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Microbiology of the stalactites from Grotta dei Cervi, Porto Badisco, Italy

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Received 2 September 1999

Accepted 15 January 2000

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Summary The active stalactites from Grotta dei Cervi, Porto Badisco, southeastern Italy, were sampled to investigate the microbial communities present in these speleothems. Sampling was carried out in a transect about 150 m long in the central gallery, where numerous Gram-positive bacteria were isolated. Actinomycetes of the genus *Streptomyces* were the most abundant, followed by members of the genus *Bacillus*. Further isolates were assigned to the genera *Amycolatopsis*, *Arthrobacter*, *Agromyces*, *Micrococcus*, *Nocardiopsis* and *Rhodococcus* of the order Actinomycetales. The ability of actinomycetes to colonize subterranean environments is discussed.

Key words *Streptomyces* · *Bacillus* · Actinomycetes · Caves · Rock art paintings

Introduction

Grotta dei Cervi is a cave which opens into the Oligocene Calcarenites at 26 m above sea level on the channel of Otranto, 370 m NE of Porto Badisco, Lecce, southeastern Italy. It was discovered on February 1, 1970. From the geomorphological point of view, the cave is the site of an ancient underground water table. The innumerable concavities which are visible on the roof of the gallery have formed by the effect of the whirling and turbulence of water.

The cave consists of a main gallery running NE-SW, from which three cavities branch off running prevalently SW-NE. The total planimetric extent is about 1550 m (the central branch alone runs for 320 m from the entrance). The eastern gallery reaches a depth of 26–28 m below ground in its final stretch. From the western gallery there is an entry to a series of galleries and passages. The central gallery, which reaches sea level at its maximum depth, is blocked, especially at the end, by a major slide and tends towards the surface above. Even the roof of the gallery reaches 15.3 m below ground level. The central gallery contains rock art paintings in red ochre showing stylized, partly faded, figures of men and animals. Black paintings, made with guano, represent cruciform and other geometric figures and sorceries which are difficult to interpret. Because of the wealth of the rock art paintings, the cave was defined by Graziosi [5] as “the shrine of Prehistory” after verifying “the sacred

character, in that religious, or rather, magic-religious ceremonies were performed”.

The knowledge of microbial communities in caves is scant [6]. Contradictory data can be found in the literature, e.g. that chemolithoautotrophic bacteria are likely to be primary producers in caves [14], or that they play an insignificant role in the cave ecosystem [3]. Microbial communities of caves usually rely on allochthonous input of organic matter which is transported from the surface by water. Dissolved organic carbon content in dripping waters from some caves is highly variable, ranging from < 5 mg C/liter to 2200 mg C/liter [10].

The biodeteriorative role of microbial communities, especially those of actinomycetes growing on hypogean environments which contain rock art or mural paintings, has been emphasized by some authors [6, 7, 11]. We investigated heterotrophic microbial communities from active stalactites from Grotta dei Cervi, Porto Badisco, Italy. This study was of particular interest because the difficult, restricted access to the cave preserves it from external contamination.

Material and methods

Sampling and sample location A sampling campaign was carried out in the last week of September 1997 to investigate the microbial populations on the different cave formations.

