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DIVERSITY OF CULTURABLE BACTERIA AND MEIOFAUNA IN THE EPIKARST OF ŠKOCJANSKE JAME CAVES (SLOVENIA)

RAZNOVRSTNOST BAKTERIJ, KI JIH LAHKO GOJIMO, IN MEIOFAVNE IZ EPIKRASA ŠKOCJANSKIH JAM (SLOVENIJA)

BARBARA GERIČ[1] & TANJA PIPAN[2] & JANEZ MULEC[2*]

[1] Department of Biology, Biotechnical Faculty, Večna pot 111, SI–1000 Ljubljana, Slovenia
[2] Karst Research Institute ZRC SAZU, Titov trg 2, p.p. 59, SI–6230 Postojna, Slovenia
[2*] Corresponding author. E-mail:janez.mulec@guest.arnes.si

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Abstract UDC: 551.44(497.4):579.68

Barbara Gerič & Tanja Pipan & Janez Mulec: Diversity of culturable bacteria and meiofauna in the epikarst of Škocjanske jame caves (Slovenia)

The epikarst zone becomes an important site for hydrogeological, geomorphological and biological investigations. From the samples at four sites in Škocjanske jame caves where water tricklets constantly drip from the cave ceiling the meiofauna were identified. The sites were screened also for the presence of culturable bacteria. The viable cell counts were supplemented with morphological and biochemical analysis of the isolates. Results show that Gram–negative bacteria prevail among culturable bacteria in the percolating epikarst waters.
Key words: bacteria, meiofauna, karst, epikarst, Škocjanske jame caves, Slovenia.

Izvleček UDK: 551.44(497.4):579.68

Barbara Gerič & Tanja Pipan & Janez Mulec: Raznovrstnost bakterij, ki jih lahko gojimo, in meiofavne iz epikrasa Škocjanskih jam (Slovenia)

Epikras postaja pomembno mesto hidrogeoloških, geomorfoloških in bioloških raziskav. Iz vzorcev pobranih s štirih mest v Škocjanskih jamah, kjer stalno kaplja voda z jamskega stropa, smo identificirali meiofavno. Na istih mestih so bili odvzeti tudi vzorci za izolacijo bakterij. Podatke o številu viabilnih celic smo dopolnili z morfološkimi in biokemijskimi lastnostmi izolatov. Izsledki kažejo, da v vzorcih prenikle vode iz epikrasa med bakterijami, ki jih lahko gojimo v in vitro razmerah, prevladujejo po Gramu negativne bakterije.
Ključne besede: bakterije, meiofavna, kras, epikras, Škocjanske jame, Slovenija.

INTRODUCTION

The epikarst represents the upper layer of the unsaturated or vadose zone, which is densely fissured due to the water's higher aggressiveness, tension release, tectonic processes and differences in temperature. Its principal characteristics are substantial storage capacity and high permeability (Petrič 2002). Due to the inaccessibility of the epikarst zone it is impossible to examine directly the biological processes within the epikarst. On the other hand it is easy to study epikarst interactions indirectly by taking samples of water which drips down the cave walls or seeps from the cave ceiling.

The epikarst zone is a habitat of a diverse meiofauna which has evolved special features for life within the dark (Holsinger 1994). Meiofauna is a complex group of animals that pass through the 0.500 mm sieve and are retained on the 0.063 mm (or 0.045 mm) sieve. Meiofauna representatives are important permanent inhabitants of the interfaces or ecotones (Schmid–Araya 1997). From samples collected from vertically dripping and seeping epikarst waters it was realized that among the scarce but species-rich meiofauna inhabiting the epikarst, copepods seem to dominate this aquatic habitat (Galassi 2001; Pipan & Brancelj 2001).

