Environmental Earth Sciences (2021) 80:665
https://doi.org/10.1007/s12665-021-09969-w

ORIGINAL ARTICLE

The role of bacterial community in the formation of a stalactite in coral limestone areas of Taiwan by 16S rRNA gene amplicon surveys

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Received: 15 September 2020 / Accepted: 1 August 2021 / Published online: 25 September 2021

Abstract

This study investigated the effect of environmental physical factors on the relative abundance of bacteria and the consequential landscape evolution in karst topography, focusing mainly on the effects of ureolytic microbial $CaCO_3$ precipitation. The narrow-sky located in the upper part of Tangshan is a small gulch of Pleistocene coralline limestone formation in southern Taiwan. A 16S-metagenomic approach was used to determine the relationship of microbial community structures on the landscapes in various habitats. Our results showed that the biomass of habitats in the opening of the gulch was two times higher than the inside where light penetration was lower. We also found that speleothems only occurred at the inner wall inside the gulch, where the environment exhibited water drips running through the surface of speleothems and less light penetration. The sequence reads of operational taxonomic units relative to urease-producing bacteria and weathering-associated bacteria from each habitat were determined by NCBI database. Our data revealed that the 16S-metagenomics of the inner wall and water samples exhibited more sequences that were similar to those of urease-producing bacteria, whereas the outer wall showed more sequences that were similar to those of weathering-associated bacteria, suggesting that bacteria facilitated the formation of limestone weathering and calcite precipitation for various habitats. The semi-quantitative PCR for determining bacterial urease gene (*ure*C) levels confirmed that the inner limestone habitat had higher *ure*C gene levels than the outer limestone habitat. This study revealed the pivotal role of microorganisms in governing the geological evolution in the lightless limestone landscape.

Keywords Limestone · 16S-metagenomic approach · Urease-producing bacteria · *ure*C gene

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Introduction

Weathering and calcite precipitation are two opposite activities that affect the dynamic changes of the karst landscape. Although weathering and calcite precipitation can occur in abiotic conditions, several lines of evidence from cave studies or laboratory data have shown that microorganisms can accelerate the reactions that promote the formation of calcium carbonate and the breakdown of calcite in situ (Castanier et al. 1999; Gat et al. 2014; Jones 2017; Lian et al. 2008; Sulu-Gambari 2011). During the breakdown of carbonate rocks, microbial colonies build up on rock surfaces, resulting in rock decomposition by acidification and moisturization onto the surfaces (Hutchens 2009; Uroz et al. 2009; Wu et al. 2017). The obtainment of nutrients from the rock surface further promotes the release of organic ligands, which in turn facilitate the release of mineral elements, thus creating a positive feedback loop (Lian et al. 2008; Uroz et al. 2007). Many studies have documented that the mineral dissolution of rocks in a flow-through system was higher in the presence of surface-attached microorganisms (Ahmed and Holmström 2015; Jacobson and Wu 2009; Seiffert et al. 2014). Many bacterial strains have been reported to have the ability to adhere to rock surfaces and establish the weatherability (Sulu-Gambari 2011). For example, *Shewanella oneidensis* can recognize silicate and oxide mineral surfaces and cause further weathering associated reactions (Lower et al. 2001). To date, many studies have categorized the bacteria of weatherability (Lian et al. 2008; Sulu-Gambari 2011; Uroz et al. 2009).

Many bacteria can induce the biomineralization processes of calcium carbonate precipitation that render the formation of stalactite. The microbial-induced reaction is mainly carried out by urease-producing bacteria in the presence of ammonium ions in the alkaline environment. The identified urease-producing bacteria have been investigated extensively (Abo-El-Enein et al. 2012; Anbu et al. 2016; Animesh and Ramkrishnan 2016; Ercole et al. 2001; Jones 2017; Wei et al. 2015). The microbial communities of karst habitats are diverse, and their components largely depend on the locations and composition of limestone (Barton and Northup 2007; Barton et al. 2010; Ortiz et al. 2014; Tomczyk-Żak and Zielenkiewicz 2015). Temperature, light intensity, and light penetration are important parameters that control the developing orientation of microbial communities. Researchers have shown that microorganisms, operating together with the local environmental conditions, play important roles in remodeling the landscapes of karst (Anbu et al. 2016; Castanier et al. 1999; Mortensen et al. 2011; Qabany et al. 2012). However, how physical factors affect microbial communities and the consequent geological changes remains unknown. Determining how microbial relative abundance shifted in response to changes in environmental factors and the consequent geological evolution can enable us to better understand the effect of microorganisms on the dynamic alterations of karst landscapes.

