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The microbial community of a biofilm lining the wall of a pristine cave in Western New Guinea

Paolo Turrini [a], Marco Tescari [a], Daniela Visaggio [a], Mattia Pirolo [a], Gabriele Andrea Lugli [b], Marco Ventura [b], Emanuela Frangipani [c,1], Paolo Visca [a,*,1]

[a] Department of Science, Roma Tre University, Viale G. Marconi 446, 00146 Rome, Italy
[b] Laboratory of Probiogenomics, Department of Chemistry, Life Sciences, and Environmental Sustainability, University of Parma, Parco Area delle Scienze 11a, 43124 Parma, Italy
[c] Department of Biomolecular Sciences, University of Urbino Carlo Bo, Urbino, Italy

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ABSTRACT

Caves are extreme environments inhabited by microbial communities adapted to thrive oligotrophic conditions. Cave microbes are organised in complex ecological networks and have developed survival strategies involving the production and release of a large variety of secondary metabolites, including antibiotic-like compounds. In this study, the structure and the metabolic features of a biofilm-like microbial community lining the walls of a pristine karst cavity (the Yumugi river cave) located in a remote region of the Western New Guinea were investigated. 16S rRNA and shotgun sequence analyses highlighted the prevalence of chemoorganotrophic phyla (*Proteobacteria, Actinobacteria, Firmicutes* and *Acidobacteria*), consistent with metabolic predictions inferred from the cave metagenome analysis. Few clinically relevant antimicrobial resistance genes were detected. A culture-based approach allowed the isolation of some heterotrophic members of the bacterial community, and antimicrobial susceptibility testing revealed an overall high level of resistance to different antimicrobials classes. Isolates presumptively representing new uncharacterized members of genus *Pseudomonas* displayed interesting antibiotic properties against Gram-positive indicator strains. Our work supports the hypothesis that caves represent a reservoir for new bacterial species and drug discovery research.

1. Introduction

Caves represent one of the most unique and poorly studied ecosystems on Earth. Karst caves are natural geological formations resulting from the seepage of water into calcareous rocks (Palmer, 1991; Gillieson, 1996). Although karst systems comprise approximately 5–20 % of the Earth's ice-free land surface, only 10 % of them have so far been explored (Engel, 2010).

Caves are considered extreme environments for life, due to the combination of unique physical-chemical conditions, including total darkness or low light level, high humidity, low stable temperature, and scarcity of nutrients (Engel, 2010). Therefore, the majority of cave microorganisms are oligotrophic species well adapted to near-starvation conditions, displaying slow growth and consequent low biotic potential. Cave microorganisms are involved in biomineralization processes, either by promoting the formation of secondary deposits called speleothems (*e.g.,* moonmilk, pool finger precipitation), or by inducing carbonate dissolution through acid secretion (Northup and Lavoie, 2001; Barton and Northup, 2007; Maciejewska et al., 2017).

Many phylogenetic studies have revealed a paradoxically broad microbial diversity (Hutchinson, 1961; Torsvik et al., 2002), which may rule out a competitive exclusion driven by such low-nutrient environments. Indeed, cave microbial communities seem to organize themselves in cooperative and mutualistic consortia, where some well-adapted species support the growth of others, to overcome the limitations imposed by the extreme cave environment (Barton and Jurado, 2007).

The broad metabolic potential of cave microorganisms has recently been recognized in microbial communities inhabiting the limestone Manao-Pee cave in Thailand, showing the ability to use a variety of nutrient sources, including inorganic sulphur and xenobiotic compounds (Wiseschart et al., 2019). Moreover, the predicted presence of biosynthetic pathways for secondary metabolites suggests an ecological

* Corresponding author.
E-mail address: paolo.visca@uniroma3.it (P. Visca).
[1] Contributed equally.

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role of these bioactive compounds in the survival and maintenance of the microbial community (Wiseschart et al., 2019). It is reasonable that the cave extreme environment positively selects for new metabolic pathways, including those for secondary metabolites endowed with antimicrobial activity, hence relevant for new drug discovery (Cheeptham et al., 2013; Ghosh et al., 2017; Rangseekaew and Pathom-aree, 2019). The reason(s) why microbial communities in low-nutrient environments produce bioactive compounds (*i.e.,* antimicrobials) is still debated. If competition is the preferred strategy for survival, antimicrobials could be used as weapons to compete for resources, but also as signals to allow cell-to-cell communication (Linares et al., 2006; Schlatter and Kinkel, 2014), or even as nutrient sources (Davies, 2006; Dantas et al., 2008).

Recent studies pointed out that more than 65 % of culturable bacterial isolates from a cave in New Mexico, which remained inaccessible and never exposed to modern antimicrobials for over 4 million years, were resistant to 3–4 classes of antimicrobials (Bhullar et al., 2012; Pawlowski et al., 2016). Thus, caves are also reservoirs of antimicrobial

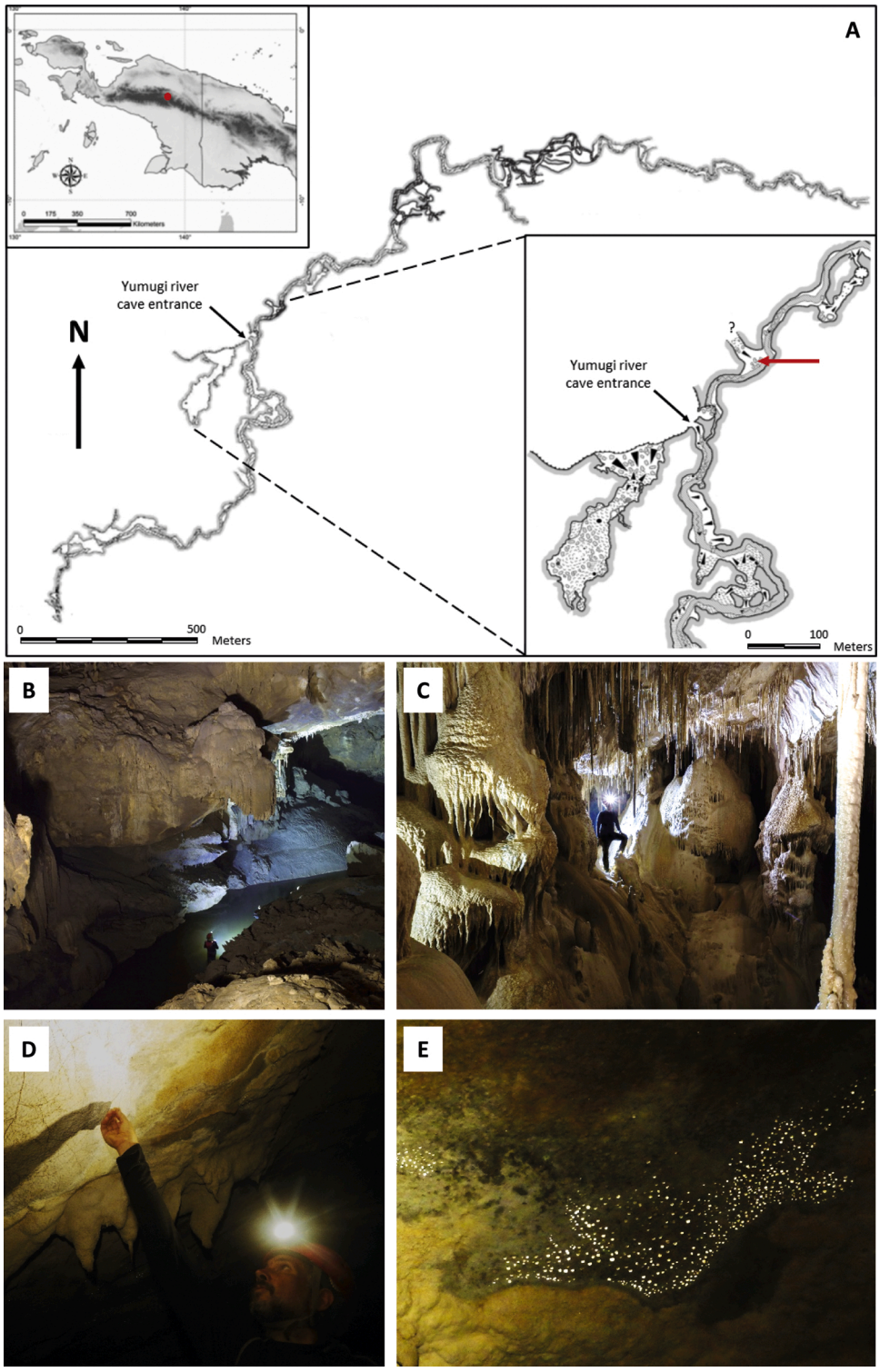

Fig. 1. Cave map and sampling site. A) Localization and map of the Yumugi river cave in Western New Guinea. Red arrow indicates the sampling site. B) Active meander where Yumugi river flows through the cave. C) Relict gallery widely adorned with calcium carbonate formations such as flowstones, stalactites, stalagmites and columns. D) General view and E) close up of the sampling site. The Yumugi river cave map was generated by: Eavis A., Fogg P., Fogg T., Mabel M., Uaga A., Hewat R., White A., during the December 1996 expedition, and by: Benassi A., Biondi T., Pasquini T., Pozzo R., Turrini P., Zampatti K., during the December 2017 expedition. Pictures were provided by the Acheloos Geo Exploring Archive (B-C, T. Biondi; D-E, K. Zampatti).

resistance genes (ARGs), generated as the result of dynamic and competitive microbial interactions that have evolved over time (D'Costa et al., 2006; Walsh and Duffy, 2013).

The anthropogenic use of antimicrobials has contributed to increasing the frequency of antibiotic resistance in both environmental bacteria and clinical pathogens, which is dramatically leading to a global health crisis (Michael et al., 2014; von Wintersdorff et al., 2016). In fact, ARGs from soil bacteria are similar to those of bacterial pathogens and are often flanked by mobile DNA elements, highlighting the role of horizontal gene transfer as the driving mechanism governing the flux of ARGs from environmental to clinically relevant bacteria (Forsberg et al., 2012; Jiang et al., 2017).

New Guinea is a biodiversity hotspot containing over 5 % of the world's biodiversity in less the 0.5 % of the total area. The extreme biodiversity of Oceanian archipelagos has long stimulated research in ecology and evolution (Marshall and Beehler, 2007). New Guinea's caves represent some of the few environments with very limited anthropogenic influence, and despite limestone covers large sections of this region, only a handful of caves has been explored in this island, limiting the knowledge of cave biology (Deharveng et al., 2007; Patoka et al., 2017). Microbial communities in New Guinea caves have never been investigated, though they may provide insights into the role played by both antimicrobials and their resistance mechanisms in a natural environment devoid of the selective pressure imposed by anthropogenic activities.

