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AIRBORNE BACTERIA CULTIVATED FROM UNDERGROUND HIBERNATION SITES IN THE NIETOPEREK BAT RESERVE (POLAND)

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Abstract

The study is the first report of cultivable bacteria present in the close vicinity of hibernating bats. Samples were collected in January 2016 in one location outside the hibernation site and in five locations underground. Samples were incubated at 7 and 37 °C. Bacteria were identified based on phenotypic tests and 16S rRNA gene analysis. Air samples collected inside of underground sites contained more propagules of bacteria (from 232 ± 28.3 to 1189 ± 124.7 bacterial colony-forming units per m^3 of air) than outdoor air samples (42 ± 12.2). In total, eight species of airborne bacteria belonging to three phyla and three orders were cultured from the samples. More species of airborne bacteria, eight, were isolated from the indoor underground air samples than from the outdoor air, two, especially in close vicinity to hibernating bats. Generally, *Actinobacteria* dominated in this study. *Paeniglutamicibacter psychrophenolicus* was isolated most frequently from samples incubated at 7 °C, and *Micrococcus luteus* from samples incubated at 37 °C. Additionally, the study was supplemented by detailed phenotypic and physiological characteristics of airborne bacteria obtained in the Nietoperek bat reserve.

Introduction

Microorganisms, especially extremophilic Bacteria and Archaea, are able to colonize all niches of the biosphere, including underground ecosystems (Rampelotto, 2013). However, because of low constant temperatures and high humidity of the air, little availability of nutrients, no light, and extensive areas of mineral surfaces, underground ecosystems are one of the most inhospitable habitats for microbial life (Jurado et al., 2010; Vanderwolf et al., 2013; Ogórek et al., 2014). Specific microclimatic conditions in underground sites, especially low temperatures (ca. from 2 °C to 10 °C) and high relative humidity (above 80 %) of air, allow bats to minimize energy during hibernation, when their food supply is scarce from late autumn to early spring (Speakman and Racey, 1989; Kokurewicz, 2004).

From August to October, before hibernation starts, bats gather in underground sites and actively fly in front of entrances to the underground. This phenomenon, known as swarming, involves circling both inside and outside the hibernacula (Fenton, 1969; Kretzschmar and Heinz, 1995). Due to their unique lifestyle, bats can provide organic nutrients and microorganisms for caves and other subterranean habitats directly from the surface of their bodies or indirectly through their guano. In addition, bat carcasses can be a source of organic matter for bacteria and fungi and a food source for arthropods (Veikkolainen et al., 2014; Kokurewicz et al., 2016; Ogórek et al., 2016a). However, it should be mentioned that other factors such as air currents, percolation via surface water, soil, and sediments, human activities, and the presence of small mammals and arthropods may also influence underground ecosystems and provide energy sources and nutrients (Chelius et al., 2009; Vanderwolf et al., 2013; Ogórek et al., 2016b).

The majority of microorganisms cannot actively grow in the subterranean ecosystems, but they could be present in aerosol form (Ogórek et al., 2016b). Bioaerosols may contain bacteria, fungi, viruses, cellular fragments, fungal spores, and byproducts of microbial metabolism such as mycotoxins, endotoxins, enterotoxins, and enzymes (Mandal and Brandl, 2011). Many reports have been published about underground airborne bacteria and fungi (Rdzanek et al., 2015; Ogórek et al., 2016a, 2016b), but only one microbiological study has been published for a large bat-hibernation site that relates to airborne fungi. It was found that the number of cultivable airborne fungi increase significantly during the hibernation season (Kokurewicz et al., 2016). There are no known similar reports regarding bacteria or bacteria and fungi in bioaerosols a short distance from hibernating bats.

The aims of the study were to determine phenotypic and genotypic diversity of airborne bacteria present close to hibernating bats, to quantify their concentrations, and to assess the pathogenic potential for bat assemblages and in the underground system of Nietoperek bat reserve.

