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Diversity of cultivable bacteria involved in the formation of macroscopic microbial colonies (cave silver) on the walls of a cave in Slovenia

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Abstract: Karstic caves often support white, yellow, grey or pink microbial colonies that are termed 'cave silver' by speleologists. Using various sample pre-treatments and culture media, a wide variety of bacteria associated with these colonies were recovered from a cave in Slovenia, Pajsarjeva jama. Decreasing the inoculum size resulted in significant increases in viable counts, while pre-treatments had the opposite effect with the exception of microwave irradiation. While all growth media yielded viable counts, the maximal counts were observed on a low-nutrient TWA medium.

Based on the 16S rRNA gene sequence of OTU representatives, the majority of the 80 isolates examined belonged to *Streptomyces* (25%), *Micrococcus* (16%) and *Rhodococcus* (10%). Other abundant groups were *Pseudomonas* (9%), *Agrobacterium* (8%), *Lysobacter* (6%) and *Paenibacillus* (5%), while members of genera *Microbacterium*, *Agrococcus*, *Arthrobacter*, *Bacillus*, *Kocuria*, *Oerskovia*, *Sphingomonas*, *Aerococcus*, and *Bosea* represented a minor portion of cultivable diversity encountered. Members of *Streptomyces* and *Agrobacterium* were common to all samples. Although these microorganisms readily form colonies under laboratory conditions, they were unrelated to abundant environmental phylotypes recovered from same samples in a previous study. However, the comparative 16S rRNA analysis showed that microorganisms highly related to the ones obtained in this study were cultivated from other subterranean environments indicating that they might represent true microbial cave dwellers.

Keywords: cave; bacteria; microbial colonisations; cultivation; 16S rRNA

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INTRODUCTION

Caves were long considered to support only rare microbial specialists (Palmer et al., 1991; Barton & Jurado, 2007). Most cavern microbiota was considered to be imported from the surface through air currents, water, sediments, animal or human vectors and thus not specialized for subterranean life (Palmer et al., 1991; Keswick et al., 1982; Cunningham et al., 1993; Northup et al., 1994; Rusterholtz & Mallory, 1994). Beginning in the 1990's, the use of molecular techniques quickly established that caves are in fact endowed with diverse microbial communities (Barton & Jurado, 2007). The microbes present within caves were found to acquire energy using a plethora of approaches. These ranged from breaking down complex surface-derived aromatic

compounds to oxidizing reduced inorganic compounds within the cave rocks. More importantly, the microbial diversity encountered was surprisingly high compared to what was expected given the amount and complexity of nutrients available in subterranean environments. This apparent violation of competitive exclusion or the 'one species-one niche' requirement (Hardin, 1960) led scientists to believe that within caves the selfish competition for resources is replaced by more cooperative and mutualistic microbial associations (Barton & Jurado, 2007).

One eye-catching example of cave microbes are those found in Karstic and in lava caves which morphologically resemble white, yellow, grey, or pink bacterial colonies. Where present, these colonies can be observed throughout the cave as distinct one-millimetre

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size spots or can form thin (up to 1 mm thick) biofilm-like coatings that can be several square meters in size. This type of microbial growth, sometimes referred to as 'cave silver', is often covered with water droplets and reflects light in characteristic fashion, hence its name.

Over the years a number of studies have focused on cave silver samples from caves in Spain, Portugal, Hawai'i, New Mexico, Czech Republic and Slovenia. DGGE fingerprinting and phylogenetic analysis of 16S rRNA genes obtained from cave silver samples from Spanish caves showed that regardless of its colour, they are dominated by members of Proteobacteria and Actinobacteria. The cave silver colonies supported various other phylotypes affiliated with Actinobacteria, Bacteroidetes, Gemmatimonadetes, Firmicutes, Planctomycetes and Nitrospira (Schabereiter-Gurtner et al., 2004; Portillo et al., 2008, 2009). An independent 16S rRNA study that focused on cave silver in Slovenia showed similar results. This study also reported the presence of Verrucomicrobia and Chloroflexi (Pašić et al., 2010), which had been previously undetected. Members of the above listed phyla dominated a morphologically similar growth in lava caves in New Mexico, Portugal and Hawai'i (Northup et al., 2008; Garcia et al., 2009; Moya et al., 2009; Northup et al., 2011). Recently, a comparative analysis of environmental 16S rRNA from samples recovered from yellow cave silver growing on the walls of caves in Spain, Slovenia, Czech Republic, Portugal and Hawai'i found that all these caves support similar colonisations and have a common core composed of actinobacterial Pseudonocardinae and Gammaproteobacteria (Porca et al., 2012).