In this work, we have investigated the microbial community of a biofilm-like layer coating the wall of a pristine cave located in the Western New Guinea, using both metagenomic and culture-based approaches. The taxonomic and metabolic profiles of the microbial community were investigated, and the presence of ARGs was detected in metagenomics sequences. Bacterial isolates were tested for susceptibility to several antimicrobials and for their ability to produce antimicrobial compounds. Our findings provide novel insights into the microbial-community structure and antimicrobial resistance potential of a still unexplored cave system.

2. Methods

2.1. Site description and sampling procedure

The sample analysed in this study was collected in the Yumugi river cave during an Italian speleological expedition organized by Acheloos Geo Exploring in Papua on December 2017 (Benassi and Pasquini, 2018). Yumugi river cave is located in the New Guinea Highlands, Jayawijaya Regency, Papua Province, GPS coordinates: S 04°02.04' E 139°00.095', 6 km North-Est of the Wamena city in the Baliem Valley (Fig. 1A). The Yumugi river cave was discovered in 1996 by a British-Australian speleological team, but it has so far experienced very little human impact, due to its isolate location in a mountainous area far from inhabited centres, and to cave protection policies of the local population. The only human activity since then has been reported in July 2017, when a Czechoslovakian team of scientists has documented the isolation of a rare endemic cave crayfish (Patoka et al., 2017). Yumugi river cave opens at 1,720 m above sea level and is a limestone cave formed by an active meander where the Yumugi river runs through the cave with a flow of about 1.5 m^3/s, until emerging at the entrance level (Fig. 1B). The cave has several relict galleries widely adorned with calcium carbonate formations such as flowstones, stalactites, stalagmites and columns (Fig. 1C). Currently, the Yumugi river cave represents the longest cave in Western New Guinea with a development plan map of about 8 km (Fig. 1A).

For this study, *ca.* 4 cm^2 of a soft biofilm-like structure were scraped off the wall of a completely dark section of the cave, located about 100 m away from the Yumugi river cave entrance, using a sterile swab (Fig. 1D). This biofilm-like layer covered extensive areas of the cave surface, forming characteristic gold or silver light-reflecting water droplets (Fig. 1E). At the time of sampling, the air temperature, which is relatively constant during the whole year, was 15 °C and the relative humidity was 100 %. The sample was collected in a sterile 15-ml tube, kept refrigerated during transport to the laboratory, where it was immediately processed.

2.2. DNA extraction, shotgun metagenomic sequencing and taxonomic analysis

Total DNA was extracted from the cave sample using the PowerSoil DNA Isolation Kit (MOBIO, CA), according to the manufacturer's recommendations. Integrity and quality of extracted DNA was determined spectrophotometrically by Microliter UV/Vis Spectrophotometer (Picodrop).

Data processing and bioinformatic procedures are provided in Supplementary Fig. S1. For shotgun metagenomic sequencing, DNA library preparation was performed using the Nextera XT DNA sample preparation kit (Illumina, San Diego, CA) according to the manufacturer's instructions. One ng input DNA was used for library preparation. The isolated DNA underwent fragmentation, adapter ligation and amplification. The ready-to-go libraries were pooled equimolarly, denaturated and diluted to a sequencing concentration of 2 pM. Illumina NextSeq 550 sequencer was used for sequencing, according to the manufacturer's instruction, using 2 × 150 bp with NextSeq Mid Output v2 Kit chemicals. Shotgun sequencing FASTQ files were processed with METAnnotatorX pipeline (Milani et al., 2018). After quality control, the 1,555,447 assembled reads were analysed for the taxonomic assignment, then assembled in 528 contigs. Contigs with length < 5,000 bp were discarded.

2.3. Environmental 16S rRNA gene amplification

Partial 16S rRNA gene sequences were amplified from the extracted DNA using primers Probio_Uni and Probio_Rev, which target the V3 region of the 16S rRNA gene (Milani et al., 2013). The PCR conditions were 5 min at 95 °C, 35 cycles of 30 s at 94 °C, 30 s at 55 °C and 90 s at 72 °C, followed by 10 min at 72 °C. Amplification was carried out by using a Verity Thermocycler (Applied Biosystems). The integrity of the PCR amplicons was analysed by electrophoresis on a 2200 TapeStation Instrument (Agilent, USA). Illumina adapter were added to the partial 16S rRNA gene-specific amplicons, which were further processed using the 16S Metagenomic Sequencing Library Preparation Protocol (Part #15044223 Rev. B – Illumina), as previously reported (Mancabelli et al., 2016). Further amplifications were carried out using a Verity Thermocycler (Applied Biosystems). The integrity of the PCR amplicons was analysed by electrophoresis on a 2200 TapeStation Instrument (Agilent Technologies, USA). DNA concentration of the amplified sequence library was determined by the Qubit quantification system (Life Technologies, USA). Amplicons were diluted to 4 nM, and 5 µl of diluted DNA amplicons were mixed to prepare the pooled final library. Each step of the library preparation was performed using HiPure Molecular Biology Grade Water (GE Healthcare, USA). A negative control was sequenced in order to verify that any contamination did not occur during the amplification and sequencing phases. Briefly, the negative control was processed as a normal sample (see above), but HiPure Molecular Biology Grade Water was used instead of a DNA sample. Furthermore, sequencing performance was validated using a synthetic mock community of eight known organisms employing the ZymoBIOMICS HMW DNA Standard D6322 (Zymo Research, USA). Sequencing was performed using an Illumina MiSeq sequencer with MiSeq Reagent Kit v3 chemicals, in order to obtain 2 × 250 bp reads (Mancabelli et al., 2016).

2.4. Environmental 16S rRNA sequence analysis

16S rRNA gene sequences raw FASTQ files were processed using QIIME (Caporaso et al., 2010), as previously described (Milani et al.,

2013). Pair-ends reads were quality filtered to retain sequences with a length between 140 and 400 bp, a mean quality score > 20 and with truncation of a sequence at the first base if a low-quality 10 bp rolling window was found. All sequences with homopolymers > 7 bp and mismatched primers were excluded. Sequences were clustered into Operational Taxonomic Units (OTUs) using QIIME with ≥ 99 % similarity (Caporaso et al., 2010), and taxonomic assignments were obtained by using the SILVA database (release 132; Quast et al., 2012). Sequences were rarefied prior to calculation of alpha diversity statistics. Alpha-indexes were calculated with QIIME. Chao1, and Shannon indexes were used for the richness of the samples and their diversity, respectively (Supplementary Fig. S2).

2.5. Analysis of shotgun metagenomic sequences

The METAnnotatorX pipeline (Milani et al., 2018) was used for *de novo* assembly and Open Reading Frames (ORFs) prediction and translation. Functional classification of the reads was performed using a custom database and the METAnnotatorX pipeline (Milani et al., 2018). Reads were classified according to the Clusters of Orthologous Groups (COG) functional category profiles based on the EggNog nomenclature (Huerta-Cepas et al., 2016) and the MetaCyc metabolic pathways classification (Caspi et al., 2016). The functional prediction of ORFs was performed by interrogating the COG and MetaCyc databases with RAPSearch2 (Zhao et al., 2012) with an e-value cutoff of 10^{-15}, a minimum alignment length of 50 amino acids and 50 % minimum identity.

RAPSearch2 (Zhao et al., 2012) was used to compare translated ORFs with the MEGARes database amino acid sequences (Lakin et al., 2017), which were previously indexed with the prerapsearch function. Sequences with a e-value < 10^{-25} were discarded. Positive hits were filtered using a coverage cut-off > 60 % amino acidic length and an identity value > 40 % in the aligned region. To confirm the assigned function, putative ARGs were compared with sequences of the Protein family (Pfam) database (El-Gebali et al., 2018) by using the HMMER software (Potter et al., 2018). Among the MEGARes positive hits, those classified as putative membrane transport proteins were compared with sequences deposited in the Transporter Classification Database (TCDB) (Saier et al., 2016).

2.6. Isolation of culturable bacteria

The swab sample from Yumugi river cave (carrying approximately 0.1 g of biofilm-like material) was suspended in 10 ml of saline (0.9 % NaCl water solution), mixed by vortexing and serially diluted up to 10^{-5}. Every dilution was then plated in duplicate on 50 % tryptic soy agar (TSA) and Reasoner's 2A agar (R2A) media supplemented with cycloheximide (50 μg/mL), to inhibit fungal growth. Plates were incubated at 22 °C for 14 days, and colony-forming units (CFUs) were enumerated and selected according to morphological characteristics, including shape, pigmentation, margin, surface and texture. Colonies initially grown on R2A were subcultured twice on 50 % TSA to ensure purity. Forty-six isolates were selected, purified and stored in 96-well microtiter plates containing 50 % tryptic soy broth (TSB) and 15 % glycerol at −80 °C, for further analyses. Temperature-dependent growth of all isolates was tested at 4 °C, 22 °C, 37 °C and 42 °C for 4 days.

2.7. Tentative identification of cave isolates

DNA from pure bacterial cultures and from the cave sample was extracted using Nucleospin Tissue kit (Macherey-Nagel), according to manufacturer's instruction and stored at −20 °C until used. The molecular identification of each bacterial isolate was performed by 16S rRNA full-length gene sequence analysis. The 16S rRNA gene was amplified with primer pairs 27f (5′ AGAGTTTGATCMTGGCTCAG 3′) and 1492 r (5′ CGGTTACCTTGTTACGACTT 3′) (Lane, 1991). The PCR reaction was carried out in 50 μl volumes containing 200 μM dNTPs, 0.5 μM of each primer, 1.25 U GoTaq G2 Flexi DNA Polymerase (Promega) and 1x Taq Buffer containing 2 mM $MgCl_2$. The following PCR conditions were used: initial denaturation at 95 °C for 5 min, followed by 25 cycles consisting of denaturation (95 °C for 30 s), annealing (57 °C for 30 s) and extension (72 °C for 90 s) and a final extension step at 72 °C for 7 min. PCR products were purified with a Wizard SV Gel and PCR Clean-Up System (Promega). The quantity and quality of the extracted DNA were tested using a Thermo Scientific™ NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Thermo Scientific) and by agarose gel electrophoresis, respectively. PCR products were stored at −20 °C until sequencing (DNA Sanger sequencing, Microsynth). Forward and reverse strands of each amplicon were sequenced in a 3730 DNA Analyzer (Applied Biosystems). Forward and reverse sequences obtained from the amplicons of each 16S rRNA gene were manually merged and resulted in sequences of *ca.* 1,400 nucleotide (nt) length. The 16S rRNA–based identification was obtained through different steps. Initially, BLASTn (Altschul et al., 1990) was used on NCBI RefSeq database to identify the closest relative species for each cave isolate and to determine the number of variable sites within their 16S rRNA sequences. Multiple isolates showing identical phenotype and 16S rRNA gene sequence were considered only once for subsequent experiments. Then, all the reference sequences for each species belonging to the identified genus were retrieved from the NCBI database and aligned with cave isolate sequences belonging to the same genus, using MEGA 7 (Kumar et al., 2016). Finally, a phylogenetic tree was generated for the entire genus comprising each cave isolate 16S rRNA gene using the neighbor-joining method, and the evolutionary distances were computed using the Kimura 2-parameters method (Kimura, 1980). Tree robustness was statistically verified by bootstrap analysis (with 1,000 replications). The accession number of sequences used to generate the phylogenetic trees are listed in Supplementary Dataset S1.

