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Culture dependent and –independent identification of microorganisms on monuments

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Overview

Summary

In 3.8 billion years of evolution microorganisms have learned to adapt to all substrates on earth (Bunyard, 1996). Besides chemical and physical factors, microorganisms inhabiting different stone materials play a key role in the biodeterioration of our Cultural Heritage. Historic buildings, artistic sculptures and other stone works in indoor and outdoor environment as well on modern as on historic objects are colonized by a great variety of organism, including bacteria, archaea, fungi, algae, lichens, mosses, plants and higher organisms (Ettenauer et al., 2010; Piñar et al., 2001a and 2001b; Heyrman et al., 1999; Schabereiter-Gurtner, 2000; Schabereiter-Gurtner et al., 2001a, 2001b and 2001c).

In order to prevent and restore our Cultural Heritage it is important to recognize and understand these microorganisms. It is necessary to gain more insight into their origin, their metabolic activities, their meanings and mechanisms in biodeterioration, the microbial community structures and last but not least the abilities of microorganisms that can be used to protect our objects of art.

In many studies all kind of materials have been investigated using a variety of different methods, grouped into cultivation-dependent and –independent methods, which not always yield coherent data. It is generally accepted that conventional cultivation methods recover less than 1% of all inhabiting bacteria from environmental samples (Amann et al., 1995; Ward et al., 1990). In spite of the disadvantages of standard cultivations assays, physiological and metabolic studies can only be done with pure cultures obtained with the classical approach. On the other hand culture-independent methods have been shown to supply a reliable method to identify and monitor the micro-biota on objects of art (Schabereiter-Gurtner et al., 2001b).

The general methodology used in this study includes as a first step the extraction of DNA from small sample amounts followed by Polymerase Chain Reaction (PCR) directed to amplify fragments of ribosomal RNA genes (16S rRNA) from *Bacteria* and *Archaea* and the further fingerprinting of the microbial community using Denaturing Gradient Gel Electrophoresis (DGGE). Additionally techniques such as conventional cultivation assays, Random Amplified Polymorphic DNA (RAPD), construction and screening of clone libraries and identification of the microorganisms using 16S rDNA sequencing were applied to perform a monitoring in the three projects presented in this thesis.

Samples subjected to investigation were derived from the Chapel of St. Virgil, Vienna (Austria) and from two ancient buildings located at the city of Granada (Spain) – the Monastery of San Jéronimo and the Royal Hospital.

The investigation performed at the Chapel of St. Virgil, in Vienna, focused on the identification of its inhabiting microorganisms, especially focusing on the halophilic members, by using cultivation assays as well as molecular techniques. Comparative sequence analysis revealed that the majority of the isolated bacterial strains belonged to the genera *Halobacillus* and *Bacillus*. Furthermore archaeal clone sequences were identified as members of the genera *Halococcus*, *Halalkalicoccus* and *Halobacterium*.

The investigation performed at the two buildings located in Granada was a part of an international collaboration with the Department of Microbiology at the University of Granada. This working group has a vast experience in the application of carbonatogenic bacteria and/or a sterile-nutritive solution on stone materials to induce a consolidation of the decayed stone materials (Rodriguez-Navarro et al., 2003; Jimenez-Lopez et al., 2007, 2008; Gonzalez-Muñoz et al., 2008).

Under laboratory conditions the dynamics in the microbial community structures on quarry and decayed ornamental carbonate stones, which received a consolidation treatment with *Myxococcus xanthus,* were investigated during a time scale of 30 days. Irrespectively of the origin of the stone the same carbonatogenic microorganisms were activated, namely *Pseudomonas sp*., *Bacillus* sp. and *Brevibacillus* sp. The monitoring of *M. Xanthus* during the time course of the experiment was done using DGGE, conventional PCR and qPCR using species-specific primers designed in this study (by Dr. Guadalupe Piñar).

As follow-up of this investigation, the microbial community structure of two historic buildings in Granada (Spain), which received three different treatments *in situ* for the consolidation of the stone, was monitored. We performed a short-term (for a period of a week) and a long-term molecular monitoring (for a period of one year) of the changes in the microbial communities occurring during and after the application of the treatments in order to evaluate the impact of such consolidation treatments. Results revealed that similar dynamics of the inhabiting micro-biota were initiated on both buildings by the applications. In the first week members of the phyla *Firmicutes* and *Proteobacteria* became activated, whereas after one year on the treated areas the order *Bacillales* (*Firmicutes*) represented the dominant group of microorganisms.

Zusammenfassung

In 3.8 Milliarden Jahren Evolution haben Mikroorganismen gelernt sich an alle möglichen Substrate auf der Erde anzupassen (Bunyard et al., 1996). Neben den chemischen und physikalischen Faktoren spielen Mikroorganismen eine entscheidende Rolle bei der biologischen Zerstörung unserer Kulturgüter. Historische Gebäude, Skulpturen und andere Kunstartefakte aus Stein, sowohl historische und moderne werden von Mikroorganismen (Bakterien, Archaea, Pilze, Algen, Flechten, Mose, Pflanzen und höheren Tieren) besiedelt (Ettenauer et al., 2010; Piñar et al., 2001a and 2001b; Heyrman et al., 1999; Schabereiter-Gurtner, 2000; Schabereiter-Gurtner et al., 2001a, 2001b and 2001c).

Um unsere Kulturgüter zu schützen und für unsere Nachkommen zu erhalten, ist es wichtig diese Mikroorganismen zu identifizieren und zu verstehen. Es ist notwendig deren Ursprung, ihre metabolischen Aktivitäten, ihr Einfluss und den Mechanismus der biologischen Zerstörung, die Strukturen der mikrobiellen Gemeinschaften und nicht zu Letzt auch deren Fähigkeiten, die genützt werden können um diese Kunstobjekte zu schützen, näher zu untersuchen.

In vielen Studien wurden die unterschiedlichsten Materialien mit verschiedensten Methoden, die man in Kultivierungs-abhängige und –unabhängige Methoden einteilen kann, untersucht. Mit den unterschiedlichen Techniken erhält man nicht immer kohärente Daten. Generell kann man durch Kultivierung nur etwa 1% der Bakterien einer Umweltprobe identifizieren (Amann et al., 1995; Ward et al., 1990). Trotzdem kann man nur mit Hilfe von Kultivierungsversuchen die metabolischen und physiologischen Eigenschaften von Reinkulturen untersuchen. Die Verwendung von molekularen Methoden erlaubt eine Identifizierung von Mikroorganismen und die kontinuierliche Überwachung von mikrobiellen Gemeinschaften auf Kunstobjekten (Schabereiter-Gurtner et al., 2001b).

Die Methodik die bei dieser Diplomarbeit zur Anwendung kam, besteht aus der Extraktion von DNA aus dem Probenmaterial, gefolgt von der Amplifizierung von Fragmenten der ribosomalen RNA Gene von Bakterien und Archaea durch PCR (Polymerase Ketten Reaktion) und der weiteren Analyse durch DGGE (Denaturing Gradient Gel Electrophosesis). Zusätzlich wurden Techniken, wie die konventionelle Kultivierung von Mikroorganismen, RAPD (Rapid Amplified Polymorphic DNA), die Konstruktion und die Analyse von Klon-Bibliotheken und die phylogenetische Identifizierung der Mikroorganismen durch 16S rDNA Sequenzierung angewendet.

Die Proben der drei durchgeführten Projekte dieser Diplomarbeit, stammten von der Virgilkapelle in Wien und von zwei historischen Gebäuden aus Granada (Spanien) – dem Kloster von San Jéronimo und dem Königlichen Hospiz.

Bei der Charakterisierung den Mikroorganismen, mit Fokus auf salzliebende/-tolerante Vertreter, von Proben der Wiener Kapelle, wurden klassische Kultivierungsversuche als auch molekulare Methoden verwendet. Durch die vergleichende Sequenzanalyse der isolierten Reinkulturen konnten die meisten Bakterien zu den Gattungen *Halobacillus* und *Bacillus* eingeordnet werden. Die Vertreter der *Archaea* waren am nähersten verwandt zu den Gattungen *Halococcus*, *Halalkalicoccus* und *Halobacterium.*

Die Verwendung von Nährmedien mit und ohne Kalziumkarbonat-produzierenden Bakterien als Festigungsmethode für verwitternde Gesteine wurde in vielen Untersuchungen bereits positiv getestet (Rodriguez-Navarro et al., 2003; Jimenez-Lopez et al., 2007, 2008; Gonzalez-Muñoz et al., 2008).

Unter Laborbedingungen wurden die Veränderungen der Bakteriengemeinschaft auf verwittertem und ursprünglichem Steinen in einem Zeitraum von 30 Tagen untersucht, die eine Behandlung mit bzw. ohne *Myxococcus xanthus* erhielten. Auf beiden Gesteinen konnte man die gleichen Kalziumkarbonat-produzierenden Bakterien nachweisen, nämlich *Pseudomonas sp*., *Bacillus* sp. und *Brevibacillus* sp. Für den Nachweis des als Biomineralisierungsagens verwendete Bakteriums, *M. xanthus*, über den Zeitraum der Untersuchung wurden DGGE, konventionelle PCR und Real time PCR (qPCR) mit Spezies-spezifischen Primern verwendet. Die Spezies-spezifischen Primer wurden in dieser Studie von Dr. Guadalupe Piñar designt.

Abschließend wurde in dieser Diplomarbeit eine *in situ* Kurz- und Langzeit-Untersuchung an zwei historischen Gebäuden in Granada (Spanien) durchgeführt. Beide Gebäude erhielten drei unterschiedliche Behandlungen, für die Festigung des Gesteins. Mit Hilfe von molekularbiologischen Methoden konnte gezeigt werden, dass auf beiden Gebäuden ähnliche Veränderungen in den bakteriellen Gemeinschaften durch die Behandlungen ausgelöst wurden. In der ersten Woche der Behandlung wurden vor allem Vertreter der Stämme *Firmicutes* und *Proteobakterien* aktiviert, wohingegen nach einem Jahr die Ordnung der *Bacillales* (*Firmicutes*) dominierte.

List of abbreviations

A	Adenin
Amp	Ampicilin
APS	Ammoniumperoxodisulfat
aw	water activity
BLAST	Basic Local Alignment Search Tool
bp	Basepairs
BDT v3.1	BIGDYE® TERMINATOR V3.1 CYCLE SEQUENCING RR-100
C	Cytosin
℃	Degree Celsius
cm	Centimeter
CTAB	hexadecyltrimethyl ammonium bromide
dATP	desoxy Adenin
dCTP	desoxy Cytosin
dH_2O	destilled water
ddNTPs	Didesoxy nucleotide
DGGE	Denaturing Gradient Gel Electrophoresis
dGTP	desoxy Guanin
dH_2O	destilled water
DNA	Desoxyribonucleinacid
dNTP	Desoxy nucleotide
dsDNA	double stranded DNA
dTTP	desoxy Thymin
EDTA	Ethylenediaminetetraacetic acid
EMBL	European Molecular Biology Laboratory
FISH	Fluorescence in situ hybridization
G	Guanin
g	Gramm
h	Hour(s)
IPTG	Isopropyl-beta-D-thiogalactopyranoside
ITS	Internal Transcribed Spacers
L	Liter
LB	lysogeny broth
M	Molar
mg	Milligramm
min	Minute(s)
ml	Milliliter
mm	Millimeter
µl	Microliter
µg	Microgramm

mm	Millimeter
mM	Millimolar
mg	Milligramm
NaCl	Soldium chloride
NCBI	National Centre for Biotechnology Information
nm	Nanometer
nt	Nucleotides
O.D.	Optical desity
o/n	Over night
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
pmol	Picomol
RAPD-PCR	Random Amplified Polymorphic DNA-PCR
rDNA	ribosomal DNA
RNA	Ribonucleic acid
Rpm	Rounds per minute
rRNA	ribosomal RNA
SDS-PAGE	Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis
SSCP	Single Strand Conformation Polymorphism
ssDNA	single stranded DNA
Strp	Streptomycine
T	Thymine
TAE	Trisacetic-EDTA
Tc	Tetracylin
TE	Tris-EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
Temp	Temperature
t-RFLP	Terminal restriction fragment length polymorphism
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
U	Units
UV	Ultraviolet
V	Volt
v/v	Volume per volume
VK	Samples taken from the Chapel of St. Virgil
vol	Volume
w/v	Weight per volume
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

1. Introduction

1.1. DETERIORATION OF STONE

The use of different stone materials as medium for artistic expressions and as building material ranges from small statues over historic monuments to all kind of different buildings from the beginning of mankind to the present. The weathering of rocks to soil is a well known degradation process essential for the evolution of life and erosion formed earth over billions of years till the present state and also in future. On the other hand the decay of culturally meaningful stone artefacts, buildings etc. represents an irreversible loss of our cultural heritage. Many different substrates used for building materials as stones, stuccos, mortars, plasters, frescos, ceramic and others, are subjected to deterioration. Weathering processes, including wind, sunlight, temperature, rain, snow and moisture are grouped into physical and chemical factors that have influence on the materials. These agents affect the stability of the rock matrix as well as oxidation and hydration reactions, dissolution of carbonates and solubilisation of some elements cause chemical corrosion of the stone-forming minerals (Keller, 1957). Additionally, anthropogenic factors (e.g. air pollution due to cars, electric utilities, heating and many more) leading to a higher atmospheric concentration of inorganic and organic compounds, that deposit on stone surfaces, contribute to the decay of exposed stone materials (Arnold, 1981 and 1993; Baedecker and Reddy, 1993; Warscheid, 2000; Koestler, 2000). Numerous investigations evaluated the role of biological agents in the deterioration of stone (Koestler et al., 1997; Urzi and Krumbein, 1994; Bock and Sand, 1993) finding that physical, chemical and biological factors act in co-association to degrade different stone materials (Koestler et al., 1994, Valentin, 1993). Microorganisms can alter the mechanisms and rates of chemical as well as physical weathering processes of stones. The identification of the inhabiting microorganisms on the building materials and the further understanding of their involvement and causes in biodeterioration have to be evaluated to find successful measures to prevent and solve the associated problems in order to restore our Cultural Heritage. At the same time it is desirable that the obtained information would be catalogued concerning the different building materials, climatic conditions, other external influences (mankind and animals) and also taking into account the different methods used to gain this knowledge (Piñar and Sterflinger, 2009).

1.2. ENVIRONMENTAL FACTORS DETERMINE THE BACTERIA PRESENT ON MINERAL MATERIALS

Biological colonization and the resulting biodeterioration processes on stone materials are strongly influenced by many environmental factors. These factors determine the type of microbes, their amounts and the overall microbial diversity present on a building material. There are a lot of environmental influences on the growth of microorganisms such as ambient and material humidity, temperature, moisture, light, pH values, the presence of organic and inorganic nutrients and the nature of the nutrient sources, oxygen and carbon dioxide concentrations, physical properties of the surface of the material and also the presence of already inhabiting microorganisms (Weirich, 1988; Valentin, 2003). The most important factor for growth is the availability of water. The water activity number (aw) helps to evaluate the possibility of microbial colonization on a certain material. Most microorganisms can grow in a water activity range from 0.6 to greater than 0.98. Bacteria generally require very humid environments (aw>0.95-0.98), whereas fungi can grow at lower water activities (aw>0.60-0.65). For that reason building materials that can absorb large amounts of water and also keep it for longer periods are usually colonized by a wide variety of bacteria and fungi, whereas breathable and fast drying materials are more difficult to inhabit and harbour a very low diversity of microorganisms.

Stone materials provide a variety of ecological niches, suitable for the colonization by photoautotrophic, chemolithotrophic and also heterotrophic bacteria. The first colonizers on mineral materials are photoautotrophic and/or chemolithotrophic microorganisms that use inorganic and/or organic substrates for their growth. The death and lysis of these primary colonizers, and also their metabolites provide nutrients for the secondary settlement by heterotrophic bacteria (Ortega-Calvo et al., 1991). Further nutrients for heterotrophic bacteria are available from dust layers, from airborne organic compounds, dripping water, animal feces (birds, bats, mosquitoes, etc.), from human beings contaminations and from compounds present in the substrate itself. This scenario shows that the micro-biota on stone materials underlies regular changes leading to fluctuations in the community dynamics. Considering this fact, it is obvious that taken samples at a certain point of time represent only a snapshot of a bacterial community during ongoing dynamics of the inhabiting micro-biota. In order to understand and recognize all microbial members on the stone, a continuous monitoring is necessary. The gained information from such a long-term monitoring can further be used to apply appropriate restoration measures.

The mineralogy, porosity, surface roughness and the capacity to take up water and organic materials of a certain material control its bio-receptivity and the risk of biodeterioration (Krumbein and Gorbushina, 1995). In addition the environmental factors mentioned above affect the type and number of the colonizing microorganisms (Tiano et al., 1995). Biofilms on decayed stones harbour a mixed, complex population, consisting of fungi, algae, filamentous and unicellular microbial populations embedded in a polymeric matrix. A great number of bacteria have been detected on different stone materials showing the dominance of Gram-positive organisms, especially spore-forming bacteria as for example *Bacillus sp*. (Tayler and May, 1991). By using culture-dependent and –independent techniques a broad variety of different bacteria have been identified that inhabit various mineral materials and objects of art: *Actinobispora, Alcaligenes, Amycolata, Aquaspirillum, Arthrobacter, Asiosporangium, Bacillus, Chromohalobacter, Cytophagales, Erythrobacter, Flavobacterium, Micrococcus, Mycobacterium, Nocardia, Paenibacillus, Porphyrobacter, Promicromonospora, Pseudomonas, Pseudonocardia, Rubrobacter, Saccharopolyspora, Salmonella, Sarcina, Sphaerobacter, Staphylococcus, Streptomonospora, Streptomyces, Thermocrismum* (Gurtner et al., 2000; Rölleke, 1996; Rölleke et al., 1996, 1998, 2000; Saiz-Jimenez, 1995, 1997; Portillo et al., 2008; Zanardini et al., 2000).

Several studies have shown that certain bacteria can tolerate high salt concentrations on stone monuments (May et al., 1999), but cultivation strategies, especially in the case of these extreme conditions with high salt concentrations, yielded only very few microorganisms present on these micro-niches.

1.2.1. Salty micro-niches on stone-works represent a habitat for extreme microorganisms

For a long period of time, the domain *Archaea* was considered as inhabitants of extreme, hostile environments (Tindal, 1992; Woese et al., 1990). Halophilic bacteria and archaea were thought to be found only in saline environments like salt lakes, salterns, solar salts, black smokers or subsurface salt formations (Vreeland et al., 1998; Rothschild and Mancinelli, 2001; Sass et al., 2001; Litchfield and Gillevet, 2002; Oren, 2002a and 2002b). Deteriorated monuments, stone works and mural paintings represent neglected environments with high salt concentrations. However, in the last years, it has been observed that these ecosystems are a common habitat for extremely salt tolerant and moderate halophilic bacteria (Rölleke et al., 1996,

1998; Piñar et al., 2001a; 2001b, 2009; Rikpa, unpublished diploma thesis, 2005; Saiz-Jimenez and Laiz, 2000; Gurtner et al., 2000; Heyrman et al., 1999; Laiz et al., 2000). Building stone, wall paintings, etc. - all porous material, particularly if they are subjected to rainwater and rising damp, contain different hygroscopic salts such as carbonates, chlorides, nitrates, sulphates, etc., which are locally concentrated or are dispersed within the porous materials (Ripka et al., 2006). As a result of changing physical parameters, these soluble salts migrate with the water through the stone and after drying out these salts are exposed to the surface of the material. This process leads to the formation of deposits of hygroscopic salts on the surface, so called salt efflorescences. The formed, extremely saline environments represent niches for the settlement of halophilic microorganisms. The microbial growth is further favoured by the ability of hygroscopic salts to retain considerable amounts of moisture. The crystallization processes, additionally mediated by the microorganisms, have destructive effects. Due to the recurring crystallization or recrystallization of some salts to different hydrates, the stone pores suffer additional pressure leading to cracking, powdering and flaking of the material (Saiz-Jimenez and Laiz, 2000).

In previous works salt-tolerant microorganisms were often overlooked due to inappropriate cultivation methods, but several investigations based on molecular techniques have demonstrated that monuments where salt crusts on the surfaces of the walls are a common phenomenon represent a habitat for halphilic microorganisms (Saiz-Jimenez and Laiz, 2000). The Chapel of St. Virgil was already subject of previous studies performed by Ripka et al. (2006) and Piñar et al. (2009). The usage of molecular methods, comprising of PCR analysis with archaea-specific primers and DGGE fingerprinting, showed that many of the obtained sequences were phylogenetically affiliated with halophilic members of the *Archaea*, namely *Halobacterium* and *Halococcus*. The results of their studies agree with the results of this Diploma Thesis, showing that the previously detected halophilic archaea represent a stable community in the Chapel of St. Virgil and that these microorganisms are commonly found on micro-niches represented by salt efflorescences.

1.3. BIOMINERALIZATION – A SOLUTION TO BIODETERIORATION?

The process of the production of minerals by living organisms is called biomineralization. This is a very widespread phenomenon and over 60 different

minerals have been reported in various organisms. These minerals often form structural features, harden or stiffen the existing tissues or are released to the exterior. Numerous studies have shown that a great variety of bacteria, isolated from different materials, induce and mediate mineralization of different minerals (Banfield and Hamers, 1997; Douglas and Beveridge, 1998; Ehrlich, 2002; Ercole et al., 2007). The ability of some bacteria to precipitate calcium carbonate, so called bacterial carbonatogenesis (Rodriguez-Navarro et al., 2003), has drawn the attention to these microorganisms from basic and applied points of view. Recently possible scientific and technological applications of bacterial biomineralization, such as a new environmental friendly and effective conservation strategy for historic buildings and stone artworks, were taken into account (Le Mètayer-Levrel et al., 1999). Various microorganisms (e.g. *Bacillus cereus*) were tested as biomineralizating agents to consolidate stone surfaces (Castanier et al., 2000; González-Muñoz et al., 2008; Orial et al., 1993; Tiano et al., 1999).

The microorganism used for consolidation treatments of stone-works in this Diploma Thesis is *Myxococcus xanthus*, an abundant gram-negative, non-pathogenic aerobic soil bacterium, belonging to the δ-subdivision of the *Proteobacteria*. The complex life circle of this bacterium involves a remarkable process of differentiation and morphogenesis (Dworkin and Kaiser, 1993) and it is able to induce extracellular precipitation of calcium carbonate. Through the metabolic activity of *M. Xanthus*, CO_2 and NH_3 are produced, resulting in an extracellular increase of pH values and $CO_{3(aq)}^{2-}$ concentration, according to the following equilibriums:

$$NH_{3(g)} + H_2O \Leftrightarrow NH_4^+{}_{(aq)} + OH^-{}_{(aq)}$$

$$CO_{2(g)} + H_2O_{(l)} \Leftrightarrow H_2CO_{3(aq)} \Leftrightarrow HCO_3^-{}_{(aq)} + H^+{}_{(aq)} \Leftrightarrow CO_3^{2-}{}_{(aq)} + 2H^+{}_{(aq)}$$

The precipitation of $CaCO_3$ occurs when a sufficient super-saturation of CO_3^{2-} is reached with respect to this equation (Rodriguez-Navarro et al., 2007):

$$Ca^{2+}{}_{(aq)} + CO_3^{2-}{}_{(aq)} \Leftrightarrow CaCO_{3(s)}$$

The newly formed calcium carbonate creates a 10-50 μm thick layer of coherent carbonate cement that roots 1 mm down into the stone, remaining the porosity of the stone unchanged.

Jimenez-Lopez et al. (2007 and 2008), Jroundi et al. (2010) and Piñar et al. (2010) studied the natural microbial communities on calcarenite stones and the community dynamics throughout a consolidation treatment with the mentioned carbonatogenic bacterium, *M. xanthus*, or with the application of a nutritional culture media. Their

results provide new possibilities for conservation treatments of stone-works: (i) The use of bacterial strains, able to induce calcium carbonate precipitation on the stone, (ii) or the mere application of an appropriate nutritive solution which supports the growth of inhabiting microorganisms with the capability to produce $CaCO_3$, offer new possibilities of environmental friendly methods to restore our cultural heritage presented by stone artworks.

1.4. METHODS FOR THE DETECTION OF MICROORGANISMS

1.4.1. Culture-dependent techniques

Traditional research in this field has been done using mainly classical cultivation methods. In order to evaluate the risk of microbial contamination and biodeterioration on historic buildings, monuments, statues and other stone art, it is important to identify and quantify the inhabiting microorganisms. The isolation and cultivation of microorganisms is usually carried out by incubation of sample material in liquid and/or solid selective media. Therefore, cultivation assays require high amounts of sample material, leading to massive problems when working with objects of art. Additionally, these classical methods are very time-consuming and require 1-6 weeks for cultivation. Quantification of the isolated microorganisms is usually done by plate count and most probable number (MPN) techniques. However, by using culture-dependent methods, problems arise during the detection of members from environmental samples. Many studies have shown that only a very small proportion of the total microbial community can be obtained (Pace, 1996; Ward et al., 1990). Even less than 1% of the inhabiting bacteria can be detected by standard cultivation techniques due to problems such as: (a) the use of unsuitable culture media; (b) insufficient incubation time; (c) the non-culturability of microorganisms under laboratory conditions due to the lack of suitable methods; (d) bacteria in a non-culturable state and (e) obligate symbiotic and/or parasitic organisms are unable to grow on bacteriological media in the absence of their host. As a consequence of these problems and limitations the obtained cell numbers are underestimated and do not provide a complete picture of the microbial diversity present on the stone. Nevertheless, the development of new culture media and the use of conventional cultivation methods are essential to obtain pure bacterial strains in order to perform physiological and metabolic studies.

Combining molecular methods with classical cultivation assays is a very good approach to characterize the microorganisms in an environmental sample and to get a better overall picture of the bacterial diversity present in different habitats. The phylogenetic information obtained with molecular techniques can be very useful to design new suitable culture media in order to cultivate the newly identified microorganisms (Piñar et al., 2001b). An overview of a protocol for the analysis of microbial communities is given in Figure 1, where culture-dependent and -independent techniques are combined.

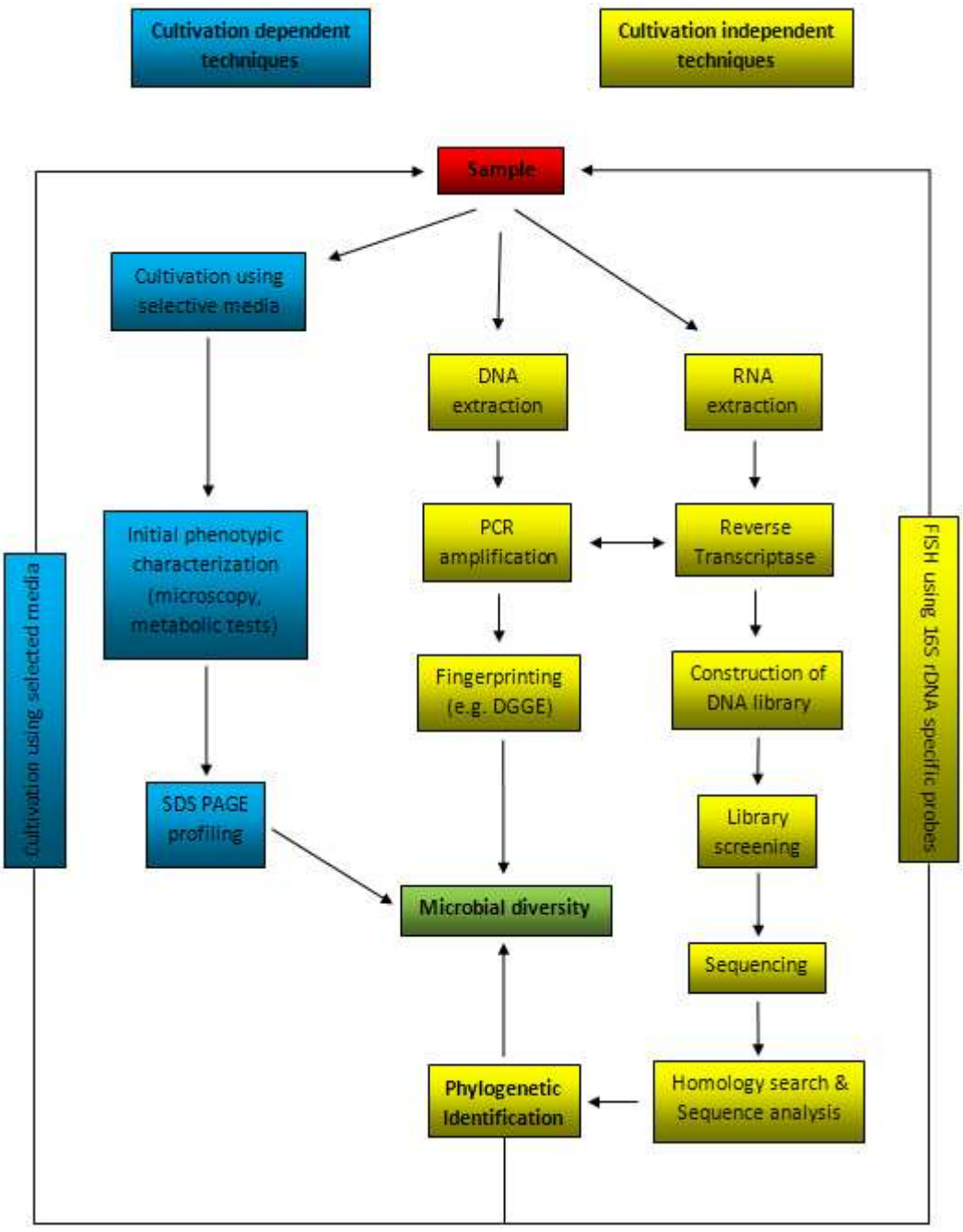


Figure 1
Schematic overview of culture dependent and –independent techniques.

1.4.2. Culture-independent techniques

In the last decades, a series of new molecular techniques for culture-independent analysis of microbial communities on environmental samples have been developed (Muyzer et al., 1993; Schabereiter-Gurtner et al., 2001b). These methods allow the detection of a wider variety of microbial species than can be obtained with conventional cultivation assays, and therefore deliver a more reliable picture of the micro-biota present on the sample of art (Laiz et al., 2003a and 2003b). The bases for most of the studies are ribosomal sequences used as phylogenetic markers (Woese, 1987). These universal marker sequences (e.g. 16S for bacteria, 18S for eukaryotes and ITS for fungi), also called molecular chronometers, are present in all organisms. The sequences of the small subunit ribosomal RNA (rRNA) genes are large sequences with many domains, containing conserved as well as variable regions. The 16S rRNAs have a high degree of functional constancy, a high copy number in each organism and can be sequenced directly. All these attributes designate the 16S rRNA as ideal marker for the study of bacteria and allow distinguishing between different organisms on all phylogenetic levels. Further, the availability of complete DNA databases for rRNA genes allows an identification and classification of the detected sequences.

One possible approach (see Figure 1 and Figure 2), using the 16S rRNA sequences of the microorganisms, is the specific PCR amplification of these RNA genes with primers targeting the conserved regions. The next step in the procedure is to display a fingerprint to visualize the complexity of the microbial community, i.e. by using Denaturing Gradient Gel Electrophoresis (DGGE). The additional construction of clone libraries containing PCR fragments of the ribosomal genes and the further screening of the clone inserts by DGGE allows a comparison of the 16S rDNA clone sequences with the fingerprint from the original community profile. The phylogenetic characterization of the individual clone inserts is done by sequencing of the 16S rDNA fragments and further comparison with databases (Schabereiter-Gurtner et al., 2001a-c).

1.4.2.1. DNA extraction and PCR amplification of target genes

The first step of the molecular approach is the extraction of nucleic acids, either DNA or RNA, from the taken samples. Generally, the protocol for the extraction of DNA, respectively RNA, has to be adapted to the sampling material, e.g. historic buildings,

paintings, different objects of art and other cultural assets. The sample amounts collected from building materials are generally very small, often even less than 1 mg, and therefore a special protocol provided by Schabereiter-Gurtner et al. (2001b) is often used to overcome this problem. Figure 2 gives a short overview of the main steps of the protocol.

Specific target genes (rRNA genes) of the extracted DNA are further amplified using the Polymerase Chain Reaction (PCR) technique. For PCR amplification two gene-specific primers are required in order to obtain a high number of copies of the desired gene fragment. Therefore, usually 25-35 cycles of melting of the DNA strands, primer annealing and primer extension are performed. Nowadays, a great variety of primers is available to target any class of microorganism (*Archaea*, *Bacteria* or *Eukarya*) in a certain micro-biota. The possibility to design primers allows a group-specific amplification of DNA (Daly et al., 2000; Petri and Imhoff, 2000) and even the identification of a single species in a microbial community.

Beside the small sample amount, another problem often occurs with samples derived from objects of art. The usual high concentration of impurities and the presence of inhibitors can complicate the molecular work. To overcome these problems two PCR reactions are performed with two sets of primer pairs. In the first round of PCR a large fragment of the 16S rDNA is targeted and multiplied. In the second round of PCR, a nested-PCR, the primers are directed to a small part within the fragment amplified in the first round. Additionally, one primer of the nested PCR has a GC clamp at its 5’ end, which stabilizes the melting behaviour in DGGE analysis (see Figure 3).

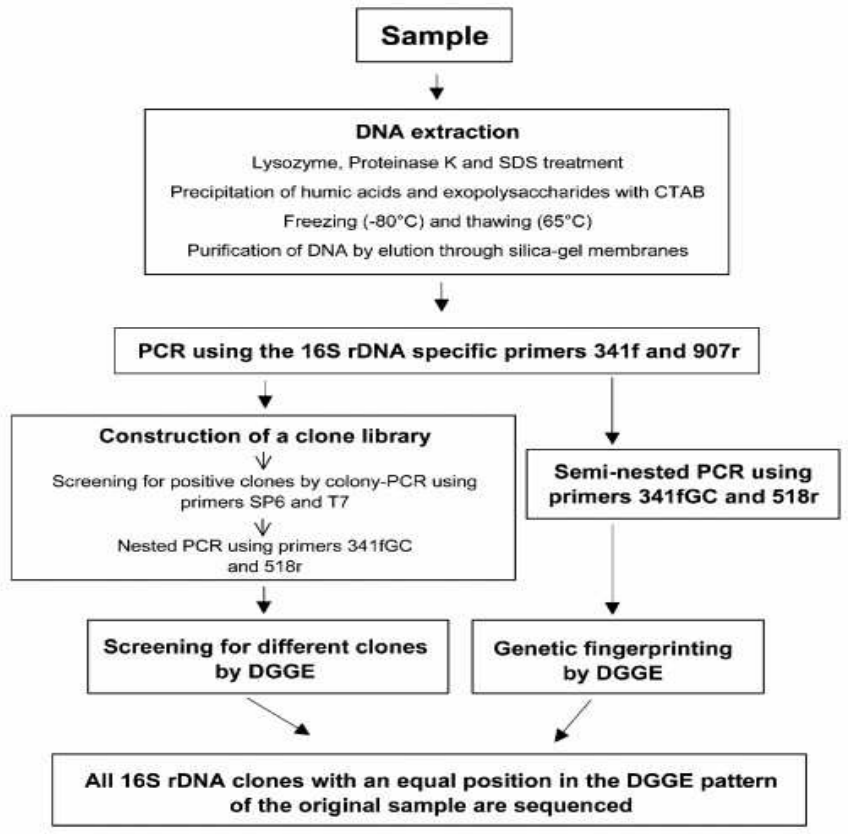


Figure 2
Detailed schematic diagram showing the experimental steps of a molecular approach to analyze the structure of microbial communities and the phylogenetic charcterization of the individual members (Schabereiter-Gurtner et al., 2001b).

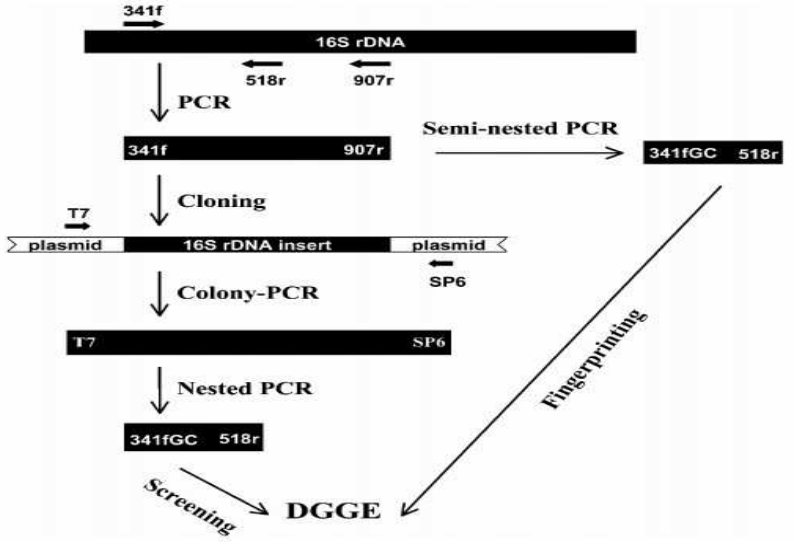


Figure 3
Schematic diagram showing the primers 341f and 907r used for the construction of 16S rDNA clone libraries. For genetic fingerprinting of bacterial communities by DGGE, the primers 341GC and 518r are used in a semi-nested PCR reaction. Screening of clones is done with the vector specific primers SP6 and T7. Positive clones are further amplified with the primers 341GC and 518r for DGGE analysis (Schabereiter-Gurtner et al., 2001b).

1.4.2.2. Genotyping techniques – Fingerprinting

In recent years, a series of different genotyping techniques have been developed, all using the products of an antecedent PCR amplification. The amplified rRNA genes from different microorganisms show a different electrophoretic mobility and reveal different patterns. As a result, the PCR products of a microbial community from an environmental sample can be visualized and differentiated by their electrophoretic migration, displaying a certain profile. This individual profile, also called microbial community fingerprint, can be compared with the profiles of other samples and it allows an evaluation and even a monitoring of environments, when samples are taken at different points of time.

Some of the established techniques for the fingerprinting of environmental samples are listed below:

- Denaturing Gradient Gel Electrophoresis (DGGE)
- Temperature Gradient Gel Electrophoresis (TGGE)
- Amplified ribosomal DNA restriction analyses (ARDRA)
- Ribosomal intergenic spacers analyses (RISA)
- Terminal-restriction fragment length polymorphisms (t-RFLP)
- Single strand conformational polymorphisms (SSCP).

1.4.2.2.1. Denaturing Gradient Gel Electrophoresis (DGGE)

In many studies, Denaturing Gradient Gel Electrophoresis (DGGE), as an electrophoretic separation method, was successfully applied for the investigation of microbial communities in environmental samples and for the screening of clone libraries (Ettenauer et al., 2010; Rölleke et al., 1996, 1998; Piñar et al., 2001a-c, 2009; Schabereiter-Gurtner et al., 2001a, 2001b; Gonzalez and Saiz-Jimenez, 2004a, 2004b; Cappitelli et al., 2007; Miller et al., 2008; Portillo et al., 2008). DGGE, as a molecular fingerprinting technique separates PCR-generated DNA products of same length but with different sequences. Based on differences in the melting behaviour of double stranded DNA fragments (Fisher and Lerman, 1979) (three hydrogen bonds between GC and only two between AT), the PCR fragments can be separated in a polyacrylamide gel containing a linear gradient of DNA denaturants (Urea and Formamide) (Muyzer et al., 1993; Muyzer and Smalla, 1998). Each bacterial species has its characteristic ribosomal DNA sequence and, as a consequence, the PCR fragments of the rRNA genes theoretically show different

band motilities in the gel. For the stabilization of the PCR fragments, one primer used for the nested PCR contains a GC-rich tail (about 40 bases) at its 5' end (GC-clamp) (Muyzer et al., 1993). During the run the temperature and the voltage are constant, at 60℃ and 200V. Depending on the PCR products, one run takes about 3.5 h for bacterial sequences and 6-12 h for ITS sequences of fungi. After the run the acrylamide gel is usually stained in an ethidium bromide solution and further visually analyzed by a UV documentation system.

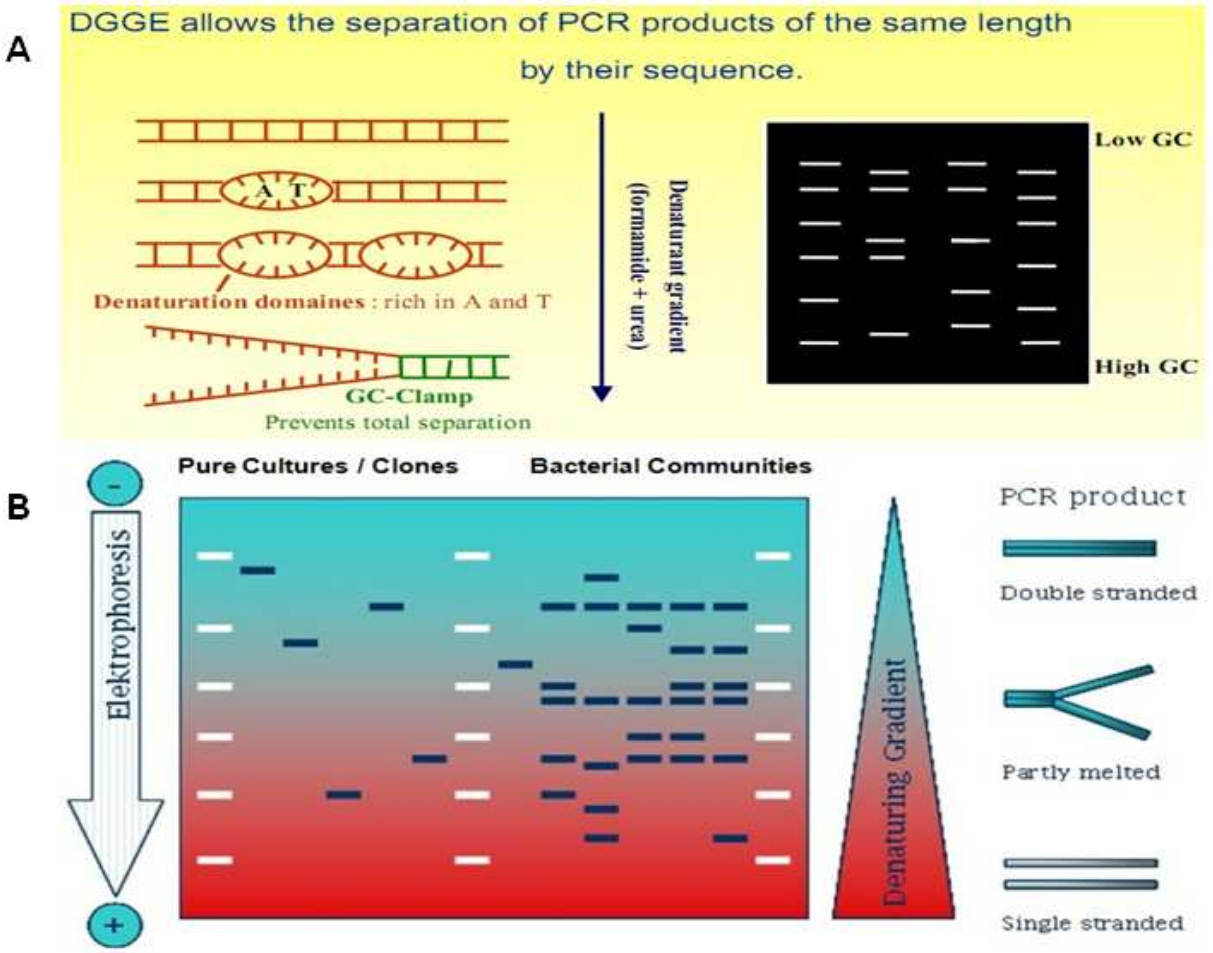


Figure 4
Schematic overview of the methodology of DGGE (Denaturing Gradient Gel Electrophoresis). **A** and **B.** The denaturing gradient comprising urea and formamide in a polyacrylamide gel melts the double stranded PCR products according to their GC content. PCR fragments with a low GC content migrate very short in the gel, whereas DNA sequences with a high amount of GC basepairs have a great motility in the acrylamide gel. The GC clamp at the 5' end stabilizes the melting behaviour of the PCR generated 16S rDNA fragments (Ripka, unpublished diploma thesis, 2005).

1.4.2.2.2. Random Amplification of Polymorphic DNA (RAPD)

A technique for genotyping of pure strains is Random Amplification of Polymorphic DNA (RAPD). The general principle of this method is based on the fact that the polymorphisms among amplification products can be used as genetic markers. Therefore, single oligonucleotides of arbitrary chosen sequence are used to prime DNA synthesis at low stringency (low annealing temperature). The oligonucleotide primers match, or partially match at different sites in the entire bacterial genome. These short primers (8-17 nucleotides) are not directed to any specific sequence but randomly hybridize to initiate DNA polymerization and as a result, strain-specific profiles of DNA products are amplified (Welsh and McClelland, 1990; Williams et al., 1990). The proximity, number and the location of the priming sites vary between different strains and produce semi-unique DNA fingerprints. A great advantage of this technique is the fact that no prior knowledge of the DNA sequence is required, and for that reason, this technique can theoretically be applied to any organism. In a series of studies, RAPD has been adapted for the genetic fingerprinting of microorganisms isolated from building materials and monuments and for the discrimination of different bacterial species (Ettenauer et al., 2010; Urzi et al., 1999; Ripka et al., 2006; Suihko et al., 2007).

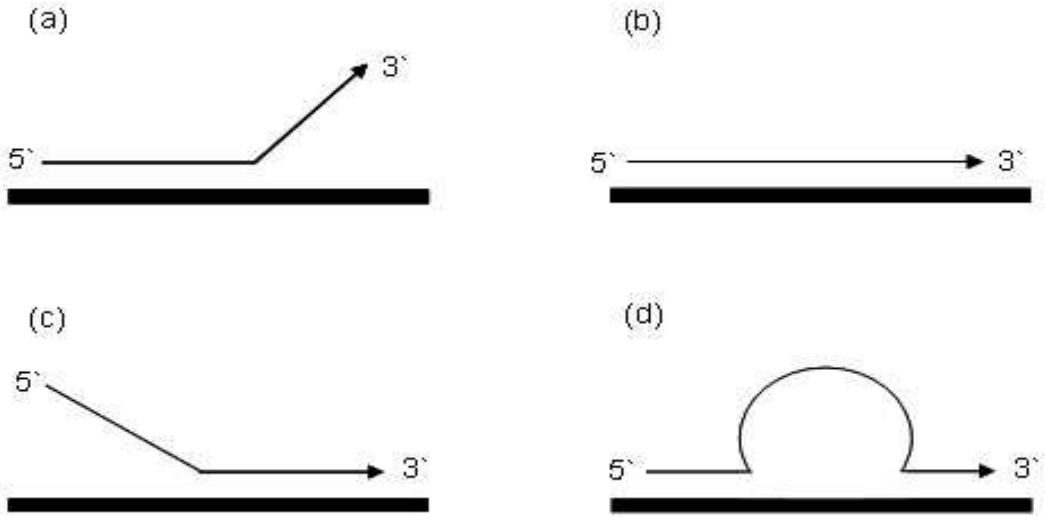


Figure 5

Primer-template interactions during DNA amplification in PCR: (a) annealing only at the 5' end, no annealing at the 3' end, no extension possible; (b) perfect annealing allows extension; (c) no annealing at the 5' end, but annealing at the 3' end, extension possible; (d) annealing at the 5' and 3' ends and formation of a loop or hairpin, extension possible (Power, 1996).

1.4.2.3. Clone libraries and sequence analysis

The different members of a microbial community must further be phylogenetically identified. Therefore, the individual bands in the DGGE fingerprint can be cut out of the gel, sequenced and compared with sequences of known species in databases. However, phylogenetic analyses of sequences obtained directly from DGGE patterns are often difficult. Sequence information obtained by direct sequencing of manually excised bands does not always allow reliable phylogenetic analyses, due to short sequence length. Furthermore, co-migration of several different 16S rDNA sequences, which have the same melting behaviour and therefore the same position in the acrylamide gel, leads to overlapping DGGE bands, which cannot be sequenced directly (Schabereiter-Gurtner et al., 2001a-c).

To overcome this problem, a more time-consuming approach is the construction of a clone library of the investigated sample. This is done by ligating the amplified 16S rDNA fragments from the environmental sample into an appropriate vector system and the further transformation of the vectors with different 16S rDNA-inserts into competent cells. The screening of the clones by DGGE is a time consuming step, but it allows a comparison of the band motility of the individual clone insert with the community fingerprint of the original sample. The individual 16S rDNA clone sequence can be associated to dominant, faint or not visible bands in the profile of the sample revealing more reliable information. Clones matching with bands from the environmental sample or showing different band migration, are selected, sequenced and compared with databases to phylogenetically identify the rDNA fragments. The length of the 16S rDNA fragment of the cloned sequence is enough to obtain an unambiguous characterization of a microorganism at the genus level. A homology search in internet databases, like EMBL (European Molecular Biology Laboratory) or NCBI (National Centre for Biotechnology Information) for known DNA sequences with the search tools FASTA or BLAST (Altschul et al., 1997; Pearson, 1994) provides detailed information on the taxonomic and phylogenetic lineage of the microorganism corresponding to the cloned sequence.

A newly developed method by Gonzalez et al. (2003), which was also tested in one part of this Diploma Thesis, allows on the one hand an efficient screening of clones and on the other hand saves costs (up to 90%) and time. In this procedure, the clones are arranged in groups of ten and further processed by PCR amplification and analysed by DGGE. If a set of clones includes a sequence of interest, the individual members of the group are analysed separately.

1.5. AIM OF THIS WORK

Microbial activity on stone-works and microbial biodeterioration of historic buildings are accepted knowledge and have been proven by many studies. Different restoration and conservation methods, including the application of bioconsolidation treatments (with and without a carbonatogenic bacterium) were developed and tested for their ability to restore our stone-made cultural heritage. The hence resulting changes in the microbial community structures on the treated buildings and the ongoing dynamics of the inhabiting micro-biota have been to date subject of very few studies.

In this Diploma Thesis, three projects were realized monitoring and evaluating the microbial communities on three different buildings, which received different treatments under laboratory as well as environmental conditions.

In the first project, culture-dependent and –independent techniques were used to investigate the inner walls of the medieval Chapel of St. Virgil in Vienna (Austria) and an artificial wall painting placed in the chapel. The salt-attacked stone surfaces of the chapel represent a habitat for extremely salt tolerant and moderate halophilic microorganisms. We investigated and monitored the cultivable and non-cultivable members of the microbial community present on the stone work after severe disturbances of the microbial environment caused by several desalination and disinfection treatments. Using Denaturing Gradient Gel Electrophoresis (DGGE), Random Amplified Polymorphic DNA (RAPD) and 16S rRNA gene sequencing, the genetic diversity of the bacterial strains, isolated from the stone work, was analyzed. The isolated strains were clustered into groups on the basis of their RAPD patterns and DGGE profiles. Representatives of each group and additional isolates were selected for phylogenetic identification using 16S rDNA sequencing. In addition, PCR-DGGE fingerprints in combination with the creation of clone libraries and sequencing analysis were used to identify and monitor the non-cultivable members of the community, the *Archaea*. The results of this investigation were compared with previous detected halophilic microorganisms found in the Chapel of St. Virgil.

The second and the third part of this thesis were implemented in cooperation with the Department of Microbiology (University of Granada, Spain).

In the first work, we investigated under laboratory conditions the bacterial communities inhabiting quarry and decayed ornamental carbonate stones before and after the treatment with a *Myxococcus xanthus*-inoculated culture medium, used for the consolidation of the stones. The dynamics of the community structure and the

prevalence of the inoculated strain, *M. xanthus,* were monitored during the time of the consolidation treatment (30 days). A molecular strategy, consisting of PCR-DGGE (Denaturing Gradient Gel Electrophoresis) analysis, construction and screening of eubacterial 16S rDNA clone libraries by DGGE and sequencing of selected clones that matched with dominant bands from the DGGE fingerprint of the original samples, was applied. Monitoring and quantification of the inoculated strain was performed by culture-dependent techniques (performed by the Spanish working group), DGGE-analysis, conventional Polymerase Chain Reaction (PCR) and quantitative real-time PCR (qPCR; performed by Dr. Guadalupe Piñar) using *M. xanthus* -specific primers designed by Dr. Guadalupe Piñar in this study.

