	K12	[692]	*Halococcus salifodinae* 16S [AJ131458.1] *Halococcus salifodinae* spec. nov., an Archaeal isolate from an Austrian salt mine	99.5	AM159639
			Halococcus sp. 16S ribosomal [Z28387.1] *Halococcus salifodinae spec. nov.*, an Archaeal isolate from an Austrian salt mine	99.5	

Table 17 Results clone library Archaea: Phylogenetic affinities of partial 16S rRNA coding sequences detected in stone work of the Chapel of St.Virgil

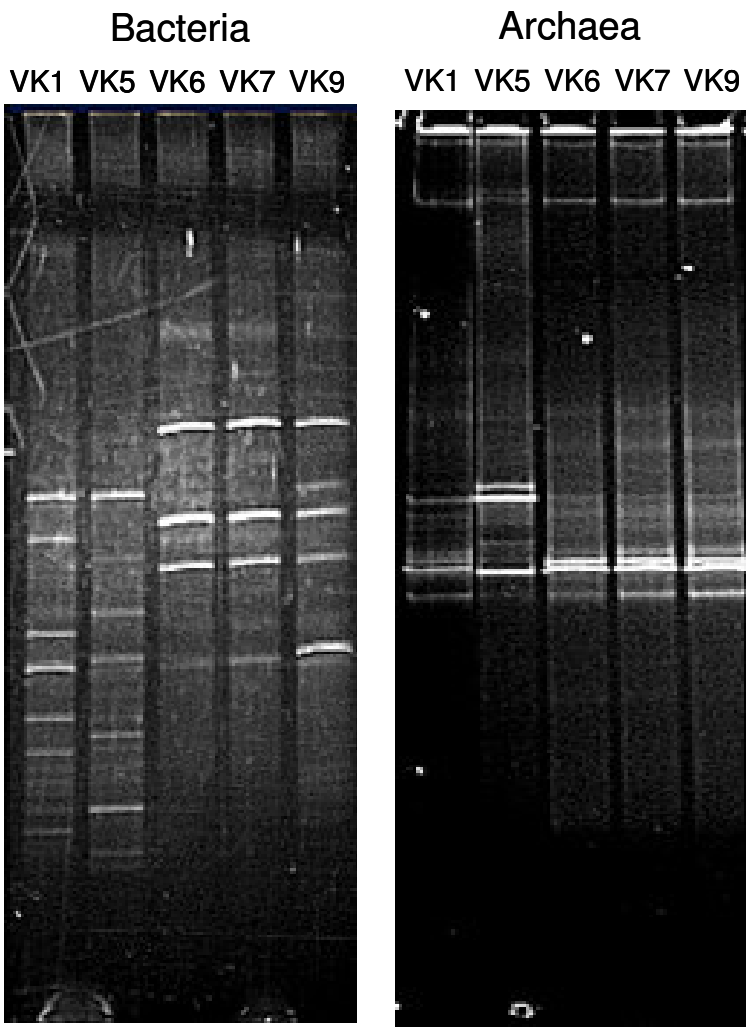


Figure 22 DGGE showing community fingerprints: **A** DGGE 30-55% showing bacterial community fingerprint; **B** DGGE 25-55% showing archaeal community fingerprint

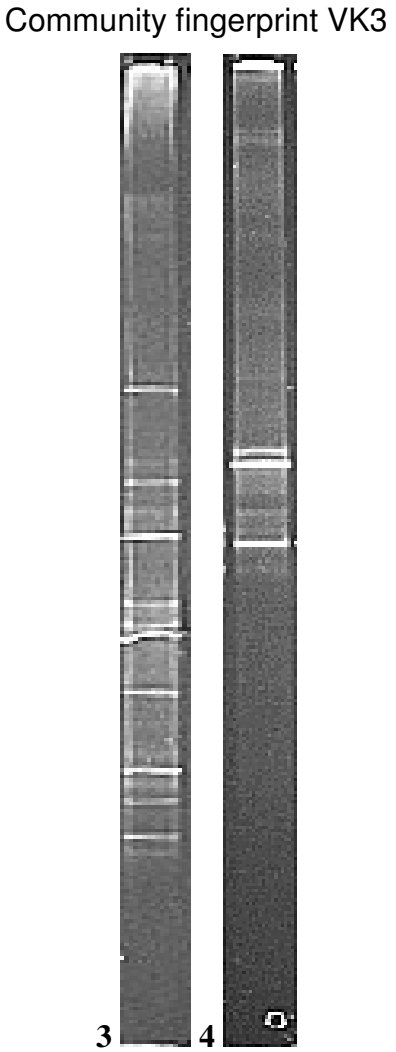


Figure 23 DGGE (30-55%) showing bacterial community fingerprint of sample VK3
Figure 24 DGGE (25-55%) showing archaeal community fingerprint of sample VK3

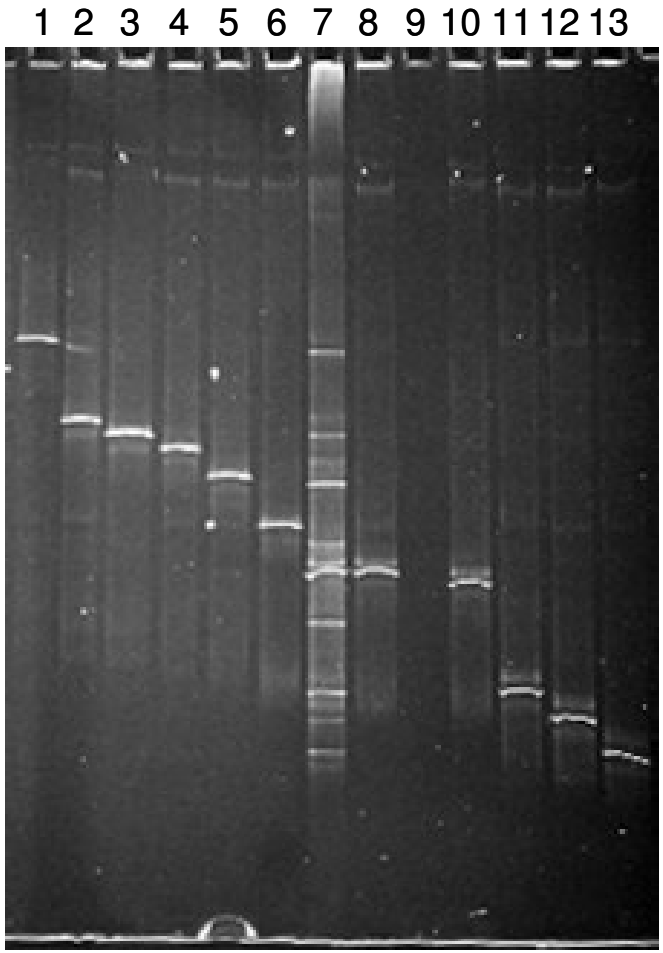


Figure 25 DGGE 30-55% showing clone library bacteria from sample VK3 Lane 1: clone 6, Lane 2: clone 25, Lane 3: clone 13, Lane 4: clone 24, Lane 5: clone 36; Lane 6: clone 10; Lane 7: community fingerprint; lane 8: clone 8; Lane 9: blank; Lane 10: clone 7; Lane 11: clone 41; Lane 12: clone 22; Lane 13: clone 39

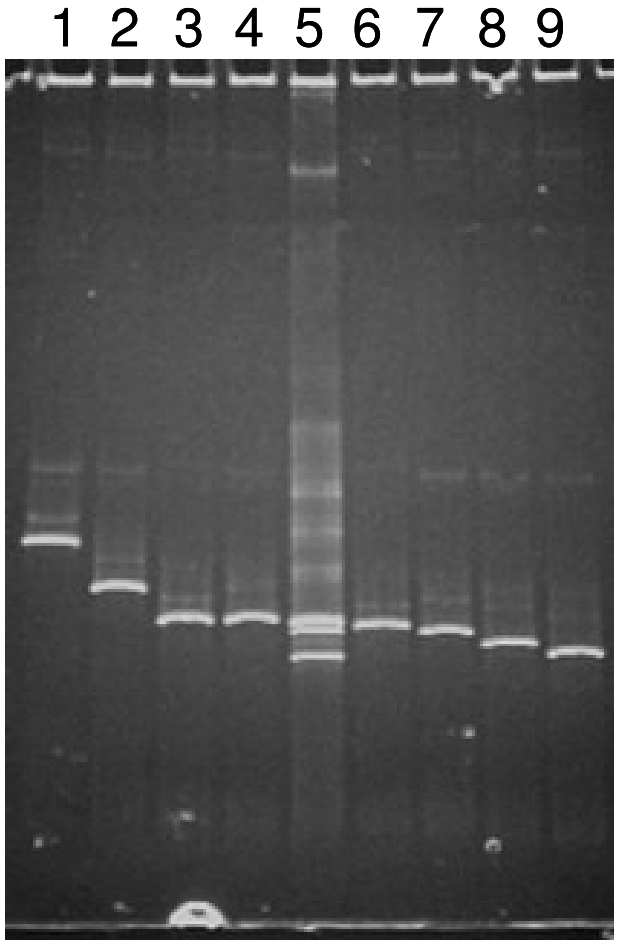


Figure 26 DGGE 25-55% showing clone library archaea from sample VK3. Lane 1: clone 45; Lane 2: clone 16; Lane 3: clone 46; Lane 4: clone 12; Lane 5: community fingerprint; Lane 6: clone 23; Lane 7: clone 14; Lane 8: clone 25; Lane 9: clone22

III. Molecular characterisation of *Halobacillus* populations isolated from different medieval wall paintings and building materials

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Running title: molecular characterization of new *Halobacillus* populations

Keywords: Wall paintings, *Halobacillus* sp, 16S rDNA, DGGE, RAPD-PCR, ITS-HHP

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Abstract

In previous studies several moderately halophilic gram-positive, spore-forming bacteria were isolated by conventional enrichment cultures from damaged medieval wall paintings and building materials. Comparative 16S rDNA sequence analyses showed that the isolates belong to the family *Bacillaceae* most closely related to species of the genus *Halobacillus*. *Halobacillus*-like bacteria could be anew isolated from the medieval Virgil`s chapel, Vienna, Austria. 16S rRNA gene sequences of the novel isolates (a total of 10) clustered with species of the genus *Halobacillus* (97.5-99.0 % similarity). A detailed molecular typing including established *Halobacillus* species was carried out in order to discriminate the new isolated strains. Several molecular typing methods were compared in this study i.e. Denaturing Gradient Gel Electrophoresis (DGGE), Random Amplified Polymorphic DNA (RAPD-PCR) and the Internal Transcribed Spacer- Homoduplex-Heteroduplex Polymorphism (ITS-HHP) fingerprinting. RAPD showed to be the most discriminatory method for differentiating the novel strains followed by DGGE profiles. In comparison ITS-HHP was least discriminative. All *Halobacillus* isolates included in this study were found to be typeable by each of the methods.

