Microbiological study of the dripping waters in Altamira cave (Santillana del Mar, Spain)

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The culturable microbial populations in dripping waters from Altamira cave were studied and compared with those of the ceiling rock. Water communities have low proportions of gram-positive bacteria, and are mainly composed of gram-negative rods and cocci (*Enterobacteriaceae* and *Vibrionaceae*), while those of ceiling rocks are mainly *Streptomyces* spp. The community differences are probably related to environmental cave conditions: high humidity, relatively low and stable temperature, water pH close to neutrality and nature of the organic matter. All these factors seem to favor colonization and long-term growth of actinomycetes over other heterotrophic bacteria on ceiling rocks.

Keywords: dripping water; *Enterobacteriaceae*; *Vibrionaceae*; *Streptomyces*; crystal formation

1. Introduction

The Altamira cave, situated on the Cantabrian cornice, Santillana del Mar, Spain, is known as the Sistine Chapel of Quaternary Art. The cave has the most important prehistoric paintings of Spain, and probably of the world, particularly the Polychromes Hall, which contains the majority of the Magdalenian paintings, about 14000 years old (Valladas et al., 1992).

The cave, in a small calcareous hill, 158 m above sea level, was discovered in 1879. Since then, Altamira has suffered a series of changes related to its structure and the increasing growth in number of visitors, in such a way that the Polychromes Hall was reduced to a small artificial chamber, with environmental conditions very different from the natural ones and leading to deterioration of the paintings.

Deterioration of paintings and microbial growth was related to the high number of visitors, which reached a daily flow of 1500 persons in the 1960s, increasing rapidly in the 1970s to 3000 daily, despite the first alarming signs of deterioration observed in the paintings. In 1976 a Commission in charge of conservation proposed the closure of the cave, but this did not happen, for various reasons, until 1979. Since 1982 visits to the cave were reduced to 45 persons per day, but the conservation problems of the paintings still remain (Hoyos, 1993).

The nature and distribution of dripping waters in Altamira have been the basis of controversy. Villar et al. (1983) selected ten dripping water points in the ceiling of the Polychromes Hall and observed that

the monthly average of dripping water is 6±1 l. However, Hoyos (1993) stated that the dripping water flux is not constant, nor are its geochemical characteristics.

A preliminary study of bacterial communities in the dripping waters in Altamira cave was carried out by Somavilla et al. (1978). *Bacillus* and *Pseudomonas* appeared as the most abundant genera with six and five species, respectively, followed by *Flavobacterium* and *Erwinia* with two species. Five sampling points were located outside the Polychromes Hall which showed higher variability (ten genera) than two sampling points inside the Hall with only three species of the genera *Bacillus* and *Erwinia*. Uruburu et al. (1981) estimated cfu (colony-forming units) of bacteria in two samples of dripping water but no identification of genera and species was provided. Hardisson et al., 1982 found differences in the cfu of bacteria between two samplings in November 1981 and February 1982. Gonzalez de los Reyes-Gavilan et al. (1984) reported that the dripping waters contained a considerable number of bacteria, which was not eliminated through rock filtration.

Recently, Groth et al. (1999) reviewed the growth of actinomycetes in caves and hypogean environments. Two caves with rock art, Altamira and Tito Bustillo, were selected as a case-study. Within about 350 actinomycetes were identified by morphological, physiological and chemotaxonomic methods in Altamira cave. Most of the actinomycetes growing on the surface of ceiling and wall rocks were colonies from 1 to 10 mm diameter, visible with the naked eye. Many isolates corresponded to strains obtained directly from the colonies. The genera *Streptomyces*, *Nocardioides*, *Amycolatopsis*, *Brevibacterium*, *Nocardia*, *Rhodococcus*, *Aureobacterium*, and the family *Micrococcaceae* were well represented. In Tito Bustillo cave, the surface of the rock was colonized by a large number of small, yellow, round colonies of about 1–2 mm. Direct isolates from the colonies were found to be *Streptomyces xanthophaeus* strains.

In this paper, the microbial population in the dripping waters of the Altamira cave is studied and compared with those of the ceiling and wall rocks using culture methods.