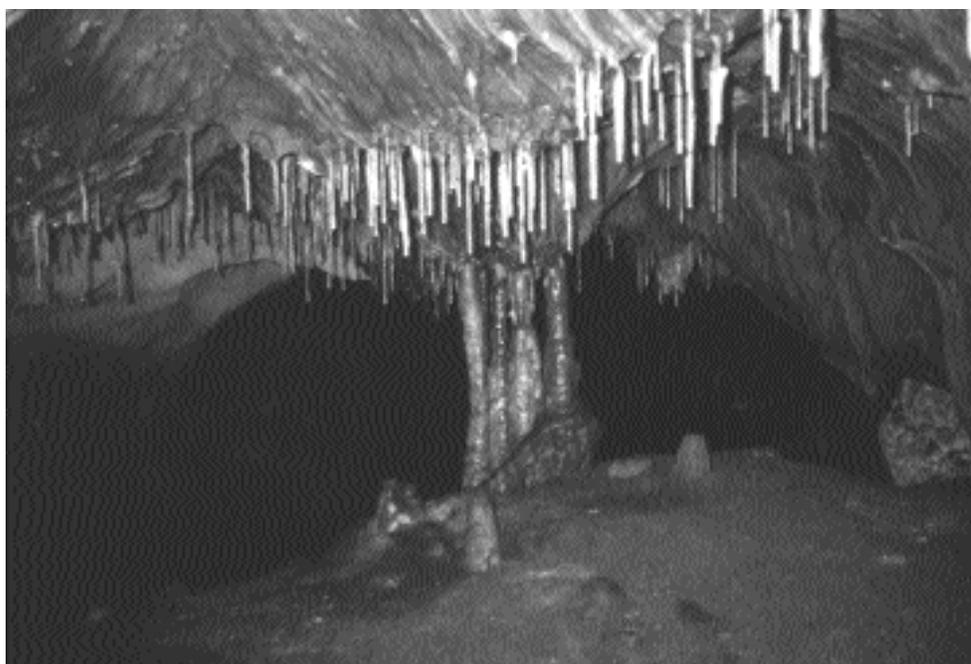


Fig. 1. Stalactite formation in sector III, Grotta dei Cervi, Porto Badisco, southeastern Italy

Active stalactites at different points along the cave (transect of about 150 m) were collected in sterile tubes and kept at 4°C for two days until microbiological analyses were performed. The sampling sites are located along the whole length of the central gallery. Two of the samples used for this study were obtained from stalactites located in the entrance hall, near the narrow passage leading to the cave galleries. These samples were influenced by external factors as they were located in a place where archaeologists had mixed the soil during excavations. There was a stalactite formation in the ceiling of sector III (Fig. 1), approximately in the middle of the central gallery. Other samples were taken from different stalactites around this area, at places which were undisturbed due to their location and position. Other cave formations or places with high guano content (some walls, soils, etc.) were not included in this study. In fact, the main purpose was to investigate both the strategies of bacteria for the colonization and the distribution of colonies on the stalactites in the presence of low organic matter inputs.

Isolation and identification Samples were suspended in a saline solution (0.9 % NaCl) and inoculated into different culture media: Tryptone soy agar (TSA), malt-yeast extract, starch-casein, glycerol-asparagin and water-agar. Compositions of the media are described elsewhere [10]. General laboratory cultivation of the coryneform isolates was done on rich medium [18] and, in the case of sporoactinomycetes, on oatmeal agar ISP-3 [17]. The plates were incubated at 28°C either for 48 h or for 8 weeks, to allow slowly-growing bacteria to develop. In some cases, cotton swabs were used to touch small areas of the stalactites with a visible growth of microorganisms. The

adhered bacteria were immediately distributed on agar plates by streaking or were suspended in buffer previously to a dilution plating.

Individual colonies were randomly isolated and purified by streak plating on TSA or any other appropriate medium to obtain pure cultures.

Characterization of the isolates We classified the isolates by morphological and chemotaxonomic methods. Physiological properties were tested using standard microbiological methods including the Biolog-identification system. In a few cases, we were able to identify isolates on the basis of Biolog database.

Total cellular fatty acid methyl esters (FAME) were analyzed by using the MIDI system in accordance with the protocols for cultures grown on solid medium and instrument specifications recommended by Microbial Identification System, Inc. Delaware, USA. This method, which allowed us to identify bacteria automatically by comparison with the Sherlock Standard Aerobe database, was used to identify bacteria other than actinomycetes. The identification of streptomycetes isolates was performed using morphological and physiological characterization as recommended by Shirling and Gottlieb [17]. Methods for chemotaxonomic characteristics were described elsewhere [7].

Cooperative growth patterns We used peptone-agar medium to grow isolates for the study of cooperative patterns. Peptone contents were 0.25, 0.5, 2, 5 and 10 g/l, respectively, and agar contents were 20 and 40 g/l. We inoculated 5 µl of a suspension containing 15×10^5 bacteria at the center of Petri plates, and incubated them for eight weeks at 28°C [1].