Being so small, free–living copepods can feed only on small food particles like bacteria, diatoms or other unicellular organisms (Walter 2001). The role of microorganisms in cave environments is still poorly understood. New microhabitats colonized by certain microbial taxa in Slovenian caves have been recently described (Mulec et al. 2002). Research of bacteria directly accompanying meiofauna in dripping water has been neglected although some data concerning enumeration of bacteria found in cave dripping waters are published. Dripping water samples from Altamira cave contained low proportion of Gram–positive bacteria. (Laiz et al. 1999). White et al. (1995) described the cave ecosystem as an open system relying on transport of organic matter from the surface as an energy base for the system. Nevertheless, the existence of chemolithotrophy based ecosystems should be considered as well. The presence of bacterial chemolithoautotrophic production in caves is usually correlated with an increased diversity of the cave meiofauna. The well–known chemolithotrophy based Movile cave ecosystem contains 48 species of cave–limited invertebrates, 33 of which are endemic to the system (Kinkle & Kane 2000).

The purpose of the present study was to establish bacterial groups from permanent tricklets in Škocjanske jame caves from which meiofauna was collected and identified (Pipan 2003).

MATERIAL AND METHODS

Sampling location

The Škocjanske jame caves (Fig. 1), situated at an elevation of 425 m above sea level, are a system of a ponor caves formed by the Reka sinking river at the contact of impermeable Eocene flysch and both Upper Cretaceous and Paleocene limestones (Mihevc 2001). In the geomorphological sense the area is divided into three bigger parts. The initial part is composed by collapse dolines, Velika dolina, Mala dolina and Okroglica. The next part is composed of a more than a 100 m high underground canyon, terminated by a siphon. In the higher level lies a dry passage Tiha jama. The complete cave system is 5800 m long and the Reka river flows through most of the Škocjanske jame caves (Kranjc et al. 1997). The vegetation above the caves is diverse, ranging from grassland and shrubs to mixed forest.

The nature and distribution of dripping water localities in Škocjanske jame caves have been previously described (Pipan & Brancelj 2001). Prior bacterial sampling some physical and chemical analyses have been conducted in a one year period to examine quality of the dripping water. Results showed temperature range from 10.3 to 12.6 °C and pH range from 7.4 to 8.2 (Pipan 2003). Four trickles (Fig. 1) in the caves were chosen for bacterial analyses due to permanent dripping water from the ceiling and high biodiversity of meiofauna found there (Table 2) (Pipan & Brancelj 2001; Pipan 2003). Considering the fact that the number of bacteria in cave dripping water changes seasonally with its peak in February (Laiz et al. 1999), we collected samples of dripping water to analyse culturable bacteria in March of 2002. Samples were aseptically collected into sterile 50 ml tubes and transferred in a cooling bag in a laboratory. Water discharge was measured simultaneously while collecting samples.

Bacterial enumeration and identification

Each sample was filtered through 0.22 μm pore filter (Sartorious, Germany) and put onto 0.1 strength nutrient agar (Difco, USA) to mimic nutrient poor cave environment. The medium contained per litre: peptic digest of animal tissue 0.5 g, 0.3 g of beef extract and 15 g agar. Agar plates were incubateded 21 days at 8 °C. After the incubation period concentration of viable bacteria was calculated as a total number of colony forming units per millilitre, cfu ml^{-1}. Enumerated colonies were streak plated and pure isolates were screened for cell morphology (Gram staining and Schaeffer staining for endospore screening) and biochemical properties: presence of oxidase and catalase,