The presence of culturable bacteria in the cave sample was further confirmed by cross-checking the presence of the 16S rRNA gene sequences in the metagenomic sequence dataset that had been used to define the bacterial community structure. A custom database was compiled by listing all OTUs obtained by sequencing the V3 region of 16S rRNA genes, and used to run a local BLASTn (Pearson, 2014) analysis using the 16S rRNA genes obtained from culturable isolates as query (Supplementary Table S1).

2.8. Genome sequencing, assembly and analysis

The draft genome sequence (WGS) of *Pseudomonas* isolates from Yumugi river (namely, YUM26, YUM27, YUM28 and YUM46) was determined using the MiSeq platform (Illumina, UK). Fastq files of Illumina paired end reads (250 bp) were used as input for the genome assembly through the MEGAnnotator pipeline (Lugli et al., 2016). SPAdes v.3.13.0 was used for de novo genome assembly with the pipeline option "–careful" and a list of k-mer sizes of 21, 33, 55, 77, 99 and 127 (Bankevich et al., 2012). Contigs were then analysed for the prediction of protein-encoding ORFs using Prodigal (Hyatt et al., 2010). Predicted ORFs were functionally annotated by means of RAPSearch2 (cutoff e-value, 10^{-5}; minimum alignment length, 20 amino acids) using the NCBI nr database (Zhao et al., 2012) coupled with hidden Markov model profile (HMM; http://hmmer.org/) interrogation of a manually curated Pfam-A database (cutoff e-value, 10^{-10}). The tRNA genes were identified using tRNAscan-SE v.1.4 (Chan and Lowe, 2019), while rRNA genes were detected using RNAmmer v.1.2 (Lagesen et al., 2007).

2.9. Multilocus sequence analysis and pairwise genome identity of *Pseudomonas* isolates

Presumptive identification of *Pseudomonas* isolates was achieved by a two-step multilocus sequence analysis (MLSA) combined with average nucleotide identity (ANI) analysis.

A preliminarily MLSA was performed on the ordered concatemers (*ca.* 3,700 nt) of available 16S rRNA, *gyrB*, *rpoD* and *rpoB* partial genes sequences of 140 representative species of genus *Pseudomonas* (Mulet et al., 2012), and of *Pseudomonas* isolates YUM26, YUM27, YUM28 and YUM46. Sequences were manually concatenated, aligned using ClustalW in MEGA7 with default settings (Kumar et al., 2016) and trimmed with Gblocks v.0.91b (Castresana, 2000). Concatemer sequences (Supplementary Table S2) were used to construct a phylogenetic tree using the neighbor-joining method, and the evolutionary distances were computed using the Kimura 2-parameters method (Kimura, 1980). Tree robustness was statistically verified by bootstrap analysis (with 1,000 replications).

Subsequently, an extended MLSA was performed using the full-length sequences of 16S rRNA, *gyrB*, *rpoD*, *rpoB* and *recA* genes retrieved from the assembled genomes of 56 members of the *Pseudomonas* groups that were more closely related to *Pseudomonas* YUM26, YUM27, YUM28 and YUM46 isolates, according to the preliminary MLSA results (Supplementary Table S3). Sequences were manually concatenated, aligned using ClustalW in MEGA7 with default alignment parameters (Kumar et al., 2016) and trimmed with Gblocks v.0.91b (Castresana, 2000). Concatemer sequences were used to construct a phylogenetic tree using the above described method and settings.

Pairwise ANI was calculated with FastANI v.1.3 (Jain et al., 2018) using the matrix illustrated in Supplementary Table S4.

2.10. Antimicrobial susceptibility testing of cave isolates

The Yumugi river cave culturable isolates were tested against a panel of 18 different antimicrobials belonging to the principal antimicrobial classes. To this aim, isolates were suspended in 500 µl of saline at $OD_{600} = 0.1$. Then, up to 10^{-6} serial dilutions were performed, and 5 µl of each dilution were spotted on 50 % TSA plates supplemented with 20 µg/mL of each antimicrobial (Bhullar et al., 2012). After 4-days incubation at 22 °C, plates were inspected for growth, and isolates were defined resistant to the antimicrobial agent if growth level was comparable between test and control plates (*i.e.,* plates containing no antimicrobials). The following antimicrobials were used: ampicillin (AMP; Sigma-Aldrich Co.), aztreonam (ATM; Sigma-Aldrich Co.), ceftriaxone (CRO; Sigma-Aldrich Co.), chloramphenicol (CHL; Sigma-Aldrich Co.), ciprofloxacin (CIP; Sigma-Aldrich Co.), clindamycin (CLI; Sigma-Aldrich Co.), colistin (CST; Sigma-Aldrich Co.), daptomycin (DAP; kindly provided by Accord Healthcare Limited), erythromycin (ERY; Sigma-Aldrich Co.), fosfomycin (FOS; Sigma-Aldrich Co.), linezolid (LZD; Sigma-Aldrich Co.), meropenem (MEM; Astra Zeneca), nalidixic acid (NAL; Sigma-Aldrich Co.), rifampicin (RIF; Sigma-Aldrich Co.), streptomycin (STR; Sigma-Aldrich Co.), tetracycline (TET; Sigma-Aldrich Co.), trimethoprim-sulfamethoxazole (SXT; Sigma-Aldrich Co.) and vancomycin (VAN; Sigma-Aldrich Co.).

2.11. Antimicrobial activity screening

The production of antimicrobial compounds by isolates from Yumugi river cave was tested on Mueller Hinton II Agar (MH, Difco, BD) plates using the cross-streak method, as previously described (Maciejewska et al., 2016). Briefly, each isolate was independently streaked across the diameter of a single plate using a 1-µl loop, and growth was allowed for 5 days at 22 °C. Then, cave isolates cultures were cross-streaked with the bacterial indicator strains (*i.e., Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 29213), previously grown in LB broth at 37 °C for 18 h, and normalized to $OD_{600} = 0.1$. Plates were then incubated at 37 °C for 24 h, and release of antibacterial compounds was determined by direct observation of the growth inhibition zone of test strains.

2.12. Data availability

16S rRNA, shotgun and whole genome sequencing data have been submitted to the NCBI Sequence Read Archive (SRA) under BioProject PRJNA616285. The 16S rRNA sequencing data for the 14 culturable isolates were submitted to NCBI GenBank under Accession No. MT259660-MT259673.

3. Results

3.1. Taxonomic analysis and metabolic profiling of the microbial community lining the wall of Yumugi river cave

Taxonomic assignment of shotgun metagenomic merged reads revealed that the Yumugi river cave sample was mainly composed by *Bacteria* (97.2 %), whereas only a small fraction of reads was assigned to *Archaea* (1.0 %), *Eukarya* (0.6 %) and viruses (1.3 %) (Fig. 2A).

To characterize the bacterial community of the Yumugi river cave in more detail, a targeted metagenomic approach was carried out, through the taxonomic assignment of the V3 hypervariable region of the 16S rRNA gene. Sequencing of the V3 region of the 16S rRNA gene generated a total of 96,516 pair-ends reads. After quality-filtering, 90,561 reads were analysed and clustered into 497 OTUs, with 99 % cut-off for sequence identity. Rarefaction curves, calculated using ten subsamplings of valid reads, were generated on Chao1 and Shannon indexes. Both curves reached the plateau, confirming that sequencing provided adequate coverage for the richness and diversity of the sample (Supplementary Fig. S2).

Overall, 16S rRNA gene sequencing revealed *Proteobacteria* (49.6 %) and *Actinobacteria* (40.4 %) as the predominant phyla (Fig. 2B). Less prevalent bacterial phyla were *Acidobacteria* (1.8 %), *Firmicutes* (1.7 %), *Chloroflexi* (1.6 %) and *Nitrospirae* (1.1 %). A minority (3.5 %) of phyla accounting for < 1.0 % of total reads were grouped as Others, while unclassified phyla were 0.4 % (Fig. 2B). At the phylum level, a similar community composition was observed by taxonomic assignment of shotgun metagenomic reads, with *Proteobacteria* (49.5 %) and *Actinobacteria* (37.6 %) dominating the microbial community (Fig. 2B). Interestingly, shotgun assignment also revealed the presence of few additional phyla, including *Planctomycetes* (2.0 %), *Bacteroidetes* (1.3 %) and *Cyanobacteria* (1.2 %).

Five classes of *Proteobacteria* were identified by 16S rRNA gene sequencing, among which *Pseudomonadales* and *Rhizobiales* predominated (25.1 % and 24.4 %, respectively). Within the *Actinobacteria* phylum, the most abundant class was *Actinobacteria* (79.4 %). A detailed taxonomic catalogue of the *Proteobacteria* and *Actinobacteria* community, as determined by 16S rRNA gene sequencing, is shown in Fig. 2 (C and D). Within these two major phyla, the taxonomic composition inferred from the 16S rRNA gene analysis showed an overall concordance with the metagenome-based taxonomy at the class level, though some differences were observed at the order level (Supplementary Fig. S3).

The microbial community was dominated by chemoorganotrophic taxa, namely *Proteobacteria*, *Actinobacteria*, *Firmicutes* and *Acidobacteria*, which altogether accounted for the 93.5 % and 90.0 % of the 16S rRNA gene sequences and shotgun reads, respectively (Fig. 2B). Members of phototrophic (*Chloroflexi*) and chemolithotrophic (*Nitrospirae*) taxa were poorly represented, accounting for 1.0–1.6 % and 0.6–1.1 %, respectively, depending on whether frequencies were calculated according to 16S rRNA or shotgun sequencing results. Similarly, nitrogen-fixing bacteria (*Bradyrhizobium*), methanol oxidizing bacteria (*Methyloceanibacter* and *Hyphomicrobium*) and sulphur oxidizing bacteria (*Beggiatoa*) were rarely detected (0.2–1.1% and 0.1–2.7% of 16S rRNA and shotgun sequencing reads, respectively; data not shown).