By standard bacteriological testing all isolates were strictly aerobic, Gram-positive, rod-shaped (singly, or in long chains) and non-motile. *Halobacillus* isolates were able to grow in the presence of 5-20% (wt/vol) NaCl. No growth was observed in the absence of NaCl. Optimum growth occurred at 37 ^{o}C in media containing 5 to 10 % (wt/vol) NaCl and with an optimum pH around 7.5-8.0.

1. Introduction

Microorganisms play a crucial role in the deterioration processes of building materials and objects of art, particularly if such materials are exposed to open air (Bock and Sand, 1993; Ciferri, 1999). The knowledge about the complexity and diversity of the bacterial communities present in such habitats has increase enormously in the last years by using molecular techniques as DGGE analysis (Rölleke et al. 1996, 1998, 1999; Gurtner et al. 2000). The information revealed by analyses of 16S rDNA sequences obtained directly amplified from environmental samples allows the detection of bacteria not yet cultivated. These data can be then used to design specific culture media for the cultivation of the previously detected microorganisms (Teske et al. 1996).

Several investigations based on culture-independent (Rölleke et al. 1996, 1999; Piñar et al. 2001a, b) as well as on culture–dependent techniques (Krumbein et al. 1991; Incerti et al. 1997; Heyrman et al. 1999; Laiz et al. 2000; Saiz-Jimenez and Laiz 2000) have demonstrated that monuments, where salt efflorescences on the surfaces are a common phenomenon as a result of changing physical parameters (Amoroso and Fassina 1983), may be an habitat for extremely salt tolerant and moderate halophilic microorganisms. The recent molecular detection and isolation of *Halobacillus* species in deteriorated wall paintings and building materials of the Catherine chapel at the castle Herberstein (Styria, Austria) (Piñar et al. 2001c) prompted further studies to focus on this group of bacteria not previously reported from stonework. Halobacilli were overlooked in the past due to the use of unsuitable culture media with inadequate salt concentration, and/or the long incubation time required for the culturability of this group of bacteria under laboratory conditions.

Recently, we cultivated from stonework samples of a medieval building in Austria (St. Virgil`s chapel) again *Halobacillus*-like bacteria. Here we report on the molecular characterisation of both *Halobacillus* wall paintings and building materials populations.

2. Materials and Methods

2.1. Description of the site and sampling.

This study was carried out in two different locations, the Catherine chapel at the castle Herberstein (Styria, Austria) and the St. Virgil Chapel (Vienna, Austria). The Catherine chapel at the castle Herberstein is decorated with numerous frescoes of the 14th century. The wall paintings were discovered and partly exposed around 1930, but restoration did not start before the 1940s. However, after restoration, the chapel was neglected and used as storage room until mid-nineties (Ochsenfarth 1998). The location is subdued to frequent outbursts of humidity at irregular intervals. Two samples were collected from different areas of the wall

paintings: sample H1 was taken below the chancel's east wall's window where a brownish biofilm was observed; sample H6 was taken from the chancel's north wall from a zone of the painting with an intense rosy discoloration. An additional sample (m7) was taken outdoors the chapel from a limewall showing also an intense rosy discoloration.

The St. Virgil Chapel, (Vienna, Austria) dates back from the beginning of the 14 century. It is located under the ruins of St. Mary Magdaleine Chapel. The St. Virgil Chapel was originally created as a tomb. After the Chapel of St. Mary Magdaleine was destroyed in 1781 by fire and demolished, the underground room was abandoned. The chapel was rediscovered in an excavation in 1972. The rectangular room is 10.5 m long and 6 m wide. The mortar and rubble walls are 1.5 m thick, containing 6 recesses with pointed arches, of which one was removed from where the present entrance is situated. They are decorated with large, red crosses painted onto white plaster. The whole chapel is covered by salt efflorescences visible by naked eye. A sample (S3) was taken directly from a salt efflorescence.

All samples were collected with a scalpel under sterile conditions as possible by scraping off surface material and plaster to a depth of 1 to 3 mm. Samples were used immediately for conventional enrichment and cultivation.

2.2. Enrichment and isolation.

Conventional enrichments were performed in 300 ml Erlenmeyer flasks containing 30 ml M2 medium (Tomlinson and Hochstein 1976) as well as Maintenance Medium (MM) (Spring et al. 1996). To avoid fungal growth, media were supplemented with 50 µg ml-1 cycloheximide (Sigma). All enrichments were incubated aerobically at room temperature (22 oC ± 3 oC) and at 37 oC in a waterbath by shaking using magnetic stirring bars. During a total period of 1 to 3 weeks, once a week aliquots of 100 µl enrichments were plated onto four different solid media: M2 medium (20 % wt/vol NaCl), M2A medium (20 % wt/vol NaCl) (Denner et al. 1994), Halococci medium (25 % wt/vol NaCl) and LB medium (Atlas 1995). All media were incubated aerobically at room temperature and at 37 oC.

2.3. Bacterial strains.

Strains K3-1, I3, I7, I3R and I3A were isolated from the chapel of castle of Herberstein (Styria, Austria). Five additional strains, named S3, S4, S20, S21 and S22, were isolated from the stonework of the chapel of St.Virgil in Vienna (Austria). All isolates were stored at −70 ^{o}C in MM broth containing 20 % (wt/vol) glycerol as cryoprotectant. Bacterial inocula for all experiments were taken from cryopreserves, streaked onto MM plates and cultivated at 37 ^{o}C for 48 h. Reference type strains of *Halobacillus* used in this study were: *H.*

karajensis DSM 14948^T, *H. halophilus* DSM 2266^T, *H. litoralis* DSM 10405^T, *H. trueperi* DSM 10404^T, *H. locisalis* DSM 16468^T and *H. salinus* JCM 11546^T (DSM-German Collection of Microorgansims; JCM-Japanese Collection of Microorganisms).

2.4. Phenotypic characterization.

Growth at different NaCl concentrations (0, 1, 5, 10, 15, 20, 25, 30% w/v) was determined on M2A agar (Denner et al. 1994); plates were incubated aerobically at 37°C for 48 h. Cellular morphology was examined on a Leitz Diaplan (Germany) phase contrast microscope. Gram staining, KOH-lysis test and conventional biochemical tests (i.e. catalase, nitrate reduction, oxidative-fermentative metabolism of glucose [O/F test], citrate utilization, hydrolysis of casein and starch) were carried out as described by Smibert and Krieg (1994). Oxidase-activity was tested using commercialised test strips (Bactident oxidase; Merck).

2.5. Molecular characterisation

2.5.1. DNA extraction and PCR analysis.

Genomic DNAs were extracted according to the protocol provided by Ausubel et al. (1991). For PCR analysis, 2x PCR Master Mix (Promega) [50 units/ml of TaqDNA Polymerase supplied in a proprietary reaction buffer (pH 8.5), 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP, 3mM $MgCl_2$] was diluted to 1x and 12.5 pmol of each primer were added. PCR was carried out in 25µl volumes and 2.5µl template was added.

Two different PCR reactions were carried out to amplify the eubacterial 16S rDNA fragments. For DGGE analysis, 200 base pairs fragments of the 16S rDNA were amplified using the eubacterial specific primer 341fGC (forward) to which a 40-base GC clamp was added to its 5' end (Muyzer et al. 1993). As reverse primer the universal consensus primer 518r (Muyzer et al. 1993) was used. For sequencing analyses, ~1500 base pairs 16S rDNA fragments were amplified using the forward primer 27f and the reverse primer 1492r (Lane 1991). PCR reactions were performed using a Robocycler (Stratagene). PCR conditions: 5 min denaturation (95 ^oC), followed by 30 cycles consisting of 1 min denaturation (95 ^oC), 1 min primer annealing (55 ^oC) and 2 min primer extension (72 ^oC), with a final extension step of 72 ^oC for 5 min.

2.5.2. Denaturing Gradient Gel Electrophoresis (DGGE) analysis

DGGE was done as previously described (Muyzer et al. 1993) using a D GENE-System (Bio-Rad) in 0.5 x TAE (20 mM Tris, 10 mM acetate, 0.5 mM Na_2EDTA; pH 7.8). Conditions used: lineal chemical gradient ranged from 25 to 60 % (100 % denaturant contains 7 M urea and 40 % vol./vol. formamide). Gel electrophoretic separation was done at 60 ^oC

and 200 V for 3.5 h. Subsequently, gels were stained with ethidium bromide and documented using a UVP documentation system.

2.5.3. 16S rDNA sequencing and phylogenetic analyses.

The individual 16S rDNA fragments (~1400 bp) were purified with the QIAquick PCR Purification Kit (Qiagen) and sequenced as described previously (Schabereiter-Gurtner et al. 2001). The obtained sequences were compared with known 16S rDNA sequences of prokaryotes contained in the Ribosomal Database Project (RDP) (Maidak et al. 1999) and the EMBL database. Pairwise evolutionary similarities and distances were computed by using the phylogeny inference package (PHYLIP) (Cole et al. 2003) of the online analysis of the RDP-II . A total of 1262 bases were compared.

Initial sequence data base search was performed by the SIMILARITY-RANK tool of the RDP and the FASTA search option (Pearson 1994).

2.5.4. Random Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR)

For RAPD analysis, PCR was performed with 10 different primers (see table 1). To the 1x diluted Mastermix (Promega) primer concentrations from 3 to 15nM were applied. PCR was carried out in 25µl volumes and 2.5µl template was added.

When using 10-nt primers PCR was performed under the following thermocycling conditions: 4 cycles of [94°C, 5min; 36°C, 5min; and 72°C, 5min], 30 cycles of [94°C, 1min; 36°C, 1min; and 72°C, 2min] followed by 10min at 72°C (Williams et al. 1990).

When primer oligos ≥17nt were used, the cycling program was 4 cycles of [94°C, 5min; 40°C, 5 min; and 72°C, 5min; low stringency amplification], 30 cycles of [94°C, 1min; 55°C, 1min; and 72°C, 2 min; high stringency amplification] and a final elongation step for 10min at 72°C (Welsh and McClelland 1990).

The whole reaction batches were run on a 2% (wt/vol) agarose gel at 160 V for 2 hours, stained with ethidium bromide and visualized with an UVP documentation system.

2.5.5. Intergenic transcribed spacer homoduplex heteroduplex PCR (ITS-HHP)

For PCR 12.5 pmol of each primer S-D-Bact-1494-a-S-20 and L-D-Bact-0035-a-A-15 (Daffonchio et al. 2003) were used. PCR was carried out in 50µl volumes to which 3.5µl template were added. PCR conditions: 4 min denaturation at 94°C, then 35 cycles [94°C, 1min; 55°C, 7min; and 72°C, 2 min] with a final extension step at 72°C for 7 min (Daffonchio et al. 1998).

