International Journal of Speleology	41(2)	125-136	Tampa, FL (USA)	July 2012

Available online at scholarcommons.usf.edu/ijs/ & www.ijs.speleo.it

International Journal of Speleology

Official Journal of Union Internationale de Spéléologie

Chemoorganotrophic bacteria isolated from biodeteriorated surfaces in cave and catacombs

Filomena De Leo[1], Agnese Iero[1], Gabrielle Zammit[2], and Clara E. Urzì[1]

Abstract:

De Leo F., Iero A., Zammit G. and Urzì C. 2012. Chemoorganotrophic bacteria isolated from biodeteriorated surfaces in cave and catacombs. International Journal of Speleology, 41(2), 125-136 Tampa, FL (USA). ISSN 0392-6672. http://dx.doi.org/10.5038/1827-806X.41.2.1

The main objective of this work was the comparative analysis of a large number of bacterial strains isolated from biodeteriorated surfaces in three different sites, namely the catacombs of St. Callistus in Rome, Italy, the catacombs dedicated to St. Agatha in Rabat, Malta and the Cave of Bats in Zuheros, Spain. Our results showed that even considering only culturable chemoorganotrophic bacteria the variability is very high, reflecting the great variety of microhabitats present. Hence any strategies to prevent, control or eliminate the biofilm-embedded microbiota from an archeological surface should take into account a number of considerations as stipulated in our study.

Keywords: biofilm; catacombs; caves; chemoorganotrophic bacteria; clustering; 16S rDNA sequencing

Received 20 October 2011; Revised 2 January 2012; Accepted 10 January 2012

INTRODUCTION

Any study of microbial communities colonizing biodeteriorated surfaces should involve a combination of several analytical techniques such as microscopy, culture techniques, biochemical tests and molecular tools, which are designed to give complementary results. Each individual technique while having its own limitations, contributes the necessary information to provide a better understanding of the microbial community as a whole and its role in the deterioration of inorganic and/or organic substrata (Urzì et al., 2003).

Culture-based techniques are selective due to the limited choice of media used for the cultivation of microorganisms, and because the viable and culturable microflora (VCM) may be restricted to 1 to 5% of the whole population (Amann, 2000). However, the culture-based approach offers the possibility to isolate and thus analyze a great number of strains (Donachie et al.,2007). It is then possible to study the isolated strains, to cluster them into operational taxonomic units (OTUs), to compare different microbial communities on the basis of types of cultivable bacteria isolated in term of richness and the frequency of isolates, distribution or relative abundance of types. Furthermore, cultivation-based techniques in association with other complementary molecular techniques give a good idea of the "microorganisms in action" because, in most cases, strains that grow in culture can prove to be metabolically active microorganisms, if the environment provides the required conditions. This is particularly true in the conservation of indoor Cultural Heritage monuments where most of the dangerous microorganisms for the artefact itself (biodeteriogens) are those that grow epilithically on the surfaces; they form a biofilm and cover areas that are the most valuable because are sculptured or painted.

Very often, in fact, stone surfaces are hidden by unaesthetic colonization due to phototrophic and chemoorganotrophic biofilms (Roldán & Hernández-Mariné, 2009), and it is a common practice to treat those surfaces with biocides in order to eradicate the biodeteriogens present (Nugari et al., 2009).

However, biocides commonly applied on valuable surfaces are not always completely successful to eradicate the complex community within the biofilm (Salvadori & Charola, 2011). Thus it is important to study the culturable fraction of microorganisms in order to test in the laboratory if the chemical compounds used are effective against the deteriogenic microflora. For this reason, when dealing with a large number of strains, it is imperative to use a reliable and easily implemented technique to group the strains into homogeneous clusters and reduce the amount of work to be carried out to characterize the isolates.

In this research study a large number of cultivable bacteria isolated from three sites were analyzed through a multi-step approach that included the frequency of types of colony, preliminary description of their micro-morphology, clusterization of all the isolates via ITS-PCR, and the identification of selected strains within each cluster.

[1]Department of Life Sciences, "M. Malpighi" University of Messina, Viale F. Stagno d'Alcontres 31, 98166 Messina, Italy; Corresponding author: Clara Urzì (clara.urzi@unime.it)

[2]Department of Physiology and Biochemistry, University of Malta, Biomedical Sciences Building, Msida, MSD2620, Malta.

MATERIAL AND METHODS

Areas of study

Sampling campaigns were carried out in the Ocean Cubicle (CSC13), inside the Catacombs of St. Callistus (Rome, Italy), in the Cave of Bats (Z1 to Z8) close to the village of Zuheros (Córdoba, Southern Spain) in the frame of the research activity carried out during the European project CATS (EVK4-2000-00028), and in different areas of St. Agatha's Crypt and Catacombs (SA) in Rabat, Malta, during the activity carried out in the framework of a COST Action G8 scientific mission (COST-STSM-G8-1435) (Zammit et al., 2009).

In both catacombs the relative humidity (RH) was always above 90% while the Mediterranean climate of the areas did not influence the inner temperature of the catacombs due to their deep location (range 15-20°C) (Albertano et al., 2003; Zammit et al., 2009). The Cave of Bats presented a RH variable from 95 (inner part of the cave) to 56% (near the exit) and an average temperature between 8 and 14ºC, depending on the area, at the time of sampling (Urzì et al., 2010). Samples were taken aseptically in correspondence to alterations on rock surface described as black spots, green patina, whitish/grey patinas with scalpel and/or adhesive tape as shown in Table 1.