2. Material and methods

2.1. Sampling and sample location

A sampling campaign was carried out to investigate the microbial population in the dripping waters of the cave of Altamira at the end of January 1997. Gonzalez de los Reyes-Gavilan et al. (1984) stated that the number of bacteria changed seasonally with a peak in February.

Dripping water from five different points along the cave (Fig. 1) were collected in sterile tubes (triplicate) and kept at 4°C until microbiological analysis was carried out two days after sampling. The points are located in the Kitchen Hall (EN-1), the gallery to the Polychromes Hall (EN-2 and 3), the Hall of the Walls (GM) and the Big Hall (GS) in a transect of about 800 m. A chemical analysis of the waters is shown in Table 1.

For comparison, two samples (P-5 and P-6) from the surface of the Polychromes Hall ceiling and one (C-EG) from the ceiling of the gallery conducting to this Hall (about 2 meters from EN-3) were studied. These points were sampled by plate impressions from the surface.

2.2. Enumeration

Petrifilm aerobic count plates were inoculated with 1 ml water sample. The plates contained a ready-made medium for enumerating total aerobic bacteria

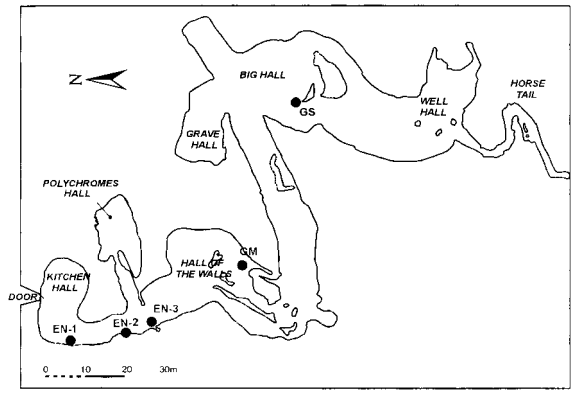

Fig. 1. Altamira cave and dripping water sampling points.

Table 1
Chemical and microbiological analysis of dripping waters

Sample	pH	T°C	CO_2 [a]	CO_3H^-	SO_4^{2-}	Cl^-	Ca^{2+}	Mg^{2+}	Na^+	K^+	CFU ml^{-1}
EN-1	7.84	13.4	17.5	307.9	38.7	12.0	88.9	12.30	8.96	2.08	210
EN-2	7.62	13.7	25.0	457.3	5.0	9.0	122.5	16.45	7.39	0.41	TNTC[b]
EN-3	7.63	14.6	17.5	405.5	53.1	38.5	121.0	18.6	21.87	9.73	75
GM	7.63	14.2	25.0	350.6	51.7	21.5	116.6	7.97	13.28	2.87	310
GS	7.62	13.5	12.5	350.6	26.3	11.5	114.3	4.08	9.29	1.50	TNTC

[a] mg l^{-1} of dissolved CO_2, all other anions and cations expressed in mg l^{-1}.
[b] Too numerous to count.

populations. According to the manufacturer (3M), the medium contains (per litre) 5 g tryptone, 2.5 g yeast extract and 1 g glucose, a cold-water-soluble gelling agent, and a tetrazolium indicator dye which facilitate colony enumeration. Counts shown in Table 1 represent the mean on at least 6 Petrifilm plates for each triplicate sampling point. The plates were incubated at 28°C for 48 h. Some water samples were inoculated directly in Sigma tryptone-soy agar (TSA) plates.

For comparison, samples from the rock surface from the Polychromes Hall and galleries were enumerated on Rodac plates prepared with different culture media: TSA, malt-yeast extract -MEY- (Laiz, 1991), starch-casein -SC- (Küster and Willians, 1964), glycerol-asparagin -GA-, humic acid-agar -HA- (Hayakawa and Nonomura, 1987), and cycloheximide-agar -CA- (Dietz and Thayer, 1980). The plates were incubated at 28°C for 48 h before enumeration and were recounted after 8 weeks to record slowly growing microorganisms.