The PCR products were run on three different kinds of gels: first, 25µl PCR product were run at 160 V for 2 hours on a 2% (wt/vol) agarose gel; second, 6% (wt/vol) acrylamide gels and mutation detection enhancement (MDE) gels were used for a better separation. For reducing band broadening 10% (vol/vol) glycerol was added to the 6% (wt/vol) acrylamide gels (Kumeda and Asao 1996). For the same reason urea was added to the MDE gel matrix as recommended by the manufacturer (Cambrex, USA). 15µl of ITS-HHP PCR products were loaded on the polyacrylamide and MDE gels and electrophoresed in 1x TBE buffer (5x TBE /l: 54g Tris; 27.5g Borat, 20 ml 0.5M EDTA; pH 8.0) for 7 hours at 100 V using a D GENE System (BioRad, Munich, Germany).

3. Results

3.1. 16S rDNA sequence analysis

16S rRNA gene sequences of all strains (n=10) isolated in this study clustered with species of the genus *Halobacillus* (97.5-99.0 % similarity), most closely to *Halobacillus trueperi* (98.6 to 99.0 % similarity) and to *Halobacillus litoralis* (98.6 to 98.9 % similarity) (Spring et al. 1996). These high sequence similarities clearly indicates that they are closely related phylogenetically but does not allow one to evaluate confidently whether they should be considered to be a single species (Fox et al. 1992). Strains isolated in this study clustered in three groups: strain S3, S4, S21 and S22 (99.8-100 % similarity); strains K3-1, I7 and S20 (99.3 to 99.7 % similarity); strains I3, I3A and I3R (100 % similarity). According to the high level of 16S rRNA gene sequence similarities among the newly isolated Halobacilli, a detailed molecular typing was carried out in order to validate the bacterial grouping.

3.2. Molecular typing

3.2.1. Discrimination of halobacilli by DGGE analysis

For grouping the bacteria, isolates were subjected to a DGGE analysis. Figure 27 shows the DGGE profiles of the individual isolates obtained in this study as well as from the reference type strains. Most of the isolates showed a single predominant band at the same position of the denaturing gel, as strains S20, K3-1, I3, I3R, I3A as well as some of the reference type strains of *H. trueperi, H. litoralis* and *H. karajensis*. A second group of strains, namely S3, S4, S21 and S22 showed similar band patterns with at least 5 dominant bands. Isolate I7 as well as reference type strains *H. halophilus*, *H. locisalis* and *H. salinus* showed unique profiles with significant differences to the strains isolated in this study.

3.2.2. Discrimination of halobacilli by RAPD-PCR analysis.

For RAPD analysis, PCR was performed with 10 different primers. Five of them were 10 nt in length and the other half ≥17 nt. They also differ in their GC content which affects the annealing behaviour. The results of RAPD-PCR analysis using the 10 nt primer 1254 (containing 70% GC) are presented in Figure 28A. By using this primer several strains showed highly similar profiles and could be clustered, as strains S3, S4 and S21 in one group, strains K3-1 and I7 in a second group and strains I3R and I3A in a third group. Strain S20 and S22 showed unique profiles, whereas strain I3 showed to be not typeable using this primer. In addition, all reference strains showed unique profiles different to those from the strains isolated in this study. The 10 nt primers 1283, 1247, 1281 and 1209 (see table 1) were used for RAPD-PCR analysis as well. However, not all strains showed to be typeable by using these primers (data not shown).

Results of RAPD-PCR analysis using longer primers (~17nt primers) showed best results when using the primer D14216 (containing 44% GC) followed by primers D14307 (55% GC) and D11344 (52% GC) (Figure 28B, 28C and 28D, respectively). By using these three primers, strain I3 showed to be typeable and was clustered with strains I3R and I3A. By using primers D8635 (67% GC) and D9355 (71% GC) not all strains showed to be typeable (data not shown). In general, by using ~17 nt primers strains could be clustered as in the case of the 10 nt primer 1254.

Test to optimise reaction conditions were carried out with the 10 nt primers as well as with the 17 nt primers. The most informative patterns were obtained when using a concentration of 15 nM of primer per 25 µl reaction, less primer resulted in fewer bands. Consequently, this concentration of primer was used for further RAPD test.

The reproducibility of RAPD patterns was tested by using the 17 nt primers and DNA preparations made from separate cultures of each strain on different days. Identical strain-specific profiles of DNA fragments were obtained from the paired DNA preparations with each primer tested (data non-shown)

3.2.3. Discrimination of halobacilli by 16S-23S rRNA gene Intergenic Transcribed Spacer fingerprinting.

The ITS homoduplex-heteroduplex polymorphism (ITS-HHP) analysis was used to investigate the nature of the strain-specific bands that allow strain discrimination in the ITS-PCR profile. The suitability of this fingerprinting method for typing purposes has been described elsewhere (Daffonchio et al. 2003). For this purpose all isolated strains obtained from wall paintings and building materials were analysed and compared with the ITS-PCR

profiles from all type strains included in this study. Figure 29A, B, C, show the ITS-HHP profiles of all isolated strains as well as from all type strains after separation in a 2% agarose gel, MDE gel 6% and acrylamide gel, respectively. All strains gave reproducible patterns. In any case, there were significant differences in between the band patterns obtained from the reference strains *H. halophilus, H. trueperi, H. litoralis*, *H. karajensis* and *H. salinus* and from the strains isolated in this study. By using agarose gel no differences were observed between the 10 isolates obtained in this study and with the reference strain *H. locisalis*. However, we could observe than when ITS-PCR products were separated in electrophoretic matrices with higher resolution than agarose, slowly migrating bands could be identified. By using MDE gels the slowly migrating bands were identified only in some of the reference type strains (*H. halophilus, H. trueperi, H. litoralis* and *H. salinus*) while by using 6% polyacrylamide gel with 10% glycerol these bands could be identified in all strains isolated in this study as well. Best results where obtained when using the last approach (6% [wt/vol] polyacrylamide gel with 10% [vol/vol] glycerol). In this way it was possible to differentiate all isolated strains from the reference type strains. However, this method seems to be not enough discriminative to differentiate our strains. Only strains K3-1 and I7 showed a light difference in the migration of the lower fingerprinting band, suggesting that the two strains are different to the other strains.

3.3. Key phenotypic characteristics and metabolic properties of the Halobacillus isolates

By standard bacteriological testing all isolates were strictly aerobic, Gram-positive, rod-shaped (singly, or in long chains) and non-motile. Biochemical characteristics positive for all strains were as follows: catalase, oxidase and hydrolysis of indole. Hydrolysis of casein was found in all isolates from the Castle of Herberstein. In addition, strains K3-1 and I7 hydrolysed starch and DNA. Hydrolysis of Tween 80 was positive in all isolates from the St. Virgil Chapel, with the exception of isolate S20. In addition, strains S4 and S21 reduced nitrate. Non of the isolates hydrolyzed gelatine.

All isolates (*n*= 10) obtained from samples H1, H6, m7 and S3 were able to grow in the presence of 5-20% (wt/vol) NaCl. The temperature range of growth was between 10 and 40 ^{o}C. Optimum growth occurred at 37 ^{o}C in media containing 5 to 10 % (wt/vol) NaCl. No growth was observed in the absence of NaCl. The pH range for growth was 7.0 -9.5 with an optimum around 7.5-8.0.

4. Discussion

By conventional enrichment techniques several Gram-positive, pigmented, moderately halophilic, spore-forming bacteria were isolated from ancient wall paintings and building materials collected at the castle of Herberstein, Styria, Austria, as well as from the St. Virgil Chapel, Vienna, Austria. 16S rRNA gene sequences of the strains isolated in this study clustered with species of the genus *Halobacillus* (97.5-99 % similarity). These high sequence similarities clearly indicates that they are closely related phylogenetically but does not allow one to evaluate confidently whether they should be considered to be a single species (Fox et al. 1992).

In a previous study (Piñar et al. 2001c) a DNA-DNA hybridization experiment was performed between three of the strains isolated from the Castle of Herberstein, namely K3-1, I3, I7 and the reference type strain *Halobacillus litoralis* DSM 10405^T. DNA reassociation values obtained with the type strain of *Halobacillus litoralis* were below the usual standard criterion of approximately 70%, which justifies that the strains K3-1, I3, and I7 were not related to this species, in fact they represented two distinct taxa (species) within the genus *Halobacillus*. Comparative 16S rRNA gene sequence analyses and estimation of phylogenetic relationships revealed that the newly isolated halophilic bacteria were closely related to each other (98.0 to 99.0 %, similarity) and to *Halobacillus litoralis* DSM 10405^T. On the other hand, DNA-DNA hybridization studies demonstrated only low levels of DNA homology between the new *Halobacillus* isolates and *Halobacillus litoralis* DSM 10405^T. Based upon the previous informations we expanded our study including further *Halobacillus* strains isolated from the Castle of Herberstein as well as several new similar isolates obtained from the St. Virgil Chapel in Vienna.

4.1. Molecular typing

Within the frame of this study several molecular methods were compared for bacterial typing (see Table 19). As a less discriminatory but rapid subtyping method DGGE analysis (Myers et al. 1985) was chosen. This technique is frequently used to characterise microbial communities (Muyzer et al. 1993; Muyzer and Smalla 1998), but has been also used to subtype bacterial species (Nielsen et al. 2000; Hein et al. 2003). Buchholz-Cleven et al. (1997) used DGGE analysis of PCR amplified 16S rDNA fragments as a first rapid means to screen the genetic diversity of different bacterial isolates after which a more detailed analysis, i.e., sequencing of the total 16S rRNA encoding gene of a few unique isolates was performed. In our study a 200 bp fragment of the V3 region of the 16S rDNA was amplified from each isolate for the detection of a possible microheterogeneity in rRNA encoding genes. Isolates

S20, K3-1, I3, I3R, I3A as well as the reference type of *H. trueperi*, *H.litoralis*, *H. locisalis* and *H. karajensis* showed a unique band in the DGGE profiles, therefore they were clustered in a group. The rest of strains showed more than one band in the DGGE melting profiles suggesting microheterogeneity in the 16S rRNA gene. Isolates S3, S4 and S21 showed the same DGGE fingerprinting and could be clustered as well. Nübel et al. (1996) observed in analysing PCR products from pure bacterial cultures the presence of more than one band in the DGGE/TGGE pattern. A more detailed analysis of this observation revealed microheterogeneity in the different rRNA operons present in different bacterial species. As indicated by the authors this finding has important consequences for the use of 16S rRNA sequence data for biodiversity estimates and phylogenetic reconstruction. Though DGGE typing in some cases agreed on the grouping formed by the other method, some of the groups formed by DGGE were subdivided by RAPD analysis.