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Material and methods

Description of the Study Area

Nietoperek bat reserve is situated in western Poland in the central part of the so-called Międzyrzecz Fortified Front (52°25′N, 15°32′E), which was built by Germans in the 1930s and during World War II. The above-ground bunkers are connected by approximately 32 km long systems of underground railway tunnels (Woźniak, 1996). To protect hibernating bats and their foraging areas around the fortifications the underground system with the surrounding surface area of 7377.37 ha became protected in November 2007 as Natura 2000 site (area code: PLH080003). The underground corridors of the MMF are one of the ten largest bat hibernation sites in the European Union, and most parts are closed for visitors during the hibernation period from October 15 to April 15. Four bat species are under protection in MMF and mentioned in Annex II of the EU Habitat Directive (Directive 92/43/EEC, 1992) (i.e., *Myotis myotis*, *Barbastella barbastellus*, *Myotis dasycneme*, and *Myotis bechsteinii*). The air temperature in the underground complex ranges from 0 °C near the entrances to 10.4 °C in rear parts. So far 12 bat species with a maximum number of 38,594 individuals have been found hibernating there. Since 1995 the dominant species has been the greater mouse-eared bat (*M. myotis*), constantly increasing in its number since 1985 (Kokurewicz et al., 2014, 2016). Generally, the population of bats in the Nietoperek bat reserve is fairly constant. For example, in January 2014 among all the locations of our study (Fig. 1), the largest number of bats (423 bats) was recorded in section 7.2 (Locations from IV to VI). On the other hand, in Location III there were recorded only several individuals, and in Location II (section 7.8) only ten bats (Kokurewicz et al., 2016).

Sampling Methods

The observations were made under the license nr.WPN-I.640 I.369.2015.JK issued by Nature Conservancy Management in Gorzów Wielkopolski. The microbial air sampler MAS100-ECO (MBV), and YPG medium (yeast extract peptone glucose: 10.0 g L^{-1} yeast extract, 20.0 g L^{-1} peptone, 20.0 g L^{-1} glucose, 15.0 g L^{-1} agar) were used for the microbiological evaluation of the air. In order to eliminate fungi and yeast from the bacteria samples, 30 g mL^{-1} of nystatin was added to the medium (Polfa, Kraków). The samples were taken on January 9, 2016 from one location situated ca. 5 m in front of the entrance of the underground tunnels of Nietoperek bat reserve and from five locations inside of them (Fig. 1). The microbial air sampler was positioned at a distance of 0.7 m from clusters of *M. myotis* (locations from IV to VII) or it was positioned 1.5 m above the level of the floor (locations from I to III). It was programmed for air sample volumes of 50 L and 100 L, and the measurement in particular sampling sites was performed in triplicates for each volume. The incubation of samples in Petri dishes with YPG medium was carried out at 7 ± 0.5 °C for 21 days and 37 ± 0.5 °C for 5 days. After incubation, bacterial colonies on the plates were counted, and the colony-forming units concentrations were expressed as CFU per cubic meter of air using the formula $X = (a \times 1000) / V$, where a is the number of colonies obtained on a Petri dish, and V is the air volume sampled (m^3). Then bacterial colonies were subcultured on YPG medium for phenotypic and molecular identification.

Phenotypic Studies

Bacterial colonies on YPG medium were subcultured on nonselective media, Nutrient agar (NA, Biocorp, Poland) and Tryptic Soy agar (TSA, Biocorp, Poland) for morphological and phenotypic analyses. Biochemical and enzymatic characteristics of bacterial isolates were determined after incubation at 25 ± 0.5 °C for 48 h by using commercial systems API 20NE and API 20 Staph (Biomerieux) with inoculation of 0.5 McF (10^8 CFU ml^{-1}) in sterile water solution of 0.85 % NaCl. The profile of substrate utilization by bacteria in oxidative and fermentative conditions was assessed according to recommended procedures using API Aux Medium (1.5 % v/v vitamin solution, 1 % v/v trace elements, 0.15 % agar, 0.2 % $(NH_4)_2SO_4$, 0.62 % NaH_2PO_4, 0.15 % KCl) and API Staph Medium (0.05 % yeast extract, 1 % bactopeptone, 0.5 % NaCl, trace elements 1 % v/v). The addition of reagents and the interpretation of reactions were done according to the manufacturer's directions. The supplementary tests, including those for cell morphology and Gram-stain reaction, oxidase and catalase production, and casein, starch, and Tween-80 utilization were performed based on standard methods (Smibert and Krieg, 1994). The ability of growth for bacteria (C_k 10^4 CFU mL^{-1}) at 25 ± 0.5 °C and 35 ± 0.5 °C and in the presence of 12.5 % NaCl at 25 ± 0.5 °C was evaluated visually based on the turbidity of Nutrient Broth (NB, Biocorp, Poland) after incubation for 1 to 3 days. The antimicrobial susceptibility to bacitracin (10UI, Becton Dickinson, Poland) and furazolidone (100 mg L^{-1}, Becton Dickinson, Poland) was determined after incubation at 25 ± 0.5 °C for 24 h to 48 h by disc diffusion test on nutrient agar with bacterial suspensions of 0.1 mL 10^8 CFU mL^{-1} in saline water solution of 0.85 % NaCl. Bacterial isolates without a zone of inhibition were defined as resistant. The ability of N_2 fixation by bacteria was detected in culture after incubation at 25 °C for 3 to 5 days by using a modified Ashby (ASH) medium (1 % mannitol, 0.5 % terhalose, 0.5 % glucose, 0.02 % K_2HPO_4, 0.02 % $Mg_2SO_4 \cdot 7H_2O$, 0.02 % NaCl, 0.01 % K_2SO_4, 0.05 % $CaCO_3$, 1.5 % agar) (Stella and Suhaimi, 2010). The growth of bacteria on this medium indicated the bacterial ability to fix N_2 from the air. The results were interpreted using the APIweb software and monographs (Lapage et al., 1968; Kocur et al., 1972, 1975; Kodama et al., 1985; Margesin et al., 2004; Gupta et al., 2004; Lalucat et al., 2006; Zhao