Because of its porous and loose property, limestone can be easily infiltrated by rainfall or groundwater to form trenches, shallow concavity, or clefts. Limestone landscapes in Taiwan are scattered all over the island and can be found in the Hengchun Peninsula, east coastal areas, central range, and southwest of Taiwan. The tectonic studies of Tangshan revealed that the upthrow consists of large lenses of Pleistocene anticline, 4 km in length and 2 km in width (Lacombe et al. 1997; Hsieh and Knuepfer 2001). The crest of Tangshan is covered with coral reef limestones (with an average thickness of 40 m), which are interbedded in clastic layers. On top of the hills, expanding vegetation coverages, coupled with erosion soil, are commonly observed in most of these limestone landscapes. The Narrow-Sky is a nickname for a mountain crack located at a limestone hill in the Tainliao district of Taiwan. The dimensions (length, width, and height) of the gulch are approximately 100 m, 2 m, and 12 m, respectively. Because of the vegetation coverage and its topographic features, the exposure of sunlight at different spots inside the gulch is different. For example, sunlight can penetrate the limestone wall of the opening through the vegetation coverage, while it is relatively dim inside the path of the gulch. Moreover, moisture and temperature are also different between the opening and the center of the gulch. The most tangible difference between the inner and outer wall of the gulch is the formation of speleothems, which are plentiful in the inner gulch and are nonexistent in the outer section. Because microbial communities are sensitive to changes in environmental physical factors, the microbial composition in different locations may have adapted to the environment according to physical factors, which may play a role in reshaping the gulch scenery.

In this study, we investigated the effect of physical factors on microbial communities in the limestone landscape. With the recent advent of next-generation sequencing (NGS) platform and computational methods, we could conduct genome studies on microbes to determine the relationship between environmental factors in their habitats, such as sunlight penetration during daytime, humidity, and pH and the relative abundance of microbes. We collected samples from limestone walls at the opening and inside the gulch, from water dripping at the inner limestone wall, and from the soil of the outer weathered limestone at the gulch opening: we collected these samples to extract DNA. Genomic DNA extracted from these samples was further subjected to the PCR amplification of 16S rRNA gene sequences using the Illumina's MiSeq system. Bioinformatics tools were employed to explore DNA reads in operational taxonomic units (OTUs). The DNA sequence in each OTU was blasted

with the sequences which is current weathering bacteria and urease-producing bacteria available in the National Center for Biotechnology Information (NCBI) database.

Materials and methods

Sample site description and the collection of samples and physical parameters

We collected samples from the limestone gulch of Tainliao (120° 21′ 19.1″ E, 22° 51′ 00.7″ N): the location is illustrated in Fig. 1. For collecting the microorganisms in the surface of limestone walls, sterile cotton swabs were used to wipe the surface areas of sampled spots. The samples were collected in a tube and sent to the laboratory to measure total organic carbon and extract DNA for subsequent 16S-metagenomics studies. The physical factors in sampling spots including illumination, temperature of the air or soil, humidity, and pH of soil were recorded.

DNA extraction and PCR for 16S-metagenomics analysis

The procedure modified from kit of Genomic DNA from soil (Macherey–Nagel) was used to extract bacterial DNA from limestone samples. The detailed procedure was described in detail in our previous study (Huang et al. 2018). In short, DNA in a bulk of soil fraction was isolated and eluted for the PCR amplification of 16S rRNA gene sequences at V3–V4 regions using Illumina's MiSeq system to create paired-end sequencing data. After NGS data analysis, the conserved DNA were performance bacterial urease gene quantitative assay. The target sequence was amplified through PCR using mixed forward and reverse primers. After separation through electrophoresis in agarose gel, PCR products with expected sizes were harvested.

