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

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Abstract: In the last few 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 Chapel of St. Virgil 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 the original medieval paintings. The salt, together with the 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 analysed 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.

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Key words: *Archaea*, DNA-fingerprints, Halobacillus, halophilic microorganisms, molecular monitoring, phylogenetic identification, salt efflorescences.

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 the 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 efflorescence (Amoroso & Fassina 1983). These extremely saline environments represent niches for the settlement of halophilic and halotolerant microorganisms (Saiz-Jimenez & 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 & 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–c, 2009; Gonzalez & 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 2001; Täubel 2001; Ripka *et al.* 2006; Piñar *et al.* 2009). The last investigation, in relation to a sampling performed on 2004, revealed 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 communities 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 efflorescence 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 Fig. 1). 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 a substrate the cellulose remaining in the fissures after the treatment. Therefore, restorers 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 fractions 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 the 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 analysed by different molecular techniques, such as random amplified polymorphic DNA (RAPD) analysis, 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.

Methods

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 (Fig. 1A). The whole chapel is covered by salt efflorescence that is visible by the naked eye. Therefore, the chapel underwent four desalination treatments (in 2001, 2002, 2005 and 2008) with wet compresses (Fig. 1B). 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 the salt efflorescence (Fig. 1C). One additional sample (VK-E) was taken by scraping off the wall material. Another sample (VK-A) was collected from some detached wall material that had trickled onto the floor (Fig. 1D). 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.

Enrichment cultures

Three different media were used for enrichment: Trypticase Soy Agar (TSA), Maintenance Medium (HMM, 10% NaCl)

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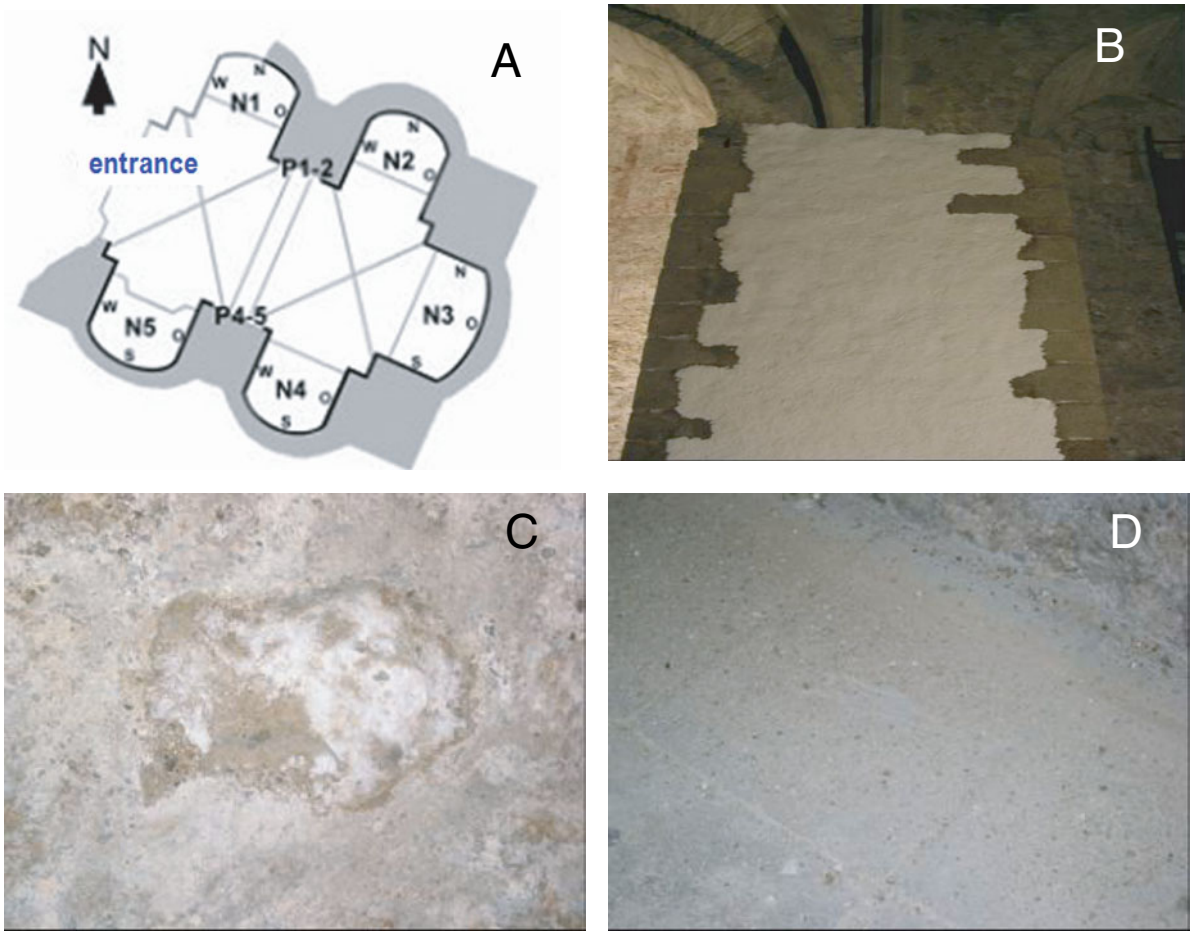