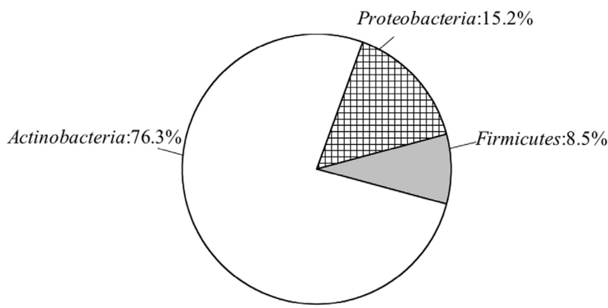
Cultural analyses

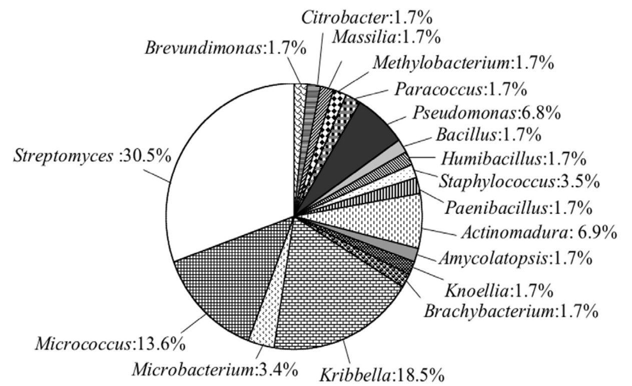
For the isolation of chemoorganotrophic microorganisms samples were processed as described by Urzì et al. (2010). The following agarized media were used: BRII medium (Bunt and Rovira, 1955 modified as reported in Urzì et al., 2001), SC (Starch Casein KNO_3 agar, Kuster & Williams, 1964) and R2A (Reasoner and Geldreich, 1985 Oxoid); in all media 0.05% cycloheximide was added to avoid/limit the growth of unwanted fungal contaminants. Incubation was carried out at 28° C up to one month to allow slow-growing strains. At the end of incubation time, enumeration of microorganisms as cfu/g of sample was carried out and randomly chosen bacterial strains (10/20 colonies per sample) were isolated on Trypticase Soy Agar (TSA, Oxoid). The bacterial isolates were maintained on TSA (Tryptone, Soy Agar) or GYM (Glucose, Yeast extract, Malt extract, Agar).

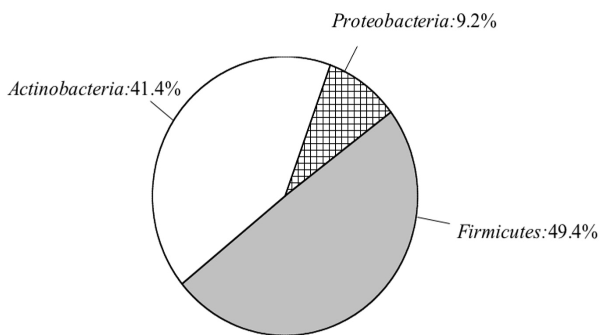
Bacterial strains were preliminarily characterized by their macro- and micro-morphology, their Gram staining, catalase and oxidase activity. The strains were then clustered on the basis of their ITS profiles. Randomly selected strains belonging to the same profile were identified through 16S rDNA partial sequencing.

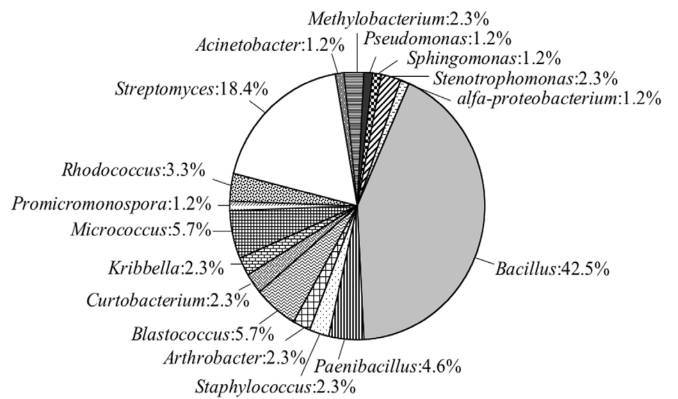
Table 1. Samples, modality of sampling type of alteration, surface in three site studied. Presence and/or absence phototrophic organisms was also considered

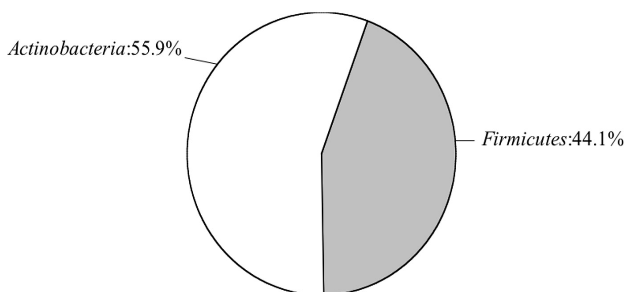
Sampling site	Alteration type	Type of substrate/ Sampling modality	Presence of phototrophs
CSC13a, CSC13b, CSC13c	Whitish/grey patina	Tufa/Scalpel	N
CSC13i, CSC13f	White patinas on top of green biofilm	Fresco/Adhesive tape	Y
CSC13h	Interface between green biofilm and white patina	Fresco/Adhesive Tape	Y
CSC13d	no apparent colonization after 6 months from biocide treatment	Fresco/Adhesive tape	Y
CSC13e,	Dark Green biofilm	Fresco/Adhesive tape	Y
CSC13g	Black spots	Tufa/Adhesive tape	Y
Z1a	Dark grey spots	Limestone/Scalpel-Adhesive tape	Y
Z1b	Red spots	Limestone/Scalpel-Adhesive tape	Y
Z3b, Z3c	Black alteration	Limestone/Scalpel-Adhesive tape	Y
Z2a, Z2b, Z3a, Z4a, Z4b, Z5a, Z5b, Z5d, Z6a, Z6b, Z7a, Z8a	Green patina	Limestone/Scalpel-Adhesive tape	Y
SA1, SA14, SA15, SA16, SA20	Reddish spots	Limestone/Adhesive tape	Y
SA2, SA3, SA8, SA9, SA13, SA17, SA18	Green patina	Limestone, Plaster/Adhesive tape	Y
SA4, SA7, SA19, SA23	Whitish/grey patina	Limestone/Scalpel-Adhesive tape	N
SA10, SA12	Dark grey spots	Plaster, Fresco/Adhesive tape	Y
SA6	Yellowish spots	Plaster/Adhesive tape	N











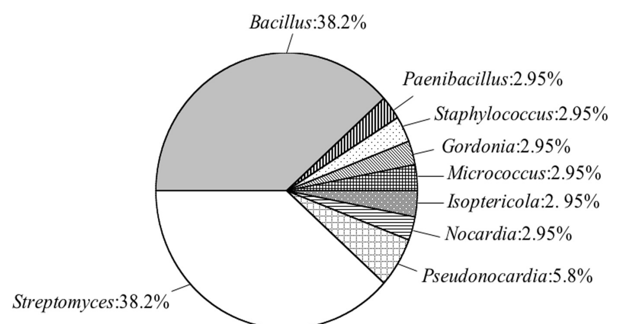


Plate 1. Graphic representation of distribution of bacteria isolated from St. Callistus catacombs (a, b), Cave of Bats (c, d), and St. Agatha catacombs (e, f). On the left the different classes of bacteria isolated (a, c and e); on the right are represented the different genera and the respective percentage of isolation (b, d and f).