DNA extraction from stalactite surfaces and PCR F-3, F-15, F-16 and F-18 were the samples used for this study. We scratched stalactite surface areas of approximately 20 mm² with a sterile razor blade to collect approximately 1 mm³ material. The material was transferred into sterile 1.5 ml microcentrifuge tubes containing 50 μ l deionized water, which were heated for 5 min at 95°C. After heating the tubes, they were incubated for 10 min at -80°C; this boiling/freezing treatment was repeated twice. We amplified 16S rDNA sequences with a GeneAmp PCR System 2400 thermocycler (Perkin Elmer-Cetus, Norwalk, Conn.), using 40 cycles of 94°C for 10 s, 48°C for 20 s, and 68°C for 40 s. The PCR reaction mixture (20 μ l) contained 50 mM Tris-HCl (pH 8.9), 50 mM KCl, 3 mM MgCl₂, 50 μ M each of dATP, dCTP, dGTP, and dTTP, 30 pmol of primer GC968 and 1401 [13], 0.5 units of Amplitaq DNA polymerase, LD (Perkin Elmer-Cetus), and 1 μ l cell lysate. Amplification products were confirmed by 1.4% agarose gel electrophoresis.

Screening of isolates for matching sequences We extracted total DNA of bacterial isolates by transferring cell material with an inoculation loop into 1.5 ml microcentrifuge tubes containing 50 μ l deionized water. The tubes were heated for 15 min at 95°C to lyse the cells and then chilled on ice. Amplification of 16S rDNA sequences was performed with a thermocycler (see above), using 35 cycles of 94°C for 10 s, 48°C for 20 s and 68°C for 40 s. The PCR reaction mixture (20 μ l) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM MgCl₂, 50 μ M each of dATP, dCTP, dGTP, and dTTP, 30 pmol of primer GC968 and 1401 [13], 0.2 units of Pro-HA DNA polymerase (Eurogentec, Seraing, Belgium), and 1 μ l cell lysate. Amplification products were confirmed by 1.4% agarose gel electrophoresis. PCR products from isolates were loaded on temperature gradient gel electrophoresis (TGGE) gels together with PCR products from DNA extracted directly from the stalactite surface.

Temperature gradient gel electrophoresis (TGGE) analysis

The Diagen TGGE system (Diagen, Düsseldorf, Germany) was used for sequence specific separation of PCR [16]. Electrophoresis was performed in a 0.8 mm-polyacrylamide gel (6% w/v acrylamide, 0.1% w/v bis-acrylamide, 8 M urea, 20% v/v formamide, 2% v/v glycerol) with 1X TA buffer (40 mM Tris-Acetate, pH 8.0) at a fixed current of 9 mA (approximately 120 V) for 16 h [4]. A temperature gradient was built up in electrophoresis direction from 37°C to 46°C. After the run, gels were silver-stained [2].

Environmental data An environmental monitoring station was installed in the cave, from which sensor No. 3 was positioned in the stretch of the gallery where we took most of the samples for the microbiological analyses. Air and rock surface temperatures in the cave, and outdoor and indoor relative humidity (RH) were measured from August 1997 to July 1998.