2.3. Identification

After enumeration, individual colonies were randomly isolated and purified by streak plating on TSA until pure cultures were obtained. Isolates were characterized by morphological and physiological properties, the latter using standard microbiological methods, API and Biolog. Identifications were performed with APILab Plus and Biolog databases.

2.4. Physiological activities

The activities of decomposition of organic compounds were calculated as in vitro activities from API test reactions. Total physiological activity of one bacterial strain was expressed as percentage of total activity of an isolate: $G_{(i)}$ %, and calculated according to Kölbel-Boelke et al. (1988) from the number of positive and negative characters of each isolate. Physiological activities of the community for digestion of specific substrates was obtained from the number of isolates with positive and negative characters.

2.5. Precipitation of salts

All isolates were tested for salt precipitation using B-4 medium composed of: 2.5 g calcium acetate, 4 g yeast extract, 10 g glucose, 15 g agar in 1 l distilled water, pH 8 (Boquet et al., 1973). Precipitation was also tested in liquid medium for the isolates with higher precipitation capacity in order to collect the crystals for further analysis. SEM and EDX, X-ray diffraction and FT-IR were routinely used for this purpose.

3. Results and discussion

3.1. Cell counts

Petrifilm plates have been widely used for the enumeration of contaminated liquids and foods (Ginn et al., 1984; Matner et al., 1990; Byrne and Bishop, 1991) and were considered a good choice for this study. Table 1 shows Petrifilm counts at the different sampling points. Dissolved-organic-carbon content in Altamira samples showed high variability depending on the dripping points and seasons. However, in the sampling period dissolved carbon was

less than 5 mg C l^{-1} (van Grieken, personal communication). The concentration of organic materials in recharge water and in aquifers is low, typically around 1 mg C l^{-1}, a concentration believed too low to support life (Leenheer et al., 1974). In Altamira dripping waters, cell counts varied from 75 cfu ml^{-1} to concentration of colonies too numerous to count. Very close sampling points also showed a high variability. A similar variability (30–690 cfu ml^{-1}) was reported by Stetzenbach et al. (1986) for a continuously working deep groundwater well. Wolters and Schwartz (1956) obtained 10–32 cfu ml^{-1} for 39 drinking water wells from which water was pumped from depths of 40–50 m. Kölbel-Boelke et al. (1988) found less than 100 cfu ml^{-1}, and in many cases even less than 50 cfu ml^{-1}, in water samples from a sandy aquifer from 5 m below the surface.

3.2. Bacteria identification

Culturable isolates from Altamira water samples contained low proportions of gram-positive (27.3%) relative to gram-negative (72.7%) bacteria (Fig. 2). It has also been shown that water communities contain low proportions of gram-positive bacteria when compared with sediments or soils (Kölbel-Boelke et al., 1988; Wilson et al., 1983).

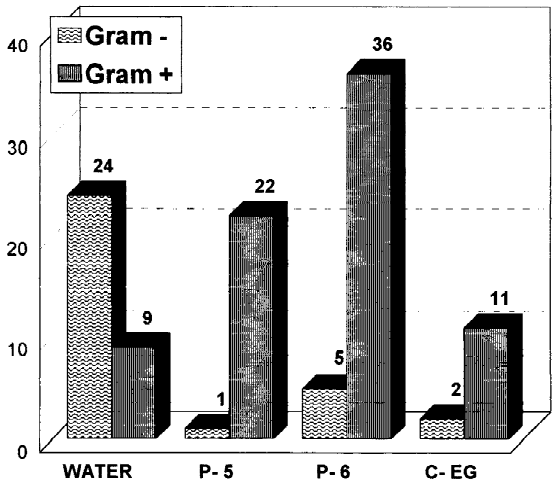

Fig. 2. Distribution of bacteria in Altamira samples. Dripping water samples are summarized under WATER, P-5 and P-6 are samples from the ceiling of Polychromes Hall and C-EG from the access gallery to Polychromes Hall.

The opposite was found in the samples from the ceiling of the Polychromes Hall and galleries. In the ceiling of the Polychromes Hall, the proportions of gram-positive bacteria were threefold higher (95.7% for P-5, 87.8% for P-6) near the paintings, or in the access gallery to Polychromes Hall (84.6% for C-EG).