Bacterial strains

180 strains were isolated and considered in this study. In particular, 59 bacterial strains were isolated from biofilm samples taken from the Ocean Cubicle (CSC13), in the St. Callistus Catacombs in Rome, Italy, 87 strains were isolated from 16 samples taken from the Cave of Bats in Zuheros, Spain and 34 strains were isolated from 19 samples taken from St. Agatha Crypt and Catacombs in Malta.

ITS-PCR

Genomic DNA was extracted from all the bacteria, as described by Rainey and co-workers (1996), after growth of the strains on TSA medium for 7 days.

Amplification of ITS was carried out using the primer pair F1492 (5'AAGTCGTAACAAGGTAGCCG3') and R188 (5'GGTACTTAGAGTTTTCAGTTC) (Gurtler & Stanisich, 1996) in a final volume of 50 µl containing 2.5 U of Taq DNA polymerase (Pharmacia Biotech, Italy), 200 mM (each) deoxynucleoside triphosphates (dNTPs), 0.2 mM of each primer in 1X reaction buffer [1.5 mM $MgCl_2$, 50 mM KCl, Tris-HCl (pH 9.0)] and 100 ng of DNA with a DNA thermal cycler 2400 (Perkin Elmer Cetus, Norwalk, USA). The profile temperature was as follows: initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min and extension at 72 °C for 90 s, and final extension at 72 °C for 7 min.

The PCR products were purified through a QIAquick PCR Purification kit according to the supplier's instructions (Qiagen, Milan, Italy) and then separated on a 2% agarose gel stained with ethidium bromide. Profiles were examined using Kodak Digital Science 1d 2.0 software and the analysis was carried out on the basis of the number and size of bands as compared to a 50bp DNA ladder marker (Invitrogen, Milan, Italy).

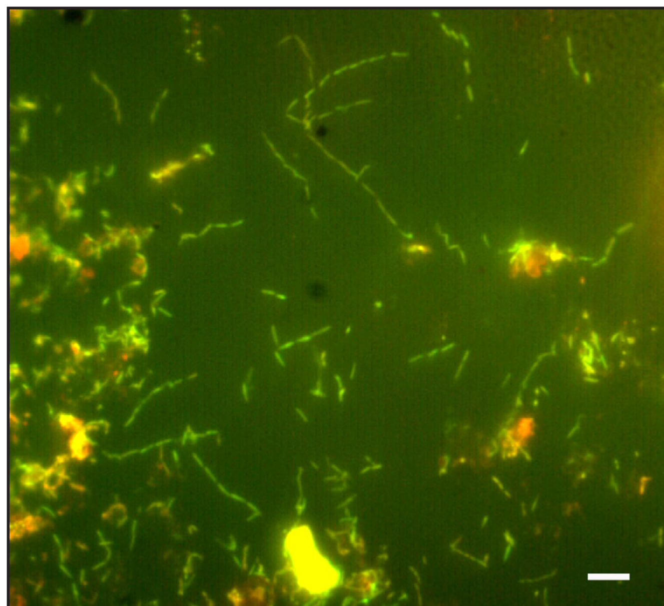


Fig. 1. Direct observation under epifluorescent microscopy of adhesive tape sample SA7 taken in Malta Catacombs. Chain of rod shaped spore forming bacteria were visible after Acridine orange staining. Inorganic material showed a bright self-fluorescence. Bar is 10 µm.

Table 2. Proteobacteria isolated from the Ocean Cubicle in St. Callistus Catacombs and from Zuheros Cave. Strains were clusterized on the basis of their ITS-PCR profile.

Nr. ITS clusters	CSC13	Zuheros*	St. Agatha	Nearest relative on the basis of 16S rDNA sequences similarity#
1		Z5d4		*Acinetobacter lwofii* FJ529917 (98.2%)
1	CSC13b 9			*Brevundimonas diminuta* X87274 (99.9%)
1	CSC13i 3			*Citrobacter sp. EU341320* (99.3%)
1	CSC13a22			*Massilia timonae* AY157761 (99.9%)
1		Z1b10		*Methylobacterium extorquens* AJ400917 (99%)
1	CSC13b2			*Methylobacterium populi* CP001029 (99.5%)
1		Z7a8		*Methylobacterium sp.* AJ400934 (99.5%)
1	CSC13d7			*Paracoccus yeeii* AY014173 (100%)
2	CSC13g4 CSC13g2			*Pseudomonas putida* AF094736 (100%)
1		Z6a7		*Pseudomonas sp.* ABO13843 (98.6%)
1	CSC13g7 CSC13i3			*Pseudomonas stutzeri* CP000304 (99.9%)
1		Z2b7		*Sphingomonas aerolata* AJ429240 (98.8%)
1		Z6a2 Z6a6		*Stenotrophomonas maltophila* AJ295673 (98.3%-100%)
1		Z1a13		*Uncultured alpha proteobacteria* AY133099 (99.2%)

Underlined strains were those identified by sequencing of 16S rDNA; (*) data taken from Urzì et al., (2010).

16S rDNA sequencing

Due to the reliability of the ITS-PCR method as already reported by Pangallo et al. (2009), identification procedures were carried out on randomly selected strains belonging to each cluster (see underlined strains in Table 2 and Appendices 1-3).

Genomic DNA was extracted as described before and amplification was carried out using the universal primers F27 (5'AGAGTTTGATCCTGGCTCAG3') and R1492 (5'CGGCTACCTTGTTACGACTT3') (Life Technologies, Italy) in a final volume of 100 ml containing 2.5 U of Taq DNA polymerase (Pharmacia Biotech, Italy), 200 mM (each) deoxynucleoside triphosphates (dNTPs), 0.2 mM of each primer in 1X reaction buffer [1.5 mM $MgCl_2$, 50 mM KCl, Tris-HCl (pH 9.0)] and 100 ng of DNA with a DNA thermal cycler 2400 (Perkin Elmer Cetus, Norwalk, USA). The profile temperature was as follows: initial denaturation at 95 °C for 2 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 90 s, and final extension at 72 °C for 7 min and cooling at 4 °C. The presence and yield of specific PCR products, approximately 1500 bp long, were visualized on agarose (1%) gel electrophoresis after staining with ethidium bromide (50 ng/ml) for 30 min at 7 V/cm.

The PCR products obtained, were purified using a QIAquick PCR Purification kit according to the supplier's instructions (Qiagen, Milan, Italy), and sequenced by GENELAB (Rome, Italy), using the universal primer F27.

Sequences obtained were compared with those published on the EMBL-EBI databank (http://www.ebi.ac.uk/fasta33/nucleotide.html) and alignment of sequences was carried using ClustalW software (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

RESULTS

83 PCR profiles were obtained after amplification of the ITS region, followed by separation of the PCR products by electrophoresis. Taxonomic assignment for each ITS-PCR profile was carried out after sequencing of the 16S rDNA of the randomly selected species from each cluster as reported in Table 2 and Appendices 1-3.