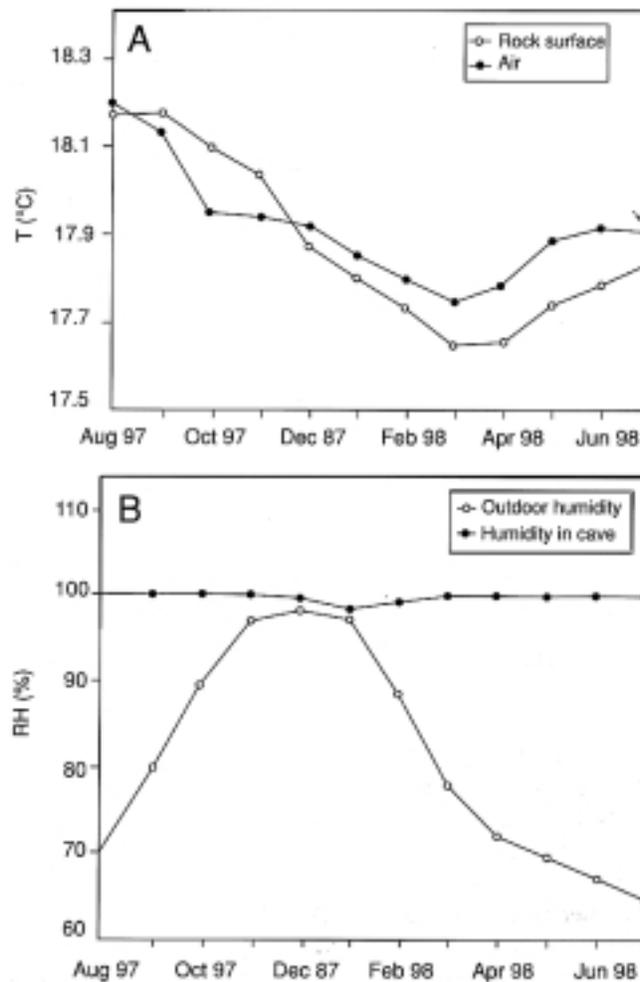


Fig. 2. (A) Monthly average temperature from August 1997 to July 1998. (B) Monthly average relative humidity (RH) from August 1997 to July 1998

Results and Discussion

Caves have relatively constant temperatures, high RH and low quantities of organic nutrients. Groth and Saiz-Jimenez [6] have suggested that the growth of actinomycetes, the most abundant microorganisms in caves, may be favored by the association of two factors: low temperatures and high RH. These environmental conditions, together with the availability of nutrients and the nature of the organic matter are major factors controlling the activity of actinomycetes in caves.

We monitored environmental variables at the Grotta dei Cervi and compared them with variables at other well known caves [9, 15]. The monthly average temperatures recorded on both internal walls and concretions during the period August 1997–July 1998 were around 18°C (Fig. 2A). They were slightly higher (18.2°C) in August and September, 1997, and fell

gradually, the lowest temperature having been 17.6°C in March. Afterwards they rose again. Annual thermal fluctuation is of 0.5°C. The air temperature in the cave was slightly lower (17.9°C) than that of the rock (18.1°C) from September to December. From December onward the reduction of the values for rock surfaces (until reaching a minimum 17.6°C) is accompanied by an increase in those for the air, which reach 17.8°C in March and 18.2°C in August and September. Annual thermal fluctuation of the rock reaches a maximum of 0.6°C. RH values recorded inside the cave reached levels very close to 100% throughout the year, with the exception of a period limited to December–February during which humidity levels fell to 98%. RH values outside the cave showed an opposite, more marked trend (Fig. 2B).

Most caves so far studied are characterized by both stable temperatures ranging from 13°C to 15°C and high RH [9, 11, 15]. These conditions favor the growth of actinomycetes [6, 7]. The mean temperature in the Grotta dei Cervi is higher than those recorded in northern Spanish caves and in French caves (13–15°C), but nearer to the range of temperatures recorded in some southern Spanish caves: 19–23°C [8]. This suggests that temperature is not a limiting factor for the growth of actinomycetes in such environments. In fact, actinomycetes are common and abundant in caves and other hypogean environments [6, 12]. They can be seen in the lower part of cave walls and in cave soils. Their growing has been related to the presence of organic matter from guano. Guano was used for most of the paintings. The composition of microbial communities growing on those habitats, where organic matter is most abundant, was not the object of the present study. Instead, we focused our study on microbial growth at places with low organic matter inputs, and we obtained many isolates from the samples taken at active stalactites at the entrance and in the central gallery. Most isolates were streptomycetes and nocardioform and coryneform actinomycetes.

Most bacterial isolates from all sampling sites were of the genus *Streptomyces* (8 strains), followed by *Bacillus* (4 strains), and *Nocardiopsis* (3 strains). We also found species of the genera *Rhodococcus*, *Agromyces*, *Arthrobacter*, *Amycolatopsis*, *Brevibacillus*, *Micrococcus*, *Staphylococcus*, *Paenibacillus*, and *Variovorax*. *Streptomyces* strains were isolated from all samples except in one stalactite. From this stalactite, we only focused on the isolation of typical white dendrite-like colonies, identified as *Nocardiopsis* spp. (Fig. 3). Irrespective of their location, most samples contained *Streptomyces* species. In samples taken from undisturbed places, however, we found a richer variety of actinomycetes and bacilli. Only two Gram-negative bacteria (*Variovorax paradoxus* and an unidentified strain) were isolated. An example of man and animal figures found in the cave is shown in Fig. 4.