16S-Metagenomics library construction and analysis

The Illumina Nextera XT index kit was used in the second-stage PCR for the addition of the index. The raw data of forward and reverse reads were aligned using CLC bio's analysis platform (Genomic Workbench v.8.5) with Q20 as a threshold to generate output fasta files. Fasta files were further processed using the sequence analysis tool USEARCH. All sequence fills were merged together, followed by removing duplicates and clustering sequences into OTUs at 97% pairwise identity with the minimum cluster size being set at 2 to construct an OTU-reference library. A comparison between samples and the reference library at a level of 97% sequence identity was made to yield an OTU table, and the number of reads in each OTU was revealed. A 16S UTAX reference database was employed for the assignment of taxonomy for query sequences in the OTU-reference library. We analyzed each habitat by aligning the data, relative abundance, and biodiversity with a heatmap

Fig. 1 The location of the limestone gulch. **A** Shows a series maps of increased scale pointing to the limestone gulch. **B** Shows the view of the gulch. Both sides of the path opening have stairs leading to the center of the gulch. We took samples from outer limestone wall of the gulch (OL, **b1**), the soil on the outer ground (OS, **b2**), the inner limestone wall of the gulch (IL, **c3**), and water dripping from the wall (WA). The inner and outer limestone walls exhibit distinct landscapes in the stalactite formation

and principal coordinate analysis (PCoA) by R software and Microsoft Excel software.

Functional bacteria analysis

To investigate urease-producing bacteria and weathering-associated bacteria in each habitat, a bioinformatics approach was used to find functional bacteria based on the similarity of DNA sequences. In this method, tables of urease-producing bacteria and weathering bacteria—including bacteria for surface recognition, surface attachment, and mineral dissolutions—were selected from previously published papers in which their corresponding 16S DNA sequences were downloaded from the NCBI database, as shown in Supplementary Tables 1 and 2. The DNA sequence tables were used as references to construct phylogenetic trees by employing the Molecular Evolutionary Genetics Analysis 7 (MEGA 7) program with the setting of parsimony, neighbor-joining, and maximum likelihood analyses. The similarity between adjacent pairs of OTU sequences and reference sequences was tested using the NCBI nucleotide BLAST program. DNA sequences with a similarity of more than 95% were defined as urease-producing bacterial lineages or weathering-associated bacterial lineages of corresponding bacteria, and their read numbers were manually selected to calculate their populations.

Semi-quantitative PCR

The *ure*C gene expression levels in all the samples from the limestone region were estimated using semi-quantitative PCR in triplicate. DNA aliquots diluted 1000× were mixed with 1.0 µL of the *ure*C gene primer set (forward primer: 5′-TGG GCC TTA AAA THC AYG ARG AYT GGG-3′; reverse primer: 5′-GGT GGT GGC ACA CCA TNA NCA TRT C-3, 0.4 µM), 5.0 µL of Fast-Run™ Taq Master Mix with Dye (Protech, Taiwan) and 16 µL of double distilled H_2O to make the final volume to 25 µL. The thermal cycling conditions employed for the PCR have been described previously (Reed 2008). After semi-quantitative PCR, 8.0 µL volume of each PCR product was subjected to electrophoresis on 1.5% agarose gel, which was then stained using 0.5 µg/mL SYBR Safe DNA stain (Sigma-Aldrich, USA). The expected size of the amplicon was 340 bp. All relative band intensities were determined using ImageJ software (Maryland, USA) and compared with the outer limestone *ure*C levels set to 100%. Statistically significant differences were estimated using Student's *t* test in R software.