Fig. 1. (A) Ground view of the Chapel of St. Virgil. (B) Treatment with wet compresses applied for desalination. (C) Salt crusts of sodium chloride on plaster. (D) Wall material detached on the floor of the chapel.

(Spring *et al*. 1996) and M2 medium (20 % NaCl) (Tomlinson & Hochstein 1976). Enrichments were conducted in 300 ml Erlenmeyer flasks containing 50 ml of medium. Flasks were incubated aerobically at room temperature (22 ± 3 °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 pellet collected was frozen at −20 °C 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 °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 °C for conservation.

Molecular characterization

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 analysis was carried out in 25 µl volumes and 2.5 µl of template was added.

For DGGE analysis, 200 base pair 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 °C), followed by 30 cycles each consisting of 1 min denaturation (95 °C), 1 min primer annealing (55 °C) and 1 min primer extension (72 °C), with a final extension step of 72 °C for 5 min.

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.

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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 programme 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 min 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 primer 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 programme was used as described for amplification of bacterial 16S rDNA.

DGGE. DGGE was carried out as previously described (Muyzer *et al.* 1993) using a D GENE-System (Bio-Rad) in 0.5 × TAE (20 mM Tris, 10 mM acetate, 0.5 mM Na2EDTA; 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% for screening of archaeal communities. Gel electrophoretic separation was carried out at 60 °C 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).

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 were at least 17 nt in length (namely, primers D14216, D11344 and D14307) (Ripka *et al.* 2006). To the 1 × 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 °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 primer oligos of at least 17 nt were used, the cycling program was four 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 & 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 min at 70 V, stained in an ethidium bromide solution (1 µg/ml; stock 10 mg/ml) for 30–45 min 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.

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 bromr-4-cloro-3-indolyl-ß-D-galactorpyranoside; 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.

16S rDNA sequencing and phylogenetic analyses. For sequencing of clone inserts, 2 × 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 °C, followed by 30 cycles consisting of 1 min denaturation at 95 °C, 1 min primer annealing at 55 °C and 2 min primer extension at 72 °C, followed by a final extension step of 5 min at 72 °C. 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 manufacturer'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 1 (bacterial strains) and Table 2 (archaeal clones).

Results and discussion

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 visualized 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 2 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 contrast, DGGE profiles derived from the enrichment 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 Fig. 2), indicating a succession in the community during the time of cultivation as mentioned above.

In summary, the results shown in Fig. 2 reveal a higher population of microorganisms in the original wall sample communities than in the corresponding enrichment cultures (see the 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 & Giovannoni 2003).

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.

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 3 shows the DGGE profiles derived from one representative strain of each of the 19 groups. Table 1 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 & Smalla, 1998).

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

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Table 1. *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	Percentage of the individual RAPD group	Strains in the same RAPD group	*DGGE cluster*	*Percentage 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%	$81^{3,D}$, $104^{3,E}$, $105^{3,E}$, $106^{3,E}$, $108^{3,E}$, $113^{3,C}$, $114^{3,E}$, $115^{3,E}$, $116^{3,E}$	*R*	*32.7%*	*23, 24, 25, 59, 64, 65, 73, 79, 81, 83, 84, 85, 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	FN435901
$85^{3,E}$	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 2. *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

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Table 2 (*cont.*)

Clone number [sequence. Length, bp]	Closest identified phylogenetic relatives [EMBL accession number]	Similarity (%)	Accession number
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 to 100%.