The second molecular typing method used in this study was the Intergenic Transcribed Spacers (ITS) between the 16S and the 23S rRNA genetic loci (Daffonchio et al. 2003). This polymorphism can be used for discriminating closely related strains in species that harbor multiple ribosomal operons that differ in length and sequence. Genera of *Bacillus*, *Brevibacillus*, *Geobacillus* and *Paenibacillus* have showed high variety in their ITS regions between the 16S and the 23S rDNA. Using specific primers targeting these regions leads to formation of Hetero- and Homoduplexes depending on whether there is a tRNA gene located in the operon or not (Daffonchio et al. 2003). Due to ITS-HHP it is possible to discriminate species and in some groups even strains of bacteria by MDE, a specific polyacrylamide matrix, or, increasing the number of cycles in PCR, using a 2% agarose gel. Since the genus *Halobacillus* is closely related to the mentioned genera we attempted to adapt this method to our purpose. However, our results show that this typing method is not the most accurate to discriminate in between the strains isolated in this study (Table 19).

As third molecular typing method, RAPD-PCR analysis was adapted for this study. This method uses an oligonucleotide of arbitrarily chosen sequence to prime DNA synthesis from pair of sites to which it is matched or partially matched, and results in strain-specific profiles of DNA products (Welsh and McClelland 1990; Willians et al. 1990). This technique showed to be the most discriminative for differentiating the strains isolated in this study. This high discriminatory potential can be attributed to its ability to determine polymorphims in the entire bacterial genome. By using short primers (~10 nt), best results were obtained with primer 1254 (70% GC) producing reproducible profiles from all strains isolated in this study. Primer 1254 showed to be the most discriminative rendering excellent patterns for the typing

of other bacteria as *H. pylori* (Akopyanz et al. 1992). In general, short primers with high GC content render best results as those with lower GC content (Power 1996). In contrast, by using longer primers (~17 nt) most informative profiles (with a higher number of bands) were obtained when using primers D14216, D11344 and D14307 with a lower CG content (in between 44 and 55%). By using primers D8635 and D9355 (GC content 67% and 71% respectively) not all strains were typeable. Longer primers yielded higher number of bands in the strain-specific profiles. This can be attributed to the additional alternative of formation of a hairpin structure or a loop when annealing occurs in the PCR reaction, producing higher number of bands (Power 1996).

4.2. Ecophysiological relevance of the new *Halobacillus* isolates

In addition to the molecular characterisation, several physiological and biochemical differences were found as well, including their different pigmentation i.e. all species of the genus *Halobacillus* described so far are crème-yellow to orange-pigmented, the newly isolated halobacilli are white and crème-rosa to red -pigmented. Furthermore, strains isolated from the castle of Herberstein hydrolysed starch (strain K3-1 and I7) and showed a strong casein hydrolysis activity. This fact may be related with the damaged showed by the paintings as for example in the last restoration (1949) the paintings were fixed with casein-water (1:10) and casein was also added to the mortars for consolidation. Furthermore, fungal growth was reported only five days after casein application and the ceiling had to be cleaned again by the restorer, washing with distilled water and fixing it with new casein.

Isolates from the St. Virgil Chapel in Vienna showed special issues differing from those of strains isolated from the Castle of Herberstein as well as reference type strains. They were able to hydrolyse Tween 80 and some of them (S4 and S20) could reduce nitrate.

Reports concerning the isolation of halotolerant microorganisms from monuments and building materials are limited (Krumbein et al. 1991; Incerti et al. 1997; Heyrman et al. 1999; Laiz et al. 2000; Saiz-Jimenez and Laiz 2000; Piñar et al. 2001c). The predominance of *Bacillus* related species could be explained by the fact that they are able to survive for long periods as spores in which their DNA is protected against damage by small, acid-soluble protein (Fairhead et al. 1993), but also by an artifact due to the selectivity of the culture media used. Heyrman et al. (1999) performed enrichments from samples collected at the chapel of Herberstein (including the samples H1 and H6) in standard media with and without the supplementation of 10% NaCl. They reported higher total counts of heterotrophic bacteria (in the range of 10^9 CFU) on media containing 10% NaCl. However, on NaCl containing media a lower colony diversity was observed. They isolated extreme halotolerant strains which

FAME-profiles showed high similarity with the genus *Halomonas*. However, Heyrman et al. (1999) did not isolate *Halobacillus* spp., maybe due to the fact that they used selective rich media, i.e. TSA, in which halobacilli are not able to grow. *Halobacillus* species have been previously isolated from hypersaline sediments and soils, Antarctic sea ice, salt lakes, marine solar saltern and from fermented foods (Spring et al. 1996; Bowman et al. 1997; Chaiyanan et al. 1999; Amoozegar et al. 2003; Yoon et al. 2003, 2004).

The isolation of *Halobacillus* species from historic buildings and monuments in two different geographical locations, Styria and Vienna, but both subjected to salt attack suggest that these new species are common inhabitants of this kind of environments.

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Primer	Sequence	%G+C
10 nt primers		
1254	CCGCAGCCAA	70
1283	GCGATCCCCA	70
1247	AAGAGCCCGT	60
1281	AACGCGCAAC	60
1290	GTGGATGCGA	60
≥ 17 nt primers		
D14216	NNNAACAGCTATGACCATG	~44
D11344	AGTGAATTCGCGGTGAGATGCCA	52
D8635	GAGCGGCCAAAGGGAGCAGAC	67
D9355	CCGGATCCGTGATGCGGTGCG	71
D14307	GGTTGGGTGAGAATTGCACG	55

Table 18 Primers tested for RAPD-PCR analysis of *Halobacillus* species

ITS-HHP	DGGE	16S rRNA GENE	RAPD-PCR
n.D.	S3, S4, S21, S22 K3-1, S20, I3, I3A, I3R	S3, S4, S21, S22 K3-1, I7, S20	S3, S4, S21 K3-1, I7
	I7	I3, I3A, I3R	I3, I3A, I3R S20 S22
NOT ACCURATE TYPING METHOD	LESS DISCRIMINATORY BUT RAPID SUBTYPING METHOD	GOOD CLUSTERING TYPING METHOD	MOST DISCRIMINATIVE TYPING METHOD

Table 19 Comparison of molecular typing methods for the clustering of the novel *Halobacillus* species. N.D.: no discrimination of strains was possible

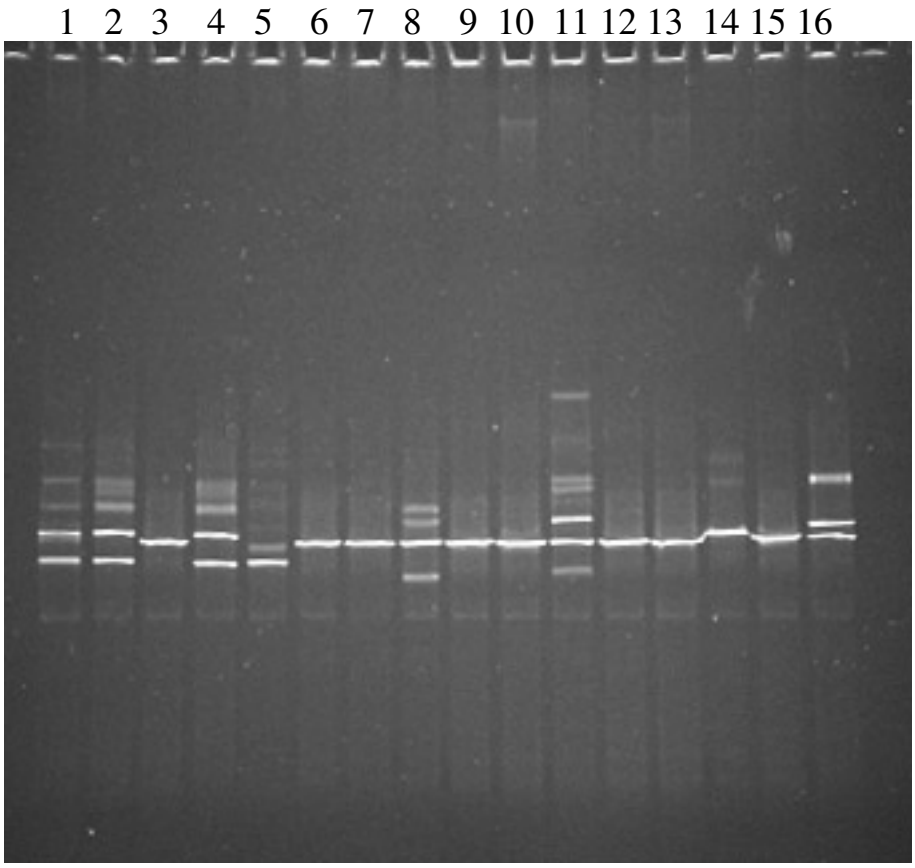

Figure 27 DGGE profiles of *Halobacillus* species and wall paintings and building materials isolates. Lane 1: isolate S3; lane 2: isolate S4; lane 3: isolate S20; lane 4: isolate S21; lane 5: isolate S22; lane 6: isolate K3-1; lane 7: isolate I3; lane 8: isolate I7; lane 9: isolate I3R; lane 10: isolate I3A; lane 11: *H. halophilus*; lane 12: *H. trueperi*; lane 13: *H. litoralis*; lane 14: *H. locisalis*; lane 15: *H. karajensis*; lane 16: *H. salinus.*