Results

General description of environmental factors

The location of the limestone gulch in Tainliao and the path to the mountain gulch are demonstrated in Fig. 1. The left panel of the figure shows the locations of Tainliao, and the right panel describes in detail where soil and karst samples were collected. The water samples were collected from the drippings of stalactites in the gulch. Various environmental parameters were assessed, namely, illumination, temperature, humidity in air, humidity in soil, and pH in soil. The illumination in the gulch was relatively low all year around, ranging from approximately 20–600 Lux in a location where reflected light is available, and ranging from approximately 5–70 Lux on the wall when measured from 9 a.m. to 6 p.m. on a shiny summer day. The illumination at the opening of the gulch ranged from approximately 100–800 Lux at a brighter location, but it ranged from approximately 60–650 (Lux) on the limestone wall. On the same day, the illumination at an open space around the gulch was approximately 8000 Lux, 150,000 Lux, 85,000 Lux, and 4000 Lux at 9 a.m., 12 noon, 3 p.m., and 6 p.m., respectively. The temperature in the inner of the gulch was 2–4 °C lower than the temperature at the opening of the gulch. The humidity in the soil versus air was 100% versus 70% ± 5% at the inner gulch and 37.5% ± 22% versus 60% ± 5% at the opening. The pH of the soil was approximately 4.4–5 at the inner gulch and approximately 6.2–6.6 at the opening. The total organic carbon content in the inner karst wall, the outer karst wall, and the soil of the outer ground was 3.9% ± 0.2%, 7.7% ± 1%, and 9.1% ± 0.5%, respectively. In short, the inner gulch was a zone of relatively lower light penetration compared with the opening of the gulch. The humidity in the air was similar in the inner and outer gulch. The relative light penetration in the outer gulch may affect the level of local biosynthesis, resulting in a higher total organic carbon content in the areas.

The microbial community structure in various karst habitats based on results of the NGS platform

The 16S-metagenomic sequence data from different habitats, namely from the outer soil, the outer karst, the inner karst, and from dripping water, contained a similar level of assembled reads that were clustered into OTUs, revealing a high variety in numbers, as shown in Table 1. The average read number was more than 400,000. The sample from the soil of the outer gulch had the highest OTUs, whereas the water sample had the lowest OTUs. Because the Shannon index of the sample from the outer gulch was the highest, the effective number of species was also the highest.

Table 1 The results of total organic carbon, the basic information from the NGS Platform, and the bacterial biodiversity in four different habitats

	OS	OL	IL	WA
TOC	9.0 ± 0.4 (%)	7.7 ± 0.9 (%)	3.9 ± 0.1 (%)	–
Reads	296,726	433,210	477,281	448,446
OTUs	2470	831	1899	467
Phyla	20	15	19	18
Shannon–Wiener index	6.2	4.1	3.9	3.6
Effective number of species	511	61	51	36

The symbols of OS, OL, IL, and WA represent sample sites of outer ground, outer limestone wall, inner limestone wall and water, respectively

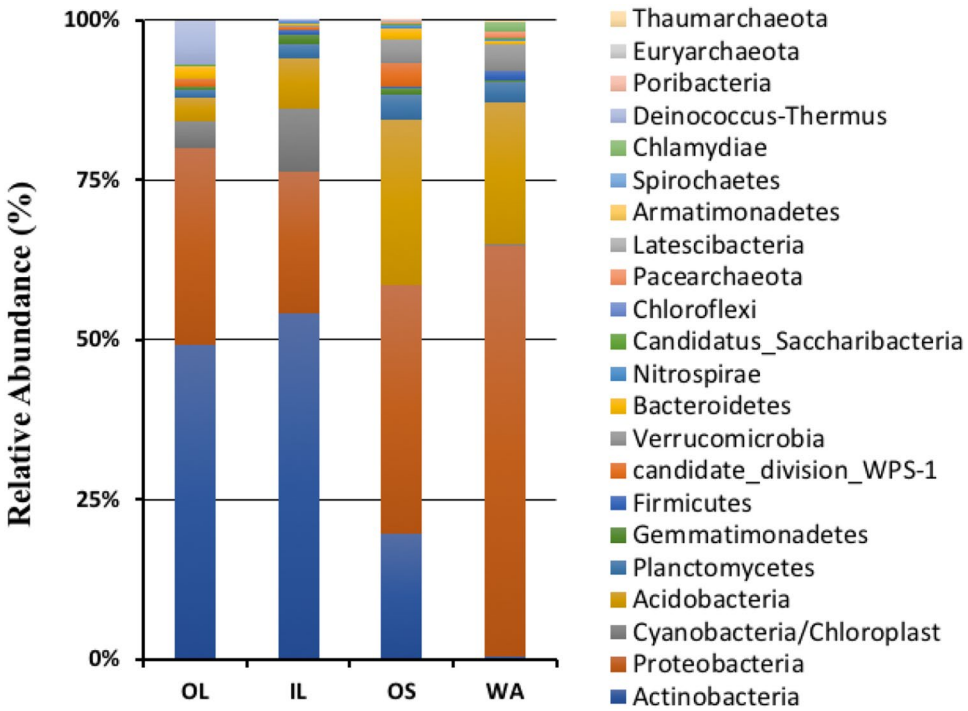

Fig. 2 The relative abundance of four various habitats from the karst landscape in Tainliao