Investigations of cave silver have included cultivation efforts, however initially there were no published reports assessing the cultivable portion of microbial diversity. They are instances where cave isolates have been reported (Mulec, 2008) and three novel species were described from white colony samples taken from an Altamira cave (Jurado et al., 2006; 2008; 2009). These corresponded to the taxa that constitute a minor portion of the community, as is frequently the case with these studies. Regardless, cultivation is an important part of the description of a microbial community as the readily cultivated rare taxa represent a reservoir of biological diversity that is seldom retrieved in molecular studies. Our present study describes the bacterial diversity of the cultivable fraction of yellow, grey, pink and white cave silver colonies that develop in Pajsarjeva jama, Slovenia which were recovered using approaches successfully utilized previously in similar subterranean systems.

MATERIALS AND METHODS

Sample collection and processing

Microbial cave wall colonies were sampled in May 2011 in a 55 m long cave, Pajsarjeva jama, located 20 km south-west of Ljubljana, Slovenia (45°49'51"N, 14°16'15"E). At the time of sampling, the air temperature was 12.0 °C, and the relative humidity was 100%. Previous cultivation-independent studies found no differences in structure and membership of microbes in cave silver samples of same colour (Porca et al., 2012). This allowed us to collect four samples that

corresponded to yellow, grey, pink and white cave silver morphotypes. We sampled areas in which the respective colour morphotype covered an area of several square centimetres. The samples were carefully scraped off with a sterile scalpel into sterile vials without touching the supporting rocks, were stored on ice and processed within 2 h after collection.

An aliquot (0.05 g) of each of the four samples was resuspended in 5 ml of 0.9% NaCl and divided into 1 ml subsamples. These were each treated as described in Niyomvong et al. (2012) in order to elicit the growth of non-*Streptomyces* Actinobacteria. The suspensions were either exposed to wet heat (water bath at 50 °C for 5 minutes); dry heat (120 °C in a hot air oven for 1 h); dry heat and phenol (1 ml of suspension was added to 9 ml of 1.5% phenol solution and was incubated at 120 °C in a hot air oven for 1 h followed by 30 minutes at room temperature); microwave irradiation (frequency of 2460 MHz and a power setting at 100 W for 45 sec); or remained untreated.

Media and cultivation conditions

Prior to plating, the treated suspensions were diluted to 10^{-3} in 0.9% NaCl. One hundred microliters of each dilution was spread onto seven different media. We chose media that contained substrates that are likely present in the natural environment and/or were previously shown to initiate the growth in a wide variety of cave organisms (Jurado et al., 2006; 2008; 2009; Niyomvong et al., 2012). Media included glycerol-asparagine agar (GAA) (ISP medium N° 5), peptone-yeast extract-brain heart infusion agar (PY-BHI) (Yokota et al., 1993), 1000-fold-diluted tryptic soy agar (TSA) (Difco, USA), starch-casein agar (SCA) (Wellington & Cross, 1983), malt-yeast extract agar (MYA) (Oxoid, USA), soil extract agar (SEA, 1.5% agar in 10% (w/v) filtrate of soil in tap water) and tap water agar (TWA, 1.5% agar in sterilized tap water). All media was supplemented with the fungal inhibitor nystatin (Sigma, USA, 100 mg/ml).

Plates were incubated at 30 °C as suggested by Laiz et al. (2003), who found that low temperatures (13°C) reduce the viable counts and the diversity of isolates related to Actinobacteria (but yield somewhat higher diversity of other Bacteria). During the five week incubation, the plates were opened periodically for colony counts. Morphologically different colonies were purified by streak-planting and were maintained on TSA or Oatmeal agar (Difco, USA) or slopes.

Statistical analysis

The Shapiro-Wilk normality test was used to determine the distribution of the data. The significance of effects of sample type, inoculum size, incubation time, sample pre-treatments and media choice on viable counts was estimated using Kruskal-Wallis test. The Mann-Whitney U test was used to identify differences among medians of viable counts obtained with individual pre-treatments and in the absence of treatment.

In order to identify growth media with significant effects on viable counts, we log₁₀ transformed the data, performed one-way ANOVA, and calculated the difference between each pair of means using Fisher's least significant difference procedure.

Phenotypic tests

The morphological properties of presumably novel isolates were studied microscopically following Gram staining. Gram staining and the determination of catalase and oxidase activities were all performed using standard procedures (Gerhardt et al., 1998). Other biochemical tests were performed by inoculation of API 20 NE, API 20E, API Coryne, API Strep and API 50CH strips (bioMérieux) according to the manufacturer's instructions.

Molecular methods

Genomic DNA isolation

Growth from stationary phase cultures was inoculated aseptically into 150 ml Erlenmeyer flasks containing 10 ml of growth medium and was incubated in a rotary shaker incubator at 22 °C for 72 hours at 200 rpm. Cultures (3 ml) were centrifuged at 12000×g for 3 minutes and were resuspended in 500 µl of TES buffer (0.3 M sucrose, 25 mM Tris-HCl pH 8.0, 25 mM EDTA pH 8.0) supplemented with 50 µg/ml RNase A. Beads ranging in size from 0.1–0.5 µm were added, the mixture was frozen in liquid N₂, and the cells were disrupted by bead-beating for five minutes. This suspension was supplemented with 4 mg/ml of lysozyme and was incubated for 1 hour at 37°C. Subsequently, 250 µl of 2% sodium dodecyl sulphate was added and the mixture was vortexed for one minute and the resulting lysate was extracted with phenol:chloroform:isoamylalcohol (25:42:1). The DNA was precipitated by adding 0.1 volume of 3 M Na-acetate (pH 4.8) and 1 volume of isopropanol. After incubating at room temperature for five minutes, the DNA was pelleted, air-dried, resuspended in TE buffer and stored at -20 °C.

