A COMPARATIVE STUDY ON THE MICROBIAL ACTIVITIES AND PHYSIOLOGICAL GROUPS OF BACTERIA IN SOME CAVES FROM PĂDUREA CRAIULUI MOUNTAINS

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Abstract: microorganisms represent a heterogeneous group, widely spread in different environments. Our goal in this study was to determine whether microorganisms are present in four particular Transylvanian caves, which are: Ungurului Cave, Izvor Cave, Lesianei Cave, and Moanei Cave, all situated in the Pădurea Craiului Mountains. All of those caves are often visited by tourists. In order to conceive this study we have collected and analyzed different samples, using enzymological and microbiological methods; the samples were taken from the floor deposits, wall deposits and sludge. The analysis has shown the presence of different types of bacteria: aerobe heterotrophic bacteria, iron-reducing bacteria, lipolytic and proteolytic bacteria, according to each analyzed sample. We also studied some enzymatic activities, such as: catalase activity, phosphatase activity, actual and potential dehydrogenase activity, as well as the non-enzymatic catalytic activity.

INTRODUCTION

Procaryotes can live in an enormous variety of ecological niches, and they are amazingly varied in their biochemistry, far more varied than eucaryotic cells are. There are organotrophic species that can make use of practically any type of organic molecule as food, from sugars or amino acids, to methane. Than, there are the phototrophic species, which can take light energy in a variety of ways, some of them generating oxygen, others not. Last, but not least, there are lithotrophic species that can feed on a plain diet of inorganic nutrients, getting their carbon from CO_2, and their energy from H_2S, H_2, Fe^{2+}, elemental sulfur or any other chemicals in the environment.

A large part of this world of microscopic organisms is virtually unexplored. Traditional methods of bacteriology have allowed us to detect only those species that can be isolated and cultured in the laboratory. But new techniques, such as DNA sequence analysis, have shown that most species can't be cultured by standard laboratory methods. According to one estimate, at least 99 % of procaryotic species are still unknown (Alberts et al., 2002).

Very few studies have been made as far as the cave microbiota is concerned, mostly because of the complications met when collecting the samples, but also because it is very difficult to establish the origin of the detected bacteria (many of them are allochthonous and accidentally brought in by tourists).

Cave environments are quite steady when regarding the temperature (usually constant), high humidity, as well as the complete lack of light (Hidreth-Werker and Werker, 1997). This particular environment has been very little explored, concerning its potential for new medical products, may be because some publications during 1950-1960 wrongly stated that caves exhibit a sterile potential in this matter. But, between 1980-1997, was discovered a large diversity of bacteria, exploring cave like Lachuguilla (New Mexico), Mammoth (Kentucky, USA), and caves from Hawaii, collecting and culturing over one thousand species (Rusterholtz and Mallory, 1994).

A large variety of bacteria belonging to the actinobacteria (actinomycetes), cyanobacteria, and even archaebacteria have been isolated from caves. Some actinomycetes give off a characteristic smell of cave entry, due to the production of a original metabolite (geosmine) (Moore ans Sullivan, 1997). Cañavaras et all. (1999) give a big importance to some actinomycetes species (*Streptomyces roseoviridis, S. flavogriseus etc*) in the process of moonmilk production. Cyanobacteria are considered active agents in some speleogenesis processes (Pentecost and Spiro, 1990; Pentecost 1994; Knauth 1994; Drăgan-Bularda and Boeraş, 2004). Northup et all. (1998) made evident in Lechuguilla cave an archaebacteria from *Crenarchaeota* group.

The diversity of the metabolic reactions explains the complexe geo-chemical importance of such(Kiss et all. (1986). Stone (1997) shows that the extracellular organic compounds produced by the microorganisms have a big importance in connecting the biological processes with the geo-chemical ones.

These results bring up the possibility of using certain bacterial products (toxins) in medicine, and obtaining new antibiotics or other kinds of medicines for curing different diseases, including cancer (Rusterholtz and Mallory, 1994).

Our aim in this study is to determine whether bacteria are present or not in the caves we have collected samples from: Ungurului Cave, Izvor Cave, Lesianei Cave, and Moanei Cave, from the Pădurea Craiului Mountains, and if there are any enzymatic activities mainly due to the microorganisms.

MATERIALS AND METHODS

Sampling. For microbiological analysis, samples were taken in aseptic conditions from different places of the four caves: floor deposits, wall deposits and sludge. The only cave which misses the sludge sample was the Lesianei Cave, and this due to the fact that there was no accessible water stream.

Sampling took place in April, the 4th and June, the 18th, 2005.