Most of the *Streptomyces* strains isolated were pigment-producing, mainly brownish, but one of them produced melanin. All strains used glucose as carbon source. Xylose and fructose were also extensively used, but three strains used inositol. From all isolates, only the species *S. roseofulvus* could be identified

We used TGGE to study the uncultivated microbial communities of stalactites and to confirm the identification of the isolates. The predominant signals found in the stalactites are double-bands resembling those of isolated *Nocardiopsis* spp. However, the location of *Nocardiopsis* spp. in the stalactites remains unclear. On the one hand, we isolated several different strains which, in Petri plates, showed cooperative growth patterns quite similar to those observed on stalactites (Fig. 5). The TGGE analysis revealed that the samples from stalactites contained bacterial DNA of limited diversity, dominated by a signal showing a *Nocardiopsis*-like double band formation. In contrast to the morphological diversity of the isolates, this signal was constant in all other stalactite samples, and corresponded to the visual similarity of the observed *Nocardiopsis* isolates. Furthermore, the signals from some isolates tested (*Nocardiopsis* spp., *S. roseofulvus*, *Streptomyces* spp. *Micrococcus luteus*, *Arthrobacter ilicis*, and *Brevibacillus laterosporus*) did not match the dominant stalactite signals. The dominant microorganism differed from the isolates, but both the peculiar morphology of its colonies and the TGGE signals suggested a close relationship with the isolates of *Nocardiopsis*.

In a recent paper, Groth et al. [7] studied the cave of Altamira, Santander, Spain. This cave, discovered in 1879, reached a daily flow of 1500 persons in the 1960s, which increased rapidly to 3,000 daily in the 1970s. Since 1982 visits to the cave have been restricted to 45 persons per day. Actinomycetes isolated from Altamira revealed a great taxonomic diversity, *Streptomyces* strains being the predominant isolates. Members of the genera *Nocardia*, *Rhodococcus*, *Nocardioides*, *Amycolatopsis*, *Saccharothrix*, *Brevibacterium*, *Microbacterium* and coccoid actinomycetes (family Micrococcaceae) have been also found in Altamira. Grotta dei Cervi was discovered 91 years later than Altamira, and the total number of visitors (entry permitted only for study purposes) has been very low. Nevertheless, both caves show similar microbiological colonization patterns.

Actinomycetes are well known for their ability to grow on very poor media and to use recalcitrant organic matter, e.g. lignocellulose residues, humic substances, etc. [6]. While guano input is appreciable on the walls of the galleries, on the ceiling of the narrow passage at the entrance, and on the soils, it is not found on the stalactites. Dripping water must contain low levels of dissolved soil organic matter, as it is inferred from the scarce vegetation on the soil above the cave and the percolation of water through 15 m of rock. This agrees with cooperative growth patterns of bacterial colonies from the stalactites (see Fig. 3). In fact, this kind of growth is often related to unfavorable environmental conditions, and the bacteria develop sophisticated modes of cooperative behavior. Ben-Jacob et al. [1] grew *Bacillus subtilis* under different growth conditions ranging from very low concentrations of nutrient (0.01 %) to rich ones (1%). The pattern represents aggregates of bacteria, which move in response to gradients in nutrient concentration. The patterns

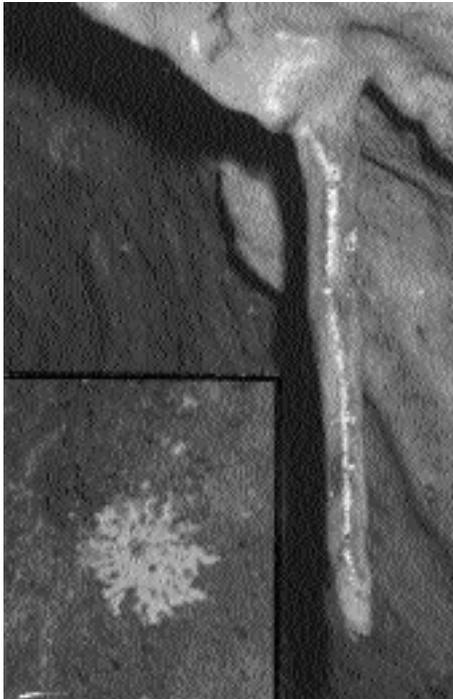


Fig. 3. Stalactite from which sample F-18 was taken. A detail of a colony (inset)