The comparative analysis of microflora evidenced that within each habitat considered, the composition, structure and distribution of strains was quite different.

In fact, on the basis of taxonomic assignment, the bacteria belonged to the Classes of Proteobacteria, Firmicutes and Actinobacteria (Plate 1 a, c, e) had a different percentage in each of the three sites. In particular the following observations were made:

Proteobacteria constituted a small fraction of the bacteria isolated. In fact, they ranged between 9.2% and 15.2% respectively in Cave of Bats and CSC13 while they were not isolated from the samples taken from St. Agatha catacombs.

Firmicutes were isolated in high percentages from the microbial community sampled from the Cave of Bats (49.4%) and St Agatha catacombs (44.1%), while in the CSC13 they were found to be present in very low percentage (8.5%).

In the catacomb sites, members of the Actinobacteria were the predominant microorganisms, especially in the catacombs of St. Callistus (76.3%), but also at St Agatha's (55.9%). At the Cave of Bats, the percentage of isolated Actinobacteria was lower than Firmicutes (41.4% vs. 49.4%).

As far as the frequency and relative abundance of genera in each site studied is concerned, the results are reported below and shown in Plate 1.

St. Callistus Catacombs

According to ITS-PCR analyses, the 59 bacterial strains fell in 30 different profiles and on the basis of the sequencing data into 26 OTUs (Plate 1a, b).

Among the Actinobacteria, the majority of strains belonged to the genera *Streptomyces* (30.5%), *Kribbella* (18.5%), *Micrococcus* (13.6%), *Actinomadura* (6.9%) and *Microbacterium* (3.4%); other genera were isolated with a lower frequency (1.7%) and their occurrence was considered to be occasional findings. Two new species of the genus *Kribbella*, namely *K. catacumbae* and *K. sancticallisti* were described from this site (Urzì et al., 2008). Among the Gram negative (15.2% of the total of isolated strains), *Pseudomonas* was the most common genus found (6.8%).

Firmicutes, namely species from the genera *Bacillus, Paenibacillus, Humibacillus,* and *Staphylococcus* were occasionally found (1.7%-3.4%).

Cave of Bats

According to ITS-PCR analyses, the 87 strains fell into 37 different profiles and 32 OTUs (Table 2, Appendices 1-3 and Plate 1c, d).

The majority of bacteria were Gram positive strains (90.6%) belonging to Firmicutes in the genera *Bacillus* (42.5%) and *Paenibacillus* (4.6%), and to Actinobacteria (41.2%) in the genera *Streptomyces* (18.4%) *Blastococcus* (5.7%), *Micrococcus* (5.7%), and *Rhodococcus* (3.3%).

Occasional found were bacteria of the genera *Arthrobacter, Curtobacterium, Kribbella, Promicromonospora* and *Staphylococcus* (2.3%). Among the Gram negatives (9.4% of total strains isolated) the strains belonging to the genera of *Methylobacterium,* (Alpha-Proteobacteria), *Pseudomonas, Sphingomonas,* and *Stenotrophomonas* (Gamma-Proteobacteria) were considered as occasional (1.2-2.3%). As reported in a previous paper (Urzì et al., 2010) it was observed that only a few species of *Bacillus* were found in almost all samples, while other bacteria were localized especially on those samples collected in places near to the entrance.

St. Agatha's Catacombs

According to ITS-PCR analyses, the 34 isolated strains clustered into 20 different profiles and 18 OTUs.

No Proteobacteria were isolated. The majority of cultured species belonged to the genus *Bacillus* (37.3%), followed by *Streptomyces* spp. (38.2%) and *Pseudonocardia* (5.8%) while *Paenibacillus, Isoptericola Gordonia, Micrococcus,* and *Nocardia* were less frequently isolated (2.95%). The spore-forming *B. aquimaris* were the most widespread.

DISCUSSION

The main aim of this study was the analysis of a large number of chemoorganotrophic bacteria colonizing three cave/catacomb sites belonging to different geographic regions in the Mediterranean area, which were characterized by well documented microclimatic parameters and biodeterioration patterns (Albertano et al., 2003; Zammit et al., 2009).

Caves and catacombs are biotopes whose surfaces are generally poor in nutrients, with relatively stable and low temperature with high relative humidity and mineral concentrations that provide various ecological niches for highly specialized microorganisms. It is a well known fact that chemoorganotrophic bacteria, mainly Actinobacteria such as *Streptomyces*, *Micrococcus*, *Arthrobacter*, *Brevibacterium*, *Nocardia* (Groth & Saiz-Jimenez, 1999; Laiz et al., 2003) are common inhabitants of stone surfaces in subterranean environments. Their presence in caves and catacombs has caused evident biodeterioration phenomena on the archaeological surfaces due to the formation of patinas and biofilms in which either phototrophic microorganisms or chemorganotrophic bacteria can prevail depending on the presence/absence of light sources (Albertano et al., 2003; Urzì et al., 2010; Zammit et al., 2011a).

However, as evidenced in the present research, apparently similar alterations (e.g. those described by whitish/grey patinas) can be due to different kind of bacteria that are often seen under the microscope and described as filamentous bacteria. In the past, these alterations were considered caused almost exclusively by the growth of different species of *Streptomyces* (Agarossi et al., 1985). We found, however, that despite their apparent homogeneity they harboured (or were due to) different types of bacteria (as shown in Table 3). Furthermore, both in caves and catacombs, it is common to find surfaces widely covered by white fluffy (cotton like) biofilm in which mineral precipitates are associated with bacteria, especially *Bacillus* (Sanchez-Moral et al., 2003; Cuezva et al., 2009), and several studies show that a number of strains may actively participate in the precipitation of mineral phases (Urzì et al. 1999; Sanchez-Moral et al., 2003; Zammit et al.

Table 3. Bacteria found in correspondence of whitish/grey patinas with no apparent cyanobacteria colonization.

	Samples	Bacterial strains
St. Callistus Catacombs	CSC13a	*Massilia timonae* *Humibacillus xanthopallidus* *Staphylococcus hominis* *Amycolatopsis lurida* ***Kribbella catacumbae*** ***Streptomyces nojiriensis*** *Streptomyces floccolosus* *Streptomyces spororaveus*
	CSC13b	*Brevundimonas diminuta* *Methylobacterium populi* *Knoellia subterranea* ***Kribbella sancticallisti*** *Microbacterium phyllosphaerae* ***Streptomyces nojiriensis*** ***Streptomyces avidinae***
	CSC13c	***Actinomadura fulvescens*** *Actinomadura cremea* ***Streptomyces avidinae***
St. Agatha Catacombs	SA4	***Streptomyces badius*** ***Streptomyces flavidofuscus*** *Streptomyces sp.*
	SA7	***Bacillus aquimaris***
	SA19	***Bacillus aquimaris*** *Nocardia uniformis* *Streptomyces chungwensis*
	SA23	***Streptomyces badius***

The dominant strains for each sample are shown in bold.