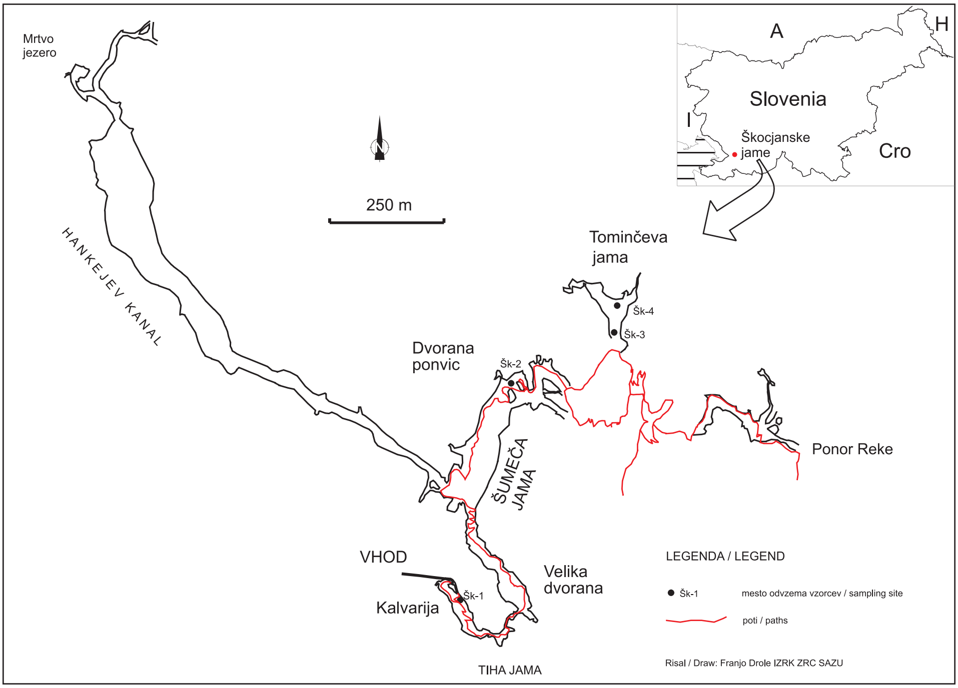

Fig. 1: Geographical location of Škocjanske jame caves in Slovenia. Sampling locations are designated on the ground plan of Škocjanske jame caves.

oxidative vs. fermentative metabolism, ability to utilize certain carbohydrates (maltose, arabinose, manitol), reduction of nitrate and N_2, hydrolise of aesculin, motility, ability to grow at 37 °C, ability to grow anaerobically (composition of atmosphere: 80 % N_2, 10 % CO_2, 10 % H_2), growth in the presence of 0/129 vibriostatic factor (2,4–diamino–6,7–diisopropilpteridine phosphate) and higher salt concentrations (1 % and 6 % NaCl concentration) as described in Holt et al. (1994). Violet pigmented bacteria were further treated to examine absorption spectrum of the pigment as described in Logan & Moss (1992).

RESULTS AND DISCUSSION

We isoleted bacteria on a nutrient–poor medium, because such a medium best represents the nutrient–poor conditions of the cave habitat. It was reported (Rusterholtz & Mallory 1994) that plate counts on nutritionally dilute media always exceeded those on the nutritionally rich media. Rusterholtz & Mallory (1994) also reported that bacteria isolated on the rich–nutrient medium are much more dificult to maintain. The direct microscopic cell counts normally exceed viable–cell counts of samples from different natural habitats by several orders of magnitude (Amann et al. 1995). Kölbel–Boelke et al. (1988) reported that in the case of sandy aquifier direct cell counts yielded numbers two to three orders of magnitude higher which could be used as an estimation relevant to dripping water.

The viable cell counts calculated on the basis of bacteria grown on a 0.22 μm pore filter (Fig. 2) ranged from 0.5 to 2.2 cfu ml^{-1} (Table 1). These values are lower comparing to data published by Laiz et al. (1999). They found in dripping waters of Altamira cave from 75 cfu ml^{-1} up to innumerable number of grown colonies. Kölbel–Boelke et al. (1988) reported less than 100 cfu ml^{-1} and in some cases even less than 50 cfu ml^{-1} in sandy aquifer samples. Concentration of bacteria varied from sample to sample which indicates the existence of small very diverse microhabitats (Kölbel–Boelke et al. 1988).