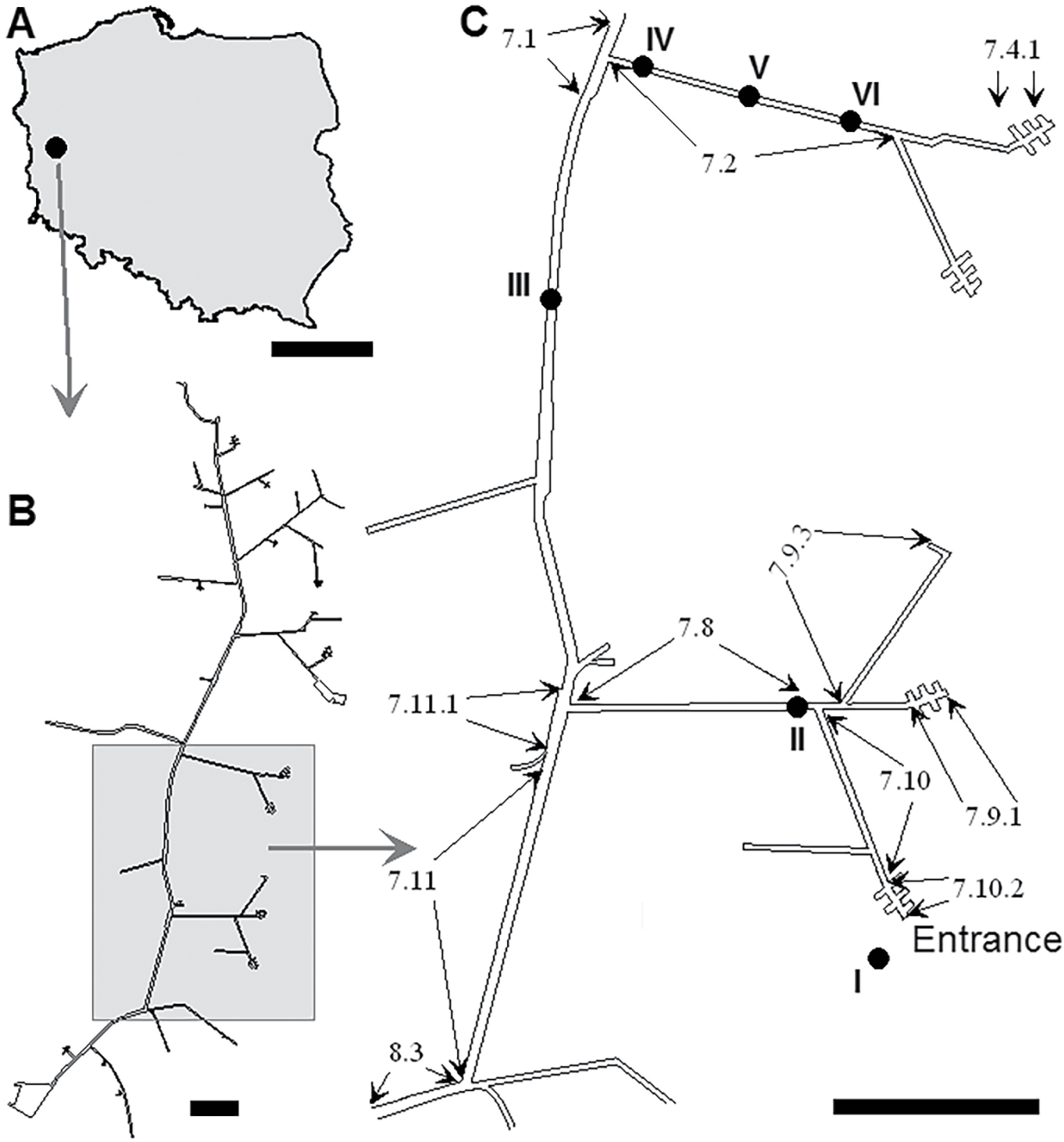

Figure 1. Geographic location of Nietoperek bat reserve in Western Poland (A). The outline of the underground fortification system (B). Sections (7.1–8.3) and sampling sites (C): location I outside the underground fortification system, and locations II to VI inside the underground fortification system. Scale bars: A = 250 km, B and C = 500 m.

et al., 2009; Pindi et al., 2010; Kim et al., 2016). Subsequently, bacterial cultures were genetically analyzed to confirm the affiliation of the species and determine the genetic diversity.