PCR amplification and sequencing

Genomic DNA of 96 isolates was used as a template in PCR amplifications as described previously (Porca et al., 2012). Sequencing of amplified 16S rRNA genes was performed at Macrogen Inc. (Seoul, Korea) using universal bacterial primers 27F and 1492R. Sequences shorter than 750 bp and with average quality values below 20 were removed from the dataset. Upon assembly in BioEdit (Hall, 1999), the final dataset contained 80 sequences. These were compared to databases available from GenBank (<http://www.ncbi.nlm.nih.gov>, BLASTN algorithm), GREENGENES (<http://greengenes.lbl.gov>, 'Compare' tool) and EzTaxon (<http://eztaxon-e.ezbiocloud.net/>, Kim et al., 2012).

Operational taxonomic unit (OTU)-based analysis and phylogenetic analysis

We used Mothur to group sequences into OTUs (Schloss et al., 2009). The sequences were aligned to Silva-based template alignment (available from http://www.mothur.org/wiki/Silva_reference_alignment); and the uncorrected pair-wise distances between aligned sequences were calculated. The sequences differing in evolutionary distances by less than 3% were grouped into OTUs using the furthest neighbour algorithm.

For phylogenetic tree reconstruction, we used MUSCLE (Edgar et al., 2004) to generate an alignment of representative isolate sequences and relevant phylogenetic data. The latter included most similar environmental sequences, most similar validly described

species, and major representatives of each taxonomic group detected. Gaps and ambiguously aligned positions were identified using CORE available from T-COFFEE (<http://tcoffee.vital-it.ch/cgi-bin/Tcoffee/tcoffee.cgi/index.cgi>) and subsequently removed from the alignment. This dataset was analysed by maximum likelihood (ML) in MEGA5 (Tamura et al., 2011) by applying a general-time-reversible (GTR) model of sequence evolution and taking among-site variation into account using a four-category discrete approximation of a Γ distribution with a portion of invariable sites using MEGA5. ML bootstrap support values were assessed by 1000 bootstrap replications.

The sequences obtained in this study were deposited in GenBank under accession numbers KC978859–KC978879.

RESULTS AND DISCUSSION

Cultivation of cave silver microorganisms on solid media

We used four sample pre-treatments and seven different growth media in order to initiate the growth of a wide spectrum of prokaryotes that constitute cave silver. Inside Pajsarjeva jama, we sampled areas where individual colour morphotypes covered areas at least one square centimetre in size. We analysed four samples that corresponded to the yellow, pink, grey, and white cave silver morphotypes, respectively. The collected data was not normally distributed (Shapiro-Wilk normality test, $W = 0.5$, $P < 0.0001$) and, therefore, we report the median (Me) and the quartile 1–quartile 3 values (Q1–Q3).

Upon five weeks of incubation at 30°C, the median viable counts did not differ if the gathered data were considered with respect to sample colour ($P = 0.69$, Kruskal-Wallis test). The median viable counts ranged from 5.8×10^3 (Q1–Q3 = 1.20×10^3 – 3.12×10^4) CFU/g (grey-coloured sample) to 4.2×10^4 (Q1–Q3 = 5.80×10^3 – 1.08×10^5) CFU/g (pink-coloured sample).

The effect of inoculum size and incubation time on viable counts

After five weeks of incubation at 30°C, we did not observe the expected 10-fold reduction in colony numbers in cultured inoculated with 10-fold fewer bacteria. In fact, the median viable counts obtained in 10^{-3} dilutions (Me = 2.50×10^5 (Q1–Q3 = 1.10×10^5 – 4.10×10^5) CFU/g) were a magnitude higher than the counts obtained with 10^{-1} diluted samples (Me = 2.25×10^4 (Q1–Q3 = 1.75×10^4 – 4.30×10^4) CFU/g). This difference was statistically significant (Kruskal Wallis test, $P < 0.001$) regardless of sample type, sample pre-treatment or growth media used. This phenomenon has been described in other cultivation studies and has been hypothesized to occur due to overcrowding (e.g. Davis et al., 2005) or antibiosis. However, in soil samples, it had no effect on the isolation of members of rarely isolated groups (Davis et al., 2005).

The growth of colonies on media was recorded for five weeks and the first visible colonies were observed at six days after inoculation. The increase in CFU was most prominent during the second and the third week of incubation. Upon the third week of incubation, the total CFU/g increased on most media by 0.9% to 9.0%,

with exception of SCA and TSA where this increase was 25% and 56%. The drying of the plates prevented us from measuring growth for any later incubation time points.