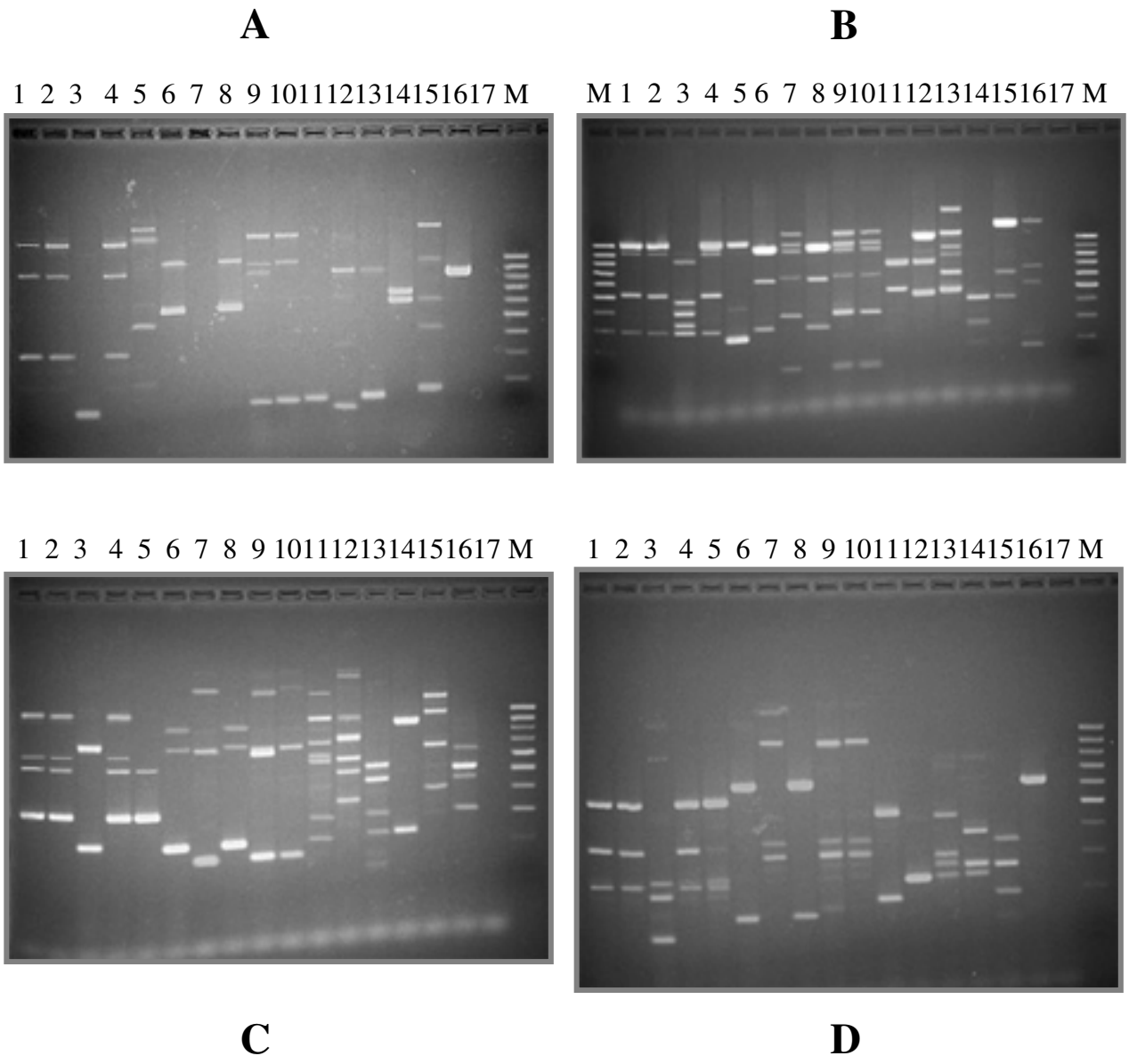

Figure 28 RAPD-PCR analyses of *Halobacillus* species and wall paintings and building materials isolates. A) RAPD profiles with the 10 nt primer 1254; B) RAPD profiles with the ~17 nt primer D14216; C) RAPD profiles with the ~17 nt primer D14307; D) RAPD profiles with the ~17 nt primer D11344. Lane 1: isolate S3; lane 2: isolate S4; lane 3: isolate S20; lane 4: isolate S21; lane 5: isolate S22; lane 6: isolate K3-1; lane 7: isolate I3; lane 8: isolate I7; lane 9: isolate I3R; lane 10: isolate I3A; lane 11: *H. halophilus*; lane 12: *H. trueperi*; lane 13: *H. litoralis*; lane 14: *H. locisalis*; lane 15: *H. karajensis*; lane 16: *H. salinus;* lane 17: negative PCR control; lane M: 100-bp ladder.

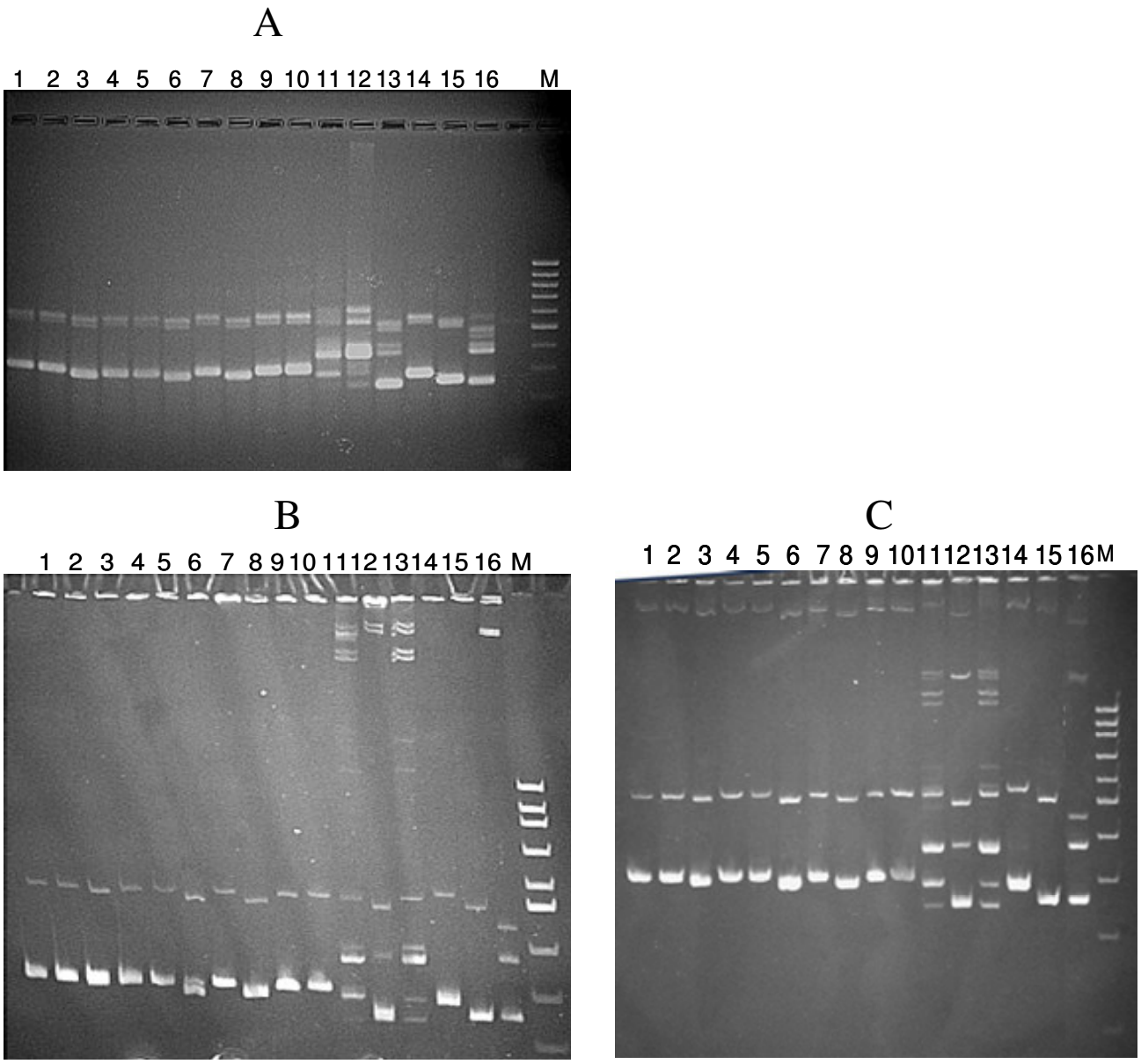

Figure 29 ITS-PCR fingerprints of *Halobacillus* species and wall paintings and building materials isolates. A) resolved by 2% agarose gel; B) resolved by 0.6% MDE gel with urea; C) resolved by 6% polyacrylamide gel with 10% glycerol. Lane 1: isolate S3; lane 2: isolate S4; lane 3: isolate S20; lane 4: isolate S21; lane 5: isolate S22; lane 6: isolate K3-1; lane 7: isolate I3; lane 8: isolate I7; lane 9: isolate I3R; lane 10: isolate I3A; lane 11: *H. halophilus*; lane 12: *H. trueperi*; lane 13: *H. litoralis*; lane 14: *H. locisalis*; lane 15: *H. karajensis*; lane 16: *H. salinus;* control; lane M: 100-bp ladder.

IV. *Halobacillus virgiliensis sp. nov., Halobacillus styriensis sp.nov.* and *Halobacillus herbersteinensis sp.nov.* isolated from medieval mural paintings and stone-works in two different locations in Austria

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1. Abstract

Ten moderately halophilic, Gram-positive, endospore-forming bacteria were isolated from damaged medieval wall paintings and building materials from the Castle of Herberstein, Styria (Austria) as well as from the medieval stonework of the Chapel of St. Virgil, Vienna, Austria. All strains were aerobic with rod-shaped cells that occurred single, in pairs or in chains. They were non-motile and a single endospore could be detected in phase contrast microscope. Growth occurred at 10-40 °C and in the pH range from 6.0-9.5. Strains isolated from the castle of Herberstein grew at salinities of 1-20% (w/v) NaCl, whereas the strains isolated from the chapel of St. Virgil did at salinities of 5-20% (w/v) NaCl, showing optimal growth at 10% (w/v) NaCl. Phylogenetic analysis based on 16S rDNA gene sequences showed that the strains isolated were closely related to species of the genus *Halobacillus* (97.5-99.0 % similarity). The microorganisms showing the closes phylogenetic relationship with the isolated strains were *Halobacillus trueperi* (98.6-99.0% similarity) and *Halobacillus litoralis* (98.6-98.9% similarity). Based on biochemical, physiological and chemotaxonomic traits, RAPD-PCR-generated genomic fingerprints and comparative 16S rDNA sequence analysis, it is proposed that the isolates from the Castle of Herberstein as well as from the Chapel of St.Virgil should be placed in the genus *Halobacillus* representing three novel species namely, *Halobacillus virgiliensis sp.nov*, *Halobacillus herbersteinensis sp.nov.* and *Halobacillus styriensis sp.nov.*.

2. Introduction

Hypersaline environments include a variety of habitats such as natural inland salt lakes, soda lakes, salt flats, evaporation ponds, deep-see basins and subsurface salt formations (Vreeland et al, 1998; Rothschild and Mancinelli 2001; Sass *et al*., 2001; Litchfield and Gillevet 2002; Oren 2002)**.** Recently, mural paintings and stoneworks have been shown to be a habitat for extremely salt tolerant and moderate halophilic bacteria (Incerti *et al*., 1997; Laiz *et al*., 2000; Saiz-Jimenez and Laiz, 2000) and archaea (Rölleke *et al*., 1998; Piñar *et al*., 2001a). Practically all porous building materials contain soluble salts due to exposure to rainwater and rising damp. Dispersed within the porous material or locally concentrated, these salts are solubilized and migrate with the water followed by drying out of the solution. This results in the formation of deposits of crystallized salts on the surface (Amoroso and Fassina, 1983) which are commonly known as efflorescences and appear to build niches for settlement of halophilic microorganisms.

In a previous work, five moderately halophilic gram-positive, spore-forming bacteria were isolated by conventional enrichment cultures from damaged medieval wall paintings and building materials located in the Catherine chapel of castle of Herberstein (Styria, Austria). DNA-DNA reassociation experiments identified the isolates as a population of hitherto unknown new *Halobacillus* species (Piñar *et al*., 2001b). In this study, five additional halophilic bacterial strains could anew be isolated from samples taken from the medieval chapel of St.Virgil (Vienna, Austria). These organisms were consider to be members of the genus *Halobacillus* from the results of preliminary partial 16S rDNA sequence analysis.