Molecular Studies

DNA was extracted according to the CTAB method (Doyle and Doyle, 1987) with minor modifications (Ogórek et al., 2012). Amplification of DNA was performed in a 25 µL reaction mixture using the 2×PCR mixture containing a Taq polymerase (0.1 U μL^{-1}), dNTP mix (2 mM), $MgCl_2$ (4 mM), 0.25 µM of each primer, rD1: AAGGAGGTGATCCAGCC,

and fD1: AGAGTTTGATCCTGGCTCAG (Weisburg et al., 1991) and 45 ng of DNA in the Biometra thermal cycler for 35 cycles. After initial denaturation for 5 min at 94 °C, each cycle comprised 30 s denaturation at 94 °C, 30 s annealing at 55 °C, 45 s extension at 72 °C, with a final extension for 7 min at 72 °C at the end of 35 cycles. The amplification product was separated on agarose gel (1.5 %), visualized by UV light, purified from the gel and sequenced by the sequencing service at Macrogen (http://dna.macrogen.com/eng/). Partial sequences were analyzed with the BLAST algorithm (http://www.ncbi.nlm.nih.gov/), aligned and compared with published 16s rRNA sequences from GenBank of the National Center for Biotechnology Information, Bethesda, MD, USA.

Results

Eight species of airborne bacteria belonging to three phyla and three orders were isolated from air samples collected in the Nietoperek bat reserve. The species diversity, eight, of airborne bacteria was higher in air samples collected inside the underground Nietoperek bat reserve than from the outdoor air, two. From indoor and outdoor sites, bacterial species such as *Paeniglutamicibacter psychrophenolicus* and *P. sulfurous* were cultured only from samples incubated at 7 °C. Other bacterial species were cultured only from samples incubated at 37 °C (Tables 1, 2).

Table 1. Airborne bacteria cultured from air samples in the Nietoperek bat reserve, and results of BLAST analysis (all E values were zero).

Isolate	Identification		Identity with Sequence from GenBank		
	Species	GenBank Accession No.	Query Cover, %	Identity, %	Accession
UWR_Bak5	*Dermacoccus nishinomiyaensis*	KY575239.1	100	93	NR_044872.1
UWR_Bak7	*Micrococcus luteus*	KY575241.1	98	91	KY317958.1
UWR_Bak4	*Micrococcus yunnanensis*	KY575238.1	98	94	KU877632.1
UWR_Bak8	*Paenibacillus polymyxa*	KY575242.1	98	95	CP011420.1
UWR_Bak1	*Paeniglutamicibacter psychrophenolicus*	KY575235.1	100	94	NR_027226.1
UWR_Bak2	*Paeniglutamicibacter sulfureus*	KY575236.1	96	92	AB046358.1
UWR_Bak6	*Pseudomonas oryzihabitans*	KY575240.1	100	91	LC191548.1
UWR_Bak3	*Pseudomonas stutzeri*	KY575237.1	91	91	CP011854.1

Overall, indoor air samples contained more propagules of bacteria than outdoor air samples, from 232 ± 28.3 to 1189 ± 124.7 and 42 ± 12.2 bacterial CFU per m^3 of air respectively. The highest number of species isolated from the indoor air was noted for location V (eight bacterial species) and the smallest number of species was noted for location II (four bacterial species). The incubation temperature of samples influenced the number of bacteria cultured from the air (Table 2). After an incubation of samples at 7 °C, the highest number of detected species was noted for location VI (Fig. 2). The bacterial species most frequently isolated from the air outside the Nietoperek bat reserve was *P. psychrophenolicus*, and from the air inside of it was *Micrococcus luteus* (Table 2).

Airborne bacteria isolated from the Nietoperek tunnels showed morphological diversification between the genus, but not within the genus, with the exception of color and colony diameter on nutrient agar medium for *Micrococcus* and *Pseudomonas* spp. All species grew at 25 °C and were resistant to furazolidone. *Paenibacillus polymyxa*, *Pseudomonas oryzihabitans* and *P. stutzeri* were also resistant to bacitracin, all other bacterial species were sensitive to it. Species *P. psychrophenolicus* and *P. sulfureus* did not grow at 35 °C, but others did. Among all tested species, only *M. luteus* showed growth in nutrient broth medium with 12.5 % NaCl. The majority of bacteria required oxygen for growth, with the exception of *P. polymyxa*, which grew in presence and absence of oxygen (Table 3).