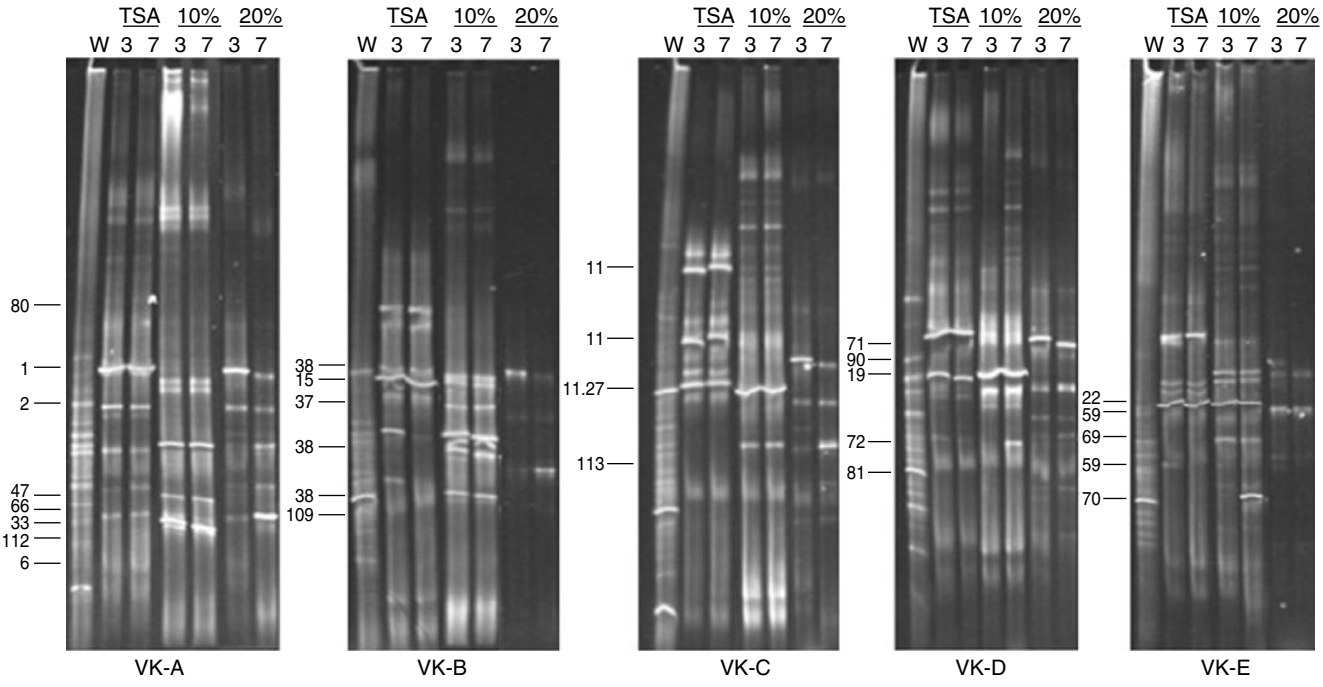

Fig. 2. DGGE fingerprints derived from the five original wall samples (VK-A, VK-B, VK-C, VK-D and VK-E (marked as W)) as well as the community DGGE fingerprints obtained from their corresponding enrichments in the three different culture media (marked as TSA, 10% and 20%) after 3 and 7 days of incubation (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. The phylogenetic affiliations of bacterial strains are showed in Table 1.

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 the 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 into 29 different fingerprint patterns (Fig. 4). Table 1 shows, in addition to the DGGE grouping,