Fig. 4. An example of man and animal figures found in the cave

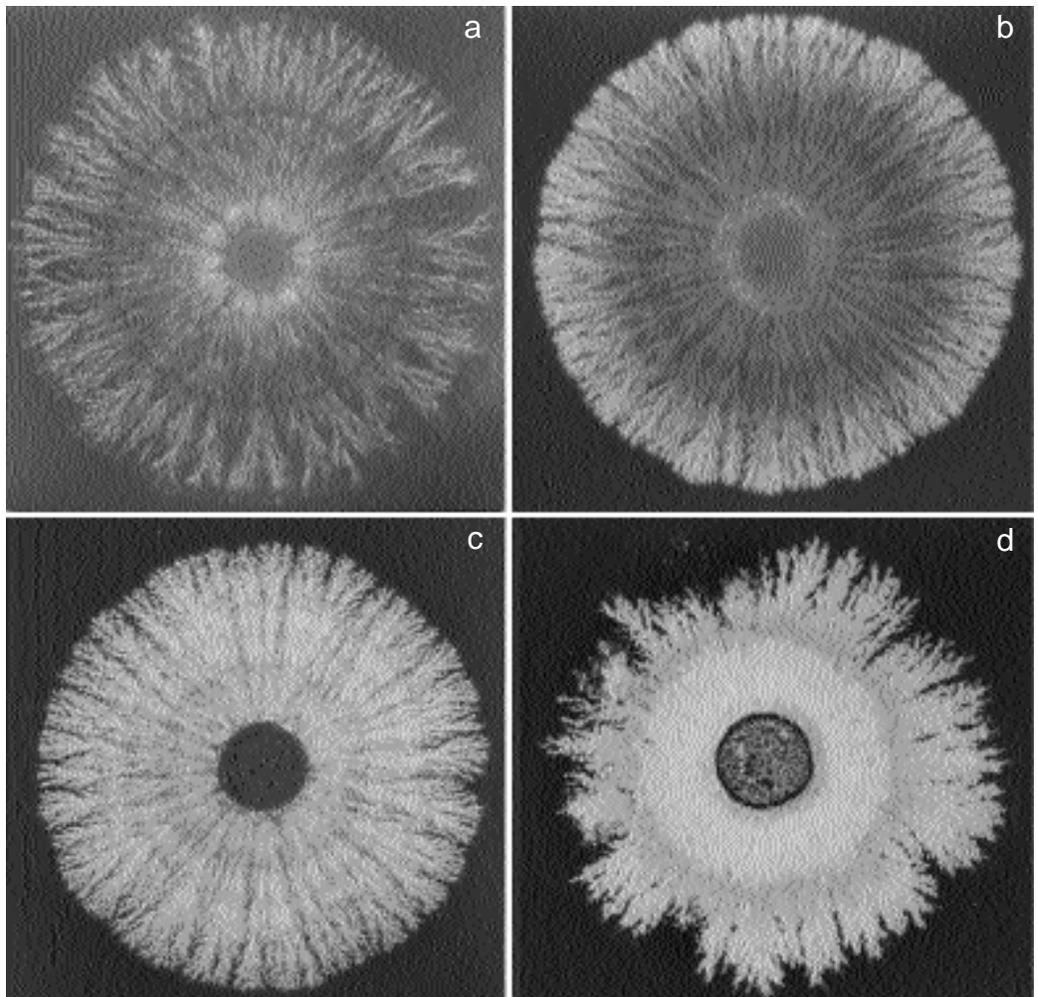


Fig. 5. Colonies of *Nocardiopsis* sp. (F-101) grown on 4% agar and four different concentrations of peptone (0.25, 2, 5 and 10%, from left to right, top and bottom)

observed by Ben-Jacob et al. at low levels of nutrient were similar to those observed for *Nocardiopsis* sp. (F-101) in the culture medium (see Fig. 5).

The preliminary application of TGGE (in comparison with classical microbial cultivation approaches) has shown that even apparently simple environmental microbial communities such as those growing on stalactites, besides the abundant colonies of *Streptomyces*, are difficult to compare. The results suggest that, on the stalactites, there are *Nocardiopsis*-like strains so far uncultivable, as well as other fast-growing relatives, which can be cultivated easily. Further use of molecular methods for cultivation-independent analysis of microbial communities in caves will broaden the understanding of this relatively unknown ecosystem.

Acknowledgments This research was supported by the European Commission, contract ENV4-CT95-0104. The Soprintendenza Archeologica della Puglia is acknowledged for the facilities for sampling in the cave.

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