The studied biochemical properties of airborne bacteria were diversified, although some trends between species belonging to the same genus were detected. Generally, species were separated into two main clusters. Based on enzymatic metabolism, isolates of *P. psychrophenolicus* and *P. sulfureus* showed the highest similarity, and they differed only in reduction of nitrate to nitrite, utilization of glucose, and phenylacetic acid (Table 4). The most extensive biochemical profile showed *P. polymyxa*, and only this species produced acetylmethylcarbinol, β-glactosidase, β-glucosidase, and utilized lactose and N-acetyl-β-glucosamine. In contrast, the narrowest range of biochemical abilities was shown by *Dermacoccus nishinomiyaensis*. Among all tested biochemical properties, calatase was produced by all tested species, whereas indole was produced by none (Table 4).

Discussion

Bats (order *Chiroptera*) occur on all continents except Antarctica. Some species inhabit polar regions and isolated oceanic islands (Jones et al., 2009). These small endothermic mammals play important roles in ecosystems and the economy, as plant pollinators, seed dispersers responsible for regeneration of tropical rain forest, predators of insect

Table 2. Average number of airborne bacteria cultured at 7 °C and 37 °C from air samples in the Nietoperek bat reserve with mean ± standard deviation of CFU per m³ (ND = not detected).

Species	Sampling Location											
	I		II		III		IV		V		VI	
	7 °C	37 °C	7 °C	37 °C	7 °C	37 °C	7 °C	37 °C	7 °C	37 °C	7 °C	37 °C
Dermacoccus nishinomiyaensis	ND	ND	ND	ND	ND	ND	ND	43 ± 9.3	ND	86 ± 14.4	ND	49 ± 12.7
Micrococcus luteus	ND	ND	ND	36 ± 6.0	ND	142 ± 13.6	ND	311 ± 45.0	ND	432 ± 63.5	ND	367 ± 37.5
Micrococcus yunnanensis	ND	ND	ND	ND	ND	65 ± 16.6	ND	96 ± 23.6	ND	126 ± 24.7	ND	103 ± 21.2
Paenibacillus polymyxa	ND	ND	ND	59 ± 8.9	ND	11 ± 3.7	ND	204 ± 33.2	ND	158 ± 24.5	ND	115 ± 24.5
Paeniglutamicibacter psychrophenolicus	29 ± 10.2	ND	98 ± 21.1	ND	146 ± 33.9	ND	171 ± 27.8	ND	197 ± 29.3	ND	227 ± 47.1	ND
Paeniglutamicibacter sulfureus	13 ± 8.5	ND	39 ± 13.9	ND	45 ± 8.4	ND	105 ± 21.2	ND	131 ± 26.4	ND	176 ± 37.6	ND
Pseudomonas oryzihabitans	ND	ND	ND	ND	ND	34 ± 4.7	ND	13 ± 6.6	ND	23 ± 7.7	ND	9 ± 3.7
Pseudomonas stutzeri	ND	ND	ND	ND	ND	ND	ND	2 ± 1.9	ND	36 ± 5.3	ND	ND

I – Outside the underground fortification system.
II to VI – Inside the underground fortification system (II = Single individuals), (III = Several individuals), (IV to VI = Large number of bats wintering in clusters).

populations, including agricultural and forest pests, and insects harmful for humans such as mosquitoes (*Culicidae*) and biting midges (*Ceratopogonidae* and *Simuliidae*) (Vilas, 2016). Moreover, most bat species are listed in the IUCN Red list of endangered species and almost half of these are considered threatened or near-threatened (Mickleburgh et al., 2002). Thus, their health and factors determining their favorable conservation status should be monitored, including microbial agents that can cause death in bats (Evans et al., 2009; Mühldorfer et al., 2010). Therefore, microbiological monitoring of bats could be a vital element of the protection of these mammals.