Among the gram-negative bacteria isolated from the dripping waters, the most abundant genus isolated was *Aeromonas*, occurring in fresh waters and sewage (Table 2). *Aeromonas hydrophila* was the species most frequently isolated both at the entrance gallery and at the Big Hall sampling point. *A. salmonicida* and *A. sobria* were the other *Aeromonas* species identified in samples obtained near the entrance of the cave. All *Aeromonas* species were identified to very good levels of confidence, but the *A. sobria* strain did not utilize citrate (80% utilization for the species).

Acinetobacter spp. were also isolated from the entrance and from the sampling points farther into the cave. *Acinetobacter* spp. comprised 54% of the total number of isolates from deep-well ground water (Stetzenbach et al., 1986). It was suggested that stimulation of growth of *Acinetobacter* sp. by low concetrations of carbon reflects the ability of this organisms to effectively utilize a variety of carbon sources and may in part explain its predominance in well water.

Quantitatively important was also the genus *Enterobacter*, widely distributed in nature and occurring in fresh water, sewage and animal and human feces, represented by the species *Enterobacter amnigenus*. This bacterium was present in the dripping waters from the gallery leading to the Polychromes Hall (EN-3). Other bacteria identified in this sampling point were *Serratia liquefaciens*, *Chromobacterium violaceum*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Janthinobacterium lividum*, *Chryseomonas luteola*, *Xanthomonas maltophilia*, *Flavimonas oryzihabitans*, and *Kingella kingae*. Four gram-negative bacteria could not be identified.

The culturable gram-positive bacteria were represented by the genus *Bacillus*. The species *B. cereus*, *B. circulans*, *B. subtilis*, *B. stearothermophilus*, and *B. polymyxa* were mainly isolated from dripping water taken at the sampling points located at the cave entrance (Table 3). A microbial community based

Table 2
Gram-negative isolates of dripping waters of Altamira cave

Group	Identification	No isolates	Origin[a]	Percentage of identification[b]
Facultatively anaerobic gram-negative rods	*Enterobacter amnigenus*	1	EN-3	91.5
	Serratia liquefaciens	1	EN-3	81.3
	Erwinia sp.	1	GM	85.8
	Aeromonas hydrophila	3	GS, EN-1,EN-2	99.6
	Aeromonas sobria	1	EN-1	74.8
	Aeromonas salmonicida	1	EN-3	99.5
	Chromobacterium violaceum	2	EN-3	99.8
Gram-negative aerobic/microaerophilic rods and cocci	*Janthinobacterium lividum*	1	EN-3	77.0[c]
	Pseudomonas fluorescens	1	EN-3	97.3
	Pseudomonas aeruginosa	1	EN-3	97.0
	Chryseomonas luteola	1	EN-3	99.8
	Xanthomonas maltophilia	1	EN-3	88.0[c]
	Flavimonas oryzihabitans	1	EN-3	81.3
	Acinetobacter sp.	3	GS, EN-1	95.0
	Kingella kingae	1	EN-3	84.0[c]
	NI[a]	4	EN-1,EN-2, GS	

[a] EN: entrance of the cave, GM: Hall of the Walls, GS: Big Hall, NI: not identified.
[b] An estimate of how closely the profile corresponds to the taxon relative to all the other taxa in the Apilab Plus data base.
[c] Identified with Biolog.

almost exclusively on *Bacillus* spp. was also found in rock art paintings from Atlanterra shelter (Gonzalez et al., 1999).

The absence of culturable actinomycetes in dripping waters agrees with the observation of Kölbel-Boelke et al. (1988). They found very few actinomycetes in 60 water and sediment samples.

Bacterial enumeration of a sample from ceiling rock surface (P-5) showed 30 cfu cm^{-2}. From twenty three isolates (Fig. 2) six were *Streptomyces* spp., and four gram-positive non-endospore producing, calcium carbonate crystal precipiting strains (Fig. 3).