The effect of sample pre-treatments on viable counts

Previous studies indicated that Actinobacteria might be particularly abundant in cave silver colonies from Pajsarjeva jama (Pašić et al., 2010; Porca et al., 2012). In order to increase the numbers and the variety of Actinobacteria in our pure cultures, we applied four different selective pre-treatments. These were previously reported to lead to growth inhibition of some and to stimulate the growth of other actinobacterial genera (wet heat, dry heat), to reduce the spreading of bacteria and fungi (wet heat, phenol) or to increase the number of non-*Streptomyces* soil actinomycetes (irradiation in a microwave) (e.g. Bredholt et al., 2008; Niyomvong et al., 2012).

The pre-treatments had a significant effect on median viable counts obtained on all counted plates (Kruskal-Wallis test, $P < 0.05$) (Table 1). The median viable counts were reduced if the samples were irradiated in a microwave, treated with dry heat and phenol, treated with dry heat or treated with wet heat. Compared to untreated samples, only microwave irradiation did not have a statistically significant effect (Mann-Whitney test, $W=864$, $P = 0.17$).

We observed no viable counts if the white cave silver sample was irradiated in a microwave. Microwave irradiation is known to induce germination of spores of some species of *Streptomyces*, *Nocardia*, *Streptosporangium*, *Lentzea*, *Micromonospora* and *Micropolyspora* (Bulina et al., 1997; Wang et al., 2013). This treatment also reduces total prokaryotic counts (Ferriss, 1984) and has recently been shown to reduce cultivability of some soil isolates affiliated with Actinobacteria, which could be the reason for the apparent absence of CFU. In similar fashion, no viable counts were observed if the pink cave silver sample was treated with dry heat and phenol, a treatment designed to select for actinobacterial genera *Micromonospora*, *Microbispora*, *Actinoplanes* and *Streptosporangium* (Seong et al., 2001). Indeed, no members of the above listed genera were obtained in pure culture with the notable exception of genus *Streptomyces*. The ability of these isolates to withstand microwave irradiation and dry heat and phenol treatment is currently under investigation.

The effect of media choice on viable counts

We based our growth media choice on previous cultivation studies involving cave microorganisms (Jurado et al., 2006; 2008; 2009; Niyomvong et al., 2012). The median viable counts obtained on different media ranged from 1.00×10^3 (Q1-Q3= 6.00×10^2 - 2.00×10^4) observed on MYA to 9.92×10^4 (Q1-Q3= 3.07×10^4 -

2.70×10^5) CFU/g observed on TWA medium (Fig. 1). The seven media types used in this study differed in their ability to elicit the formation of colonies (one-way ANOVA, $F = 2.98$, $P < 0.05$). We identified low-nutrient TWA as the only growth medium with significant effect on viable counts (Fisher's least significant difference, $P < 0.05$). This was expected given that low-nutrient media favour the growth of slow-growing species, as previously observed in various other environments (e.g. Janssen et al., 2002; Bussmann et al., 2001).

Phylogenetic placement of isolate sequences

We did a phylogenetic analysis on the representative sequences of OTUs for 80 isolates obtained in this study and relevant environmental sequences present in the GenBank database (Figs. 2, 3). The levels of diversity identified using cultivation dependent and independent approaches were notably different. Actinobacteria made up the dominant group among cultivated strains (47%) and among the environmental sequences recovered from Pajsarjeva jama cave silver samples (16%, Pašić et al., 2010). Unfortunately, we could not cultivate Actinobacterial phylotypes that were dominant in environmental 16S rRNA gene libraries (Fig. 2). Instead, the OTUs represented by 16S rRNA sequences of isolates C3 (7.5% of all isolates obtained), F2 (2.5%), C10 (2.5%), E4 (3.8%) and D11 (2.5%) were $\geq 99\%$ similar to 16S rRNA sequences of *Rhodococcus wratislaviensis*, *Rhodococcus erythropolis*, *Kocuria kristinae*, *Microbacterium paraoxidans*, and *Agrococcus citreus*, respectively. The OTUs represented by isolates A11 (16.3%), D8 (2.5%),

Fig. 1. Distribution of median viable counts on plates inoculated with cave silver samples upon five weeks of incubation at 30°C with respect to growth medium. The box-plots show minimum, maximum, the 25 and 75 percentile and median. The growth medium with significant effect on viable counts (Fisher's least significant difference, $P < 0.05$) is indicated by an asterisk.

Table 1. Total viable counts (CFU/g) obtained from pretreated cave silver samples.