The following specific *media* have been used:

Medium for aerobe heterotrophic bacteria: agar nutritive broth; sterilized at 121ºC, for 30 min. Number of bacterial group was expressed as CFU/ ml (colony forming units) (Drăgan-Bularda, 2000).

Medium for iron-reducing bacteria: 3g K_2HPO_4; 0.8g KH_2PO_4; 0.2g $MgSO_4$; 0.2g KCl; 20g glucose; 5g peptone; 0.5g Fe_2O_3; 0.1g $MnSO_4$; 1000ml distilled water. Sterilization was done at 105ºC, for 60 min. Most probable number (MPN) was established on the reaction with L,L-dipiridill for ions of Fe 2+ (see Alexander, 1965).

Medium for lipolytic bacteria (nutritive agar media containing Tween 80): 10g peptone (Bacto); 5g NaCl; 0.1g $CaCl_2$; 10g Tween 80; 15g Bacto-agar; 1000ml distilled water; pH = 7.8; Sterilized at 121 °C, for 20 min. MPN was established on the basis of precipitation of CaCl2 aroud of the colonies (Stoica et al., 2002).

Medium for proteolytic bacteria: 30g gelatine; 0.1g $FePO_4$; 1000ml distilled water; pH 7.0 – 7.2; sterilized at 110 ºC, for 20 min. MPN was established on the basis of appear gelatine liquefaciens (Aaronson, 1970).

Catalase activity was determined using a technique based on KAPPEN's method (1993). We took 1 g of material, added 10 ml of distilled water and 2 ml of H_2O_2 3 %. This mixture was incubated at 20 ºC, for one hour. The catalase activity is calculated from the difference between the active samples and the inactivated ones, and is expressed in mg H_2O_2/1 g material/5 h/20 ºC.

Dehydrogenase activity was determined using CASIDA et al. method (1964). The mixture consisted of 1 g of material, 0.5 ml TTC 3 % and 2 ml distilled water, for the actual activity. For the potential activity, instead of 2 ml of water we used only one and added another ml of glucose 3 %. Incubation took place at 37 ºC, for 72 h. The activity was measured in mg formazan/1 g material/72 h/37ºC.

Phosphatase activity was determined using method of Kramer and Erdely (Drăgan-Bularda, 2000) using a mixture consisting of 1 g of material and 10 ml of disodic fenilphosphate solution 0.5 %. For incubation took more than 2 h, we added 2 ml of benzene. Incubation took place at 37 ºC, for 3 days. After incubation, we added ammonium alaun solution, borax tampon (pH 9.4) and Gibbs reagent.The intensity of the blue color shows the intensity of the reaction. Phosphatase activity was measured in mg phenol/1 g material/72 h/37ºC.

Non-enzymatic catalytic activity was established also using KAPPEN's method: 1 g material, 2 ml H_2O_2 3 %, 10 ml distilled water, but in this case samples underwent a thermic inactivation. Non-enzymatic catalytic activity also expressed in mg H_2O_2/g material/5 h/20ºC).

RESULTS AND DISCUSSION

Results regarding the physiological groups of bacteria are shown in table 1, 2 and 3.

Table 1. The presence of aerobe heterotrophic bacteria

Sample origin	Sample	No. bacteria / g cavematerial
Ungurului Cave	1. floor deposits	4.5
	2. wall deposits	2
	3. sludge	17
Izvor Cave	1. floor deposits	6.8
	2. wall deposits	0
	3. sludge	14
Lesianei Cave	1. floor deposits	20
	2. wall deposits	0
Moanei Cave	1. floor deposits	21
	2. wall deposits	0
	3. sludge	17

Presence of aerobe heterotrophic bacteria is very poorly represented (table 1), which demonstrates a low existing amount of organic matter. Samples from wall deposits, excepting Ungurului Cave, did not even exhibit aerobe heterotrophic bacteria. These results evidence the lack of any source of organic pollution.

Iron-reducing bacteria are very irregular: samples of floor deposits from Ungurului cave contain a high concentration of this group of bacteria, while some other samples contain only a limited number of iron-reducing bacteria, and others do not exhibit any, as shown in table 2.