P-6 yielded 27 cfu cm^{-2}, from which 41 isolates were obtained (56% with clear aerial mycelia). Seven isolates were identified as *Streptomyces* spp. and one as *Nocardioides* sp. Other bacteria found were *Erwinia amylovora*, *Chryseomonas luteola*, *Pseudomonas fluorescens* and *Bacillus sphaericus*. Four *Streptomyces* spp. precipitated crystals in B-4 medium (Fig. 3). Thirteen isolates were obtained from C-EG sample, among which *Streptomyces rishiriensis*, *S. flavogriseus*, *S. xanthophaeus* and *Streptomyces* sp. were identified in addition to *Flavimonas oryzihabitans* and *Xanthomonas* sp. These data clearly demonstrate that dripping water

Table 3
Gram-positive isolates from dripping waters of Altamira cave

Identification	No isolates	Origin[a]	Percentage of identification[b]
Bacillus cereus	3	EN-3	99.9
Bacillus polymyxa	1	EN-3	99.3
Bacillus circulans	2	EN-3	91.6
Bacillus stearothermophilus	1	GM	99.9
Bacillus subtilis	1	EN-1	80.5
NI[a]	1	GM	

[a] EN: entrance of the cave, GM: Hall of the Walls, NI: not identified.
[b] An estimate of how closely the profile corresponds to the taxon relative to all the other taxa in the Apilab Plus data base.

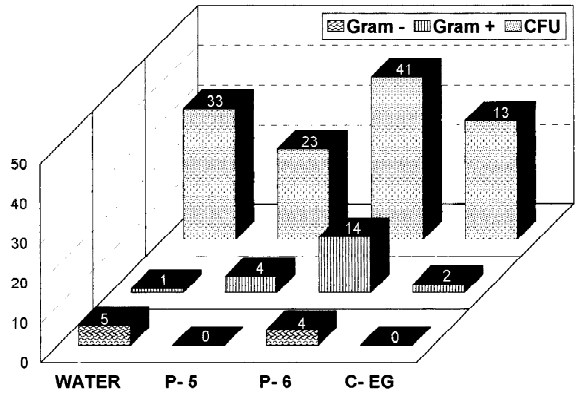

Fig. 3. Distribution of bacteria forming calcium carbonate. Explanation as in Fig. 2. Histograms represent number of calcium carbonate-producing gram-positive and gram-negative bacteria with respect to cfu.

microbial communities were different from those of rock.

3.3. Physiological activities

Dripping water isolates were tested for their ability to degrade different substrates. Table 4 shows total physiological activity of the dripping water isolates. Isolates from the families *Enterobacteriaceae* and *Vibrionaceae*, distributed homogeneously in all samples, showed the higher values, followed by some *Bacillus* species. Physiological activities of the isolates for digestion of specific substrates is shown in Table 5, expressed as percentage frequency of positive characters. This table compares the ten substrates that had been utilized with highest frequencies by the isolates of the water community. All gram-

Table 4
Physiological activity of dripping water isolates

Group	Bacterial strain	G (i) %[a]
Facultatively	*Enterobacter amnigenus*	47.8
anaerobic	*Serratia liquefaciens*	47.8
gram-negative rods	*Erwinia sp.*	34.8
	Aeromonas hydrophila	47.8
	Aeromonas sobria	60.9
	Aeromonas salmonicida	17.4
	Chromobacterium violaceum	23.9
Gram-negative	*Janthinobacterium lividum*	13.0
aerobic/microaerophilic	*Pseudomonas fluorescens*	30.4
rods and cocci	*Pseudomonas aeruginosa*	26.1
	Chryseomonas luteola	30.4
	Xanthomonas maltophilia	26.1
	Flavimonas oryzihabitans	8.7
	Acinetobacter sp.	4.4
	Kingella kingae	13.0
	NI[b]	4.4–60.9
Endospore-forming	*Bacillus cereus*	23.5
gram-positive rods	*Bacillus polymyxa*	47.5
	Bacillus circulans	46.8
	Bacillus subtilis	41.0
	Bacillus stearothermophilus	24.6
	NI[b]	24.6

[a] G(i)%: percentage of total activity of a bacterial strain.
[b] NI: not identified.