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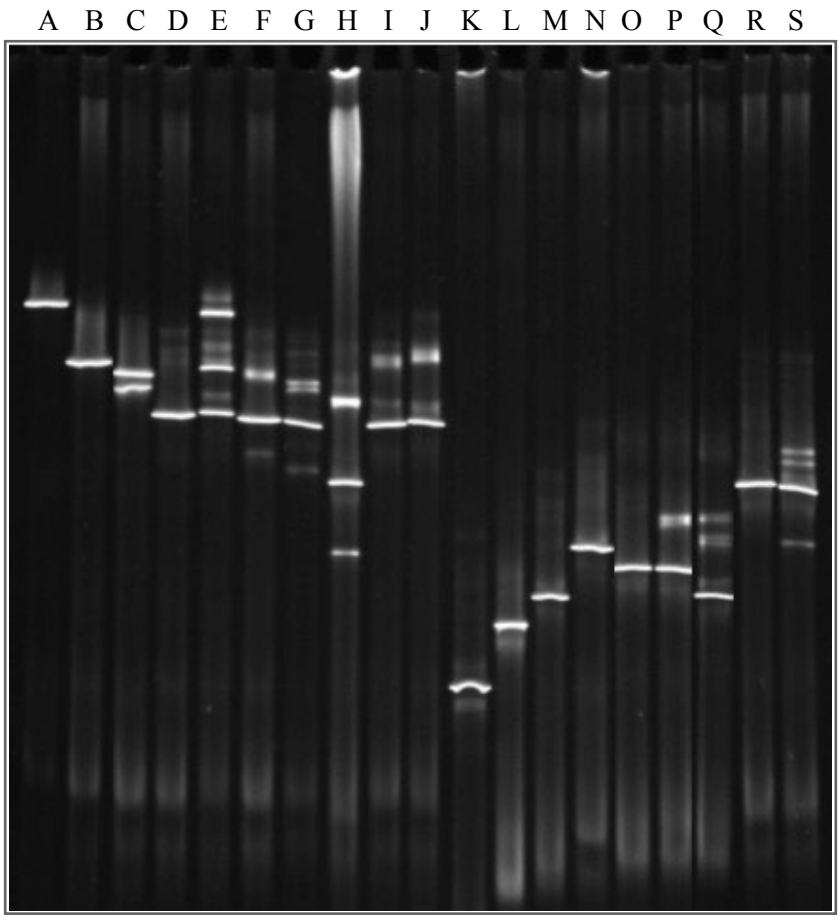

Fig. 3. DGGE-profiles derived from one representative strain of each of the 19 DGGE groups. Lane A: strain 80, lane B: strain 71, lane C: strain 1, lane D: strain 2, lane E: strain 11, line F: strain 29, line G: strain 31, line H: strain 38, line I: strain 22, line J: strain 15, Line K: 110, line L: 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.

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 the basis of the DNA diversity in the entire bacterial genome, therefore offering a broad spectrum of genetic variation (Welsh & McClelland 1990; Williams *et al*. 1990).

Phylogenetic analyses of selected bacterial isolate. 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 Fig. 4) 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 1.

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 *Firmicutes* 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*

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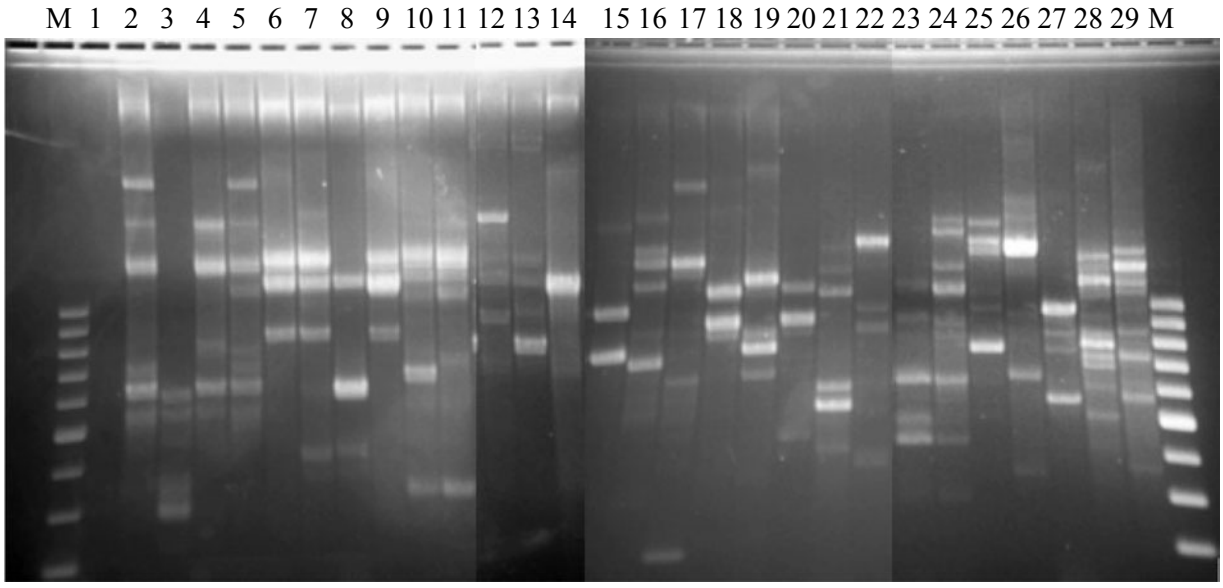

Fig. 4. RAPD-PCR patterns of representative strains of the 29 RAPD groups. Line 1: strain 34 representing group I; line 2: strain 41 representing group II; line: strain 47 representing group III; line 4: strain 66 representing group IV; line 5: strain 109 representing group V; line 6: strain 86 representing 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.

genus accounted for 35.6% of the isolated strains, being related with seven different species (see Table 1). 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 the 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).