In the last part of this Thesis, the autochthonous micro-biota of two ancient buildings in Granada (the Monastery of San Jéronimo and the Royal Hospital of Granada, Spain) which afterwards received an *in situ* bioconsolidation treatment, consisting of the application of a nutritional solution (with and without *Myxococcus xanthus*) and sterile water as control, was investigated. A continuous short- and long-time monitoring of the changes in the bacterial community structure on the two buildings was performed. Therefore, pellet samples obtained from enrichment cultures conducted in the first week of the treatment and stone samples taken after six and twelve months, were analyzed using molecular techniques. DGGE analysis of PCR amplified 16S rDNA fragments of the samples, construction of clone libraries and screening of the clones using DGGE profiling were used to select different clones for phylogenetic characterization by 16S rDNA sequencing of the clone inserts. The monitoring of the carbonatogenic bacterium used for the consolidation treatment (*M. xanthus*) was done by PCR-DGGE analysis and PCR amplification with species-specific primers. The evaluated results show the dynamics in the bacterial community structures on each differently treated area on both buildings in a short-term (during the first week) and further during the whole time of the investigation (one year). Finally, the structural changes in the micro-biota on the two buildings that received the same treatments were compared with each other.

2. Materials and Methods

2.1. SAMPLING

2.1.1. The Chapel of St. Virgil

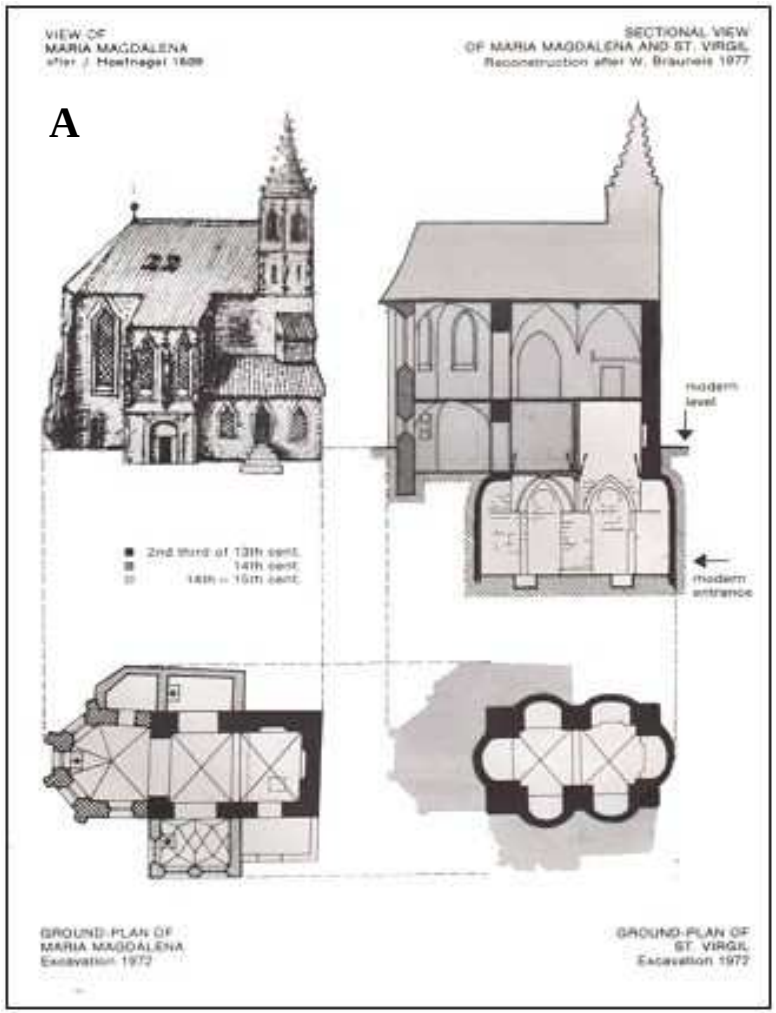
The Chapel of St. Virgil is located under the ruins of St. Mary Magdalene Chapel next to the Dome of St. Stephan in the 1st district of Vienna (Austria). The architecture of the underground crypt can be dated back to the early 13th century, when Frederick II. (1230-1246), the last Duke of the House of Babenberg, was the ruler of Vienna. He built the chapel as a Crypt for St. Coloman, the patron of the new diocese. However, the chapel was not mentioned in the chronicles till the year 1307, when a burial chapel of the Viennese family Chrannest was mentioned in a document. This chapel, used as the family burial place, was located at the Cemetery of St. Stephan, had a few altars and one was dedicated to the Saint Virgil – giving the chapel its name.

In the Middle Ages, the Dome of St. Stephan was surrounded by a large cemetery. The two-story Chapel of St. Mary Magdalene was used for consecrations and requiem masses, having the Chapel of St. Virgil lying directly under this chapel. A vertical shaft, connecting these two buildings, was constructed in 1378. After the family Chrannest suffered extinction, the chapel was used for requiem masses and became a meeting room for different brotherhoods. In the year 1781, the St. Mary Magdalene Chapel was destroyed by a fire and demolished. The underground Chapel of St. Virgil was filled with rubble and fell into oblivion. It was rediscovered during the construction of the Vienna underground railway in 1973 (Feuchtmüller, 1978).

Especially because of the original arches and the motif of the red cross in a red circle, the chapel can be dated to the beginning of the 13th century. The east-west orientated rectangular room lies approximately 12 meters beneath the square of the Dome of St. Stephan (Figure 6 A). It is 10.5 m long, 6 m wide and has 6 rectangular niches with pointed arches, of which one of the recesses was removed and represents the present entrance to the chapel. The mortar and rubble walls are 1.5 m thick, white plastered and painted with red lines (Figure 6 B). On the ceiling of each recess is a red cross in a circle. The ground of the chapel consists of firm loam with a 1 m deep round well with unknown function. The entrance of the chapel is still unclear. Possibly the underground room could be reached through a trap door from the St. Mary Magdalene Chapel above (Hartl, 1995).

The Chapel of St. Virgil has survived with only few damage and provides a good glance into the world of the Middle Ages. But water migrates horizontally through the walls from the surrounding soil, carrying a lot of soluble salts that crystallize, so that the whole room is covered with salt efflorescenses (Figure 6 D and E), visible by naked eye. This phenomenon is causing material losses and destruction of original medieval fineries and paintings (Figure 6 C).

Figure 6
A. Picture of the Chapel of St. Virgil showing the location and ground plan. **B.** Red wheeled cross painted onto white plaster on the wall of the chapel. **C.** Detached stone material that trickled off the wall to papier-mache. **D** and **E.** Typical salt efflorescence on the stone work of the chapel.



2.1.2. The Monastery of San Jerónimo

The Monastery of San Jerónimo is located southwest from the Hospital de San Juan de Dios in Granada. It was founded in 1492 by the Catholic kings and consists of a church, two cloisters with gardens that are decorated with fountains and orange trees and some additional rooms. The construction works were started in 1496 and finished in 1547. Francisco and Jacobo Florentino were the two Italian master architects who commenced building in 1525, followed by another master architect Diego de Siloé who continued the work after the death of Jacobo in 1528. Doña María Manrique, who also financed the construction works of the church, first conceived it as a pantheon for her deceased husband Gonzalo Fernández de Córdoba, "El Gran Capitán". In the splendid Capilla Mayor is the burial place of Gonzalo and his wife, whose kneeling statues are also flanking the high altar.

The portal and façade, having a very artistic decoration by Diego de Siloé, and the tower of the Monastery belong to the first Renaissance and the walls of the church are completely covered with 18th century wall paintings. The Monastery has a Latin cross plan with one nave covered by Gothic vaults and side chapels. Jacobo Florentino carried out the transept and the major chapel. In the altarpiece of the major altar (1570-1605), which extends from the front of the apse to the vault, Renaissance and Mannerist characteristics are realized. The altarpiece consists of four bodies with several valuable sculptures, relieves and carvings that represent scenes from Christ' life, the Apostles, saints and angels. The beauty of the Monastery courtyards, gardens and the cloisters are also amazing.

The richly decorated Monastery of San Jerónimo was plundered by the French and was eventually closed down in 1835 to be converted into military barracks, especially for cavalry. Restoration of what was left of the buildings commenced in 1958 with the contributions of the University of Granada with the Hieronymite Order. In 1977, nuns could again move into the Monastery of San Jerónimo. Nowadays, it is a wonderful place for tourists and it gains insight into the historical architecture of the 16th and 17th century. Therefore, the Monastery of San Jerónimo is protected by the Spanish Historical Heritage Legislation and it has been recognized as World Heritage by the UNESCO.

Figure 7
A. Picture of the Monastery of San Jerónimo. **B.** Photograph of the entrance. **C.** Inside view, showing the two cloisters of San Jerónimo.

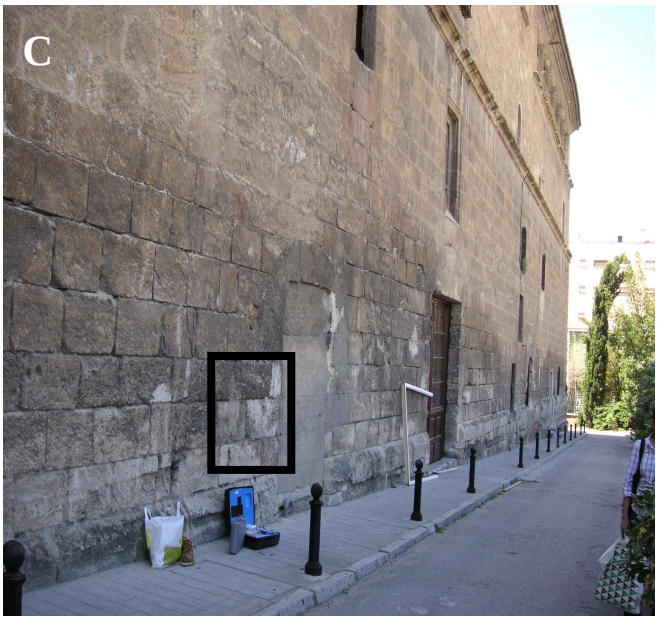
2.1.3. The Royal Hospital of Granada

The Royal Hospital (local name: Hospital Real) is located Northwest of the Plaza del Triunfo in Granada, on Calle Ancha de Capuchinos. It was built during the Catholic kings' reign and commissioned by the Catholic Monarchs Isabel and Fernando on 15th September 1504. The construction was started in 1511 under the design of the architect Enrique Egas, who had created the Hospital of Santiago de Compostela a few years earlier. Enrique Egas' work was based on a simplified model used by the Italian Antonio Filarete for the Ospedale Maggiore of Milan. The work was interrupted after the death of Ferdinand, the Catholic Monarch, and was restarted in 1522 by the emperor Carlos V. The front façade was not finished until the first half of the 17th century and so figuratively speaking the building combines a mixture of different styles: Gothic, Renaissance and Mudejar. It can be described as a Gothic representation of a Renaissance structure with incorporation of Spanish Mudejar building techniques.

The ground plan of the building has the form of a Greek cross: The inside of the building is divided by the cross into four equal parts. The portal was made by García de Pradas with marble from the Sierra Elvira. On top of four Corinthian columns at the façade is a statue with an image of Virgin Mary flanked by statues of the founders Isabel and Fernando built by Alonso de Mena (Figure 8 A). At the outside of the portal, the coat of arms of the Catholic kings and Carlos V is also present. Inside, in the lower floor, the naves are covered by alfarjes, whereas in the upper floor by mocarabs. The Royal Hospital has many rooms with ceilings decorated in Mudejar but also in Renaissance style. Worth seeing are the cloister, the staircase and the Mudejar-vault of the church.

The Royal Hospital was one of the first and one of the few civil monuments in Granada and was situated outside the city walls. The building was used for a variety of purposes, but originally it served as a hospital for the poor, pilgrims and for injured soldiers during the conquest of Granada against the Arabs. After 1536, it was used as a prison for insane people, later as a place to treat people who suffered syphilis and venereal diseases from all over Spain. Nowadays, the Royal Hospital houses the offices of the University of Granada, including the Rectorate, administrative buildings and the main library of the University. It has become a cultural centre which is often used for ceremonies, exhibitions and other cultural activities.

Figure 8
A. Front view of the portal of the Royal Hospital of Granada constructed by García de Pradas, showing the figure of Virgin Mary, the flanking statues of the founders and the coat of arms of the Catholic kings and Carlos V. **B.** Photograph of the cloister of the Royal Hospital. **C.** Picture of an outside wall of the building where the application of the treatment was performed (marked with a black rectangle).



2.2. DNA EXTRACTION

In the present study, two different DNA extraction protocols were used: Thereof, one allows the extraction of PCR-amplifiable DNA from samples derived from stone material, respectively from mixed cultures. This protocol was especially designed to cope with the problems of PCR inhibitors and small sample amounts taken from ancient buildings. A mixture of plenty different archaeal, bacterial and fungal DNAs can be gained. The second protocol was used for the DNA extraction from pure bacterial strains. Both methods allow PCR amplification of the obtained DNA and further DGGE fingerprint analysis.

2.2.1. DNA extraction from soil material, objects of art or enrichment cultures

This protocol was used to extract the DNA from stone material/objects of art/enrichment cultures (Schabereiter-Gurtner et al., 2001a).

Small amounts (1-200 mg) of sample material were mixed with 100 µl DNA extraction buffer 1 [150 mM Na_2EDTA, 225 mM NaCl; pH 8.5] and 45 µl lysozyme (stock: 50 mg/ml) (Tsai and Olson, 1991) to get a total volume of 150 µl. After vortexing the samples were incubated at 37℃ for 30 minutes with agitation in an Eppendorf Thermomixer Compact (minimum 700 rpm). Then 3 µl of prewarmed 25 % SDS (sodium dodecyl sulfate) and 3 µl proteinase K (stock: 20 mg/ml) were added to get a final concentration of 100 mM Na_2EDTA, 150 mM NaCl, 15 mg/ml lysozyme, 0.5% SDS and 400 µg/ml proteinase K (pH 8.5). This solution was incubated for one hour at 37℃ with agitation (≥700 rpm). Every 10 minutes the samples were additionally vortexed to destroy the cells mechanically. After the incubation, 50 µl of prewarmed (90℃) DNA extraction buffer 2 [100 mM Na_2EDTA, 400 mM Tris-HCl, 400 mM Na_2phosphate buffer (pH 8.0), 5.55 M NaCl, 4 % CTAB (hexadecyltrimethyl ammonium bromide); pH 8.0] (Sambrook et al., 1989; Zhou et al., 1996) and 9 µl of prewarmed 25 % SDS were added. After incubation at 65℃ for one hour - with agitation (≥700 rpm) and vortexing every 15 minutes, the samples were subjected to three cycles of freezing and thawing (-80℃ and 65° C for 6-8 minutes) to break the cells mechanically and release the DNA. The samples were centrifuged for 5 minutes at 13.000 rpm in a microfuge and the supernatant was transferred in a new Eppendorf tube. Further DNA extraction was carried out with 200 µl chloroform-isoamyl alcohol (24:1 v/v; BioRad) to remove the CTAB/protein/polysaccharide

complex. During a vortexing step, a white interface formed and after another centrifugation step (5 minutes at 13.000rpm, at room temperature) the DNA in the supernatant was transferred into a new tube. The aqueous phase containing the DNA was further purified with the QIAamp Viral RNA Mini Kit.

2.2.2. DNA purification using the the QIAamp viral RNA mini kit

The QIAamp Viral RNA Mini Kit removes several PCR-inhibitors and contains carrier RNA, which facilitates the elution of small amounts of RNA as well as DNA. The sample (about 140 µl) was pipetted into a clean 1.5 ml Eppendorf tube and 560 µl Buffer AVL containing the carrier RNA was added to the sample. After mixing by vortexing and an incubation at room temperature (15-35℃) for 10 minutes, 560 µl of cold ethanol (96-100%) were pipetted to the sample and vortexed. 630 µl of the sample was applied to a QIAamp spin column and centrifuged at 6000 x g (8.000 rpm) for one minute. The flow through was discarded and the rest of the sample was applied to the collection tube. Again, the sample was centrifuged and the flow-thru was discarded into the waste. The bound DNA on the spin column was washed with 500 µl of buffer AW1. The flow-thru, after another centrifugation step (6000 x g for 1 min), was discarded, and 500 µl of buffer AW2 were used for a second washing step. Once more the spin column with the collection tube was centrifuged for one minute at 6000 x g and the flow-thru discarded. To remove the rest of the washing buffer the sample was centrifuged a second time for two minutes at full speed. For the elution of the purified DNA, the QIAamp spin column was placed in a clean 1.5 ml microfuge tube. The DNA was eluted three times with 100 µl preheated (80℃), autoclaved, ultra pure water from Sigma to obtain three different eluents per extraction. The DNA in the flow-thru from all three elution steps was stored at -20℃ and was further tested for PCR-amplifiable DNA with 16S rDNA specific primers.

2.2.3. DNA extraction from pure bacterial cultures (Ausubel et al., 1991)

This protocol was used to prepare genomic DNA from pure bacterial strains.

For the extraction of the DNA, the biomass can be derived from fresh liquid over night cultures or the bacterial strains can be grown on plates. About 1-2 ml of culture material from a fresh over night culture were centrifuged for 15 minutes at 13.000 rpm in a microfuge and the pellet was resuspended in 540 µl 1x TE buffer [10 mM

Tris-HCl, 1 mM EDTA; pH 8.0] and 50 µl lysozyme (stock: 50 mg/ml). Respectively, if the biomass is derived from plates, from the freshly cultured bacterial lawn, one loop of each strain was resuspended in 540 µl 1x TE buffer and 50 µl lysozyme (stock: 50 mg/ml). After 20 minutes incubation at 37℃ with ag itation in an Eppendorf Thermomixer Compact (≥500rpm), 11.5 µl prewarmed 25% SDS and 6 µl proteinase K (stock: 20 mg/ml) were added to get a final concentration of 0.5% SDS and 200 µg/ml proteinase K. In a following incubation step of one hour at 37℃ with agitation, the cell walls and membranes were broken. Afterwards 100 µl 5M NaCl and 80 µl CTAB/NaCl solution were added. The CTAB binds the proteins and polysaccharides and the concentration of NaCl had to be higher than 0.5 M, because otherwise a CTAB-DNA complex would have formed. The samples were incubated for 10 minutes at 65℃ (≥500rpm) and afterwards, the equal volume (0.8 ml) chloroform-isoamyl alcohol (24:1, v/v; BioRad) was added, vortexed till a white interface was formed and then centrifuged for 5 minutes at 13.000 rpm. The Chloroform-Isoamyl alcohol removes the CTAB/protein/polysaccharide complex. The aqueous supernatant containing the DNA was transferred into a new clean microfuge tube. Further extraction was carried out with the equal volume (~0.7 ml) Phenol/Chloroform/Isoamyl alcohol (25:24:1, v/v; BioRad) with the aqueous supernatant. After another centrifugation step (5 minutes at 13.000 rpm, at room temperature), the supernatant (~0.5-0.6 ml) was transferred into a new Eppendorf tube. 0.6 volumes (~300 µl) ethanol (96%; -20℃) we re added and precipitated over night at -20℃. On the next day, the samples were c entrifuged (15 minutes at 13.000 rpm; at room temperature), the ethanol discarded and the pellet was dried at 40-50℃ till the Ethanol was completely removed. Finally, the DNA was resuspended in 100 µl autoclaved ultra pure water from Sigma.

Every sample was tested by PCR using specific eubacterial, respectively archaeal primers.

2.3. PCR AMPLIFICATION

2.3.1. PCR amplification of eubacterial 16S rDNA fragments from environmental samples

For all PCR reactions 2x PCR Master Mix from Promega [50 units/ml of TaqDNA Polymerase supplied in a appropriate reaction buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, 3 mM $MgCl_2$] was diluted to 1x and 12.5 pmol/µl

of each primer (stock: 50 pmol/µl) were added. Primer sequences used in this study are shown in Table 1. Two different PCR reactions were performed to amplify eubacterial 16S rDNA fragments for genetic fingerprinting by DGGE. A first Polymerase Chain Reaction round, amplifying the nucleotide positions 341-926 of the *Escherichia coli* 16S rRNA gene, was carried out with primer 341f (Muyzer et al., 1993 and 1995), and 907r, a universal primer for 16S rDNA (Teske et al., 1996). The PCR was carried out in a total volume of 25 µl and 2 µl template of the extracted DNA were added. The second round, a semi-nested PCR amplifying a 200-bp DNA fragment (nucleotide positions 341-534 in *E. Coli* 16S rRNA gene) for DGGE analysis, was performed with the primers 341fGC and 518r (Neefs et al., 1990). A 40-base GC clamp at the 5' end of the forward primer stabilizes the melting behavior of the DNA fragments in DGGE analysis (Muyzer et al., 1993). The nested PCR was performed in a total volume of 100 µl, separated on two tubes to 50 µl and 3 µl template of the first round of PCR were added to each tube. The two 50 µl PCR products of each sample were pooled in a clean 1.5 ml Eppendorf tube and precipitated over night at -20℃ in 1 ml ethanol (9 6 %, -20℃). The next day, the PCR reaction was centrifuged for 15 minutes at 14.000 x g. The ethanol was discarded and the DNA pellet was dried on a thermoblock at 40-50℃. The dry DNA pellet was resuspended in 20 µl autoclaved ultra-pure water (Sigma).

PCR was performed in a MJ Research PTC-200 Peltier Thermal Cycler with the following thermocycling program: the DNA was denaturated for 5 min at 95℃, followed by 30 cycles each consisting of 1 min denaturation at 95℃, 1 min annealing of the primers at 55℃ and 1 min extension at 72℃. 5 min at 72℃ were used as final extension step. 7 µl of each PCR product were run on a 2% (w/v) agarose gel for ~35 min at 110 V, stained in an ethidium bromide solution [1 µg/ml; stock: 10 mg/ml] for 15-25 minutes and visualized by a UVP documentation system (BioRad Transilluminator, Universal Hood; Mitsubishi P93D-printer).

For each Master Mix, a negative control was carried out to exclude the possibility of false-positive PCR results through cross-contaminations.

2.3.2. PCR amplification of eubacterial 16S rDNA fragments from pure bacterial strains

PCR was carried out as described above using the same Master Mix and PCR conditions. For the PCR amplification of the pure bacterial isolates, only the semi-nested PCR with the primers 341fGC and 518r was carried out (Nübel et al., 1996).

2.3.3. PCR amplification of archaeal 16S rDNA fragments from environmental samples

For the amplification of archaeal 16S rDNA fragments, the same Master Mix and conditions as described above were used. Also two PCR protocols were necessary to obtain sufficient product for DGGE. The first reaction using the primer pairs ARC344 (forward) (Raskin et al., 1994) and ARC915 (reverse) (Raskin et al., 1994) were used to specifically amplify a 590 bp fragment of the archaeal 16S rDNA. Primer sequences used in this study are shown in Table 1. 3 µl of the DNA directly extracted from the stone material were used as template DNA. The second round of PCR, amplifying a 200 bp fragment, was performed with the archaea specific primer ARC344 and the universal primer 518r (Neefs et al., 1990). A 40-base GC clamp at the 5' end of the reverse primer stabilizes the melting behaviour of the DNA fragments in DGGE analysis (Muyzer et al., 1993). The same PCR volumes as described for eubacterial PCR were used for the 1st and 2nd round PCR for the archaeal PCR amplification. PCR was performed in a MJ Research PTC-200 Peltier Thermal Cycler with the following thermocycling conditions for the 1st round: DNA denaturation for 5 min at 95℃, followed by 40 cycles each consisting of 1 min denaturation at 95℃, 1 min annealing at 60℃ and 1 min primer extension at 72℃. 5 min at 72℃ were used as final extension step (Piñar et al., 2001a). For the 2nd round of PCR, the same thermocycling program was used as described for the amplification of eubacterial DNA.

2.3.4. PCR amplification of *Myxococcus xanthus* DNA

Samples from Granada treated with *Myxococcus xanthus* were screened for the presence of *M. xanthus*-DNA. The strain specific primers Frz799 and Frz1147 (Piñar et al., 2010) were used to amplify a 349 bp fragment of the *frz*ABCD gene. The PCR conditions consisted of 5 min denaturation at 95℃, followed by 35 cycles, each consisting of 1 min denaturation at 95℃, 1 min annealing of the primers at 56℃ and 1 min primer extension at 72℃, with a final extension step of 5 min at 72℃ (Piñar et

al., 2010). Aliqouts of PCR product were analysed on 2% agarose gels for 35 minutes at 110 V, stained with ethidium bromide [1 µg/ml; stock: 10 mg/ml] for 15-25 minutes and visualized by a UVP documentation system.

Phylogenentic group	Primer	Target region	Nucleotide sequence (5‘-3‘)	Reference
eubacteria	341f	341-457	CCTACGGGAGGCAGCAG	Muyzer et al., 1993
eubacteria	341CG	341-457	CGCCCGCCGCGCGCGGCGGGC GGGGCGGGGGCACGGGGGGCC TACGGGAGGCAGCAG	Muyzer et al., 1993
Universal	907r	907-926	CCCCGTCAATTCATTTGAGTTT	Teske et al., 1996
Universal	518r	518-534	ATTACCGCGGCTGCTGG	Neefs et al., 1990
Universal	518GC			Muyzer et al., 1993
Universal	1492r	1492-1513	TACGG(C/T)TACCTTGTTACGACT T	Lane, 1991
Universal	27f	8-27	AGAGTTTGATCCTGGCTCAG	Edwards et al., 1989
Universal	985r	968-985	GTAAGGTTCTTCGCGTT	Heuer et al., 1997
archaea	ARC344		ACGGGGAGCAGCAGGCGCGA	Raskin et al., 1994
archaea	ARC915		GTGCTCCCCCGCCAATTCCT	Raskin et al., 1994
CLONING	SP6		ATTTAGGTGACACTATAGAATAC	Promega
CLONING	T7		TAATACGACTCACTATAGGG	Promega
M. xanthus	Frz799		GTGGACCTCCTCTTCTTCGACAT C	Piñar et al., 2010
M. xanthus	Frz1447		GACATGTGTCTCAGTGCCTTCCT T	Piñar et al., 2010
RAPD	D1254	arbitrary	CCGCAGCCAA	Ripka et al., 2006
RAPD	D14216	arbitrary	(AGCT)(AGCT)(AGCT)AACAGCTA TGACCATG	Ripka et al., 2006
RAPD	D11344	arbitrary	AGTGAATTCGCGGTGAGATGCC A	Ripka et al., 2006
RAPD	D14307	arbitrary	GGTTGGGTGAGAATTGCACG	Ripka et al., 2006

Table 1
Sequences and target regions of primers used in this Diploma Thesis

2.4. FINGERPRINT ANALYSIS BY DGGE - DENATURING GRADIENT GEL ELECTROPHORESIS

For genetic fingerprinting of environmental samples 2 x 50 µl of semi-nested PCR products were pippeted together in a clean 1.5 ml microfuge tube and precipitated in 1 ml 96% ethanol overnight at –20℃. After a centri fugation step (15 minutes at 14.000 x g), the ethanol was discarded and the DNA pellet was dried at 40-50℃. The

DNA was resuspended in 20 µl autoclaved ultra pure water (Sigma), supplemented with 5 µl Loading Dye Solution (Fermentas) and further separated by DGGE.

For the discrimination of 16S rDNA clone sequences using DGGE, two rounds of PCR were necessary. After the first round with the vector specific primers SP6 and T7 (Promega) (see section 2.8. Screening of clones (archaeal and eubacterial)), a second round using the primers 341GC and 518r in a total reaction volume of 25 µl was performed. The PCR products, mixed with 5 µl Loading Dye Solution (Fermentas) were applied on the DGGE.

The gels for the screening of archaeal and bacterial communities were run in 0.5 x TAE buffer [20 mM Tris, 10 mM acetate, 0.5 mM Na_2EDTA; pH 8.0] for 3.5 hours at 200 V and 60℃ in a BIORAD-DCODE^{TM} – Universal Mutation Detection System (Muyzer et al., 1993).

For screening of bacterial communities, a chemical gradient ranging from 30 to 55% of urea and formamide in an 8% (w/v) polyacrylamide gel (BioRad, Munich, Germany) was used to separate the DNA bands. The gels were stained in an ethidium bromide solution [1 µg/ml; stock: 10 mg/ml] for 15-25 minutes and visualized by a UVP documentation system (BioRad Transilluminator, Universal Hood; Mitsubishi P93D-printer).

A denaturing gradient from 25-60% of urea and formamide was appropriate to screen the archaeal communities. The same running conditions were used as for the genetic fingerprinting of bacterial samples (Piñar et al., 2001a).

Solutions for DGGE:

Two acrylamide stock solutions (acrylamide-N,N-methylene bisacrylamide, 37:1, v/v; BioRad) containing 0 and 80% of denaturants were prepared for pouring the gels (Table 2). By mixing the two solutions in proper proportions in a gradient mixer (Sigma) all desired concentrations of high-acryl to low-acryl can be made (Table 3) (Ripka, unpublished Diploma Thesis, 2005).

0% urea and formamide	40 ml	40% Acrylamide (Biorad)
	2 ml	TAE (50x)
	158 ml	Deionised water
Σ = 200ml		
80% urea and formamide	40 ml	40% Acrylamide (Biorad)
	2 ml	TAE (50x)
	60 ml	Deionised water
	64 ml	99.9% Formamide deionised
	67.2 g	Urea
Σ = 200 ml		

Table 2
Stock solutions for DGGE

Example:

Separating zone for a 30-55% gradient:

For the 30% solution, 6.25 ml of the 0% acrylamide stock solution were mixed together with 3.75 ml of the 80% acrylamide stock solution in a 15 ml falcon tube, having a total volume of 10 ml. The 55% solution was prepared by mixing 3.13 ml of the 0% acrylamide stock solution with 6.88 ml of the 80% acrylamide stock solution. The polymerisation was started by adding 50 µl APS (10%) and 7 µl TEMED - as catalyst for the polymerisation - to the two denaturing solutions. Both falcon tubes were shortly mixed and at the same time loaded into the two tubes of the gradient mixer. 10 ml of the 30% solution were mixed with 10 ml of 55% solution in an Acrylamide Gradient Mixer (Sigma) and pumped with a CHEMAP AG pump between the two clean glass plates.

Stacking zone:

For the stacking zone on top of the gel, 7 ml of 0% solution were mixed together with 30 µl APS (10%) and 4 µl TEMED. This solution was pumped onto the separating zone.

Gradient	0% Lösung	80% Lösung	Mix	Denat.Gel - 10mL	Slots 7mL
20	7,50	2,50	APS 10%	50	30
25	***6,88***	***3,13***	TEMED	7	4
30	**6,25**	**3,75**			
35	5,65	4,38			
40	5,00	5,00			
45	4,38	5,63			
50	3,75	6,25			
55	**3,13**	**6,88**			
60	***2,50***	***7,50***			
65	1,88	8,13			
70	1,25	8,75			
75	0,63	9,38			
80	0,00	10,00			

Table 3
Ratios of different mixtures to get all desired concentrations of acrylamide for DGGE solutions

2.5. ELUTION OF CUT BANDS FROM POLYACRYLAMIDE GELS

After completion of electrophoresis, staining of the gels and gel documentation, the intensely stained bands of interest were excised out of the gel using a clean scalpel. The cut bands were put in fresh Eppendorf tubes, taking care that the tube was not contaminated with ethidium bromide. An incubation step with agitation (300 rpm) at 37℃ over night in 500 µl autoclaved ultra pure wat er (Sigma) (or elution buffer [0.3 M NaCl, 3 mM EDTA, 30 mM Tris (pH 7.6)]) to elute the DNA, was followed by adding 500 ml Phenol/Chloroform/Isoamyl alcohol (25:24:1, v/v;BioRad) (or 250 µl Phenol and 250 µl Chloroform). The samples were mixed and centrifuged for 15 minutes at 14.000 rpm and the supernatant was transferred into a clean Eppendorf tube. The supernatant was precipitated in 1 ml ethanol (96%, -20℃) for one hour at -20℃. Afterwards, the sample was centrifuged (15 minutes at 13.000 rpm at room temperature), the ethanol discarded and the DNA dried at 40-50℃. The DNA pellet was resuspended in 15 µl autoclaved ultra pure water (Sigma) and 2 µl were directly used as a template for a new 25 µl PCR reaction with the primers used for DGGE (e.g. 341GC/518r). After analyzing the PCR products on a 2% agarose gel, the samples were run in a DGGE to prove that this was only one band. If this was the case, the DNA sample could be used as a template for a new PCR for sequencing.

2.6. RAPD-PCR (RANDOM AMPLIFIED POLYMORPHIC DNA - POLYMERASE CHAIN REACTION)

RAPD-PCR analysis was used to discriminate between all bacterial isolates from the Chapel of St. Virgil which partly showed an identical position in the DGGE fingerprint. These clustered isolates were subjected to further discrimination using RAPD-typing. For RAPD analysis, PCR was carried out with 4 different primers (Table 1). One of them was 10-nt in length (1254) (Ripka et al., 2006) and the other three ≥17-nt (namely: primers D14216, D11344 and D14307) (Ripka et al., 2006; Ripka, unpublished diploma thesis, 2005). The 1x diluted Master Mix (Promega) was used to perform RAPD-PCR with primer concentrations ranging from 1-50 pmol/µl. To the 25 µl PCR reaction volume 2.5 µl templates DNA were added. The following thermocycling program was used for the 10-nt primer: 4 cycles of [94°C, 5 min; 36°C, 5 min; and 72°C, 5 min], 30 cycles of [94°C, 1 min; 36°C, 1 min; and 72°C, 2 min] followed by 10 min at 72°C (Williams et al., 1990). When using the ≥17-nt primers, the cycling program was 4 cycles of [94°C, 5 min; 40°C, 5 min; and 72°C, 5 min; low stringency amplification], 30 cycles of [94°C, 1 min; 55°C, 1 min; and 72°C, 2 min; high stringency amplification] and a final elongation step for 10 min at 72°C (Welsh and McClelland, 1990). The whole 25 µl RAPD-PCR reaction volumes were run with 4 µl Loading Dye Solution (Fermentas) on a 2% (w/v) agarose gel for ~160-240 minutes at 70 V, stained in an ethidium bromide solution [1 µg/ml; stock: 10 mg/ml] for 20-35 minutes and visualized by a UVP documentation system (BioRad Transilluminator, Universal Hood; Mitsubishi P93D-printer). Isolates showing the same RAPD profiles were clustered into the same groups and representatives were selected for phylogenetic identification by means of sequencing.

2.7. CONSTRUCTION OF 16S RDNA CLONE LIBRARIES

2.7.1. Preparation of competent *E.coli* cells

2.7.1.1. *E.coli Xl1-Blue tet*^{R} (Nishimura et al., 1990)

A pre-culture was inoculated over night in 3 ml LB liquid media with tetracycline (10 µg/ml). 0.5 ml of the o/n- *E. coli* culture were pipette into 50 ml of medium A (LB broth supplemented with 10 mM $MgSO_4 \times 7\ H_2O$ and 0.2% glucose) and incubated (about 2.5 – 3 hours) with aeration until the mid-log phase (0.5 – 0.8 O.D. at 660 nm). The measuring of the OD_{660} was done with a Pharmacia Biotech Ultrospec 1000E –

UV/Visible Spectrophotometer. At least 1 ml of medium A should be kept in a sterile Eppendorf tube at 4℃ or on ice. From now on the cells had to be kept on ice or on 4℃. Following the incubation, the cells with an OD in the desired limits were put on ice for 10 minutes. In a refrigerated centrifuge (Beckman coulter avanti j-20xp Centrifuge with a JA17 rotor) the cells were centrifuged at 4℃ for 15 minutes at 3.500rpm (~1.500 x g). The supernatant was discarded and the cell pellet was gently resuspended in 0.5 ml of medium A (pre-cooled on ice). Additionally, 2.5 ml of pre-cooled (4℃) storage medium B [36% glycerine, 12% PEG (Polyethylene glycol), 12 mM $MgSO_4$ x 7 H_2O, added to LB broth (pH 7) and sterilized by filtration through a 0.22 µm filter] were added and mixed without vortexing. The competent cells were divided into aliquots of 0.1 ml in Eppendorf tubes and immediately stored at -80℃ until further usage.

2.7.1.2. $CaCl_2$/$RbCl_2$ competent cells – MOPS protocol

A pre-culture of *E. coli* was inoculated over night in 3ml LB media with tetracycline (10 µg/ml). 0.5 ml of the o/n-culture were added to 200 ml LB media (with tetracycline) and incubated (about 2.5 – 3 hours) with aeration until the exponential phase (0.5 – 0.8 O.D. at 660 nm). The measuring of the OD_{660} was done with a Pharmacia Biotech Ultrospec 1000E – UV/Visible Spectrophotometer. From this step on the cells had to be kept cool. The cells in the exponential growth phase were centrifuged for 10 minutes at 3.500rpm (~1.500 x g) at 4℃. Afterwards, the supernatant was discarded and the cells were resuspended in 10 ml pre-cooled MOPS I media. For 10 minutes the cells were kept on ice before the next centrifugation step (3.500rpm for 10 min at 4℃). Once more the supernatant was poured off and the cell pellet was resuspended in 10 ml pre-cooled MOPS II media. After 10 minutes on ice, the cells were span again (3.500rpm for 10 min at 4℃) and the upper phase was decanted. Finally 720 µl pre-cooled MOPS II media and 450 µl of 50% glycerol were added to the *E. coli* cells. After another 10 minutes on ice aliquots of 100µl were transferred into clean 1.5 ml Eppendorf tubes and immediately frozen at -80℃.

Reagents:

MOPS I: 100 mM MOPS
10 mM $CaCl_2$ x $2H_20$
10 mM $RbCl_2$

Dissolve in 400 ml dH_2O, adjust the pH to 7.0 with KOH, fill up to 500 ml and sterilize by autoclaving.

MOPS II: 100 mM MOPS
70 mM $CaCl_2$ x $2H_20$
10 mM $RbCl_2$

Dissolve in 400 ml dH_2O, adjust the pH to 6.5 with KOH, fill up to 500 ml, sterilize by autoclaving.

2.7.2. Amplification of the 16S rDNA fragments for cloning

For the construction of clone libraries for each sample 2 x 3 µl DNA templates were amplified in 2 x 50 µl volumes using the primers 341f and 907r for bacteria, respectively the primers ARC344 and ARC915 for archaea. The PCR products were commingled and 6-7 µl of the 100 µl were analyzed on a 2% agarose gel. Afterwards, the PCR products were purified using the QIAquick PCR Purification Kit (Qiagen), following the protocol of the manufacturer, and eluted with 25-50 µl autoclaved ultra pure water (Sigma). The purified DNA was analysed on a 2% agarose gel, stained in an ethidium bromide solution and documented with an UVP documentation system.

2.7.3. QIAquick PCR Purification Kit

Purification of PCR products was done using the QIAquick PCR Purification Kit. Five volumes of buffer PB1 (500 µl) were added to one volume of PCR product (100 µl) and mixed by vortexing. The yellow mixture was applied to a QIAquick spin column in a 2 ml collection tube. If the colour of the mixture appeared orange or violet, 10 µl of 3 M sodium acetate (pH 5.0) were added, mixed and the colour turned yellow again. To bind the DNA on the column, the sample was centrifuged for one minute at 13.000 rpm. The flow-thru was poured off and the DNA was washed with 750 µl buffer PE. After centrifuging for one minute at 13.000 rpm, the flow-thru was discarded again and, to remove the rest of the washing buffer, an additional centrifugation step was done (one minute at 13.000 rpm). To elute the DNA, the

QIAquick spin column was transferred in a clean 1.5 ml Eppendorf tube and 25-50 µl of autoclaved ultra pure water (Sigma) were added onto the centre of the QIAquick membrane. After 1-2 minutes the column was centrifuged for one minute at 13.000 rpm. The purified DNA was analyzed on a 2% agarose gel, stained in an ethidium bromide solution and documented with an UVP documentation system.

2.7.4. Ligation protocol

5.5 µl of purified PCR product were used as a ligation template into the pGEM®-T Vector System from Promega following the instructions of the manufacturer.

7.5 µl Rapid Ligation Buffer 2 x were pippeted in a clean 1.5 ml Eppendorf tube. Then, 1 µl pGEM®-T vector (50 ng) and 1 µl T4 DNA ligase (3 Weiss units/µl) were added to the buffer. Finally, 5.5 µl of the purified PCR product were added to receive a total volume of 15 µl. The ligation mixture was mixed by gently pipetting up and down a few times and, afterwards, incubated over night at 4℃ for the maximum number of transformants.

2.7.5. Transformation protocols

The ligation products were transformed into three different kind of competent cells: *E.coli* XL1-BlueTc, MOP competent *E.coli* cells and One shot TOP10 cells (Invitrogen). All cells allow the identification of recombinants (white colonies) on an indicator LB medium containing ampicilline (100 µg/ml), tetracycline (10 µg/ml) or streptomycine (25 µg/ml), X-Gal (5-bromo-4-chloro-3-indolyl-ß-D-galactorpyranoside; 0.1mM) and IPTG (isopropyl-ß-D-thiogalactopyranoside: 0.2 mM; not necessary in the case of One shot TOP10 cells) (see Table 4) (Sambrook et al., 1989).

Plates for transformation	End concentration	Stock concentration
LB media		
Ampicilline	100 µg/ml	100 mg/ml
Streptomycine	25 µg/ml	25 mg/ml
Tetracycline	10 µg/ml	10 mg/ml
IPTG	0.2 mM	1 M (80 µl in 400 ml)
X-Gal	0.1 mM	2% (dilute 1000 folder)

Table 4
Concentration of the used antibiotics in the LB media for transformation analysis

2.7.5.1. E.coli XL1-BlueTc, MOP cells

Aliquots of the competent cells were taken from -80℃ and put on ice. Immediately, 10 µl of the ligation were transferred to 100 µl of competent cells and incubated for one hour on ice (without mixing of the cells). The cells were heat-shocked for 90 seconds at 42℃ and immediately put back on ice for five minutes. Afterwards, 500 µl LB media without any antibiotics were added, so the cells could easily grow. The cells were incubated on a thermomixer (Eppendorf Thermomixer Compact) at 37℃ with agitation (300rpm) for one hour. Thereafter, the cells were centrifuged at low speed (5000 rpm) for 8 minutes. About 200-300 µl of the supernatant were discarded and 100 µl each were platted on two plates containing LB-media with ampicillin (100 µg/ml), tetracycline (10 µg/ml), X-Gal (0.1 mM) and IPTG (0.2 mM) (Sambrook et al., 1989). The inverted plates were incubated over night at 37℃.

On the next day, white colonies were picked from the plates with sterile tooth sticks and transferred into clean Eppendorf tubes containing 40 µl 1x TE buffer [10 mM TrisHCl, 1 mM EDTA; pH 8.0] for PCR analysis and also plated on new LB-plates with the appropriate antibiotics, incubated o/n at 37℃ and further sealed and stored at 4℃.

2.7.5.2. One Shot TOP10 cells (Invitrogen)

One 50 µl vial containing the competent cells for each ligation/transformation was taken from -80℃ and put on ice. The ligation reaction was span briefly and 5 µl were transferred to the competent cells without mixing by pipetting. The remaining ligation

reaction was further stored at -20℃. The competent cells and the ligation reaction were mixed by tapping gently. After 30 minutes incubation on ice, the cells were heat-shocked for 30 seconds at 42℃ on a thermoblock and immediately put on ice for additional two minutes. Then, 250 µl of pre-warmed S.O.C. medium (Invitrogen) was added to each transformation reaction and the vials were incubated at 37℃ for one hour at 225-300 rpm in a shaking incubator. After this step, 2 x 30 µl of the transformation reaction were platted on plates, preheated to 37℃, containing LB media with ampicillin (100µg/ml), streptomycin (25 µg/ml) and X-Gal (0.1 mM) and incubated over night at 37℃ (see Table 4). The next day, the white colonies were harvested and resuspended in a microfuge tube containing 40 µl 1x TE buffer. To store the clones for a few weeks, the colonies were also plated on an additional plate with LB media, ampicillin and streptomycin, incubated o/n, sealed with parafilm and stored at 4℃.

2.8. SCREENING OF CLONES (ARCHAEAL AND EUBACTERIAL)

About 50 to 150 white clones from each clone library were picked with sterile tooth sticks and resuspended in 40µl 1x TE buffer [10 mM Tris-HCl, 1 mM EDTA; pH 8.0]. The cells were mechanically destroyed by three cycles of freezing at -80℃ and thawing at 65℃ (for 6-8 minutes). Screening of the clone libraries by PCR and DGGE was performed as follows: To amplify the inserts, the vector specific primers SP6 and T7 were utilized and 3 µl of the lysed cells were directly used as template for a 25 µl PCR reaction. For PCR, the following thermocycling program was used: 5 minutes denaturation at 95℃, followed by 35 cycles each consisting of 1 min denaturation at 95℃, 1 min annealing of the primers at 46℃ and 1 min extension at 72℃. The final extension step was performed with 5 min at 72℃. About 7µl of the PCR products were loaded on a 2% agarose gel, stained with ethidium bromide and visualized by a UVP documentation system. Positive clones produced a 760 bp PCR product. It contained the 16S rDNA insert (600 bp) and the two 80 bp flanking regions of the plasmid. Negative clones, missing the insert, produced a 160 bp product from the flanking regions of the plasmid (Schabereiter-Gurtner, 2000).

A nested PCR using the primers for DGGE analysis (341GC/518r for eubacteria, respectively ARC344/518rGC for archaea) was performed in a total reaction volume of 25 µl. The PCR products were directly run on 30-55% (respectively on 25-60%) polyacrylamide gels containing 25 slots. The band motility of the clones were visually

compared with one another and with the band pattern of the original sample. Inserts of clones matching with the most intense bands and with faint bands of the DGGE fingerprint of the original were selected for sequencing.

2.9. SEQUENCING AND IDENTIFICATION OF CLONES AND BACTERIAL ISOLATES

Cycle sequencing is a method for performing enzymatic extension reactions for DNA sequencing using fluorescent dye-labeled dideoxynucleotides (terminators). The 2', 3'-dideoxynucleotides are incorporated at 3'-end of the growing DNA chain, the elongation of which is terminated selectively. The cycle sequencing reaction uses the BigDye®Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) that combines AmpliTaq DNA Poliymerase, dye terminators and other components required for the polymerase chain reaction.

For sequencing of clone inserts, a 100 µl PCR product was generated with primers SP6 and T7 and 3 µl of the lysed clones in TE buffer were added as template. The 2 x 50 µl PCR reaction volumes were pooled and purified with a QIAquick PCR Purification Kit (Qiagen). The PCR products were electrophoresed in a 2% agarose gel for 35 minutes at 110V, stained with ethidium bromide [1 µg/ml; stock: 10 mg/ml] for 15-25 minutes and visualized by a UVP documentation system (BioRad Transilluminator, Universal Hood; Mitsubishi P93D-printer).

Sequencing of the selected bacterial isolates was performed by producing a 100 µl (2 x 50 µl) PCR product with the bacterial primers 27f and 1492r (Edwards et al., 1989; Lane, 1991). 3 µl of the extracted DNA were used as template DNA. The thermocycling program consisted of 5 minutes denaturation, followed by 30 cycles of 1 minute denaturation at 95℃, 1 minute primer anne aling at 55℃ and 2 minutes primer extension at 72℃. 5 minutes at 72℃ were us ed as a final extension step. The obtained products were purified using the QIAquick PCR Purification Kit (Qiagen) and analyzed by electrophoresis in 2% (w/v) agarose gels.

Following the gel electrophoreses, sequencing reactions were prepared by cycle sequencing with 10 pmol/µl primer concentrations (SP6 or T7, or both for clones; 27f, 1492r and 958r or 907r for bacterial isolates) in 20 µl reaction volume. 10 µl dH_2O, 6 µl BDT v3.1 buffer (buffer 10x with EDTA), 2 µl BDT v3.1 Mix were pippeted together in a 200µl or 500 µl PCR reaction tube. 1 µl of the primer and finally 1 µl of the purified template DNA were added to the cycle sequencing reaction. The following

thermocycling program was used: 25 cycles were performed, each cycle consisted of 30 seconds denaturation at 96℃, followed by 15 sec onds annealing at 50℃ and an extension step with 60℃ for 4 minutes. After cycle sequencing, the samples were purified using the GE Healthcare AutoSeq G-50 Dye Terminator Removal Kit (GE Healthcare) following the manufacturers protocol.

2.9.1. GE Healthcare AutoSeq™ G-50 Dye Terminator Removal Kit (GE Healthcare)

The GE Healthcare Kit allows rapid dye terminator removal from sequencing reactions by the process of gel filtration. The resin in the column of the tube was resuspended by vortexing and after snapping off the bottom of the closure and loosening the cap one quarter, the column was placed in a supplied collection tube. After a pre-spin step (one minute at 2000 x g), the collection tube was replaced by a clean microcentrifuge tube and the sample (20 µl) was carefully applied to the centre of the resin. The purified sample was collected in the microfuge tube after spinning the column for one minute at 2000 x g (GE Healthcare).

2.10. DRYING OF THE DNA (SPEED VAC) AND SEQUENCING PREPARATIONS

The purified DNA was dried in a vacuum centrifuge (Speed Vac) for approximately 30-40 minutes and then prepared for the injection into an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems).

The dried samples were dissolved in 25 µl formamide (Applied Biosystems) and vortexed. To collect the liquid at the bottom of the tube, the samples were span for a few seconds with maximum 8.000 x g. The samples were heated up to 95 ℃ for 3 minutes in a thermal cycler, and afterwards put on ice for 2 minutes. The samples were vortexed for another time, spun down and put back on ice for 2 minutes. 12 µl of each primed sample were pippeted in a 96-well reaction plate, taking care that no air bubbles in the well could interfere with the measurement of the genetic analyzer. After applying all samples, the septa was laid on top of the 96-well plate and put into the instrument. The run settings were adjusted according to the instrument settings.

2.11. PHYLOGENETIC IDENTIFICATION

In order to find homologous sequences, the DNA sequences obtained from the 3100-Avant Genetic Analyzer were compared with the sequence database, NCBI (National Centre for Biotechnology Information), respectively EMBL (European Molecular Biology Laboratory). To search for close evolutionary relatives the programs BLAST, respectively FASTA were used (Altschul et al., 1997; Pearson, 1994). BLAST (the Basic Local Alignment Search Tool) performs pairwise sequence alignment between the query and target sequence and calculates the statistical significance of matches. The ribosomal sequences of all clones and bacterial isolates have been deposited at the EMBL nucleotide database.

2.12. CULTIVATION ASSAYS - ENRICHMENT CULTURES AND ISOLATION OF BACTERIAL STRAINS

Conventional cultivation methods were used to grow bacteria isolated from stone samples of the Chapel of St. Virgil. Enrichment cultures were performed in 300 ml Erlenmeyer flasks containing 30 ml TSA, Maintenance media (Spring et al., 1996) and M2 media (Tomlinson and Hochstein, 1976). Stone material from each sampling point was incubated in all three media. The culture media in the flasks were supplemented with pre-weighted stone slabs and aerobically incubated at 21℃, respectively 28℃ on a shaking incubator (160-175rp m). During a total period of 2 weeks, twice a week aliquots of 100 µl from each flask were extracted, serial diluted and plated onto the same solid media. Plates were incubated aerobically at the same temperature as the corresponding enrichment culture for 1-2 weeks depending on the growth of the microorganisms. All colonies showing different colour and/or morphology, plus some randomly chosen colonies, were transferred to new culture plates to obtain pure cultures. Pure isolates were cultivated in liquid media until the exponential growth phase and further stored in 70% glycerol at -80℃ for conservation.

Lysogeny broth was used for cloning assays. After autoclaving the appropriate amount of antibiotics (respectively X-Gal and IPTG) were added.

Lysogeny broth (LB) - media: universal media, for 1000 ml (Bertani, 1951; Miller, 1972)

Yeast extract	5 g
Tryptone Peptone	10 g
NaCl	10 g
(Agar	15 g)

Fill up with destilled water to 1000 ml

Trypticase Soy Agar (TSA) - media: universal media, for 1000 ml

Tryptic Soy Broth	30 g
(Agar	15 g)
Destilled water	1000 ml

Autoclaved

Maintenance media (HMM 10% NaCl): for 1000 ml (Spring et al., 1996)

NaCl	100 g
$MgSO_4$ x 7 H_2O	5 g
Peptone from Casein	5 g
Yeast extract	3 g
(Agar	15 g)

Fill up with destilled water to 1000 ml

pH 7.5

M2 media (20% NaCl) for 1000 ml (Tomlinson and Hochstein, 1976)

NaCl	200 g
Tris	12 g
Hycase or Caseinpeptone	5 g
Yeast extract	5 g
(Agar	20 g)

Fill up with destilled water to 950 ml

pH 7.5

Add 50 ml of solution I (separately sterilized) to 950 ml of M2 media.