It should be stressed that samples for this study were taken from trickles with constant dripping water. Using the highest cfu ml^{-1} value (2.2 for cave trickle Šk–2; Table 1) and discharge value for the same trickle gives us 15.4 cfu min^{-1}, which means that each minute this amount of culturable bacteria is released into open space of the cave. Taking into account a simple relation for this example 22 176 cfu are released in 24 hours, 155 232 cfu in a week and 8 094 240 cfu in a year, from just one trickle. The presented situation could be explained as an »epikarst chemostat«: consisting of introduction of allochtonous nutrients from the cave surface, microbial multiplication in the epikarst

Trickle	Bacterial concentration (cfu ml^{-1})	Discharge (ml min^{-1})	Bacterial discharge (cfu min^{-1})	Meiofauna No. of specimens
Šk–1	1.2	9.5	11.4	35
Šk–2	2.2	7.0	15.4	250
Šk–3	0.53	6.3	3.3	180
Šk–4	0.5	5.2	2.6	40

Table 1: Counts of viable bacterial cells (cfu ml^{-1}), measured discharge of percolating water (ml min^{-1}), discharge of bacteria calculated as cfu min-1 and corresponding total number of animal individuals collected in one year sampling period.

and release of these microbes into the open cave space (Fig. 2.).

In the epikarst habitat not just intramicrobial interactions are present but also interactions with higher organisms. Bacteria represent along with other unicellular organisms (Walter 2001) the first link in a food chain of the epikarst meiofauna. Release of microbes into the cave space could serve also as a food source for the organisms which live in the cave.

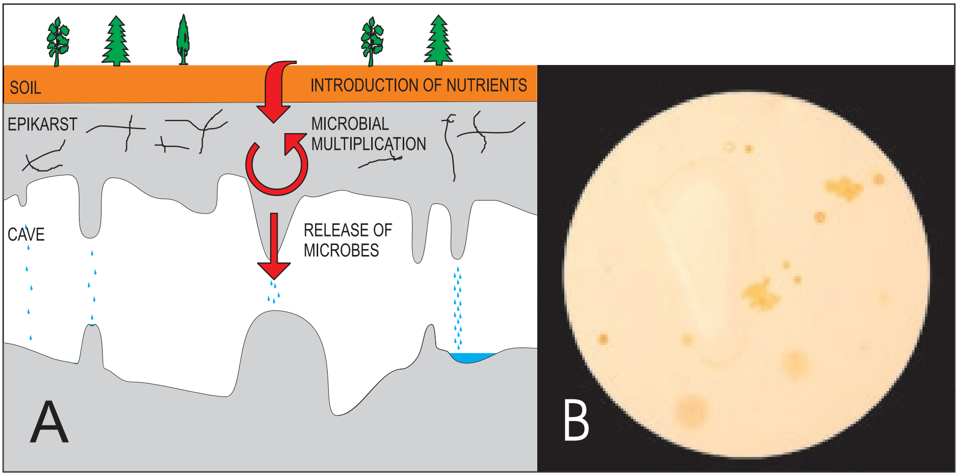

Fig. 2: A: Schematic representation of the epikarst zone. Introduction of allochtonous nutrient source from the surface, multiplication of organisms in the epikarst and their release into a cave are designated. B: Bacterial colonies isolated from the epikarst water on 0.22 μm filter (filter size 50 mm in diameter). (Photo: Bojan Otoničar)

Animal taxon	**Trickle**			
	Šk–1	**Šk–2**	**Šk–3**	**Šk–4**
Turbellaria	0	3	8	0
Nematodes	5	91	56	18
Oligochaeta	1	4	15	4
Acarina	0	1	4	1
Ostracoda	5	0	0	0
Copepoda & nauplia	23	149	82	14
Collembola	0	1	1	0
Diptera, larvae	1	1	14	3
Total number	**35**	**250**	**180**	**40**

Table 2: List and total number of animal taxa collected from cave dripping water from Škocjanske jame caves.