The aim of the present study was to determine the exact taxonomic position of all ten strains by using the combination of phenotypic, chemotaxonomic, fatty acid analysis (to be done), as well as a detailed genetic analysis including RAPD-PCR fingerprints and phylogenetic analysis based on nearly complete 16S rDNA sequence relatedness. On the basis of the results presented here, these new strains should be placed in the genus *Halobacillus* representing three novel species, for which we proposed the names *Halobacillus virgiliensis sp.nov*., *Halobacillus herbersteinensis sp.nov and Halobacillus styriensis sp. nov***.**

3. Materials and Methods

3.1. Description of the site and sampling.

This study was carried out in the Catherine chapel at the castle Herberstein (Styria, Austria). The chapel is decorated with numerous frescoes of the 14th century. The wall paintings were discovered and partly exposed around 1930, but restoration did not start before the 1940s. However, after restoration, the chapel was neglected and used as a storage room until mid-nineties. The location is subdued to frequent outbursts of humidity at irregular intervals.

Two samples were collected from different areas of the wall paintings: sample H1 was taken below the chancel's east wall's window where a brownish biofilm was observed; sample H6 was taken from the chancel's north wall from a zone of the painting with an intense rosy discoloration. An additional sample (m7) was taken outdoors the chapel from a limewall showing also a intense rosy discoloration.

The St. Virgil Chapel, (Vienna, Austria) dates back from the beginning of the 14 century. It is located under the ruins of St. Mary Magdaleine Chapel. The St. Virgil Chapel was originally created as a tomb. After the Chapel of St. Mary Magdaleine was destroyed in 1781 by fire and demolished, the underground room was abandoned. The chapel was rediscovered in an excavation in 1972. The rectangular room is 10.5 m long and 6 m wide. The mortar and rubble walls are 1.5 m thick, containing 6 recesses with pointed arches, of which one was removed from where the present entrance is situated. They are decorated with large, red crosses painted onto white plaster. The whole chapel is covered by salt efflorescences visible by naked eye. A sample (S3) was taken directly from a salt efflorescence.

All samples were collected with a scalpel under sterile conditions as possible by scraping off surface material and plaster to a depth of 1 to 3 mm. Small amount of samples were used immediately for cultivation. Aliquots used for molecular analyses were kept at -80^{o}C until their use.

3.2. Enrichment and isolation.

Conventional enrichments were performed in 300 ml Erlenmeyer flasks containing 30 ml M2 medium (Tomlinson and Hochstein 1976) as well as Maintenance Medium (MM) (Spring *et al*., 1996). To avoid fungal growth, media were supplemented with 50 µg ml^{-1} cycloheximide (Sigma). All enrichments were incubated aerobically at room temperature (22 ^{o}C ± 3 ^{o}C) and at 37 ^{o}C in a waterbath by shaking using magnetic stirring bars. During a total period of 1 to 3 weeks, once a

week aliquots of 100 µl enrichments were plated onto four different solid media: M2 medium (20 % wt/vol NaCl), M2A medium (20 % wt/vol NaCl) (Denner et al., 1994), Halococci medium (25 % wt/vol NaCl) and LB medium (Atlas, 1995). All media were incubated aerobically at room temperature and at 37 °C.

3.3. Bacterial strains.

Strains K3-1, I7, I3, I3R and I3A were isolated from the chapel of castle of Herberstein (Styria, Austria). Five additional strains, named S3, S4, S20, S21 and S22, were isolated from the stonework of the chapel of St.Virgil in Vienna (Austria). All isolates were stored at −70 °C in MM broth containing 20 % (wt/vol) glycerol as cryoprotectant. Bacterial inocula for all experiments were taken from cryopreserves, streaked onto MM plates and cultivated at 37 °C for 48 h. Reference type strains of *Halobacillus* used in this study were: *H. karajensis* DSM 14948^T, *H. halophilus* DSM 2266^T, *H. litoralis* DSM 10405^T, *H. trueperi* DSM 10404^T, *H. locisalis* DSM 16468^T and *H. salinus* JCM 11546^T (DSM-German Collection of Microorganisms; JCM-Japanese Collection of Microorganisms).

3.4. Phenotypic characterization.

Cell morphology was examined by phase contrast microscopy with a Leitz Diaplan microscope. Gram reaction was determined by staining and was confirmed by the KOH-lysis test as described previously (Smibert and Krieg, 1994). Conventional biochemical testings were carried out as described by Smibert and Krieg (1994) including tests for: catalase, nitrate reduction, oxidative-fermentative metabolism of glucose (O-F test), citrate utilization, hydrolysis of casein and starch. Oxidase-activity was tested using Bactident oxidase test strips (Merck) according to the manufacturer's instructions.

Urease activity was tested as described by OXOID handbook (OXOID GmbH; 6th edition, 2003). Acid production from carbohydrates was determined on phenolred agar as described by Atlas (2004). For Gelatine hydrolyse 12% (w/v) gelatine was added to medium maintenance (Bast, 2001). Enzymatic activities were tested using API Zym test strips (biomerieux) following the instructions of the manufacturer. Therefore cell mass was resuspended in 10% saline to reach a McFarland standard of 5. The strips were incubated for 4.5 hours at 37°C.

Strains were grown and maintained in a complex medium (MM) (Spring *et al*., 1996). Growth at different NaCl concentrations (0, 1, 5, 10, 15, 20, 25, 30% w/v) was

determined on plates containing M_2A medium (Denner *et al.*, 1994). All media were incubated aerobically at 37°C. Growth at various temperatures (4-50°C) and at different pH values (pH 5 to 9.5) was determined in MM media containing 10% NaCl.

3.5. Molecular characterization

Genomic DNAs were extracted according to the method described previously by Ausubel et al. (1991). For PCR analysis, 2x PCR Master Mix (Promega) [50 units/ml of TaqDNA Polymerase supplied in a proprietary reaction buffer (pH 8.5), 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP, 3mM $MgCl_2$] was diluted to 1x and 12.5 pmol of each primer were added. PCR was carried out in 25µl volumes and 2.5µl template was added.

For sequencing analyses, ~1500 base pairs 16S rDNA fragments were amplified using the forward primer 27f and the reverse primer 1492r (Lane 1991). PCR reactions were performed using a Robocycler (Stratagene). PCR conditions: 5 min denaturation (95 ^{o}C), followed by 30 cycles consisting of 1 min denaturation (95 ^{o}C), 1 min primer annealing (55 ^{o}C) and 2 min primer extension (72 ^{o}C), with a final extension step of 72 ^{o}C for 5 min.

The individual 16S rDNA fragments were purified with the QIAquick PCR Purification Kit (Qiagen) and sequenced as described previously (Schabereiter-Gurtner et al. 2001). The obtained sequences were compared with known 16S rDNA sequences of prokaryotes contained in the Ribosomal Database Project (RDP) (Maidak et al. 1999) and the EMBL database. Pairwise evolutionary similarities and distances were computed by using the phylogeny inference package (PHYLIP) (Cole et al. 2003) of the online analysis of the RDP-II . A total of 1262 bases were compared.

Initial sequence data base search was performed by the SIMILARITY-RANK tool of the RDP and the FASTA search option (Pearson 1994).

The sequences obtained in this study have been assigned the accession numbers AM161499-AM161508.

For RAPD analysis, PCR was performed with 10 different primers. Five of them were 10 nt in length (namely: primers 1254, 1283, 1247, 1281 and 1290) and the other half ≥17 nt (namely: primers D14216, D11344, D8635, D9355 and D14307) (Ripka et al, 2005). To the 1x diluted Mastermix (Promega) a primer concentration of 15nM was applied. PCR was carried out in 25µl volumes and 2.5µl template was added.

When using 10-nt primers PCR was performed under the following thermocycling conditions: 4 cycles of [94°C, 5min; 36°C, 5min; and 72°C, 5min], 30 cycles of [94°C,

1min; 36℃, 1min; and 72℃, 2min] followed by 10min at 72℃ (Williams et al. 1990). When primer oligos ≥17nt were used, the cycling program was 4 cycles of [94℃, 5min; 40℃, 5 min; and 72℃, 5min; low stringency amplification], 30 cycles of [94℃, 1min; 55℃, 1min; and 72℃, 2 min; high stringency amplification] and a final elongation step for 10min at 72℃ (Welsh and McClelland 1990).

The whole reaction batches were run on a 2% (wt/vol) agarose gel at 160 V for 2 hours, stained with ethidium bromide and visualized with an UVP documentation system.

4. Results

4.1. Phenotypic characterisation

After three weeks of enrichment cultures it was possible to isolate 5 different strains from the castle of Herberstein, Styria (Austria), a red-pigmented bacterial strain designated as K3-1 from sample H6, three crème-pigmented bacterial strains designated as I3, I3A and I3R from sample H1 and an additional white to yellow-pigmented strain named I7 from sample m7. Phenotypical characterisation of strains are shown in Table 20.

Strains I3, I3A and I3R were able to grow in M2A or MM in the presence of 1-20% (wt/vol) NaCl, whereas strain K3-1 and I7 did at a range of 5-20% (wt/vol) NaCl. All strains grew at temperature range of 10-40 ^{o}C. Optimum growth occurred in media containing 5 to 10 % NaCl at 37 ^{o}C. No growth was observed in the absence of NaCl. The pH range for growth was 6.0 -9.5 with an optimum around 7.5-8.0. Growth at higher pH values was not assessed because of the formation of precipitates of unknown chemical composition in the medium.(Antunes *et al.*, 2003)

By standard bacteriological testing all bacterial isolates were aerobic, Gram-positive, rod-shaped (singly, or in long chains) and non-motile. Biochemical characteristics positive for all strains were as follows: catalase, oxidase, hydrolysis of casein. Strains K3-1 and I7 hydrolysed starch and were slightly DNase positive. I3, I3R, I3A were DNase negative. Characteristics negative for all strains were: indol, urease; citrate utilization; nitrate reduction; hydrolysis of DNA, Tween 80 and gelatine. Acid production from glucose, maltose, D-mannose and sucrose was detected for strains I3, I3A and I3R showing that this strain was different to the other two (Table 21). No acid production was detected for strains K3-1 and I7.

Five additional strains, named S3, S4, S20, S21 and S22, were isolated from the stonework of the chapel of St.Virgil in Vienna (Austria). Cells were rod shaped, single or in chains. They were all gram positive and aerobic. Colonies were smooth, circular, crème to red coloured, entire, opaque and approx. 2mm in diameter after 2days incubation at 37°C on MM plus 10% (w/v) NaCl. The isolates were able to grow on M2A media containing 5-20% (w/v) NaCl with an optimum at 10% (w/v) NaCl. The temperature range for growth was 10-40°C. There was no growth at pH values under a pH of 6 and over a pH of 9.5. The optimum was around 7.5-8.0 (Table 20)

No motility could be determined using SIM agar or MM plus 0.3% Agar. Hydrolysis of casein, Starch and Gelatine showed to be negative in strains S3, S4, S21 and S22, all of which were positive hydrolysing Tween 80. S20 was able to hydrolyse casein but not to hydrolyse Tween80 showing to be different to the rest of the isolates.