Sample	Wet heat (a)	Dry heat (b)	Dry heat and phenol (c)	Electromagnetic wave (d)	No treatment (e)	Total number of isolates
Yellow	1.4×10^3	2.0×10^2	2.0×10^4	2.0×10^6	5.9×10^6	12
Gray	2.2×10^4	2.0×10^4	2.0×10^2	2.1×10^6	7.7×10^6	29
White	4.4×10^3	2.0×10^2	5.6×10^4	ND	2.7×10^7	15
Pink	2.0×10^4	4.0×10^4	ND	5.3×10^6	1.9×10^7	24
ND – no colonies were detected upon eight-week incubation at 30°C.						80

Fig. 2. ML tree showing the phylogenetic position of OTU representatives (prefix 'cave bacterium PJ') belonging to Actinobacteria. Numbers at nodes (>75%) indicate percentages of 1000 bootstrap resamplings. Sequences with prefix 'uncultured actinobacterium clone PJM' originate from a previous study (Pašić et al., 2010).

D12 (2.5%), G4 (1.3%) and F3 (23.8%) shared 95%-97% sequence identity with validly described species and likely represent novel taxa. Their phenotypic characteristics are presented in Table 2.

In similar fashion, the environmental 16S rRNA sequences affiliated with Proteobacteria differed from those obtained through the cultivation approach (Fig. 3). While environmental 16S rRNA sequences were related to Chromatiales (23.4%) and Xanthomonadales (7.9%), the cultivated taxa (OTU representatives A9 (1.3% of isolates obtained in this study), C11 (7.5%) and C1 (6.3%)) represented novel phylotypes within genera *Pseudomonas* and *Lysobacter*. Isolate sequences affiliated with Alphaproteobacteria shared ≤97% sequence identity with known species of genera *Sphingomonas* (B3, 2.5%), *Bosea* (C5, 1.3%) and *Agrobacterium* (A3, 6.3%). We further cultivated members of *Firmicutes*, a group that

was not found in Pajsarjeva jama using a cultivation-independent approach. Their 16S rRNA sequences were not closely related (95%-97% sequence identity) to validly described species of genera *Paenibacillus* (E2, 5%), *Bacillus* (E7, 1.3%; H9, 1.3%) and *Aerococcus* (F10, 1.3%). Phenotypic characteristics of the presumably novel taxa listed above are presented in Table 2.

The majority of cave isolates reported here were obtained on multiple media. *Microbacterium*, *Micrococcus*, *Oerskovia*, *Paenibacillus*, *Pseudomonas*, *Rhodococcus*, *Streptomyces*, *Agrobacterium*, *Lysobacter* and *Arthrobacter* species were obtained from PY-BHI, SCA, SEA, TSA and TWA. A group of isolates belonging to genus *Pseudomonas* was obtained on GAA and MYA media. *Bacillus* species were obtained only on TSA and SCA. Finally, isolates belonging to the genera *Bosea*, *Kocuria* and *Sphingomonas* were isolated only on TWA

Table 2. Phenotypic features of presumably novel isolates detected in this study.

Isolate name	PJC1	PJA9	PJC11	PJE2	PJB3	PJC5	PJA3	PJA11
Proposed phylogenetic affiliation	<i>Lysobacter</i> sp.	<i>Pseudomonas</i> sp.	<i>Pseudomonas</i> sp.	<i>Paenibacillus</i> sp.	<i>Sphingomonas</i> sp.	<i>Bosea</i> sp	<i>Agrobacterium</i> sp.	<i>Micrococcus</i> sp.
Colony pigmentation on TSA medium	Pale-yellow	White	White	White	White	Cream	White	Yellow
Cell shape	Rods	Rods	Rods	Rods	Rods	Rods, straight to curved	Rods	Cocci
Gram stain	Negative	Negative	Negative	Positive	Negative	Negative	Negative	Positive
Oxidase	Negative	Positive	Positive	Positive	Negative	Positive	Positive	Positive
Catalase	Positive	Positive	Positive	Negative	Negative	Positive	Positive	Negative
Biochemical characteristics (API 20NE)								
	Glucose fermentation	Glucose fermentation	Esculin hydrolysis	Reduction of nitrates to nitrites	Esculin hydrolysis	Reduction of nitrates to nitrogen	Esculin hydrolysis	Glucose fermentation
	Gelatin hydrolysis	Arginin hydrolysis		Urea hydrolysis e		Indole production	β -galactosidase	Gelatin hydrolysis
	Esculin hydrolysis	Urea hydrolysis		Esculin hydrolysis				
Assimilation of (API 20NE)								
	D-glucose	D-glucose	D-glucose	D-mannitol	D-glucose	D-mannose	D-glucose	D-glucose
		L-arabinose	L-arabinose	N-acetyl-glucosamine	L-arabinose	Potassium gluconate	L-arabinose	D-maltose
		D-mannose	D-mannose	D-mannitol			D-mannose	Phenylacetic acid
		D-mannitol	D-mannitol	Potassium gluconate			D-mannitol	
		Potassium gluconate	N-acetyl-glucosamine	Adipic acid			N-acetyl-glucosamine	
		Capric acid	potassium gluconate	Malic acid			D-maltose	
		Adipic acid	Capric acid	Trisodium citrate			Malic acid	
		Malic acid	Malic acid					
		Trisodium citrate	Trisodium citrate					
Acid production from (API 50CH)								
	Glycerol	Galactose	D-glucose	Glycerol	Glycerol	Ribose	Ribose	Galactose
	Ribose	D-glucose	Esculin	Erythritol	Erythritol	Esculin	Amidon	D-glucose
	Galactose	D-fructose		L-arabinose	L-arabinose		β -gentiobiose	D-fructose
	D-glucose	D-mannose		Ribose	Ribose		D-fucose	D-mannose
	D-fructose	Maltose		D-xylose	D-xylose		L-fucose	N-acetyl glucosamine
	D-mannose	Lactose		Galactose	Adonitol		Esculin	Maltose
	Mannitol	Saccharose		D-glucose	β methyl-xyloside			Lactose
	Sorbitol	Trehalose		D-fructose	β methyl-xyloside			Saccharose
	Esculin			D-mannose	Galactose			Trehalose
	Maltose			N-acetyl glucosamine	D-glucose			D-tyranose
	Lactose			Arbutine	D-fructose			D-tyranose
	Saccharose			Esculin	D-mannose			Esculin
	Trehalose			Salicine	L-sorbose			
	Gluconate			Maltose	Sorbitol			
	Esculin			Lactose	N-acetyl glucosamine			
				Amidon	Amygdaline			
				Glycogene	Cellobiose			
				β -gentiobiose	Lactose			
				Esculin	Maltose			
					Saccharose			
					Trehalose			
					Esculin			