Most of airborne bacterial species isolated a short distance from bats hibernating in the Nietoperek belonged to the *Actinobacteria* phylum. This phylum is widely distributed in the environment and represents one of the largest taxonomic units; it includes five subclasses and fourteen suborders. *Actinobacteria* are also one of the most dominant groups of the skin microbiota in humans, various amphibian species, fish, and bats (Larsen et al., 2013; Kueneman et al., 2014; Hamm et al., 2017). Members of this phylum are well adapted to survive long periods and grow well in nutrient-depleted environments (Barton et al., 2004). Therefore, *Actinobacteria* represent one of the most abundant microbiota from the underground ecosystems where bats hibernate (Northup et al., 2011). However, it should be noted that only fewer than 1% of bacteria from this phylum can be detected by using culture-based methods (Zang et al., 2013). This study supports literature reports that *Actinobacteria* exhibit a wide variety of morphologies as well as diverse physiological and metabolic properties, such as the production of enzymes (Ventura et al., 2007). Other species of airborne bacteria isolated from the Nietoperek belonged to the *Firmicutes* and *Proteobacteria*. These phyla are the cosmopolitan group of environmental bacteria, especially in the colder parts of the world (Teixeira et al., 2010). *Firmicutes* and *Proteobacteria*, like *Actinobacteria*, can colonize the body of bats and their guano, but they are less dominant than the *Actinobacteria* phylum (Hoyt et al., 2015; Banskar et al., 2016). Some species of *Proteobacteria* probably play an important role in maintaining interactions in microbial communities of the skin of bats, decreasing or preventing development and growth of undesirable or pathogenic microbes. For example, *Pseudomonas* spp. isolated from the skin of bats can inhibit *in vitro* the growth of the white-nose syndrome fungus *Pseudogymnoascus destructans* (Hoyt et al., 2015).

The study was conducted during winter, and the bacterial species most frequently isolated from the air samples incubated at 7 °C was *Paeniglutamicibacter psychrophenolicus* (basonym: *Arthrobacter psychrophenolicus*), which is a Gram-positive, non-endospore-forming, non-motile aerobic bacterium (Margesin et al., 2004). *P. psychrophenolicus* belongs to the *Actinobacteria* phylum and cannot grow at temperatures of 35 °C because

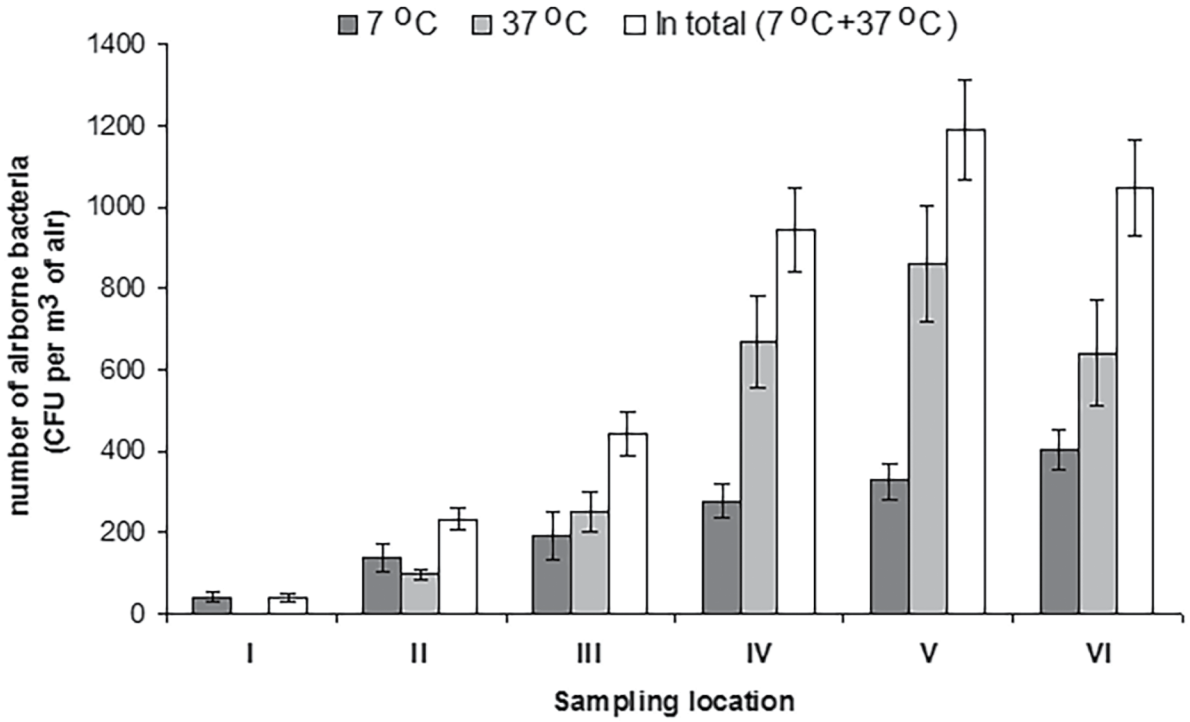

Figure 2. Total number of airborne bacteria (CFU per m^3 ± standard deviation) cultured from the Nietoperek bat reserve. Sampling location I was outside the underground fortification system, locations II to VI were inside the underground fortification system (Figure 1). II had single individuals, III had several individuals, and IV–VI had large numbers of bats wintering in clusters.

of its facultative psychrophilic characteristics (Margesin et al., 2004; Ventura et al., 2007). Cold-adapted species belonging to this genus are widespread among bacteria found in subterranean sites, glacier silts, or Arctic and Antarctic environments (Loveland-Curtze et al., 1999; Juck et al., 2000; Stibor et al., 2003). The ability of *P. psychrophenolicus* and *P. sulfureus* to survive in colder temperatures is probably one of the main reasons that these bacterial species were isolated only from the outdoor air samples in this study.