All strains were urease and DNase negative and Indol positive. S4 and S21 showed to reduce nitrate. No acid production from carbohydrates could be detected for any of the strains (Table 21).

4.2. Enzymatic activities.

Enzymatic activity results from all strains are shown in Table 22**.** All Strains showed positive results for Esterase (C4) and Esterase lipase (C8). Most investigated strains, accept I3, I3A and I3R were positive for Alkaline phosphatase. K3-1, I7, I3, *H.truperi, H.halophilus, H.salinus* and *H.locisalis* showed beta-galactosidase activity. K3-1, I7 and S21 were Naphtohol-AS-BI-phosphohydrolase positive. I3 and all reference strains accept *H.karajensis* gave positive results for α –glucosidase.

The API zym test strips showed low enzymatic activity. As API ZYM test strips are applied to standard organisms like *E.coli* they may not work equally for halophilic bacteria. The strains were resuspended in 10% saline which may affect the test and inhibit proper results.

4.3. Genotypic analysis.

In a previous study (Ripka *et al*., 2005), a detailed molecular typing including established *Halobacillus* species was carried out in order to discriminate the new strains isolated in this study. Several molecular typing methods were compared i.e. Denaturing Gradient Gel Electrophoresis (DGGE), Random Amplified Polymorphic DNA (RAPD-PCR) and the Internal Transcribed Spacer- Homoduplex-Heteroduplex

Polymorphism (ITS-HHP) fingerprinting. RAPD showed to be the most discriminatory method for differentiating the novel strains.

For RAPD analysis, PCR was performed with 10 different primers. Five of them were 10 nt in length and the other half ≥17 nt. They also differ in their GC content which affects the annealing behaviour. RAPD-PCR analysis by using 10 nt primers demonstrated that not all strains showed to be typeable by using these primers (data non shown). Results of RAPD-PCR analysis using longer primers (~17nt primers) showed best results when using the primer D14216 (Figure 30). By using this primer several strains showed highly similar profiles and could be clustered, as strains S3, S4 and S21 in one group, strains K3-1 and I7 in a second group and strains I3, I3A and I3R in a third group. Strain S20 and S22 showed unique profiles. According to the high level of RAPD-PCR profile similarities among the grouped isolated strains, the almost complete 16S rDNA of the bacterial strains was sequenced to validate the bacterial grouping.

16S rRNA gene sequences of all strains isolated in this study clustered with species of the genus *Halobacillus* (97.5-99.0 % similarity), most closely to *Halobacillus trueperi* (98.6 to 99.0 % similarity) and to *Halobacillus litoralis* (98.6 to 98.9 % similarity) (Spring et al. 1996). These high sequence similarities clearly indicates that they are closely related phylogenetically but does not allow one to evaluate confidently whether they should be considered to be a single species (Fox et al. 1992). To verify their phylogenetic positions evolutionary distances were calculated and a phylogenetic tree was constructed (Figure 31). Strains isolated in this study clustered in three groups: strain S3, S4, S21 and S22 (99.8-100 % similarity); strains K3-1, I7 and S20 (99.3 to 99.7 % similarity); strains I3, I3A and I3R (100 % similarity).

4.4. Description of *Halobacillus virgiliensis sp.nov.*

This species comprise 4 different strains, S3, S4, S21 and S22. Cells are gram positive rods, occurring single, in pars or in short chains. Non-motile, spherical or ellipsoidal endospores are produced in the central or subterminal position. Colonies are pigmented (rose to crème), circular, opaque and entire. Growth occurs in 5-20% NaCl (w/v); 10% (w/v) is optimal for growth. No growth occurs in the absence of NaCl. Growth occurs at 10-40°C. No growth under pH 6 and above pH 9.5 was detected. Strictly aerobic, catalase and oxidase are produced. Starch, casein, gelatine, indole and urease are negative. Nitrate is reduced to nitrite. Tween 80 is

hydrolysed. No acid is produced from Glucose, maltose, D-galactose, D-xylose, D-mannose, D-fructose and sucrose.

4.5. Description of *Halobacillus herbersteinensis sp.nov*.

This species comprises 3 different strains, K3-1, I7 and S20**.** Cells are gram positive rods, occurring single, in pairs or in short chains. Non-motile, spherical or ellipsoidal endospores are produced in the central or subterminal position. Colonies are pigmented (red to crème-white), circular, opaque and entire. Growth occurs in 5-20% NaCl (w/v); 10% (w/v) is optimal for growth. No growth occurs in the absence of NaCl. Growth occurs at 10-40°C. No growth under pH 6 and above pH 9.5 was detected. Stirctly aerobic, catalase and oxidase are produced. Gelatine, indole and urease are negative. Nitrate is not reduced to nitrite. Casein is positive for all strains, whereas starch is negative for strain S20, Tween 80 is not hydrolysed. No acid is produced from Glucose, maltose, D-galactose, D-xylose, D-mannose, D-fructose and sucrose.

4.6. Description of *Halobacillus styriensis sp.nov*.

This species comprises 3 different strains, I3, I3A and I3R. Cells are gram positive rods, occurring single, in pairs or in short chains. Non-motile, spherical or ellipsoidal endospores are produced in the central or subterminal position. Colonies are pigmented (crème), circular, opaque and entire. Growth occurs in 1-20% NaCl (w/v); 10% (w/v) is optimal for growth. No growth occurs in the absence of NaCl. Growth occurs at 10-40°C. No growth under pH 6 and above pH 9.5 was detected. Strictly aerobic, catalase and oxidase are produced. Gelatine, indole and urease are negative. Nitrate is not reduced to nitrite. Casein is positive and starch is negative for all strains, Tween 80 is not hydrolysed. Acid production is observed from glucose, maltose, D-mannose and sucrose.

5. References

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	S3	S4	S20	S21	S22	K3-1	I3	I7	I3A	I3R	(1)[A]	(2) [A]	(3) [B]	(4) [C]	(5) [D]	(6)[E]
Cell morphology	Rods, single or in chains	Rods,single or in chains	Rods, single or in chains	Rods, single or in chains	Rods, single or in chains	Rods	Rods	Rods	Rods	Rods	Rods, single	Rods,single	Oval or cocci	Rods, single	Rods, single	Rods, single
Spore shape	E/S	E/S	E/S	E/S	E/S	E/S	E/S	E/S	E/S	E/S	E/S	E/S	S	S/E	E/S	E/S
Spore position	C/ST	C/ST	C/ST	C/ST	C/ST	C/ST	C/ST	C/ST	C/ST	C/ST	C/ST	C/ST	C/ST	C/ST	C/ST	C/ST
Colony color	creme	Crème -rosa	salmon	Crème -rosa	white	Red	Crème	White	Crème	Crème	orange	orange	Orange	White	Light Orange	Pale Orange
Temperature range	10-40	10-40	10-40	10-40	10-40	10-40	10-40	10-40	10-40	10-40	10-43	10-44	15-40	10-49	10-42	10-45
Growth at/in																
45°C	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
pH 5.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
pH 6.0	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
NaCl concentration for growth (%, w/v):																
- Range (4 days)	5-20	5-20	5-20	5-20	5-20	5-20	1-20	5-20	1-20	1-20	0.5-25	0.5-30	2-20	1-24	1-10	0.5-20
- Optimum	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Hydrolysis of																
- Casein	-.	-	+	-	-	+	+	+	+	+	-	-	+	+	-	+
- Starch	-	-	-	-	-	(+)	-	+	-	-	-	-	+	+	+	-
- Gelatine	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+
- Tween 80	+	+	-	+	+	-	-	-	-	-	-	-	-	-	+	+
- DNase	-	-	-	-	-	+	-	+	-	-	+	+	-	+	-	+
Nitrate Reduction	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Motility	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	+

Table 20 Table showing phenotypical characteristics of isolates S3, S4, S20, S21, S22, K3-1, I3, I7, I3A, I3R and the reference strains (1) *H.litoralis* ASM 10405T, (2) *H.trueperi* DSM 10404T, (3) *H.halophilus* DSM2266T, (4) *H.karajensis* DSM14948T, (5) *H..locisalis* DSM16468T and (6) *H.salinus* JCM11546T; E-elipsoid; S-spherical; C-central; ST-subterminal; T-terminal

[A] Data from Spring et al. (1996) [B] Data from Claus et al. (1983) and Spring et al.(1996)
[C] Data from Amoozegar et al. (2003) [D] Data from Yoon et al. (2004)
[E] Data from Yoon et al. (2003)

	S3	S4	S20	S21	S22	K3-1	I3	I7	I3A	I3R	(1) [A]	(2) [A]	(3)[B]	(4) [C]	(5) [D]	(6) [E]
Acid production:																
Glucose	-	-	-	-	-	-	+	-	+	+	+	+	-	+	+	+
Maltose	-	-	-	-	-	-	(+)	-	(+)	(+)	+	+	-	+	-	+
D-Galactose	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+
D-Xylose	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
D-Mannose	-	-	-	-	-	-	(+)	-	(+)	(+)	-	-	-	-	-	+
D-Fructose	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	+
Sucrose	-	-	-	-	-	-	+	-	(+)	(+)	+	+	-	+	+	+

Table 21 Acid production from different sugars isolates S3, S4, S20, S21, S22, K3-1, I3, I7, I3A, I3R and the reference strains (1) *H.litoralis* ASM 10405T, (2) *H.trueperi* DSM 10404T, (3) *H.halophilus* DSM2266T, (4) *H.karajensis* DSM14948T, (5) *H..locisalis* DSM16468T and (6)*H.salinus* JCM11546T

[A] Data from Spring *et a*l. (1996)
[B] Data from Claus *et al*. (1983) and Spring *et al*.(1996)
[C] Data from Amoozegar *et a*l. (2003)
[D] Data from Yoon *et a*l. (2004)
[E] Data from Yoon *et a*l. (2003)

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
K3-1	-	+	+	+	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-
I7	-	+	+	~	-	-	-	-	-	-	~	+	-	+	-	-	-	-	-	-
I3	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-
I3A	-	-	+	+	-	~	-	-	-	-	-	-	-	-	-	-	-	-	-	-
I3R	-	~	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S3	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S4	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S20	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S21	-	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
S22	-	+	+	~	-	-	-	-	-	-	-	-	-	-	-	~	-	-	-	-
H.litoralis	-	+	+	+	-	-	-	-	-	-	-	~	-	-	-	+	-	-	-	-
H.trueperi	-	+	+	+	-	-	-	-	-	-	~	~	-	+	-	+	-	-	-	-
H.halophilus	-	+	+	~	-	+	-	-	-	-	-	-	-	+	-	+	-	-	-	-
H.locisalis	-	+	+	+	-	-	-	-	-	-	-	~	-	+	-	+	~	-	-	-
H.salinus	-	+	+	+	-	-	-	-	-	-	-	~	-	+	-	+	+	-	-	-
H.karajensis	-	~	+	+	-	~	~	-	-	-	-	~	-	-	-	-	-	-	-	-