Bacterial group	**No. of isolates**	**Identification**
Enterobacteriaceae	3	
Gram–negative microaerophillic/aerobic rods and cocci	3	
Gram–positive rods	6	
Pseudomonadaceae	1	*Pseudomonas fluorescens*
Vibrionaceae	3	*Plesiomonas* spp
Violacein producing strains	2	*Iodobacter* sp *Janthinobacterium viridum*

Table 3. Bacterial diversity in the epikarst water from Škocjanske jame caves.

In Škocjanske jame caves we collected from the same four trickles eight different animal taxa (Table 2; Pipan 2003). In addition to copepods in the percoaltion water samples there were numerous speciments of Nematodes and few speciments of Turbellaria, Oligochaeta, Acarina, Ostracoda, Collembola and dipteran larvae. The highest release of bacteria as nutrient source from the epikarst (trickle Šk–2) could explain the highest number of collected meiofauna individuals in one year period, i.e. 250 individuals of seven different taxonomic groups of which Copepoda and Nematoda were the most abundant (Table 2). Seven species of Copepoda were identified. Three species (from genera *Moraira* and *Parastenocaris*) are recognized as new for science. Their morphological features and distribution indicate they are obligate epikarst species (Pipan 2003).

Bacteria isolated from dripping water were divided into six groups and some were identified to the species level. Culturable isolates contained high proportion of Gram–negative bacteria: i.e. Enterobacteriaceae, Gram–negative microaerophillic/aerobic rods and cocci, Pseudomonadaceae, Vibrionaceae, Violacein producing strains; relative to, i.e. Gram–positive rods (Table 3). Our findings are in accordance with the results published by Laiz et al. (1999) who showed that cave water communities contain low proportions of Gram–positive bacteria with the highest incidence of Enterobacteriaceae and Vibrionaceae and practicly no culturable actinomycetes.

Composition of the microbial community may be attributable to the environmental factors of the habitat such as: relatively low and stable water temperature, pH close to neutrality and nutrient limitation. Important culturable bacterial group in caves are Pseudomonadaceae as experienced also by Arroyo et al. (1997) and Mulec et al. (2002). Bacterial community from the epikarst habitat analysed in this study is typical water since it contains low proportion of Gram–positive and quite a lot of motile bacteria (Kölbel–Boelke et al. 1988). Besides water and air flow, gravity is an important agent in introducing the nutrients in the epikarst habitat.

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Povzetek

Epikras zavzema vedno pomembnejše mesto znanstvenega proučevanja, zato ga je treba razčleniti z geološkega kot tudi biološkega stališča. Zaradi nedostopnosti epikraške cone ni mogoče neposredno spremljanje biološkega dogajanja, lahko pa opazujemo ekološke interakcije posredno, s proučevanjem prenikle vode, ki odseva dogajanja v tem habitatu. Epikras je pomemben habitat podzemeljske favne, redkih in še neopisanih vrst, med katerimi prevladujejo ceponožni raki. Pomemben vir hranil tem organizmom predstavljajo drobni enocelični organizmi, tudi bakterije.

V prispevku smo na primeru Škocjanskih jam dokazali prisotnost nekaterih predstavnikov favne in spremljajoče bakterijske mikrobiote. Na istih mestih v jami, kjer so curki prenikle vode prisotni tekom celega leta, smo odvzeli vzorce za identifikacijo favne in za izolacijo bakterij, ki jih lahko gojimo v in vitro razmerah. Podatke o številu viabilnih bakterijskih celic, ki so bile v razponu od 0,5 do 2,2 cfu ml^{-1}, smo dopolnili še s podatki o morfoloških in biokemijskih lastnostih izolatov.

Okoljski pogoji, kot sta nizka temperatura, vrednost pH v območju nevtralnosti pogojujejo sestavo bakterijske združbe v epikrasu. Vnos alohtonih snovi v epikras, razmnoževanje bakterij, zapletne interakcije med organizmi, izpiranje bakterij iz epikrasa v jamo spominja na dogajanje v kemostatu. V vzorcih prenikle vode iz Škocjanskih jam prevladujejo bakterije značilne za vodne združbe z visokim deležem po Gramu negativnih bakterij.