The assembled Yumugi river cave metagenomic sequences contained 1,555,447 assembled reads and 5,526 ORFs. Metagenomic classification into COG categories revealed good agreement between functional

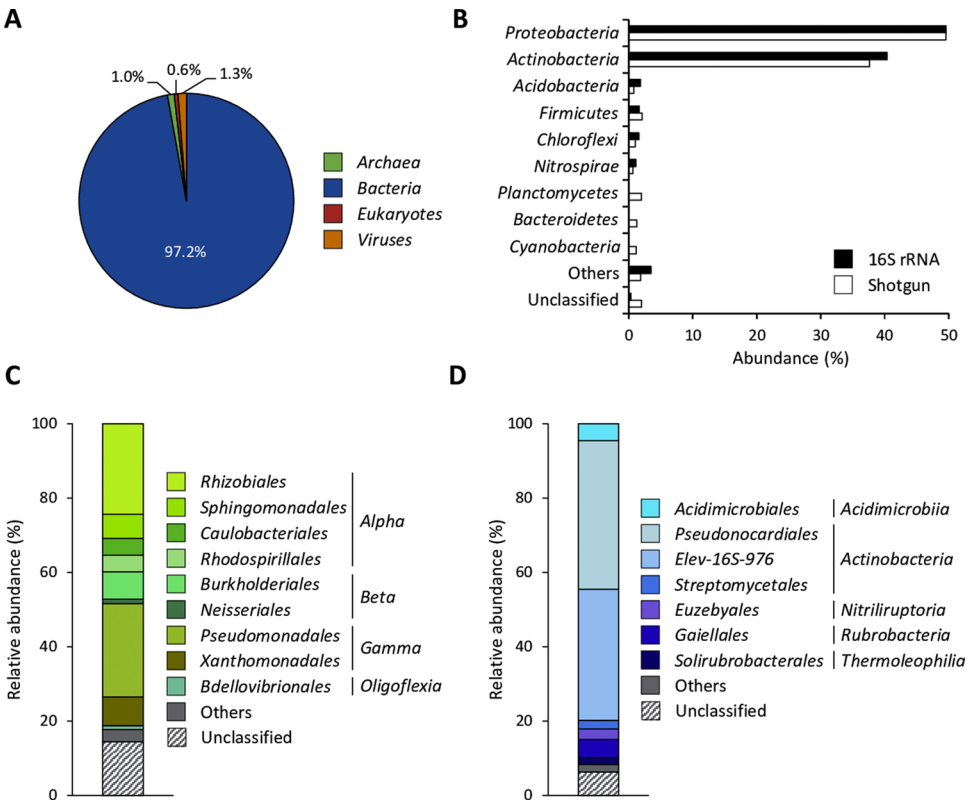

Fig. 2. Taxonomic profiles of the biofilm-like microbial community lining the walls of Yumugi river cave. Microbial community composition at the domain level, as determined by shotgun analysis (A). Comparative analysis of the bacterial community composition at phylum level, as inferred from shotgun and 16S rRNA gene sequence analyses (B). Relative composition of *Proteobacteria* (C) and *Actinobacteria* (D) phyla at class and order levels, as determined by 16S rRNA gene sequencing. Others indicates the sum of the relative percentages of phyla (B) or orders (C, D) that represented less than 1.0 % of the total reads.

predictions for reads and ORFs (Fig. 3A). Excluding the "Unknown" and "General functions" categories, it was observed that "Amino acid transport and metabolism" and "Replication, recombination and repair" formed the core functional categories of the bacterial community, accounting for 18.9 % and 19.6 % of reads and ORFs, respectively (Fig. 3A). Interestingly, *ca.* 3.0 % of reads and ORFs were assigned to the "Secondary metabolites biosynthesis, transport and catabolism", whereas both "Cell motility" and "Cell cycle control, cell division, chromosome partitioning" categories were poorly represented (< 2.0 % of reads and ORFs) (Fig. 3A).

Metabolic pathway prediction through the MetaCyc hierarchical classification system clustered the majority of reads (54.1 %) in the "Biosynthesis" class (Fig. 3B), which includes anabolic pathways shared by nearly all taxa. Two classes, namely "Generation of precursor metabolite and energy" and "Degradation, utilization and assimilation", which account for the 24.7 % and 15.2 % of the reads, respectively, were inspected in more detail to link metabolic pathways with specific bacterial trophisms (Fig. 3B). Apart from "Energy production" and "Generation of precursors metabolites" which include common pathways of all taxa, "Organic compound degradation" was the largest group (46.1 %), consistent with the chemoorganotrophic metabolism of the dominant taxa (Fig. 3B). "Inorganic compound metabolism" and "C_1 compound metabolism" accounted for 7.5 % and 3.2 % of pathways, respectively, which is in good agreement with the low prevalence of chemolitotrophic and methylotrophic taxa in the sample (Fig. 3B). Likewise, pathways related to primary production, including "CO_2 fixation" and "Photosynthesis", were poorly represented (4.0 % and 1.1 %, respectively; Fig. 3B), consistent with the scarcity of autotrophic and phototrophic taxa in the biofilm. A similar metabolic profile was inferred from categorization of predicted ORFs according to the MetaCyc hierarchical classification (Supplementary Fig. S4). A detailed analysis of chemoorganotrophic metabolism, characteristic of ≥ 90 % of taxa and exemplified by the "Organic compound degradation" category, showed carbohydrate, aromatic compound, carboxylate and aminoacid degradation as the principal metabolic pathways (Supplementary Fig. S4), primarily associated with bacteria belonging to the *Pseudonocardiales* (37.9 %) and *Streptomycetales* (13.1 %) orders.

3.2. Antimicrobial resistome in the Yumugi river cave

Interrogation of the MEGARes database retrieved 19 ORFs annotated as potential ARGs, whose function was further checked using the Pfam database (Table 1). Hits identified as putative ABC transporters were also confirmed by the use of the TCDB (Supplementary Table S5).

All ARGs were identified in contigs assigned to the *Actinobacteria* phylum, which was mostly represented by the *Pseudonocardiales* order (16/19 ARGs; Table 1). The majority of ARGs (7/19) encoded for efflux transporters, namely *drrA* and *macB* (four and three ARGs, respectively), which are predicted to mediate the export of antimicrobials across the cell surface, thereby reducing intracellular antimicrobial concentration. The *drrA* gene encodes for the nucleotide binding domain of an ATP binding cassette (ABC) transporter, which forms a multidrug efflux pump (Choudhuri et al., 2002), whereas the *macB* gene encodes for the inner membrane component of an ABC transporter that confers resistance to macrolides (Greene et al., 2018).

Six ARGs were involved in two-component regulatory systems, consisting of the drug sensor and the response regulator that control the expression of the cognate ARG. Of these, three shared similarities with *mtrA* and one with the *prmA* gene, all encoding response regulators controlling the expression of cell surface gene products, some of which are required for cell envelope permeability and drug resistance (Möker et al., 2004; Cangelosi et al., 2006; Chen and Groisman, 2016). Another ARG predicted to encode for a response regulator was the *arlR* gene, that induces resistance by controlling the expression of the *norA* promoter, encoding a multi-drug transporter system in *Staphylococcus aureus* (Fournier et al., 2000). The last ARG of this group was *vanS*, which acts as drug sensor of the *vanSR* two-component signal transduction system and is involved in vancomycin resistance (Novotna et al., 2016).

Three ARGs were involved in drug-target modifications, namely the *rbpA* and *tsnR* genes (one and two ARGs, respectively). The *rbpA* gene

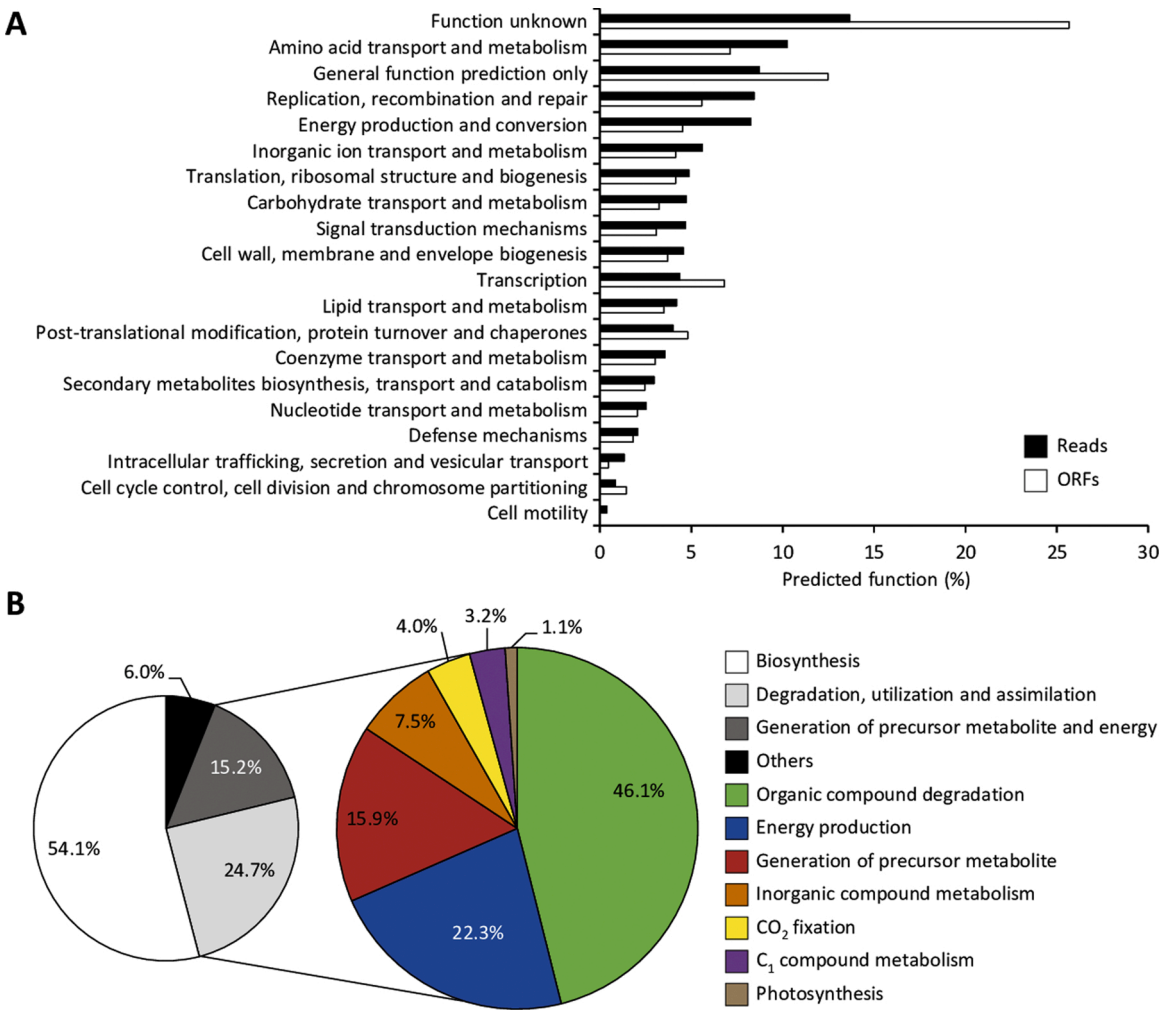

Fig. 3. Metabolic profiling of the Yumugi river cave microbial community. (A) Classification of reads and Open Reading Frames (ORFs) in the Clusters of Orthologous Groups (COG) functional categories. (B) Metabolic pathway prediction of reads through the MetaCyc hierarchical classification. The pie chart on the right provides details of the metabolic pathways clustered in the "Generation of precursor metabolite and energy" and "Degradation, utilization and assimilation" classes.

encodes a bacterial RNA polymerase-binding protein, which binds to the sigma subunit of RNA polymerases, and confers resistance to rifampicin (Newell et al., 2006), while the *tsnR* gene encodes a thiostrepton resistance factor which interacts with the ribosome to reduce the antimicrobial effect (Smith et al., 1995).