Solution I: for 100 ml

$MgCl_2$ x 7 H_2O	40 g
KCl	4 g
$CaCl_2$ x 2 H_2O	0.4g

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4. Manuscripts

I. Cultivation and molecular monitoring of halophilic microorganisms inhabiting an extreme environment presented by a salt-attacked monument

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II. Bacterial community dynamics during the application of a *Myxococcus xanthus*-inoculated culture medium used for consolidation of ornamental limestone

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III. Bio-consolidation of ornamental porous limestones: molecular identification and monitoring of the bacteria colonising and remaining on the stones

Manuscript in preparation

I. Cultivation and molecular monitoring of halophilic microorganisms inhabiting an extreme environment presented by a salt attacked monument

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1. ABSTRACT

In the last years several investigations, based on culture-dependent and –independent techniques, have shown that salt-attacked stone surfaces present a habitat for extremely salt tolerant and moderate halophilic microorganisms. The inner walls of the St. Virgils chapel in Vienna (Austria) are an example of this phenomenon. Salt crusts cover most of the wall surfaces and salt crystallization in the porous space of the stone is causing decohesion of material and destruction of original medieval paintings. The salt, together with oligotrophic conditions, creates a very special and extreme habitat for halotolerant and halophilic microorganisms.

In this study we investigate and monitor the cultivable and non-cultivable members of the microbial community present on the stonework of the medieval Chapel of St. Virgil after several severe disturbances of the microbial environment caused by desalination and disinfection treatments. With this finality, a combination of culture-dependent and –independent techniques was selected. The genetic diversity of a total of 104 bacterial strains isolated from the stone samples was analyzed by Denaturing Gradient Gel Electrophoresis (DGGE), Random Amplified Polymorphic DNA (RAPD) analysis and 16S rRNA gene sequencing. Strains were distributed over 29 groups on the basis of their RAPD patterns. Only 19 groups were differentiated by DGGE. Comparative sequence analyses showed that the isolated strains belong to related species of the genera *Halobacillus* (47.1%), *Bacillus* (35.6%), *Acinetobacter* (4.8%), *Halomonas* (3.9%), *Nesterenkonia* (2.9%), *Paucisalibacillus* (2.9%), *Paenibacillus* (1%), *Staphylococcus* (1%) and *Exiguobacterium* (1%).

In addition, polymerase chain reaction DGGE fingerprints, in combination with the creation of clone libraries and sequencing analyses, were used to monitor and identify *Archaea*, the non-cultivable fraction of the microbial community. The detected archaeal sequences were closely related to different uncultured archaeons as well as to the cultured genera *Halococcus* and *Halalkalicoccus* and *Halobacterium*.

Cultivation and molecular analyses revealed the presence of highly specialized microorganisms that were able to thrive and survive after several desalination and disinfection treatments in the extreme environment presented by the salt-attacked Chapel of St. Virgil.

2. INTRODUCTION

The ability of halotolerant and halophilic microorganisms – especially haloarchaea – to survive under extreme conditions and under high doses of ultraviolet (UV) radiation make them suitable organisms for the study of potential microscopic life in extraterrestrial environments and for the study of biological response to simulated Martian conditions (Hansen, 2007). Halobacteria and haloarchaea have been isolated from 250-million-year old rock salts, thus suggesting their long-term survival and the possibility of haloarchaeal survival in the Martian surface environment (Stan-Lotter et al., 2004; Fendrihan et al., 2009). Terrestrial materials such as stone works, wall paintings and building materials, particularly if they are subjected to rainwater and rising damp, contain a variety of hygroscopic salts such as carbonates, chlorides, nitrates and sulphates. These soluble salts migrate within the capillary water through the stone and, as a result of changing physical parameters, their crystallization results in the formation of deposits of hygroscopic salts on the surface of these materials, the so-called salt efflorescences (Amoroso and Fassina, 1983). These extremely saline environments represent niches for the settlement of halophilic and halotolerant microorganisms (Saiz-Jimenez and Laiz, 2000). Studies based on conventional cultivation techniques have shown that halophilic microorganisms are often overlooked due to problems such as: (a) the use of unsuitable culture media; (b) inappropriate salt concentration; (c) insufficient incubation time; and (d) the non-culturability of these microorganisms under laboratory conditions (Giovannoni et al., 1990; Head et al., 1998; Hugenholtz et al., 1998; Rappé and Giovannoni, 2003). More recent investigations based on molecular techniques (especially those that amplify ribosomal gene fragments by the Polymerase Chain Reaction (PCR) and Denaturing Gradient Gel Electrophoresis (DGGE) analysis of PCR products) (Rölleke et al., 1996, 1998; Piñar et al., 2001a, 2001b, 2001c, 2009; Gonzalez and Saiz-Jimenez, 2005; Ripka et al., 2006) have increased our knowledge about the complexity and diversity of the microbial communities of salt-attacked monuments.

The Chapel of St. Virgil (Vienna) represents a good example of a highly osmotic environment and has been subjected to several investigations (Klose, unpub. Diploma thesis, Univ. Vienna, 2001; Täubel, unpub. Diploma thesis, Univ. Vienna, 2001; Ripka et al., 2006; Piñar et al., 2009). The last investigation, in relation to a sampling performed on 2004, reveals a great abundance of salts - primarily sodium

chloride but also traces from gypsum, sodium sulphate and sodium nitrate - in the surfaces of the chapel's walls (Piñar et al., 2009). Furthermore, a very specialized microbial community thriving on the walls of the chapel was identified. The fungal community showed a relatively low diversity as compared to other rock surfaces, which was explained by their lower tolerance towards salt stress and the possible competition with faster growing bacteria. In contrast, results showed a high bacterial diversity consisting of members of moderately halophilic bacteria. In addition, a community of extremely halophilic archaea was detected, supporting the hypothesis of co-habitation of moderately halophilic bacteria and neutrophilic halophilic archaea on this hypersaline environment. The bacterial and archaeal communties detected at the Chapel of St. Virgil showed striking similarities with those found in other hypogean monuments situated at different geographical locations, confirming the widespread distribution of some halotolerant/halophilic microorganisms such as *Rubrobacter* sp., *Halobacillus* sp., and *Halococcus* sp. on the hypersaline environments represented by salt-attacked monuments.

As a consequence of all these investigations, and because the salt efflorescences and the crystallization pressure caused by them was assumed to be the most important destructive factor of the original medieval fineries and plaster, several desalination treatments were carried out during the period from 2002 to 2008. Water soaked cellulose compresses were applied by restorers for the desalination of the walls (see Figure 9). As a consequence of this treatment, the development of the cellulose degrader fungus *Stachybotrys chartarum* on the plaster and rock surfaces – a fungus that is normally found on damp cellulose in indoor environments – was found. The proliferation of this fungus was due to its ability to use as substrate the cellulose remaining in the fissures after the treatment. Therefore, restores decided to apply additional disinfection treatments using a formaldehyde-releaser ("Antimoss") in the years 2004 and 2005.

In the present study, we monitored the diversity of the micro-biota inhabiting the stonework of the medieval chapel of St. Virgil after the application of the desalination and disinfection treatments applied on the walls. With this aim, samples were collected in 2008 after the last desalination treatment from different areas of the walls. Samples were investigated to monitor the cultivable and non-cultivable fraction of the microbial community inhabiting the stonework that were able to survive the treatments.

A strategy combining culture-dependent and –independent techniques was chosen. PCR-DGGE fingerprint analyses of the 16S rDNA fragments derived from enrichment cultures as well as from the original wall samples were compared with each other to estimate the cultivable fraction of the bacterial community. The genetic diversity of the cultivable bacteria isolated from the stone samples was analyzed by different molecular techniques, such as Random Amplified Polymorphic DNA (RAPD) analysis, Denaturing Gradient Gel Electrophoresis (DGGE) analysis and 16S rRNA gene sequencing. Strains were distributed into clusters and representative isolates from each cluster were subjected to sequencing and phylogenetic identification. In addition, PCR-DGGE fingerprints, in combination with the creation of clone libraries, were used to monitor the non-cultivable fraction of the microbial community, as the archaeal domain.

3. METHODS

3.1. Sampling

The Chapel of St. Virgil, Vienna (Austria) dates back to the beginning of the 14th century. The rectangular room is 10.5 m long and 6 m wide (Figure 9A). The whole chapel is covered by salt efflorescences that is visible by naked eye. Therefore, the chapel underwent four desalination treatments (in 2001, 2002, 2005 and 2008) with wet compresses (Figure 9B). In addition, two disinfection treatments with a formaldehyde-releaser were carried out in December 2004 and July 2005. Three samples (VK-B, VK-C and VK-D) were carefully collected directly from salt efflorescences (Figure 9 C). One additional sample (VK-E) was taken by scrapping off the wall material. Another sample (VK-A) was collected from some detached wall material that had trickled onto the floor (Figure 9 D). All samples were collected with sterile scalpels and vials by scraping off surface material and plaster to a depth of 1 to 4 mm.

Samples were divided into four aliquots. Three aliquots were used within a few hours for conventional enrichment and cultivation on three different media and one was used for molecular analyses.

3.2. Enrichment cultures

Three different media were used for enrichment: Trypticase Soy Agar (TSA), Maintenance Medium (HMM, 10% NaCl) (Spring et al., 1996) and M2 medium (20% NaCl) (Tomlinson and Hochstein, 1976). Enrichments were conducted in 300 ml Erlenmeyer flasks containing 50 ml of medium. Flasks were incubated aerobically at room temperature (22^{o}C ± 3^{o}C) by shaking at 200 rpm (HT TR-225 Infors AG, Switzerland) over a total period of 1 week. After 3 and 7 days of incubation, 1 ml of each flask was collected and centrifuged for 15 min at 14.000 g. The collected pellet was frozen at -20 ℃ for further molecular analyses. In addition, aliquots of 100 µl enrichments were serial diluted and plated onto the same solid media. All media were incubated aerobically at room temperature and at 28^{o}C for 1 day to 2 weeks, depending on the growth of the microorganisms. The cell morphology was examined on an Olympus SZX9 phase contrast microscope. Cells showing different morphology and appearance were transferred to new culture plates to obtain pure cultures. Pure isolates were cultivated in fresh media until exponential growth occurred to be finally stored in 70% glycerol at -80 ℃ for conservation.

3.3. Molecular characterization

3.3.1. DNA extraction from bacterial strains and PCR analysis

Genomic DNAs were extracted according to the protocol provided by Ausubel et al. (1991). PCR reactions were executed in a MJ Research PTC-200 Peltier Thermal Cycler using PCR Master Mix (Promega, Mannheim, Germany). For PCR analysis, Master Mix was diluted as recommended by the manufacturers and 12.5 pmol of each primer were added. PCR was carried out in 25 µl volumes and 2.5 µl of template were added.

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 Guanine-Cytosine (GC) clamp was added to its 5' end (Muyzer et al. 1993). As a reverse primer, the universal consensus primer 518r (Muyzer et al., 1993) was used. The PCR conditions were as follows: 5 min denaturation (95^{o}C), followed by 30 cycles each consisting of 1 min denaturation (95^{o}C), 1 min primer annealing (55^{o}C) and 1 min primer extension (72^{o}C), with a final extension step of 72^{o}C for 5 min.

3.3.2. DNA extraction from stone material and PCR analyses

DNA was directly extracted from the stone samples, as well as from the pellets collected from the enrichment cultures according to the protocol provided by Schabereiter-Gurtner et al. (2001). PCR reactions were executed as described above.

For the amplification of bacterial 16S rDNA fragments, primers 341f (Muyzer et al. 1993) and 907r (Teske et al., 1996) were used. For genetic fingerprints, a semi-nested PCR was performed with primers 341fGC and 518r. For the semi-nested PCR, 100 µl volumes were separated into two tubes to which 50 µl and 3.5 µl of template were applied to each tube. PCR reactions were performed with the thermocycling program mentioned above. Then 10 µl of each PCR product were run in a 2% (w/v) agarose gel for ~35 min at 110 V, stained in an ethidium bromide solution (1 µg/ml; stock: 10 mg/ml) for 15-25 minutes and visualized by a gel documentation system (BioRad Transilluminator, Universal Hood; Mitsubishi P93D-printer).

For the amplification of archaeal 16S rDNA fragments, PCR analyses were carried out as for bacteria, but primers ARC344 (forward) and ARC915 (reverse) (Raskin et al., 1994), specific for archaeal 16S rDNA, were used under the following thermocycling conditions: 5 min denaturation at 95° C, followed by 40 cycles consisting of 1 min denaturation at 95° C, 1 min pr imer annealing at 60° C and 1 min extension at 72° C, with a final extension step of 72° C for 5 min (Piñar et al., 2001a).

For genetic fingerprints, a semi-nested PCR was performed with primers 518r carrying a GC clamp at its 5' end (Muyzer et al., 1993) and the *Archaea* specific primer ARC344. The same thermocycling program was used as described for amplification of bacterial 16S rDNA.

3.3.3. Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was carried out 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). The following conditions were used: a linear chemical gradient ranging from 30 to 55% (100% denaturant contains 7 M urea and 40% v/v formamide) for screening of bacterial communities and from 25 to 60% fro screening of archaeal communities. Gel electrophoretic separation was carried out at 60℃ and 200 V for 3.5 h. Subsequently, gels were stained with an ethidium bromide solution

(1 µg/ml; stock: 10 mg/ml) for 15 min and visualized by a UVP documentation system (BioRad Transilluminator, Universal Hood; Mitsubishi P93D-printer).

3.3.4. RAPD-PCR

For RAPD analysis, the PCR was performed with four different primers. One of them was 10 nt in length (1254) and the other three at least 17 nt in length (namely, primers D14216, D11344 and D14307) (Ripka et al., 2006). To the 1x diluted Master Mix (Promega), primer concentrations from 1 to 12.5 pmol /µl were applied. The PCR was carried out in 25 µl and 2.5 µl of template were added.

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

When primer oligos of at least 17nt were used, the cycling program was four cycles of [94℃, 5 min; 40℃, 5 min; and 72℃, 5 min; low stringency amplification], 30 cycles of [94℃, 1 min; 55℃, 1 min; and 72℃, 2 mi n; high stringency amplification] and a final elongation step for 10 min at 72℃ (Wel sh and McClelland, 1990).

The whole reaction batches were run with 4 µl loading dye solution (Fermentas) in a 2% (w/v) agarose gel for ~130-160 minutes at 70 V, stained in an ethidium bromide solution (1 µg/ml; stock: 10 mg/ml) for 30-45 minutes and visualized by a UVP documentation system (BioRad Transilluminator, Universal Hood; Mitsubishi P93D-printer). The GeneRuler™ 100 bp DNA Ladder from Fermentas was used as a size marker.

3.3.5. Creation of archaeal clone libraries and screening by DGGE

Clone libraries containing the archaeal 16S rDNA fragments were created as described by Piñar et al. (2001a) with modifications as follows. The ligation products were transformed into One Shot TOP10 cells from Invitrogen (according to the manufacturer's protocol), which permits the identification of recombinants (white colonies) on an indicator LB medium containing ampicilline (100 µg/ml), streptomycine (25 µg/ml) and X-Gal (5 bromo-4-chloro-3-indolyl-ß-D-galactopyranoside; 0.1 mM) (Sambrook et al., 1989). Clones were screened on

DGGE, as described by Schabereiter-Gurtner et al. (2001). The clones displaying different fingerprints were selected for sequencing.

3.3.6. 16S rDNA sequencing and phylogenetic analyses

For sequencing of clone inserts, 2 x 50 µl PCR reaction volumes using primers SP6 and T7 and 3 µl of template were conducted as described by Schabereiter-Gurtner et al. (2001).

For sequencing analyses of bacterial isolates, ~1500 base pair 16S rDNA fragments were amplified using the forward primer 27f and the reverse primer 1492r (Lane 1991). 100 µl PCR reaction volumes, separated into two tubes to 50 µl and 3 µl of the extracted DNA were conducted. The thermocycling programme used was as follows: 5 min denaturation at 95℃, followed by 30 cycles consisting of 1 min denaturation at 95℃, 1 min primer annealing at 55℃ and 2 min primer extension at 72℃, followed by a final extension step of 5 min at 72℃. The products obtained were purified using the QIAquick PCR Purification Kit (Qiagen) and analysed by electrophoresis in 2% (w/v) agarose gels. The ABI PRISM 3100-Avant Genetic Analyser (Applied Biosystems) was used and the samples were prepared according to the manufacture's protocol.

Comparative sequence analyses was performed by comparing pairwise insert sequences with those available in the online databases (accessible to the public) provided by the National Centre for Biotechnology Information using the BLAST search program (Altschul et al., 1997). The ribosomal sequences of archaeal clones and bacterial isolates have been deposited at the EMBL nucleotide database under the accession numbers listed in Table 5 (bacterial strains) and Table 6 (archaeal clones).

4. RESULTS AND DISCUSSION

4.1. Enrichment cultures

One aliquot of all five collected samples in this study were subjected to direct DNA extraction, prior to any cultivation assay, to visualize the microbiota inhabiting the original wall samples. The three additional aliquots of the collected samples were used to perform conventional enrichment cultures on three different media (see the

Method section) at salinities ranging from 0 to 20% NaCl (w/v). After 3 and 7 days of cultivation, aliquots of 1 ml of each flask were removed for DNA extraction and PCR amplification using bacteria- and archaea-specific primers. DGGE fingerprint analyses were conducted with the amplified DNA of both the original wall samples and the culture enrichments to obtain information on the diversity (bacteria and archaea) present on the original samples as well as on the culturability of the visualised microorganisms.

By using archaea-specific primers, it was possible to amplify the DNA directly extracted from all the wall samples (data not shown). However, only bacteria and no archaea present on the wall samples could be isolated from the enrichment cultures.

By using bacterial universal primers, it was possible to amplify the DNA directly extracted from all the wall samples as well as that extracted from all enrichment cultures. Figure 10 shows the bacterial DGGE fingerprints derived from the five original samples compared with the community fingerprints obtained from their corresponding enrichments in the three different culture media after 3 and 7 days of incubation.

Fingerprints derived from the original samples were shown to be rather complex, with one to ten dominant bands and many faint bands. DGGE profiles obtained from the TSA enrichment cultures of all five samples showed the same band patterns after 3 and 7 days of incubation. However, in some cases, it was possible to observe a change in the intensity of some bands (as for sample VK-B). This fact reflects the dynamics of the bacterial community in the enrichment during the time course of the incubation, leading to a decrease of some bacterial species whereas others become the predominant species in the enrichments. In the contrary, DGGE profiles derived from enrichments cultures containing NaCl (10% and 20%) showed some differences after 3 and 7 days of incubation. More bands appeared after 7 days of incubation (as for sample VK-D and VK-E in 10% NaCl) indicating that the microorganisms able to grow at these NaCl concentrations need, in general, longer incubation times. For samples VK-A, VK-B and VK-C, it was also possible to see differences in the intensity of the DGGE-bands in the enrichment cultures containing 20% NaCl. An increase in the intensity of the DGGE band at the lower position of the gel after 7 days of incubation correlated with a decrease of the DGGE band at the higher position of the gel (see Figure 10), indicating a succession in the community during the time of cultivation as mentioned above.

In summary, the results shown in Figure 10 reveal a higher population of microorganisms in the original wall sample communities than in the corresponding enrichment cultures (see marked bands at the DGGE fingerprints derived from the original wall samples in the figure), reflecting the inability to obtain all microorganisms present on a given sample by culture-dependent techniques. Furthermore, the figure shows the differences in the bacterial populations that can be cultivated when using different salinities in the selected media. These results validate those obtained in previous studies, where cultivation methods and molecular techniques provide variable constitutions of the investigated microbial community (Busse et al., 1996; Laiz et al., 2003). An explanation for this fact is the use of unsuitable culture media, non-appropriate incubation times and, last but not least, the non-culturability of many microorganisms under laboratory conditions (Giovannoni et al., 1990; Ward et al., 1990; Head et al., 1998; Hugenholtz et al., 1998; Rappé and Giovannoni, 2003).

4.2. Isolation of bacterial strains

A total of 104 isolates differing in morphology and appearance could be cultivated from the five collected samples. A total of 27 pure strains were isolated (26%) from sample VK-A, 14 strains from sample VK-B (13.5%), 16 strains from sample VK-C (15.4%), 14 strains from sample VK-D (13.5%) and 33 pure strains from sample VK-E (31.7%).

Summarized over all 104 cultured strains, 25 isolates were derived from TSA media (24%), 32 strains could be obtained from 10% NaCl media (30.8%) and 47 cultures grew on 20% NaCl media (45.2%). In order to make a pre-selection of the bacterial isolates to be sequenced and further characterized, a genotyping strategy was applied.

4.2.1. Discrimination by DGGE analyses

The genetic diversity of the 104 isolated strains from the stone material was primary analysed by DGGE. To this end, a 200 bp fragment of the V3 region of the 16S rDNA was amplified from all 104 bacterial isolates and subjected to DGGE fingerprint analysis. The resulting individual band patterns were compared by visual inspection.

Results showed that the bacterial strains could be grouped according to their different band migration into 19 clusters. Figure 11 shows the DGGE profiles derived

from one representative strain of each of the 19 groups. Table 5 lists the strains which were grouped together by DGGE. Most of the strains displayed a single band in the denaturing gel (DGGE-groups A, B, D, K, L, M, N, O and R). Two dominant bands were visible for strains belonging to group C. Some strains showed multiple bands (groups E, F, G, H, I, J, P, Q and S) indicating micro-heterogeneity in the genes encoding 16S rRNAs of these strains (Nübel et al., 1996; Muyzer and Smalla, 1998).

4.2.2. Discrimination by RAPD analyses

All 104 bacterial isolates were additionally investigated using RAPD-PCR analyses for a more accurate discrimination. RAPD-PCR analyses were performed with four different primers as mentioned in the Method section. One of them (primer 1254) was 10 nt in length and contained 70% GC; the other three primers were at least 17 nt and contained 44-55% GC. The differences in their GC content may affect annealing behaviour (Power 1996). From the primers used in this study, the 23 nt primer D11344, at a concentration of 12.5 pmol per PCR, showed the most informative RAPD-PCR profiles (higher band numbers). Less primer concentration resulted in fewer RAPD bands. When using the other primers (1254, D14307 and D14216) not all isolates could be identified by type (data not shown). In general, the 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).

RAPD-PCR profiles derived from primer D11344 grouped the 104 isolated strains in 29 different fingerprint patterns (Figure 12). Table 5 shows, in addition to the DGGE grouping, the RAPD grouping of the 104 isolates obtained. In conclusion, 57 of all the 104 cultivated strains (54.8%) were divided into the same groups by DGGE and RAPD analysis.

Comparing these two molecular techniques for genotyping of bacterial strains, it turns out that RAPD analysis is the more discriminatory method. DGGE fingerprinting has been successfully used for sub-typing of microbial species before (Ward et al., 1990; Amann et al., 1995; Buchholz-Cleven et al., 1997; Hein et al., 2003; Ripka et al., 2006). However, DGGE is only able to discriminate genetic variations occurring within the 200 bp of the 16S rDNA region analysed. Otherwise, RAPD profiling

reveals more detailed results and sub-classifies more strains on the species level. The explanation is that RAPD-PCR uses an oligonucleotide of arbitrarily chosen sequence to prime DNA synthesis from pairs of sites to which it is partially or completely matched. As a result, DNA profiles are obtained that allow discrimination at the subspecies level on basis of the DNA diversity in the entire bacterial genome, therefore offering a broad spectrum of genetic variation (Welsh and McClelland, 1990; Williams et al., 1990).

4.2.3. Phylogenetic analyses of selected bacterial isolates

One member of each RAPD cluster was selected for identification by 16S rDNA sequencing. Also isolate 34, not able to be identified by type with primer D11344 in RAPD analysis (see Figure 12) was selected for identification by 16S rDNA sequencing. A comparison of sequences obtained from the isolated strains with sequences of known bacteria in the EMBL database using the search tools FASTA and BLAST is summarized in Table 5.

Similarity values to sequences from the EMBL database ranged from 95% to 99%. Generally, most isolates were affiliated with cultured bacterial strains but also with some uncultured cloned sequences. Results from 16S rDNA sequence analysis revealed that all 104 strains were affiliated with species of nine genera within three phyla: *Firmicutes,* of the order *Bacillales* (number of isolates, *n*=92, representing 88.5% of all isolated strains), *Proteobacteria* (*n*=9, 8.7%) and *Actinobacteria* (*n*=3, 2.9%).

Within the *Firmicute*s phylum, a total of six different genera could be identified, the genus *Halobacillus* being the most abundant (with 47.1% of all isolates). The *Bacillus* genus accounted for 35.6% of the isolated strains, being related with seven different species (see Table 5). The *Paucisalibacillus* genus accounted for 2.9% of the total strains and, in addition, the genera *Exiguobacterium*, *Paenibacillus* and *Staphylococcus*, each of them accounting for 1% of the total strains, were identified. Within the *Gamma Proteobacteria,* two different genera were identified as the genera *Acinetobacter* (4.8%) and *Halomonas* (3.8%). Finally, 2.9% of strains were shown to be closely related to the genus *Nesterenkonia* within the phylum *Actinobacteria*.

It is worth noting that most of the bacterial strains isolated from the Chapel of St. Virgil could grow under moderate to high salt concentrations. Moderately halophilic bacteria are microorganisms that grow optimally in media containing 3%-15% (w/v)

salt (Ventosa et al. 1998), such as the *Halomonas* sp., *Halobacillus* sp., *Nesterenkonia* sp. (Romano et al. 1996; Spring et al. 1996; Li et al. 2008) and some species of the genus *Bacillus*, such as *B. aquimaris*, *B. simplex* and *B. psychrotolerans* (Heyrman and Swings 2001; El-Rahman et al. 2002; Yoon et al. 2003) identified in this study. *Paucisalibacillus globulus*, grows optimally in media containing 1% NaCl, although it can grow in media containing 0-8% NaCl as well (Nunes et al., 2006).

4.2.4. Comparison of the bacterial diversity present at the Chapel of St. Virgil detected in this study with the diversity obtained prior to disinfection treatments in previous investigations.

The diversity of the micro-biota inhabiting the walls of the Chapel of St. Virgil was the subject of previous investigations, as mentioned in the Introduction (Ripka, unpub. Diploma Thesis, Univ. Vienna, 2005; Ripka et al., 2006; Piñar et al., 2009). In both studies, samples were taken in 2004, before the application of biocides on the walls of the chapel.

In order to monitor the consequences of these disinfection and desalination treatments on the micro-biota inhabiting the chapel, we performed the sampling for this study in the year 2008.

Results show that strains isolated in the present study were affiliated to the same phyla as the sequences and isolates obtained from the previous investigations. Thirty-eight strains isolated in this study (36.5 %) belonging to six RAPD groups (VI, VII, VIII, IX, X and XI) were shown to be affiliated with five strains (strains S3, S4, S20, S21 and S22) previously isolated from the Chapel of St. Virgil in the sampling of 2004 (Ripka et al. 2006). All 38 strains showed high score similarities with *Halobacillus*-related species.

Forty-three isolates (from RAPD groups XVIII, XX, XXI and XXIII) (41.3%) were affiliated to sequences obtained in other studies concerning medieval mural paintings. They matched with sequences from bacteria isolated from mural paintings in the Servilia tomb at the Necropolis of Carmona, Seville (Spain) (Heyrman and Swings 2001; Heyrman et al. 2005).

Sequences from 23 strains (22.1%) showed no correlation in the database to sequences derived from stone works, ancient paintings or buildings materials.

In general, the microbial diversity showed to be higher in 2004. Two genera within the *Gamma-Proteobacteria* could be detected in the present study, such as *Acinetobacter* and *Halomonas*. In 2004, *Acinetobacter*-related species could be identified as well but, in addition, two more genera belonging to this class were detected, *Enterobacter* and *Salinisphaera*. Within the *Actinobacteria, Nesterenkonia* species were isolated in the present study, whereas none of the genera previously identified in 2004 belonging to this phylum (*Rubrobacter* and *Jiangella*) could be isolated. Furthermore, no members of the *Bacteriodetes,* previously identified at the Chapel of St. Virgil could be isolated.

The decrease in the total biodiversity can be due to the treatments performed in the Chapel of St. Virgil, leading to a shift of the bacterial community inhabiting the chapel. However, it is worth noting that both investigations were based on different strategies, that of 2004 using a combined strategy of culture-dependent (Ripka et al., 2006) and -independent techniques (Piñar et al., 2009), and the one of 2008 using conventional culture-dependent techniques. As already shown in Figure 10, with conventional enrichment cultures it is not possible to obtain all members of a microbial community, but only those which are able to grow on the supplied culture media (Giovannoni et al., 1990; Ward et al., 1990; Head et al., 1998; Hugenholtz et al., 1998; Rappé and Giovannoni, 2003). This fact could also reflect the differences in the results obtained at both sampling times. Nevertheless, the dominance of *Halobacillus* and *Bacillus* sp., irrespectively of desalination and disinfection treatments, shows that these organisms might, due to their ability to survive unfavourable conditions as spores (Nicholson, 2002) and by their osmotic adaption (Lazar, 1971; Incerti et al., 1997), overcome these restoration treatments in the best way and represent the dominant species thriving on the chapel's walls over the period of time (2004-2008).

4.3. Monitoring and phylogenetic identification of non-culturable *Archaea*

As mentioned above, the archaeal members of the micro-biota inhabiting the Chapel of St. Virgil could not be isolated with the conventional cultivation methods used in this study. However, it was possible to amplify the pure DNA extracts obtained from all five original wall samples by using archaea-specific primers (data not shown), confirming the presence of *Archaea* as inhabitants of the chapel. Therefore, molecular techniques were chosen to monitor and identify this microbial domain. The

amplified DNA was further subjected to DGGE fingerprint analysis to visualize the biodiversity of the archaeal domain obtained in this study (Figure 13 A) and, in addition, to perform a comparision with the archaeal community detected in the sampling of 2004 (Figure 13 B). The community fingerprints derived from samples collected in 2008 showed one to four dominant bands and some other faint bands (Figure 13 A), strongly resembling the archaeal fingerprints obtained in the sampling of 2004 (Figure 13 B).

In order to obtain an accurate phylogenetic identity of the individual members of the archaeal community present in the Chapel of St. Virgil in 2008, clone libraries were performed as described in the Methods section. Inserts of clones producing PCR products, which showed different motility behaviour and matched with bands from the original wall sample in DGGE, were selected for sequencing. A total of 41 clones were sequenced and compared with known archaeal sequences contained at the EMBL database using the search tools FASTA and BLAST. Clones showed high score similarity values ranging from 94% to 100% (Table 6). The cloned sequences affiliated with three genera of the *Halobacteria* (number of clones, *n*=30, representing 73.2% of all sequenced clones), namely *Halococcus* (*n*=15, 36.6%), *Halobacterium* (*n*=13, 31.7%) and *Halalkalicoccus* (*n*=2, 4.9%).

Within the genus *Halococcus*, 15 clones (36.6%) were identified. Thereof, eight clones (K4-57, K4-59, K4-100, K6-51, K6-61, K6-75, K10-1 and K14-46) showed the highest score similarity values (96-100%) with *H. dombrowskii* (19.5%). Five clones (clones K6-60, K6-71, K6-77, K6-91 and K10-6) were affiliated (96- 99% similarities) with the type strain *H. salifodinae* (12.2%). Clone K4-86 was affiliated (97% similarity) with *H. morrhuae* (2.4%), and one clone (K6-56) was closest related (99%) to *Halococcus sp*. (2.4%). Within the genus *Halobacterium*, 13 clones (31.7%) were identified (K4-56, K4-62, K4-79, K4-81, K4-85, K4-92, K4-99, K5-68, K5-72, K5-81, K5-83, K10-2 and K10-16), all of them showing the highest score similarities (95-99%) with uncultured members of this genus. Within the genus *Halalkalicoccus*, two clones (4.8%) were identified. Clone K4-54 was affiliated (97% similarity) with *H. tibetensis* (2.4%) and clone K4-97 showed highest score similarity (98%) with *Halalkalicoccus sp.* (2.4%).

The remainder of the sequenced clones (*n*=11, 26.8%), clones K4-80, K4-98, K5-77, K10-7, K10-33, K14-2, K14-3, K14-9, K14-20, K14-26 and K14-27, showed the highest score similarities (94-99%) with uncultured archaeons.

Comparative sequence analyses showed that 22 (53.6%) of the sequenced clones, namely clones K4-56, K4-57, K4-59, K4-62, K4-79, K4-81, K4-86, K4-99, K4-100, K5-72, K6-51, K6-60, K6-61, K6-71, K6-75, K6-77, K6-91, K10-1, K10-2, K10-6, K10-16 and K14-46 (see Table 6) were phylogenetically related to sequences already detected in the Chapel of St. Virgil in 2004 (Ripka, unpub. Diploma Thesis, Univ. Vienna, 2005). These results show that, despite of the desalination and disinfection treatments carried out at the chapel, the archaeal community inhabiting this monument remained stable.

Nine clones (22%), clones K4-80, K10-7, K10-33, K14-2, K14-3, K14-9, K14-20, K14-26 and K14-27, were phylogenetically related to archaeal sequences identified in two other salt-attacked monuments (Piñar et al. 2001c). The remaining ten clones (24.4%), clones K4-54, K4-85, K4-92, K4-97, K4-98, K5-68, K5-77, K5-81, K5-83 and K6-56, showed correlation to sequences detected in other environments different to salt-attacked monuments (Table 6).

5. CONCLUSIONS:

The Chapel of St. Virgil (Vienna) is a good example of a salt-attacked monument, offering a habitat for extreme and moderate halophilic microorganisms. The microbiota inhabiting the Chapel consists of a stable community (bacteria and archaea) highly adapted to their specific saline habitat. In spite of severe disturbance of the osmotic environment by several desalination treatments and two disinfection treatments with formaldehyde, the microbial community has been shown to be very similar to that identified in previous studies (Ripka, unpub. Diploma Thesis, Univ. Vienna, 2005; Ripka et al., 2006; Piñar et al., 2009).

The results obtained in this study from conventional cultures show the dominance of *Halobacillus* and *Bacillus* sp. These spore-forming bacteria are ubiquitous in the environment, and their endospores represent some of the hardiest and longest-lived cells on Earth (Nicholson et al., 2000; Nicholson, 2002). In addition, results obtained from molecular analyses show a very stable community of haloarchaea. These two groups of microorganisms, i.e. spore-forming bacteria and haloarchaea, present a common characteristic, namely their remarkable resilience against disturbance of their environment, which makes them interesting candidates as model for studying

life under extreme conditions in general, and life under Martian conditions in particular (Fajardo-Cavazos and Nicholson, 2006).

Finally, this study shows that the combination of molecular techniques - DGGE analysis, RAPD PCR analysis, construction of clone libraries and sequencing of 16S rDNA fragments – with cultivation techniques offers a viable method for monitoring the diversity of cultivable and non-cultivable microorganisms after any disturbation of their environment.

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Figure 9
(A) Ground view of the Chapel of St. Virgil. (B) Treatment with wet compresses applied for desalination (C) Salt crusts of sodium chloride on plaster (D) Wall material detached on the floor of the chapel.

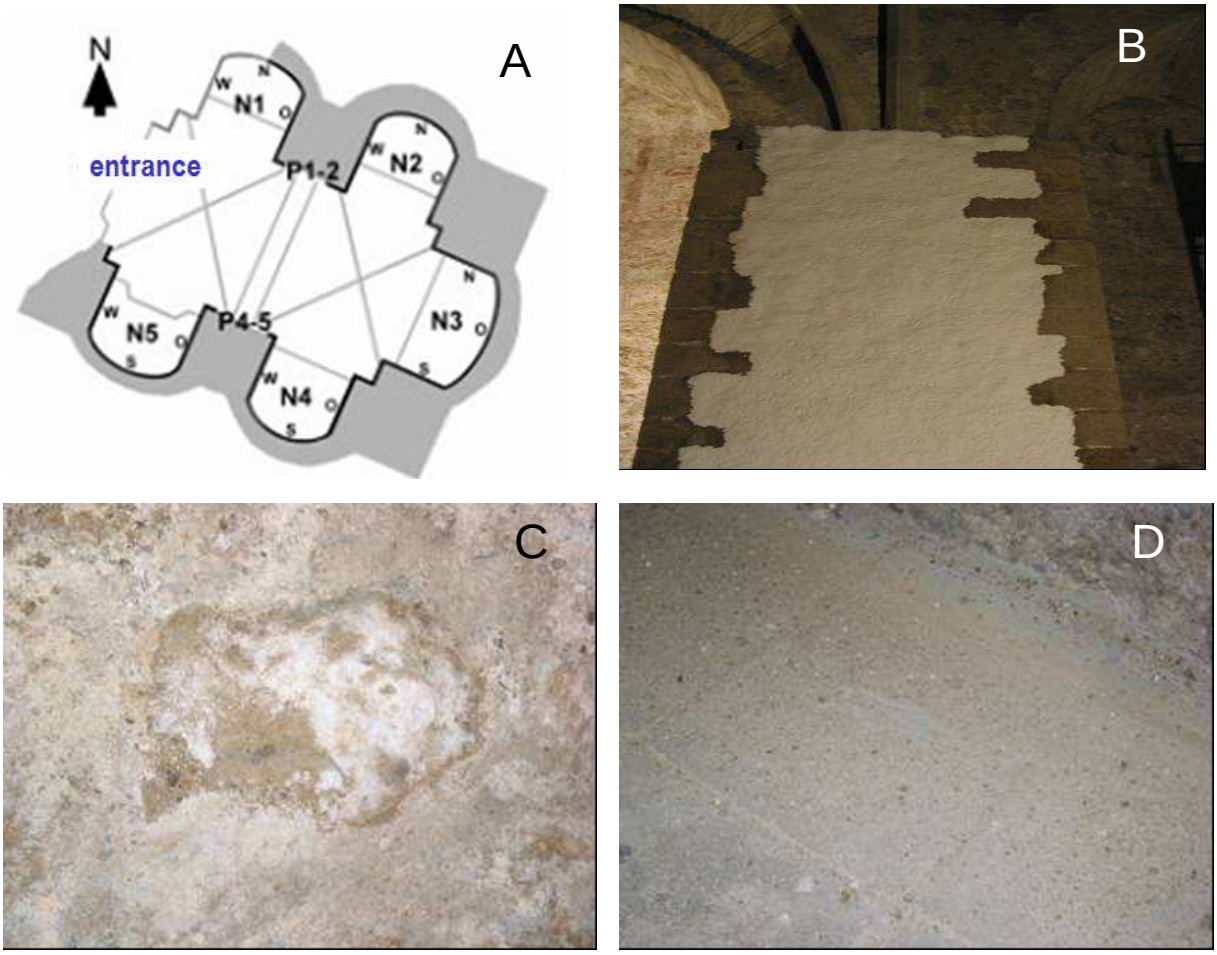


Figure 10

DGGE-fingerprints derived from the five original wall samples [VK-A, VK-B, VK-C, VK-D and VK-E (marked as W)] as well as the community DGGE fingerprints obtained from their corresponding enrichments in the three different culture media (marked as TSA, 10% and 20%) after 3 and 7 days of incubation (marked as 3 and 7). DGGE-bands matching the sequenced bacterial strains are marked with the corresponding strain number on the DGGE fingerprint derived from the original wall samples. Bands of some isolated strains are not visible in the DGGE fingerprint of the original wall samples. Strains 11 and 38 showed three bands in DGGE profiling. Phylogenetic affiliations of bacterial strains are showed in Table 5.

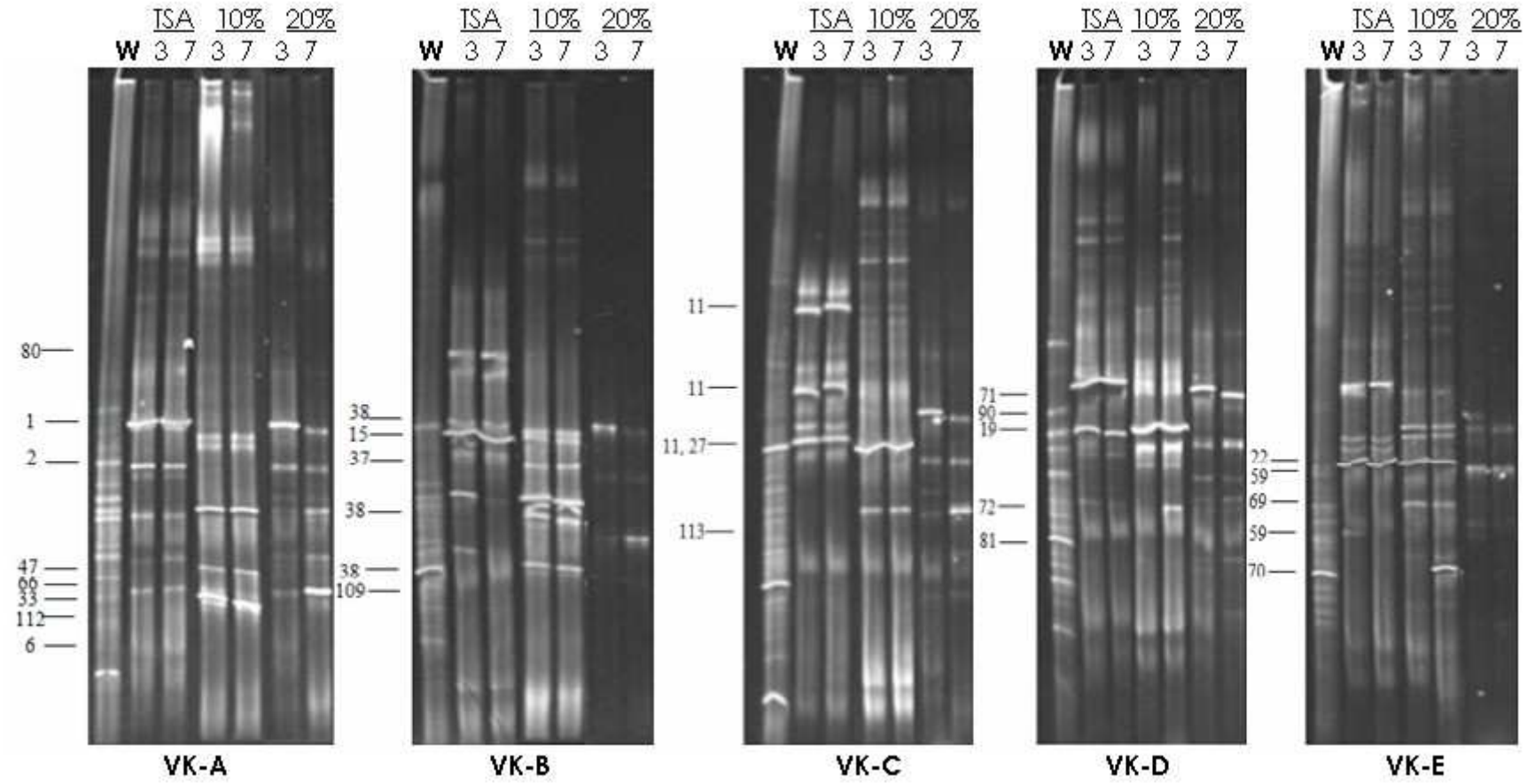


Figure 11
DGGE-profiles derived from one representative strain of each of the 19 DGGE groups. Lane A: strain 80, lane B: strain 71, lane C: strain 1, lane D: strain 2, lane E: strain 11, line F: strain 29, line G: strain 31, line H: strain 38, line I: strain 22, line J: strain 15, Line K: 110, line L: strain 6, line M: strain 33, line N: strain 70, line O: strain 41, line P: strain 89, line Q: strain 66, line R: strain 86, line S: strain 107.

A B C D E F G H I J K L M N O P Q R S

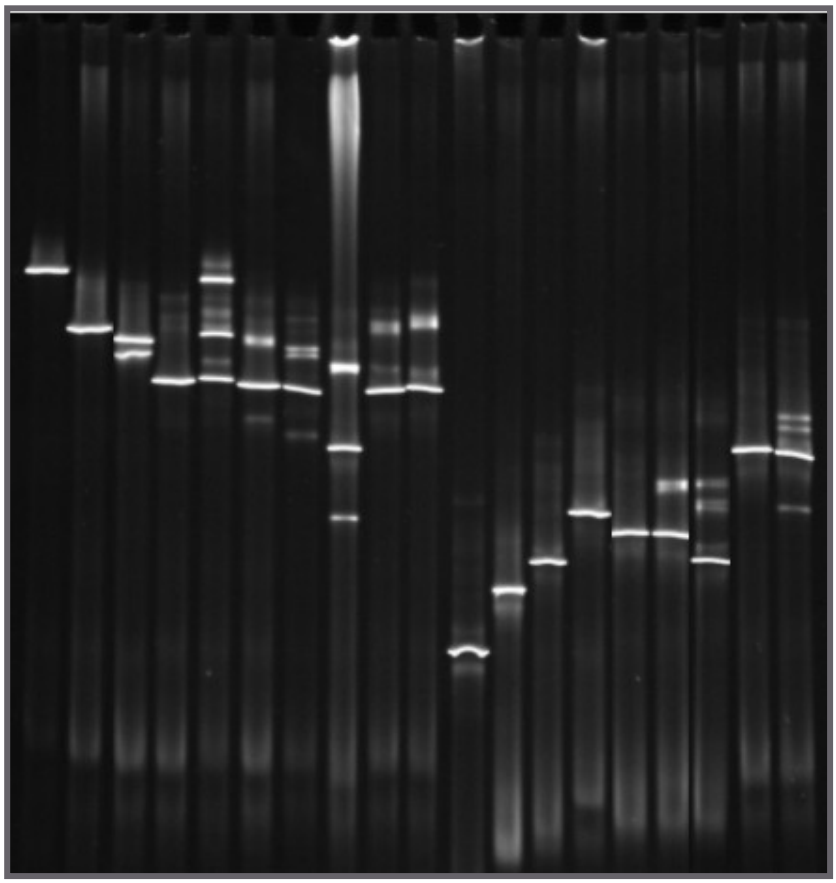


Figure 12
RAPD-PCR patterns of representative strains of the 29 RAPD groups. Line 1: strain 34 representing group I; line 2: strain 41 representing group II; line : strain 47 representing group III; line 4: strain 66 representing group IV; line 5: strain 109 representing group V; line 6: strain 86 representing group VI; line 7: strain 107 representing group VII; line 8: strain 58 representing group VIII; line 9: strain 95 representing group IX; line 10: strain 72 representing group X; line 11: strain 85 representing group XI; line 12: strain 118 representing group XII; line 13: strain 119 representing group XIII; line 14: strain 6 representing group XIV; line 15: strain 1 representing group XV; line 16: strain 2 representing group XVI; line 17: strain 11 representing group XVII; line 17: strain 11 representing group XVII; line 17: strain 11 representing group XVII; line 17: strain 11 representing group XVII; line 18: strain 27 representing group XVIII; line 19: strain 29 representing group XIX; line 20: strain 32 representing group XX; line 21: strain 38 representing group XXI; line 22: strain 22 representing group XXII; line 23: strain 21 representing group XXIII; line 24: strain 20 representing group XXIV; line 25: strain 80 representing group XXV; line 26: strain 71 representing group XXVI; line 27: strain 4 representing group XXVII; line 28: strain 112 representing group XXVIII; line 29: strain 70 representing group XXIX; Lines M: 100-bp ladder.

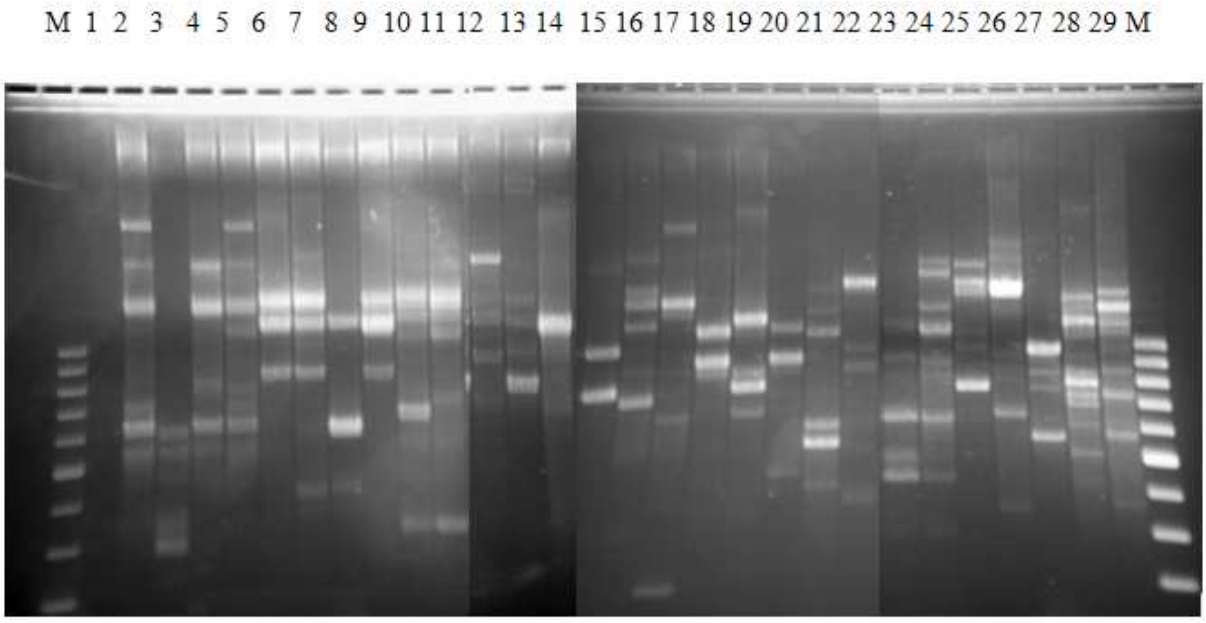


Figure 13
DGGE-profiles derived from the archaeal community inhabiting the walls of the Chapel of St. Virgil (VK-A, VK-B, VK-C, VK-D, VK-E) in the sampling of 2008 (A) and from samples VK1 and VK5 from sampling of 2004 (B) (Piñar et al., 2009). Clones matching with intense bands at the DGGE-profile are indicated on the figure. The phylogenetic affiliations of the clones are showed in Table 6.

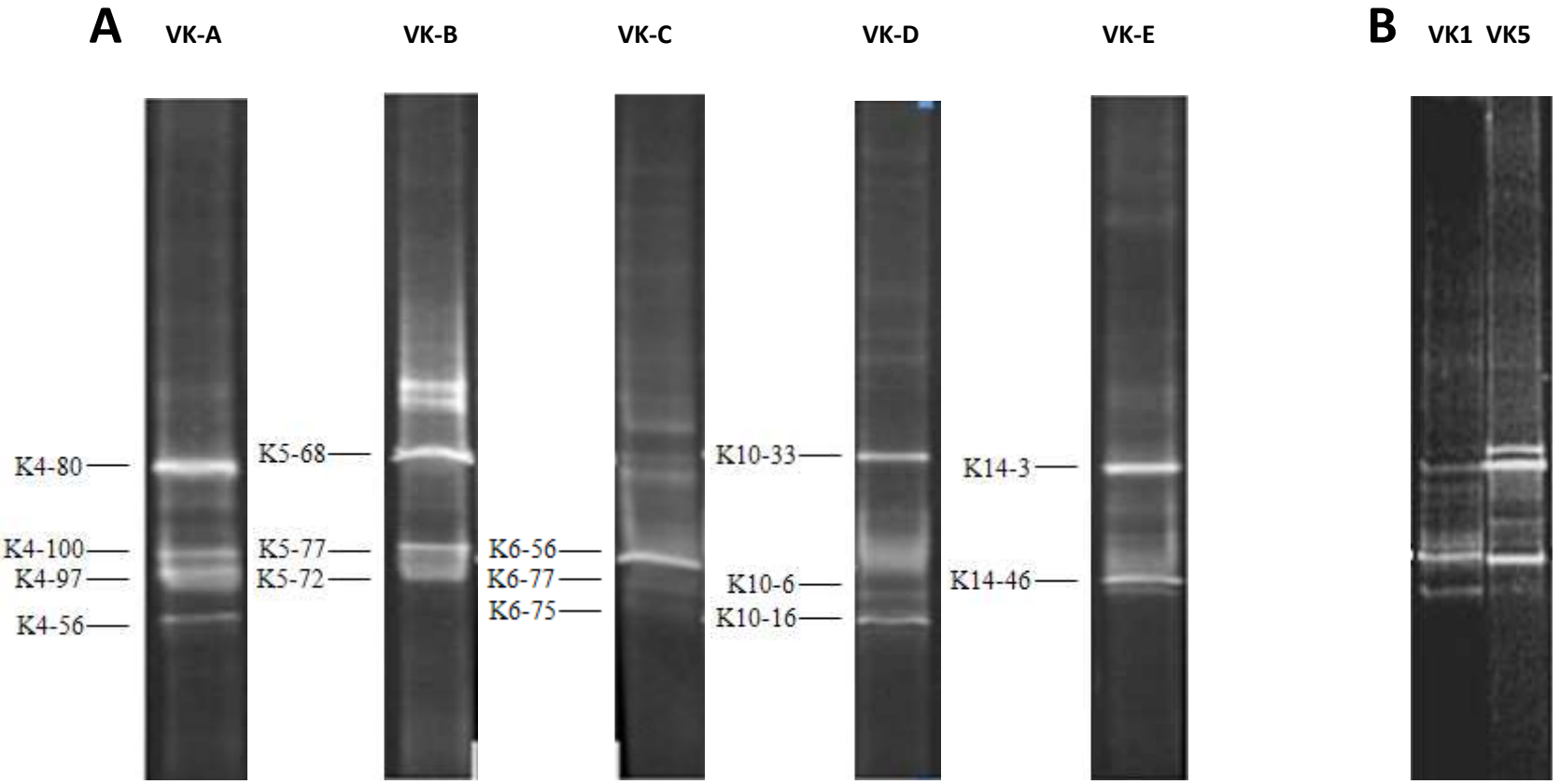


Table 5
Molecular characteristics, grouping of the isolates according to RAPD and DGGE analysis and phylogenetic classification of bacterial strains from the Chapel of St. Virgil: The superscript numbers 1, 2 and 3 indicate the media the samples were derived from. The origin of the isolated strains is described by the superscript letters A-E.