2011b). We found different species of *Bacillus* or closely related spore-forming bacteria (Table 3) growing in chains and resembling filament-like structures (Fig. 1). The biomediated precipitation of mineral phases along the cells, as already demonstrated by different authors (Sanchez-Moral et al., 2003; Cañaveras et al., 2006, Zammit et al., 2011b) may explain the cotton-like aspect of this kind of alteration.

The high number of spore forming bacteria isolated from the Cave of Bats may be explained by an occasional organic input present on the surface due to medium impact of visitors in this site (20.000 visitors year $^{-1}$) (Fernández-Cortes et al., 2008) and the low RH in most areas of this cave, which may induce sporulation. In St. Agatha catacombs spore forming bacteria belonging to the species *B. aquimaris* (Fig. 1), never found before in similar sites, were the most common and widely spread in the whole Catacombs while in Cave of Bats species such as *B. simplex* were the most widespread on the surfaces.

Due to the fact that members of the Proteobacteria were isolated only occasionally it is very difficult to hypothesize a biodeteriorative role. Their growth on lithic surfaces is related to the amount of water available. In the St. Callistus catacombs strains belonging to the Proteobacteria were isolated at a lower percentage with respect to the Actinobacteria and they seem to be unrelated to human contamination and be of environmental origin, intimately associated to the presence of cyanobacteria (Bruno et al., 2006). In fact, Proteobacteria are often associated with debris from cyanobacterial biofilms, and due to their metabolic versatility they may use several byproducts for their metabolic requirements (Albertano & Urzì, 1999; Berg et al., 2009). Their number is relatively low in presence of metabolically active phototrophic microflora, while their amount increases dramatically in stress conditions such as after biocide treatments (Urzì et al., 2012).

Recently, the analysis and identification of microorganisms colonizing cultural heritage sites has been prolific through the application of molecular techniques for the rapid analysis of the microbial communities inhabiting a given environment (Schabereiter-Gurtner et al., 2002a, b; Laiz et al., 2003; Urzì et al,. 2003; Gonzalez & Saiz Jimenez, 2005).

In this study we decided to focus our investigation to the portion of bacterial microflora that was able to grow in culture. In fact, in some cases, culture-dependent techniques deserve more credit because once it has been established the role of a particular bacterium or a bacterial consortium in biodeterioration processes by a polyphasic approach in which microscopy, molecular tools and cultivation are combined, further study on the isolates can help answering some important questions: a) which strain is the responsible of specific alteration pattern? and, b) if more than one species is isolated in correspondence of he same pattern: how do the different members of this biodeteriorative community interact to form that alteration? In addition, during biocide and any other cleaning treatments, the use of isolated strains can help answer other important questions: i) how do bacteria behave in response to the treatment? and, ii) is the dose of treatment (either physical or chemical) effective for all the strains?

In addition, Fluorescent In Situ Hybridization (FISH) carried out with a combination of probes prepared according to the isolated strains may allow to answer to the two first questions, while experiments carried out both in laboratory conditions as well as in the field could help answer the biocide questions. Furthermore, through the isolation of bacterial strains, it becomes possible to discover strains with interesting taxonomies (Groth et al., 2005, 2006; Jurado et al., 2005a, 2005b; Urzì et al. 2008; Zammit et al., 2010, 2011a), and also strains with biotechnological potential including applications in art conservation.

In conclusion, the alteration of surfaces observed in the three sites and described as dark spots, green biofilms or white to grey veils/patinas were caused by the colonization of highly diverse bacterial associations (Albertano et al., 2003; Urzì et al., 2010; Zammit et al., 2009; 2011a,b)

According to Curtis and Sloan (2004), this diversity observed depends also on the so-called 'metacommunity'. This means that each microbial community will depend on the pioneer "microbe" that succeeds to colonize a given surface first; it will control the further process of formation and assessment of whole microbial community, hence it is clear that every process of colonization is different from the others. This fact may be the reason why even in samples taken only from a very short distance apart, the variability within the same species was very high (Urzì et al., 2010). In our opinion, this genetic variability within the species colonizing a particular site may be associated to a variety of different responses to biocide resistance that should be taken into account in the planning of conservation strategies of stone surfaces in subterranean environments.

ACKNOWLEDGEMENTS

This work was supported by the EU Programs EESD, the European Commission FP6 project CATS (contract EVK4-CT2000-00028), the COST G8 scientific mission (COST-STSM-G8-1435) and the University of Messina through Progetti di Ricerca di Ateneo P.R.A. years 2008-2010.

The Authors would like to express their gratitude for the kind cooperation of dott.ssa R. Giuliani of the "Pontificia Commissione di Archeologia Sacra (CdV)", at the Christian catacombs of Rome, to Fr. V. Camilleri MSSP, curator of St. Agatha Crypt and Catacombs in Malta and all the staff of Cave of Bats, in Spain.

The Authors would like to express a special thank to Prof. C. Saiz Jimenez and Prof. Diana Northup for their careful revision of the text and their valuable help to improve the manuscript.

REFERENCES

Agarossi G., Ferrari R. & Monte M. 1985 - *Microbial biodeterioration in the hypogea: the subterranean neo-pythagorean basilica of Porta Maggiore in Rome*. In: Furlan V. (Ed)., *5th International Congress on Deterioration and Conservation of Stone*. Lausanne: Presses Polytechniques Romandes, **2**: 597-605.

Albertano P. & Bruno L., 2003 - *The importance of light in the conservation of hypogean monuments*. In: Saiz-Jimenez C. (Ed)., *Molecular Biology and Cultural Heritage*. Lisse: Swets & Zeitlinger: 171-177.

Albertano P., Moscone D., Palleschi G., Hermosín B., Saiz-Jimenez C., Sánchez-Moral S., Hernández-Mariné M., Urzì C., Groth I., Schroeckh V., Saarela M., Mattla Sandholm T., Gallon JR., Graziottin F., Bisconti F. & Giuliani R., 2003 - *Cyanobacteria attack rocks (CATS): control and preventive strategies to avoid damage caused by cyanobacteria and associated microorganisms in Roman hypogean monuments*. In: Saiz-Jimenez C. (Ed)., *Molecular Biology and Cultural Heritage*. Lisse: Swets & Zeitlinger: 151-162.