Two ARGs were found to be involved in antibiotic modification, namely the *aac(2')* and the *aph(6)*, both conferring resistance to the aminoglycosides class of antibiotics. The *aac(2')* gene encodes a N-acetyltransferases that transfers an acetyl group from acetyl-CoA to the aminoglycoside drug (Aínsa et al., 1997), whereas the *aph(6)* gene encodes an O-phosphotransferase which transfer a phosphate from ATP to the 3'' hydroxyl group on aminoglycosides (Ramirez and Tolmasky, 2010).

Finally, one ARG was closely related to the *sul2* gene, which encodes for an alternative dihydropteroate synthetase, resistant to inhibition by sulphonamides (Sköld, 2000).

3.3. Culture-based isolation and taxonomic characterization of Yumugi river cave bacteria

An aliquot of the Yumugi river cave biofilm was used for culture-based investigations. Two different growth media were used for this purpose: *i)* a low-nutrient medium (R2A) and *ii)* a higher-nutrient medium (50 % TSA). On these media, the total amount of culturable aerobic bacteria population was *ca.* 10^6 CFU/g of cave material. Based on morphological differences, 46 isolates were selected. Analysis of the 16S rRNA gene was performed in order to identify each of the 46 isolates. Despite the apparent morphological difference of colonies, 16S rRNA gene sequence analysis assigned the isolates to 14 clusters composed indistinguishable members. One representative isolate for each cluster was used for subsequent identification (Supplementary Table S6); twelve isolates were Gram-negative and two were Gram-positive (Table 2). Their occurrence in the cave sample was confirmed by cross-check with the metagenomic data of the V3 hypervariable region of the 16S rRNA gene (Fig. 2B). All 16S rRNA sequences from the 14 isolates matched with OTUs present in the targeted metagenomic dataset (100 % identity), except for isolate YUM25, which showed an identity value of 94.0 %. This discrepancy might be dependent on the under-representation (0.08 % relative abundance) of this particular sequence in the cave sample (Supplementary Table S1). Results of 16S rRNA sequencing indicated that cave isolates belonged to three phyla, namely *Proteobacteria*, *Actinobacteria* and *Firmicutes*, gathering five classes, seven orders and eight genera (Table 2). Interestingly, isolates YUM14, YUM21, YUM26 and YUM27 exhibited 16S rRNA gene sequence identity $\leq$ 98.7 % with their closest relative, suggestive of their belonging to putative novel species according to current taxonomic criteria (Stackebrandt and Ebers, 2006).

Concerning species classification, some important features arose for isolates presumptively assigned to the *Aminobacter* and *Ensifer* genera

Table 1
ARGs identified in the Yumugi river cave metagenome.

ARG	ORF - ID	Genus (order)[a]	MEGARes				PFAM	Predicted function	Resistance mechanism[b]
			Accession no.	E-value (Log)	Identity (%)	Coverage (%)	E-value (Log)		
aac (2')	484 - 4	*Streptomyces* (*Streptomycetales*)	U72714.1	−45.8	50.0	96.0	−11.4	N-acetyltransferases	DI
aph(6)	199 - 7	*Streptosporangium* (*Streptosporangiales*)	U70376.2	−114.4	69.1	97.5	−71.3	O-phosphotransferases	DI
arlR	271 - 7	*Saccharothrix* (*Pseudonocardiales*)	NC_002758	−34.7	40.4	79.5	−29.2	Response regulator	RR/DE
							−21.2	Transcriptional regulator	
drrA	24 - 7	*Kutzneria* (*Pseudonocardiales*)	AM709783.1	−46.1	45.1	62.5	−29.3	ABC transporter	DE
drrA	46 - 9	*Saccharothrix* (*Pseudonocardiales*)	AM709783.1	−42.1	43.1	65.3	−26.9	ABC transporter	DE
							−10.1	ATPase	
drrA	501 - 6	*Micromonospora* (*Micromonosporales*)	AM709783.1	−75.2	52.9	90.7	−25.3	ABC transporter	DE
drrA	52 - 12	*Pseudonocardia* (*Pseudonocardiales*)	AM709783.1	−43.3	46.0	60.3	−23.9	ABC transporter	DE
macB	369 - 6	*Allokutzneria* (*Pseudonocardiales*)	AY768532.1	−43.5	44.8	86.0	−31.0	ABC transporter	DE
macB	388 - 7	*Pseudonocardia* (*Pseudonocardiales*)	AY768532.1	−25.8	42.9	61.6	−12.4	FtsX-like permease	DE
macB	92 - 7	*Saccharopolyspora* (*Pseudonocardiales*)	AY768532.1	−55.7	46.6	96.1	−30.9	ABC transporter	DE
mtrA	135 - 3	*Actinosynnema* (*Pseudonocardiales*)	CP003248.2	−41.6	45.5	77.4	−30.6	Response regulator	RR/MP
							−24.0	Transcriptional regulator	
mtrA	314 - 2	*Saccharopolyspora* (*Pseudonocardiales*)	CP003248.2	−100.2	79.6	100.0	−29.8	Response regulator	RR/MP
							−25.3	Transcriptional regulator	
mtrA	369 - 3	*Allokutzneria* (*Pseudonocardiales*)	CP003248.2	−42.7	42.0	98.3	−27.7	Response regulator	RR/MP
							−24.5	Transcriptional regulator	
pmrA	372 - 2	*Kutzneria* (*Pseudonocardiales*)	JQ340367	−37.7	41.4	96.1	−23.1	Response regulator	RR/MP
							−23.5	Transcriptional regulator	
rbpA	257 - 7	*Actinoalloteichus* (*Pseudonocardiales*)	HQ203032.1	−39.5	69.1	96.5	−40.6	RNA-pol. binding protein	DTM
sulII	490 - 2	*Saccharopolyspora* (*Pseudonocardiales*)	FJ968160.1	−32.0	41.7	64.3	−82.9	Dihydropteroate synthases	DTB
tsnR	218 - 1	*Pseudonocardia* (*Pseudonocardiales*)	AL123456	−30.9	56.2	99.2	−10.4	rRNA methylase	DTM
tsnR	421 - 1	*Kutzneria* (*Pseudonocardiales*)	AL123456	−28.9	55.5	91.5	−21.5	rRNA methylase	DTM
vanS	488 - 1	*Kutzneria* (*Pseudonocardiales*)	NG_051735.1	−44.9	43.2	93.8	−17.1	ATPase	DS/DTM
							−12.1	Histidine kinase	

[a] Taxonomic assignment based on optimum BLASTn alignment of the whole contig with the best hit from the NCBI non-redundant database.
[b] DE, drug efflux; DI, drug inactivation; DS, drug sensor; DTB, drug-target bypass; DTM, drug-target modification; MP, membrane permeability; RR: response regulator.

(Table 2). Indeed, the 16S rRNA gene of YUM01 had high sequence similarity (99.9 % identity, 1 mismatch) with both *Aminobacter aganoensis* TH-3^T and *Aminobacter niigataensis* DSM 7050^T, whereas YUM09 was closely related (99.2 % identity, 10 mismatches) to both *A. aganoensis* and *A. niigataensis*, and also to *Carbophilus carboxidus* ATCC 51424^T (99.2 % identity, 10 mismatches). Concerning cave isolates assigned to the *Ensifer* genus, the 16S rRNA gene of YUM15 had high sequence similarity with *Ensifer adhaerens* NBRC 100388^T (99.0 % identity, 13 mismatches), whereas YUM16 was closely related to *E. adhaerens* (99.4 % identity, 8 mismatches) and *Ensifer sesbaniae* CCBAU 65729^T (99.4 % identity, 8 mismatches; Table 2).

To corroborate the taxonomic classification of Yumugi river cave isolates, a phylogenetic tree was constructed by aligning their 16S rRNA sequences to those of reference strains of representative species belonging to the corresponding genus (Supplementary Dataset S1 and Fig. S5). Given the availability of very few *Jeongeupia* validated species, sequences of related taxa were also incorporated in the phylogenetic analysis (Chen et al., 2013). Phylogenetic analysis confirmed the taxonomic allocation of all isolates, with few exceptions. Among these, YUM09 (putative *Aminobacter* spp.) fell into a distinct branch outside the *Aminobacter* genus (Urakami et al., 1992) (Fig. 4A). Likewise, phylogenetic analysis revealed that 16S rRNA gene sequences of YUM15 and YUM16 (putative *Ensifer* spp.) were located in a separate branch from *Ensifer adhaerens* (Fig. 4B). Of note, within genus *Pseudomonas*, YUM26 and YUM28 were located in a different branch relative to their closest neighbour *Pseudomonas kilonensis* and *Pseudomonas migulae*, respectively (Supplementary Fig. S5).

All cave culturable isolates were mesophiles, with few exceptions (Supplementary Table S6). Isolates belonging to genera *Aminobacter, Brevundimonas, Lysobacter, Jeungepia* grew only at 22 °C, whereas isolates belonging to the *Pseudomonas* and *Ensifer* genera grew between 4 °C and 22 °C. Isolate YUM12 (*Brevibacterium epidermidis*) grew at a broad temperature range (4−37 °C), whereas isolate YUM13 (*Brevundimonas diminuta*) was the only cave isolate able to grow from 4 to 42 °C.

Table 2
Culturable bacteria isolated from the Yumugi river cave sample.