Representative Strains from RAPD group	RAPD group	Percent of the individual RAPD group	Strains in the same RAPD group	*DGGE cluster*	*Percent of the individual DGGE cluster*	*Strains in the same DGGE cluster*	Closest related type strain on basis of 16S rRNA gene sequence	Similarity %	Accession numbers of the sequences submitted to the EMBL database
$34^{2, A}$	I	1%	-	*P*	*2.9%*	*89, 92*	*Halobacillus sp.* [AB166985]	98	FN435895
$41^{2, A}$	II	1%	-	*O*	*4.8%*	*47, 62, 63, 109*	*Halobacillus sp.* [AM990738]	99	FN435899
$47^{2, A}$	III	1%	-	*O*	*4.8%*	*41, 62, 63, 109*	*Halobacillus sp.* [AB166985]	99	FN435900
$66^{3, A}$	IV	4.8%	$67^{3, A}$, $68^{3, A}$, $89^{2, A}$, $92^{2, A}$	*Q*	*2.9%*	*67, 68*	*Halobacillus sp.* [AB166985]	99	FN435907
$109^{3, B}$	V	2.9%	$62^{3, B}$, $63^{3, B}$	*O*	*4.8%*	*41, 47, 62, 63*	*Halobacillus sp.* [AM990738]	99	FN435913
$86^{3, E}$	VI	12.5%	$24^{3, E}$, $25^{3, E}$, $59^{3, E}$, $73^{3, E}$, $79^{3, E}$, $83^{3, E}$, $84^{3, E}$, $87^{3, E}$, $88^{3, E}$, $97^{3, E}$, $99^{3, E}$, $100^{3, E}$	*R*	*32.7%*	*23, 24, 25, 59, 64, 65, 72, 73, 79, 81, 83, 84, 85, 87, 88, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 108, 111, 113, 114, 115, 116*	*Halobacillus sp.* [EU868841]	99	FN435909
$107^{3, E}$	VII	1%	-	*S*	*3.8%*	*58, 60, 61*	*Halobacillus sp.* [EU868841]	99	FN435912
$58^{3, E}$	VIII	2.9%	$60^{3, E}$, $61^{3, E}$	*S*	*3.8%*	*60, 61, 107*	*Halobacillus herberstensis* [AM161503]	98	FN435903
$95^{3, C}$	IX	2.9%	$102^{3, C}$, $103^{3, C}$	*R*	*32.7%*	*23, 24, 25, 59, 64, 65, 72, 73, 79, 81, 83, 84, 85, 86, 87, 88, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 108, 111, 113, 114, 115, 116*	*Halobacillus herberstensis* [AM161503]	99	FN435910
$72^{2, D}$	X	9.6%	$81^{3, D}$, $104^{3, E}$, $105^{3, E}$, $106^{3, E}$, $108^{3, E}$, $113^{3, C}$,	*R*	*32.7%*	*23, 24, 25, 59, 64, 65, 73, 79, 81, 83, 84, 85, 86, 87,*	*Halobacillus styriensis* [AM161506]	99	FN435901

			$114^{3, E}$, $115^{3, E}$, $116^{3, E}$			*88, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 108, 111, 113, 114, 115, 116*			
$85^{3, E}$	XI	7.7%	$23^{3, E}$, $64^{3, C}$, $65^{3, C}$, $96^{3, C}$, $98^{3, E}$, $101^{3, C}$, $111^{3, C}$	*R*	*32.7%*	*23, 24, 25, 59, 64, 65, 72, 73, 79, 81, 83, 84, 86, 87, 88, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 108, 111, 113, 114, 115, 116*	*Halobacillus styriensis* [AM161506]	99	FN435908
$118^{3, A}$	XII	1%	-	*K*	*2.9%*	*110, 119*	*Nesterenkonia halobia* [EU647699]	99	FN435921
$119^{3, A}$	XIII	1.9%	$110^{3, A}$	*K*	*2.9%*	*110, 118*	*Nesterenkonia halophila* [AY820953]	98	FN435922
$6^{1, A}$	XIV	1%	-	*L*	*1%*	-	*Exiguobacterium sp.* [EF177690]	99	FN435882
$1^{1, A}$	XV	4.8%	$3^{1, A}$, $5^{1, A}$, $7^{1, A}$, $8^{1, A}$	*C*	*9.6%*	*3, 4, 5, 7, 8, 9, 10, 43, 44*	*Bacillus psychrotolerans* [NR. 025408]	99	FN435880
$2^{1, A}$	XVI	1%	-	*D*	*1%*	-	*Bacillus pichinotyi* [AF519460]	98	FN435881
$11^{1, C}$	XVII	3.8%	$12^{1, C}$, $13^{1, C}$, $14^{1, C}$	*E*	*3.8%*	*12, 13, 14*	*Bacillus megaterium* [DQ093582]	99	FN435883
$27^{2, C}$	XVIII	2.9%	$28^{2, C}$, $36^{2, C}$	*F*	*10.6%*	*28, 29, 30, 35, 36, 37, 46, 93, 94, 117*	*Bacillus sp. 19500* [AJ315068]	99	FN435891
$29^{2, D}$	XIX	6.7%	$30^{2, D}$, $35^{2, D}$, $46^{2, D}$, $93^{2, D}$, $94^{2, D}$, $117^{2, D}$	*F*	*10.6%*	*27, 28, 30, 35, 36, 37, 46, 93, 94, 117*	*Bacillus aquimaris* [DQ432010]	99	FN435892
$32^{2, E}$	XX	3.8%	$31^{2, E}$, $37^{2, B}$, $69^{3, E}$	*G*	*2.9%*	*31, 69*	*Bacillus sp. 19500* [AJ315068]	99	FN435894
$38^{2, B}$	XXI	5.8%	$39^{2, B}$, $40^{2, B}$, $45^{2, B}$, $48^{2, B}$, $49^{2, B}$	*H*	*5.8%*	*39, 40, 45, 48, 49*	*Bacillus sp. 19500* [AJ315068]	96	FN435897
$22^{1, E}$	XXII	1%	-	*I*	*1.9%*	*21*	*Bacillus mycoides* [EU221418]	99	FN435889
$21^{1, E}$	XXIII	5.8%	$15^{1, B}$, $16^{1, B}$, $17^{1, B}$, $18^{1, B}$, $19^{1, D}$	*I*	*1.9%*	*22*	*Bacillus simplex* [AJ628746]	99	FN435888
$20^{1, D}$	XXIV	1%	-	*J*	*5.8%*	*15, 16, 17, 18, 19*	*Paenibacillus polymyxa* [EF634026]	99	FN435887
$80^{1, A}$	XXV	1%	-	*A*	*1%*	-	*Staphylococcus sp.* [NR 027519]	99	FN435890
$71^{2, D}$	XXVI	2.9%	$90^{2, D}$, $91^{2, D}$	*B*	*2.9%*	*90, 91*	*Paucisalibacillus globulus* [AM114102]	95	FN435898
$4^{1, A}$	XXVII	4.8%	$9^{1, A}$, $10^{1, A}$, $43^{1, A}$, $44^{1, A}$	*C*	*9.6%*	*1, 3, 5, 7, 8, 9, 10, 43, 44*	*Acinetobacter lwoffii* [DQ289068]	99	FN435915

112[2, A]	XXVIII	2.9%	33[2, A], 42[2, A]	*M*	*2.9%*	*33, 42*	*Halomonas sp.* [AB166895]	99	FN435919
70[2, E]	XXIX	1%	-	*N*	*1%*	-	*Halomonas pantelleriensis* [NR_026298]	98	FN435918

1 … isolated from TSA media

2 … isolated from 10 % NaCl media

3 … isolated from 20 % NaCl media

A … strain isolated from stone sample VK-A

B … strain isolated from stone sample VK-B

C … strain isolated from stone sample VK-C

D … strain isolated from stone sample VK-D

E … strain isolated from stone sample VK-E

Table 6

Phylogenetic affiliations of archaeal sequences detected in stone work of the Chapel of St.Virgil: The best match with sequences from the EMBL database is listed for every clone. K4 is related to sample VK-A, K5 is related to sample VK-B, K6 is related to sample VK-C, K10 is related to sample VK-D and K14 is related to sample VK-E.

Clone number [sequence length, bp]	Closest identified phylogenetic relatives [EMBL accession number]	Similarity (%)	Accession number
K4-54 [553]	*Halalkalicoccus tibetensis strain DS12 16S ribosomal RNA gene, partial sequence* [AF435112]	97	FN433758
K4-56 [554]	*Uncultured Halobacterium sp*. partial 16S rRNA gene, *clone K22* [AM159640] previously detected on mural paintings of the St. Virgil Chapel, Austria.	97	FN433759
K4-57 [555]	*Halococcus dombrowskii partial 16S rRNA gene, clone K23* [AM159645] previously detected on mural paintings of the St. Virgil Chapel, Austria, [AJ420376]	99	FN433760
K4-59 [555]	*Halococcus dombrowskii partial 16S rRNA gene, clone K23* [AM159645] previously detected on mural paintings of the St. Virgil Chapel, Austria, [AJ420376]	100	FN433761
K4-62 [555]	*Uncultured Halobacterium sp. clone K14* [AM159641] previously detected on mural paintings of the St. Virgil Chapel, Austria.	99	FN433762
K4-79 [555]	*Uncultured Halobacterium sp. clone K14* [AM159641] previously detected on mural paintings of the St. Virgil Chapel, Austria.	95	FN433763
K4-80 [555]	*Uncultured archaeon H1-K2* [AJ291415] detected in two disparate deteriorated ancient wall paintings.	99	FN433764
K4-81 [556]	*Uncultured Halobacterium sp.* partial 16S rRNA gene, *clone K14* [AM159641] previously detected on mural paintings of the St. Virgil Chapel, Austria.	99	FN433765
K4-85 [554]	*Uncultured Halobacteria archaeon clone A110* [EU328127] from saline soils	98	FN433766
K4-86 [552]	*Halococcus morrhuae* partial 16S rRNA gene, *clone K45* [AM159643] previously detected on mural paintings of the St. Virgil Chapel, Austria.	97	FN433767
K4-92 [553]	*Uncultured Halobacteria archaeon clone A110* [EU328127] from saline soils	94	FN433768
K4-97 [555]	*Halalkalicoccus sp.* [DQ373058] isolate from in Aci Lake, Salda Lake, Seyfe Lake	98	FN433769
K4-98 [552]	*Uncultured archaeon clones* [EF020500; EF020600; EF020685; EF021735; EF022714; EF022874] from soil microbial diversity associated with trembling aspen	94	FN433770
K4-99 [555]	*Uncultured Halobacterium sp.* partial 16S rRNA gene, *clone K14* [AM159641] previously detected on mural paintings of the St. Virgil Chapel, Austria.	99	FN433771
K4-100 [551]	*Halococcus dombrowskii* partial 16S rRNA gene, *clone K23* [AM159645] previously detected on mural paintings of the St. Virgil Chapel, Austria, [AJ420376]	98	FN433772
K5-68 [555]	*Uncultured Halobacteria archaeon clone A110* [EU328127] from saline soils	98	FN433773
K5-72 [553]	*Uncultured Halobacterium sp.* partial 16S rRNA gene, *clone K22* [AM159640] previously detected on mural paintings of the St. Virgil Chapel, Austria.	95	FN435847

K5-77 [555]	*Uncultured archaeon clone A101-21* [AY940004] from asphalt seeps	99	FN435848
K5-81 [554]	*Uncultured Halobacteria archaeon clone A110* [EU328127] from saline soils	98	FN435849
K5-83 [554]	*Uncultured Halobacteria archaeon clone A110* [EU328127] from saline soils	98	FN435850
K6-51 [555]	*Halococcus dombrowskii* partial 16S rRNA gene, *clone K23 and K25* [AM159645; AM159642] previously detected on mural paintings of the St. Virgil Chapel, Austria, [AJ420376]	98	FN435851
K6-56 [555]	*Halococcus sp. strain BIHTY10/11 and strain 004/1-2* [AM902586, AM902588] isolated from rock salt, Austria	99	FN435852
K6-60 [555]	*Halococcus salifodinae* partial 16S rRNA gene, *clone K12 and K46* [AM159639; AM159644] previously detected on mural paintings of the St. Virgil Chapel, Austria.	98	FN435853
K6-61 [555]	*Halococcus dombrowskii* partial 16S rRNA gene, *clone K23 and K25* [AM159645], [AM159642] previously detected on mural paintings of the St. Virgil Chapel, Austria, [AJ420376]	98	FN435854
K6-71 [556]	*Halococcus salifodinae* partial 16S rRNA gene, *clone K12 and K46* [AM159639; AM159644] previously detected on mural paintings of the St. Virgil Chapel, Austria.	96	FN435855
K6-75 [556]	*Halococcus dombrowskii* partial 16S rRNA gene, *clone K23 and K25* [AM159645; AM159642] previously detected on mural paintings of the St. Virgil Chapel, Austria, [AJ420376]	96	FN435856
K6-77[555]	*Halococcus salifodinae* partial 16S rRNA gene, *clone K12 and K46* [AM159639; AM159644] previously detected on mural paintings of the St. Virgil Chapel, Austria, [AJ131458]	98	FN435857
K6-91[554]	*Halococcus salifodinae* partial 16S rRNA gene, *clone K12 and K46* [AM159639], [AM159644] previously detected on mural paintings of the St. Virgil Chapel, Austria, [AJ131458]	99	FN435858
K10-1 [555]	*Halococcus dombrowskii* partial 16S rRNA gene, *clone K23* [AM159645] previously detected on mural paintings of the St. Virgil Chapel, Austria, [AJ420376]	99	FN435867
K10-2 [551]	*Uncultured Halobacterium sp.* partial 16S rRNA gene, *clone K14* [AM159641] previously detected on mural paintings of the St. Virgil Chapel, Austria.	99	FN435868
K10-6 [556]	*Halococcus salifodinae* partial 16S rRNA gene, *clone K12 and K46* [AM159639], [AM159644] previously detected on mural paintings of the St. Virgil Chapel, Austria.	99	FN435869
K10-7 [554]	*Uncultured archaeon clone H1-K2* [AJ291415] detected in two disparate deteriorated ancient wall paintings.	98	FN435870
K10-16 [555]	*Uncultured Halobacterium sp.* partial 16S rRNA gene, *clone K14* [AM159641] previously detected on mural paintings of the St. Virgil Chapel, Austria.	99	FN435871
K10-33 [555]	*Uncultured archaeon clone H1-K2* [AJ291415] detected in two disparate deteriorated ancient wall paintings.	99	FN435872
K14-2 [553]	*Uncultured archaeon clone H1-K2* [AJ291415] detected in two disparate deteriorated ancient wall paintings.	98	FN435873
K14-3 [552]	*Uncultured archaeon clone H1-K2* [AJ291415] detected in two disparate deteriorated ancient wall paintings.	98	FN435874
K14-9 [552]	*Uncultured archaeon clone H1-K2* [AJ291415] detected in two disparate deteriorated ancient wall paintings.	98	FN435875
K14-20 [555]	*Uncultured archaeon clone H1-K9* [AJ291418] detected in two disparate deteriorated ancient wall paintings.	97	FN435876
K14-26 [555]	*Uncultured archaeon clone H1-K2* [AJ291415] detected in two disparate deteriorated ancient wall paintings.	99	FN435877
K14-27 [554]	*Uncultured archaeon clone H1-K2* [AJ291415] detected in two disparate deteriorated ancient wall paintings.	99	FN435878
K14-46 [555]	*Halococcus dombrowskii* partial 16S rRNA gene, *clone K23* [AM159645] previously detected on mural paintings of the St. Virgil Chapel, Austria.	100	FN435879

Similarity ranges from 94-100%

ANNEX TO MANUSCRIPT I

MONITORING AND PHYLOGENETIC IDENTIFICATION OF NON-CULTURABLE *ARCHAEA* PRESENT ON AN ARTIFICIAL WALL PAINTING PLACED AT THE CHAPEL OF ST. VIRGIL, VIENNA.

Inside the frame of a project started 11 years ago by Dr. Busse (University of Veterinary, Vienna, Austria), Prof. Lubitz (University of Vienna, Austria), and the Academy of Building Arts (Vienna, Austria), an artificial wall painting was created by members of the master-school for restoration and conservation of the Academy. The wall painting was created following the fresco technique using five different pigments. The finality of this wall painting was to serve as a support to be inoculated with different strains formally isolated from other damaged medieval wall paintings. The inoculated painting could be, thereafter, used as a model to investigate how this "artificial" microbial community develops with the time. Eighty different investigation areas were delimitated in the painting. Defined areas were inoculated with a combination of different bacterial strains (cocktail α, β, γ) and finally, the painting was exposed in the Chapel of St.Virgil, Vienna. After two months of exposure, three different biocides (Metatin101, Metatin 58-10 and Metatin 58-10/101), commonly used in restoration, were applied with a brush (for details see diploma thesis of Klose, 2001).

As mentioned in Chapter I, the archaeal members of the micro-biota inhabiting the Chapel of St. Virgil could not be isolated with the conventional cultivation methods used in this study. Therefore, molecular techniques were chosen to monitor and identify this microbial domain.

Four samples, named VK-F, VK-G, VK-H and VK-I were collected from the artificial wall painting placed in the chapel of St. Virgil, Vienna (see Figure 14 and Table 7) by scrapping off material from the painting with a sterile scapel. These four samples were investigated for the presence of archaea by amplifying the extracted DNA with specific-archaeal primers. PCR showed to be positive for samples VK-F and VK-G, indicating the presence of this microbial Domain on the painting (data not shown). From samples VK-H and VK-I no archaeal DNA could be amplified with primers ARC344 and ARC915. Therefore only amplified DNA from samples VK-F and VK-G) was further subjected to DGGE fingerprint analysis (Figure 15 A) to visualize the biodiversity of the archaeal domain colonizing the painting and in addition, to compare with the archaeal community detected in the artificial wall painting in the

sampling of 2004 (Ripka, unpublished Diploma Thesis, University of Vienna, 2005). The community fingerprints derived from samples collected in 2008 (Figure 15 A) showed to be very similar, consisting of one dominant band and a few faint bands, strongly resembling the archaeal fingerprints obtained in the sampling of 2004 (Figure 15 B).

In order to obtain a phylogenetic identity of the individual member of the archaeal community colonizing the artificial wall painting in 2008, clone libraries were performed as described in the section of methods. Inserts of clones producing PCR products, which showed different motility behaviour and matched with bands from the original wall sample in DGGE, were selected for sequencing. A total of 14 clones were sequenced and compared with known archaeal sequences contained at the EMBL database using the search tools FASTA and BLAST. Clones showed high score similarity values ranging from 97% to 99% (Table 9).

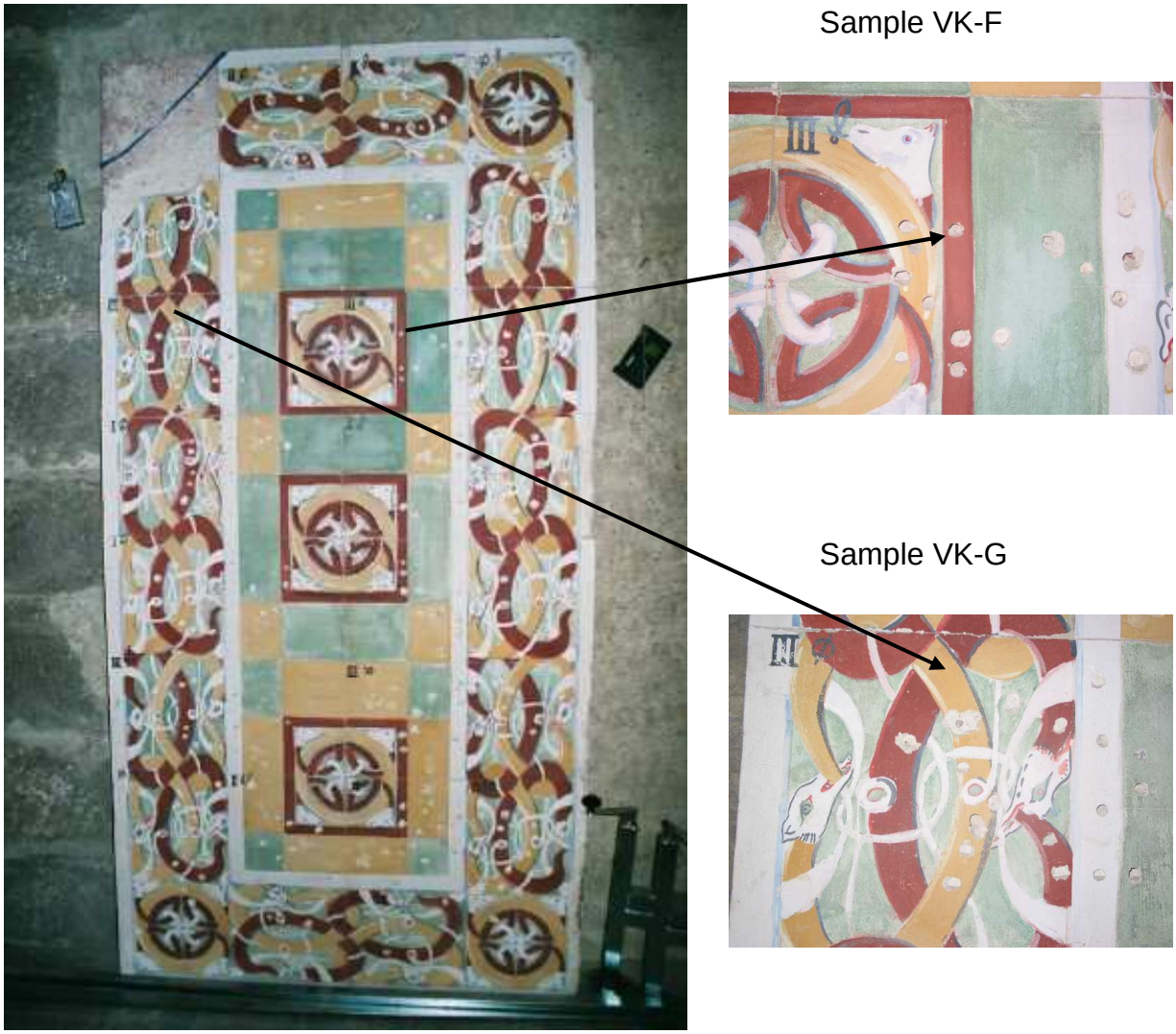
The cloned sequences derived from this study affiliated with non-cultivable members of the archaeal Domain. The sequence analysis revealed that nine sequences affiliated with uncultured clones of the genus *Halobacterium* (64.3%). Two clones affiliated with an uncultured *archaeon* (14.3%). Three further clones, representing each 7.1%, could be identified as uncultured *Crenarchaeon*, uncultured *Euryarchaeote* and as the cultivable *Halococcus morrhuae*.

Comparative sequence analyses showed that ten of the sequenced clones (71.4%), (see Table 9) were phylogenetically related to sequences already detected in the artificial wall painting in 2004 (Ripka, unpublished Diploma thesis, University of Vienna, 2005). Two clones (14.3%) were phylogenetically related to archaeal sequences identified in wall paintings located at two disparate salt attacked monuments, the Catherine Chapel of the Castle of Herberstein (Austria) and the Tomb of Servilia of the Roman Necropolis of Carmona (Spain) (Piñar et al. 2001; Gonzalez et al., 2004). The resting two clones (14.3%) showed correlation to sequences detected in other environments different to salt attacked monuments or mural paintings (Table 9), as clone K2-45, related to an uncultured *crenarchaeon* clone sequence isolated from a thermal spring in the Austrian Central Alps (Weidler et al., 2007) and clone K2-55 related to a sequence identified from brine-seawater interface of the Shaban Deep in the Red Sea (Eder et al., 2002).

The artificial wall painting in the Chapel of St. Virgil was not treated with cellulose wet compresses or formaldehyde and so it is not very surprising that in this sampling

nearly two-thirds of the sequenced clones showed affiliations to microorganisms already found on this mural painting in the year 2004. Additionally it has to be mentioned that only a mixture of bacterial strains was applied onto the artificial wall painting at the beginning of the original project in 1998. No archaeal microorganisms were inoculated on the mural painting. These micro-biota on the paintings surface emerged during the exposure in the Chapel. It is most likely that surface material trickled off the walls and the roof of the chapel onto this artificial wall painting leading to the colonization of these microorganisms on an additional habitat in the chapel presented by this artificial wall painting. Comparison of the sequencing results obtained from the samples taken from wall material and the artificial painting in this study, showed that 71.4 % of the identified clones from the mural painting yielded the same highest match as the samples taken from the chapel's walls.

Figure 14
Artificial wall painting placed in the Chapel of St. Virgil and indicated sampling points of samples VK-F and VK-G.



Quadrant	Sample	Treatment
III β	VK-F Sample taken with a sterile scalpel from a hole with red pigments	Cocktail β, Biocide Metatin 58-10
III Ø	VK-G Sample taken with a sterile scalpel from an area with yellow pigments	No cocktail , Biocide Metatin 58-10

Table 7
Origin of samples taken from the artificial wall painting

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

Table 8
Phylogenetic affiliation of inoculated strains on the artificial wall painting placed in the Chapel of St. Virgil (Klose, 2001, unpublished Diploma Thesis)

Figure 15
A. DGGE fingerprints derived from the samples taken from the artificial wall painting in the Chapel of St. Virgil in the year 2008 (VK-F and VK-G). Clones matching with intense bands are indicated in the figure. Phylogenetic affiliations of the clones are shown in Table 9. DGGE was performed with a 25-65% polyacrylamid gradient and run for 3.5 h at 200V and 60℃. **B.** DGGE profiles from the sampling of 2004 (Ripka, 2005, unpublished Diploma Thesis). DGGE gels contained 25-55% denaturants and were run for 3 h at 200V and 60℃.

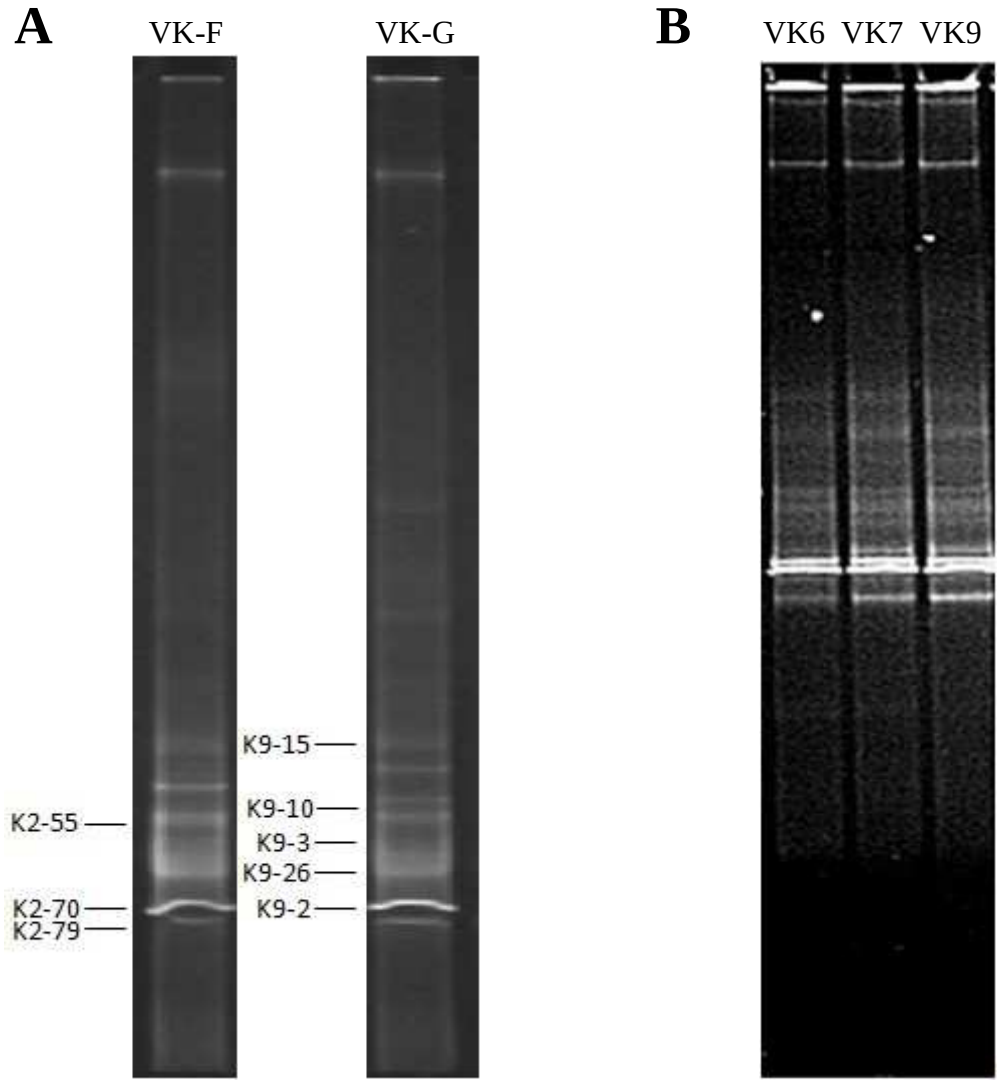


Table 9
Phylogenetic affiliations of archaeal sequences detected in the artificial wall painting located at the Chapel of St.Virgil: The best match with sequences from the EMBL database is listed for every clone. K2 is related to sample VK-F and K9 represents the clones derived from sample VK-G.

Clone no [seq. length bp]	Closest identified phylogenetic relatives [EMBL accession number]	Similarity (%)	Accession number
K2-45 [546]	*Uncultured crenarchaeon* 16S rRNA gene, *clone FJQBAA3* [AM039532] from a Thermal Spring in the Austrian Central Alps, an Evidence of Ammonia-Oxidizing Crenarchaeota	98	FN435846
K2-51 [552]	*Uncultured Halobacterium sp.* partial 16S rRNA gene*, clone K14* [AM159641] previously detected on mural paintings of the St. Virgil Chapel, Austria.	99	FN433753
K2-52 [555]	*Uncultured Halobacterium sp.* partial 16S rRNA gene*, clone K14* [AM159641] previously detected on mural paintings of the St. Virgil Chapel, Austria.	99	FN433754
K2-55 [557]	*Uncultured euryarchaeote clone ST-3K67A* [AJ347778] from brine-seawater interface of the Shaban Deep, Red Sea	96	FN433755
K2-70 [555]	*Uncultured Halobacterium sp.* partial 16S rRNA gene*, clone K14* [AM159641] previously detected on mural paintings of the St. Virgil Chapel, Austria.	99	FN433756
K2-79 [556]	*Uncultured Halobacterium sp.* partial 16S rRNA gene*, clone K14* [AM159641] previously detected on mural paintings of the St. Virgil Chapel, Austria.	99	FN433757
K9-2 [555]	*Uncultured Halobacterium sp. partial 16S rRNA gene, clone K14 [AM159641] previously detected on mural paintings of the St. Virgil Chapel, Austria.*	99	FN435859
K9-3 [555]	*Halococcus morrhuae partial 16S rRNA gene, clone K16 [AM159638]] previously detected on mural paintings of the St. Virgil Chapel, Austria.*	98	FN435860
K9-10 [555]	*Uncultured Halobacterium sp. partial 16S rRNA gene, clone K14 [AM159641] previously detected on mural paintings of the St. Virgil Chapel, Austria.*	99	FN435861
K9-15 [551]	*Uncultured archaeon clone H1-K2 [AJ291415] detected in two disparate deteriorated ancient wall paintings.*	99	FN435862
K9-24 [555]	*Uncultured archaeon H6-K5 [AJ291421] detected in two disparate deteriorated ancient wall paintings*	97	FN435863
K9-26 [556]	*Uncultured Halobacterium sp. partial 16S rRNA gene, clone K14 [AM159641] previously detected on mural paintings of the St. Virgil Chapel, Austria.*	98	FN435864
K9-35 [555]	*Uncultured Halobacterium sp. partial 16S rRNA gene, clone K22 [AM159640] previously detected on mural paintings of the St. Virgil Chapel, Austria.*	99	FN435865
K9-41 [555]	*Uncultured Halobacterium sp. partial 16S rRNA gene, clone K14 [AM159641] previously detected on mural paintings of the St. Virgil Chapel, Austria.*	99	FN435866

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II. Bacterial community dynamics during the application of a *Myxococcus xanthus*-inoculated culture medium used for consolidation of ornamental limestone

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Running title: consolidation of ornamental limestone by a *M. xanthus*-inoculated culture medium

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1. ABSTRACT

In this study, we investigated under laboratory conditions the bacterial communities inhabiting quarry and decayed ornamental carbonate stones before and after the application of a *Myxococcus xanthus*-inoculated culture medium, used for consolidation of the stones. The dynamics of the community structure and the prevalence of the inoculated bacterium, *M. xanthus,* were monitored during the time course of the consolidation treatment (30 days). For this purpose, we selected a molecular strategy combining fingerprinting by DGGE (denaturing gradient gel electrophoresis) with the screening of eubacterial 16S rDNA clone libraries by DGGE and sequencing. Quantification of the inoculated strain was performed by quantitative real-time PCR (qPCR) using *M. xanthus*-specific primers designed in this work.

Results derived from DGGE and sequencing analysis showed that, irrespectively of the origin of the stone, the same carbonatogenic microorganisms were activated by the application of a *M. xanthus* culture. Those microorganisms were *Pseudomonas* sp., *Bacillus* sp. and *Brevibacillus* sp. The monitoring of *M. xanthus* in the culture media of treated stones during the time course experiment showed disparate results depending on the applied technique. By culture-dependent methods, the detection of this bacterium was only possible the first day of the treatment, showing the limitation of these conventional techniques. By PCR-DGGE analysis, *M. xanthus* was detected during the first 3-6 days of the experiment. At this time, the population of this bacterium in the culture media varied between 10^8-10^6 cells m^{-1}, as showed by qPCR analyses. Thereafter, DGGE analyses showed to be not suitable for the detection of *M. xanthus* in a mixed culture. Nevertheless, qPCR analysis using specific primers for *M. xanthus* showed to be a more sensitive technique for the detection of this bacterium, revealing a population of 10^4 cells m^{-1} in the culture media of both treated stones at the end of the consolidation treatment.

The molecular strategy used in this study is proposed as an effective monitoring system to evaluate the impact of the application of a bacterially induced carbonate mineralization as restoration/conservation treatment for ornamental stones.

2. INTRODUCTION

Conventional treatments, both inorganic as well as organic, used for the protection and consolidation of ornamental stones have yielded poor results due to the incompatibility between the treated stone and the nature of the material applied in the treatment. In many cases, treatments have in fact proved harmful by accelerating the alteration (13). In contrast, consolidation methods based on the biomineralization capability of some microorganisms have shown to be more successful than traditional methods. Several groups have investigated the potential of different microorganisms to be used as biomineralizating agents (12, 13, 53). In this regard, for instance, Castanier et al. (6) and Orial et al. (33) used *Bacillus cereus* to consolidate stone surfaces. They found that *B. cereus* was able to induce extracellular precipitation of calcium carbonate on decayed limestones. This calcium carbonate was compatible with the substrate and significantly reduced the water absorption of the treated stone. However, the layer of the new cement induced by *B. cereus* consisted of only few microns.

Alternatively, Rodriguez-Navarro et al. (42) proposed another bacterial conservation method based on the use of *Myxococcus xanthus*. This method appears to be more effective than that proposed by Castanier et al. (6). The Gram negative, non-pathogenic, common soil bacterium, *M. xanthus* (δ-subdivision of the *Proteobacteria*), is able to induce the extracellular precipitation of calcium carbonate (42). The metabolic activity of *M. xanthus* results in the production of CO_2 and NH_3. Extracellular ammonia release raises pH values and therefore $CO_{3(aq)}^{2-}$ concentration, according to these equilibria:

$$NH_{3(g)} + H_2O \Leftrightarrow NH_4^+{}_{(aq)} + OH^-{}_{(aq)} \quad (1)$$

$$CO_{2(g)} + H_2O_{(l)} \Leftrightarrow H_2CO_{3(aq)} \Leftrightarrow HCO_3^-{}_{(aq)} + H^+{}_{(aq)} \Leftrightarrow CO_3^{2-}{}_{(aq)} + 2H^+{}_{(aq)} \quad (2)$$

Precipitation of a calcium carbonate phase occurs when a sufficient supersaturation is reached with respect to this phase (43):

$$Ca^{2+}{}_{(aq)} + CO_3^{2-}{}_{(aq)} \Leftrightarrow CaCO_{3(s)} \quad (3)$$

The newly formed calcium carbonate creates a coherent carbonate cement of 10–50 μm coating the treated stones. Moreover, this cement is rooted down to a depth of 1 mm while, at the same time, the porosity of the stone remains unaltered (42).

Recently, Jimenez-Lopez et al. (18, 19) investigated the role of the natural microbial community inhabiting decayed and quarry calcarenites when either a sterile specific culture medium or a culture inoculated with M. xanthus was applied to consolidate

both kind of stones. Authors compared the chemical evolution of the culture medium, calcium carbonate precipitation and stone consolidation on both decayed and quarry calcarenites during the consolidation treatment. The culture media used in these studies were partially selective due to the introduction of only peptones as a source of carbon and nitrogen, thus enhancing, among the microbial community inhabiting the stone, the growth of chemoorganotrophic bacteria that make use of the amino acids as a source of these both macronutrients. The oxidative deamination of the amino acids induced an increase on the pH of the system as a consequence of the release of NH3, thus creating favorable conditions for calcium carbonate precipitation. Jimenez-Lopez et al. (18, 19) demonstrated that the bacteria activated from the natural microbial community of the stone were able to induce the precipitation of CaCO3 cement that was compatible with the substrate and consolidated the porous limestone without pore plugging. These results were improved when M. xanthus was present.

Nevertheless, and with the goal of the potential application of a *M. xanthus*-inoculated culture medium to treat ornamental stone *in situ*, it is necessary to develop a sustainable monitoring system to evaluate the effects on the microbial community inhabiting a stone during the application of such a treatment. This monitoring will allow to develop a more efficient consolidation method and to reduce the time needed for the treatment, thus reducing costs and the risk of potential contaminations. Therefore, in the present study we propose the application of a molecular strategy, as monitoring system, to investigate a) the microbial communities inhabiting both quarry and decayed ornamental porous limestone prior any consolidation treatment, b) the dynamics of the community structure during the application of a *M. xanthus*-inoculated culture medium and c) the prevalence of the inoculated strain, *M. xanthus,* on such stone in which the autochthonous microbiota has not been eliminated.

The proposed molecular strategy combines fingerprinting by DGGE (denaturing gradient gel electrophoresis) with the screening of eubacterial 16S rDNA clone libraries by DGGE and sequencing. Furthermore, the quantification of *M. xanthus* is performed by quantitative real-time PCR (qPCR) using *M. xanthus*-specific primers designed in this work.

3. METHODS

3.1. Stone slabs, inoculum and culture medium

Two sets of porous limestone slabs (2 x 5 x 0.5 cm) cut out from “quarry calcarenite” and “decayed calcarenite” were used. The quarry calcarenite was collected from La Escribanía (Escúzar, Spain). The decayed calcarenite was collected from a large, thoroughly decayed pinnacle once placed at the Granada Cathedral (Spain) and substituted during one restoration intervention performed over the early nineties of the twentieth century (for more details about both types of calcarenite, see references 18, 19). One piece of each set of slabs from quarry (Q) and decayed (D) stone, here referred as non-treated stone samples, was reserved for molecular analyses prior the treatment.

The microorganism used as inoculum was *Myxococcus xanthus* (strain number 422, Spanish Type Culture Collection, Burjasot, Valencia, Spain), cultured in liquid CT (42), incubated at 18.85 $rad \cdot s^{-1}$ for 48 h at 28℃.

Biomineralization test was conducted in M-3P culture medium with the following composition: 1 wt % $Ca(CH_3COO)_2 \cdot 4H_2O$, 0.2 wt % $K_2CO_3 \cdot 1/2H_2O$, 10 mM phosphate buffer in distilled water, pH 8 (42). Carbohydrates were excluded from the culture medium, while 1 wt % Bacto Casitone was introduced as the sole source of carbon and nitrogen. Removal of carbohydrates excludes the growth of microorganisms that produce organic acids as the result of their metabolism, and enhances the growth of bacteria that use amino acids as C and N source.

3.2. Experimental procedure

Sixteen 250 ml Erlenmeyer flasks containing 100 ml of M3-P medium filtered and sterilized were used in the experiment. Eight of these flasks were dedicated to runs involving quarry calcarenite slabs and eight to decayed calcarenite ones. In those runs involving quarry stone, one slab of non-sterile quarry calcarenite was introduced on each of the eight flasks. The same holds true for runs involving decayed calcarenite.

Out of each group of eight, four of the Erlenmeyer flasks were inoculated with 2 ml of *M. xanthus* inoculum (cell density ~ 3×10^8 cells ml^{-1}), and the remaining four Erlenmeyer flasks were kept sterile, acting as a controls. Therefore, four replicas of each experiment were carried out. Three of these replicas were used to study both the chemistry of the culture medium and the consolidation achieved on the stones,

showed in the studies performed by Jimenez-Lopez et al. (18, 19). The remaining one replica was solely devoted to the molecular analysis of both the culture medium and the stones, showed in the present study. No sampling other than that specified below was performed on this replica to avoid potential contamination.

All Erlenmeyer flasks were incubated in the dark at 28ºC for 30 days in a rotary shaker at 5.97 rad·s^{-1}. Shaking was performed to ensure enough homogenization and aeration of the flask. At predetermined time intervals (0, 1, 2, 3, 6, 8, 10, 15, 20, 25 and 30 days) an aliquot of 2 ml of culture media was collected from the Erlenmeyer flasks under aseptic conditions, centrifuged at 1571.05 rad·s^{-1} for 5 minutes and the pellet was stored at –80 ºC for further molecular analyses of the microbiota growing in the culture medium. At the end of the experiment, treated quarry (referred as TQ) and decayed (referred as TD) stone slabs were collected, rinsed twice using distilled water and dried in an oven at 40 ℃ for 48 h. A pie ce of each stone was separated and stored in a sterile Petri dish for molecular analyses.

The presence of *M. xanthus* was also investigated by a culture-dependent approach. A volume of 0.5 ml of the culture medium in which the stone (both quarry and decayed) was being treated was collected at each time intervals mentioned above during the time course experiment. Serial decimal dilutions were performed, and three Petri dishes containing M3-P solid culture medium (liquid M3-P medium supplied with 1.8% agar-agar, Difco) were inoculated with 0.1 ml of each dilution.

3.3. DNA extraction and PCR analyses

Genomic DNA was extracted directly from stone slabs (non-treated and treated) as well as from the 2 ml collected from the culture media of treated samples as described by Schabereiter-Gurtner et al. (48). Briefly, cells were lysed enzymatically with lysozyme and proteinase K, then treated with a SDS-based lysis at 65oC in a final buffer concentration of 100 mM Na2EDTA, 100 mM Tris-HCl, 100 mM Na2phosphate buffer (pH 8.0), 1.5 M NaCl, 1% CTAB (hexadecyltri-methyl ammonium bromide) and 1.5% SDS. Samples were next subjected to 3 cycles of freezing (−80oC) and thawing (65oC) to break cells mechanically. Afterwards the DNA was purified with the QIAamp Viral RNA Mini Kit (Qiagen) as recommended by the manufacturers.

For PCR analysis, 2 × PCR Master Mix (Promega) [50 units ml-1 of Taq DNA polymerase supplied in a proprietary reaction buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, 3 mM MgCl2] was diluted as recommended by

the manufacturers, and 12.5 pmol of each primer were added. PCR was carried out in 25 µl volumes, and 2 µl DNA template was added.

Two different PCR reactions were carried out to amplify eubacterial 16S rDNA fragments. In a first amplification, DNA was amplified with the primer-pairs 341f/907r (26, 52) or 341f/1492r (26). For DGGE analysis, 200-bp fragments of the 16S rDNA were amplified with a nested PCR using the eubacterial specific primer 341f-GC (forward) to which a 40-base GC clamp was added to its 5' end (31). As reverse primer, the universal consensus primer 518r (32) was used. PCR reactions were performed using a Robocycler (Stratagene). PCR conditions were as described by Schabereiter-Gurtner et al. (48).

DNA of *Myxococcus xanthus* was amplified with the specific primer pair Frz799 (5'-GTGGACCTCCTCTTCTTCGACATC-3') and Frz1147 (5'-GACATGTGTCTCAGTGCCTTCCTT-3') designed in this study with the Primer3 program (http://primer3.sourceforge.net/) (46), which specifically amplifies 349 bp of the *frz*ABCD gene. The *frz* genes are involved in aggregation and fruiting body formation of *M. xanthus* (8). Primers were further tested for specificity with the PrimerBlast program (46).

The specificity of the PCR assay was examined by using bacterial DNAs of other bacterial taxons, including the α-*Proteobacterium Methylobacterium rhodesianum* (environmental isolate), the *β-Proteobacterium Nitrosomonas* sp. (environmental isolate), the γ- *Proteobacteria Pseudomonas stutzeri* (DSM 5190) and *Ps. putida* (DSM 291), the δ-*Proteobacteria Desulfovibrio desulfuricans* (environmental isolate) and *Myxococcus xanthus* (CECT422), the *Firmicutes Bacillus megaterium* (DSM 32), *B. pumilus* (DSM 27), *B. flexus* (DSM 1320) *and Brevibacillus brevis* (environmental isolate) and the *Actinobacteria Kocuria rosea* and *Micrococcus luteus* (environmental isolates).

PCR conditions: 5 min denaturation (95℃), followed by 35 cycles consisting of 1 min denaturation (95℃), 1 min primer annealing (56℃) and 1 min primer extension (72℃), with a final extension step of 72℃ for 5 m in.

3.4. DGGE analysis

DGGE was performed as previously described (31) using a D-Code system (Bio-Rad) in 0.5 × TAE (20 mM Tris, 10 mM acetate, 0.5 mM Na_2EDTA; pH 7.8). The optimized lineal chemical gradient used ranged from 30% to 55% [100% denaturants contains 7 M urea and 40% (vol vol^{-1}) formamide]. Gels electrophoretic separation was performed at a constant temperature (60℃) and 200 V for 3.5 h. Subsequently, gels were stained in an ethidium bromide solution (1 µg ml^{-1}) for 15 minutes and visualized by a UVP documentation system (BioRad Transilluminator, Universal Hood; Mitsubishi P93D-printer).

3.5. Cloning of PCR products and screening of inserts by PCR-DGGE analysis

Five microliters of the purified PCR product (QIAquick PCR purification kit, Qiagen), amplified with primers 341f and 907r, were cloned directly with the pGEM-T Vector System (Promega, Mannheim, Germany), following the protocol of the manufacturer. The ligation product was subsequently transformed into E. coli XLI-Blue which allows blue-white screening, and plated on LB medium containing ampicilline (100 µg ml-1), tetracycline (10 µg ml-1), X-Gal (0.1 mM) and IPTG (0.2 mM) (47).

The obtained clone libraries were screened by comparing the migration of reamplified inserts by Denaturing Gradient Gel Electrophoresis (DGGE) analyses (48). Clones showing different positions in DGGE were selected for sequencing.

3.6. Sequencing and comparative sequence analysis

The selected clones were purified and sequenced as previously described (48). Briefly, 100 ml PCR product generated with primers SP6 and T7 were purified with a QIAquick PCR Purification Kit (Qiagen) and sequenced with a LI-COR DNA sequencer Long Read 4200 (30). Sequencing reactions were carried out by cycle sequencing with the SequiTherme system EPICENTRE with 2 pmol fluorescently labelled primer T7 and 5 U SequiTherm thermostable DNA polymerase. The obtained sequences were compared with known sequences by using the FASTA search option (34) for the EMBL database to search for close evolutionary relatives. Accession numbers are listed in Panel B of Figure 16 and Figure 17.

3.7. Quantification of M. xanthus by Real-Time PCR

For qPCR reactions, 2 × PCR Master Mix (SensiMix dT, Quantace) was diluted as recommended by the manufacturers. The specific primer pair Frz799/Frz1147 was used and 12.5 pmol of each primer were added. SYBR Green was added at the concentration recommended by manufacturers and PCR was carried out in 10 µl volumes containing 2 µl DNA as template. PCR reactions were performed using the Rotor-Gene 6000 (Corbett Research). PCR conditions: 10 min denaturation (95℃), followed by 55 cycles consisting of 1 min denaturation (95℃) and 1.45 min primer annealing and extension (60℃), with a final extension step of 72℃ for 5 min. Fluorescent measurements were recorded during each extension step. The amplification program was immediately followed by a melt program consisting of a gradual increase from 60^{o}C to 95^{o}C at a rate of 0.5℃ with intervals of 10 seconds. The Rotor Gene 6 software (Corbett Research) was used to establish C_t values for each sample.

The efficiency of the assay was determined by performing the standard curve on the basis of 10-fold serial dilutions of *M. xanthus* DNA in the range of 600 ng to 60 fg per PCR reaction. These DNA concentrations are equivalent to 3.9×10^7 to 3.9 bacteria, if estimated that approx. 10.4 fg of DNA by a genome size of 9,454 kbp (7) corresponds to one bacterium. Vegetatively growing cells of *M. xanthus* contain one to two copies of the genome, but upon entry into stationary phase, the chromosome copy number drops to a single copy. Of particular interest, fruiting body-derived myxospores contain a specific two-chromosome DNA complement with both origin and terminus regions localized to the periphery of the myxospore (54). This means that each bacterium contains in average 1.5 genomes (54) and this would correspond to approx. 15.5 fg of DNA per bacterium. Each standard was quantified in triplicate.

For the quantification of *M. xanthus* DNA on treated samples, 2µl out of 100µl undiluted DNA extracts were added to each PCR reaction as template DNA. The 100 µl DNA were extracted from the 2 ml sample collected from each Erlenmeyer at different times, as mentioned above. This information allows us to calculate the concentration of DNA per ml of sample, and hence the number of *M. xanthus* cells per ml of sample.

4. RESULTS

4.1. PCR-DGGE analysis of the microbiota inhabiting non-treated and treated calcarenite stones

The microbiota inhabiting quarry and decayed ornamental calcarenites were investigated by PCR-DGGE analysis. Two different primer pairs, 341f/907r and 341f/1492r (see section of Methods) were used to amplify the 16S rRNA gene. DNA could be successfully amplified from all stone samples by using both primer combinations, resulting in nearly identical DGGE fingerprints (data not shown), indicating the reproducibility of the method. However, the amplification of DNA using primers pair 341f/907r allowed a better visualization of faint bands on each sample and therefore, this primer combination was used in further PCR-DGGE analysis.