Albertano P. & Urzì C., 1999 - *Structural interactions among epilithic cyanobacteria and heterotrophic microorganisms in Roman Hypogea*. Microbial Ecology, **38**: 244-252. http://dx.doi.org/10.1007/s002489900174

Amann R., 2000 - *Who is out there? Microbial aspects of diversity*. Systematic and Applied Microbiology, **23**: 125-136. http://dx.doi.org/10.1016/S0723-2020(00)80039-9

Berg K.A., Lyra C., Sivonen K., Paulin L., Suomalainen S., Tuomi P. & Rapala J., 2009 - *High diversity of cultivable heterotrophic bacteria in association with cyanobacterial water blooms*. The ISME Journal, **3**: 314-315. http://dx.doi.org/10.1038/ismej.2008.110

Bruno L., Billi D., Urzì C. & Albertano A., 2006 - *Genetic characterization of epilithic cyanobacteria and their associated bacteria*. Geomicrobiology Journal, **23**: 293-297. http://dx.doi.org/10.1080/01490450600760732

Bunt J.S. & Rovira A.D., 1955 - *Microbiological studies of some subantartic soils*. Journal of Soil Science, **6**: 119-128. http://dx.doi.org/10.1111/j.1365-2389.1955.tb00836.x

Cañaveras J.C., Cuezva S., Sanchez-Moral S., Lario J., Laiz L., Gonzalez J.M. & Saiz-Jimenez C., 2006 - *On the origin of fiber calcite on moonmilk deposits*. Naturwissenschaften, **93**: 27-32. http://dx.doi.org/10.1007/s00114-005-0052-3

Cuezva S., Sanchez-Moral S., Saiz-Jimenez C. & Cañaveras J.C., 2009 - *Microbial communities and associated mineral fabrics in Altamira Cave, Spain*. International Journal of Speleology, **38**: 83-92.

Curtis T.P. & Sloan W.T., 2004 - *Prokariotic diversity and its limits: microbial community structure in nature and implications for microbial ecology*. Current Opinion in Microbiology, **7**: 221-226. http://dx.doi.org/10.1016/j.mib.2004.04.010

Donachie S.P., Foster J.S. & Brown M.V., 2007 - *Culture clash: Challenging the dogma pf microbial diversity*. The ISME Journal, **1**: 97-102. http://dx.doi.org/10.1038/ismej.2007.22

Fernández-Cortés A., Calaforra J.M., Martín-Rosales W., González-Rios, M.J. 2008. *Cavidades turísticas de Andalucía*. In: Calaforra Chordi J.M & Berrocal Pérez J.A. (Eds)., *El Karst de Andalucía*. Sevilla: Consejería de Medio Ambiente de la Junta de Andalucía: 105-116.

González J.M. & Saiz-Jiménez C., 2005 - *Application of molecular nucleic acid-based techniques for the study of microbial communities in monuments and artworks*. International Microbiology, **8**: 189-194.

Groth I. & Saiz-Jimenez C., 1999 - *Actinomycetes in hypogean environments*. Geomicrobiology Journal, **16**: 125-136. http://dx.doi.org/10.1080/014904599270703

Groth I., Schumann P., Schuetze B., Gonzalez J.M., Laiz L., Saiz-Jimenez C. & Stackebrandt E., 2005 - *Isoptericola hypogeus sp. nov., isolated from the Roman catacomb of Domitilla*. International Journal of Systematic and Evolutionary Microbiology, **55**: 1715-1719. http://dx.doi.org/10.1099/ijs.0.63632-0

Groth I., Schumann P., Schutze B., Gonzalez J.M., Laiz, L., Suihko M-L. & Stackebrandt E., 2006 - *Myceligenerans crystallogenes sp. nov., isolated from Roman catacombs*. International Journal of Systematic and Evolutionary Microbiology, **56**: 283-287. http://dx.doi.org/10.1099/ijs.0.63756-0

Gurtler V. & Stanisich V.A., 1996 - *New approaches to typing and identification of bacteria using the 16S-23S rDNA spacer region*. Microbiology, **142**: 3-16. http://dx.doi.org/10.1099/13500872-142-1-3

Kuster E. & Williams S.T., 1964 - *Selection of media for isolation of Streptomyces*. Nature, **22**: 928-929. http://dx.doi.org/10.1038/202928a0

Jurado V., Groth I., Gonzalez J.M., Laiz L., Schuetze B. & Saiz-Jimenez C., 2005a -*Agromyces italicus sp. nov., Agromyces humatus sp. nov. and Agromyces lapidis sp. nov., isolated from Roman catacombs*. International Journal of Systematic and Evolutionary Microbiology, **55**: 87125-13675. http://dx.doi.org/10.1099/ijs.0.63414-0

Jurado V., Laiz L., Gonzalez J.M., Hernandez-Marine M., Valens M. & Saiz-Jimenez C. 2005b - *Phyllobacterium catacumbae sp. nov., a member of the order 'Rhizobiales' isolated from Roman catacombs*. International Journal of Systematic and Evolutionary Microbiology, **55**:1487-1490. http://dx.doi.org/10.1099/ijs.0.63402-0

Laiz L., Piñar G., Lubitz W. & Saiz-Jimenez C., 2003 - *Monitoring the colonization of monuments by bacteria: cultivation versus molecular methods*. Environmental Microbiology, **5**: 72–74. http://dx.doi.org/10.1046/j.1462-2920.2003.00381.x

Nugari M.P., Pietrini A.M., Caneva G., Imperi F. & Visca P., 2009 - *Biodeterioration of mural paintings in a rocky habitat: The Crypt of the Original Sin (Matera, Italy)*. International Biodeterioration & Biodegradation, **63**: 705-711. http://dx.doi.org/10.1016/j.ibiod.2009.03.013

Pangallo D., Chovanová K., Drahovska H., De Leo F. & Urzì C., 2009 - *Application of fluorescence internal transcribed spacer-PCR (f-ITS) for the cluster analysis of bacteria isolated from air and deteriorated fresco surfaces*. International Biodeterioration & Biodegradation, **63**: 868-872. http://dx.doi.org/10.1016/j.ibiod.2009.04.011

Rainey F.A., Ward-Raney N., Kroppenstedt R.M. & Stackebrandt E., 1996 - *The genus Nocardiopsis represents a phylogenetically coherent taxon and a distinct actinomycete lineage: proposal of Nocardiopsaceae fam. nov.* International Journal of Systematic Bacteriology, **46**: 1088-1092. http://dx.doi.org/10.1099/00207713-46-4-1088

Reasoner D.J. & Geldreich E.E., 1985 - *A new medium for the enumeration and subculture of bacteria from potable water.* Applied and Environmental Microbiology, **49**: 1-7.