Table 5
Comparison of the 10 substrates digested with highest frequencies by isolates from dripping waters

Gram-negative isolates		Gram-positive isolates	
Substrate[a]	Isolates using substrate (% of total)	Substrate[a]	Isolates using substrate (% of total)
ADH	54.2	GLU	100.0
GLU	50.0	FRU	100.0
ARA	41.7	MAL	100.0
CIT	41.7	ESC	100.0
GEL	41.7	CEL	88.9
SAC	37.5	AMD	77.8
MEL	29.2	GLYG	77.8
AMY	25.0	GEL	77.8
RHA	20.8	RIB	66.7
INO	8.3	MNE	66.7

[a] ADH: arginine, CELlobiose, GELatine, MALtose, RIBose; AMD: starch, CITrate, GLUcose, MELibiose, SACcharose, AMYgdalin, ESCulin, GLYcoGen, MaNnosE, ARAbinose, FRUctose, INOsitol, RHAmnose.

positive isolates were able to use glucose, fructose, maltose and esculin, however, gram-negative isolates showed comparatively lower frequencies for glucose (50%), arabinose (41.7%), saccharose (37.5%), and melibiose (29.3%). In general, gram-positive bacteria presented higher positive reactions for carbon and nitrogen sources than gram-negative bacteria, with the exception of rhamnose, lysine, ornithine and arginine. The data demonstrate that endospore-forming gram-positive rods degraded monosaccharides with the highest frequencies, but higher frequency of occurrence of tested physiological activities were usually found among facultatively anaerobic gram-negative rods.

3.4. Crystal formation

In the ceiling and walls of Altamire cave some formations of calcite, aragonite, and hydromagnesite can be found (Cañaveras et al., 1999). The presence of aragonite and hydromagnesite are highly suggestive of a microbial-mediated precipitation. To this end, crystal formation with all isolated bacteria from dripping waters and ceiling rock was tested (Fig. 3). The bacteria producing crystals in a B-4 medium were *Acinetobacter* sp., *Serratia liquefaciens*, *Chryseomonas luteola*, *Xanthomonas maltophilia*, *Flavimonas oryzihabitans* and *Bacillus cereus*. In dripping waters, gram-negative bacteria producing crystals amounted to 15.2% of the population detected, higher than in rock (from 9.8 to 0%). Among gram-positive bacteria, the most common genus either from water or rock samples was *Bacillus*, with *B. cereus* in water and *B. sphaericus* in rock as isolates. Considering the total number of isolates, the percentage was much higher in the P-6 rock sample than in dripping water and other rock samples. In fact, the total percentage of crystal-producing bacteria was 18.2% for dripping waters, 43.9% for P-6, 17.4 for P-5 and 15.4% for C-EG.

Cañaveras et al. (1999) suggested that in Altamira cave, some calcium and magnesium crystal deposits originated by the action of bacteria. The bacteria isolated either from dripping waters or rock showed the ability to produce crystals and therefore could play a role in the deposition of calcium carbonate polymorphs on the rock surface. Interestingly, Went (1969) found a fungus regularly associated with the active tip of stalactites where crystallization of calcium carbonate occurred on hyphae suspended from the stalactite wall. He reported that the hyphae function both as crystallization nuclei and as attachment, without which individual crystals would be eliminated by the falling drop.

3.5. Crystal analysis

From the three *Acinetobacter* sp. strains isolated, one grew very fast and produced a large amount of crystals surrounding the colonies, which were visible

after 24 h. After one week the crystals were distributed all over the plate (Fig. 4). This isolate was only able to use acetate and pyruvate but not citrate or glucose, the other two strains also used citrate.

Crystals were collected from the monolayer surface of the most active *Acinetobacter* sp. after 20 days culture for structural and morphological analysis. Calcium carbonate polymorphs were identified by FT-IR spectroscopy (Falini et al., 1996), SEM, and confirmed by X-ray diffraction. The main crystals were vaterite (85%) and calcite (15%). Calcite was precipitated by different genera of bacteria (Ben Omar et al., 1997), aragonite by *Acinetobacter* strains (Del Moral et al., 1987). Vaterite is highly unstable and is rarely found. Lowenstam (1981) only reported vaterite in *Rhodophyta*, in addition to a few animal taxa.