A) Quarry stone

DGGE fingerprints derived from non-treated (Q) and treated (TQ) quarry calcarenite are shown in Figure 16. The profiles showed a low number of dominant bands, representing the diversity of the 16S rDNA sequences due to different bacterial types. Five different dominant bands were visible on the profile of non-treated stone (lane Q, bands 1-5), and after 30 days of treatment, the profile derived from the treated quarry stone revealed the dominance of only three bands (lane TQ, bands 11-13).

To obtain detailed phylogenetic information about the members of the community inhabiting non-treated and treated quarry calcarenite, the stone slab samples Q and TQ were used for the construction of clone libraries. Sequence results obtained from inserted clones showed percent sequence similarities between 94.8% and 100% with sequences from the EMBL (see panel B of Figure 16).

Sequences of clones derived from non-treated quarry stone (Q) corresponding to DGGE-bands 1-4 were phylogenetically affiliated with genera of the cultivated *β-Proteobacteria*, namely *Diaphorobacter*, *Acidovorax*, *Imtechium* and *Comamonas*, respectively. Sequence corresponding to DGGE-band 5 was phylogenetically affiliated with a cultivated genus of the *α-Proteobacteria*, namely *Sphingomonas*.

Fingerprint derived from treated quarry calcarenite (TQ) showed three dominant bands, DGGE-bands 11 and 12 related to species of genus *Brevibacillus* and DGGE-band 13 related to *Bacillus sp.* In addition, two faint bands (DGGE-bands 8 and 9), corresponding to *Pseudomonas sp.* could be also detected inhabiting the stone slab.

B) Decayed stone

DGGE fingerprints derived from non-treated (D) and treated (TD) decayed calcarenite are shown in Figure 17. As for quarry calcarenite, the profile derived from non-treated decayed stone showed four to five different dominant bands (lane D, bands 1-4). DGGE profile of treated decayed stone showed only two dominant bands (lane TD, bands 12 and 13) and few faint bands (as band 14).

Clone libraries derived from stone slab samples D and TD allowed the phylogenetic identification of the community members inhabiting the stone. Comparative sequence analysis showed percent sequence similarities between 95% and 100% with sequences from the EMBL (see panel B of Figure 17).

Cloned sequences derived from sample D corresponding to DGGE-bands 1-3 were phylogenetically affiliated with genera of the cultivated *γ-Proteobacteria* *Moraxellaceae*, of the genus *Acinetobacter*. Clone corresponding to DGGE-band 4 was phylogenetically affiliated with a cultivated *β-Proteobacteria* of the genus *Imtechium*. Furthermore, a fifth additional band was observed at the bottom of the fingerprint of sample D (see Figure 17). However, this DGGE-band could not be further characterized, even after several cloning attempts. Additional efforts to get this band by cutting it out of the gel yielded no results by directly sequencing.

After 30 days of treatment, the DGGE-fingerprint derived from the stone slab sample TD showed two dominant bands (bands 12 and 13) both phylogenetically related to *Bacillus* sp. In addition, it was possible to detect a faint band (band 14), also visible at the supernatant sample of day 30, related to the genus *Exiguobacterium*.

4.2. Bacterial community dynamics during the treatment of calcarenites with a *M. xanthus*- inoculated culture medium

A) Quarry stone

Figure 16 shows the original DGGE fingerprints obtained from aliquots taken from the Erlenmeyer flasks over the time course experiment (30 days). As expected, immediately after the application of the *M. xanthus*-inoculated culture (lane 0), a unique dominant band was observed (DGGE-band 6) corresponding to *M. xanthus*. From the first day on, it was possible to see the appearance of dominant DGGE-bands, such as band 7, corresponding to *Bacillus* sp. which dominated the first three

days of the experiment. At this time, DGGE-bands 8 and 9, both corresponding to *Pseudomonas* sp., appeared. The intensity of the bands 8 and 9 decreased during the time course experiment, while three other bands gradually showed up (DGGE-bands 10-12) corresponding to *Brevibacillus* sp.

B) Decayed stone

Regarding decayed stone, a similar behaviour was observed with respect to the succession of the microbial community during the application of the *M. xanthus*-inoculated culture (Figure 17). Immediately after the application of the treatment (lane 0), an unique dominant band was observed (DGGE-band 5) corresponding to *M. xanthus*. The DGGE-profile from the first day of the experiment (lane 1) showed the appearance of five additional dominant bands. DGGE-bands 6, 8-10 corresponded to sequences of *Pseudomonas* sp., and DGGE-band 7 to *Naxibacter varians*. These bands were stable until the third day of the experiment. During the 6^{th} and 15^{th} day, DGGE-profiles showed a shift in the microbial community and some bands disappeared. Then after, two DGGE-bands (bands 9 and 11) corresponding to *Pseudomonas* sp. and *Brevibacillus* sp., respectively, dominated until the end of the experiment.

4.3. Monitoring and quantification of *Myxococcus xanthus* during the 30 days of treatment

To monitor the dynamic followed by the inoculated bacterium, *M. xanthus,* as well as the potential competition between this strain and those activated from the stone during the treatment, different methods were applied and compared.

Culture-dependent assays yielded positive results only from aliquots taken from the culture medium the first day of the treatment, showing a limitation of these conventional techniques. Therefore, culture-independent methods were chosen for the monitoring of *M. xanthus*. Three different molecular approaches were used, as described below.

First, PCR-DGGE analysis of amplified 16S rDNA, directly extracted from aliquots of treated samples, using universal bacterial primers. DNA of *M. xanthus* was loaded on the gel as marker (lane M of Fig. 1 and 2). DGGE-profiles showed the presence of *M. xanthus* in quarry calcarenite samples during the first 3 days of treatment (DGGE-band 6 Figure 16), and in the decayed calcarenite samples during the first 6 days of

treatment (DGGE-band 5 Figure 17). Afterwards, it was not possible to visualize a DGGE-band corresponding to the DNA of *M. xanthus* in the fingerprints of the treated samples.

To overcome this problem, a second molecular approach was developed to increase the sensitivity in the level of detection of *M. xanthus* based on the use of species-specific primers. For this purpose, the *frz* locus of *M. xanthus* was selected as target sequence to be amplified, and the specific primer pair Frz799/Frz1147 was designed, which amplifies 349 bp of the *frz*ABCD gene. To test the specificity of the primer pair Frz799/Frz1147, a PCR was set up using as template DNA from *M. xanthus* as well as from other different bacterial taxons (see section of Methods). Only the DNA of *M. xanthus* could be amplified using this primer combination, indicating the specificity of primers Frz799/Frz1147. Furthermore, stone slabs and aliquots of samples treated during 30 days with culture medium inoculated and non-inoculated with *M. xanthus* (see section of Methods) were tested by PCR analysis using the specific primer pair Frz799/Frz1147 for the presence/absence of *M. xanthus*. Samples treated with a culture medium non-inoculated with *M. xanthus* (acting as a control) showed no amplification of the DNA extracted from either the stone slab or the culture medium, demonstrating the specificity of the primers. Nevertheless, the DNA extracted from the culture medium of samples inoculated with *M. xanthus* could be amplified by using this specific primer pair throughout the experiment (30 days). However, it is worth noting that the amplification of the DNA extracted from stone slabs treated with a *M. xanthus*-inoculated culture was not possible.

The third molecular approach was the quantification of the DNA of *M. xanthus* in the treated samples during the time course experiment by quantitative real-time PCR analysis (qPCR). The specific primers pair Frz799/Frz1147 was used and the efficiency of the assay was determined by performing the standard curve on the basis of 10-fod serial dilutions of *M. xanthus*, as mentioned in section of methods. In a range between 600 ng and 60 fg, a slope of -3.3 was obtained in the standard curve, indicating an efficiency of 0.99% and a R^2 of 0.99. C_t values were inconsistent at a DNA concentration under 60 fg per reaction.

Optimization tests for qPCR were performed with the prepared serial dilutions of purified *M. xanthus* DNA. Different concentrations of $MgCl_2$ (ranging from 3 to 5 mM) and of primers (ranging from 12.5 pmol to 1.25 pmol) were tested. Results showed no significant differences when using different concentrations of $MgCl_2$. However, the

concentration of primers showed to be a critical point, the lower the primers concentration the higher the C_t values, indicating a delay to reach the minimum number of PCR cycles needed to obtain significant fluorescence signals. Consequently, the higher concentration of primer (12.5 pmol) and the lower concentration of MgCl2 (3 mM) tested were kept for the further analyses of samples.

The C_t values of reactions derived from treated samples throughout the experiment, as well as the related DNA concentration determined in qPCR are given in Table 10. Since samples were run in duplicate, each value represents the average of two individual reactions.

Our results show that the concentration of *M. xanthus* DNA in the treated quarry and decayed stone samples, immediately after the immersion of the slabs in the inoculated culture medium were 2580 and 2701 ng ml^{-1} sample, corresponding to 1.6 $\times 10^8$ and 1.7 $\times 10^8$ cells ml^{-1} sample, respectively. After three days of treatment, the *M. xanthus* DNA concentration decreased strongly in the culture medium of treated quarry stone (from 617 ng ml^{-1} sample at day 3 to 1 ng ml^{-1} sample at day 6), what is in accordance with the results obtained with DGGE analysis, where no DGGE-band corresponding to *M. xanthus* was detected after the third day of treatment (see Fig.1). In the culture medium of treated decayed stone, the DNA concentration decreased gradually between days 3-6 of the experiment (from 545 ng ml^{-1} sample at day 3 to 58 ng ml^{-1} sample at day 6), which is also in accordance with the results obtained with DGGE analysis, where it was still possible to detect a faint band corresponding to *M. xanthus* at the sixth day of treatment (see Fig.2). At the end of the treatment (30 days) it was possible to detect 0.37 ng DNA of *M. xanthus* per ml sample of both treated quarry and decayed stones (Table 10), what is equivalent to 2.2 $\times 10^4$ cells ml^{-1} sample.

5. DISCUSSION

In a previous investigation to this study, Jimenez-Lopez et al. (18, 19) developed a consolidation treatment for ornamental stones based on the application of a sterile specific culture medium or a medium inoculated with *Myxococcus xanthus,* as calcium carbonate precipitating-bacteria. Both treatments succeed in the activation of bacteria inhabiting the stones, with a potential for calcium carbonate precipitation. Nevertheless, the effects of the application of a culture medium to a stone that has its own microbiota, as well as the effects of the introduction of a new strain on such

community, have been to date not clarified. Therefore, as follow up of the studies mentioned above, in this work we investigated and compared the microbiota inhabiting quarry and decayed ornamental carbonate stones before and after the application of a culture medium inoculated with *M. xanthus* as stone consolidation treatment. Furthermore, the bacterial community dynamics during the application of such a treatment, as well as the prevalence of the inoculated strain, *M. xanthus*, were monitored by using a molecular strategy.

5.1. Identification of the microbiota inhabiting non-treated and treated stones

Comparing the detected autochthonous microbiota of non-treated quarry and decayed calcarenites, species belonging to the β-Proteobacteria dominated on quarry calcarenite while γ-Proteobacteria dominated on decayed calcarenite. In summary, sequences derived from the non-treated quarry calcarenite (Q) were related to cultivated members of the β-Proteobacteria, family Comamonadaceae, namely Diaphorobacter (17, 21, 22), Acidovorax (16) and Comamonas (57). These sequences were also phylogenetically affiliated with uncultured bacterial clones found in ground-waters from a deep mine (27) and activated sludge (21). Sequence corresponding to DGGE-band 3 was phylogenetically affiliated with Imtechium assamiensis strain BPTSA16, isolated from a warm spring of Assam in India (not validly published). This clone showed also similarities with different uncultured bacterial clones related with degradation process (50), benzene and monochlorobenzene contaminated ground-water (1) and activated sludge (56). Sequence corresponding to DGGE-band 5 was phylogenetically affiliated with the α-Proteobacteria, family Sphingomonadaceae, namely Sphingomonas, as well as with an uncultured bacterium clone associated with a Geological Substrata in a Pristine Aquifer (5). In general, these environments are consistent with the geological/hydrological setting of the quarry from which the carbonate stone was extracted (18, 19).

Furthermore, sequences derived from the non-treated decayed calcarenite (D) revealed high similarities with the cultivated *γ-Proteobacteria Moraxellaceae*, of the genus *Acinetobacter*, (29, 37). Some of the sequences related to *Acinetobacter* sp. showed phenanthrene degrading activities (4). Clone corresponding to DGGE-band 4 was phylogenetically affiliated with the genus *Imtechium*, as well as with different

uncultured bacterial clones involved in degradation process (1, 56). These results are consistent with the origin as well as the exposure of the decayed calcarenite to urban contaminants.

It is worth noting the presence of *Imtechium assamiensis* in the microbiota colonising both non-treated calcarenites. As mentioned above, this bacterium was isolated from a warm spring. This habitat is more in accordance with the physico-chemical environment of the quarry calcarenite, suggesting that the presence of this bacterium in the decayed stone is a remnant of the quarry from which the stone was once collected.

After 30 days of treatment, sequences derived from the treated quarry (TQ) calcarenite were related to species of genus *Brevibacillus*, *Bacillus* (14, 28) and *Pseudomonas* (40). Sequences derived from the treated decayed (TD) calcarenite were related to species of genus *Bacillus*. In addition weak bands corresponding to *Brevibacillus* and *Exiguobacterium* (41) genera were detected on the stone slab. These results indicate that these few bacterial genera were the fraction of the total bacterial community optimally activated during the treatment and able to dominate on the treated stone, thus being detectable by PCR-DGGE (31). As demonstrated by Jimenez-Lopez et al. (18, 19) all activated bacteria, once isolated on solid M3-P culture medium, were able to produce considerable amount of calcium carbonate. This observation is in agreement with the results from other authors regarding the potential for calcium carbonate precipitation of some species of *Bacillus* and *Pseudomonas* genera as *B. sphaericus* (15), *B. cereus* (6), *B. thuringiensis* and *B. pumilus* (3), *P. halophila* (39) and *P. fluorescens* (2).

5.2. Bacterial community dynamics during the incubation of the stone slabs with a *M. xanthus*-inoculated culture

The bacterial community dynamics in the culture media of both quarry and decayed calcarenites, showed in Fig. 1 and 2, indicated the activation of very specific groups of organisms throughout the treatment. DGGE fingerprints derived from the quarry calcarenite culture (Fig. 1) showed a bacterial succession, where species belonging to *Bacillus* were quickly activated by the application of the culture medium and dominated during the first three days of the experiment. Afterwards, an activation of *Pseudomonas* sp. inhibited the further proliferation of *Bacillus* sp. and this species dominated during the days 3-15 of the experiment. Finally, from day 6 on, species

belonging to *Brevibacillus* were activated and dominated until the end of the experiment. The proliferation of *Brevibacillus* sp. produced a gradual disappearance of *Pseudomonas* sp. These results suggest an antagonistic behaviour among the bacteria activated, probably triggered by the lack of nutrients. However, a more detailed study, which is beyond the scope of the present one, would be needed to prove the causes of such an antagonism. In fact, it has been shown that *Pseudomonas* is able to produce membrane vesicles (MVs) filled with periplasmic components with a predatory role in natural ecosystems (20). MVs are capable of lysing a variety of gram-negative and gram-positive bacteria, as *Bacillus* sp (20), increasing the availability of nutrients. In addition, there are works reporting that sporulating bacteria develop predation behaviours in mixed cultures under nutritional stress, by using an antibacterial factor (23). This fact could explain the observation of the gradual disappearance of *Pseudomonas* sp. when *Brevibacillus* sp. proliferated in the medium.

Similarly, DGGE fingerprints showed a bacterial succession during the time course experiment when a culture of *M. xanthus* was applied to decayed calcarenite (Figure 17). In this case, *Pseudomonas* sp. dominated during the first 15 days of the experiment. At this time, the activation of *Brevibacillus* sp. and *Bacillus* sp. induced a shift in the population of *Pseudomonas* sp., and those sporulating bacteria dominated until the end of the experiment. This phenomenon could be also explained by the predation behaviour of sporulating bacteria under nutrient stress mentioned above (23).

An interesting finding is that, irrespectively of the origin of the stone (quarry versus decayed), the same microorganisms were activated by the application of a *M. xanthus*–inoculated culture for consolidating the stone. Those microorganisms were *Pseudomonas* sp., *Bacillus* sp. and *Brevibacillus* sp. These bacteria are commonly found in natural environments and have also been found in ornamental rocks from different locations (55). They are aerobic chemoorganotrophs and can grow in culture media containing aminoacids, and within the pH ranges and temperature of our experiments. Some of them were spore-forming bacteria, as *Brevibacillus* sp. and *Bacillus* sp. (49, 51). *Pseudomonas* sp. produces extracellular polysaccharides which provide protection against a variety of environmental stresses, such as UV radiation, pH shifts, osmotic shock and desiccation (40). These characteristics could explain why the latter strains were activated and detected either due to their ability to form

spores or to produce extracellular polysaccharides and, therefore, to their resistance to dry conditions on the stones.

5.3. Comparison among the bacteria detected in the culture medium during the application of a *M. xanthus* culture and the bacteria colonising the stone

Comparing the bacteria detected in the culture medium with those detected in the stone it is worth noting that the bacteria detected in the non-treated stone were not detected in the culture medium and vice versa.

The first observation can be explained by the fact that the identified non spore-forming bacteria, detected in non-treated stones, could enter into dormant stages and were not reactivated by the provided culture media, since it has already been reported that dormant microorganisms are unable to grow on standard culture media (45). Therefore, bacteria detected on the non-treated stone were probably non-activated, or although activated, those bacteria may not be abundant enough to be detected by the electrophoretic techniques used in this study (31).

Regarding the second observation, bacteria detected in the culture medium could be non-detected in the stone for several reasons. One thereof is that spore-forming bacteria, as the detected *Bacillus* and *Brevibacillus,* could be initially in the non-treated stone and later activated by the culture medium. In this case, those bacteria may not be detected in the stone due to some limitations of molecular techniques. In fact, several works reported on bias for the extraction of DNA from spores (24, 25) and on the preferential amplification of some sequences, excluding *Bacillus*-16S rDNA (10, 38). This could explain why these bacteria were not detected in the non-treated stone but were detected later on in the culture medium.

Finally, some bacteria present in the culture medium were not detected in the stone after the treatment. This can be explained by the calcification of the bacteria into the stone, as showed by Jimenez-Lopez et al. (18, 19), thus preventing the extraction of DNA. In this regard, it is important to consider the case of *Pseudomonas,* which was detected in the culture medium of decayed calcarenite (see Figure 17), but not in the decayed stone neither in non-treated or treated ones. It is known that *Pseudomonas* is very active inducing the precipitation of calcium carbonate (3), thus most *Pseudomonas* cells present in the stone may be calcified, leaving no enough population to be detected by molecular techniques (31). Furthermore, there are works

showing difficulties in DNA extraction due to the strong binding of DNA to certain mineral surfaces (9, 44).

5.4. Monitoring and quantification of *M. xanthus* during the time course experiment

Our results highlights the limitations of culture-dependent techniques for the monitoring of *M. xanthus* in a mixed culture, which yielded positive results only the first day of the treatment. This fact can be related to the greater generation time of *M. xanthus* compared to that of the other activated bacteria (19). Once those bacteria become activated, their growth overlaps that of *M. xanthus*, hindering its detection by cultivation techniques. To overcome this problem, we used molecular techniques to monitor the inoculated strain, *M. xanthus*. However, some limitations were detected by using PCR-DGGE analysis, even if this technique has been successfully applied for the analysis of microbial communities on monuments and building materials (11, 35, 36). Therefore, specific primers were designed in this study to amplify the DNA of *M. xanthus*, which allow the detection of the targeted microorganism, even if there are differences in the relative abundance avoiding competition during PCR. The primer pair Frz799/Frz1147 was selected, which amplifies 349 bp of the frzABCD gene. By using this specific primer-pair, we succeed in the detection and quantification of *M. xanthus* in the culture medium of samples treated with this bacterium during the time course experiment (30 days). Our qPCR analysis showed to be reliable, with a standard curve having a correlation coefficient R^2 value of 0.99 and a slope of -3.3. These values are inside the recommended limits (58). Furthermore, results derived from qPCR analysis correlated well to those derived from PCR-DGGE analysis. DGGE profiles showed that, under the conditions given in our experiments, the detection of *M. xanthus* in a mixed culture was not possible by this fingerprinting technique after the first 3-6 days of the experiment (for quarry and decayed stone, respectively). At this time, the population of this bacterium was below 10^6 bacteria ml^{-1} of sample (see Table 10). The limitation of PCR-DGGE analyses can be explained by the several factors affecting the quantities of PCR products obtained for fingerprinting techniques when using universal primers as i.e. the differential or preferential amplification of rRNA genes by PCR (38), differences in the genome size and the copy number of 16SrRNA genes (10) and the relative abundance of the target sequence in the total microbial community (31). The qPCR

performed with the specific primers for *M. xanthus* showed to be a very sensitive technique for the detection of this bacterium in a mixed culture, revealing a population of 10^4 cells m^{-1} in the culture media of both treated stones at the end of the consolidation treatment. However, the amplification of the DNA of *M. xanthus* from the stone slabs was not possible after the 30 days of treatment, even by using the specific primer pair. This can be explained by the calcification of *M. xanthus* into the stone (18, 19), preventing the extraction of DNA from this bacterium

5.5. Relevance of the present study for the optimization of the consolidation treatment

Results obtained in this work provide new data to optimize the consolidation method for both decayed and quarry calcarenite, based on the application of a culture medium inoculated with *M. xanthus,* already described (18, 19, 42). In these previous studies, authors observed that a pH increase and calcium consumption from the culture media occurred due to the bacterial metabolic activity, mainly within the 15 first days of the experiment. Calcium carbonate precipitation occurred earlier and was more noticeable in those experiments inoculated with *M. xanthus* (18, 19). This fact showed that the presence of *M. xanthus* accelerates the production of new calcium compared to the *M. xanthus*-free experiments, since the achievement of the necessary super-saturating conditions for calcium carbonate precipitation in the latter experiments were delayed until the microbial community inhabiting the stone was activated.

Taking together the data mentioned above with the results obtained in this study, our DGGE fingerprints showed that the microbiota activated within the first stages of the treatment was dominated by *Bacillus* sp. and *Pseudomonas* sp. in the case of quarry calcarenite (Fig. 1), and by *Pseudomonas* sp. in the case of decayed calcarenite (Fig. 2). Therefore, those bacteria, along with *M. xanthus* are probably responsible for the consolidation achieved in the treated stone within the first week. Furthermore, our qPCR analyses showed that the population of *M. xanthus* during the first 3 days of the experiment varied between 10^8 -10^7 bacteria ml^{-1}, indicating that this bacterium would be the main responsible for the precipitation of calcium carbonate within the first hours-days, until *Pseudomonas* sp. and *Bacillus* sp. become activated by the culture medium. It is not clear, however, the role of *Brevibacillus* sp. on the precipitation of calcium carbonate at the later stages of the experiment (last 15 days). The almost absence of calcium in the culture medium at these time intervals may

have prevented further calcium carbonate precipitation by *Brevibacillus* sp., since the saturation of the system with respect to calcite was very close to equilibrium (19). This data is important, since it demonstrates that even though other bacteria may be activated as a result of the consolidation treatment, an uncontrolled calcium carbonate precipitation, which could be potentially problematic, does not occur, since such a precipitation is limited by the calcium concentration in the culture medium used for the treatment.

Based on these results, it seems clear that the decisive time frame for the conservation treatment is the first 7 days. Therefore, the treatment proposed by Rodriguez-Navarro et al. (42) and Jimenez-Lopez et al. (18, 19) to consolidate ornamental carbonated stones may be optimised by significantly reducing the time of the treatment, which also reduces noticeably the costs.

6. CONCLUSIONS

In summary, this study highlights the advantages of the application of a molecular strategy to monitor the microbial community dynamics occurring when a restoration treatment, based on the use of a culture medium inoculated with *M. xanthus* as biomineralizating agent, is applied to protect and consolidate ornamental stones.

The molecular strategy used in this study allow to monitor the dynamics of the natural microbial community present on the stones, as well as the inoculated strain, *M. xanthus*, during the time course of the consolidation treatment. Results reveal that the culture medium used in this study activates the same group of calcium carbonate producing-bacteria, irrespectively of the origin of the stone. The earlier activated *Pseudomonas* and *Bacillus* sp. may be responsible, together with the inoculated *M. xanthus*, of the calcium carbonate precipitation during the first days of the treatment. This offers the possibility of reducing the time of the consolidation treatment to a period of a week, with the consequent reduction of costs. Furthermore, the proposed molecular strategy, together with data derived from physical and chemical analyses, may facilitate the understanding of benefits obtained from the application of a bacterially-induced carbonate mineralization as restoration/conservation treatment for ornamental stones.

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9. LEGEND FOR FIGURES

Figure 16

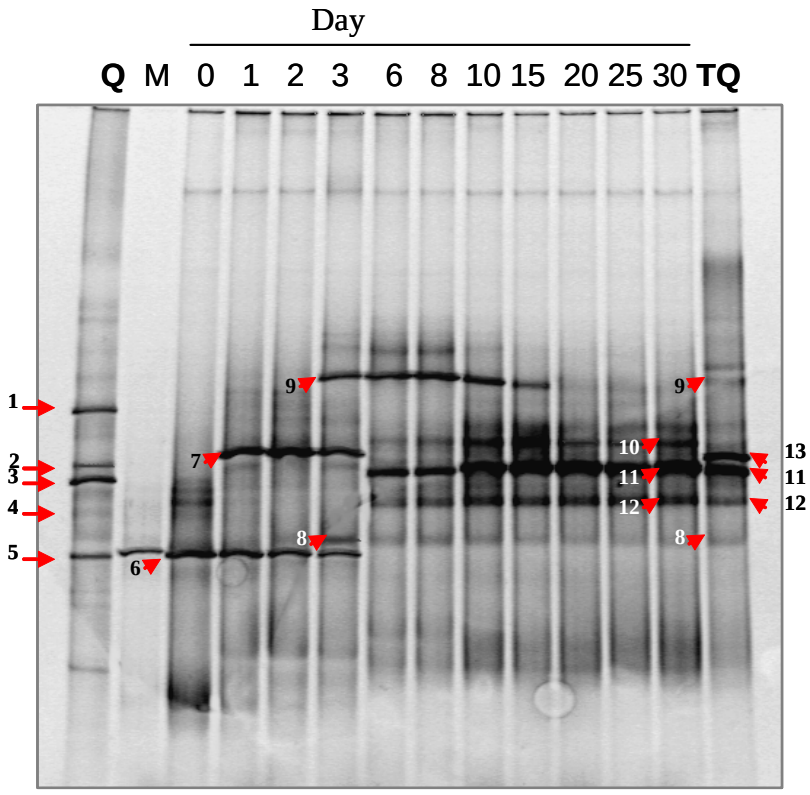
PCR-DGGE profiles representing the succession in the bacterial community structure, as well as the monitoring during the time course experiment of *Myxococcus xanthus* on quarry calcarenite. DNA was directly extracted from non-treated (Q) and treated (TQ) stone slabs, as well as from aliquots taken from the culture medium of treated samples during the time course experiment, and subsequently amplified with the 16S rRNA primers pair 341f/907r. Numbers of lanes indicate sampling days. Lane M: marker of *M. xanthus*. DGGE-bands identified from the 16S rDNA clone libraries are numbered and are indicated by arrowheads. These bands are described in the Panel B. Closest relative, as determined by comparative sequence analysis, level of identity with this relative, clone designation and accession number for each band are summarized in Panel B.

Figure 17

PCR-DGGE profiles representing the succession in the bacterial community structure, as well as the monitoring during the time course experiment of *Myxococcus xanthus* on decayed calcarenite. DNA was directly extracted from non-treated (D) and treated (TD) stone slabs, as well as from aliquots taken from the culture medium of treated samples during the time course experiment, and subsequently amplified with the 16S rRNA primers pair 341f/907r. The description for lane numbers and for panel B are as indicated for Figure 16.

Figure 16

A



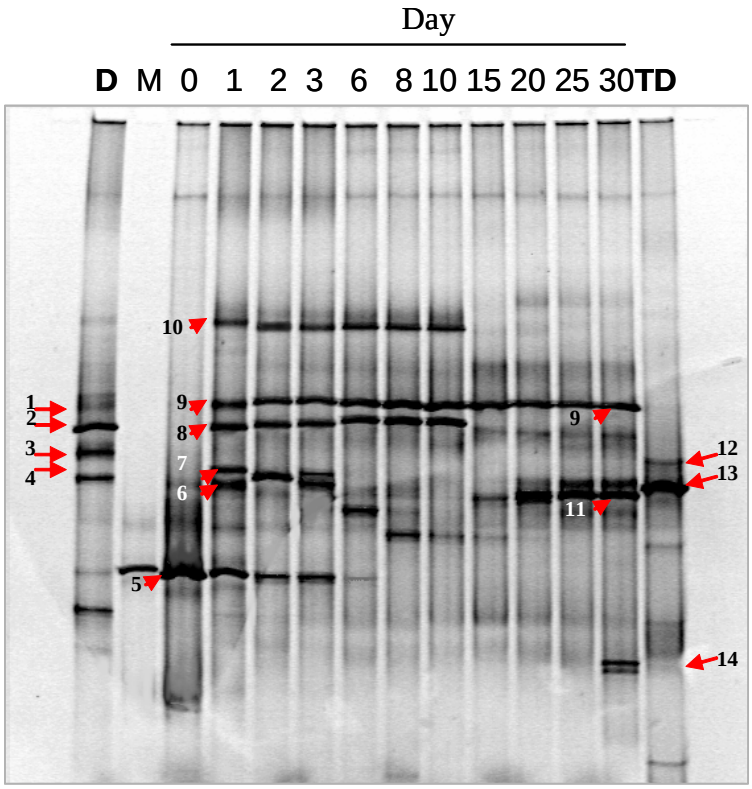
Q: non-treated quarry stone / TQ: treated quarry stone after 30 days of treatment

B

DGGE Band	Closest identified phylogenetic relatives (EMBL accession numbers)	Similarity %	Clones	Accession n°
1	*Diaphorobacter sp* [AB076855; AB076856; AB064317]	99.5	CLA-K13	AM940953
2	*Acidovorax sp.* [DQ111771], [AJ534865], [AM084034; AM084010]	99.3	CLA-K8	AM940952
3	*Imtechium assamiensis sp* [AY544767]	95.7	CLA-K7	AM940951
4	*Comamonas aquatica sp.*[AJ430345; AJ430346; DQ187385]	99.6	CLA-K24	AM940954
5	*Sphingomonas sp.* [AF411072]	97.8	CLA-K4	AM940949
6	*Myxococcus xanthus* [CP000113]	100	CL1.3-K6	FM882256
7	*Bacillus sp.* [AE017355], [CP000001], [AE016877], [AE016879], [AE017334]	99.7	CL1.3-K10	AM947006
8	*Pseudomonas sp.* [AF063219], [AF067960], [EF198403].	94.8	CL1.3-K4	AM947007
9	*Pseudomonas sp.* [AY017341], [AJ312172], [AJ312173], [AJ312174], [AY683883], [AY683884], [EF025737].	99.8	CL1.3-K17	AM947008
10	Low G+*C* Gram-positive bacterium D-N(1)-3C [AB074677] *Brevibacillus sp.* [AY591911], [AB112712]	99.5 99.3	CL2L-K7	AM940960
11	*Brevibacillus sp.* [AY591911], [AB112712] [AF378234] Low G+*C* Gram-positive bacterium D-N(1)-3C [AB074677]	99.5 99.5	CL2S-K14	AM949681
12	*Brevibacillus sp.* [AF378234] Low G+*C* Gram-positive bacterium D-N(1)-3C [AB074677]	99.7 99.3	CL2S-K6	AM949679
13	*Bacillus licheniformis sp.* [AE017333], [CP000002]	99.3	CL2S-K8	AM949680

Figure 17

A



D: non-treated decayed stone /TD: treated decayed stone after 30 days of treatment

B

DGGE Band	Closest identified phylogenetic relatives (EMBL accession numbers)	Similarity %	Clones	Accession n°
1	*Acinetobacter junii* [AB101444], [AY881242]	99.6	CLB-K4	AM940955
2	Acinetobacter junii [AB101444]	99.1	CLB-K22	AM940958
3	*Acinetobacter sp* [X81659], [DQ837531], [AM401576], [EF111219]	95.0	CLB-K7	AM940956
4	*Imtechium assamiensis* [AY544767]	99.3	CLB-K18	AM940957
5	*Myxococcus xanthus* [CP000113]	100	CL2.3-K5	FM882257
6	*Pseudomonas stutzeri* [U65012], [U65012]	97.6	CL2.3-K12	AM947009
7	*Naxibacter varians* [AM774587]	98.8	CL2.3-K19	AM947010
8	*Pseudomonas stutzeri* [AF063219], [AF067960]	99.5	CL2.3-KJ	AM947011
9	*Pseudomonas sp.* [AY017341], [AJ312172], [AJ312173], [AJ312174], [AY683883], [AY683884], [EF025737].	99.8	CL2.3-K13	AM947012
10	*Pseudomonas stutzeri* [AF063219], [AF067960].	99.8	CL2.3-LK7	AM947013
11	*Brevibacillus sp.* [AF378230]; [AB215102]; [AB112721]; [AB116134]; [AJ586382]; [EF139656]; [DQ350827], [AF252328]	99.6	CL16L-K9	AM940970
12	*Bacillus silvestris sp.* [AJ006086] [EU249562]	99.3	CL16S-K9	AM949683
13	*Bacillus silvestris sp.* [AJ006086] [EU249562]	99.3	CL16S-K13	AM949684
14	*Exiguobacterium sp*. [DQ530521], [DQ019166]	98.5	CL16S-K7	AM949682

Table 10
Quantification by real time PCR of *M. xanthus* DNA on the culture media used for the treatment of quarry and decayed ornamental stone slabs during 30 days.

Sampling day	C_t*	**[DNA] (ng/reaction)**	**Cells/ reaction**	**[DNA] (ng ml^{-1})**	**Cells ml^{-1}**
Q.0	9.1 + 0.007	103.2	6.6×10^6	2580	1.6×10^8
Q.1	9.2 + 0.077	97.0	6.2×10^6	2425	1.5×10^8
Q.2	10.2 + 0.007	47.1	3×10^6	1176.5	7.5×10^7
Q.3	11.2 + 0.007	24.7	1.6×10^6	617	4×10^7
Q.6	20.4 + 0.049	0.04	2.6×10^3	1	6.5×10^4
Q.8	20.5 + 0.070	0.04	2.6×10^3	1	6.5×10^4
Q.10	20.4 + 0.042	0.04	2.6×10^3	1	6.5×10^4
Q.15	19.9 + 0.070	0.06	3.8×10^3	1.5	9.6×10^4
Q.20	20.5 + 0.021	0.04	2.6×10^3	1	6.5×10^4
Q.25	21.6 + 0.070	0.02	1.2×10^3	0.45	3×10^4
Q.30	21.9 + 0.127	0.015	9×10^2	0.37	2.2×10^4
D.0	9.0 + 0.007	108.4	6.9×10^6	2701	1.7×10^8
D.1	10.5 + 0.452	39.7	2.6×10^6	991.5	6.4×10^7
D.2	11.4 + 0.106	20.5	1.3×10^6	513.5	3.3×10^7
D.3	11.3 + 0.007	21.8	1.4×10^6	545	3.5×10^7
D.6	14.6 + 0.007	2.3	1.5×10^5	58	3.7×10^6
D.8	17.9 + 0.014	0.22	1.4×10^4	5.5	3.5×10^5
D.10	18.1 + 0.170	0.2	1.3×10^4	5.0	3.2×10^5
D.15	18.4 + 0.014	0.16	1×10^4	4.0	2.5×10^5
D.20	19.9 + 0.127	0.06	3.7×10^3	1.45	9.3×10^4
D.25	22.6 + 0.042	0.009	5.5×10^2	0.22	1.4×10^4
D.30	21.9 + 0.007	0.015	9×10^2	0.37	2.2×10^4

*****Mean values of two individual reactions /Standard deviation (n=2)

Q: quarry stone

D: decayed stone

III. BIO-CONSOLIDATION OF ORNAMENTAL POROUS LIMESTONES: MOLECULAR IDENTIFICATION AND MONITORING OF THE BACTERIA COLONISING AND REMAINING ON THE STONES

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1. Abstract

In the last years, bacterial bio-mineralization has been the subject of many studies, especially its application for the consolidation of decayed ornamental stone and therefore proposing an environmental friendly method to restore our cultural heritage. Studies performed under laboratory conditions on calcarenite stones have successfully reported of using a *Myxococcus xanthus*-inoculated culture media as bio consolidation agent, as well as the potential of using a sterile nutritive media, of a specific composition, able to activate the autochthonous microorganisms inhabiting the stone, capable to induce calcium carbonate precipitation. Both approaches yielded a sufficient consolidation of the stone material. Nevertheless in these studies the microbial community of the treated stone was only examined for a period of maximum 30 days. Here we analyzed the autochthonous micro-biota of two ancient buildings in Granada (Spain) during the *in situ* application of three different consolidation treatments, consisting of a nutritional solution (inoculated and non-inoculated with *Myxococcus xanthus*) and sterile water as a control. To evaluate the dynamics of the microbial communities, pellet samples gained from enrichment cultures conducted during the first week and stone samples, collected after six and twelve months of the treatment, were analyzed using a molecular strategy. PCR amplification of 16S rRNA sequences, construction of clone libraries and discrimination of different clones by DGGE analysis followed by sequencing was

used to gain insight into the microbial diversity present on the differentially treated stones at various points of time. Results of this short- and long-term monitoring showed that on both buildings similar dynamics were triggered by the treatments. 16S rDNA sequencing revealed the dominant occurrence of members belonging to the *Firmicutes* and *Proteobacteria* in the first week, whereas after one year the order *Bacillales* of the phylum *Firmicutes* was the predominantly detected group of microorganisms. The monitoring of *M. xanthus* was done using PCR-DGGE analysis as well as PCR amplification using species specific primers.

The molecular strategy applied in this study allowed a continuous monitoring of the dynamics in the bacterial community structures through the treatments and a detection of the inoculated strain along the time course of the experiment.

2. Introduction

Stone consolidation based on the biomineralization capability of some microorganisms has shown to be a successful method in conservation. There are several works focusing on the potential of different microorganisms to be used as biomineralization agents (Castanier et al., 2000; Gonzalez-Muñoz et al., 2008a; Gonzalez-Muñoz, 2008b; Tiano et al., 1999).

Jimenez-Lopez et al. (2007; 2008) investigated, under laboratory conditions, the role of the microbial community inhabiting ornamental calcarenites when either a sterile specific culture medium or a culture inoculated with *M. xanthus*, as calcium carbonate precipitating bacteria, was applied to consolidate the stones. The used culture media contained only peptones as a source of carbon and nitrogen, showing to be partially selective and able to promote the growth of chemoorganotrophic bacteria that make use of the amino acids as a source of these both macronutrients. Authors demonstrated that a fraction of the microbial community inhabiting the stone was able to induce the precipitation of $CaCO_3$ cement that was compatible with the substrate and consolidated the porous limestone without pore plugging. In these studies, authors observed that bacterial metabolic activity was present during the thirty days of treatment. However, the precipitation of $CaCO_3$ occurred earlier in those experiments inoculated with *M. xanthus* (within the first 3 to 7 days) compared to those non-inoculated (15 days). No significant precipitation occurred afterwards.

In a follow-up study, Piñar et al. (2010) investigated, under laboratory conditions, the bacterial communities inhabiting ornamental limestones during the application of a *M.*

xanthus inoculated-culture as consolidation treatment. The dynamics of the bacterial communities as well as the prevalence of the inoculated strain, *M. xanthus,* on such stones in which the autochthonous micro-biota was not eliminated, was monitored by molecular means during a month. Authors showed that the micro-biota activated within the first stages of the treatment was dominated by *Bacillus* sp. and *Pseudomonas* sp, both of them well known for their capability of $CaCO_3$ precipitation. Therefore, those bacteria, along with *M. xanthus* were probably responsible for the consolidation achieved in the treated stone within the first days. Putting together the results of Jimenez-Lopez et al. (2007; 2008) and Piñar et al. (2010) it seems clear that the decisive time frame for the conservation treatment was during the first 7 days. The significant time reduction of the treatment led to a noticeable cost cut.

Finally, the consolidation treatment developed by Jimenez-Lopez et al. (2007; 2008) optimized to be applied for a total period of a week, has been applied by Jroundi and co-workers (2010) to two monumental buildings *in situ.* The two locations selected are the Monastery of San Jerónimo (Diego de Siloé, 1525) and the Royal Hospital (Enrique Egas, 1511-1522), both dated back to the 16th century and placed in the city of Granada, Spain.

The results shown in the present study are part of this first *in situ* application study in the frame of an international collaboration with the Department of Microbiology (Faculty of Sciences, University of Granada, Spain). The application of the conservation treatment and sampling were performed from the Spanish working group. Thereafter, samples were sent to the Institute for Applied Microbiology (Vienna) for molecular analysis. In the case of the Monastery of San Jerónimo, the enrichment cultures gained during the first week of the application of the treatment were further analysed by conventional culture-dependent techniques (Jroundi et al., 2010).

With the obtained samples we conducted a short-term monitoring, the period corresponding to the first week of the treatment application, as well as a long-term monitoring, performed six and twelve months after the treatment, of the bacterial communities inhabiting the stones by using a molecular strategy. The monitoring (short- and long-term) of any stone conservation treatment, when it is applied to buildings *in situ*, should be a requisite to evaluate the real consequences of the application of such a treatment. With this purpose, the goals of the present study were to investigate and compare a) the microbial communities inhabiting ornamental

limestone from two different buildings prior any consolidation treatment, b) the dynamics of the community structure during the first week of the application of three consolidation treatments (short-term monitoring), c) the impact of the three consolidation treatments on the inhabiting micro-biota after one year (long-term monitoring) and d) to monitor the prevalence of the inoculated strain, *M. xanthus,* on both buildings during a period of a year.

The molecular strategy applied, combined the fingerprinting by denaturing gradient gel electrophoresis (DGGE), construction and screening of 16S rDNA clone libraries by DGGE and sequencing of selected clones.

3. Materials and methods

3.1. Stone-consolidation treatment

The decayed porous limestone (calcarenite) building blocks selected for the consolidation treatments in this study are part of an upright wall exposed for centuries to outdoors weathering in the church of San Jerónimo Monastery and the Royal Hospital, both placed in Granada, Spain. Both buildings were built using a buff coloured bioclastic calcarenite stone which was extracted from Santa Pudia's quarries (Escúzar, Granada). The stone material has an average porosity of ca. 28% and a limited degree of cementation (micritic and sparitic cement) that makes it highly susceptible to weathering. In the case of the Monastery of San Jerónimo, the area selected for treatment has a southeast orientation and is localised about 20 m over ground level. Treated sectors on the Royal Hospital of Granada are located on the ground floor of an outer wall with adjacent pavement and street. The stone from both buildings shows extensive granular disintegration (sanding), surface pitting, and a general loss of surface relief (Figure 18, Figure 19 and Figure 20).

The selected areas for the stone-consolidation treatment were delineated in three sectors as shown in Figure 18 and Figure 19. Thereof each sector was treated differently: Sectors A and D, the control areas, were treated with sterile distilled water; Sector B and E were inoculated with *Myxococcus xanthus* (strain number 422 from Spanish Type Culture Collection) – these areas received an inoculum of this strain in exponential growth phase (~ 10^9 cells/ml) (see Rodriguez-Navarro et al., 2003 for details on the inoculum preparation) in conjunction with a sterile nutritive solution M-3P [1% Bacto Casitone, 1% $Ca(CH_3COO)_2 \cdot 4\ H_2O$, 0.2% $K_2CO_3 \cdot 1/2\ H_2O$ in

a 10 mM phosphate buffer, pH 8] (Rodriguez-Navarro et al., 2003); and to Sectors C and F only the sterile M-3P nutritional solution was applied. The application of the solutions was carried out using the spray technique as described by Tiano et al., 2006 (Figure 20 A). The treatment application was repeated twice every day during the seven days of treatment to maintain the stone adequately damp. The *M. xanthus* inoculum was applied twice only the first day of the treatment on Sector B and E and the remaining days sterile M-3P was applied once a day. The total volume of solutions employed to the stone in all three sectors, was 1-1.5 ml/cm^2. The treated areas were covered with an aluminium foil, respectively a plastic foil, to avoid desiccation and direct impact of sunlight on the stone surface (Figure 20 B, C and D). The foils were not in direct contact with the stone surface to enable air circulation during the seven days of treatment plus three more days until the applied solutions evaporated and the stones become fully dried.

3.2. Sampling

3.2.1. Stone sampling

Pieces of stone were taken prior the application of any treatment to analyze the autochthonous micro-biota inhabiting the stone (here referred as non-treated stone). After the treatment, further stone samples were collected six months (only in the case of the Monastery of San Jerónimo) and one year later. Stone slabs were introduced in 1 ml sterile distilled water, mixed with hand, and aliquots of water were taken to perform serial dilutions and incubations in different selective media (for details see Jroundi et al., 2010). The remaining water was dried at 37^{o}C in sterile tubes and sealed with a 45 µm pore size filter. Dried stone slabs were frozen until their analysis. Two stone samples were taken from each area and mixed together prior to DNA extraction. From the Monastery of San Jerónimo sample SJ-NT-A was taken from the sector A, sample SJ-NT-B from sector B and sample SJ-NT-C was collected from sector C (see Figure 18 A). From the Royal Hospital sample RH-NT was taken before the treatment. After one year samples SD12-1 and SD12-2 were collected from sector D, sample SE12 from the sector E and sample SF12-1 and SF12-2 from sector F (see Figure 19 A).

3.2.2. Sampling to obtain bacterial pellets

Sterile and absorbent filter paper (AFP, ANOIA, Spain) pieces (ca. 1 x 2 cm in size), that can absorb a volume of 15 µl/cm^2 of solution were used to collect the bacteria from the microbial community stimulated to growth as a result of the application of the nutritional solution (as shown in the Figure 18 B and Figure 19 B). As soon as the treated areas were humidified at the first and last day (day 8) of treatment, the paper pieces were placed for 5 minutes on several spots (from 1 to 9 and 19 to 27, see Figure 18 B and Figure 19 B) of the stone blocks surface. To each aseptically collected AFP piece, 1 ml of sterile M-3P nutritive solution was added immediately and transported to the laboratory for further investigation. In order to get representative results of the bacterial communities (both air-borne and bacteria inhabiting the stone) normally settling in the calcarenite stone material, samples were collected from three different spots of each of the three sectors. The sampling locations are shown in Figure 18 B for San Jerónimo and Figure 19 for the Royal Hospital. The absorbent paper strips were subsequently introduced into Erlenmeyer flasks containing M-3P medium and incubated for 24 hours. After this incubation time, the enrichment cultures were centrifuged and the pellets were frozen for further molecular analysis.

From the Monastery of San Jerónimo after the first week of the treatment the pellet samples PA1 and PA8 were derived from sector A, samples PB1 and PB8 from sector B and samples PC1 and PC8 from sector C. From the Royal Hospital the pellet samples PD1-1, PD1-2 and PD8-1 and PD8-2 could be obtained on the first and eighth day after the treatment from subsectors D-1, respectively D-2. Samples PE1 and PE8 from sector E and samples PF1-1, PF1-2, and PF8-1 and PF8-2 were derived on the first and eighth day after the application of the media from subsectors F-1, respectively F-2.

3.3. DNA extraction and PCR analysis

DNA from stone slabs as well as from the collected bacterial pellets was extracted according to the protocol described by Schabereiter-Gurtner et al. (2001). This protocol was used because it was especially designed to cope with problems such as PCR inhibitors and small sample amounts that can occur with samples taken from ancient buildings.

For all PCR reactions 2x PCR Master Mix from Promega [50 units/ml of TaqDNA Polymerase in a supplied reaction buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, 3 mM $MgCl_2$] was diluted to 1x and 50 pmol/µl of each primer were applied to the reaction volumes. Two PCR reactions were performed to amplify eubacterial 16S rDNA fragments for genetic fingerprinting by DGGE: The first round PCR reaction was carried out with primer 341f (Muyzer et al., 1993), and 907r, universal primers for 16S rDNA (Teske et al., 1996) in 25 µl total reaction volume and 2 µl template DNA from the stone materials. The second round PCR was performed with the primers 341fGC and 518r (Neefs et al., 1990), amplifying a 200-bp DNA fragment for DGGE analysis. At the 5' end of the forward primer was an additional 40-base GC clamp that stabilizes the melting behaviour of the DNA fragments in DGGE analysis (Muyzer et al., 1993). This PCR round was performed in a total volume of 100 µl (2 x 50 µl) and 3 µl template of the first round of PCR were added. The PCR products were pooled, precipitated in ethanol and 20 µl were loaded onto the DGGE gel. PCR was performed in a MJ Research PTC-200 Peltier Thermal Cycler with the following thermocycling program: the DNA was denaturated for 5 min at 95℃, followed by 30 cycles each consisting of 1 min denaturation at 95℃, 1 min annealing of the primers at 55℃ and 1 min extension at 72℃. 5 min at 72℃ were used as final extension step (Schabereiter-Gurtner et al., 2001). 7 µl of each PCR product were run on a 2% (w/v) agarose gel for about 35 min at 110 V, stained in an ethidium bromide solution [1 µg/ml; stock: 10 mg/ml] for 15-25 minutes and visualized by a UVP documentation system (BioRad Transilluminator, Universal Hood; Mitsubishi P93D-printer). A negative control was carried out in all PCR reactions, where no template was added, to exclude the possibility of cross-contaminations.

3.4. Fingerprint analysis by DGGE - Denaturing Gradient Gel Electrophoresis

For DGGE fingerprinting of the bacterial communities of the stone samples, the pooled DNA from the 2nd round PCR, supplemented with Loading Dye Solution (Fermentas), was run on gels in 0.5 x TAE buffer [20 mM Tris, 10 mM acetate, 0.5 mM Na_2EDTA; pH 8.0] for 3.5 hours at 200 V and 60℃ in a BIORAD-DCODE™ – Universal Mutation Detection System (Muyzer et al., 1993).

A chemical gradient ranging from 30 to 55% of urea and formamide in an 8% (w/v) polyacrylamide gel (BioRad, Munich, Germany) was used to separate the DNA bands from the different bacterial members. Staining of the denaturant

polyacrylamide gels was done in a 1 µg/ml ethidium bromide solution [stock: 10 mg/ml] for 15-25 minutes and afterwards visualized by a UVP documentation system (BioRad Transilluminator, Universal Hood; Mitsubishi P93D-printer).

3.5. Construction of 16S rDNA clone libraries

For the construction of clone libraries 2 x 3 µl DNA templates of each sample were amplified in 2 x 50 µl reaction volumes using the universal primers 341f and 907r. Aliquots of PCR products were supplemented with Loading Dye Solution (Fermentas), analysed on a 2% agarose gel and further purified using the QIAquick PCR Purification Kit (Qiagen, following the protocol of the manufacturer). The purified DNA was again visualized on a 2% agarose gel, stained in an ethidium bromide solution and documented with an UVP documentation system.

5.5 µl of the purified PCR product were used as ligation template for the pGEM – T easy Vector system (Promega) following the instructions of the manufacturer. One shot TOP10 cells (Invitrogen) were used for the transformation reactions. These cells allow the identification of recombinants (white colonies) on an indicator LB medium containing ampicilline (100 µg/ml), streptomycine (25 µg/ml) and X-Gal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside; 0.1mM) (Sambrook et al., 1989).

3.6. Screening of the bacterial clones

About 50 to 150 white clones from each clone library were harvested, resuspended in 1x TE-buffer [10 mM TrisHCl, 1 mM EDTA; pH 8.0] and the cells were mechanically destroyed by three cycles of freezing and thawing. In order to amplify the inserts, the vector specific primers SP6 (5'-ATTTAGGTGACACTATAGAATAC-3', Promega) and T7 (5'-TAATACGACTCACTATAGGG-3', Promega) were used and 3 µl of the lysed cells were added as template DNA for a 25 µl PCR reaction. The following thermocycling program was used: 5 minutes denaturation at 95℃, followed by 35 cycles consisting of 1 min denaturation at 95℃, 1 min annealing of the primers at 46℃ and 1 min extention at 72℃. A final extension step was performed at 72℃ for 5 min. The quality of PCR products was verified by gel electrophoresis through 2% agarose gels.