Cave isolate	Identical isolates (no.)[a]	Length (nt)	Closest relative(s)	Accession no.	Identity (%)	Mismatches (no.)	Phylum (class, order)
YUM01	8	1335	*Aminobacter aganoensis* TH-3^T	NR028876	99.9	1	*Proteobacteria* (α-*proteobacteria*, *Rhizobiales*)
			Aminobacter niigataensis DSM 7050^T	NR025302	99.9	1	
YUM09	3	1330	*Aminobacter aganoensis* TH-3^T	NR028876	99.2	10	*Proteobacteria* (α-*proteobacteria*, *Rhizobiales*)
			Aminobacter niigataensis DSM 7050^T	NR025302	99.2	10	
			Carbophilus carboxidus ATCC 51424^T	NR104931	99.2	10	
YUM12	1	1377	*Brevibacterium epidermidis* ATCC 35514^T	NR029262	99.1	12	*Actinobacteria* (*Actinobacteria*, *Micrococcales*)
YUM13	1	1292	*Brevundimonas diminuta* NBRC 12697^T	NR113602	99.6	5	*Proteobacteria* (α-*proteobacteria*, *Caulobacterales*)
YUM14	1	1303	*Brevundimonas viscosa* F3^T	NR117900	98.7	16	*Proteobacteria* (α-*proteobacteria*, *Caulobacterales*)
YUM15	4	1347	*Ensifer adhaerens* NBRC 100388^T	NR113893	99.0	13	*Proteobacteria* (α-*proteobacteria*, *Rhizobiales*)
YUM16	1	1348	*Ensifer adhaerens* NBRC 100388^T	NR113893	99.4	8	*Proteobacteria* (α-*proteobacteria*, *Rhizobiales*)
			Ensifer sesbaniae strain CCBAU 65729^T	NR133053	99.4	8	
YUM20	1	1374	*Jeongeupia naejangsanensis* BIO-TAS4-2^T	NR116772	99.4	8	*Proteobacteria* (β-*proteobacteria*, *Neisseriales*)
YUM21	4	1402	*Lysobacter niastensis* GH41-7^T	NR043868	98.5	20	*Proteobacteria* (γ-*proteobacteria*, *Xantomonadales*)
YUM25	1	1425	*Macrococcus equipercicus* H8h3^T	NR044926	99.6	5	*Firmicutes* (*Bacilli*, *Bacillales*)
YUM26	1	1440	*Pseudomonas kilonensis* DSM 13647^T	NR028929	98.1	26	*Proteobacteria* (γ-*proteobacteria*, *Pseudomonadales*)
YUM27	1	1392	*Pseudomonas glarae* KMM 9500^T	NR145562	97.6	33	*Proteobacteria* (γ-*proteobacteria*, *Pseudomonadales*)
YUM28	18	1398	*Pseudomonas migulae* NBRC 103157^T	NR114223	99.4	8	*Proteobacteria* (γ-*proteobacteria*, *Pseudomonadales*)
YUM46	1	1395	*Pseudomonas resinovorans* ATCC 14235^T	NR112062	99.2	11	*Proteobacteria* (γ-*proteobacteria*, *Pseudomonadales*)

[a] Number of isolates showing identical 16S rRNA gene sequence.

3.4. Phylogenetic characterization of *Pseudomonas* isolates from Yumugi river cave

From a taxonomical perspective, the genus *Pseudomonas* is one of the most complex bacterial genera and is currently the genus of Gram-negative bacteria with the 345 species (Peix et al., 2018; https://www.bacterio.net/). Since 16S rRNA gene sequences do not provide accurate discrimination between *Pseudomonas* species, MLSA has become the preferred method for establishing the phylogenetic relationships within this genus (Mulet et al., 2012; Lalucat et al., 2020). To refine the taxonomy of *Pseudomonas* isolates from Yumugi river cave, the ordered concatemers of the 16S rRNA, *gyrB*, *rpoD* and *rpoB* partial genes sequences (*ca.* 3,700 nt) of 140 representative species of genus *Pseudomonas* were retrieved from the dataset of Mulet et al. (2012), and compared with DNA concatemers assembled from the complete genome sequences of four *Pseudomonas* isolates from Yumugi river cave (Supplementary Table S2). The four-gene-based MLSA assigned YUM28 and YUM46 to the *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* groups, respectively (Supplementary Fig. S6). Within the *P. fluorescens* group, YUM28 was closely related the *Pseudomonas mandelii* subgroup, whereas YUM46 was related to the *Pseudomonas resinovorans* group (Supplementary Fig. S6), which has recently been separated from the *P. aeruginosa* group (Lalucat et al., 2020). YUM26 and YUM27 did not cluster within a definite *Pseudomonas* group (Supplementary Fig. S6), being intermingled between the *Pseudomonas anguilliseptica* and *Pseudomonas putida* groups.

To improve the phylogenetic resolution, MLSA was extended to the full length DNA sequences of the 16S rRNA, *gyrB*, *rpoD*, *rpoB* and *recA* genes for members of the most closely related *Pseudomonas* groups (Supplementary Fig. S6). The recombinase alpha subunit *recA* gene sequence was included to improve the MLSA discriminatory power. DNA concatemers (10,558 nt) were generated and analysed for a total of 42 species and 56 strains belonging to the *P. anguillaseptica*, *P. putida* and *P. resinovorans* groups, and to the *P. mandelii* subgroup within the *P. fluorescens* group (Supplementary Table S3), in addition to DNA concatemers of the Yumugi river cave isolates. Extended MLSA allowed to generate a phylogenetic tree (Fig. 5A) showing similar topology and increased resolution compared with the 4-gene-based MLSA tree (Supplementary Fig. S6). Moreover, by combining MLSA with ANI results (Fig. 5A,B; Supplementary Table S4), YUM28 could tentatively be identified as *P. frederiksbergensis*, since closely related to the *P. frederiksbergensis* ERDD5:01 reference strain (Fig. 5A), and showing an ANI value within the intraspecies variation range (86.7 %; range 85.7 %–88.5 %; Fig. 5B; Supplementary Table S4). Although clustering in the *P. resinovorans* group, YUM46 showed ANI values (87.2 %–87.4 %) lower than the intraspecies variation value determined for *P. resinovorans* (88.3 %; Fig. 5B; Supplementary Table S4), thus precluding species-level identification. Likewise, both MLSA and ANI results did not allow to assign YUM26 and YUM27 to any of the validly published *Pseudomonas* species (Fig. 5).

3.5. Antimicrobial resistance of Yumugi river cave isolates

All isolates were tested for their resistance to 18 antimicrobial agents belonging to different classes, grouped according to their target (Fig. 6). To screen for robust resistance, each drug was tested at the single concentration of 20 μg/mL (Bhullar et al., 2012), and resistance was indicated by evident bacterial growth on antimicrobial-supplemented plates

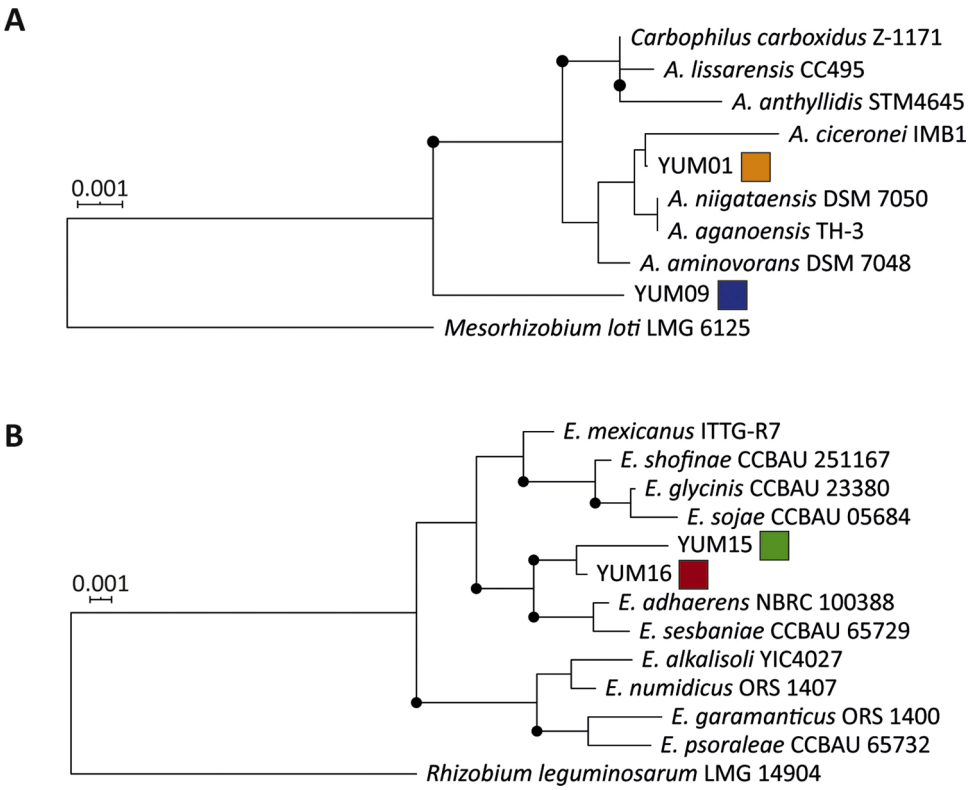

Fig. 4. Rooted neighbor-joining trees of the 16S rRNA sequences of Yumugi river cave isolates and the reference sequences of type strains of genus *Aminobacter* (A) and *Ensifer* (B). Bootstrap values ≥70 % (1,000 replications) are illustrated by filled circles at the end of branches. The scale bar indicates the sequence divergence. *Mesorhizobium loti* and *Rhizobium leguminosarum* sequences were used as outgroup of *Aminobacter* and *Ensifer*, respectively.

after 4 days of incubation at 22 °C.

All isolates were resistant to three or more different antimicrobial classes, excluding isolate YUM25, which was only resistant to ATM and NAL (Fig. 6). Most of the Gram-negative isolates showed high resistance levels (up to 10 antimicrobials for YUM13 and YUM15), whereas the Gram-positive bacteria YUM12 and YUM25 were resistant to five and two antimicrobials, respectively (Fig. 6). Overall, 55.4 % and 26.2 % of isolates were resistant drugs targeting cell wall synthesis and to inhibitors of bacterial protein synthesis, respectively. Few isolates (8.9 %) were resistant to drugs affecting RNA or DNA synthesis (Fig. 6).

3.6. Antimicrobial activity screening

Caves are unique environments where it is possible to discover potentially new bioactive compounds. Although the majority of antimicrobial metabolites derive from Gram-positive *Actinobacteria*, Gram-negative bacteria have become increasingly recognised as rich and underexplored sources of novel antimicrobial compounds endowed with therapeutic potential (Berdy, 2005; Masschelein et al., 2017). Culturable isolates from the Yumugi river cave were therefore screened for their potential antimicrobial activity by the cross streak method (Maciejewska et al., 2016), using *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 29213 as indicator strains. Among all cave culturable isolates, YUM26, YUM28, and YUM46 showed antibacterial activity against *B. subtilis*, while only YUM28 also inhibited the growth of *S. aureus* (Fig. 7). Of note, all these isolates belonged to genus *Pseudomonas* (Table 2). No antimicrobial activity was observed against *P. aeruginosa* or *E. coli* indicator strains (data not shown).