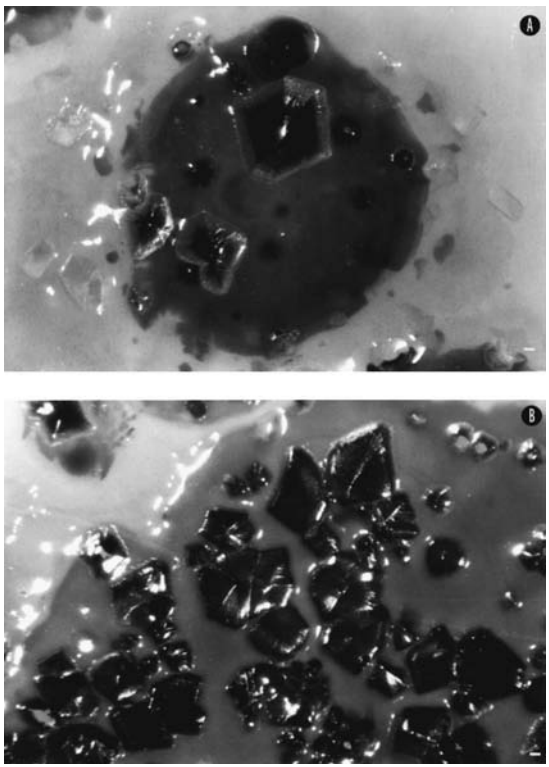

Fig. 4. (a) and (b). Crystals of calcium carbonate produced by an *Acinetobacter* sp. strain in B-4 medium. Dark crystals are due to the addition of a tetrazolium indicator dye. Bar is 0.125 mm.

4. Concluding remarks

In general, cultural bacterial communities from ground waters have low proportions of gram-positive bacteria. This was also the trend in dripping waters from Altamira cave. These communities are mainly composed of gram-negative rods and cocci (*Enterobacteriaceae* and *Vibrionaceae*) while those of rocks were mainly *Streptomyces* spp. (Groth et al., 1999). This difference cannot be ascribed to a difference in culture media composition (Petrifilm for water and TSA for rock isolation) as water samples were also inoculated directly in TSA with similar results. In addition, most of the bacteria isolated have the ability to precipitate crystals in vitro and probably in natural habitats. These findings support the assert by Cañaveras et al. (1999) that aragonite and hydromagnesite formations in Altamira cave is a biologically-mediated process.

Actinomycetes are well known for their ability to grow on very poor media (Lechevalier and Lechevalier, 1967) and streptomycetes exist for extend periods as resting arthrospores that germinate in the occasional presence of exogenous nutrients (Goodfellow and Williams, 1983). Dissolved organic carbon content in dripping waters is highly variable, from less than 5 mg C l^{-1} in winter to about 2200 mg C l^{-1} in late spring. Dissolved organic matter from soil, which is the origin of the organic carbon found in the dripping waters, contain aliphatic organic acids and phenolic compounds (Saiz-Jimenez and Hermosin, 1999). Guggenberger and Zech (1994) reported that water-soluble organic matter from soils is composed of polymeric lignocellulose degradation products. Both lignocellulose and humic materials are almost selectively degraded by actinomycetes (Crawford et al., 1983; McCarthy, 1987; Ball et al., 1989; Ball et al., 1990; Kontchou and Blondeau, 1992; Dari et al., 1995) and even humic acid is used in an actinomycete isolation medium (Hayakawa and Nonomura, 1987).

Differences in community composition may be attributable to environmental conditions in the cave: high humidity ($>95\%$), relatively low and stable temperature (around 13°C), water pH close to neutrality, and cyclic nutrient limitations. In addition, factors influencing attachment of bacteria (mi-

croroughness, substratum chemistry and pH, fluid dynamic, etc.) have to be considered. All this seems to favor long-term colonization and selective growth of actinomycetes on cave surfaces over other heterotrophic bacteria present in dripping waters.

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