The relative abundance of OTUs in different habitats, which contained 22 phyla in total had shown in Fig. 2. Our results revealed that the soil sample from the outer gulch had the highest alpha diversity, whereas the water sample had the lowest alpha diversity. Four major phyla, namely Proteobacteria, Acidobacteria, Actinobacteria, and Cyanobacteria, accounted for 80% of total microbial species in all the groups. Moreover, Cyanobacteria was present in both limestone walls and was absent in water and soil habitats. Although Actinobacteria can be found in freshwater habitats, our results revealed that they accounted for only < 0.4% of the relative abundance in the karst dripping water. The right panel of Fig. 3 shows the heatmap of OTUs in various habitats. Our data revealed that microorganisms around the gulch were considerably diverse, and the OTU pattern of the water sample was markedly different from those of other samples. The habitats of the outer gulch, outer soil, and outer karst wall more closely resembled each other than the outer and inner karst wall. These findings suggest that the effect of light penetration and moisture overwhelmed the effects of chemical compositions in the karst walls. The right panel of Fig. 3 shows the PCoA distribution of dominated OTUs in the environment, indicating that the distribution of bacteria in the karst gulch was considerably diverse. Many unique OTUs were present in the water habitat (blue square). Although the number of each OTU between the samples of the outer karst wall and soil more closely resembled (Fig. 3, right panel), the PCoA showed that the distribution of many dominant OTUs in inner (orange cross) and outer (green cross) karst walls was adjacent to each other, suggesting that the sequences of dominant species in these two habitats were similar to each other.

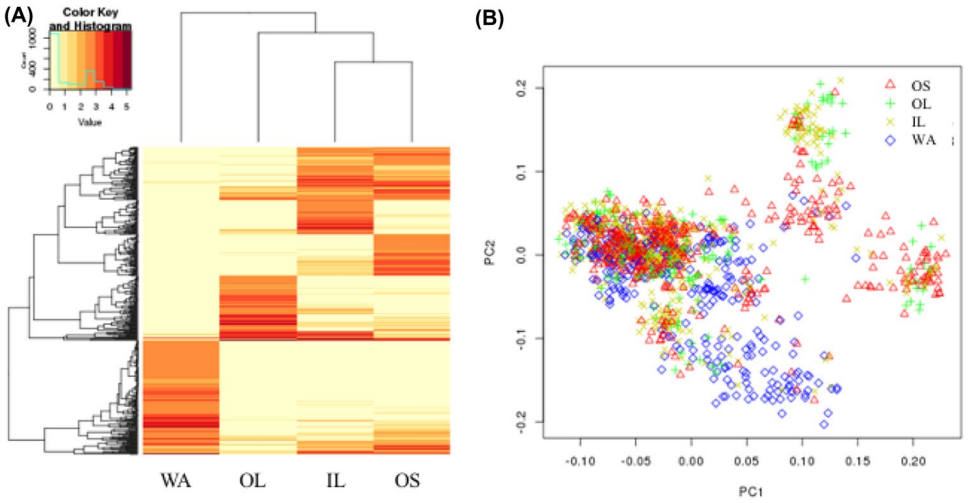

Fig. 3 (**A**) The heatmap of OTUs Based on the read number in different habitats in Tainliao. (**B**) The PCoA distribution based on the distance calibration from DNA sequences of OTUs in four habitats in Tainliao

Table 2 The taxonomy of OTUs and the bacterial references with the sequencing similarity higher than 95% to weathering-associated bacteria