Roldán M. & Hernández-Mariné M., 2009 - *Exploring the secrets of the three-dimensional architecture of phototrophic biofilms in caves.* International Journal of Speleology, **38**: 41–53.

Sanchez-Moral S., Bedoya J., Luque L., Cañaveras J.C., Jurado V., Laiz L. & Saiz-Jimenez C., 2003 – *Biomineralization of different crystalline phases by bacteria isolated from Catacombs.* In: Saiz-Jimenez C. (Ed). -*Molecular Biology and Cultural Heritage.* Lisse: Swets & Zeitlinger: 179-185.

Sanchez-Moral S., Luque L., Cuezva S., Soler V., Benavente D., Laiz L., Gonzalez J.M. & Saiz-Jimenez C., 2005 - *Deterioration of building materials in Roman catacombs: the influence of visitors.* Science of the Total Environment, **349**: 260-276. http://dx.doi.org/10.1016/j.scitotenv.2004.12.080

Salvadori O. & Charola E., 2011 - *Methods to prevent biocolonization and recolonization: an overview of current research for architectural and archeological heritage.* In: Charola E., McNamara C., Koestler R.J. (Eds).. *Biocolonization of stone: Control and preventive methods*: *Proceedings from the MCI Workshop Series.* Washington: Smithsonian Institution Scholarly Press: 37-50.

Schabereiter-Gurtner C., Saiz-Jimenez C., Piñar G., Lubitz W. & Rölleke S., 2002a - *Altamira cave Paleolithic paintings harbor partly unknown bacterial communities.* FEMS Microbiology Letters, **211**: 7-11. http://dx.doi.org/10.1111/j.1574-6968.2002.tb11195.x

Schabereiter-Gurtner C., Saiz-Jimenez C., Piñar G., Lubitz W. & Rölleke S., 2002b - *Phylogenetic 16S rRNA analysis reveals the presence of complex and partly unknown bacterial communities in Tito Bustillo cave, Spain, and on its Paleolithic paintings.* Environmental Microbiology, **4**: 392-400. http://dx.doi.org/10.1046/j.1462-2920.2002.00303.x

Urzì C., Garcia-Valles M.T., Vendrell M. & Pernice A., 1999 - *Biomineralisation processes of the rock surfaces observed in field and in laboratory.* Geomicrobiology Journal, **16**: 39-54. http://dx.doi.org/10.1080/014904599270730

Urzì C., Brusetti L., Salamone P., Sorlini C., Stackebrandt E. & Daffonchio D., 2001 - *Biodiversity of Geodermatophilaceae isolated from altered stones and monuments in the Mediterranean basin.* Environmental Microbiology, **3**: 471-479. http://dx.doi.org/10.1046/j.1462-2920.2001.00217.x

Urzì C., De Leo F., Donato P. & La Cono V., 2003 - *Study of microbial communities colonizing hypogean monument surfaces using nondestructive and destructive sampling methods.* In: Koestler R.J., Koestler V.R., Charola A.E. & Nieto-Fernandez F.E. (Eds)., *Art, Biology, and Conservation: Biodeterioration of Works of Art.* New York: The Metropolitan Museum of Art: 316-327.

Urzì C., De Leo F. & Schumann P., 2008 - *Kribbella catacumbae sp. nov. and Kribbella sancticallisti sp. nov., isolated from whitish-grey patinas in the catacombs of St. Callistus in Rome, Italy.* International Journal of Systematic and Evolutionary Microbiology, **58**: 2090-2097. http://dx.doi.org/10.1099/ijs.0.65613-0

Urzì C., De Leo F., Bruno L. & Albertano P., 2010 - *Microbial diversity in Paleolithic caves: a study case on the phototrophic biofilms of the Cave of Bats (Zuheros, Spain).* Microbial Ecology, **60**: 116- 129. http://dx.doi.org/10.1007/s00248-010-9710-x

Urzì C., Nasso R., Bruno L., De Leo F., Krakova L., Pangallo D. & Albertano P., (in press) - *Studio della diversità microbica su superfici di Catacombe prima e dopo interventi di trattamento con biocidi.* Proceedings AIAR, Palermo.

Zammit G., De Leo F., Urzì C. & Albertano P., 2009 - *A non-invasive approach to the polyphasic study of biodeteriogenic biofilms at St Agatha Crypt and Catacombs at Rabat, Malta.* In: Meli G. (Ed)., *Scienze e Patrimonio Culturale nel Mediterraneo – Diagnostica e Conservazione*: *Atti del III Convegno Internazionale di Studi La materia e i segni della Storia.* Palermo: Quaderni Palazzo Moltalbo, **15**: 323-327.

Zammit G., Kaštovský J. & Albertano P., 2010 - *A first cytomorphological and molecular characterisation of a new Stigonematalean cyanobacterial morphotype isolated from Maltese catacombs.* Algological Studies, **135**: 1-14.

Zammit G., Billi D., Shubert E., Kaštovský J. & Albertano P., 2011a - *The biodiversity of subaerophytic phototrophic biofilms from Maltese hypogea.* Fottea, **11**: 187–201.

Zammit G., Sánchez-Moral S. & Albertano P., 2011b - *Bacterially mediated mineralisation processes lead to biodeterioration of artworks in Maltese catacombs.* Science of the Total Environment, **409**: 2773-2782. http://dx.doi.org/10.1016/j.scitotenv.2011.03.008

Appendix 1. Firmicutes isolated from the Ocean Cubicle in St. Callistus Catacombs, Zuheros Cave and St. Agatha Catacombs. Strains were clusterized on the basis of their ITS-PCR profile.