4. Discussion

This work is intended to characterize a biofilm lining the walls of a pristine cave located in a remote area of the highland mountains of Western New Guinea (Indonesia). Due to the inherent difficulties to operate in this impervious site, a single biofilm sample from the wall of the cave was collected during the cave exploration in late 2017 (Benassi and Pasquini, 2018). On visual inspection, the sampled site was coated with a smooth biofilm overlaid by water droplets reflecting gold- or silver-coloured light when illuminated. Similar biofilm structures covered large areas of the cave walls and ceiling, and probably correspond to water condensation zones, which are known to form near the entrance of limestone caves (Pašić et al., 2009; Porca et al., 2012). While analysis of a single sampling site and the inability to determine the physical/chemical characteristics of the biofilm due to the small sample size are inherent limitations, this work represents the first investigation of the microbial community in a pristine limestone cave of Western New Guinea. Therefore, the Yumugi river cave microbiome could be considered as a case study to gain novel insights into the structure, composition and metabolic properties of a microbial community thriving a pristine environment with no anthropic impact.

Shotgun metagenome sequencing showed the massive prevalence of *Bacteria*, though the relative scarcity of *Archaea*, *Eukarya* and viruses could be due to the use of a DNA extraction procedure that is not specific for these (micro)organisms. Consistent with previous studies (Engel, 2010; Tomczyk-Żak and Zielenkiewicz, 2016), 16S rRNA gene sequence analysis highlighted *Proteobacteria* and *Actinobacteria* as the predominant phyla, both comprising cosmopolitan bacteria endowed with high diversity in terms of number of classes and orders. Taxonomic assignment of shotgun metagenomic reads confirmed the results of 16S rRNA gene analysis at both phylum and class levels, while discrepancies were observed at the order level, probably due to the differences in databases, algorithms and methodological approaches used for 16S rRNA and metataxonomic profiling.

Taxonomic analysis and metabolic predictions inferred from shotgun metagenomic data highlighted the prevalence of chemoorganotrophic

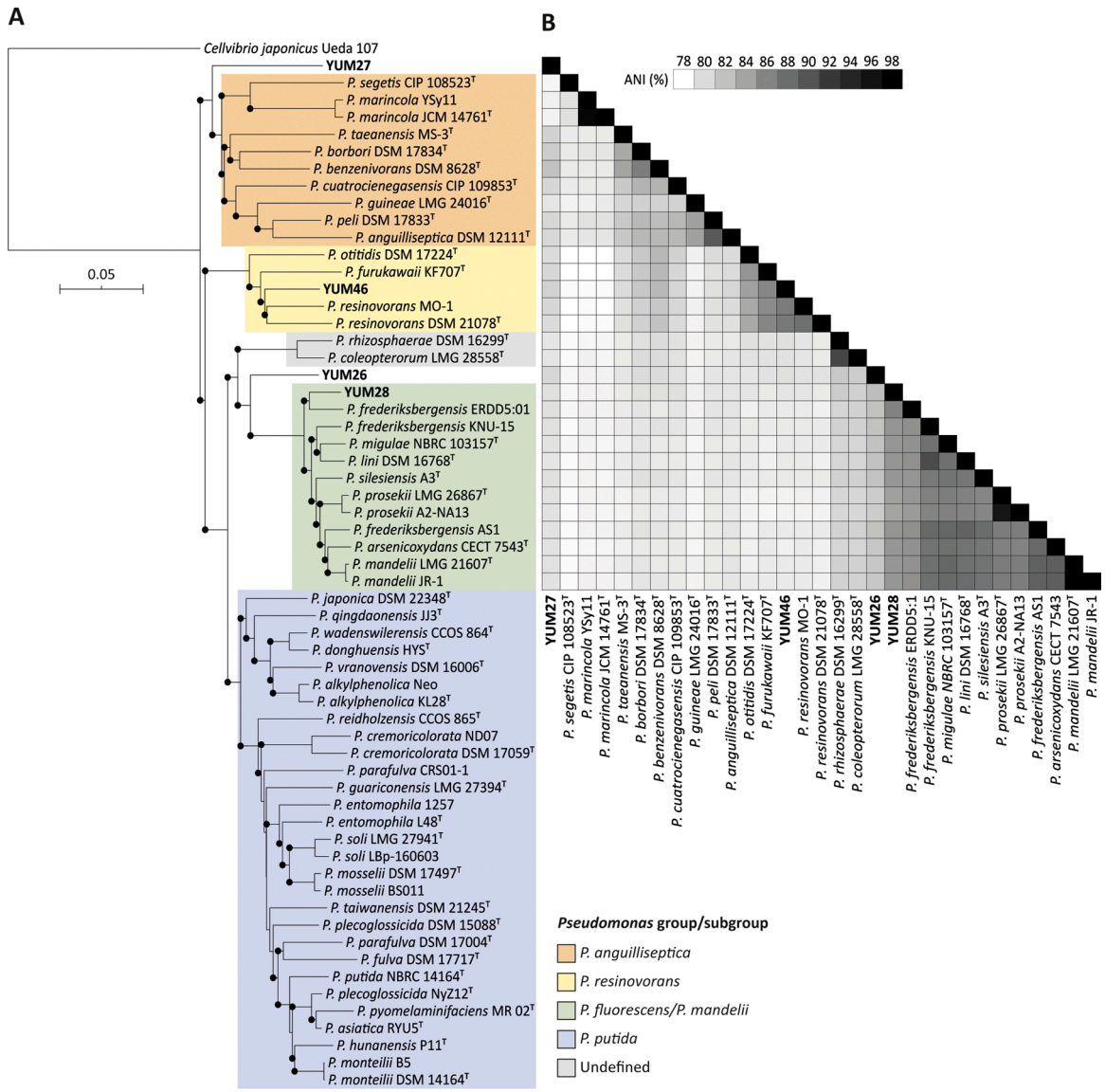

Fig. 5. Phylogenetic analysis of neighbour members of *Pseudomonas* groups associated with *Pseudomonas* spp. Yumugi river cave isolates. A) Rooted neighbor-joining trees based on the concatemers of five genes (16S rRNA, *gyrB*, *rpoD*, *rpoB* and *recA*). Bootstrap values ≥70 % (1,000 replications) are illustrated by filled circles at the end of branches. The scale bar indicates the sequence divergence. Groups and subgroups are defined according to Mulet et al. (2012) and Lalucat et al. (2020). *Cellvibrio japonicus* was used as outgroup. B) Heatmap of pairwise average nucleotide identity (ANI) values of Yumugi river cave isolates and their closest members of *Pseudomonas* groups. Yumugi river cave isolates are highlighted in bold. Strains are ordered in the ANI matrix (B) to match the order defined by the neighbor-joining phylogeny (A).

phyla, whereas chemolithoautotrophic and phototrophic microorganisms were poorly represented in the cave microbiome. The scarcity of primary producers suggests that the microbial community is not self-sustained, and should therefore be fed by organic matter coming from outside the cave, as reported from previous studies on limestone cave ecosystems (Chelius and Moore, 2004; Simon et al., 2007). The Yumugi river passing through the cave, water condensation and percolation through fractures in the limestone rock and movement of cave animals could represent major ways of organic matter delivery to the biofilm lining the cave walls (Barton and Jurado, 2007; Simon et al., 2007). This setting greatly differs from what observed in semiarid and arid cave environments, where organic carbon inputs are minimal and microbial communities are characterised by the abundance of chemolithoautotrophic *Bacteria* and *Archaea*, which sustain the growth of chemoorganotrophs by thriving on mineral nutrients present in the rocks (Ortiz et al., 2014).

A previous study on water droplets biofilm-like microbial communities obtained from limestone caves located in Spain, Czech Republic, and Slovenia (Porca et al., 2012), revealed a core microbial community formed by *Pseudonocardiales*, *Chromatiales* and *Xanthomonadales*, with a minority of *Nitrospirae*. Our results are in line with these findings, with the exception of *Chromatiales* that were not detected in our sample. Notably, *Pseudonocardiales* and *Nitrospirae* have recently been proposed as indicator taxa of limestone cave bacterial communities and pristine ecological conditions, respectively (Alonso et al., 2019; Zhu et al., 2019). Interestingly, we found a prevalence of *Pseudonocardiales* that have the potential to degrade not only carbohydrates and amino-acids, but also aromatic compounds, that are common constituents of soils and intermediates of plant litter conversion to humic acids (de Menezes et al., 2012). In caves, aromatic compounds are transported by drip water and provide energy and carbon sources to sustain the indigenous bacterial communities (Marques et al., 2019).

Members of *Cyanobacteria* and *Chloroflexi* were also identified, although with low frequency. The presence of phototrophs has already