Table 2. The presence of iron-reducing bacteria

Sample origin	Sample	No.bacteria / g cave material
Ungurului Cave	1. floor deposits	29
	2. wall deposits	15
	3. sludge	0
Izvor Cave	1. floor deposits	12
	2. wall deposits	0
	3. sludge	18
Lesianei Cave	1. floor deposits	21
	2. wall deposits	0
Moanei Cave	1. floor deposits	18
	2. wall deposits	0
	3. sludge	24

We limited our study regarding the lipolytic and proteolytic bacteria only to the Ungurului Cave, as a result of their low presence here (table 3). Lipolytic and proteolytic bacteria were detected only in sludge samples. The presence of a limited number of lipolytic and proteolytic bacteria, and only in some particular sampling sites, indicates a low potential of the sample, concerning organic matter.

Table 3. The presence of lipolytic and proteolytic bacteria in Ungurului Cave

Sample	Lipolytic bacteria	Proteolytic bacteria
1. floor deposits	0	0
2. wall deposits	0	0
3. sludge	12	17

Table 4. The catalase and dehydrogenase (actual and potential) activities on cave materials

Sample origin	Sample	Catalase activity (mg H_2O_2 / g material / 5 h / 20 ºC)	Dehydrogenase activity (mg formazan/1 g material/72h /37ºC)	
			Actual	Potential
Ungurului Cave	1. floor deposits	2.38	0.065	0.109
	2. wall deposits	2.43	0.091	0.200
	3. sludge	2.45	0.036	0.063
Izvor Cave	1. floor deposits	4.21	0.095	0.405
	2. wall deposits	0.90	0.023	0.105
	3. sludge	2.31	0.085	0.386
Lesianei Cave	1. floor deposits	2.30	0.058	0.315
	2. wall deposits	1.19	0.079	0.185
Moanei Cave	1. floor deposits	2.45	0.110	0.310
	2. wall deposits	1.07	0.055	0.099
	3. sludge	1.75	0.091	0.277

The catalase activity had a low intensity (table 4). Samples from the Ungurului Cave exhibited the most regular values regarding this activity. Samples from the other caves showed a higher irregularity concerning catalase activities, but still the values are limited.

The potential dehydrogenase activity is more intense than the actual one (table 4), which indicates the stimulating action that the carbon source (glucose) has upon the enzymatic synthesis of the germs in the samples. The low values recorded on this particular activity demonstrates a limited microbial potential.

Table 5. Phosphatase and non-enzymatic catalytic activities

Sample origin	Sample	Phosphatase activity (mg fenol/1 g material/72 h/37ºC)	Non-enzymatic catalytic activity (mg H_2O_2/g material/5 h/20ºC)
Izvor Cave	1. floor deposits	0.311	0.03
	2. wall deposits	0.070	0.09
	3. sludge	0.235	0.51
Lesianei Cave	1. floor deposits	0.210	2.70
	2. wall deposits	0.115	1.90
Moanei Cave	1. floor deposits	0.205	3.17
	2. wall deposits	0.090	0.98
	3. sludge	0.119	2.05

Phosphatase activity has registered extremely low values in all samples, which shows that phosphor is very poorly existed in these caves. Also the non-enzymatic catalytic activity was very weak, showing that compounds such as humic acids, iron oxides, etc. are usually absent in caves environment.

This study shows that bacteria are present in all samples taken into analysis: floor and wall deposits, and sludge. In comparison with other studies, we have detected the presence of aerobe heterotrophic bacteria, but we can not certainly state that these microorganisms are autochthonous. They might have been accidentally brought into these caves by the tourists, or the personnel who administrate them (Molnar, 1961).

Iron-reducing bacteria could be detected in particular samples. This result was somehow expected, as it is well known that iron oxides can exist in cave environments, being an alternative food source for microorganisms.

Enzymatic activities have also been detected and measured. Results, although poor, were nevertheless positive, as far as the catalase, dehydrogenase, phosphatase and non-enzymatic catalytic activities are concerned. The results were normal, as we refer to an extreme environment.

The presence of these enzymatic activities indicates that microbial communities do exist in caves.

CONCLUSIONS

Microorganisms exist in all analyzed samples (floor and wall deposits, sludge), but in limited strength;

The bacteria which could be distinguished using particular culture methods were: iron-reducing bacteria, lipolytic and proteolytic bacteria, and aerobe heterotrophic bacteria;

The presence of aerobe heterotrophic bacteria in this extreme environment is not necessarily convincing, as they could come from other places, and accidentally be brought here, especially if we take into account the negative results registered by other authors;

Aerobe heterotrophic bacteria are weakly represented here, fact that shows a limited existence of organic compounds in this environment and, therefore a lack of any organic polluting source;

The presence of iron-reducing bacteria indicates the existence of iron oxides in caves, as well as remains of organic matter;

This analysis and the positive results of the enzymological tests denotes the presence of microorganisms in caves, both at the time being and the past.

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