Table 22 Enzymatic acitivies- results of API ZYM test: 1 Control, 2 Alkaline phosphatase ,3 Esterase (C4), 4 Esterase Lipase (C8), 5 Lipase (C14), 6 Leucine arylamidase, 7 Valine arylamidase, 8 Cystine arylamidase, 9 Trypsin, 10 α-chymotrypsin, 11 Acid phosphatase, 12 Naphtohol-AS-BI-phosphohydrolase, 13 α-galactosidase, 14 β-galactosidase, 15 β-glucuronidase, 16 α –glucosidase, 17 β-glucosidase, 18 N-acteyl-β-glucosaminidase, 19 α-mannosidase, 20 α-fucosidase; +, postive; -, negative; ~, insecure

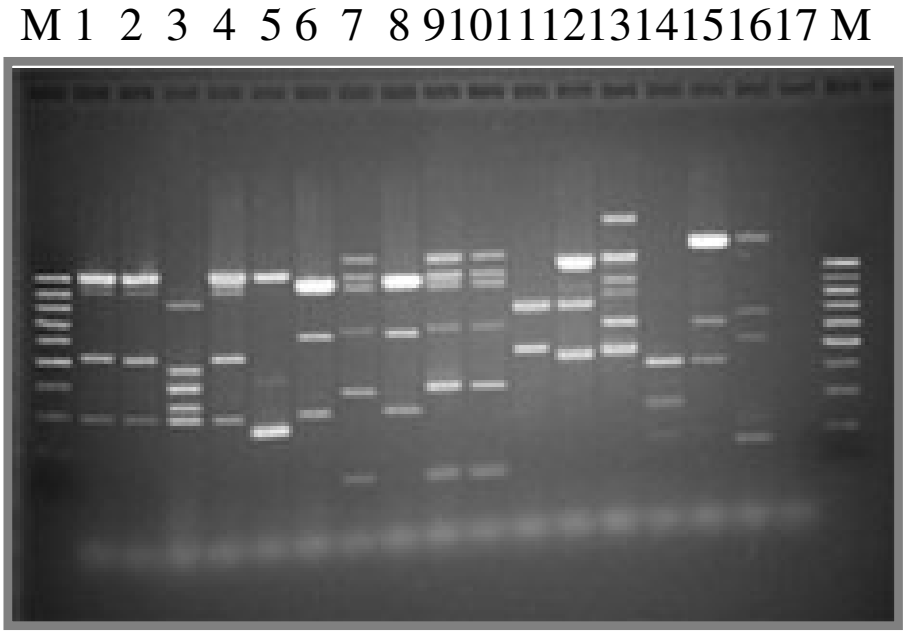

Figure 30 RAPD profiles with the ~17 nt primer D14216: Lane 1: isolate S3; lane 2: isolate S4; lane 3: isolate S20; lane 4: isolate S21; lane 5: isolate S22; lane 6: isolate K3-1; lane 7: isolate I3; lane 8: isolate I7; lane 9: isolate I3R; lane 10: isolate I3A; lane 11: *H. halophilus*; lane 12: *H. trueperi*; lane 13: *H. litoralis*; lane 14: *H. locisalis*; lane 15: *H. karajensis*; lane 16: *H. salinus;* lane 17: negative PCR control; lane M: 100-bp ladder

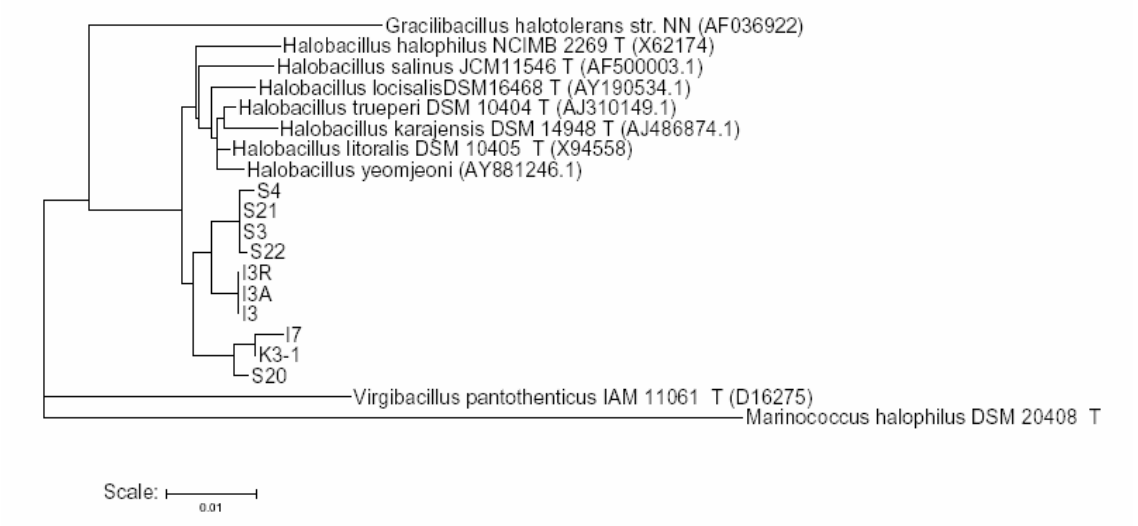

Figure 31 Neighbor-joining tree based on 16S rDNA sequences showing the phylogenetic position of strains S3, S4, S20, S21; S22, K3-1; I3; I7;I3A; I3R and the reference strains *H.litoralis* ASM 10405T, *H.trueperi* DSM 10404T, *H.halophilus* DSM2266T, *H.karajensis* DSM14948T, *H..locisalis* DSM16468T and *H.salinus* JCM11546T as well as some other related taxa.

Molecular characterisation of *Halobacillus* populations isolated from different medieval wall paintings and building materials

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Fig.1: Chapel of St. Virgil: Red wheeled cross painted onto white plaster

Description of the Site of Sampling

Colonization of objects of art by microorganisms can cause different kinds of biodeterioration such as discolouration of materials, the formation of crusts on surfaces and the loss of material, which leads to structural damage. Salt efflorescences which build a niche for extremely salt tolerant and moderately halophilic microorganisms, are a common phenomenon on the surfaces of stone works and mural paintings as a result of changing physical parameters. These microorgansims are related with biodeterioration in Cultural Heritage.

The present study was carried out in two different locations, the Catherine chapel at the Castle Herberstein (Styria, Austria) and the St. Virgil Chapel (Vienna, Austria). The Catherine chapel at the Castle Herberstein is decorated with numerous frescoes of the 14th century (Fig. 2). The location is subdued to frequent outbursts of humidity at irregular intervals. Samples were taken from wall paintings. The St. Virgil Chapel dates back from the beginning of the 14 century. It is located under the ruins of St. Mary Magdaleine Chapel. The whole chapel is covered by salt efflorescences visible by naked eye (Fig.1). A sample was taken directly from salt efflorescence.

Fig.2: Castle Herberstein: Fresco showing Jesus Christ

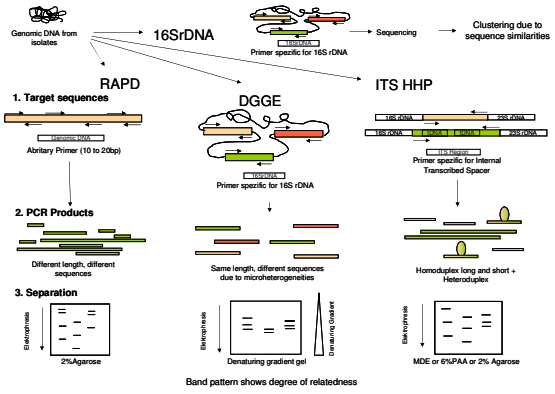
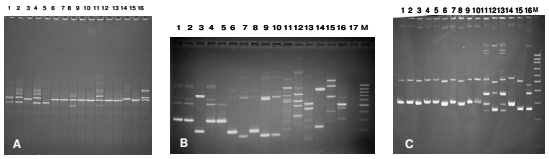


Figure 3: Schematic overview showing molecular methods for typing of bacterial species

Materials and Methods

A total of ten moderately halophilic, gram-positive, spore-forming bacterial strains were isolated by conventional enrichment cultures from wall paintings in the Castle of Herberstein (K3-1, I7, I3, I3R and I3A) (Piñar *et al.* 2001) and from salt efflourescences on the stonework of the Chapel of St. Virgil (S3, S4, S20, S21 and S22). Comparative 16SrDNA sequencing showed that the isolates belong to the family *Bacillaceae* most closely related to the genus *Halobacillus*.

The isolates were subjected to 16S rDNA sequencing (Schabereiter-Gurtner *et al.* 2001) and to a detailed molecular typing including established *Halobacillus* species (Fig.3). Several molecular methods were compared in this study for typing of isolates i.e. Denaturing Gradient Gel Electrophoresis (DGGE) (Muyzer *et al.* 1993), Random Amplified Polymorphic DNA (RAPD-PCR) (Williams *et al.* 1990; Welsh and McClelland 1990) and the Internal Transcribed Spacer-Homoduplex-Heteroduplex Polymorphism (ITS-HHP) fingerprinting (Daffonchio *et al.* 1998) .



Results and Discussion

All *Halobacillus* isolates included in this study were found to be typeable by 16SrDNA, ITS-HHP, RAPD and DGGE (Fig.4). ITS-HHP analysis showed only poor results for discriminating between the new isolates but differentiation on species level was possible as all reference strains gave band patterns different from those of the novel strains. DGGE showed to be a rapid subtyping method. Due to microheterogeneities in the V3 region of the 16SrDNA the isolates from St.Virgil could be discriminated from those gained from the stonework of the Castle of Herberstein. Results obtained from 16SrDNA sequencing showed that the isolates could be divided into three groups thus being a good clustering typing method. The greatest distinctions could be made when using RAPD analysis. Result revealed that strains S3, S4 and S21 are similar as well as K3-1 to I7 and I3 to I3A and I3R. S20 and S22 were different from the others.

By standard bacteriological testing the strains isolated from the castle of Herberstein hydrolysed starch (strain K3-1 and I7) and showed a strong casein hydrolysis activity. As casein is often used to fix mural paintings or is added to the mortars for consolidation these *Halobacilli* are involved in biodeterioration of cultural heritage. This fact should be considered in restoration processes.

Figure 4: Discrimination of *halobacilli* strains using different typing molecular methods. A: DGGE analysis; B: RAPD-PCR using primer D14703; C: ITS-HHP analysis and D: 16S rDNA phylogenetic affiliations. Lines: 1: S3; 2: S4; 3: S20; 4: S21; 5: S22; 6: K3-1; 7: I3; 8: I7; 9: I3R; 10: I3A; 11: *H. halophilus*; 12: *H. trueperi*; 13: *H. litoralis*; 14: *H. locisalis*; 15: *H. karajensis*; 16: *H. salinus*; 17: negative PCR control; M: 100-bp ladder.

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Figure 32 Poster presented on IBBS-13 in Madrid September, 2005

5. Legend of Figures and Tables

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