Comparison of the Culturable Bacterial Flora from Three Microenvironments from Pečina v Borštu Cave (South-West Slovenia)

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Abstract

Pečina v Borštu cave is situated in the karst area of Matarsko podolje in the south-west part of Slovenia. The cave was formed in Upper Cretaceous limestone which is in some parts of the cave extremely weathered. We have analysed samples from three different microenvironments in this cave: silver flashing droplets, water from a small pond and weathered limestone bedrock of the cave wall. In the water sample approximately 10^4 colony forming units (CFU) per millilitre were detected, whereas the weathered limestone contained approximately 10^6 CFU per millilitre. Fluorescent pseudomonads were found in all three environments. In the silver droplets a single species of a fluorescent pseudomonad was isolated. The bacterial flora in other two environments was more diverse; Gram negative nonfermentative bacteria and actinomycetes were isolated from both samples. The water contained also violet pigmented bacteria and Gram positive cocci.

Introduction

Pečina v Borštu cave is situated about 100 m above a blind valley called Jezerina where a smaller stream sinks at the contact between Eocene flysch rocks and Paleocene and Cretaceous limestones (ŠIKIĆ et al., 1972). The cave is about 250 m long and it follows the S – N direction. Active water flow is not present anymore but in some parts of the cave after atmospheric precipitation the water percolation through cave ceiling is very strong. The average annual temperature in inner parts of the cave is about 10°C but near the entrance of the cave variation of the temperature corresponds to outside changes of the temperature. Cave walls are covered by flowstone and speleothems of different ages and forms. Almost all cave walls are extremely weathered and may be seen just in cases where flowstone was not precipitated or was already corroded. Weathered zone on limestone walls varies from few millimetres to some centimetres. In the cave clastic deposits originated from flysch background are found. The main component of the deposit is quartz mixed with some clay minerals.

The karst caves are extreme environments (PEDERSEN, 2000) with constant temperatures and humidity, but generally with low nutrient input. In a single cave different microenvironments can be found. The aim of this study was to determine variability of bacterial microflora from three different niches within a single cave.

We have selected Pečina v Borštu cave because of the presence of special microenvironments: silver flashing droplets, water from a small pond and weathered limestone bedrock of the cave wall.

Silver flashing droplets are famous among speleologists as “cave silver”. Water droplets are formed near the cave entrance where cold and warm air mix and its presence is connected to condensation. We decided to take this sample to clear up if this fluorescence is connected with biological activity.

Second tested microenvironment was pond filled with water, unusual because of calcite rafts formation on its surface. This pond is periodically filled up with water controlled by raining regime. During the rain period there could be a niche for biofilm foundation and eventual calcite precipitation promoted by present microorganisms.

Weathered limestone could be referred to as one kind of “moonmilk”. This term is used to describe aggregates of microcrystalline substances of varying composition. Moonmilk could be formed as disintegration of bedrock and speleothems or as a mixed deposition of calcite crystals and water (HILL & FORTI, 1997). The extent of microbial involvement in the formation of speleothems and in weathering process of limestone is still being discussed. Microbes possess biochemical pathways which can contribute to the dissolution and mobilization of carbonates (ATLAS & BARTHA, 1997).

We have measured *in situ* pH by using Testo® pH-meter system with special electrode. Measurements of pH were made in silver flashing droplets (pH range from 7,66 to 7,65), in water of the pond (pH from 7,51 in the bottom to 7,80 on the water surface). Weathered zone of limestone is periodical wetted by percolating water and its wetness depends directly on atmospheric precipitation at surface. The pH of weathered limestone measured at the same time varies from 7,38 to 8,25 (almost at all measured points the pH values varies in range from 7,8 to 8,2; only in very damp sample the pH was 7,38) at constant temperature 10,6 °C. The value 7,38 is still in range of light aggressiveness, the other values indicate supersaturation of water solution in pores of weathered limestone zone. The pH laboratory measurements in the case of weathered limestone was also carried out and ranged from 8,0 up to 8,2.