Of particular interest in this study are phenotypic characteristics and the role of *Micrococcus luteus*, because it was the most-isolated species from the air samples incubated at 37 °C. *M. luteus*, in contrast to *P. psychrophenolicus*, grows at 35 °C and is a typical Gram-positive, obligate-aerobic bacterium. This species is present in soil, water, and undergrade environments and on the skin of humans and other animals (Kocur et al. 1972; Ventura et al., 2007; Rdzanek et al., 2015). Generally, *M. luteus* is considered a non-pathogenic saprophyte, but some strains may occasionally act as opportunists. For example, this species can cause septic arthritis, pneumonia, urinary tract infection in an immune-deficient person, meningitis, and native and prosthetic valve endocarditis (Kocur et al., 1972; Fosse et al., 1985).

Other bacterial species isolated from the air a short distance from hibernating bats are mainly considered as saprophytic organisms, but in some situations can be pathogenic. For example, *Dermacoccus nishinomiyaensis* is found in water and on the skin of mammals and is considered to be non-pathogenic (Kocur et al., 1975). However, according to recent reports, this species can cause wound infections (Shah et al., 2015). Generally, *Pseudomonas oryzihabitans* is described as a saprophyte of humans and other warm blooded animals (Giacometti et al., 1998; Molinari et al., 2003). In recent years, a nosocomial pathogenic potential has been discussed, because infections usually occur in cases referring to contaminated catheters, peritoneal dialysis, bacteremia, or even sepsis patients (Lucas et al., 1994; Verhasselt et al., 1995; Marín et al., 2000). Thus, *P. oryzihabitans* infection is strongly associated with presence of bacterial biofilms, previous trauma or surgery, and immunocompromised hosts. Reports of its pathogenic potential in humans are rare (Lin et al., 1997). *Micrococcus yunnanensis* is a novel actinobacterium that was isolated from surface-sterilized plants (Zhao et al., 2009). It is likely that it is an endophytic bacterium, which naturally colonizes the internal tissue of plants (Pisarska and Pietr, 2015). *Paenibacillus polymyxa*, formerly known as *Bacillus polymyxa*, is a plant growth promoting rhizobacterium that is used for the biocontrol of plant diseases (Ash et al., 1993; Dijksterhuis et al, 1999). For example, it is used against the plant-parasitic nematodes, and phytopathogenic fungi (Caruso et al., 1984). Additionally, this species is antagonistic to human pathogenic bacteria and fungi *in vitro* (Seldin et al., 1999). *Pseudomonas stutzeri* is a typical soil bacterium that is widely distributed in the environment (Lalucat et al., 2006). It can be an opportunistic

Table 3. Phenotypic analysis and supplementary diagnostic test results of airborne bacteria isolated in the Nietoperek bat reserve.

Characteristic	***Dermacoccus nishinomiyaensis*** UWR_Bak5	***Micrococcus luteus*** UWR_Bak7	***Micrococcus yunnanensis*** UWR_Bak4	***Paenibacillus polymyxa*** UWR_Bak8	***Paeniglutamicibacter psychrophenolicus*** UWR_Bak1	***Paeniglutamicibacter sulfureus*** UWR_Bak2	***Pseudomonas oryzihabitans*** UWR_Bak6	***Pseudomonas stutzeri*** UWR_Bak3
Colonies on AN at 25 °C								
Color	Orange-Yellow	Yellow	Bright Yellow	Pale White	Yellowish-Grey	Yellowish-Grey	Non-Pigment	Pale Yellow
Diameter (mm) after 48 h	1.0	0.5	1.0	1.0	1.0–2.0	1.0–2.0	3.0	2.0–3.0
Shape	Circular, entire, convex, smooth, glistening, non-transparent.	Circular, entire, convex, smooth, non-glistening, non-transparent.	Circular, entire, convex, smooth, non-glistening, non-transparent.	Flate, smooth glistening, non-transparent.	Circular, entire, convex, smooth, glistening, non-transparent.	Circular, entire, convex, smooth, glistening, non-transparent.	Flate, transparent non-glistening slightly wrinkled.	Flate, transparent non-glistening slightly wrinkled.
Bacteria Cell								
Shape	Spherical	Spherical	Spherical	Rod-Like	Rod-Like	Rod-Like	Rod-Like	Rod-Like
Arrangement	Irregular Clusters	Tetrads	Tetrads	Clusters	Pleomorphic	Pleomorphic	Single	Single
Gram Stain	Positive	Positive	Positive	Positive	Positive	Positive	Negative	Negative
Growth Temperature								
25 °C	+	+	+	+	+	+	+	+
35 °C	+	+	+	+	–	–	+	+
Growth in NB with 12.5 % NaCl	–	+	–	–	–	–	–	–
Growth Condition[a]	OA	OA	OA	FA	OA	OA	A	OA
Susceptibility to Agents[b]								
Bacitracin 10UI	S	S	S	R	S	S	R	R
Furazolidone 100 µg mL^{-1}	R	R	R	R	R	R	R	R