medium and isolates related to *Agrococcus* only on SEA. The pre-treatments did not increase the diversity of isolates obtained in pure culture. In fact, only a single isolate was obtained exclusively from a pre-treated sample (microwave irradiation, a non-*Streptomyces* actinomycete). Some differences in diversity of pure cultures were observed if the isolates were grouped

with respect to sample colour (Table 3). Twelve isolates originated from the yellow sample, 29 from grey, 15 from white and 24 from a pink coloured sample. Common to all samples were the abundant groups belonging to the genera *Agrobacterium* and *Streptomyces*, while isolates belonging to *Rhodococcus*, *Pseudomonas*, *Paenibacillus*, *Micrococcus* and *Arthrobacter* were present in at least

Table 2 (continued). Phenotypic features of presumably novel isolates detected in this study.

Isolate name	PJH9	PJE7	PJD12	PJD8	PJF3	PJG4	PJF10
Proposed phylogenetic affiliation	<i>Bacillus</i> sp.	<i>Bacillus</i> sp.	<i>Oerskovia</i> sp.	<i>Arthrobacter</i> sp.	<i>Streptomyces</i> sp.	<i>Streptomyces</i> sp.	<i>Aerococcus</i> sp.
Colony pigmentation on TSA medium	White	White	Pale yellow	White	Cream	White	White
Cell shape	Rods	Rods	Branched filaments	Rods to cocci	Filaments	Filaments	Cocci
Gram stain	Positive	Positive		Positive	Positive	Positive	Positive
Oxidase	Positive	Positive	Positive	Positive	Positive	Positive	Negative
Catalase	Negative	Positive	Negative	Positive	Positive	Negative	Negative
Biochemical characteristics (API 20E)			(API Coryne)				(API Strep)
	Fermentation of D-glucose	Citrate utilisation	Nitrate reduction	Nitrate reduction	Alkaline phosphatase	Alkaline phosphatase	Pyrolidonyl arylamidase
	Fermentation of arabinose	Urea hydrolysis	β -galactosidase	Pyrolidonyl arylamidase	β -galactosidase	β -galactosidase	Acidification of lactose
		Fermentation of D-glucose	α -glucosidase	Alkaline phosphatase	N-acetyl β -glucosaminidase	N-acetyl β -glucosaminidase	Acidification of trehalose
		Fermentation of D-mannose	N-acetyl β -glucosaminidase	β -galactosidase	Fermentation of D-mannitol	Gelatin hydrolysis	Acidification of inuline
		Fermentation of saccharose	β -glucosidase	Gelatin hydrolysis	Fermentation of D-maltose	Fermentation of D-maltose	α -haemolysis
			Gelatin hydrolysis	Fermentation of D-glucose			
			Fermentation of D-glucose	Fermentation of ribose			
			Fermentation of ribose	Fermentation of xylose			
			Fermentation of xylose	Fermentation of maltose			
			Fermentation of maltose	Fermentation of mannose			
				Fermentation of saccharose			
Acid production from (API 50CH)							
	Glycerol	L-arabinose	Glycerol	Glycerol	Glycerol	Rhamnose	Ribose
	D-glucose	Ribose	Erythritol	Erythritol	L-xylose	Esculin	Galactose
	D-fructose	Galactose	L-arabinose	Ribose	Adonitol	Maltose	D-glucose
	Mannitol	D-glucose	D-arabinose	D-xylose	D-mannose	Trehalose	D-fructose
	N-acetyl glucosamine	D-fructose	Ribose	L-xylose	L-sorbose	Melezitose	D-mannose
	Maltose	D-fructose	D-xylose	Adonitol	Mannitol	D-tagatose	Salicine
	Saccharose	Salicine	Galactose	β -methyl xyloside	α methyl-D-mannoside	Gluconate	Cellobiose
	Trehalose	Cellobiose	D-glucose		N-acetyl glucosamine	5 ceto-gluconate	Maltose
	D-raffinose	Maltose	D-fructose		Arbutine		Lactose
	D-turanose	Lactose	D-mannose		Salicine		Saccharose
	Esculin	Saccharose	N-acetyl glucosamine		Cellobiose		Trehalose
		Trehalose	Amygdaline		Maltose		Inuline
		β -gentiobiose	Arbutine		Melibiose		
		D-fucose	Esculin		D-raffinose		
		L-fucose	Cellobiose		D-turanose		
		2 ceto-gluconate	Maltose		D-arabitol		
		2 ceto-gluconate	Saccharose		Gluconate		
			Trehalose		5 ceto-gluconate		
			Amidone				
			Glycogene				
			Xylitol				
			β -gentiobiose				
			D-turanose				
			D-lyxose				
			Gluconate				
			Esculin