For nested PCR the primers 341GC and 518r were used in a total reaction volume of 25 µl. The PCR products were supplemented with Loading Dye Solutions

(Fermentas) and directly applied onto 30-55% polyacrylamide gels. DNA fingerprints were compared by visual inspection of the banding patterns. The band positions of the bacterial clones were compared with one another and with the DGGE profile of the original samples. Inserts of clones matching with the most intense bands and with faint bands of the DGGE fingerprint of the original were selected for sequencing.

Storage of the selected bacterial clones was performed using two different protocols: 1. the plates after transformation on which the individual clones were streaked out, were kept at 4 ºC on sealed LB-plates after incubation for 24 h at 37 ºC, and 2. transferred to fresh LB liquid medium and incubated overnight at 37 ºC to be stocked in glycerol at – 80 ºC as bacterial suspensions.

3.7. 16S rDNA sequencing and phylogenetic analyses

For sequencing of selected clone inserts, PCR reactions were performed with total volumes of 100 µl, separated into 2 x 50 µl. The primers SP6 and T7, specific for the vector, and 3 µl template DNA were used for the amplification. The thermocycling program as described for the screening of clones was used. PCR products were pooled and purified using the QIAquick PCR Purification Kit (Qiagen). The PCR products were electrophoresed on a 2% agarose gel for 35 minutes at 110 V, stained with ethidium bromide [1 µg/ml; stock: 10 mg/ml] for 15-25 minutes and visualized by a UVP documentation system (BioRad Transilluminator, Universal Hood; Mitsubishi P93D-printer).

Sequencing was done using the ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems) using a Big Dye terminator v3.0 cycle sequencing kit as supplied by the manufacturer. The DNA samples were prepared according to the manufactures protocol.

Comparative sequence analyses was done by comparing pair-wise inserts sequences with those available in the online databases provided by the NCBI (National Centre for Biotechnology Information), respectively EMBL (European Molecular Biology Laboratory) using the BLAST and FASTA search programs (Altschul et al., 1997; Pearson, 1994). The ribosomal sequences of the bacterial clones have been deposited at the EMBL nucleotide database.

3.8. PCR-Amplification of *M. xanthus*-DNA with specific primers

Stone- as well as pellet samples derived from the sectors treated with *Myxococcus xanthus* of both buildings (Sectors B and E) were screened for the presence of *M. xanthus*-DNA. The strain specific primers Frz799 (5'-GTGGACCTCCTCTTCTTCGACATC-3') and Frz1147 (5'-GACATGTGTCTCAGTGCCTTCCTT-3') (Piñar et al., 2010) were used to amplify a 349 bp fragment of the *frz*ABCD gene. The PCR conditions consisted of 5 min denaturation at 95℃, followed by 35 cycles, each c onsisting of 1 min denaturation at 95℃, 1 min annealing of the primers at 56℃ and 1 min primer extension at 72℃, with a final extension step of 5 min at 72℃ (Piñar et al., 2010). Aliqouts of PCR product were analysed on 2% agarose gels for 35 minutes at 110 V, stained with ethidium bromide [1 µg/ml; stock: 10 mg/ml] for 15-25 minutes and visualized by a UVP documentation system (Figure 23 A and B).

4. Results

4.1. Experimental procedure

On both buildings the selected areas for application of the stone-consolidation treatments were divided into three sectors. Each sector received a different treatment (Figure 18 and Figure 19). The control sectors of the Monastery of San Jéronimo and the Royal Hospital (A and D, respectively) were treated with sterile distilled water. Sectors B and E were treated with a *Myxococcus xanthus*-inoculated nutritive solution M-3P and finally sectors C and F only received the sterile M-3P nutritional solution. The solutions were applied using the spray technique as shown in Figure 20 A and were further covered with aluminium, respectively plastic foil to avoid dehydration of the treated areas.

Stone samples were collected from each sector prior the application of any treatment in order to analyze the autochthonous micro-biota inhabiting the non-treated stones of both buildings. Thereafter, and with the purpose to perform a long-term monitoring, from both historical monuments stone samples were collected one year after the treatment. In the case of San Jerónimo additional sampling was possible six months after the application. Due to restoration works at the Monastery of San Jerónimo it was not possible to take stone samples from sector A after one year.

The non-treated and treated stone samples obtained from all three sectors of both monuments (see Figure 18 and Figure 19) were subjected to DNA extraction as described in the section of Materials and methods. All DNA extracts were amplifiable using the universal bacterial primers 341f and 907r (data not shown). The micro-biota inhabiting the ornamental stones of the Monastery of San Jerónimo and Royal Hospital were further investigated by a molecular strategy combining PCR-DGGE analyses, construction of clone libraries from 16S rDNA and identification of clones by sequencing of the 16S rDNA inserts of selected clones. To implement a continuous monitoring and a comparison of the micro-biota inhabiting the stone of the two buildings during the time course of the treatment, clone sequences were deposited in the EMBL database and search results showing the highest similarity ranking were hunted for matches with clone sequences derived from the same or other sectors at previous or the same points of time of both buildings. Additional clone sequences which could also be observed during the FASTA search, but showing less than the highest similarity ranking were not taken into account.

Furthermore, during the first week of the treatment enrichment cultures were conducted with pieces of the collected stones as mentioned in the section of Materials and methods. These enrichments were analysed by using the same molecular strategy as mentioned above. This short-term monitoring allows inferring which members of the microbial community are able to be quickly activated by the different treatments and therefore be the responsible for the production of calcium carbonate within the first week.

4.2. PCR-DGGE and sequence analysis of the micro-biota inhabiting non-treated and treated stones of the Monastery of San Jerónimo

DGGE fingerprints derived from non-treated (SJ-NT) and treated (SA6, SB6, SB12, SC6, SC12) stones of the Monastery of San Jerónimo are shown in Figure 21 A. The DNA of *Myxococcus xanthus*, strain 422 was loaded in the gel as a control. DGGE analysis revealed that the autochthonous micro-biota inhabiting the non-treated stone (samples SJ-NT-A, SJ-NT-B and SJ-NT-C representing sector A, B and C, respectively) was nearly identical for all three sectors (data not shown), and therefore sample SJ-NT-C was selected as representative non-treated stone for further investigations, named as SJ-NT at the Figure 21 A.

The profiles derived from the non-treated stone showed a low diversity of the 16S rDNA sequences. Only three different dominant bands (bands 1, 2 and 4) and three additional faint bands (bands 3, 5 and 6) were visualized on DGGE. Thereafter, the application of all three treatments led to a shift in the bacterial composition present on the stone in all sectors.

Surprisingly, the sector A treated with water also showed a shift in the microbial community. Although the three dominant bands visible in the non-treated stone remained dominant after 6 months of the treatment, two additional dominant bands (bands 9 and 11) as well as further faint bands (bands 7, 8, 10 and 12) appeared at the upper position of the gel (Figure 21 A).

Sectors B and C showed a stronger shift in their bacterial composition. The community-fingerprint of sector B after 6 months of the treatment resembled very much the pattern observed before the treatment, but also showing additional bands at the upper position of the gel (bands 19-23). A long term monitoring showed a further shift in the community after one year with the appearance of additional, partially dominant bands (bands 29, 30, 33-36) whereas some bands vanished (bands 19-24). In sector C (treated with M-3P medium) the bacterial community also shifted after 6 and 12 months with the appearance and disappearance of bands. After 6 months bands 43-47 newly emerged in the DGGE profile of this sector, vanishing again in the sample after one year, thereof only band 43 represented a dominant band, whereas all others were barely visible as faint bands. One year after the treatment, a strong dominant band emerged at the top of the DGGE-gel (band 48), whereas all other dominant bands attenuated or completely disappeared.

In order to obtain detailed phylogenetic information of the bacteria colonising the stones on all three sectors, clone libraries were constructed from all stone samples shown in Figure 21 A. The patterns of a total of 54 cloned 16S rDNA fragments were compared to the patterns in the original profiles by DGGE, and clones corresponding to dominant bands and also clones showing different band motility were sequenced.

Sequence results obtained from inserted clones showed similarities between 93% and 100% with sequences from the EMBL. In total, a number of 12 different genera were identified from all stone samples of San Jerónimo. The relative abundance of the detected genera (number of clones) and their distribution on the different samples over the time course experiment are showed in Table 11 A and Table 14.

4.2.1. Phylogenetic analysis of sequenced clones derived from non-treated stone – *Sample SJ-NT*

From the non-treated stone of San Jerónimo six different clones were selected for sequencing. They belonged to the *Proteobacteria* and the *Firmicutes*. Clones 1 and 2 matched with dominant bands from the original fingerprint and were identified as *Bacillus silvestris* and *Delftia tsuruhatensis*, respectively. Under these two DGGE-bands one clone, matching a weak band, affiliated with an *uncultured Xanthomonas clone.* The third dominant band from the non-treated stone (band 4) was identified as *Agrobacterium tumefaciens*. Further two clones running under this dominant band and only visible as faint bands in the original DGGE profile could be associated to the *β-Proteobacteria*, namely *Comamonas testosteroni* and *Comamonas sp.* (bands 5 and 6).

4.2.2. Phylogenetic analysis of sequenced clones derived from treated stones

Control Sector A - water treatment

After six months – Sample SA6

Half a year after the application of sterile, distilled water on the control sector, 12 clones could be identified as members of the *Proteobacteria*, *Firmicutes* and *Bacteroidetes.* Clones 7 and 8 showed no band in the original sample, but 16S rDNA sequence analysis identified them as an *uncultured Flavobacteriaceae bacterium* and *Pseudomonas stutzeri*, respectively. The newly occurring DGGE-band 9 showed similarities with an *uncultured Bacteroidetes bacterium* clone. Phylogenetic analysis revealed that DGGE-band 10 was related to an *uncultured Bacillus sp.* clone. DGGE-bands 11 and 12, running directly under the previous bands, associated with two *uncultured Pseudomonas sp.* clones. Clone 13 showed the same band migration as band 1 from the non-treated stone, but showed higher similarities to an *uncultured Planococcaceae bacterium* clone. Directly underneath, DGGE-bands 14 and 15 showed to be phylogenetically related to uncultured clones of *Bacillus sp.* and *Delftia sp.*, respectively. Only as faint band visible in the DGGE profile was an *uncultured Rhizobiaceae bacterium* clone (band 16). Bands 17 and 18 were identified both as members of the *Comamonadaceae* family, namely *uncultured Comamonas sp*. clones.

Sector B – *Myxococcus xanthus*-inoculated culture treatment

After six months – Sample SB6

From this sector clones belonging to four different phyla could be identified. Out of the 10 sequenced clones only three showed a dominant band in the fingerprint profile (bands 24-26). All others were merely visible as faint bands. The short migrated clone 19 in the acrylamide gel could be grouped to the *Bacteroidetes*. The only member of this phylum was related to an *uncultured Flavobacteriaceae* clone. 16S rDNA sequence analysis of clone 20 proofed the relatedness to an *uncultured Bacillus sp.* clone. Within the *Proteobacteria*, members of the *α-, β-* and *γ-Proteobacteria* were found; all as uncultured bacterium clones. The detected sequences affiliated with an *uncultured Pseudoalteromonas sp.* (band 21), an *uncultured Rhizobiaceae bacterium* (band 26), and an *uncultured Comamonas sp*. (band 27). Four clones (bands 22-25) running underneath each other were grouped into the phylum *Firmicutes*. Thereof only band 25 was related to a cultured *Bacillus sp.*, whereas all other three clones showed affiliations with 16S rDNA sequences of *uncultured Bacillus sp.* clones. The most dominant band of this stone sample (band 26) could be associated with an *uncultured Rhizobiaceae bacterium* clone. Showing the same band motility as band 18 from the original sample, clone 27 could also be identified as an *uncultured Comamonas sp*. clone. The only member of the *Actinobacteria* showed the longest band migration in the gel and was phylogenetically related to a cultured *Corynebacterium sp.* (band 28).

After one year – Sample SB12

One year after the application of a *Myxococcus xanthus*-inoculated culture on the stone, all sequenced clones showed affiliations to the family *Bacillales* within the phylum *Firmicutes*. The type strains *Bacillus humi* (band 29) and *Bacillaceae bacterium MOLA 662* (band 30) matched with the two highest dominant bands. Alternately following several *Bacillus sp*. (bands 31, 33, 35 and 37) and *uncultured Bacillus sp*. (bands 32, 34 and 36) were identified on this sector.

Sector C – M-3P sterile nutritive medium

After six months – Sample SC6

Six months after the application of M-3P sterile nutritive medium on the stone, all sequenced clones clustered into the phylum *Firmicutes*. Clone 38, matching with the higher migrating of the two dominant bands, was identified as *Bacillus seohaeanensis*. Two clone sequences running under this band showed affiliations to *Virgibacillus necropolis* and *Virgibacillus carmonensis* (bands 39 and 40), respectively. The majority of the isolated clones were related to *uncultured Planococcaceae bacterium* clones (bands 41-47).

After one year – Sample SC12

Seven clones related to the phyla *Proteobacteria* and *Firmicutes* were detected after one year from this sector. A new occurring dominant band at the top of the gel could be classified as an *uncultured Planomicrobium sp.* clone (band 48), as well as the three following bands (bands 49-51). Belonging to the family *Comamonadaceae*, one clone was identified as an *uncultured Comamonas sp*. (band 52). Barely visible as faint bands the clones 53 and 54 were detected and 16S rDNA sequence analysis showed their relatedness to *uncultured Bacillus sp.* clones.

4.3. PCR-DGGE and sequence analysis of the micro-biota inhabiting non-treated and treated stones of the Royal Hospital of Granada

DGGE fingerprints derived from non-treated (RH-NT) and treated (SD12-2, SE12, SF12-1 and SF12-2) stones collected from the Royal Hospital (Granada) are shown in Figure 22 A. As in Figure 21 A, the DNA of *Myxococcus xanthus*, strain 422 was loaded in the gel as a control.

DGGE analysis revealed that the autochthonous micro-biota inhabiting the non-treated stone of the Royal Hospital showed *per se* a higher diversity of 16S rDNA sequences than that of the Monastery of San Jerónimo. Five different dominant bands (bands 2, 11, 12, 15and 18) and many other faint bands (bands 1, 3-10, 13, 14 and 16-18) were visible in the band pattern. Thereafter, as in the case of San Jerónimo, the application of the consolidation treatments led to a visible change in the microbial community structure on the different sectors.

DGGE profiles derived from the water-treated stone samples of sector D, divided into sub-sectors D-1 and D-2 after one year (samples SD12-1 and SD12-2) were nearly

identical (data not shown), and therefore sample SD-12-2 was chosen as representative stone sample for further studies, named as SD12 in Figure 22 A. As observed at the Monastery of San Jéronimo, the mere application of distilled water on the control sector led to a shift of the inhabiting micro-biota on the stone. Three new strong bands appeared in the DGGE profile (bands 21, 24 and 27). Several faint bands, matching with faint bands from the non-treated stone, could be observed in the lower part of the gel, whereas many weak bands in the upper position diminished or completely disappeared.

Sector E showed only two dominant bands (bands 40 and 42) on DGGE profiles after one year. The fingerprint showed a strong depletion on the band counts and only few faint bands were visible. Especially on top of the acrylamide gel no faint bands were detected.

A long term monitoring of the microorganisms on sector F-1, respectively sector F-2, showed a shift in the community structure on the stone after the application of the sterile M-3P media. DGGE profiles derived from both subsectors differed from each other as well as from the one of the non-treated stone. From sample SF12-1 only two dominant bands (bands 46 and 54) were visualized, which showed no matches with bands from the autochthonous micro-biota. Numerous faint bands between these dominant bands were observed (bands 47-53). In sample SF12-2, four different dominant bands (bands 58, 60, 61) occurred. Despite many attempts it was not possible to gain a clone matching with the lowest dominant band of this sample. Additionally on top of the acrylamide gel two new clones could be gained (bands 55 and 56), which were slightly visible in the original sample. Further, the faint DGGE-bands 62 to 64, running in the lower part of the gel from sample SF12-2 were detected.

Clone libraries from all stone samples showed in Figure 22 A were constructed to phylogenetically identify the microorganisms present on the non-treated and treated stones. Selected clones were sequenced and compared with the EMBL nucleotide database. A total of 64 sequences were obtained showing similarities between 93 and 100% with sequences from EMBL. Summed up, 26 different genera were identified from all stone samples of the Royal Hospital of Granada. The distribution and the relative abundance of the isolated clones are shown in Table 12 A and Table 14.

4.3.1. Phylogenetic analysis of sequenced clones derived from non-treated stone - *Sample RH-NT*

Eighteen obtained 16S rDNA sequences from the non-treated stone belonged to three different phyla: the *Proteobacteria*, the *Firmicutes* and the *Actinobacteria*. The first two identified clone sequences, of which band 2 represented a dominant band of the sample fingerprint, affiliated with *uncultured bacterium clones.* A very weak band, running slight under this dominant band was classified as *Pseudomonas sp.* (band 3). *Streptococcus bovis* was phylogenetically most affiliated to the 16S rDNA insert of clone 4. In regular distances the following twelve clones could be detected in the DGGE. To the subdivision of the *β*- and *γ-Proteobacteria* the clones 5 to 7 were grouped. FASTA search results revealed them as *Neisseria sp*., *Pseudoalteromonas issachenkonii* and *Pseudoalteromonas tetraodonis*, respectively. The DGGE-bands 8 to 12 could all be clustered into the phylum *Firmicutes.* Out of these clone sequence, - except band 10, which affiliated with *Brevibacillus brevis* - all clones were identified as *Bacillus sp*. Under the two most dominant *Bacillus* bands (bands 11 and 12), members of the *α- – Agrobacterium tumefaciens – β- –Comamonas testosteroni* and *γ-Proteobacteria –Enterobacter sp*. – (bands 13-15), were only visible as faint bands in the acrylamide gel of the stone sample. With a short distance to the previous bands three more clones were identified at the bottom of the gel. All of the three 16S rDNA sequences could be grouped into the phylum *Actinobacteria* and affiliated with *Micrococcus sp., Kocuria rosea* and *Corynebacterium testudinoris* (bands 16-18).

4.3.2. Phylogenetic analysis of sequenced clones derived from treated stone one year after the treatment

Control Sector D – water treatment - *Sample SD12*

16S rDNA sequences derived from 20 clones showed similarities with the same three phyla already identified on the non-treated stone. Two bands, running at the top of the acrylamide gel could be associated with *Bacterium J10* and *Pseudomonas brenneri* (bands 19 and 20). Matching with the upper dominant band, an *uncultured Actinobacterium* clone (band 21) was identified, followed by *Pseudomonas alcaligenes* (band 22), which stopped slightly after this dominant band in the denaturing gel. Clone 23 was affiliated with an *uncultured Planococcaceae bacterium* clone. Clones 24 and 27, corresponding to sequences of *Delftia acidovorans* and *Bosea eneae*, respectively matched with the two strongest dominant bands in the

sample. Between these bands two members of the *Proteobacteria*, *Porphyrobacter dokdonensis* (band 25) and an *uncultured Delftia sp.* clone (band 26), could be isolated. Downwards of the second dominant band of this sample a series of clones (bands 28-36), matching with faint bands, were observed. All of them could be grouped into the phyla *Firmicutes* and *Proteobacteria*, showing 16S rDNA sequence similarities with cultured bacteria but also to uncultured clone sequences. An *uncultured Planococcaceae bacterium* clone (band 28), *Bacillus sp.* (band 29), *Hydrogenophaga defluvii* (band 30), *Stenotrophomonas maltophilia* (band 31), *Thiobacillus denitrificans* (band 32), an *uncultured Rhizobiaceae bacterium* clone (band 33), *Methylobacterium aminovorans* (band 34), an *uncultured Planococcaceae bacterium* clone (band 35) and an *uncultured Exiguobacterium sp.* clone (band 36) were phylogenetically identified by their 16S rDNA sequence. The last two clones of this sample were both clustered to the phylum *Actinobacteria*. Clone 37, had the same band migration as the last dominant band of this sample, and was related to *Brachybacterium arcticum*. Showing the greatest band motility in the DGGE, an *uncultured Actinobacterium* clone was visualized.

Sector E – *Myxococcus xanthus*-inoculated culture media treatment – *Sample SE12*

One year after the treatment with a *Myxococcus xanthus*-inoculated culture media all seven obtained clone sequences affiliated with the phylum *Firmicutes*. Sequences showed high similarities with uncultured *Bacillus sp*. (bands 39, 40, 42-44). Thereof clones 40 and 42 matched with the two dominant bands in the DGGE profile of the sample treated with the bacterial-inoculated culture. Clones affiliating with cultured strains showed relations to a *Bacillus sp.* (band 41) and *Bacillus niacin* (band 45).

Sector F – M-3P sterile nutritive medium – *Sample SF12-1 and SF12-2*

From the two stone samples treated with the sterile nutritive solution, altogether 19 clones mostly belonging to the *Firmicutes* were isolated. From the subsector F-1 all clones had the highest similarity ranking in the FASTA search with uncultured clone sequences. An *uncultured Bacillus sp.* clone (band 46) matched with the first dominant band of sample SF12-1. The following, numerous faint bands highly affiliated with: an *uncultured Planococcus sp.* clone (band 47), *uncultured Planococcaceae bacterium* clones (bands 48, 51-54), an *uncultured Planomicrobium*

sp. clone (band 49) and an *uncultured Firmicutes bacterium* clone (band 50). Thereof clone 54 matched with a dominant band of the original sample.

From the subsector F-2, three out of the ten 16S rDNA clone sequences showed similarities to cultured bacteria. Having the shortest band motility in the acrylamide gel the clones 55 and 56 could be classified as *Desemzia sp.* and *Planomicrobium koreense*. Slight above the first dominant band of the sample, an *uncultured Planococcaceae bacterium* clone (band 57) was detected. Clones, matching with the three dominant bands, were all identified as *uncultured Planococcaceae bacterium* clones (bands 58, 60 and 61). Clone 59, located between the first two dominant bands was related to a *Planococcus sp.* Three faint bands at the lower part of the gel affiliated with an *uncultured Planococcaceae bacterium* clone (band 62), an *uncultured bacterium* clone (band 63) and an *uncultured Kocuria sp.* clone (band 64).

4.4. Short-term monitoring - Enrichment cultures

As mentioned in the Introduction, and based on the results obtained in our previous works, it seems clear that the decisive time frame for the consolidation of the stones is during the first 7 days after the application of the treatment. Therefore, enrichments cultures were performed during the first week of the treatment – on the 1st and 8th day - in order to visualize the fraction of the bacterial community able to actively grow after the application of the different treatments.

Bacterial pellets were gained from the conducted enrichment cultures as mentioned in the section of Materials and methods. The obtained pellets were further investigated by PCR-DGGE analyses in order to visualize the fraction of the bacteria growing on the cultures. Pellets were subjected to DNA extraction as described in the section of Materials and methods. All DNA extracts showed to be amplifiable using the universal bacterial primers 341f and 907r. DGGE-profiles derived from the actively growing bacteria are showed in Figure 21 B, for the Monastery of San Jerónimo and in Figure 22 B for the Royal Hospital. Clone libraries were generated from each enrichment culture and clones were identified by 16S rDNA sequencing and comparison with already known sequences from the EMBL-, respectively NCBI database. In order to make a short time monitoring and to compare the microbial communities in the enrichment cultures of the two buildings during the first week of the treatment, clone sequences were deposited in the EMBL database and FASTA search results with the highest similarity were looked for matches with 16S rDNA

clone sequences derived from pellet samples or non-treated stone samples of the same or other sectors of both buildings. Clone sequences which had a lower similarity ranking were not taken into account.

4.4.1. PCR-DGGE analysis of the micro-biota growing in the enrichment cultures conducted during the first week of the consolidation treatment at the Monastery of San Jerónimo

After one day of the treatment with water, it was possible to see the activation of certain members of the bacterial community present on sector A. The DGGE profile of sample PA1 showed an increase of dominant bands (bands 55-57, 59) Thereof two bands (bands 56 and 59) showed the same motility as dominant bands from the non-treated stone. Further faint bands appeared through the activation with water. The DGGE profile obtained from the enrichment culture conducted eight days after the water application, resembled very much the pattern obtained from the one conducted the 1st day. The dominant bands from the first day were still present, except the one, not identified band from the first day which vanished again. An additional band (band 64), already observed in the non-treated stone, occurred on the eighth day.

The application of a *Myxococcus xanthus*-inoculated culture on the stone promoted the growth of other members of the bacterial community. The DGGE profile showed the establishment of four new dominant bands (bands 71, 74, 77 and 79) on the first day of the treatment. Only band 69 resembled a dominant band from the non-treated stone sample. Numerous faint bands, detected on the first day, vanished again till the eighth day, showing at this time a very similar DGGE profile to that from the non-treated stone.

The treatment with the M-3P sterile media promoted the growth of at least four different bacterial species, two of them visible at the DGGE-profile as dominant bands (bands 84 and 85). After 8 days of the treatment, DGGE profiles derived from the enrichment cultures conducted at this time revealed the growth of a dominant species detected as band 88 on the gel.

In order to obtain a phylogenetic identification of the bacteria growing on the enrichment cultures during the first week of the treatment, clone libraries were performed from all samples shown in Figure 21 B. 16S rDNA sequence analysis of 35 selected clones and their comparison with sequences of the NCBI-, respectively

EMBL database was done using the search tools FASTA and BLAST. As for sequences from stone samples, all clones from pellet samples were deposited at the database. Similarities ranged from 92-100% to known sequences and 13 different genera were observed in all pellet samples from San Jerónimo (see Table 11 B and Table 13).

A. Control Sector A – water treatment - *Samples PA1 and PA8*

Within the first week of the treatment, sequenced clones showed to be phylogenetically affiliated with the phyla *Proteobacteria, Firmicutes* and *Bacteroidetes*. From sample PA1 seven clone sequences could be obtained. Clones 55 and 56, matching with dominant bands from the sample, were related to the phylum *Bacteroidetes*, namely to a *Sphingobacterium sp.* and to *Flavobacterium mizutaii*, respectively. *Brevundimonas diminuta* (clone 57) had the same migration in the acrylamide gel as the third dominant band of this sample. Slight underneath, clone 58 could be classified as an *uncultured Brevundimonas sp.* clone. The last identified clones (bands 59-61) were all associated with the *α-Proteobacteria*, represented by *uncultured Rhizobiaceae bacterium* clones.

On the eighth day of the treatment clone 62, identified as an *uncultured Comamonas sp.* clone, matched with the highest dominant band of the fingerprint of sample PA8. Showing the same motility as the second dominant band in denaturing gel, clone 63 was related to an *uncultured Bacillus sp.* clone. Despite many attempts the following dominant band could not be obtained from the clone libraries. Slightly underneath, three bands (bands 64-66) were identified as *uncultured Delftia sp.* clones. Two additional clones, running a little bit longer in the acrylamide gel (bands 67 and 68) were associated with an *uncultured Rhizobiaceae bacterium* clone and an *uncultured Comamonas sp.* clone, respectively.

B. Sector B – *Myxococcus xanthus*-inoculated culture treatment - *Samples PB1 and PB8*

The DGGE profiles derived from enrichment cultures conducted with the *Myxococcus xanthus*-inoculated culture treated stone showed clearly different profiles after one and eight days of the treatment. Clones obtained from the first day showed sequence similarities to the *Firmicutes* and the *Actinobacteria*. Clones matching with the dominant band 69 as well as the faint bands 72 were related to the 16S rRNA

sequences of *uncultured Bacillus sp.* clones. Not visible in the DGGE fingerprint of the sample, clone 70 was related to an *uncultured bacterium clone*. Bands 71 and 73 were related to the 16S rRNA sequence of a *Bacillus sp.* and to *Bacillus selenatarsenatis*, respectively. A *Planomicrobium glaciei* type strain was related to the sequence of the dominant band 74, and two additional clones (bands 76 and 77) were both characterized as *uncultured Planococcaceae* clones. Two clones were members of the *Actinobacteria*, namely *Arthrobacter psychrolactophilus* (band 75) and an *uncultured Kocuria sp.* clone (band 79). Band 78, a barely visible band underneath, was another member of the *Firmicutes* and was identified as *Solibacillus silvestris* (band 78).

All three obtained clones from the enrichment culture performed at the eighth day were characterized after nucleic acid sequencing and database comparison as members of the *β-Proteobacteria*. The most dominant band (band 81) and the two surrounding faint bands (bands 80 and 82) were identical to *uncultured Comamonas sp.* clones.

C. Sector C – M-3P sterile nutritive medium - *Samples PC1 and PC8*

16S rDNA sequences of the seven clones obtained from the enrichment cultures of this sector could be associated to the phyla *Proteobacteria* and *Firmicutes*. The two higher migrating bands of sample PC1, were related to cultured bacteria of the *Bacillales* family, represented by *Bacillus koreensis* (band 83) and *Bacillus firmus* (band 84). Matching with the lower dominant band, the 16S rDNA sequence of clone 85 showed the relatedness to an *uncultured Rhizobiaceae bacterium* clone.

On the eighth day of the treatment a faint band in the upper position of the gel (band 86) was identified as an *uncultured bacterium clone*. Bands 87-89 were all clustered into the phylum *Firmicutes*. Thereof the clone insert of the most dominant band (band 88) was related to a cultured *Solibacillus silvestris* strain, whereas the 16S rDNA sequences of the faint bands were related to *uncultured Bacillus sp.* clones.

4.4.2. PCR-DGGE analysis of the micro-biota growing in the enrichment cultures conducted during the first week of the consolidation treatment at the Royal Hospital of Granada.

Enrichment cultures derived from the first and eighth day of the water treatment from sector D-1 and D-2 were analysed by DGGE. The fingerprint profiles derived from the

enrichments of both subsectors were nearly identical at both points of time (data not shown) and therefore only the profiles from subsector D-1 are shown in Figure 22 B. Additional clone libraries were performed with enrichment samples of both subsectors and proofed the homogeneous inhabiting micro-biota of sectors D-1 and D-2 (data not shown). For subsectors F-1 and F-2 DGGE profiles displayed different community structures and therefore clone libraries were performed from all samples of this sector.

The water treatment showed to activate the growth of only few bacterial species on sector D. On the first day only two dominant bands (bands 67 and 72) were visible. Band 2 from the non-treated stone resembled with the upper dominant band (band 67) of sample PD1-1. All other dominant and faint bands from the non-treated stone vanished through the application of the water. Seven days later the activation of the growth of new bacteria render the appearance of another three new dominant bands (78, 80 and 81) and a few faint bands, whereas the previous dominant bands (bands 67 and 72) completely disappeared.

The application of the *M. xanthus*-inoculated culture promotes growth and consequently the appearance of three dominant bands (bands 82 and 85) on the first day. Numerous attempts to isolate the third dominant band were without success. Till the eighth day three new dominant bands (bands 86, 89 and 92) emerged, whereas all dominant bands from the first day vanished. All of these bands matched with faint bands from the non-treated stone of the building.

On the sector treated with sterile nutritive solution, differences between the two subsectors were detected. On subsector F-1 three dominant bands (bands 95, 97 and 99) and some faint bands showed different positions in the gel as the two dominant bands (bands 104 and 106) from sector F-2. A shift in the microbial community from the enrichments from the first to the eighth day was observed on both subsectors. Three dominant bands (bands 108, 109 and 111), which showed a different migration in the acrylamide gel as the bands from the first day were visible in sector F-1. Sector F-2 also displayed three dominant bands (bands 116, 118 and 122), but as is sector F-1, they did not resemble with the dominant bands detected on the first day. Generally no bands from sector F matched with each other; on both subsectors the previous detected bands vanished until the second sampling and new dominant bands appeared.

Clone libraries were performed from all pellet samples shown in Figure 22 B. To identify the 63 selected clones, the 16S rDNA inserts were sequenced, compared and deposited in databases, as described above. The 16S rDNA sequences displayed 89-100% relatedness to know sequences and a total number of 13 different genera were detected (Table 12 B and Table 13).

A. Control Sector D – water treatment - *Samples PD1-1 and PD8-1*

During the first week of the treatment with sterile distilled water, the sequenced clones showed correlation to the phyla *Proteobacteria* and *Firmicutes.* The most dominant band from the first day (band 67) as well as band 68, which could not be seen in the original fingerprint of sample PD1-1, could be associated with *uncultured bacterium* clones. The three direct environing bands (clones 65, 66 and 69) and also bands 70 and 71 were confirmed to be *Pseudomonas aeruginosa* strains. 16S rDNA sequence analysis of clone 72 proved its relatedness to an *uncultured Brevundimonas sp.* clone.

Out of the nine clone sequences obtained from the enrichment culture performed at the eighth day after the application of the treatment, only one clone, which showed the lowest band motility in the acrylamide gel, was related to a cultured bacterium, namely *Acinetobacter radioresistens* (band 73). Also, no band could be seen in the fingerprint profile of the original sample matching with the clones 74, 75 and 76. Bands 74 and 75 showed the highest similarity with an *uncultured Rhizobiaceae bacterium* clone and an *uncultured Delftia sp.* clone, respectively. Bands 76 and 78 were related to *uncultured Comamonas sp.* clones. Bands 77, 79-81 were identified *uncultured Exiguobacterium sp.* clones.

B. Sector E – *Myxococcus xanthus*-inoculated culture treatment - *Samples PE1 and PE8*

During the first week of treatment with a *M. xanthus*-inoculated culture, eleven different clones showed to be related to the phyla *Proteobacteria*, *Firmicutes* and *Actinobacteria*. On the first day all clone inserts were identical in their sequence to partial rRNA genes of different *Proteobacteria*. Bands 82-84 had similarities to *uncultured Delftia sp.* clones. Clone 85 matched in its band migration with the lower dominant band. 16S rDNA sequences analysis allowed the association of this clone to an *uncultured Stenotrophomonas sp.* clone. One additional dominant band

between the two described above, could not be further characterized, even after several cloning attempts.

From sample PE8, derived from the enrichment culture performed at the eighth day of the treatment, clone 86 ran in the same position as the upper dominant band. Sequencing and phylogenetic analysis revealed that the partial 16S rDNA sequence of this band was related to an *uncultured Arthrobacter sp.* clone. A cultured *Brevundimonas diminuta* strain (band 87) and an *uncultured Stenotrophomonas sp.* clone (band 88) could be obtained from the clone libraries, which only displayed a weak band in the gel. Three clones, all identified as *uncultured Comamonas sp.* clones (bands 89-91), showed very similar band motility in the acrylamide gel. Thereof the highest running clone 89 correlated with a dominant band from the sample. The lower dominant band in the gel matched in its migration with clone 92 that could be phylogenetically characterized as an *uncultured Exiguobacterium sp.* clone.

C. Sector F – M-3P sterile nutritive solution – *Samples PF1-1, PF1-2, PF8-1 and PF8-2*

DGGE profiles derived from enrichment cultures performed with samples of different subsectors and days displayed disparate band profiles. On sector F-1, most of the clones were members of the *Proteobacteria*. Only clone 97 was a relative of the *Firmicutes*, namely *Bacillus firmus* strain. Six clones (matching with bands 93-96, 99 and 100) were all identified as members of the *γ-Proteobacteria*, namely associated with *Pseudomonas monteilii* (bands 93 and 96), an *uncultured Pseudomonas sp.* clone (band 94), *Pseudomonas putida* (band 95), *Pseudomonas sp.* (band 99) and *uncultured Stenotrophomonas sp.* clone, (band 100). Three additional clones (matching with bands 98, 101 and 102) were affiliated with members of the *α-Proteobacteria*, related to an *uncultured Brevundimonas sp.* clone (band 98), an *uncultured Brevundimonas sp.* clone and an *uncultured Rhizobiaceae bacterium* clone (band 101 and 102, respectively).

From subsector F-2 only uncultured members of the *Proteobacteria* were detected. The first dominant band of the sample PF1-2 and the slight above running band (bands 103 and 104) were both identified as *uncultured Delftia sp.* clones. Bands 105-107 were all classified as *uncultured Stenotrophomonas sp.* clones.

At the eight day of the treatment from the subsector F-1 eight clones were detected. 16S rDNA analysis allowed the grouping of these clones into the phyla *Firmicutes*, *Proteobacteria* and *Actinobacteria*. Matching the top dominant band, clone 108 showed relatedness to an *Exiguobacterium sp.* Clone 109, matching with the second dominant band, had high similarity rankings to an *uncultured Planococcaceae bacterium* clone. A cultured member of the *Firmicutes*, namely *Planococcus kazaiensis*, was the next relative that could be associated with clone 110. The less-dominant band at the bottom of the gel was characterized as an *uncultured Comamonas sp.* clone (band 111). Having a great motility in the denaturing gel, the bands 112-114 were all related to *uncultured Exiguobacterium sp.* clones. The band running at the bottom of the gel, an *uncultured Arthrobacter sp.* clone (band 115) did not display a band in the original fingerprint of sample PF8-1.

On the eighth day of the treatment the twelve obtained clones from sample PF8-2 were all members of the *Firmicutes* and the *β-Proteobacteria*. The dominant band at the upper position of the gel matched with clone 118 that was identified as an *uncultured Planococcus sp.* clone. Further clones were related to *uncultured Planococcaceae sp.* clones (bands 116, 119 and 120) and also to a cultured *Planococcaceae bacterium* (band 117). Slight under these faint bands, a clone (band 121) showing no band in the original DGGE profile was identified as an *uncultured bacterium clone*. 16S rDNA sequences of clone inserts 122-124 were related to *uncultured Comamonas sp.* clones. As in the profile from the first day, at the bottom of the acrylamide gel three 16S rDNA sequences could be identified as *uncultured Exiguobacterium sp.* clones (bands 125-127).

4.5. Detection of the inoculated *M. xanthus* during the time course experiment

To monitor the prevalence of the inoculated strain on sectors treated with the *M. xanthus*-inoculated culture (sectors B and E) two different molecular methods were used: First, PCR-DGGE analysis of amplified 16S rDNA, directly extracted from treated stone slabs and from pellets derived from enrichment cultures of treated samples, using universal bacterial primers. The amplified 16S rDNA of *M. xanthus* (strain 422) was loaded on the DGGE-gel as positive control (line M.x. of Figure 21 and Figure 22). No DGGE-band corresponding to the DNA of *M. xanthus* could be clearly identified on the profiles derived from treated samples during the short- and

long-term monitoring (see Figure 21 A-B, Figure 21 B-B and Figure 22 A-E, Figure 22 B-E). Furthermore, the screening of clone libraries derived from samples of sectors B and E did not yield positive results for sequences related to this microorganism. As a consequence, a second molecular method was applied for a more sensitive detection of *M. xanthus.* With this purpose, species-specific primers, which allow the identification of the targeted microorganism were chosen to specifically amplify the *frz* locus of *M. xanthus*. The primer pair Frz799/Frz1147 - amplifying a 349 bp fragment of the *frz*ABCD gene - was selected. Amplified *M. xanthus*-DNA with primers Frz799 and Frz1147 was loaded on the 2% agarosegel as positive control (see Figure 23). This molecular method allowed the detection of *M. xanthus* in pellet samples PB1 and PB8 (both from sector B) from San Jéronimo as well as in pellet sample PE1 derived from sector E from the Royal Hospital. All these samples were taken in the first week of the treatment. It is worth noting that the detection of *M. xanthus* was not possible for a long-term period of time

5. Discussion

Rodriguez-Navarro et al., 2003 and Jimenez-Lopez et al. (2007; 2008) used a sterile culture medium or a medium inoculated with *Myxococcus xanthus* for the consolidation of ornamental stones under laboratory conditions. Results of these studies showed that despite the presence/absence of *M. xanthus*, there is an activation of the inhabiting bacteria that have the potential to produce new calcium carbonate on the stone. Piñar and co-workers (2010) investigated the effects of the application of a *M. xanthus*-inoculated culture on the micro-biota inhabiting non-sterile limestones, as a conservation treatment. Furthermore, authors monitored the prevalence of the inoculated strain for a period of a month by using a molecular strategy

As a pursuing study, a first *in situ* application, to test the conservation treatments mentioned above, was performed onto two Spanish buildings which date back to the 16th century. Both buildings are built of calcarenite stone extracted from the same quarry (Escúzar, Granada), are located in the same city and therefore underlie the same climatic conditions. The selected areas on the buildings received three different applications: (i) treatment with sterile water as a control; (ii) treatment with a *Myxococcus xanthus*-inoculated culture, and (iii) treatment with a sterile M-3P nutritive solution.

In order to evaluate the impact of the conservation treatments mentioned above on the autochthonous microbial community inhabiting the stones, in the present study we perform a reliable molecular monitoring of the bacterial community dynamics occurring during and after the application of such treatments. First, a short-term monitoring of pellets gained from enrichment cultures conducted during the first week of the treatment, to detect which bacteria are quickly activated by the application of the three different treatments, and second, a long-term monitoring (after 6 and 12 months) to infer the impact of the treatments on the microbial communities remaining on the stones after this long period of time.

5.1. Identification of the autochthonous micro-biota on non-treated stone

Comparing the autochthonous micro-biota of non-treated stone from both buildings, the DGGE fingerprints obviously showed a far more complex community structure of the Royal Hospital of Granada, than that of the Monastery of San Jerónimo.

On the non-treated stone from the Monastery of San Jerónimo six clones were sequenced from the clone library and the associated 16S rDNA sequences were related to microorganisms belonging to two phyla, namely the *Firmicutes* and the *Proteobacteria* (α-, β- and γ-subdivisions). In the case of the Royal Hospital eighteen harvested clones could be grouped to the *Firmicutes*, the *Proteobacteria* (with the same subdivisions) and into an additional phylum, the *Actinobacteria*. Except *Delftia* and *Xanthomonas*, all identified genera from the Monastery could be retrieved on the non-treated stone from the Royal Hospital. Further ten genera were newly found on this building, namely *Pseudomonas*, *Streptococcus*, *Neisseria*, *Pseudoalteromonas*, *Brevibacillus*, *Enterobacter*, *Micrococcus*, *Kocuria* and *Corynebacterium*. The greater amount of detected bacteria on the Royal Hospital can be explained by the location of the treated area. The ground floor situated sectors are more exposed to environmental influences, as traffic, animals, humans, et cetera, and therefore the possibility and the availability of additional nutrients, supporting the growth of different microorganisms is increased.

From the Monastery species belonging to the *Proteobacteria* (n=percentage of isolated clones belonging to this phylum, n=83.3%) dominated on the stone sample, but also one member of the *Firmicutes* (n=16.7%) could be isolated. This clone sequence, derived from DGGE band 1, affiliated with the *Bacillales*, family *Planococcaceae*, namely *Bacillus* (synonym: *Solibacillus*). It showed N-

acylhomoserine lactone-degrading capacities and could be isolated from the leaf surface of *Solanum tuberosum* (Morohoshi et al., 2009). All other detected clones could be associated with the *α-, β-* and *γ-Proteobacteria* subdivisions and 16S rDNA sequences derived from clone inserts were related to mostly cultured bacterial strains, namely *Agrobacterium*, *Comamonas* and *Delftia*. Representing the α-subdivision of the *Proteobacteria* and belonging to the *Rhizobiaceae* family, the detected *Agrobacterium* (n=16.7%; band 4) was linked to root nodules of *Pueraria lobata* Ohwi in China Three clone sequences (bands 2, 5 and 6) revealed high similarities to the family *Comamonadaceae*, namely to the genus *Comamonas* and to *Delftia*. Both *Comamonas sp.* (n=33.3%; DGGE bands 5 and 6) were isolated from soil in China and one of them showed phenol-degrading activities. The identified *Delftia sp.* (n=16.7%; band 2) was previously described from soil material in China and further characterization proved its potential to degrade aniline. Partial ribosomal sequence of DGGE band 3 displayed highest similarities to an *uncultured Xanthomonas* clone. The only representative of the *γ-Proteobacteria* was isolated from a sewage sample from the Shengli Oilfields in China.

The more complex DGGE profile of the non-treated stone from the Royal Hospital also reflected in the number of isolated clones and their affiliations to three different phyla, namely *Firmicutes* (n=44.4%), *Proteobacteria* (n=38.9%) and *Actinobacteria* (n=16.7%). The bands 1 and 2 were both highly similar to *uncultured bacterium isolates*, and also associated with other representatives of the *Firmicutes*. These, yet not further characterized bacteria were isolated from indoor dust (Rintala et al., 2008) in Finland and from a molasses-feeding sulphate-reducing bioreactor in China. Belonging to the family *Streptococcaceae*, namely *Streptococcus* (Schlegel et al., 2003), band 4 was identified due to high 16S rDNA sequence similarities. The sequence corresponding to DGGE band 10 was phylogenetically related to the *Paenibacillaceae* family, namely *Brevibacillus*. Further four members (bands 8, 9, 11 and 12) of the *Firmicutes* showed relatedness to the *Bacillaceae*, namely to the genus *Bacillus*. The first two Bacilli were isolated from anthropogenic soil in Sicily, the third was connected to a pharmaceutical product contaminant from Rio de Janeiro, and the forth *Bacillus sp.* was originally identified as an antibiotic-active bacteria associated with *Saccharina latissima* from the Baltic Sea (Germany) (Wiese et al., 2009). As the only member of the *α-Proteobacteria*, namely *Agrobacterium*, clone 13 could be isolated from Lespedeza root nodules in South Korea. Two 16S

rDNA sequences of clone inserts (bands 5 and 14) were characterized as members of the *β-Proteobacteria*, families *Neisseriaceae* and *Comamonadaceae*, namely *Neisseria* - which was previously detected in urban wastewater treatment plants in Portugal - and *Comamonas* (Ma et al., 2009). The most dominant subdivision of the phylum *Proteobacteria*, the *γ-Proteobacteria*, was represented by four clone sequences (bands 3, 6, 7 and 15). DGGE band 3, had the highest similarities in the database search to the *Pseudomonadaceae*, namely *Pseudomonas*, isolated from ocean water. To the family *Pseudoalteromonadaceae*, two *Pseudoalteromonas sp.* were grouped due to their 16S rDNA sequence similarities (bands 6 and 7). Clone 15, the third member of this subdivision, was characterized belonging to the *Enterobacteriaceae*, namely *Enterobacter*. This microorganism, showing antibacterial activity, was isolated from soil in Pakistan. The phylum *Actinobacteria* was represented by three sequences of isolated clones (bands 16-18). The partial ribosomal sequences matched with the families *Micrococcaceae* (*Micrococcus* and *Kocuria*), and *Corynebacteriaceae* (*Corynebacterium*). The *Micrococcaceae* members were isolated as a seedborne bacterial endophyte from *Phaseolus vulgaris* in Mexico, and as an extremely tolerant bacterium from a clean-room floor of the Kennedy Space Center in the United States of America. The detected *Corynebacterium sp.* was associated with a tortoise.

5.2. Short-term monitoring: microorganisms actively growing during the first week of the treatment.

A. Monastery of San Jéronimo

Sector A – Samples PA1 and PA8

On the first day in the pellet samples from sector A, treated with sterile water, three 16S rDNA sequences were identified that showed sequence similarities to cultured bacteria: Two members of the *Bacteroidetes* (n=28.6%), namely *Sphingobacterium* (band 55) and *Flavobacterium* (band 56) and one representative of the *α-Proteobacteria* (n=71.4%; *Brevundimonas*) (band 57). The *Sphingobacterium sp.* was previously isolated as a psychrotrophic bacterium from raw milk (Hantsis-Zacharov et al., 2007), whereas the *Flavobacterium sp.* was characterized as an arsenic resistant microorganisms from the Ascotan Lake in the Atacama wetland, Chile. DGGE band 57 matched in the BLAST search with a *Brevundimonas sp.* that was derived from

the Aran-Bidgol, a hypersaline lake in Iran. Further four clones were characterized from this sample that affiliated to clone sequences derived from other pellet samples, respectively from the non-treated stone of San Jerónimo or the Royal Hospital (for details see Table 11 B). These 16S rDNA sequences were all clustered into the α-subdivision of the *Proteobacteria*, namely *Brevundimonas*, respectively *Rhizobiaceae* (bands 58-61).

On the eighth day, from the enrichment cultures nearly all isolated clone sequences were related to already found 16S rDNA sequences of clones from other pellet samples or from non-treated stone of both buildings. Clustering the clones into the phyla *Proteobacteria* (n=85.7%) and *Firmicutes* (n=14.3%), the sequences could be associated to the genera *Comamonas* (bands 62 and 68), *Bacillus* (band 63), *Delftia* (bands 64, 65 and 66) and *Rhizobiaceae* (band 67). 16S rDNA sequence of one clone insert was associated with an *uncultured Bacillus sp.* (band 63) derived from a quarry stone sample from Granada, Spain (Piñar et al., 2010). This quarry is the same as which the stones investigated in the present study originated from.

The application of water onto the non-treated stone activated bacteria belonging to the *Bacteroidetes* on the first day. Members of the *β*- and *γ-Proteobacteria*, which could be found on the non-treated stone, gave way to representatives of the α-subdivision of this phylum. During the first week of the treatment the dominance of the phylum *Proteobacteria* was observed. The members of the *Bacteroidetes*, detected on the first day, vanished again till the eighth day and instead a *Firmicutes* representative emerged at the end of the week. Members of the *β-Proteobacteria*, not detected on the first day, were activated again on the eighth day and then dominated over the *α-Proteobacteria*.

Sector B – Samples PB1 and PB8

Species belonging to the *Firmicutes* (81.8%) dominated on the first day over two representatives of the phylum *Actinobacteria* (18.2%). These two isolated clone sequences were identified as an *Arthrobacter sp.* (Loveland-Curtze et al., 1999; band 75) and an *uncultured Kocuria sp.* clone (band 79), which had the closest phylogenetic relatedness to an *Actinobacteria* clone isolated from the non-treated stone of the Royal Hospital. Nine partial ribosomal sequences were grouped to the *Firmicutes*. Four cultivated relatives could be found, belonging to the families *Bacillaceae* and *Planococcaceae*, namely two *Bacillus sp.* (bands 71 and 73), one

Planomicrobium sp. (band 74) and a *Solibacillus sp.* (band 78). The members of the *Bacillaceae* associated with the necrotic tissue of farmed *Apostychopus japonicus* and with a soil sample from euphractica forest of Tarim River Valley in China. One of the cultivated *Planococcaceae* was isolated from a glacier (Zhang et al., 2007; band 74) and the other showed to have N-acylhomoserine lactone-degrading capacities (Morohoshi et al., 2009). DGGE bands 69, 70, 72, 76 and 77 had the highest similarity ranking with cloned sequences from the EMBL database. Thereof band 70 was related to an *uncultured bacterium clone*, derived from argali sheep feces in Mongolia (Ley et al., 2008), whereas the resting four *Fimicutes* clones (belonging to the *Planococcaceae* and *Bacillaceae*) revealed closest similarities to sequences from other pellet samples, respectively non-treated stone, identified in this study.

At the end of the week only three clones (bands 80, 81 and 82) were isolated from sector B, all belonging to the *β-Proteobacteria* (n=100%) and all of them were closely related to sequences from other pellet-, respectively non-treated stone samples of this study.

Short time monitoring on this sector showed that all members of the *Proteobacteria* from the non-treated stone vanished through the application of a *Myxococcus xanthus* inoculum. Instead dormant *Bacillales* members were activated and dominated on this sector on the first day. In addition, representatives of the *Actinobacteria*, not detectable on the non-treated stone, also emerged on the first day. Till the eighth day a dramatic shift, also visible in the DGGE profiles of both samples (Figure 21 B), could be observed. This change led to the complete disappearance of the *Firmicutes* and the *Actinobacteria*. The *β-Proteobacteria*, namely *Comamonas sp.* became activated again and dominated on this sector.

Sector C – Samples PC1 and PC8

Generally, samples from sector C showed a very simple community fingerprint in the DGGE. This presumption was assured by the little amount of different clones that could be obtained from both samples. On the first day two cultured members of the *Firmicutes* (n=66.7%), associated with the family *Bacillaceae*, and one cloned sequence of the *α-Proteobacteria* (n=33.3%) was detected. Clones 83 and 84 were phylogenetically related to different *Bacillus sp*. that were isolated from Arctic seawater (band 83) and as a heterotrophic nitrifying bacteria from a shrimp culture

pond in India. The identified *uncultured Rhizobiaceae bacterium* clone (band 85) had the closest similarity to a clone sequence of a pellet sample derived from sector A.

Seven days later the inhabiting micro-biota of sector C consisted of members of the *Firmicutes* (n=75%) and the *β-Proteobacteria* (n=25%). Associated with the *Proteobacteria* was the 16S rDNA sequence of DGGE band 86, that was previously described by Schabereiter-Gurtner et al. 2004 and was isolated from Paleolithic paintings and surrounding rock walls in two Spanish caves (Llonin and La Garma). One out of the three *Bacillales* members affiliated with a cultured *Solibacillus* (band 88) with N-acylhomoserine lactone-degrading activity (Morohoshi et al., 2009). Two clones (bands 87 and 89) showed affiliations to clone sequences of pellet samples from sector B.