Nr. ITS clusters	CSC 13	Zuheros*	St. Agatha	Nearest relative on the basis of 16S rDNA sequences similarity*
3			SA8col1 SA17col1 SA14col1 SA10col1 SA19col3 Sa19col2 SA7col1 SA7col2	*Bacillus aquimaris* GU112997 (99.5 %)
1			SA12Acol1	*Bacillus cereus* CP001177 (99.7%)
1			Sa8col2	*Bacillus endophyticus* AF295302 (99%)
2		Z5b1 Z5b3		*Bacillus firmus* EF032672 (98.5%)
1		Z3b2		*Bacillus licheniformis* FJ458451 (99.5%)
1		Z3a2		*Bacillus mycoides* AB021192 (98.7%)
1		Z5b2 Z5b5		*Bacillus pseudofirmus* EU315248 (99.9%)
2		Z1a11, Z2a7, Z2a8, Z3a1, Z3a3, Z3a4, Z3a6, Z3a7, Z3b1, Z3b4, Z3b5, Z3b6, Z3c2, Z3c3, Z4a2, Z4a4, Z4a5, Z4a6, Z4a7, Z4a8, Z4a9, Z4b3, Z4b5, Z4b6, Z5a1, Z5d2, Z6a4 Z4b1 Z4b2		*Bacillus simplex* (DQ514314 98-100%)
1			SA1col1 SA1col2	*Bacillus sp.*
1	CSC13h1	Z6b8, Z8a6		*Bacillus subtilis* (AB0184886 100%)
1			SA6col1	*Bacillus amyloliquefaciens* CP00560 (99.6%)
1	CSC13a13			*Humibacillus xanthopallidus* AB282888 (99.7%)
1			SA9col1	*Paenibacillus arenae* AY839867 (98.7%)
1	CSC13h2			*Paenibacillus agaridevorans* AJ345023 (97.6%)
3		Z2a1, Z2a3, Z4a1, Z4a3		*Paenibacillus sp* DQ444989 (99.2%)
1			SA2col1	*Staphylococcus epidermidis* CP000029 (100%)
3	CSC13a21, CSC13f4	Z5a2, Z5a3		*Staphylococcus hominis* L37601 (99.7%)

Underlined strains were those identified by sequencing of 16S rDNA; (*) data taken from Urzì et al., 2010.

Appendix 2. Actinobacteria isolated from the Ocean Cubicle in St. Callistus Catacombs, Zuheros Cave and St. Agatha Catacombs. Strains were clusterized on the basis of their ITS-PCR profile. Streptomyces strains are reported in Appendix 3.

Nr. ITS clusters	CSC13	Zuheros*	St. Agatha	Nearest relative on the basis of 16S rDNA sequences similarity*
1	CSC13b15 CSC13b17			*Actinomadura fulvescens* AJ420137 (98.7%)
1	CSC13b16 CSC13b19			*Actinomadura cremea* EU741181 (98.2%)
1	CSC13a12			*Amycolatopsis lurida* (99.3%)
1		Z1a7, Z1b7		*Arthrobacter agilis* X80748 (99.1%)
2		Z1b5, Z1b8 Z1b12, Z8a2 Z1b2		*Blastococcus saxobsidiens* AJ316574 (BC 412) (98.4%)
1	CSC13g6			*Brachybacterium conglomeratum* AY167842 (98.8%)
1		Z1b11 Z1b13		*Curtobacterium flaccumfaciens* AY167854 (99.8%)
1			SA17col2	*Gordonia soli* AY995560 (97.6%)
1			SA15col1	*Isoptericola variabilis* FJ527726 (100%)
1	CSC13b11			*Knoellia subterranea* EU867301 (98.2%)
2	CSC13a1 CSC13a2 CSC13a4 CSC13a5, CSC13a6 CSC13a9, CSC13a14	Z1b3		*Kribbella catacumbae* AM778575 (98.8-100%)^
1		Z2b1		*Kribbella flavida* AF005017 (97.1%)
1	CSC13b3, CSC13b17 CSC13b18			*Kribbella sancticallisti* AM778577 (99.8-100%)^
1	CSC13b6, CSC13b10			*Microbacterium phyllosphaerae* EF143430 (98.3%)
4	CSC13e2, CSC13f1 CSC13f2, CSC13i1 CSC13i3, CSC13b14 CSC13g1 CSC13g5	Z2a2, Z3b3 Z7a1	SA2col2	*Micrococcus luteus* AJ536198 (99.1%-100%)
1		Z2a4 Z2b4		*Micrococcus lylae* X80750 (98.1%)
1			SA19col1	*Nocardia uniformis* AF430044 (98,6%)
1		Z6b7		*Promicromonospora enterophila* X83807 (98.9%)
1			SA13col1 Sa16col1	*Pseudonocardia carboxidivorans* AF430044 (100%)
1		Z1a3		*Rhodococcus corynebacterioides* AY167850 (99.6%)
1		Z7a10		*Rhodococcus erythropolis* AY281121 (99.1%)
1		Z1a4		*Rhodococcus fascians* DQ870746 (98.8%)

Underlined strains were those identified by sequencing of 16S rDNA; (*) data taken from Urzì et al., 2010. (^) strains described by two of the Authors (Urzì et al., 2008).

Appendix 3. Streptomyces isolated from the Ocean Cubicle in St. Callistus Catacombs, Zuheros Cave and St. Agatha Catacombs. Strains were clusterized on the basis of their ITS-PCR profile.

Nr. ITS clusters	CSC13	Zuheros*	St. Agatha	Nearest relative on the basis of 16S rDNA sequences similarity*
1	CSC13b20, CSC13c5, CSC13c7, CSC13c9			*Streptomyces avidinae* AB184395 (99.5%)
1			SA4col2, SA4col3, Sa23col5, SA4col4, Sa23col2, SA8col3, Sa9col2	*Streptomyces badius* AY999783 (99.7-100%)
1		Z7a4, Z7a5, Z7a6		*Streptomyces caeruleus* EF178698 (99.5%)
1			SA19col4	*Streptomyces chungwensis* AY382292 (99.7%)
1			SA4col1, SA4col1f	*Streptomyces flavidofuscus* AY999914 (100%)
1		Z1a8, Z2a5, Z2a6, Z2b6, Z2b8, Z2b9, Z2b10		*Streptomyces flavolimosus* EF688620 (100%)
1	CSC13d3 CSC13d6			*Streptomyces mutomycini* AAJ781357 (99.5%)
1	CSC13d2 CSC13d4	Z1a1, Z1a9		*Streptomyces griseus* AY207610 (99.6%)
1		Z6b9, Z7a13, Z7a12, Z7a3		*Streptomyces laceyi* AY094367 (98.2%)
3	CSC13a4, CSC13a7 CSC13a11, CSC13a21 CSC13a23, CSC13a27 CSC13a24 CSC13b18			*Streptomyces nojiriensis* AJ781355 (99.8%-100%)
1			SA18col1	*Streptomyces peucetis* AB045887 (99.5%)
1	CSC13a12			*Streptomyces floccolosus* AB8427297 (97.6%)
1			SA3col1, SA4col6	*Streptomyces sp.* AJ315072 (99.5%)
1	CSC13a3			*Streptomyces sporoFIXME*

Underlined strains were those identified by sequencing of 16S rDNA; (*) data taken from Urzì et al., 2010.