Fig. 3. ML tree showing the phylogenetic position of OTU representatives (prefix 'cave bacterium PJ') belonging to Proteobacteria and Firmicutes. Numbers at nodes (>75%) indicate percentages of 1000 bootstrap resamplings. Sequences with prefixes 'uncultured gamma proteobacterium clone PJM' and 'uncultured alpha proteobacterium clone PJM' originate from a previous study (Pašić et al., 2010).

two different samples. The remaining isolates were sample-specific and represented a minor portion of diversity encountered.

In 2010, Jurado et al. reported on the widespread occurrence of pathogenic and opportunistic microorganisms in caves. The caves were found to support a number of pathogenic bacteria that included species of *Micrococcus*, *Pseudomonas*, *Bacillus*, *Stenotrophomonas* and *Aureimonas*. All these were found to cause infections of the respiratory organs and eyes in new-borns, children and elderly (Jurado et al., 2010). More recently, Bhullar et al. (2012) reported that cave microbes are often highly resistant to commercial antibiotics, despite their long-term isolation from the surface. The results of this study add to the growing understanding of potential

health risks associated with cave tourism. A number of bacterial isolates obtained in this study are affiliated with pathogenic bacteria, namely with *Bacillus idriensis*, *Pseudomonas simiae*, *Kocuria kristinae*, *Microbacterium paraoxydans*, and *Rhodococcus erythropolis*. All these species were reported to cause bacteraemia in humans and apes (Laffineur et al., 2003; Ma et al., 2005; Ko et al., 2006; Vela et al., 2006; Baba et al., 2009).

On the source of cave microbes

A major goal of this study was to contribute to the current knowledge on the source of cave microbes. To this aim, we compared our sequences to a subset of 12301 16S rRNA sequences that originated from cave environments and that were present in GenBank

database on January 29th, 2013 (Table 3). We found that isolate and environmental 16S rRNA sequences $\geq 97\%$ similar to OTUs sequences recovered in this study were reported from the walls and ceilings of a number of subterranean environments that differ widely in geology, e.g. from Pajsarjeva jama in Slovenia (Porca et al., 2012) formed though dissolution of limestone by carbonic acid, from Lechuguilla cave, formed by dissolution of limestone by sulfuric acid (Johnston et al., 2011), and from lava tubes formed by volcanic activity (Northup et al., 2011). Furthermore, members of genera *Bacillus*, *Micrococcus*,

Arthrobacter, *Paenibacillus*, *Streptomyces*, *Oerskovia*, and *Microbacterium* were recently reported in an aerobiological study, which examined human-induced environmental disturbances in Ardales cave in Spain (Fernandez-Cortes et al., 2011). This study concluded that cave microbes are likely dispersed as spores or attached to particles through action of wind and turbulence or through the impact of splashes of cave water droplets (Fernandez-Cortes et al., 2011).

It is interesting that the representative 16S rRNA sequences of OTUs affiliated with *Rhodococcus*,

Table 3. The similarities of 16S rRNA sequences of OTU representatives detected in this study to 16S rRNA sequences present in GenBank. Presumably novel taxa ($\leq 97\%$ sequence identity to cultivated species) are indicated in bold.