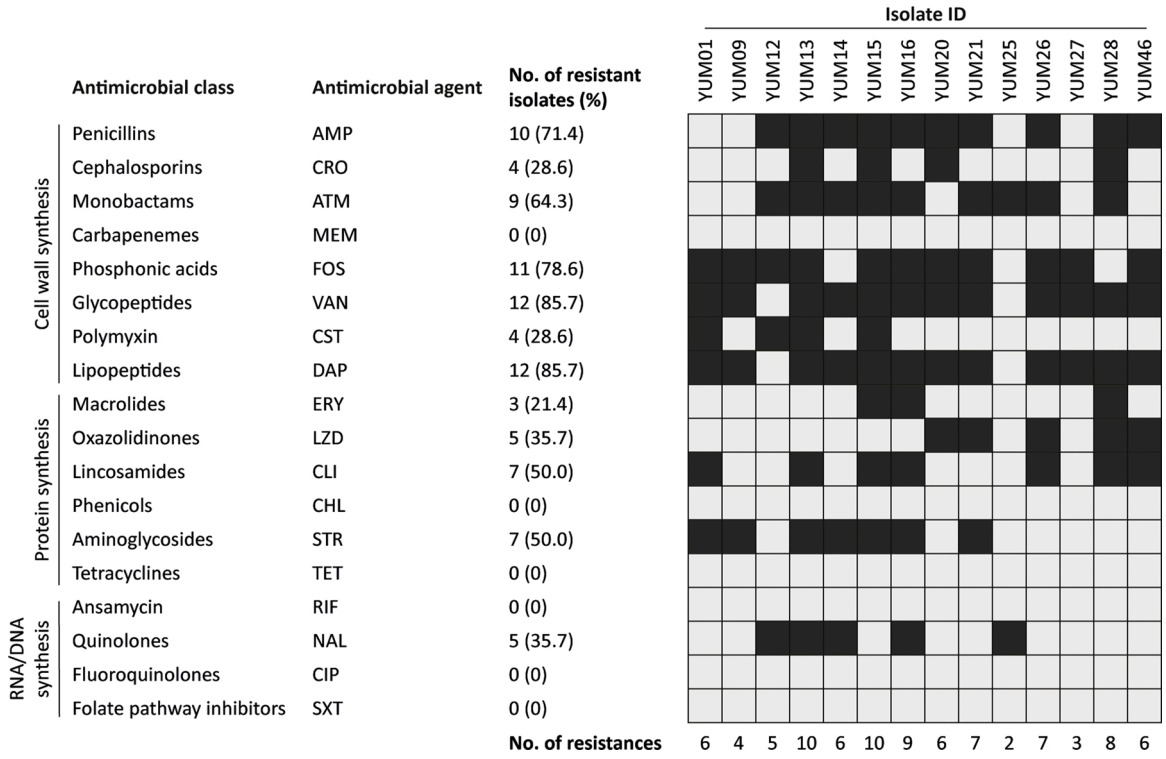

Fig. 6. Antimicrobial resistance profile of isolates from the Yumugi river cave. Black squares denote resistance. AMP, ampicillin; ATM, aztreonam; CHL, chloramphenicol; CIP, ciprofloxacin; CLI, clindamycin; CRO, cefriaxone; CST, colistin; DAP, daptomycin; ERY, erythromycin; FOS, fosfomycin; LZD, linezolid; MEM, meropenem; NAL, nalidixic acid; STR, streptomycin, SXT, sulfamethoxazole-trimethoprim; RIF, rifampicin; TET, tetracycline; VAN, vancomycin.

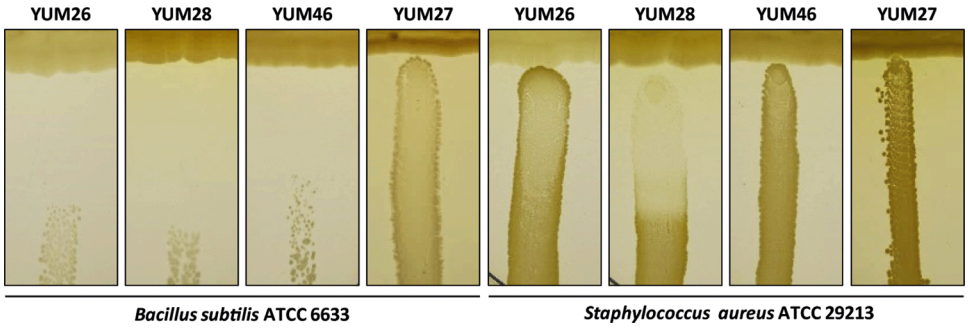

Fig. 7. Production of antibacterial compounds by isolates from the Yumugi river cave. Producer strains (YUM26, YUM28, YUM46) were streaked along the diameter of a Petri dish and incubated for 5 days at 22 °C. Then, plates were cross streaked with *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 29213 indicator strains, and further incubated at 37 °C for 24 h. Strain YUM27 was chosen as non-producer. Pictures are representative of five independent experiments, all providing similar results.

been documented in the soil of a subterranean limestone cave in the western Thailand (Wiseschart et al., 2019), suggesting that these bacteria might gain access to the cave and survive in a dark environment. Interestingly, phototrophic *Cyanobacteria* inhabiting cave entrances synthetize *f*- and *d*-type chlorophylls to harvest near-infrared radiation and sustain oxygenic photosynthesis by using light reflected from the limestone rock fundament of the caves (Behrendt et al., 2020).

To gain more insights into the bacterial community of Yumugi river cave, we have exploited a culture-based approach that allowed the identification of 14 non-duplicate bacterial isolates. While metagenomic analysis revealed comparable representativeness of *Proteobacteria* and *Actinobacteria* (49.6 % and 40.4 %, respectively), analysis of the culturable component revealed that 12 out of 14 isolates belonged to the *Proteobacteria* phylum, while the remaining two isolates belonged to the *Actinobacteria* and *Firmicutes*, respectively. This unbalance might be due to the choice of enrichment media that favoured fast-growing microorganisms such as *Proteobacteria*, whereas *Actinobacteria* require sample pre-treatments (*e.g.*, desiccation) and specific isolation media. Moreover, cave bacteria need a cooperative strategy to grow and survive; hence, some isolates might have been lost with serial passages required for the isolation of pure cultures.

However, culture-dependent analyses were consistent with previous reports showing that *Proteobacteria* inhabit diverse ecological niches in moist environments (Spain et al., 2009) and represent the most prevalent group in cave ecosystems (Tomczyk-Żak and Zielenkiewicz, 2016; De Mandal et al., 2017; Oliveira et al., 2017). Success of cave *Proteobacteria* colonization may be attributed, at least in part, to their ability to degrade a wide range of organic compounds for energy production (Tomczyk-Żak and Zielenkiewicz, 2016). Among culturable isolates, YUM12 and YUM20 were identified as *Brevibacterium epidermidis* and *Jeungeupia naejangsanensis*, respectively, which are both able to degrade cellulose (Yoon et al., 2010; Habe et al., 2015). Similarly, culturable members of genera *Aminobacter* (YUM01 and YUM09) and *Ensifer* (YUM15 and YUM16) are known to be involved in the degradation of potentially toxic molecules (Zhou et al., 2013; Horemans et al., 2017). Interestingly, isolates YUM15, YUM16 and YUM21 are closely related to bacterial predators *Ensifer adhaerens* and *Lysobacter niastensis*, known for producing bioactive compounds, including lytic enzymes effective against prokaryotic and eukaryotic microorganisms (Casida, 1982; Xie et al., 2012).

Although the collection of culturable bacteria from Yumugi river cave is limited to 14 isolates, four of them (YUM14, YUM21, YUM26, YUM27) showed ≤ 98.7 % identity with their closest neighbour (*i.e., Brevundimonas viscosa, L. niastensis, P. kilonensis, Pseudomonas glarae*), suggesting that they could represent new species according to (Stackebrandt and Ebers, 2006). Moreover, three isolates (YUM09, YUM15 and YUM16) branched apart from their closest neighbour in phylogenetic trees, hampering species assignment.

Since 16S rRNA gene sequences do not provide accurate discrimination between *Pseudomonas* species (Mulet et al., 2012; Lalucat et al., 2020), genomes of culturable isolates assigned this genus (YUM26, YUM27, YUM28 and YUM46) were *de novo* sequenced in order to perform MLSA and pairwise genome identity (*i.e.*, ANI) with a collection of reference *Pseudomonas* species (Mulet et al., 2012). Despite the refined phylogenetic analyses, none of the isolates could unequivocally be assigned to any of the validly published *Pseudomonas* species. This reflects the high endemism present in the Yumugi river cave microbial community, presumably resulting from biogeographic isolation of Western New Guinea and physical segregation of the sampling site.

Due to geological isolation, the Yumugi river cave is located in an unpolluted area of Western New Guinea, thus representing an ideal setting to study microbial communities that are not subjected to the selective pressure for antimicrobial resistance due to anthropogenic contamination. The *in silico* resistome analysis revealed the presence of ARGs encoding for several mechanisms of antimicrobial resistance, including drug efflux, drug inactivation, drug-target modification and drug-target bypass. Of note, all ARGs were assigned to the *Actinobacteria*, a predominant phylum of the cave community. This reinforces the notion that members of the *Actinobacteria* phylum not only are major antibiotic producers, but also constitute a reservoir of ARGs that could eventually be transferred to pathogenic species (Forsberg et al., 2012; Pehrsson et al., 2013; Jiang et al., 2017).

About one-fourth of ARGs detected in the biofilm metagenome, namely *aac(2')*, *aph(6)*, *rbpA* and *tsnR*, belong to the drug inactivation and drug target modification classes, which represent highly specific evolutionary adaptations to evade the cytotoxic action of antibiotics. However, the majority of ARGs identified in our study encoded for drug-efflux transporters, which are known to mediate the export of toxic compounds across the cell wall, and are regarded as the most common and ancient resistance mechanism to toxic compounds (Martinez et al., 2009; Van Goethem et al., 2018). This is likely to reflect the competitive interactions among species of the bacterial community, though their detection in *Actinobacteria* raises the possibility that ARGs are also involved in self-protection from endogenously produced antibiotics (Davies, 2006).

Although originating from an environment unpolluted by anthropic activity, Yumugi river cave bacterial isolates showed phenotypic resistance to a large number of antimicrobials, given that almost all of them were unsusceptible to three or more antimicrobial classes. The majority of resistances were observed for antimicrobials targeting cell wall and cytoplasmic membrane, especially among isolates belonging to the *Proteobacteria* phylum. These microorganisms are coated with an outer membrane that not only shells the cell from dangerous molecules, but could be also endowed with efflux pumps that can effectively reduce the intracellular concentration of several drugs (Nikaido and Pagès, 2012).

For three culturable isolates an evident antimicrobial activity against Gram-positive test species (*i.e.*, *B. subtilis* and/or *S. aureus*) was documented. All these isolates were assigned to genus *Pseudomonas*, which is known to produce bioactive metabolites (Gross and Loper, 2009), including molecules that play important roles in the biological control of phytopathogenic bacteria and fungi, as well as in bioremediation (Berdy, 2005; Isnansetyo and Kamei, 2009; Desriac et al., 2013). Further research is needed to characterize the chemical structure and mechanism of action of the bioactive compounds produced by *Pseudomonas* spp. isolates from Yumugi river cave.

5. Conclusion

The metagenome-based investigation of a microbial biofilm sample from the Yumugi river cave revealed a remarkable bacterial diversity, with prevalence of chemoorganotrophic phyla including *Proteobacteria, Actinobacteria, Firmicutes* and *Acidobacteria*. Culture-based studies highlighted the presence of numerous unassignable taxonomic entities among the community members. ARGs prediction and results of *in vitro* susceptibility tests showed that resistance to several antimicrobial classes was frequent, albeit the antibiotic selective pressure due to anthropogenic activities was predictably negligible. However, some taxa composing the community are renowned antibiotic producers, and antimicrobial activity screenings confirmed the presence of antibiotic producers among the Yumugi river cave isolates. These findings support the hypothesis that caves represent a reservoir for the discovery of new bacterial species and bioactive metabolites.

Author statements

EF, PT and PV designed the study, PT participated to the speleological expedition and sampled the site, DV, PT and MT performed laboratory experiments, GAL, MT and MV performed DNA sequencing, GAL, MP, MT, and PT performed the bioinformatic analyses, EF, MV and PV supervised the study, and all Authors contributed to data analysis and drafted the manuscript.

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Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2020.126584.

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