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 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

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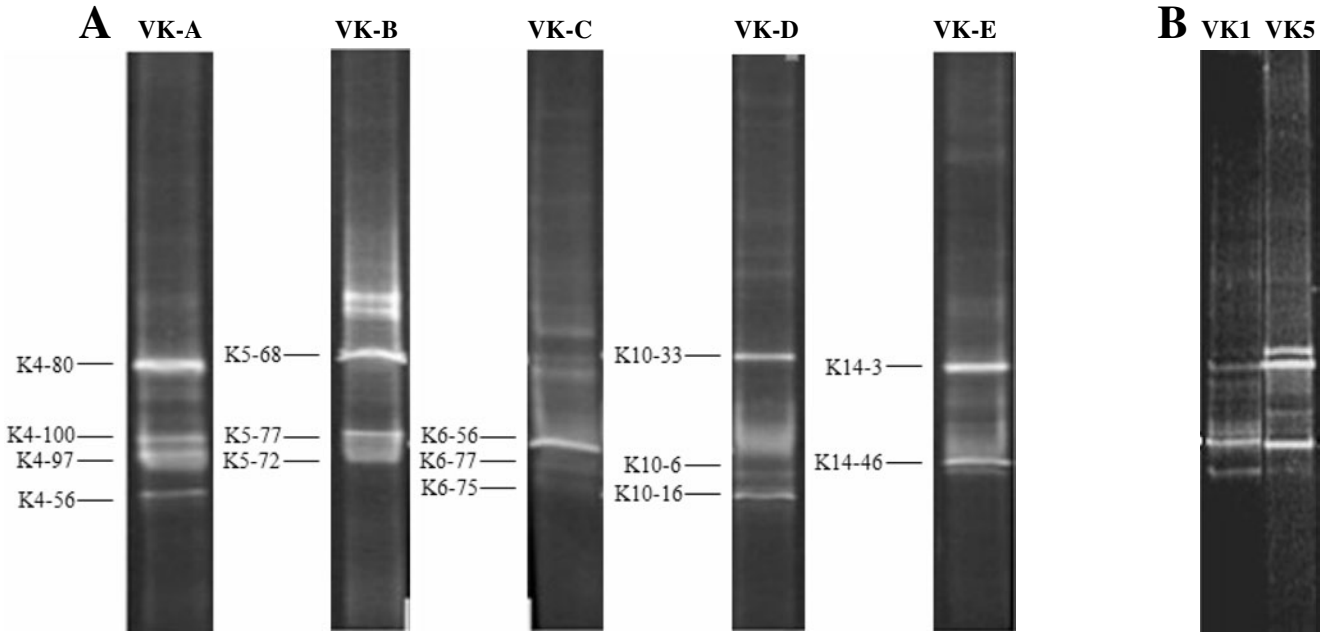

Fig. 5. DGGE profiles derived from the archaeal community inhabiting the walls of the Chapel of St. Virgil (VK-A, VK-B, VK-C, VK-D, VK-E) in the sampling of 2008 (A) and from samples VK1 and VK5 from sampling of 2004 (B) (Piñar *et al*. 2009). Clones matching with intense bands on the DGGE profile are indicated on the figure. The phylogenetic affiliations of the clones are showed in Table 2.

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 & 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 building materials.

In general, the microbial diversity were 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 could 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 that of 2008 using conventional culture-dependent techniques. As already shown in Fig. 2, 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 & 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., irrespective 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 investigated period of time (2004–2008).

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 (Fig. 5A) and, in addition, to perform a comparison with the archaeal community detected in the sampling of 2004 (Fig. 5B). The community fingerprints derived from samples collected in 2008 showed one to four dominant bands and some other

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faint bands (Fig. 5A), strongly resembling the archaeal fingerprints obtained in the sampling of 2004 (Fig. 5B).

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 2). 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 closely 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 2), were phylogenetically related to sequences already detected in the Chapel of St. Virgil in 2004 (Ripka 2005). These results show that, despite 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 2).

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 micro-biota inhabiting the Chapel consist 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 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 a 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 disturbance of their environment.

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