Isolate name	Isolate obtained from sample*	Description of the most similar sequence	GenBank accession number	% Identity	Query coverage %	Most similar sequence origin	GenBank reference
PJ A3	Y, G, W, P	<i>Agrobacterium</i> sp. LC034	JN863451	98.8	97.0	Lechuguilla Cave, USA	Johnston et al., 2012
PJ A9	P	<i>Pseudomonas fluorescens</i> R124	M001561	98.3	85.5	Orthoquartzite cave surface	Barton et al., 2012, unpublished
PJ A11	G, W, P	<i>Micrococcus</i> sp. LC402	JQ014545	99.2	96.5	Lechuguilla Cave, USA	Johnston et al., 2012
PJ B3	G	Uncultured bacterium clone CC01f45b11	JQ675472	97.6	100	Carlsbad Caverns, New Mexico Room, USA, water sample	Hughes et al., 2012, unpublished
PJ C1	G	Uncultured bacterium clone GA21498a09	JN607002	97.5	97.9	White microbial mat from lava tube wall, Gruta das Agulhas, Terceira, Azores, Portugal	Northup et al., 2011
PJ C10	Y	<i>Kocuria rosea</i> strain MM1-1	JX144942	97.0	98.0	Biofilm in Mawmsai cave, India	Banerjee and Joshi, 2013
PJ C11	Y, P	Uncultured bacterium clone YCaltP17B3	HE602891	98.9	100	Macroscopic yellow colony cave wall sample from Altamira cave, Spain	Porca et al., 2012
PJ C3	Y, G, P	Uncultured <i>Rhodococcus</i> sp. clone EC34BH03	JN032369	96.6	97.1	Cave	Johnston et al., 2012
PJ C5	P	<i>Bosea</i> sp. LC484	JQ014392	98.1	99.0	Lechuguilla Cave, USA	Johnston et al., 2012
PJ D11	G	<i>Agrococcus</i> sp. LC476	JQ014288	99.0	93.8	Lechuguilla Cave, USA	Johnston et al., 2012
PJ D12	P	<i>Oerskovia paurometabola</i> strain 0410ARD15N_4	FR848425	98.8	96.3	Air from a cave	Fernandez-Cortes et al., 2011
PJ D8	G, P	<i>Arthrobacter mysorens</i> strain DSM 12798T	AJ617482	98.2	92.3	Alpine ice cave	Margolin et al. 2003, unpublished
PJ E2	Y, G	Uncultured bacterium clone GTM1017c09	JN600577	94.9	92.3	Tan microbial mat from lava tube wall, Gruta Terra Mole, Terceira, Azores, Portugal	Northup et al., 2011
PJ E4	P	<i>Microbacterium</i> sp. GGC-P14	FJ348038	99.3	95.5	Cave coralloid speleothem, Wayne county, Kentucky, USA	Banks et al., 2010
PJ E5	G	<i>Agrobacterium</i> sp. LC034	JN863451	95.9	96.8	Lechuguilla Cave, USA	Johnston et al., 2012
PJ E7	Y	Uncultured <i>Bacillus</i> sp. clone WeeA_A07	JN032377	98.4	95.3	Cave	Johnston et al., 2012
PJ F10	P	<i>Bacillus</i> sp. GGC-P5A1	FJ348046	92.0	93.0	Cave coralloid speleothem, Wayne county, Kentucky, USA	Banks et al., 2010
PJ F2	Y, G	<i>Rhodococcus</i> sp. LC412	JN863511	99.5	90.5	Lechuguilla Cave, USA	Johnston et al., 2012
PJ F3	Y, G, W, P	<i>Streptomyces</i> sp. strain 1912	EU073049	99.6	95.9	Balatini cave, Derebucak, Konya, Turkey	Isik et al., 2007, unpublished
PJ G4	Y	<i>Streptomyces sampsonii</i> strain PM350_DR016	JQ422172	96.8	97.7	Helmcken Falls Cave, Wells Gray Provincial Park, BC, Canada	Cheeptham et al., 2013
PJ H9	W	<i>Firmicutes</i> bacterium 00YJ4	EU810836	99.2	98.65	Cave of Doña Trinidad, Ardales, Malaga, Spain	Stomeo et al., 2008, unpublished

*Abbreviations Y, W, G and P correspond to yellow, white, grey and pink cave microbial colony sample.

Paenibacillus, *Agrobacterium*, *Aerococcus* and *Streptomyces* (16.4% of isolates) corresponded to organisms more distantly related (92.0-96.8% sequence identity) to cave organisms. However, these organisms were related ($\geq 97\%$ sequence identity) to freshwater (*Rhodococcus*, *Paenibacillus*), soil (*Agrobacterium*, *Streptomyces*), or host-associated (*Aerococcus*) bacteria.

CONCLUSION

We isolated 80 morphologically different bacterial isolates from samples of microbial colonies that develop on the walls of Pajsarjeva jama in Slovenia. Viable counts increased with a decrease in inoculum size and were reduced if samples were exposed to pre-treatments. All growth media yielded viable counts, however these were highest on a low-nutrient TWA medium. We obtained 21 species-level OTU and found *Agrobacterium* and *Streptomyces* in all samples. We found a number of isolates was unrelated to validly described species. Although the obtained isolates likely represent a minor portion of diversity, they appear to be widespread in subterranean environments, judging from the distribution of highly related 16S rRNA gene sequences.

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