The microbial development on this sector, treated with the nutritive solution, showed a slight dominance of members of the *Firmicutes* over the whole week. Before the application of the treatment this phylum was only represented by one clone sequence and the later application of the M-3P media activated this group of microorganisms. During the whole week, bacteria belonging to the *Proteobacteria* could be found, but compared to the non-treated stone their total number decreased by 80%. In contrast to the other sectors, no new group of microorganisms different from those already detected on the non-treated stone was activated by the media.

As mentioned in the Introduction section, Jroundi et al. (2010) performed a parallel investigation, by using conventional cultivation assays, of the micro-biota activated on all treated sectors at the Monastery of San Jerónimo during the first week of the application of the treatments. Comparing the results obtained in the present study, concerning the molecular analyses of pellets samples of the Monastery of San Jerónimo, with those obtained by Jroundi et al. (2010), that is, molecular versus conventional cultivation techniques, it is worth to note that at the family level 60% of the cultured microorganisms could also be detected with molecular techniques. Species of seven cultivated genera could not be detected by molecular means in the pellet samples, namely *Sphingomonas*, *Diaphorobacter*, *Variovorax*, *Acidovorax*, *Stenotrophomonas* and *Pseudomonas*. Nevertheless, two new genera, not isolated by using cultivation assays, namely *Sphingobacterium* and *Flavobacterium* were detected by molecular techniques in this study. Jroundi et al. (2010) isolated a total of 116 bacterial strains, which were tested on solid M-3P medium for their ability to precipitate $CaCO_3$. Except three strains, 113 isolates apparently showed a high

capacity to produce calcium carbonate after two days of incubation. These results proved that the application of the conservation treatments boosted the growth of calcium carbonate producing bacteria. Due to the similarities between the sequences obtained from isolated strains (Jroundi et al, 2010), and our sequenced clones obtained by culture-independent techniques, it is reasonable to postulate that the microorganisms identified by molecular methods are also able to produce calcium carbonate.

B. Royal Hospital of Granada

Sector D – Samples PD1-1 and PD8-1

Sector D was divided into two subsectors, and both received the same treatment with sterile water. From both subsectors DNA was extracted and analyzed by DGGE. The fingerprint profiles from both subsectors showed the same band migration on the first and on the eighth day of treatment (data not shown). To prove, that the inhabiting micro-biota of the subsectors is identical, clone libraries were constructed with enrichment cultures from both sampling points. 16S rDNA sequencing and comparison with databases identified clones, having the same band motility in the acrylamide gel, belonging to the same genera. Therefore it was assumed that samples derived from subsectors which received the same treatment – as samples PD1-1 and PD1-2, respectively PD8-1 and PD-8-2, and showing the identical DGGE profiles, inhabited the same microorganisms.

On the first day of the water treatment eight isolated clones were all related to the phylum *Proteobacteria* (n=100%). Five 16S rDNA clone inserts were associated to cultured members of the *γ-Proteobacteria*, namely *Pseudomonas* (bands 65, 66, 69, 70 and 71). Sequence corresponding to DGGE band 65 was isolated from oil yielded water in China and showed hydrocarbon-degrading activity. Clone 66 was linked to a cultured *Pseudomonas sp.* extracted from human sputum. To strains isolated from a clinic in India, respectively from sugar beet molasses in Iran, sequences of bands 70 and 71 were most affiliated. One sequence affiliated with an *uncultured Brevundimonas sp.* (band 72), thus representing the only member of the α-subdivision of the *Proteobacteria*. The isolated clone showed similarities to a clone detected in a pellet sample from sector E of this building (for details see Table 12 B). Further two sequences were phylogenetically identified as *uncultured bacterium clones* (bands 67 and 68), also matching with sequences from other *γ-*

Proteobacteria. They were previously isolated from human tracheal aspirates of intubated patiens, who received an antibiotic treatment (Flanagan et al., 2007).
On the eighth day only one clone represented the *γ-Proteobacteria* and showed relatedness to a cultured bacterium strain, namely *Acinetobacter* (Ibrahim et al., 1997; band 73). All other eight isolated sequences were closer related to clone sequences derived from samples of this study (see Table 12 B). They affiliated with the genera: *Rhizobiaceae* (band 74), *Delftia* (band 75), *Comamonas* (bands 76 and 78) and *Exiguobacterium* (bands 77, 79, 80 and 81). In summary, the most abundant phylum was *Proteobacteria* (n=55.6%) followed by *Firmicutes* (n=44.4%).
The application of sterile water highly stimulated the growth of *Pseudomonas sp.* (*γ-Proteobacteria*) on the first day. Even though this genus was already present on the non-treated stone, its dominance on the first day was enormous. Additionally only uncultured members of the *Proteobacteria* were detected. Intriguingly, the broad range of detected microorganism from the non-treated stone disappeared and gave way to the *γ-Proteobacteria*, who dominated at this time point. Till the eighth day the dominance of the *Proteobacteria* remained, with the occurrence of members from the other subdivisions of this phylum, but also members of the *Firmicutes* were strongly activated.

Sector E – Samples PE1 and PE8
From the sector treated with the *M. xanthus*-inoculated culture four clone sequences were affiliated to 16S rDNA sequences from other pellet-, respectively non-treated stone samples of the two investigated buildings of this study. Comparison with the database allowed a clustering of the clone sequences into the phylum *Proteobacteria* (n=100%). Thereof the subdivisions *β-* and *γ-Proteobacteria* were represented by the genera *Delftia* (bands 82, 83 and 84) and *Stenotrophomonas* (band 85).
At the end of the week the detected bacterial community of this sector was represented by members of the *Proteobacteria* (n=71.4%), the *Actinobacteria* (n=14.3%) and the *Firmicutes* (n=14.3%). As on sector D, the *α-*, *β-* and *γ-* subdivisions of the *Proteobacteria* also dominated on this sector. 16S rDNA sequences of DGGE band 87, associated with the only cultured bacterium from this sector, namely *Brevundimonas*. This strain was isolated from a hypersaline lake (Aran-Bidgol) in Iran. The remaining clone sequences were closest related to sequences from other sectors, being characterized as the following genera:

Arthrobacter (band 86), *Stenotrophomonas* (band 88), *Comamonas* (bands 89, 90 and 91) and *Exiguobacterium* (band 92).

The community dynamics on this sector showed that the application of *M. xanthus*-inoculated culture led to a stronger activation of members of the *Proteobacteria*. This phylum dominated on the first day, whereas all other genera, detected on the non-treated stone vanished. This dominance remained till the end of the week. Additionally the evolvement of members from the other subdivisions of the *Proteobacteria* and also of the phyla *Firmicutes* and *Actinobacteria* was detected.

Sector F – Samples PF1-1, PF1-2, PF8-1 and PF8-2

As already observed on the other two sectors of this building, on the first day the phylum *Proteobacteria* dominated on both subsectors, F-1 and F-2. On sector F-1, nine out of ten isolated clone sequences were related to the α- and γ-subdivisions of this phylum (n=90%). Thereof four 16S rDNA sequences correlated to cultured bacteria, namely *Pseudomonas sp*. DGGE band 93 was associated with a strain isolated from rhizosphere soil. Phylogenetically related to a strain isolated from activated sludge of sewage treatment plant in China was the sequence of clone 95. The 16S rDNA inserts of clones 96 and 99 showed similarities to cultured *Pseudomonas sp.* The second isolate was further characterized as heterotrophic nitrifying bacteria, derived from the sediment of a shrimp culture pond in India.

Five sequences showed close relatedness to clones identified on other sectors of these buildings. They were grouped to the genera *Pseudomonas* (band 94), *Brevundimonas* (band 98 and 101) *Stenotrophomonas* (band 100) and *Rhizobiaceae* (band 102). One member of the *Firmicutes* (n=10%), belonging to the family *Bacillaceae* could be found in the pellet. The cultured *Bacillus* strain, matching with band 97, was previously described from a hypersaline lake (Aran-Bidgol, Iran).

On the other subsector, F-2, a complete dominance of the *Proteobacteria* (n=100%) was observed. Four of the five isolated clone sequences were associated with clones already described in this study. Clustered into the subdivisions *β*- and *γ*-*Proteobacteria*, the partial ribosomal sequences were grouped into the genera *Delftia* (bands 103 and 104) and *Stenotrophomonas* (bands 105, 106 and 107). Clone 105 showed sequence similarities to an *uncultured Stenotrophomonas sp.*, isolated from an environmental soil sample from the Pindari Glacier of Himalaya, India.

The microbial diversity found on the subsectors on the eighth day was higher than the one observed on the first day. From sector F-1, bacteria belonging to the *Firmicutes* (n=75%), *β-Proteobacteria* (n=12.5%) and *Actinobacteria* (n=12.5%) were detected. The isolated sequences associated with the *Proteobacteria* (*Comamonas*; band 111) and *Actinobacteria* (*Arthrobacter*; band 115) had high similarity rankings with clone sequences of other pellet samples, respectively with the non-treated stone samples of the Monastery of San Jerónimo or the Royal Hospital. Four *Firmicutes* members also showed relatedness to clone sequences derived from this study, namely band 109 (*Planococcaceae*) and bands 112-114 (*Exiguobacterium*). 16S rDNA analysis revealed cultured members of the *Bacillales* as closest relatives of the sequence corresponding to DGGE band 108 (*Exiguobacterium sp.*), which was isolated from sea water of the North Western Mediterranean Sea in France. Clone 110 was related to a *Planococcus sp.* that was derived from a cold dessert of Northern Himalaya, India.

On the other subsector, F-2, representatives of the *Firmicutes* (n=75%) dominated over the *β-Proteobacteria* (n=25%). Three clone inserts (bands 122, 123 and 124) were phylogenetically related to *uncultured Comamonas sp. clones*, detected in other sectors of the two buildings. From the nine isolated *Firmicutes* species, seven 16S rDNA sequences, grouped to the genera *Planomicrobium* (bands 116 and 119), *Planococcus* (bands 118 and 120) and *Exiguobacterium* (bands 125, 126 and 127), were all associated with clones already found in this study. As related to an *uncultured bacterium clone* that was isolated from a marine sediment of the Antarctica (Rodrigues et al., 2009), clone 121 could be identified. Clone 117 harbored a sequence that had the highest similarity match to a cultured *Planococcaceae* bacterium. This strain was isolated from a soil sample of North Delhi, India.

Summing up, the application of the M-3P media during the first week of the treatment on both subsectors, visibly activated the growth of bacteria belonging to the *Proteobacteria*. Representatives of all other phyla detected on the non-treated stone could not be found on the first day. During the week the micro-biota on the stone further shifted, leading to an attenuation of the *Proteobacteria* members and instead the *Firmicutes* increased dramatically and took the dominant position in the enrichment cultures. Interestingly, despite the different DGGE profiles from samples of the two subsectors, the isolated clones of the different subsectors correlated with

each other on the family- and partly on the genus level. Thus the matching results of the subsectors and it was possible to describe a similar community development during the first week of the treatment.

C. Comparison of the micro-biota detected in the pellet samples from both buildings

In the pellet samples from the Monastery of San Jerónimo 35 different clones could be isolated from six clone libraries, being grouped into thirteen different genera. Out of nine clone libraries performed with samples from the Royal Hospital of Granada 63 clone sequences, clustered into thirteen different genera were detected (see Table 13). Generally on both buildings members of the phyla *Proteobacteria*, *Firmicutes* and *Actinobacteria* were observed. Members of the *Bacteroidetes* could only be isolated from the Monastery of San Jerónimo. Twenty-one characterized 16S rDNA clone sequences from the Monastery showed the highest relatedness to clone sequences derived from the non-treated stone or from pellet samples of other sectors of one of the two buildings. In the case of the Royal Hospital forty-four such sequences were identified. The occurrence of the same clone sequences on different sectors showed that despite the different applications on the stone the microorganisms inhabiting it were the same. It also showed that the different treatments on the buildings led to similar microbial communities inhabiting the stone on the different sectors. Table 13 gives a broad overview of the detected genera and the relative amount of the observed clones sequences from the pellet samples of both buildings.

5.3. Long-term monitoring: Identification of the remaining microorganisms on treated stone

A. Monastery of San Jerónimo

Sector A – Sample SA6

Six months after the applications of the sterile water on sector A, clones harvested from the stone sample affiliated with the phyla *Proteobacteria* (n=58.3%), *Firmicutes* (n=25%) and *Bacteroidetes* (n=16.7%). Two clone sequences showed to be related to *uncultured Bacteroidetes sp.* (bands 7 and 9). Thereof clone 9 corresponded to a sequence isolated from raw milk, whereas clone 7 was associated to a 16S rDNA

sequence detected on sector B after six months. The three observed *Firmicutes* representatives also showed the highest similarity matches to clone sequences identified from the two buildings investigated in this study, namely to the genus *Bacillus* (bands 10 and 14) and to a *Planococcaceae* member (band 13). Clones being phylogenetically closest related to the *Proteobacteria*, were distributed to the α-, β- and γ-subdivisions of this phylum. The only representative of the α-subdivision (*Rhizobiaceae*), clone 16, was characterized as a relative of clone sequences derived from other samples examined in this study. Clustered to the β-group, the genera *Delftia* (band 15) and *Comamonas* (bands 17 and 18) were found, also matching with 16S rDNA sequences of previously identified clones of both buildings. Out of the three detected *γ-Proteobacteria*, two clone inserts (bands 11 and 12) had high sequence identities to *uncultured Pseudomonas sp. clones*. Sequence corresponding to DGGE band 12, was related to clone 11, which was previously described by Piñar et al. in 2010 and was derived from decayed ornamental porous limestone in Granada. One clone sequence affiliated to a cultured *Pseudomonas sp.* (band 8). This strain was isolated from the anoxic sub-surface of mud breccia from the Capt. Arutyunov mud volcano in the Gulf of Cadiz, Spain.

Identified clone sequences and also the DGGE profile of the sample taken after half a year showed that the water treatment did not change the biodiversity of the micro-biota inhabiting the stone. The detected microorganism belonged to the same phyla and the same genera as those found on the non-treated stone and on the pellet samples from the first week. In summary, the bacterial diversity on the stone treated with water remains stable, however, compared to the non-treated stone additional bands in the DGGE fingerprint were visible and more clone sequences (100% more) were obtained from the clone libraries.

Sector B – Samples SB6 and SB12

After six months, the identified clone sequences of the micro-biota inhabiting the stone of sector B, treated with a *M. xanthus*-inoculated culture, could be associated with four different phyla, namely *Firmicutes* (n=50%), *Proteobacteria* (n=30%), *Actinobacteria* (n=10%) and *Bacteroidetes* (n=10%). Comparison of the obtained 16S rDNA from clone inserts with databases showed the relatedness of all *Firmicutes* members to the family *Bacillaceae*, namely *Bacillus*. Thereof only the sequence of clone 25 was related to a cultured strain, isolated from biodeteriorated mural

paintings in the Servilia tomb (Necropolis of Carmona, Seville, Spain) (Heyrman et al., 2001). The other four partial 16S rDNA sequences (bands 20, 22, 23 and 24) had higher similarities to *uncultured Bacillus sp.* which were derived from this study. Equally the representatives of two *Proteobacteria*, showed affiliations to sequences obtained during this project. The associated genera were *Rhizobiaceae* (band 26) and *Comamonas* (band 27). An *uncultured Pseudoalteromonas* sp. clone (band 21) was derived from the Bering Sea. As closest related to an *uncultured Flavobacteriaceae bacterium* clone from the non-treated stone of San Jerónimo, DGGE band 19 could be identified. Belonging to the *Actinobacteria*, clone 28 revealed high similarities with a cultured *Corynebacterium sp.* (Pascual et al., 1995).

After one year of monitoring, all nine detected clones on this sector turned out to be related to the phylum *Firmicutes* (n=100%), family *Bacillaceae*, genus *Bacillus*. Sequences of DGGE bands 32, 34 and 36 had the best match in the database search with uncultured clone sequences derived from the Monastery of San Jerónimo. Clone 29 was related to a cultured *Bacillus sp.* derived from soil of Dokdo island. The cloned 16S rDNA sequence from DGGE band 30 was associated with the sponge *Petrosia ficiformis* collected along the North Western Mediteranean Sea. 16S rDNA sequences of two *Bacillus* strains isolated from deep-sea hypersaline anoxic sediments of Brine Lake (Sass et al., 2008), correlated with the clone inserts of bands 31 and 37. A Manganese (II)-oxidizing *Bacillus sp.* from hydrothermal sediments and plumes in Guaymas Basin (Dick et al., 2006) was the next relative of clone 33. Phylogenetically affiliated to a *Bacillaceae* strain extracted from the organics- and methane-rich Core sediments of Shimokita Peninsula in Japan (Kobayashi et al., 2008) was the sequence of clone 35.

In summary, in this sector a slight shift in the microbial community present on the stone occurred during the first six months. Beside nearly all genera found on the non-treated stone, additional members of the *Firmicutes*, as observed in the pellet samples, could be detected. This phylum dominated at this point of time on the stone (50% of the detected clones), whereas the number of identified *Proteobacteria* decreased (from 83.3% to 30%). Additional representatives of the phylum *Actinobacteria*, already detected in the pellet samples, could be isolated after six months. Newly activated/detected was a member of the *Bacteroidetes*, which was not prior, found on this sector. After one year, the micro-biota on this sector further shifted, leading to the complete dominance of the phylum *Firmicutes*.

Representatives of all previously detected phyla disappeared and interestingly only members of the *Bacillaceae* family were observed (100% of the detected clones).

Sector C – Samples SC6 and SC12

Half a year after the application of the sterile nutritive media on this sector all isolated clone sequences were affiliated to the phylum *Firmicutes* (n=100%). Clone inserts of DGGE bands 38, 39 and 40 were related to cultured *Bacillaceae*, genus *Bacillus*, respectively *Virgibacillus*. 16S rDNA sequence of clone 38 associated with a halotolerant *Bacillus sp.* that contains L-lysine in its cell wall was isolated from a salt mine in South Korea (Lee et al., 2006). The two *Virgibacillus sp.*, related to bands 39 and 40, were isolated from biodeteriorated mural paintings in the Servilia tomb (Necropolis of Carmona, Seville, Spain) (Heyrman et al., 2001 and 2003). The remaining seven clone sequences detected on this sector were all grouped into the family *Planococcaceae* according to the sequences similarities in the BLAST search. All clones (bands 41-47) showing different band migration in the acrylamide gel were close related to sequences derived from this building, partly also matching with each other.

After one year the phylum *Firmicutes* (n=85.7%) remained dominant on this sector. One member of the β-subdivision of the *Proteobacteria* (n=14.3%), family *Comamonadaceae*, genus *Comamonas* emerged. Six out of seven identified 16S rDNA sequences from this sector showed the highest affiliation to cloned sequences derived from isolates of the two investigated buildings in this study. Clones 48-51 were associated with the genera *Planomicrobium*, clone 53 with *Bacillus* and clone 52 was related to *Comamonas*. Clone 54 was phylogenetically related to an environmental sample from Italy. This *uncultured Bacilli* bacterium was isolated from oxic rice field soil (Lueders et al., 2004).

This sector showed a great microbial shift after the application of the treatment. On the non-treated stone of this building a dominance of the phylum *Proteobacteria* (83.3%) was detected, who continually disappeared throughout the time course of the experiment. In the pellet samples the number of the found *Proteobacteria* already decreased (from 33.3% on the first day to 25% on the eighth day), whereas the *Firmicutes*, only present at 16.7% percent in the non-treated stone sample, showed up. After six months of the treatment 100% of the detected sequences showed to be related to the *Firmicutes.* The dominance of the *Firmicutes* phylum remained stable

after one year of the treatment, when nearly all found clones (85.7%) were associated with this phylum. Only one representative of the *Proteobacteria*, which was already found on the non-treated stone, emerged again.

B. Royal Hospital of Granada

Sector D – Sample SD12

From sector D, treated with sterile distilled water, 20 clones could be isolated after one year. Database comparisons grouped the sequences into the phyla *Proteobacteria* (n=60%), *Firmicutes* (n=25%) and *Actinobacteria* (n=15%). The identified ribosomal sequences associated to cultivated bacterial strains as well as to uncultured clone sequences. Matching with the α- subdivision, clone 25 was associated with the family *Sphingomonadales*, namely *Porphyrobacter*, isolated from sea water (Yoon et al., 2006). Also grouped into this subdivision, 16S rDNA sequence of band 27 was related to the family *Bradyrhizobiaceae*, namely *Bosea* (Dadhwal et al., 2009). The third member of the *α-Proteobacteria* closest affiliated to an *uncultured Rhizobiaceae bacterium* clone (band 33) detected in pellet samples and also on the Monastery of San Jerónimo. Six sequences of clone inserts were clustered into the β-subdivision, namely clones 19, 24, 26, 30, 32 and 34. The associated genera were *Bacterium J10*, *Delftia*, *Hydrogenophaga* (Kämpfer et al., 2005), *Thiobacillus* (Kelly et al., 2000) and *Methylobacterium* (Knief et al., 2008). Phylogenetic analysis assigned the sequences of clones 20, 22 and 31 to the *γ-Proteobacteria*, namely to *Pseudomonas* (bands 20 and 22) and *Stenotrophomonas* (band 31). The identified *Pseudomonas sp.* were related to Spacecraft clean rooms, respectively to a fluoranthene-degrading bacteria isolated from long-term petroleum-wastewater-irrigated paddy fields. Detected on forest soil was the related *Stenotrophomonas sp.* of clone 31. Four of the five detected *Firmicutes* members showed affiliations to uncultured clone sequences derived from this study. The clone sequences were grouped into the family *Planococcaceae* (bands 23, 28 and 35) and to the genus *Exiguobacterium* (band 36). Sequences corresponding to the insert of clone 29 had as the closest relative a cultured *Bacillus sp.* isolated from a landfill site near a contaminated stream in Gorwa Industrial Estate, Baroda, Gujarat, India (Desai et al., 2009). Belonging to the *Actinobacteria*, from the sequences of the clones 21, 37 and 38, only band 37 affiliated with a cultured member of this phylum, namely *Brachybacterium* isolated from the Etruscan tomb of Mercareccia (Tarquinia, Italy).

Related to *uncultured Actinobacteria* clones were sequences of bands 21 and 38. Both associated to ancient cultural heritages: One clone sequences was derived from a natural shelter containing prehistoric paintings (Portillo et al., 2008), whereas the other was isolated from the Altamira Cave in Spain (Portillo et al., 2009).

Comparative sequence analysis showed that after one year on the sector treated with water, microorganisms belonging to the same phyla as detected on the non-treated stone were found again. The application of water boosted the growth of representatives of the phylum *Proteobacteria* during the first week, whereas members belonging to the *Actinobacteria* vanished. One year later the primordial micro-biota recaptured the stone again and all three originally found phyla could be detected again.

Sector E – Sample SE12

After one year of the application treatment with a *M. xanthus*-inoculted culture, sequence analyses of the obtained clones confirmed that the phylum *Firmicutes* (n=100%) was the dominant group of microorganisms on this sector. All characterized clone sequences showed affiliations to the family *Bacillaceae*, namely *Bacillus*. Thereof five clone inserts (bands 39, 40, 42, 43 and 44) had high sequence similarities to *uncultured Bacillus sp.* which were all described in this study. 16S rDNA sequences corresponding to clones 41 and 45 were related to different *Bacillus sp.*, from which one was isolated from a spacecraft assembly.

The application of the bacterial inoculum led to a shift in the very heterogeneous micro-biota colonizing the non-treated stone of the building. The *Proteobacteria* became the dominant phylum during the first week (81.8%). Representatives of the *Actinobacteria* as well as of the *Firmicutes* disappeared on the first day and were only rarely detected at the end of the week (together 28.6% of the total obtained clones). As observed in the Monastery of San Jerónimo, after one year of the treatment, all prior detected phyla, except the *Firmicutes*, completely vanished from the stone. Interestingly all characterized clone sequences were associated to the genus *Bacillus*, which was also the most dominant representative of this phylum on the non-treated stone.

Sector F – Samples SF12-1 and SF12-2

The micro-biota detected on both subsectors treated with the sterile nutritive solution was very similar to sector E. On sector F-1 all nine harvested clones showed affiliations to uncultured members of the phylum *Firmicutes* (n=100%). Except clone 50, which was related to an uncultured *Firmicutes* from glacial snow of a glacier in the Tibetan Plateau all other detected clones were related to sequences previously detected in samples of this study. Thereof the 16S rDNA inserts of clones 47-49 and 51-54 were phylogenetically grouped to the family *Planococcaceae*. Clone 46 contained a ribosomal fragment that had the best match to an *uncultured Bacillus sp.* clone.

Sector F-2 was also dominated by bacteria belonging to the phylum *Firmicutes* (n=80%). Only 20% of the detected clones showed relatedness to uncultured strains of the phylum *Actinobacteria*. Sequence corresponding to clone 55 was identified as a member of the family *Carnobacteriaceae*, namely *Desemzia*. This bacterium was originally detected in dewatered biosolids after mesophylic anaerobic digestion. Associated with cultured species of the family *Planococcaceae* were the sequences of clones 56 and 59. The cultured strains were derived from soil of Lop Nur region in Xinjiang, China, respectively from a human biopsy. 16S rDNA sequences of clones 57, 58, 60-62 showed the highest similarity ranking in the database search to *uncultured Planococcaceae clones.* The related clone sequences were all isolated from other sectors of this, respectively from the other building investigated in this project. Equally sequence of clone 64 correlated with an *uncultured Kocuria sp.* from the non-treated stone. Clone 63 matched with another uncultured representative of the *Actinobacteria*, isolated from argali sheep feces in Mongolia (Ley et al., 2008).

Sequences analyses from the detected clones on sector F showed, as on sector E, a strong dominance of the phylum *Firmicutes* after one year. The heterogeneous community from the non-treated stone vanished by the application of the M-3P media. In the pellet samples the *Proteobacteria* dominated on the first day (93.3%).Thereafter, during the first week, members of the *Firmicutes* (75%) showed up, whereas the *Proteobacteria* decreased (20%). After one year, members of the *Proteobacteria* were not detectable on the stone. As observed on sector E, the *Firmicutes* was the dominant phylum of this sector (89.5%), but in contrast to the sector treated with *M. xanthus*-inoculated culture, the characterized clone sequences were associated with the family *Planococcaceae*, instead of the genus *Bacillus*.

5.4. Comparison of the micro-biota found on the two buildings

A total of 54 clones were obtained from clone libraries conducted with the stone samples of the Monastery of San Jerónimo. 16S rDNA analysis clustered the sequences into twelve genera, belonging to the phyla *Firmicutes*, *Proteobacteria* (α-, β- and γ-subdivisions), *Actinobacteria* and *Bacteroidetes*. Sixty-four clones were obtained from the stone samples of the Royal Hospital of Granada, which showed to be associated to twenty-six genera of the phyla *Proteobacteria* (α-, β- and γ-subdivisions), *Firmicutes* and Actinobacteria. Table 14 shows a general overview of the detected genera and the relative amount of the observed clones from the stone samples of both buildings.

Out of the six genera detected on the non-treated stone of San Jerónimo, only the genus *Xanthomonas* could not further be detected in the treated stone samples of this building. Additionally to the bacterial genera inhabiting the non-treated stone, species belonging to the genera *Planomicrobium*, *Planococcus*, *Pseudomonas*, *Flavobacterium*, *Pseudoalteromonas*, *Corynebacterium* and *Virgibacillus* were activated and detected on the stone samples after 6, respectively 12 months.

In the case of the Royal Hospital of Granada, the genera, *Streptococcus*, *Neisseria*, *Pseudoalteromonas*, *Brevibacillus*, *Enterobacter*, *Micrococcus* and *Corynebacterium*, identified on the non-treated stone, were never detected again on pellet samples or treated stones of this building. Newly emerged and detected sequences from treated stone samples affiliated with the genera *Desemzia*, *Brachybacterium*, *Methylobacterium*, *Thiobacillus*, *Hydrogenophaga*, *Bosea*, *Porphyrobacter*, *Exiguobacterium*, *Planococcus*, *Planomicrobium*, *Stenotrophomonas* and *Delftia*. 16S rDNA sequences of microorganisms from six genera were found on both buildings, namely *Planomicrobium*, *Planococcus*, *Bacillus*, *Pseudomonas*, *Delftia* and *Rhizobiaceae.*

As for the pellet samples, many clone inserts derived from stone samples along the monitoring showed the highest similarities to 16S rDNA sequences of clones previously identified on the non-treated stone, in the pellet samples or in the case of San Jerónimo in the samples taken after six months. From San Jerónimo 32 clones showed to match with sequences previously detected on this building and, respectively 25 clones from the Royal Hospital showed similarities with other clones previously detected at that building. It is worth to note that only members of the genus *Bacillus* were detectable through the whole time course of the investigation on

all sectors of both buildings. Sequencing results of clones also showed that some clones detected on the Monastery of San Jerónimo could also be found on the Royal Hospital and vice versa. This might be due to the fact that the stone material from both buildings is derived from the same quarry and that both buildings are located in the same city. These results also prove that the application of the treatment leads to the establishment of a stable bacterial community mainly consisting of members of the phyla *Firmicutes* and *Proteobacteria.*

Some of the detected bacteria were spore-forming bacteria, as *Bacillus* sp*.* (Shida et al., 1996; Sneath, 1986). *Pseudomonas* sp. produces extracellular polysaccharides which provide protection against various environmental stresses, such as UV radiation, pH shifts, osmotic shock and desiccation (Roberson et al., 1992). These characteristics could explain why *Bacillus sp.*, once activated on the stone were detectable throughout the whole time course of the experiment. Due either to their ability to form spores or to produce extracellular polysaccharides they can resist dry conditions on the stones. The bacteria activated in our experiments are commonly found in natural environments and have also been found in ornamental rocks from different locations. Urzi et al. (1999) and Boquet et al. (1973) reported that members of the micro-biota inhabiting decayed stones, respectively most heterotrophic soil bacteria have a potential for calcium carbonate production. For numerous bacterial taxa, like *Bacillus* (Castanier et al., 2000; Baskar et al., 2006), *Pseudomonas* (Baskar et al., 2006) it has been shown that they are able to precipitate minerals in different media and in nature. Therefore the use of an appropriate media, like M-3P nutritive solution, can activate those bacteria and simultaneously the composition of the media limits the growths of acid-producing bacteria, which are undesirable because the acid dissolves the stone (Rodriguez-Navarro et al., 2003 and 2007).

In the first *in situ* application of the treatments mentioned in this study, Jroundi et al. (2010) showed that a significant strengthening of the stone was achieved through the treatments. Furthermore, they show that for an effective stone consolidation it is not necessary to apply an *M. xanthus*-inoculated medium, but only the sterile nutritive solution. These results agree with those from Jimenez-Lopez et al. (2007; 2008) who found that the application of an appropriate nutritional medium to non-sterilized carbonate stone slabs leads to the formation of a coherent cement of calcium carbonate produced by the inhabiting bacteria within the stone pores.

In this study the application of the *M. xanthus*-inoculated media, respectively the sterile nutritive solution led to shifts in the bacterial compositions on the stones. On both buildings similar dynamics could be observed leading to a great dominance of bacteria belonging to the *Firmicutes* phylum. On the contrary, the micro-biota inhabiting the control sector, treated with water, remained stable when samples were monitored for a long-term, showing to be not altered by this treatment.

The short-term monitoring applied in this study shows that the application of a specific bacterium - as a biomineralization agent - on the stone is not necessary to induce the growth of carbonatogenic bacteria present on the stone. The application of the mere sterile nutritive medium activates the inhabiting bacteria on the stone, which are able to produce calcium carbonate. Furthermore, the long-term monitoring revealed that the applied conservation treatments (media inoculated or not inoculated with *M. xanthus*) altered the autochthonous micro-biota remaining on the stone after the treatment, leading to the disappearance of a heterogeneous microbial community, which is replaced by a homogeneous group of microorganisms known for their ability to precipitate calcium carbonate.

5.5. Detection of the inoculated strain- *M. xanthus*

PCR-DGGE has been successfully applied for the screening, identification and monitoring of microbial communities on monuments and stone material (González et al., 2005; Piñar et al., 2001a and 2001b, 2009, Ettenauer et al, 2010). However, as already showed by Piñar et al. 2010, our results show the limitations of using PCR-DGGE analysis to detect *M. xanthus* in a mixed population, as it has been observed in typical environmental samples. No band corresponding to the band of *Myxococcus xanthus* was observed on the DGGE-profiles derived from sectors treated with the *M. xanthus*-inoculated culture (see DGGE profiles of sectors B and E) of both buildings. The construction of clone libraries and the isolation of different clones did not yield positive results for the detection of this microorganism. This can be explained by the difficulties of extracting the large genome of *M. xanthus* (9,454 kbp) (Chen et al., 1991) from an environmental sample. The differences in the relative abundance of DNA fragments from variable microorganisms compete in a PCR reaction, making it much more difficult to amplify this specific DNA (Muyzer et al., 1993 and unpublished data from Ettenauer et al., 2010). Additionally to the differences in genome size and copy number of 16S rRNA genes and the hence resulting variable relative

abundance of the targeted sequence in the total microbial community, the differential or preferential amplification of rRNA genes by PCR using universal primers (Reysenbach et al., 1992) makes it even more difficult to monitor *Myxococcus xanthus*. To overcome these limitations another approach using species specific primers (Piñar et al., 2010) were used to detect the microorganism used as an inoculum in this study. The primer pair Frz799/Frz1147, which amplify a 349 bp fragment of the frzABCD gene of *Myxococcus xanthus* were used in a conventional polymerase chain reaction (see section of Materials and methods). By using these species specific primers, it was possible to detect the targeted microorganism in the pellets derived from enrichment cultures of samples treated with the *M. xanthus*-inoculated culture the first day of the treatment, at both buildings (see Figure 23 A and B) and after 8 days in the case of the Monastery of San Jerónimo. Further investigations using the species specific primer pair for quantitative real-time PCR (qPCR) analysis of the treated samples will be performed in order to quantify the total amount of the *Myxococcus xanthus* DNA. This more sensitive method might also discover traces of target DNA in samples taken at later time points during the monitoring (Piñar et al., 2010).

It is worth noting that cultivation assays performed by Jroundi et al. (2010) with enrichment cultures from the Monastery of San Jerónimo could not supply positive results for the cultivation of *M. xanthus* in the first week of the treatment. Those results were expectable due to the longer generation time of *M. xanthus* compared to that of other activated bacteria (Jimenez-Lopez et al., 2008) and the hence resulting higher cell numbers of the competitively bacteria. Once other bacteria are activated, their growths overlap that of *M. xanthus* and thus hinder its detection using conventional cultivation methods. Additionally their effects (e.g. mineral precipitation) become more noticeable than that of *M. xanthus*.

6. Conclusions

Finally, this study once more highlights the advantages of the application of molecular techniques, comprising of PCR-DGGE analysis, construction and screening of clone libraries and sequencing of 16S rDNA fragments, to identify and monitor microbial community dynamics. Using these methods it is possible to gain insight into the natural micro-biota inhabiting deteriorated monumental limestones before, during and after different consolidation treatments.

The short-term monitoring performed in this study with enrichment cultures conducted during the first week of the treatment reveals that, irrespectively of the origin of the stone, the same group of microorganisms were activated by the application of the consolidation treatments (media inoculated and non-inoculated with *M. xanthus*). A dominant occurrence of members belonging to the *Firmicutes* and *Proteobacteria* during this period of time was observed, what is in agreement with results of previous investigations.

Furthermore, the long-term monitoring reveals similar dynamics in the community structures on both buildings. Once more, it is worth noting that, irrespectively of the origin of the stone and of the conservation treatment applied, members of the *Firmicutes* phylum dominated at the remaining micro-biota colonising the stones after one year of the treatments.

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9. Legend of Figures:

Figure 18
Sectors used for treatments: **A.** Stones of the wall of the apse of the church of San Jerónimo Monastery (Granada, Spain), showing the three areas which were selected for the treatments: Sector A, treated with water as a control, Sector B inoculated with *M. xanthus* (strain number 422 from Spanish Type Culture Collection) the first day and with M-3P culture medium the rest of the days, Sector C was only treated with liquid M-3P culture medium. **B.** Schematic representation of the sectors used for the treatments, indicating the sizes of the three areas. The numbers 1 to 9 and 19 to 27 correspond to the sampling points for cultivation studies. Crosses correspond to the sampling points of the stone samples. Numbers in brackets indicate the taken samples from the sector, which were mixed together and were further analysed by molecular techniques. Sector A: PA1 (1, 2, 3), PA8 (19, 20 and 21); Sector B: PB1 (4, 5, 6), PB8 (22, 23 and 24) and Sector C: PC1 (7, 8, 9), PC8 (25, 26 and 27).

Figure 19
Sectors used for treatments: **A.** Stones of the wall of the Royal Hospital (Granada, Spain), showing the three areas which were selected for the treatments: Sector D – divided into the subsectors D-1 and D-2, treated with water as a control, Sector E inoculated with *M. xanthus* (strain number 422 from Spanish Type Culture Collection) the first day and with M-3P culture medium the rest of the days, Sector F, also divided into the subsectors F-1 and F-2, was only treated with liquid M-3P culture medium. **B.** Schematic representation of the sectors used for the treatments, indicating the sizes of the three areas. The numbers 1 to 9 and 19 to 27 correspond to the sampling points for cultivation studies. Crosses correspond to the sampling points of the stone samples. Numbers in brackets indicate the taken samples from the sector, which were mixed together and were further analysed by molecular techniques. Sector D-1: PD1-1 (8), PD8-1 (26); Sector D-2: PD1-2 (9) and PD8-2 (27); Sector E: PE1 (1, 2, 3), PE8 (19, 20 and 21); Sector F-1: PF1-1 (4, 5, 6), PF8-1 (22, 23, 24) and Sector F-2: PF1-2 (7), PF8-2 (25).

Figure 20
A. Application of the different solutions on the stone material using the spray technique as described by Tiano et al., 2006. **B** and **C.** Treated areas were covered with an aluminum foil, respectively a plastic foil, to avoid exsiccation of the stone material. The foil remained for ten days on the wall till the stone became fully dried. B and C show the treated walls of the Monastery of San Jerónimo, and **D** shows the covered wall of the Royal Hospital.

Figure 21
DGGE fingerprint profiles of stone and pellet samples derived from the Monastery of San Jerónimo showing the succession in the bacterial community structure, as well as the monitoring during the time course experiment. DGGE was run for 3.5 hours at 200V at 60℃ using a chemical gradient ranging from 30 to 55% of urea and formamide. The samples were loaded according to their corresponding sector. Capital letters A, B and C correspond to the different treated areas: sectors A (water treatment), sector B (*Myxococcus xanthus*) and sector C (M-3P media). Line M.x.: *Myxococcus xanthus* strain 422 DNA for comparision. Line SJ-NT: stone sample before treatment. **A.** DGGE fingerprints of the stone samples. Line SA6: stone sample from sector A after six months. Line SB6: stone sample from sector B after six months. Line SB12: stone sample from sector B after one year. Line SC6: stone sample from sector C after six months. Line SC12: stone sample from sector C after one year. **B.** DGGE fingerprints of the pellet samples. Line PA1: pellet sample from sector A from the 1^{st} day of treatment. Line PA8: pellet sample from sector A from the 8^{th} day of treatment. Line PB1: pellet sample from sector B from the 1^{st} day of treatment. Line PB8: pellet sample from sector B from the 8^{th} day of treatment. Line PC1: pellet sample from sector C from the 1^{st} day of treatment. Line PC8: pellet sample from sector C from the 8^{th} day of treatment. Dominant, faint- and in the DGGE

profile of the original sample not visible bands, identified from the 16S rDNA clone libraries were numbered and marked with arrowheads. The bands are explained in the Table 11 A and B.

Figure 22

DGGE fingerprint profiles of stone and pellet samples derived from Royal Hospital showing the succession in the bacterial community structure, as well as the monitoring during the time course experiment. DGGE was run for 3.5 hours at 200V at 60℃ using a chemical gradient ranging from 30 to 55% of urea and formamide. The samples were loaded according to their corresponding sector. Capital letters D, E and F correspond to the different treated areas: sectors D (water treatment), sector E (*Myxococcus xanthus*) and sector F (M-3P media). Line M.x.: *Myxococcus xanthus* strain 422 DNA for comparision. Line RH-NT: stone sample before treatment. **A.** DGGE fingerprints of the stone samples. Line SD12: stone sample from sector D after one year. Line SE12: stone sample from sector E after one year. Line SF12-1: stone sample from sector F-1 after one year. Line SF12-2: stone sample from sector F-2 after one year. **B.** DGGE fingerprints of the pellet samples. Line PD1-1: pellet sample from sector D-1 from the 1st day of treatment. Line PD8-1: pellet sample from sector D-1 from the 8th day of treatment. Line PE1: pellet sample from sector E from the 1st day of treatment. Line PE8: pellet sample from sector E from the 8th day of treatment. Line PF1-1: pellet sample from sector F-1 from the 1st day of treatment. Line PF1-2: pellet sample from sector F-2 from the 1st day of treatment. Line PF8-1: pellet sample from sector F-1 from the 8th day of treatment. Line PF8-2: pellet sample from sector F-2 from the 8th day of treatment. Dominant, faint- and in the DGGE profile of the original sample not visible bands, identified from the 16S rDNA clone libraries were numbered and marked with arrowheads. The bands are explained in the Table 12 A and B.

Figure 23

Screening for *Myxococcus xanthus* DNA in the extracted DNA from the stone- and pellet samples derived from the Monastery of San Jerónimo and the Royal Hospital of Granada. For amplification of *M. xanthus*-DNA the strain specific primers Frz799 and Frz1147 were used. Aliqouts of PCR products were analysed on a 2% agarose gel, stained in an ethidium bromide solution and photographed. Sample names are written on top of the lines and are explained in the text.

A. Samples from the Monastery of San Jerónimo. Line M: molecular marker (100 bp DNA Ladder, Fermentas). Line M.x.: *Myxococcus xanthus* strain 422 DNA as positive control. Line SJ-NT: stone sample before treatment. Line PB1: pellet sample from sector B from the 1st day of treatment. Line PB8: pellet sample from sector B from the 8th day of treatment. Line SB6: stone sample from sector B after six months. Line SB12: stone sample from sector B after one year. **B.** Samples from the Royal Hospital. Line M: molecular marker (100 bp DNA Ladder, Fermentas). Line M.x.: *Myxococcus xanthus* strain 422 DNA as positive control. Line RH-NT: stone sample before treatment. Line PE1: pellet sample from sector E from the 1st day of treatment. Line PE8: pellet sample from sector E from the 8th day of treatment. Line SE12: stone sample from sector E after one year.

Figure 18

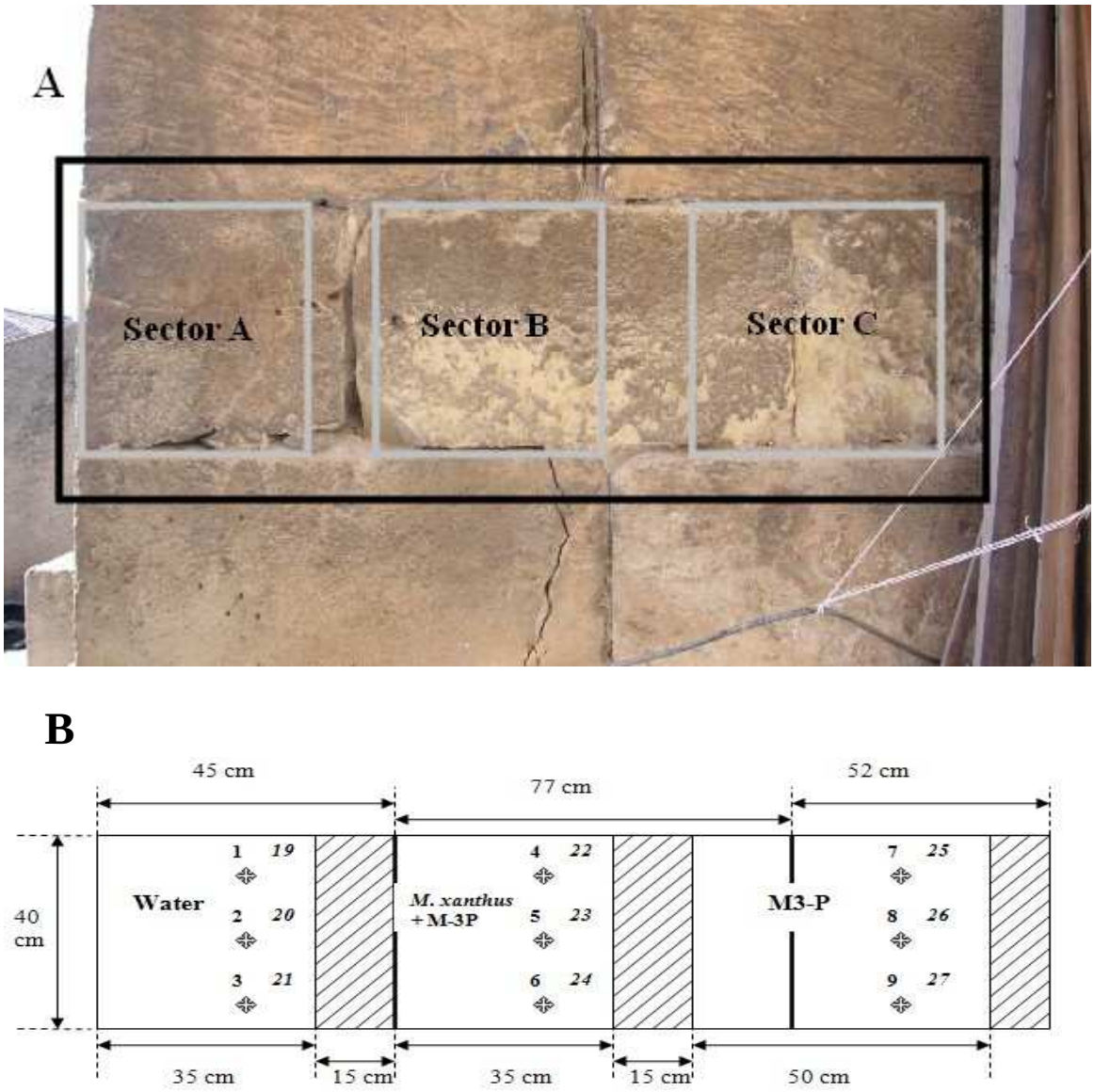
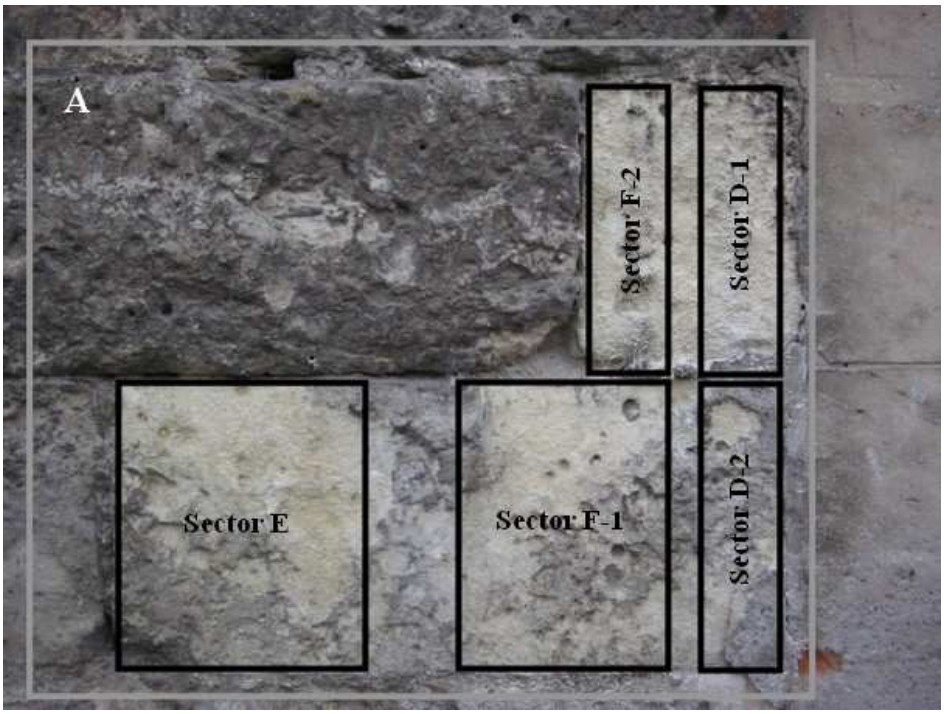

A
Sector A
Sector B
Sector C
B
45 cm
77 cm
52 cm
40 cm
Water
M. xanthus +M-3P
M3-P
1 19
2 20
3 21
4 22
5 23
6 24
7 25
8 26
9 27
35 cm
15 cm
35 cm
15 cm
50 cm

Figure 19


A
Sector F-2
Sector D-1
Sector E
Sector F-1
Sector D-2

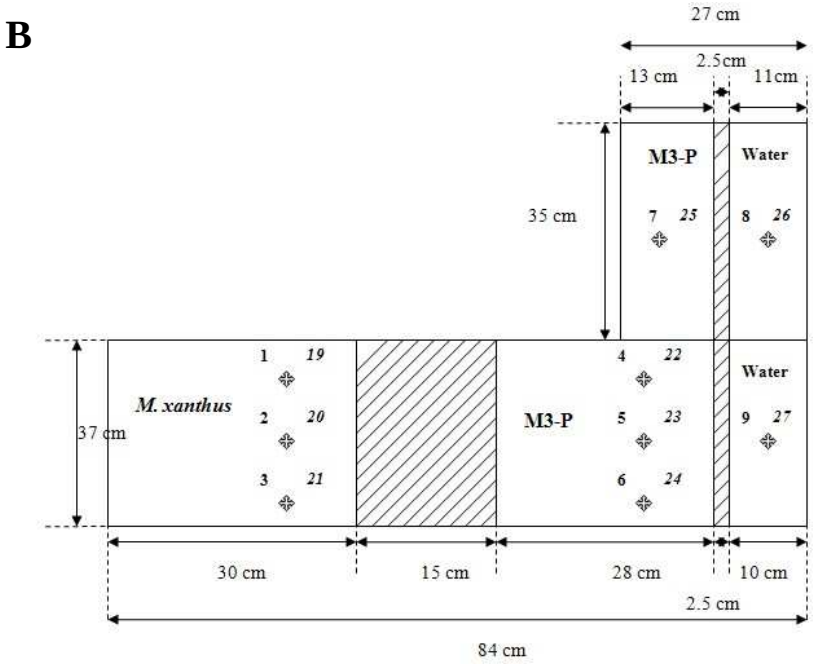

B
27 cm
2.5cm
13 cm
11cm
35 cm
M3-P
Water
7 25
8 26
1 19
2 20
3 21
M. xanthus
37 cm
4 22
5 23
6 24
M3-P
Water
9 27
30 cm
15 cm
28 cm
10 cm
2.5 cm
84 cm

Figure 20

Figure 21

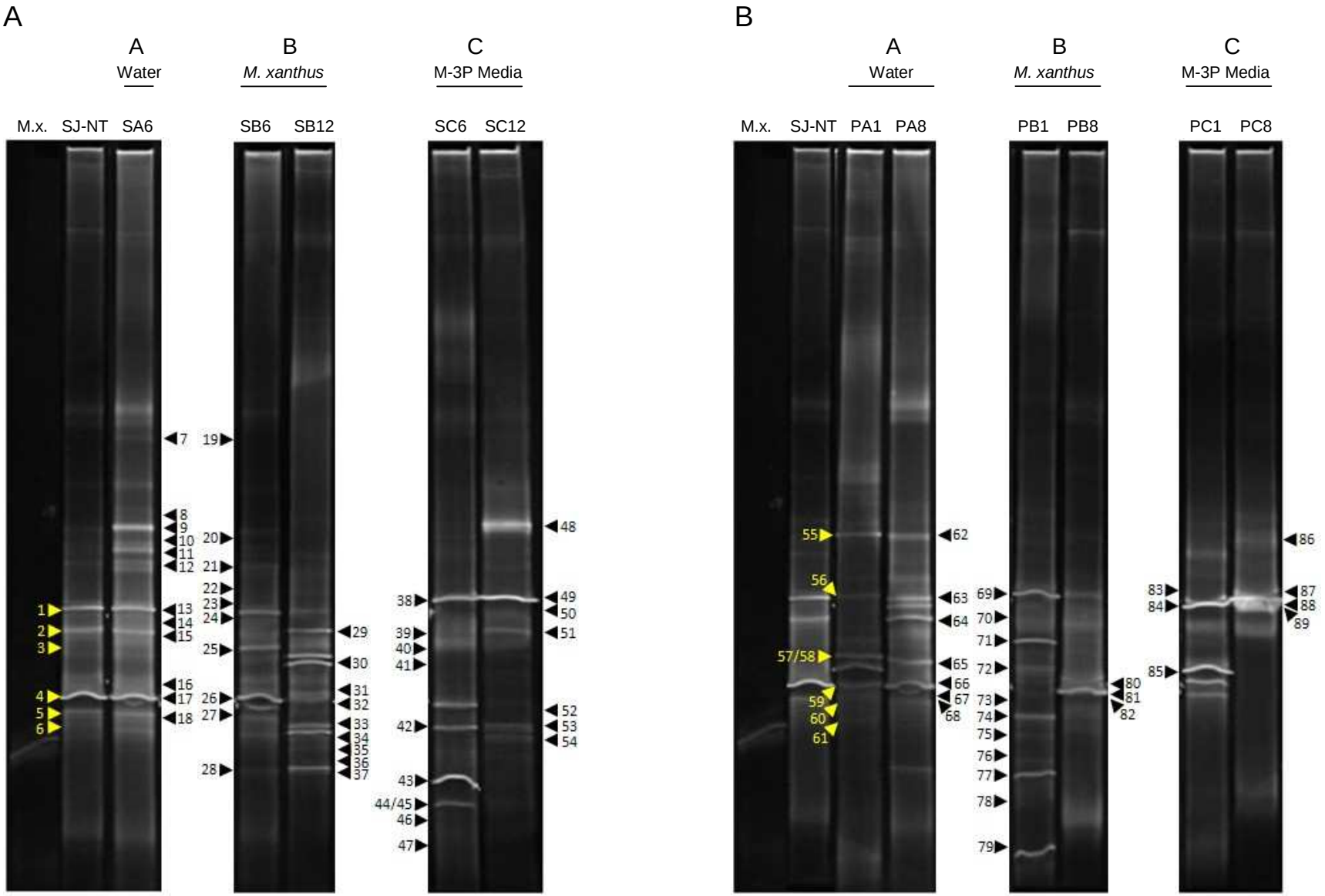

A
A
Water
B
M. xanthus
C
M-3P Media
M.x.
SJ-NT
SA6
SB6
SB12
SC6
SC12
B
A
Water
B
M. xanthus
C
M-3P Media
M.x.
SJ-NT
PA1
PA8
PB1
PB8
PC1
PC8

Table 11

Explanation of marked and numbered bands from Figure 21 A and B. Dominant, faint- and in the DGGE profile of the original sample not visible bands, identified from the 16S rDNA clone libraries were numbered and marked with arrowheads. The bands are explained in this table, describing the clone designation, the sequence length, the closest relative – as determined by comparative sequence analysis, the phylum of the clone, the similarity ranking, the accession numbers of each identified clone and additional comments concerning the relativeness of this clone to sequences of clones found on other sectors or on the Royal Hospital of Granada at different time points. **A.** Discription of the clones from non-treated and treated stone samples. **B.** Discription of clones derived from pellet samples.