Material and Methods

Determination of Total Bacterial Count

Samples were aseptically taken from the cave and transferred into the laboratory. Several cultivation media were tested for bacterial growth: (1) Prep medium contained 1 % weathered bedrock, 0,1 % yeast extract and 1,5 % agar. It was used to mimic the nutrient content of the weathered limestone. (2) King B (Difco, USA) is standard isolation medium that supports good growth of most heterotrophic bacteria.

Samples were diluted in a physiological solution and 0,1 ml of appropriate dilutions were inoculated on the plates. Plates were incubated at 10°C. After 14 days the bacterial colonies were counted and total number of culturable bacteria was calculated.

Characterization of Pure Cultures

On the primary plates all different morphotypes of bacterial colonies were selected and streaked onto fresh King B medium until the pure culture was obtained. Cultures were grouped according to the following tests: presence of oxidase and catalase, growth temperature, oxidative vs. fermentative metabolism, production of fluorescent pigments, motility, gelatinase, nitrate reduction, degradation of several carbohydrates and degradation of amino acids (MAC FADDIN, 1980).

Optical microscopy was also performed to determine Gram staining and cell morphology. For violet pigmented cultures the absorption spectra was determined as described in BOARD *et al.* (1992).

Results and Discussion

Cell Count of Culturable Bacteria

Cell count was determined only for water and weathered limestone, but not for silver flashing droplets due to small amount of the sample. (Table 1)

Weathered limestone contained more bacteria than the pond water. Interestingly, bacteria from limestone sample preferred Prep medium (prepared with weathered limestone), whereas the growth of pond water bacteria was better on King B medium. Obviously the bacteria in weathered limestone sample are well adapted to special nutrients available in such microenvironment.

Microbial Diversity Among Three Distinctive Samples

Bacteria isolated in pure culture were divided into five groups (Table 1).

Fluorescent pseudomonads were characterized by production of fluorescent pigment on King B medium. All of them are motile Gram negative rods, with oxidative metabolism, but differ in utilisation of sugars and amino acids.

Oxidative Gram negative bacteria with no production of fluorescent pigments were defined as Gram negative nonfermentatives.

The third group represents violet pigmented strains, which were Gram negative rods with fermentative metabolism. Regarding to absorption spectra the pigment was violacein (BOARD *et al.*, 1992). Therefore, the strains most likely belong to the genus *Chromobacter*.

The remaining two groups of Gram positive cocci and Gram positive irregular rods were characterized by their microscopic morphology.

The bacterial microflora in three studied microenvironments from Pečina v Borštu cave differs not only in cell count, but also in the community structure. In the silver flashing droplets we isolated only one strain of bacteria belonging to the fluorescent pseudomonads group. However, the presence of these bacteria is not a reason for fluorescence as droplets show no fluorescence under UV light. In the case of pond sample we isolated quite heterogeneous bacterial microflora, belonging to different groups (Table 1). Weathered limestone microflora was also heterogeneous. Fluorescent pseudomonads seem to be prevalent microorganisms which can be due to their versatile metabolic pathways.

Table 1 - Bacterial counts and their diversity in three different cave microenvironments

	HABITAT (microniche)		
	SILVER FLASHING DROPLETS	POND	WEATHERED LIMESTONE
BACTERIAL COUNT	**COLONY FORMING UNITS (CFU) / ml**		
King B	ND	$2,5\times10^4$	$4,0\times10^4$
Prep medium	ND	$1,0\times10^4$	$1,1\times10^4$
BACTERIAL GROUPS	**NUMBER OF ISOLATED STRAINS**		
FLUORESCENT PSEUDOMONADS	1	6	5
GRAM NEGATIVE NONFERMENTATIVES		6	2
GRAM NEGATIVE FERMENTATIVES *(violet pigment)*		2	
GRAM POSITIVE COCCI		1	
GRAM POSITIVE IRREGULAR RODS *(Actinomycetes like)*		4	4

ND – not done

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