Symbols: AN – nutrient agar; NB – nutrient broth; S – sensitive; R – resistance; "+" growth; "–"no growth;
a Type of growth condition: "A" as aerobic, "OA" as obligate aerobe, "FA" as facultative anaerobe;
b Disk Diffusion Assay

Table 4. Biochemical properties of airborne bacteria isolated in the Nietoperek bat reserve (Symbols of reaction: "+" positive, "−" negative).

Characteristic	*Dermacoccus nishinomiyaensis* UWR_Bak5	*Micrococcus luteus* UWR_Bak7	*Micrococcus yunnanensis* UWR_Bak4	*Paenibacillus polymyxa* UWR_Bak8	*Paeniglutamicibacter psychrophenolicus* UWR_Bak1	*Paeniglutamicibacter sulfureus* UWR_Bak2	*Pseudomonas oryzihabitans* UWR_Bak6	*Pseudomonas stutzeri* UWR_Bak3
Production of:								
Catalse	+	+	+	+	+	+	+	+
Oxidase	+	+	−	−	−	−	−	+
Acetylmethylcarbinol	−	−	−	+	−	−	−	−
Indole	−	−	−	−	−	−	−	−
Enzymes								
Urease	+	+	−	−	+	+	−	−
Nitrogenase	−	−	−	+	−	−	+	+
Nitrate reduction	−	−	−	+	+	−	−	+
Arginine ihydrolase	−	−	−	−	−	−	−	−
Alkaline hosphatase	−	+	+	−	−	−	−	−
α-glucosidase	−	+	+	+	+	+	−	−
β-glactosidase	−	−	−	+	−	−	−	−
β-glucosidase	−	−	−	+	−	−	−	−
Hydrolysis of:								
Skimmed Milk	+	+	−	+	−	−	−	−
Gelatine	+	−	+	+	−	−	−	−
Starch	−	−	−	+	−	−	−	+
Tween80	−	−	+	+	−	−	−	+
Utilization of:								
Glucose	+	−	+	+	−	+	+	+
Trehalose	+	+	+	+	+	+	+	−
Arabinose	+	−	−	+	−	−	+	−
Lactose	−	−	−	+	−	−	−	−
Mannose	−	+	+	+	−	−	+	−
Mannitol	−	−	−	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+
Sucrose	−	+	−	+	−	−	−	−
Gluconate	−	−	−	+	+	+	+	+
N-acetyl-β-glucosamine	−	−	−	+	−	−	−	−
Citrate	−	−	−	−	+	+	+	+
Capric acid	−	−	+	−	−	−	+	+
Adipic acid	−	−	+	−	−	−	−	−
Malic acid	−	−	+	−	+	+	+	+
Phenylacetic acid	−	−	+	−	+	−	−	−
Trehalose	+	+	+	+	+	+	+	−

pathogen for humans, however, *P. stutzeri* infection is rare (Noble and Overman, 1994; Reisler and Blumberg, 1999). Under certain circumstances, these bacterial species could act as potential opportunists affecting the health status of individual bats such as in individuals that are weakened by disease or malnutrition or have been traumatized by martens or cats or during the flight.

Conclusions

This study is the first that describes cultivable airborne bacteria present in the environment of hibernating bats. Bacterial communities showed differences in terms of composition and abundance among the different parts of the Nietoperek underground corridors, and bacterial growth was dependent on the incubation temperature of samples as well as on the number of bats. Results indicate that indoor air samples contained more propagules of bacteria and more bacterial species than outdoor air samples. Moreover, hibernating bats may be good vectors of bacteria in underground sites because more species of airborne bacteria were isolated from the samples in close vicinity to them. *Actinobacteria* dominated in this study. *Paeniglutamicibacter psychrophenolicus* was isolated most frequently from samples incubated at 7° C, and *Micrococcus luteus* from samples incubated at 37 °C. Some of the species found in the study can cause opportunistic infections in humans and animals. To better understand the microbial composition and relevance of specific bacteria, further investigations in underground sites could provide more insights into the complex ecosystems and should be regularly performed.

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