A Stone samples

Band/ Clone	Name of Clone	Lenght [bp]	Closest identified phylogenetic relatives [EMBL accession numbers]	Order	Similarity (%)	Accession no.	Comment[a]
SJ-NT							
1	K44	[589]	*Bacillus silvestris* strain: StLB099 [AB478975]	Firmicutes	97	FN434413	
2	K43	[587]	*Delftia tsuruhatensis* strain BA-S3 [EU857415]	Beta-Proteobacteria	99	FN434411	
3	K39	[589]	*Uncultured Xanthomonas sp. clone SL-13* [FJ863119]	Gamma-Proteobacteria	99	FN434412	
4	K4	[562]	*Agrobacterium tumefaciens* strain ISSDS-425 [FJ785222]	Alpha-Proteobacteria	99	FN434408	
5	K28	[587]	*Comamonas testosteroni strain F4* [FJ967838]	Beta-Proteobacteria	99	FN434410	
6	K22	[587]	*Comamonas sp. P3-3* [EU107758]	Beta-Proteobacteria	99	FN434409	
SA6							
7	K42	[580]	*Uncultured Flavobacteriaceae bacterium* clone K38 [FN434386]	Bacteroidetes	99	FN434434	*19*
8	K14	[587]	*Pseudomonas stutzeri strain CpA_a17* [FN397900]	Gamma-Proteobacteria	96	FN434427	
9	K24	[580]	*Uncultured Bacteroidetes bacterium clone Z324* [EU029401]	Bacteroidetes	100	FN434433	
10	K16	[589]	*Uncultured Bacillus sp.* clone K34 [FN434384]	Firmicutes	100	FN434430	*20*
11	K38	[588]	*Uncultured Pseudomonas sp.* clone KJ [AM947011]	Gamma-Proteobacteria	100	FN434429	
12	K26	[588]	*Uncultured Pseudomonas sp. clone K38* [FN434429]	Gamma-Proteobacteria	99	FN434428	*11*
13	K35	[588]	*Uncultured Planococcaceae bacterium* clone K5 [FN435967]	Firmicutes	100	FN434431	**119**
14	K36	[590]	*Uncultured Bacillus sp.* clone K20 [FN434407]	Firmicutes	100	FN434432	*63*
15	K9	[587]	*Uncultured Delftia sp.* clone K43 [FN434411]	Beta-	100	FN434425	*2*, *64*, **75**, **104**

				Proteobacteria			
16	K30	[562]	*Uncultured Rhizobiaceae bacterium* clone K12 [FN434406]	Alpha-Proteobacteria	99	FN434435	*4*, *26*, *59*, *67*, *85*, **102**
17	K7	[587]	*Uncultured Comamonas sp*. clone K1 [FN434373]	Beta-Proteobacteria	100	FN434424	*27*, *80*, **89**, **90**, **91**
18	K11	[587]	*Uncultured Comamonas sp.* clone K9 [FN434385]	Beta-Proteobacteria	99	FN434426	*5*, *6, 17*, *27*, *80*, *81*, *82*, **89**, **90**, **91**, **111**, **122**,**123**, **124**
SB6							
19	K38	[580]	*Uncultured Flavobacteriaceae bacterium* clone K42 [FN434434]	Bacteroidetes	99	FN434386	*7*
20	K34	[589]	*Uncultured Bacillus sp.* clone K16 [FN434430]	Firmicutes	100	FN434384	*10*
21	K44	[587]	*Uncultured Pseudoalteromonas sp.* clone JL-BS-J47 [AY664287]	Gamma-Proteobacteria	100	FN434387	
22	K3	[589]	*Uncultured Bacillus sp*., clone K39 [FN434378]	Firmicutes	99	FN434381	*23*, *24*, *69*, *87*, *88*
23	K2	[589]	*Uncultured Bacillus sp.* clone K1 [FN434376]	Firmicutes	99	FN434380	*22*, *24*, *69*, *87*, *88*
24	K22	[589]	*Uncultured Bacillus sp*. clone K35 [FN434393]	Firmicutes	99	FN434383	*22*, *23*, *69*, *87*, *88*
25	K7	[591]	*Bacillus sp. 19493 and 19495* [AJ315061], [AJ315063]	Firmicutes	99	FN434382	
26	K46	[562]	*Uncultured Rhizobiaceae bacterium* clone K12 [FN434406]	Alpha-Proteobacteria	100	FN434388	*67*, *85*
27	K9	[587]	*Uncultured Comamonas sp.* clone K7 [FN434424]	Beta-Proteobacteria	100	FN434385	*17*, *80*, **89**, **90**, **91**
28	K48	[690]	*Corynebacterium sp. strain NCTC 20294* [X84680]	Actinobacteria	99	FN434389	
SB12							
29	K17	[601]	*Bacillus humi strain DS58* [EU834274]	Firmicutes	99	FN555699	
30	K21	[592]	*Bacillaceae bacterium MOLA 662* culture collection MOLA:662 [AM990775]	Firmicutes	98	FN555700	
31	K35	[591]	*Bacillus sp. BS29 HS-2008 isolate BS29 HS-2008* [AM950294]	Firmicutes	98	FN555702	
32	K25	[589]	*Uncultured Bacillus sp. clone K63* [FN555698]	Firmicutes	99	FN555701	*53*
33	K49	[590]	*Bacillus sp. GB02-39A* [DQ079003]	Firmicutes	99	FN555703	
34	K53	[589]	*Uncultured Bacillus sp. clone K63* [FN555698]	Firmicutes	99	FN555705	*53*
35	K69	[589]	*Bacillus sp. JAM-FM1401* [AB362279]	Firmicutes	98	FN555707	
36	K61	[591]	*Uncultured Bacillus sp. clone K40* [FN434395]	Firmicutes	97	FN555706	*33*, *34*, *53*, *73*
37	K52	[590]	*Bacillus sp. DS10 HS-2008 isolate DS10 HS-2008* [AM950309]	Firmicutes	97	FN555704	
SC6							
38	K3	[590]	*Bacillus seohaeanensis strain BH724* [AY667495]	Firmicutes	98	FN434414	
39	K25	[590]	*Virgibacillus necropolis type strain LMG 19488T* [NR_025472] (reviewed REFSEQ: AJ315056)	Firmicutes	99	FN434419	*40*
40	K27	[590]	*Virgibacillus carmonensis type strain LMG 20964T* [NR_025481] (reviewed REFSEQ: AJ316302)	Firmicutes	99	FN434420	*39*
41	K9	[588]	*Uncultured Planococcaceae bacterium clone K25* [FN435961]	Firmicutes	99	FN434415	**110**

42	K10	[588]	*Uncultured Planococcaceae bacterium* clone K12 [FN434417]	Firmicutes	99	FN434416	*43*, *44*, *45*, *46*, *47*, *74*, *76*
43	K37	[589]	*Uncultured Planococcaceae bacterium* clone K17 [FN434391]	Firmicutes	99	FN434423	*42*, *44*, *46*, *47*, *74*
44	K12	[588]	*Uncultured Planococcaceae bacterium* clone K34 [FN434421]	Firmicutes	99	FN434417	*42*, *43*, *45*, *46*, *47*, *74*, *76*
45	K14	[587]	*Uncultured Planococcaceae bacterium* clone K3 [FN434390],	Firmicutes	99	FN434418	*42*, *44*, *46*, *47*, *74*, *76*
46	K36	[588]	*Uncultured Planococcaceae bacterium* clone K37 [FN434423]	Firmicutes	99	FN434422	*42*, *43*, *44*, *45*, *47*, *74*, *76*
47	K34	[588]	*Uncultured Planococcaceae bacterium* clone K10 [FN434416]	Firmicutes	99	FN434421	*42*, *43*, *44*, *45*, *46*, *74*, *76*
SC12							
48	K25	[590]	*Uncultured Planomicrobium sp.* clone K4 [FN555638]	Firmicutes	99	FN555696	**23**, *49*
49	K22	[588]	*Uncultured Planomicrobium sp.* clone K35 [FN434431]	Firmicutes	99	FN555694	*13*, **23**, *48*, **48**, **116**, **117**, **119**
50	K43	[590]	*Uncultured Planomicrobium sp.* clone K16 [FN435968]	Firmicutes	98	FN555697	*13***, 23**, **48**, *48*, *49*, **116**, **117**, **119**
51	K24	[567]	*Uncultured Planomicrobium sp.* clone K42 [FN435970]	Firmicutes	99	FN555695	*13***, 23**, *48*, *49*, *50*, **116**, **117**, **118**, **119**
52	K18	[599]	*Uncultured Comamonas sp.* clone K7 [FN434424]	Beta-Proteobacteria	93	FN555693	*5*, *6*, *17*, *18*, *27*, *80*, *81*, *82*, **89**, **90**, **91**, **111**, **122**, **123**, **124**
53	K63	[589]	*Uncultured Bacillus sp.* clone K25 [FN555701]	Firmicutes	99	FN555698	*32*
54	K13	[592]	*Uncultured Bacilli bacterium* clone H5Ba08 [AY360560]	Firmicutes	98	FN555692	

B Pellet Samples

PA1							
55	K58	[580]	*Sphingobacterium sp. H168* [EF204462]	Bacteroidetes	99	FN434369	
56	K30	[580]	*Flavobacterium mizutaii* isolate Ch4 [AM286271]	Bacteroidetes	99	FN434368	
57	K34	[396]	*Brevundimonas diminuta* strain B34 [GU397389]	Alpha-Proteobacteria	100	FN434364	
58	K19	[561]	*Uncultured Brevundimonas sp.* clone K8 [FN435947]	Alpha-Proteobacteria	96	FN434363	**72**, **87**
59	K47	[562]	*Uncultured Rhizobiaceae bacterium* clone K12 [FN434406]	Alpha-Proteobacteria	99	FN434367	*4, 67*, *85*, **102**
60	K14	[561]	*Uncultured Rhizobiaceae bacterium* clone K4 [FN434408]	Alpha-Proteobacteria	99	FN434365	*4*, *59*, *67*, *85*, **102**
61	K40	[561]	*Uncultured Rhizobiaceae bacterium* clone K6 [FN434372]	Alpha-	99	FN434366	*4*, *59*, *67*, *85*, **102**

				Proteobacteria			
PA8							
62	K6	[562]	*Uncultured Comamonas sp.* clone K22 [FN434409]	Beta-Proteobacteria	100	FN434401	*6*, **14**, **123**
63	K20	[590]	*Uncultured Bacillus sp.* clone K10 [AM947006]	Firmicutes	100	FN434407	
64	K55	[587]	*Uncultured Delftia sp.* K43 [FN434411]	Beta-Proteobacteria	100	FN434405	*2*, **75**, **104**
65	K52	[562]	*Uncultured Delftia sp.* K3 [FN435975]	Beta-Proteobacteria	97	FN434403	*2*, *64*, **75**, **82**, **83**, **84**, **103**, **104**
66	K54	[562]	*Uncultured Delftia sp.* K35 [FN428833]	Beta-Proteobacteria	98	FN434404	**83**, **103**
67	K12	[562]	*Uncultured Rhizobiaceae bacterium* clone K6 [FN434372]	Alpha-Proteobacteria	100	FN434406	*85*
68	K13	[587]	*Uncultured Comamonas sp.* clone K1 [FN434373]	Beta-Proteobacteria	98	FN434402	*5*, *6*, *80*, *81*, *82*, **89**, **90**, **91**, **111**, **122**, **123**, **124**
PB1							
69	K35	[589]	*Uncultured Bacillus sp.* clone K1 [FN434376]	Firmicutes	99	FN434393	*87*, *88*,
70	K42	[588]	*Uncultured bacterium clone AS3_aao18d01* [EU466607]	Firmicutes	98	FN434397	
71	K41	[590]	*Bacillus sp. BUS 07-20* strain BUS 07-20 [AM931044]	Firmicutes	97	FN434396	
72	K28	[589]	*Uncultured Bacillus sp.* clone K40 [FN434395],	Firmicutes	98	FN434392	*72*
73	K40	[589]	*Bacillus selenatarsenatis strain S3-3* [FJ373030]	Firmicutes	99	FN434395	
74	K17	[723]	*Planomicrobium glaciei* strain 0423 [EU036220]	Firmicutes	99	FN434391	
75	K53	[581]	*Arthrobacter psychrolactophilus* strain D2 [AF134181]	Actinobacteria	98	FN434400	
76	K3	[589]	*Uncultured Planococcaceae* clone K17 [FN434391]	Firmicutes	99	FN434390	*74*
77	K38	[588]	*Uncultured Planococcaceae bacterium* clone K3 [FN434390]	Firmicutes	97	FN434394	*74*, *76*
78	K43	[588]	*Solibacillus silvestris* strain: StLB099 [AB478975]	Firmicutes	96	FN434398	
79	K24	[570]	*Uncultured Kocuria sp.* clone K34 [FN555669]	Actinobacteria	99	FN434399	**17**
PB8							
80	K1	[587]	*Uncultured Comamonas sp*. clone K20 [FN435948]	Beta-Proteobacteria	100	FN434373	**89**, **90**, **91**
81	K38	[587]	*Uncultured Comamonas sp.* clone K22 [FN434409]	Beta-Proteobacteria	99	FN434375	*5*, *6*, *80*, *82*, **89**, **90**, **91**, **111**, **122**, **123**, **124**
82	K26	[587]	*Uncultured Comamonas sp.* clone K28 [FN434410]	Beta-Proteobacteria	99	FN434374	*5*, *6*, *80*, *81*, **89**, **90**, **91**, **111**, **122**, **123**, **124**

PC1							
83	K37	[589]	*Bacillus koreensis* [FJ889614]	Firmicutes	99	FN434371	
84	K3	[588]	*Bacillus firmus strain CHN8* [EU443751]	Firmicutes	99	FN434370	
85	K6	[562]	*Uncultured Rhizobiaceae bacterium* clone K12 [FN434406]	Alpha-Proteobacteria	100	FN434372	*67*
PC8							
86	K44	[587]	*Uncultured bacterium* clone C9-K19 [AJ421093]	Beta-Proteobacteria	92	FN434379	
87	K1	[589]	*Uncultured Bacillus sp.* clone K35 [FN434393]	Firmicutes	99	FN434376	*69*, *88*
88	K39	[589]	*Solibacillus silvestris* strain: StLB099 [AB478975]	Firmicutes	99	FN434378	
89	K10	[589]	*Uncultured Bacillus sp.* clone K41 [FN434396]	Firmicutes	94	FN434377	*71*

[a] Italic numbers correspond to clones from the Monastery of San Jerónimo (Figure 21), bold numbers to the Royal Hospital (Figure 22) of Granada, respectively. The figures display the band numbers of clones that showed the highest similarity ranking in the database search.

Figure 22

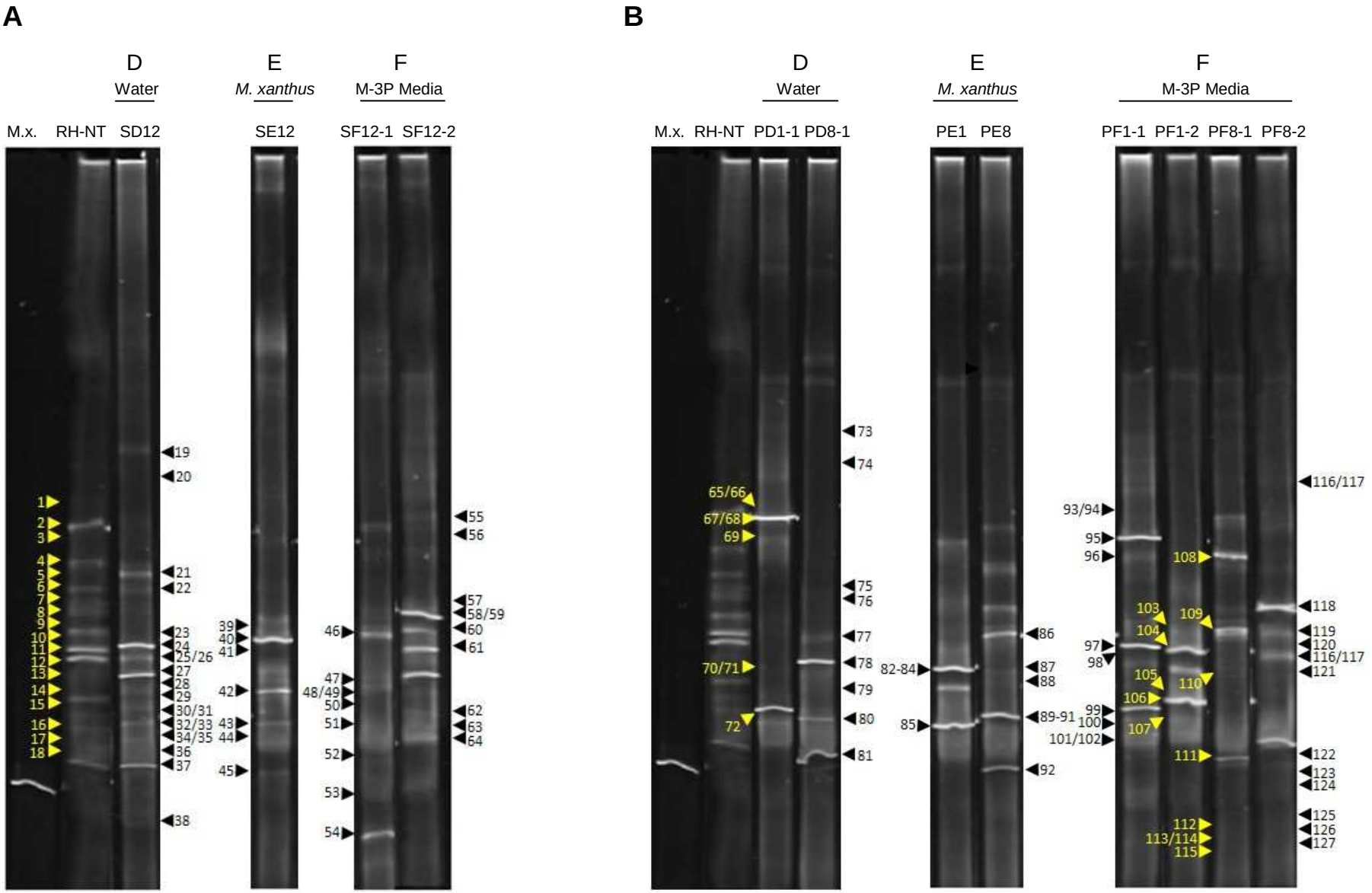

A
D
Water
E
M. xanthus
F
M-3P Media
M.x.
RH-NT
SD12
SE12
SF12-1
SF12-2
B
D
Water
E
M. xanthus
F
M-3P Media
M.x.
RH-NT
PD1-1
PD8-1
PE1
PE8
PF1-1
PF1-2
PF8-1
PF8-2

Table 12

Explanation of marked and numbered bands from Figure 22 A and B. Dominant, faint- and in the DGGE profile of the original sample not visible bands, identified from the 16S rDNA clone libraries were numbered and marked with arrowheads. The bands are explained in this table, describing the clone designation, the sequence length, the closest relative – as determined by comparative sequence analysis, the phylum of the clone, the similarity ranking, the accession numbers of each identified clone and additional comments concerning the relativeness of this clone to sequences of clones found on other sectors or on the Monastery of San Jerónimo of Granada at different time points. **A.** Discription of the clones from non-treated and treated stone samples. **B.** Discription of clones derived from pellet samples.

A Stone Samples

Band/Clone	Name of Clone	Length [bp]	Closest identified phylogenetic relatives [EMBL accession numbers]	Order	Similarity (%)	Accession no.	Comment
RH-NT							
1	K51	[591]	*Uncultured bacterium* isolate BF0002B008 [AM697058]	Firmicutes	98	FN555675	
2	K33	[591]	*Uncultured bacterium clone C69* [GQ169663]	Firmicutes	99	FN555668	
3	K37	[588]	*Pseudomonas sp. L4nov* [GU184339]	Gamma-Proteobacteria	99	FN555671	
4	K55	[589]	*Streptococcus bovis NCDO2127* [AF429766]	Firmicutes	99	FN555676	
5	K58	[589]	*Neisseria sp. GRW38 [*FJ502346]	Beta-Proteobacteria	99	FN555679	
6	K68	[588]	*Pseudoalteromonas issachenkonii* strain KOPRI 22718 [GQ885144]	Gamma-Proteobacteria	99	FN555680	
7	K30	[589]	*Pseudoalteromonas tetraodonis* strain: Do-17 [AB257325]	Gamma-Proteobacteria	99	FN555667	
8	K69	[588]	*Bacillus sp. T10T* isolate T10T [AM983523]	Firmicutes	99	FN555681	
9	K23	[590]	*Bacillus sp. T33T* isolate T33T [AM983515]	Firmicutes	99	FN555666	
10	K41	[591]	*Brevibacillus brevis NBRC 100599 DNA* [AP008955]	Firmicutes	98	FN555673	
11	K44	[591]	*Bacillus sp. 3461BRRJ* [FJ215797]	Firmicutes	99	FN555674	
12	K36	[590]	*Bacillus sp. L244* isolate L244 [AM913937]	Firmicutes	99	FN555670	
13	K56	[562]	*Agrobacterium tumefaciens* strain BLN4 [GQ181060]	Alpha-Proteobacteria	98	FN555677	
14	K20	[587]	*Comamonas testosteroni CNB-2* [CP001220]	Beta-Proteobacteria	98	FN555664	
15	K38	[589]	*Enterobacter sp. CMG24314* [EU162036]	Gamma-Proteobacteria	99	FN555672	
16	K57	[571]	*Micrococcus sp. CCGE3063* [EU867326]	Actinobacteria	98	FN555678	
17	K34	[570]	*Kocuria rosea strain 2P03AA* [EU977667]	Actinobacteria	99	FN555669	
18	K22	[581]	*Corynebacterium testudinoris strain CCUG 41823* [NR_025434] (reviewed REFSEQ: AJ295841)	Actinobacteria	99	FN555665	

SD12							
19	K71	[588]	*Bacterium J10* [FJ418599]	Beta-Proteobacteria	99	FN555656	
20	K32	[591]	*Pseudomonas brenneri* strain ES-138S [FN393788]	Gamma-Proteobacteria	99	FN555644	
21	K8	[589]	*Uncultured actinobacterium* clone A7-01QJH [EU434888]	Actinobacteria	93	FN555640	
22	K6	[588]	*Pseudomonas alcaligenes strain Y34* [FJ830845]	Gamma-Proteobacteria	99	FN555639	
23	K4	[588]	*Uncultured Planococcaceae bacterium* clone K16 [FN435968]	Firmicutes	99	FN555638	*13*, *48*, **48**, *49*, **116**, **117**, **118**, **119**, **120**
24	K54	[588]	*Delftia acidovorans* strain CC [GQ466172]	Beta-Proteobacteria	99	FN555650	
25	K13	[564]	*Porphyrobacter dokdonensis strain DSW-74* [DQ011529]	Alpha-Proteobacteria	99	FN555641	
26	K43	[587]	*Uncultured Delftia sp.* clone K3 [FN435975]	Beta-Proteobacteria	99	FN555648	*2*, *15*, *64*, **75**, **84**, **104**,
27	K66	[563]	*Bosea eneae strain DS29* [EF519707]	Alpha-Proteobacteria	99	FN555652	
28	K67	[588]	*Uncultured Planococcaceae bacterium* clone K25 [FN435961]	Firmicutes	99	FN555653	*41*, **48**, **51**, **110**
29	K34	[589]	*Bacillus sp. G2DM-33* [DQ416793]	Firmicutes	99	FN555645	
30	K46	[587]	*Hydrogenophaga defluvii* strain BSB 9.5 [NR_029024] (reviewed REFSEQ: AJ585993)	Beta-Proteobacteria	99	FN555649	
31	K70	[590]	*Stenotrophomonas maltophilia strain IMER-B3-10* [FJ772079]	Gamma-Proteobacteria	99	FN555655	
32	K2	[588]	*Thiobacillus denitrificans strain NCIMB 9548* [NR_025358] (reviewed REFSEQ: AJ243144)	Beta-Proteobacteria	98	FN555637	
33	K69	[562]	*Uncultured Rhizobiaceae bacterium* clone K56 [FN435924]	Alpha-Proteobacteria	98	FN555654	*4*, *16*, *26*, *59*, *61*, *67*, *85*, **102**
34	K35	[562]	*Methylobacterium aminovorans* [AM910532]	Beta-Proteobacteria	99	FN555646	
35	K57	[589]	*Uncultured Planococcaceae bacterium* clone K36 [FN434422]	Firmicutes	99	FN555651	*44*, *46*, *47*
36	K37	[591]	*Uncultured Exiguobacterium sp*. clone K27 [FN435981]	Firmicutes	99	FN555647	**77**, **79**, **92**, **112**, **113**, **126**
37	K29	[569]	*Brachybacterium arcticum strain DFVB11* [EU249576]	Actinobacteria	99	FN555643	
38	K28	[570]	*Uncultured actinobacterium clone 2053* [EF188664]	Actinobacteria	100	FN555642	
SE12							
39	K30	[590]	*Uncultured Bacillus sp*. clone K6 [FN555628]	Firmicutes	98	FN555659	**12**, **46**
40	K49	[589]	*Uncultured Bacillus sp.* clone K36 [FN434432]	Firmicutes	99	FN555657	*14*, *63*
41	K29	[589]	*Bacillus sp. H2(2009)* [GQ292772]	Firmicutes	98	FN555660	
42	K54	[593]	*Uncultured Bacillus sp*. clone K11 [FN555674]	Firmicutes	98	FN555658	**11**, **12**, *25*, **43**,
43	K2	[592]	*Uncultured Bacillus sp*. clone K36 [FN555670]	Firmicutes	99	FN555661	**12**

44	K59	[590]	*Uncultured Bacillus sp.* clone K20 [FN434407]	Firmicutes	97	FN555663	*14*, *63*
45	K1	[589]	*Bacillus niacini strain SAFN-019* [AY167811]	Firmicutes	98	FN555662	
SF12-1							
46	K6	[590]	*Uncultured Bacillus sp.* clone K30 [FN555659]	Firmicutes	98	FN555628	**12**, **39**
47	K70	[588]	*Uncultured Planococcus sp*. clone K33 [FN435966]	Firmicutes	98	FN555636	*13*, **118**, **119**, **120**
48	K52	[588]	*Uncultured Planococcaceae bacterium* clone K5 [FN435967]	Firmicutes	99	FN555634	*13*, **23**, **28**, *41*, *49*, **119**,
49	K26	[588]	*Uncultured Planomicrobium sp*. clone K70 [FN555691]	Firmicutes	98	FN555631	*13*, *41*, **28**, **48**, **59**, **110**, **119**,
50	K27	[587]	*Uncultured Firmicutes bacterium clone 15* [EU647532]	Firmicutes	99	FN555632	
51	K53	[588]	*Uncultured Planococcaceae bacterium* clone K25 [FN435961]	Firmicutes	99	FN555635	**28**, *41*, **110**,
52	K50	[590]	*Uncultured Planococcaceae bacterium* clone K37 [FN434423]	Firmicutes	95	FN555633	**35**, *42*, *43*, *44*, *45*, 46, 47, **53**, **54**, *74*, *76*,
53	K9	[587]	*Uncultured Planococcaceae bacterium* clone K36 [FN434422]	Firmicutes	99	FN555629	*42*, *44*, *45*, *46*, *47*, *76*
54	K13	[588]	*Uncultured Planococcaceae bacterium* clone K12 [FN434417]	Firmicutes	99	FN555630	*44*, *46*, *47*
SF12-2							
55	K63	[592]	*Desemzia sp. M341* [FJ513868]	Firmicutes	98	FN555688	
56	K69	[589]	*Planomicrobium koreense* strain WT024 [GQ152129]	Firmicutes	99	FN555690	
57	K39	[589]	*Uncultured Planococcaceae bacterium* clone K67 [FN555653]	Firmicutes	98	FN555684	*13*, **23**, **28**, *41*, **48**, *49*, **110**, **116**, **119**
58	K50	[590]	*Uncultured Planococcaceae bacterium* clone K4 [FN555638]	Firmicutes	98	FN555686	*13*, **23**, **48**, **119**
59	K70	[589]	*Planococcus sp. Smarlab 3302355* [AY538695]	Firmicutes	99	FN555691	
60	K23	[587]	*Uncultured Planococcaceae bacterium* clone K16 [FN435968]	Firmicutes	98	FN555683	**116**
61	K4	[613]	*Uncultured Planococcus sp*. clone K25 [FN435961]	Firmicutes	99	FN555682	**28**, **110**
62	K66	[591]	*Uncultured Planococcaceae bacterium* clone K13 [FN555630]	Firmicutes	98	FN555689	**35**, *42*, *43*, *44*, *45*, *46*, *47*, **53**, **54**, *74*, *76*
63	K48	[569]	*Uncultured bacterium clone AS3_aao17a08* [EU466525]	Actinobacteria	94	FN555685	
64	K60	[570]	*Uncultured Kocuria sp.* clone K34 [FN555669]	Actinobacteria	99	FN555687	**17**

B Pellet Samples

PD1-1							
65	K8	[572]	*Pseudomonas aeruginosa* strain WJ-1 [FJ948174]	Gamma-Proteobacteria	98	FN435939	
66	K2	[585]	*Pseudomonas aeruginosa* strain PSCMST1 [GU723304]	Gamma-Proteobacteria	99	FN435944	
67	K10	[588]	*Uncultured bacterium clone* P7D1-570 [EF509311]	Gamma-Proteobacteria	100	FN435940	

68	K43	[587]	*Uncultured bacterium clone* P5D23-386 [EF511691]	Gamma-Proteobacteria	100	FN435942	
69	K29	[588]	*Pseudomonas aeruginosa* strain LMG 1242T [Z76651]	Gamma-Proteobacteria	100	FN435945	
70	K31	[626]	*Pseudomonas aeruginosa* strain AS2 [GU447238]	Gamma-Proteobacteria	100	FN435941	
71	K42	[452]	Pseudomonas aeruginosa strain AM02 [GQ483504]	Gamma-Proteobacteria	100	FN435946	
72	K18	[561]	*Uncultured Brevundimonas sp*. clone K8 [FN435947]	Alpha-Proteobacteria	99	FN435943	**87**
PD8-1							
73	K29	[589]	*Acinetobacter radioresistens* strain M 17694T [Z93445]	Gamma-Proteobacteria	99	FN435978	
74	K26	[566]	*Uncultured Rhizobiaceae bacterium* clone K56 [FN435924]	Alpha-Proteobacteria	89	FN435974	*4*, *59*, *60*, *67*, *85*, **102**
75	K3	[587]	*Uncultured Delftia sp.* clone K6 [FN435934]	Beta-Proteobacteria	100	FN435975	*2*, *64*, **104**
76	K42	[430]	*Uncultured Comamonas sp.* clone K20 [FN555664]	Beta-Proteobacteria	100	FN435977	*5*, *6*, **14**, *62*, *80*, *82*, **89**, **90**, **91**, **123**, **124**,
77	K27	[589]	*Uncultured Exiguobacterium sp.* clone K7 [FN435971]	Firmicutes	100	FN435981	**92**, **113**, **126**
78	K1	[589]	*Uncultured Comamonas sp.* clone K47 [FN435954]	Beta-Proteobacteria	92	FN435976	*5, 6, 68, 80*, *81*, *82*, **89**, **90**, **91**, **111**, **122**, **123**, **124**
79	K36	[589]	*Uncultured Exiguobacterium sp.* clone K33 [FN435958]	Firmicutes	99	FN435982	**77**, **92**, **112**, **113**, **126**
80	K14	[589]	*Uncultured Exiguobacterium sp.* clone K6 [FN435953]	Firmicutes	98	FN435980	**77**, **79**, **92**, **112**, **113**, **126**
81	K12	[587]	*Uncultured Exiguobacterium sp.* clone K32 [FN435957]	Firmicutes	95	FN435979	**77**, **79**, **92**, **112**, **113**, **126**
PE1							
82	K26	[579]	*Uncultured Delftia sp.* clone K3 [FN435975]	Beta-Proteobacteria	100	FN428835	*2*, *64*, **75**, **104**
83	K35	[565]	*Uncultured Delftia sp.* clone K43 [FN434411]	Beta-Proteobacteria	100	FN428833	*2*, *64*, **75**, **82**, **104**
84	K33	[587]	*Uncultured Delftia sp.* clone K6 [FN435934]	Beta-Proteobacteria	99	FN435923	*2*, *64*, **75**, **104**
85	K31	[589]	*Uncultured Stenotrophomonas sp.* clone K2 [FN435937]	Gamma-Proteobacteria	100	FN428834	**106**
PE8							
86	K39	[461]	*Uncultured Arthrobacter sp.* clone K5 [FN435955]	Actinobacteria	99	FN435952	**115**

87	K8	[562]	*Brevundimonas diminuta* strain B34 [GU397389]	Alpha-Proteobacteria	99	FN435947	
88	K41	[564]	*Uncultured Stenotrophomonas sp.* clone K49 [FN435932]	Gamma-Proteobacteria	99	FN435951	**85**, **100**, **106**, **107**
89	K20	[587]	*Uncultured Comamonas sp.* clone K1 [FN434373]	Beta-Proteobacteria	100	FN435948	*80*, **90**, **91**
90	K40	[587]	*Uncultured Comamonas sp.* clone K20 [FN435948]	Beta-Proteobacteria	100	FN435949	*80*, **89**, **91**
91	K46	[587]	*Uncultured Comamonas sp.* clone K40 [FN435949]	Beta-Proteobacteria	100	FN435950	**89**, **90**
92	K6	[587]	*Uncultured Exiguobacterium sp.* clone K33 [FN435958]	Firmicutes	100	FN435953	**77**, **79**, **113**, **126**
PF1-1							
93	K8	[588]	*Pseudomonas monteilii* strain GAPP1 [GU396289]	Gamma-Proteobacteria	99	FN435928	
94	K66	[588]	*Uncultured Pseudomonas sp.* clone K1 [FN435927]	Gamma-Proteobacteria	99	FN435930	**95**
95	K1	[588]	*Pseudomonas putida* strain X16 [GU335250]	Gamma-Proteobacteria	100	FN435927	
96	K14	[588]	*Pseudomonas monteilii* strain SB 3091 [GU191925]	Gamma-Proteobacteria	99	FN435929	
97	K48	[589]	Bacillus firmus strain D8 [GU397391]	Firmicutes	99	FN435933	
98	K36	[587]	*Uncultured Brevundimonas sp.* clone K18 [FN435943]	Alpha-Proteobacteria	99	FN435925	**72**, **87**
99	K29	[561]	Pseudomonas sp. CHN14 [EU600783]	Gamma-Proteobacteria	98	FN435931	
100	K49	[588]	*Uncultured Stenotrophomonas sp.* clone K2 [FN435937]	Gamma-Proteobacteria	99	FN435932	**85**, **106**, **107**
101	K9	[555]	*Uncultured Brevundimonas sp.* clone K8 [FN435947]	Alpha-Proteobacteria	97	FN435926	**72**, **87**
102	K56	[562]	*Uncultured Rhizobiaceae bacterium* clone K12 [FN434406]	Alpha-Proteobacteria	99	FN435924	*4*, *59*, *67*, *85*
PF1-2							
103	K34	[559]	*Uncultured Delftia sp.* clone K26 [FN428835]	Beta-Proteobacteria	99	FN435935	*2*, *64*, **75**, **82**, **83**, **84**, **104**,
104	K6	[587]	*Uncultured Delftia sp.* clone K55 [FN434405]	Beta-Proteobacteria	100	FN435934	*2*, *64*, **75**
105	K7	[587]	*Uncultured Stenotrophomonas sp.* clone P4s-68 [GQ329143]	Gamma-Proteobacteria	93	FN435936	
106	K2	[589]	*Uncultured Stenotrophomonas sp.* clone K31 [FN428834]	Gamma-Proteobacteria	100	FN435937	**85**
107	K36	[588]	*Uncultured Stenotrophomonas sp.* clone K49 [FN435932]	Gamma-Proteobacteria	99	FN435938	**85**, **100**, **106**

PF8-1							
108	K30	[589]	*Exiguobacterium sp. MOLA 29* culture collection MOLA:29 [AM990805]	Firmicutes	99	FN435956	
109	K8	[588]	*Uncultured Planococcaceae bacterium* clone K5 [FN435967]	Firmicutes	98	FN435960	**116**, **118**, **119**, **120**
110	K25	[588]	*Planococcus kazaiensis strain K01-30* [AY260168]	Firmicutes	99	FN435961	
111	K47	[587]	*Uncultured Comamonas sp.* clone K40 [FN435949]	Beta-Proteobacteria	99	FN435954	*5*, *6*, *80*, *81*, *82*, **89**, **90**, **91**, **122**, **123**, **124**,
112	K32	[589]	*Uncultured Exiguobacterium sp.* clone K27 [FN435981]	Firmicutes	99	FN435957	**77**, **79**, **92**, **113**, **126**
113	K33	[589]	*Uncultured Exiguobacterium sp.* clone K6 [FN435953]	Firmicutes	100	FN435958	**77**, **92**, **126**
114	K48	[589]	*Uncultured Exiguobacterium sp.* clone K14 [FN435980]	Firmicutes	97	FN435959	**77**, **79**, **80**, **92**, **112**, **113**, **126**
115	K5	[510]	*Uncultured Arthrobacter sp.* clone K39 [FN435952]	Actinobacteria	99	FN435955	**86**
PF8-2							
116	K16	[588]	*Uncultured Planomicrobium sp.* clone K42 [FN435970]	Firmicutes	99	FN435968	**117**, **119**
117	K42	[588]	Planococcaceae bacterium NR201 [DQ520830]	Firmicutes	99	FN435970	
118	K33	[588]	*Uncultured Planococcus sp.* clone K1 [FN435965]	Firmicutes	99	FN435966	**119, 120**
119	K5	[588]	*Uncultured Planococcaceae bacterium* clone K16 [FN435968]	Firmicutes	99	FN435967	**116**, **117**, **118**, **120**
120	K1	[588]	*Uncultured Planococcus sp.* clone K33 [FN435966]	Firmicutes	99	FN435965	**118**, **119**
121	K39	[588]	*Uncultured bacterium* clone D02_EX [EU735523]	Firmicutes	97	FN435969	
122	K9	[587]	*Uncultured Comamonas sp.* clone K47 [FN435954]	Beta-Proteobacteria	99	FN435963	*5*, *6*, *80*, *81*, *82*, **89**, **90**, **91**, **111**, **123**, **124**
123	K3	[587]	*Uncultured Comamonas sp.* clone K20 [FN435948]	Beta-Proteobacteria	99	FN435962	*5*, *6*, *80*, *81*, *82*, **89**, **90**, **91**, **111**, **122**, **124**
124	K47	[587]	*Uncultured Comamonas sp.* clone K46 [FN435950]	Beta-Proteobacteria	99	FN435964	5, 6, 80, 81, 82, **89, 90, 91, 111, 122, 123**
125	K37	[589]	*Uncultured Exiguobacterium sp.* clone K27 [FN435981]	Firmicutes	98	FN435973	**77, 79, 92, 112, 113, 126,**
126	K7	[589]	*Uncultured Exiguobacterium sp.* clone K6 [FN435953]	Firmicutes	100	FN435971	**77, 92, 113**
127	K24	[589]	*Uncultured Exiguobacterium sp.* clone K37 [FN435973]	Firmicutes	97	FN435972	**125**

[a] Italic numbers correspond to clones from the Monastery of San Jerónimo (Figure 21), bold numbers to the Royal Hospital (Figure 22) of Granada, respectively. The figures display the band numbers of clones that showed the highest similarity ranking in the database search.

Table 13

Genera detected in the pellets of enrichment cultures derived from the Monastery of San Jerónimo and the Royal Hospital of Granada. Figures in the table show the numbers of sequenced clones related to the corresponding genus. Percentages in brackets indicate to proportion of the clones to the total sequenced clones of the related sample. SJ stands the Monastery of San Jerónimo; RH for the Royal Hospital of Granada.

Pellet samples		**Non-treated**		**Treatments**													
				Water				***M. xanthus***				**M-3P media**					
	Building	SJ	RH	SJ	SJ	RH	RH	SJ	SJ	RH	RH	SJ	SJ	RH	RH	RH	Rh
	Time of sampling	0	0	1st day	8th day	1st day	8th day	1st day	8th day	1st day	8th day	1st day	8th day	1st day	1st day	8th day	8th day
	Sample / Genera	SJ-NT	RH-NT	PA1	PA8	PD1-1	PD8-1	PB1	PB8	PE1	PE8	PC1	PC8	PF1-1	PF1-2	PF8-1	PF8-2
Alpha-Proteobacteria	*Rhizobiaceae/ Agrobacterium*	1 (16.7%)	1 (5.6%)	3 (42.9%)	1 (14.3%)		1 (11.1%)					1 (33.3%)		1 (10%)			
	Brevundimonas			2 (28.6%)		1 (12.5%)					1 (14.3%)			2 (20%)			
Beta-Proteobacteria	*Comamonas*	2 (33.3%)	1 (5.6%)		2 (28.6%)		2 (22.2%)		3 (100%)		3 (42.9%)					1 (12.5%)	3 (25%)
	Delftia	1 (16.7%)			3 (42.9%)		1 (11.1%)			3 (75%)					2 (40%)		
	Neisseria		1 (5.6%)														
	Uncultured beta proteobacteria												1 (25%)				
Gamma-Proteoabcteria	*Acinetobacter*						1 (11.1%)										
	Pseudomonas		1 (5.6%)			5 (62.5%)								5 (50%)			
	Stenotrophomonas									1 (25%)	1 (14.3%)			1 (10%)	3 (60%)		
	Enterobacter		1 (5.6%)														
	Pseudoalteromonas		2 (11.1%)														
	Xanthomonas	1 (16.7%)															
	Uncultured gamma proteobacteria					2 (25%)											
Actinobacteria	*Arthrobacter*							1 (9.1%)			1 (14.3%)					1 (12.5%)	
	Micrococcus		1 (5.6%)														

	Kocuria		1 (5.6%)					1 (9.1%)									
	Corynebacterium		1 (5.6%)														
Firmicutes	*Bacillus*	1 (16.7%)	4 (22.2%)		1 (14.3%)			4 (36.4%)				2 (66.7%)	2 (50%)	1 (10%)			
	Solibacillus							1 (9.1%)					1 (25%)				
	Exiguobacterium						4 (44.4%)				1 (14.3%)					4 (50%)	3 (25%)
	Planococcaceae							3 (27.3%)								2 (25%)	5 (41.7%)
	Brevibacillus		1 (5.6%)														
	Streptococcus		1 (5.6%)														
	Uncultured bacterium		2 (11.1%)					1 (9.1%)									1 (8.3%)
Bacteroidetes	*Flavobacterium*			1 (14.3%)													
	Sphingobacterium			1 (14.3%)													
Total number of clones		6 (100%)	18 (100%)	7 (100%)	7 (100%)	8 (100%)	9 (100%)	11 (100%)	3 (100%)	4 (100%)	7 (100%)	3 (100%)	4 (100%)	10 (100%)	5 (100%)	8 (100%)	12 (100%)

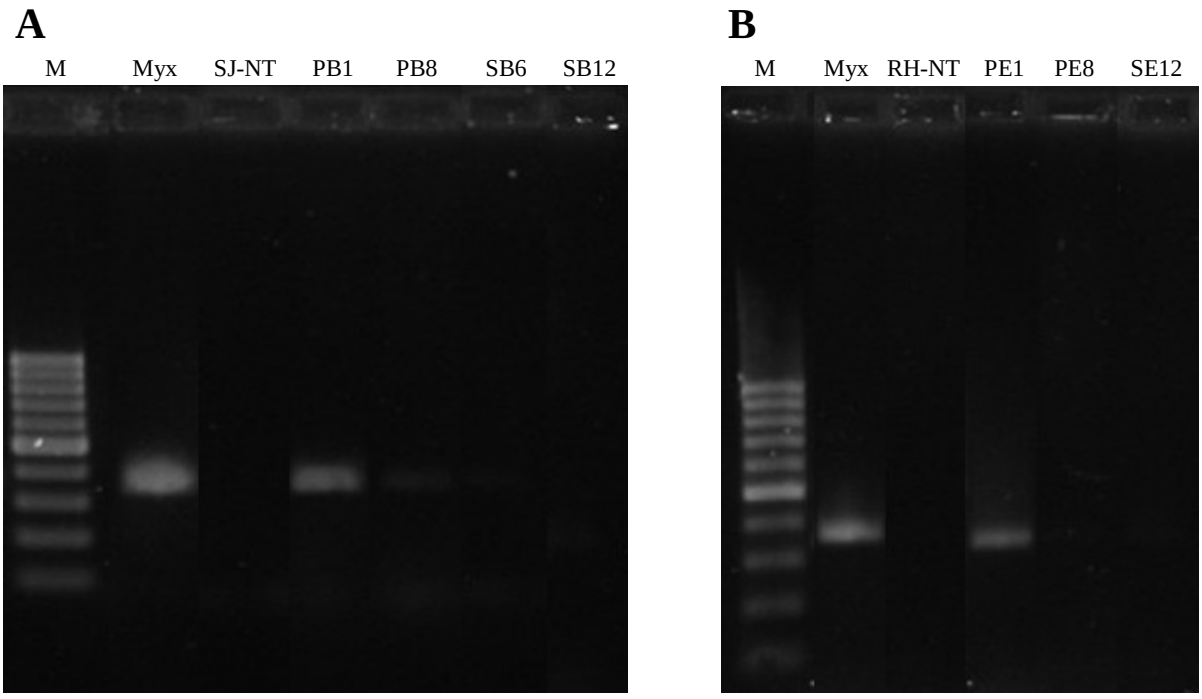
Table 14

Genera detected on the stone of the Monastery of San Jerónimo and the Royal Hospital of Granada. Figures in the table show the numbers of sequenced clones related to the corresponding genus. Percentages in brackets indicate to proportion of the clones to the total sequenced clones of the related sample. SJ stands the Monastery of San Jerónimo; RH for the Royal Hospital of Granada.

Stone samples		**Non-treated**		**Treatments**								
				Water		***M.xanthus***			**M-3P media**			
	Building	SJ	RH	SJ	RH	SJ	SJ	RH	SJ	SJ	RH	RH
	Time of sampling	0	0	6 months	12 months	6 months	12 months	12 months	6 months	12 months	12 months	12 months
	Sample / Genera	SJ-NT	RH-NT	SA6	SD12	SB6	SB12	SE12	SC6	SC12	SF12-1	SF12-2
Alpha-Proteobacteria	*Rhizobiaceae/Agrobacterium*	1 (16.7%)	1 (5.6%)	1 (8.3%)	1 (5%)	1 (10%)						
	Porphyrobacter				1 (5%)							
	Bosea				1 (5%)							
Beta-Proteobacteria	*Comamonas*	2 (33.3%)	1 (5.6%)	2 (16.7%)		1 (10%)				1 (14.3%)		
	Delftia	1 (16.7%)		1 (8.3%)	2 (10%)							
	Thiobacillus				1 (5%)							
	Methylobacterium				1 (5%)							
	Hydrogenophaga				1 (5%)							
	Neisseria		1 (5.6%)									
	Bacterium J10				1 (5%)							
Gamma-Proteobacteria	*Pseudomonas*		1 (5.6%)	3 (25%)	2 (10%)							
	Stenotrophomonas				1 (5%)							
	Enterobacter		1 (5.6%)									
	Pseudoalteromonas		2 (11.1%)			1 (10%)						
	Xanthomonas	1 (16.7%)										
Actinobacteria	*Micrococcus*		1 (5.6%)									
	Kocuria		1 (5.6%)									1 (10%)
	Corynebacterium		1 (5.6%)			1 (10%)						
	Brachybacterium				1 (5%)							
	Uncultured actinobacteria				2 (10%)							1 (10%)
Firmicutes	*Bacillus*	1 (16.7%)	4 (22.2%)	2 (16.7%)	1 (5%)	5 (50%)	9 (100%)	7 (100%)	1 (10%)	2 (28.6%)	1 (11.1%)	

	Exiguobacterium				1 (5%)							
	Virgibacillus								2 (20%)			
	Planococcaceae			1 (8.3%)	3 (15%)				7 (70%)	4 (57.1%)	7 (77.8%)	7 (70%)
	Brevibacillus		1 (5.6%)									
	Desemzia											1 (10%)
	Streptococcus		1 (5.6%)									
	Uncultured bacterium		2 (11.1%)								1 (11.1%)	
Bacteroidetes	*Flavobacterium*			1 (8.3%)		1 (10%)						
	Uncultured Bacteroidetes bacterium			1 (8.3%)								
Total number of clones		6 (100%)	18 (100%)	12 (100%)	20 (100%)	10 (100%)	9 (100%)	7 (100%)	10 (100%)	7 (100%)	9 (100%)	10 (100%)

Figure 23



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Präsentationen

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Piñar, G., Jimenez-Lopez, C., Sterflinger, K., Ettenauer, J., Jroundi, F., Fernández-Vivas, A., Gonzalez-Muñoz, M. T. 2010. Bacterial community dynamics during the application of a *Myxococcus xanthus*-inoculated culture medium used for consolidation of ornamental limestone. Microbial Ecology. In press DOI 10.1007/s00248-010-9661-2.