Classification	OTU	Reference of bacteria	Sequence ID	Identities	Taxonomy
Alphaproteobacteria	karst949	*Labrys* sp.	LC372609.1	398/407 (98%)	Labrys
	karst918	*Sphingomonas anadarae*	AB261013.1	394/405 (97%)	Sphingomonas
	karst12	*Sphingomonas* sp.	AF385529.1	400/406 (99%)	Sphingomonas
	karst1757	*Sphingomonas* sp.	AF385529.1	393/406 (97%)	Sphingomonas
	karst2759	*Sphingomonas sanguinis*	D13726.1	385/403 (96%)	Asticcacaulis
	karst1653	*Aminobacter* sp.	AB905480.1	391/408 (96%)	Ensifer
	karst341	*Aminobacter* sp.	FM886907.1	401/407 (99%)	Ensifer
	karst1961	*Rhizobium leguminosarum*	D14513.1	401/407 (99%)	Rhizobium
Betaproteobacteria	karst578	*Janthinobacterium* sp.	AM071372.1	424/433 (98%)	Massilia
	karst3282	*Janthinobacterium* sp.	AB252072.1	417/429 (97%)	Massilia
	karst5	*Collimonas* sp.	FR729923.1	419/432 (97%)	Noviherbaspirillum
	karst1267	*Collimonas* sp.	FR729923.1	413/437 (95%)	Ralstonia
Gammaproteobacteria	karst1216	*Enterobacter*	AB616140.1	431/433 (99%)	Enterobacter
	karst397	*Citrobacter rodentium*	AB682287.1	415/432 (96%)	Escherichia/Shigella
	karst3185	*Shewanella morhuae*	AB205576.1	421/433 (97%)	Shewanella
	karst595	*Pseudomonas stutzeri*	AJ006107.2	431/435 (99%)	Pseudomonas
	karst2678	*Pseudomonas fluorescens*	FJ972536.1	422/433 (97%)	Pseudomonas
	karst464	*Pseudomonas* sp.	AJ417069.1	425/434 (98%)	Pseudomonas
Gram-positive	karst1814	*Pimelobacter simplex*	AY509240.1	411/423 (97%)	Nocardioides
	karst2873	*Arthrobacter oxydans*	LN774480.1	395/413 (96%)	Arthrobacter
	karst175	*Streptomyces lividans*	AB184695.1	403/411 (98%)	Streptomyces
	karst372	*Mycobacterium colombiense*	AM062764.1	401/423 (95%)	Mycobacterium
	karst3247	*Mycobacterium colombiense*	AM062764.1	407/427 (95%)	Mycobacterium
	karst1108	*Mycobacterium colombiense*	AM062764.1	401/424 (95%)	Mycobacterium
	karst1032	*Mycobacterium colombiense*	AM062764.1	406/425 (96%)	Mycobacterium
	karst2144	*Mycobacterium ratisbonense*	AJ271863.1	393/413 (95%)	Mycobacterium
	karst101	*Mycobacterium* sp.	X84978.1	408/411 (99%)	Mycobacterium
	karst1188	*Bacillus subtilis*	AB018487.1	417/434 (96%)	Bacillus
	karst1300	*Bacillus mycoides*	AB547222.1	432/432 (100%)	Bacillus
	karst47	*Pimelobacter simplex*	AY509240.1	399/411 (97%)	Nocardioides
	karst886	*Streptomyces lividans*	AB184826.1	399/409 (98%)	Streptomyces
	karst2729	*Streptomyces lividans*	AB184695.1	398/413 (96%)	Streptomyces
	karst2914	*Kocuria polaris*	AJ278868.1	403/426 (95%)	Arthrobacter

Distribution of weathering bacteria in the karst gulch

The OTUs of habitats with sequences that had > 95% similarity to reference sequences of weathering bacteria had shown in Table 2. Supplementary Table 1 shows bacteria collected in previous studies, indicating that they were capable of promoting the functions of weathering in rocks. Most of the weathering-associated bacteria in habitats belonged to the phyla Proteobacteria. Although 220 species of weathering-associated bacteria were used as references, only < 10% of them showed similarity to OTU sequences in the karst habitats in this study. The relative abundance of weathering-associated bacteria in each habitat is shown in Fig. 4. The sample from the inner karst gulch contains the last portions of bacteria relative to weathering-associated bacteria. The dominant genus in the rock and soil of weathering-associated bacteria in the karst gulch was *Sphingomonas* whereas *Noviherbaspirillum* was the unique genus in the water sample. The existence of weathering-associated bacteria in water suggested that water plays a role in mediating the propagation of weathering bacteria (Ahmed and Holmström 2015). Because studies have revealed that microorganisms suspended in liquid can still lead to the dissolution of elements from rocks, *Noviherbaspirillum* can facilitate the weathering process of the inner karst wall. However, the relative impact of these bacteria on the weathering process remains unclear.

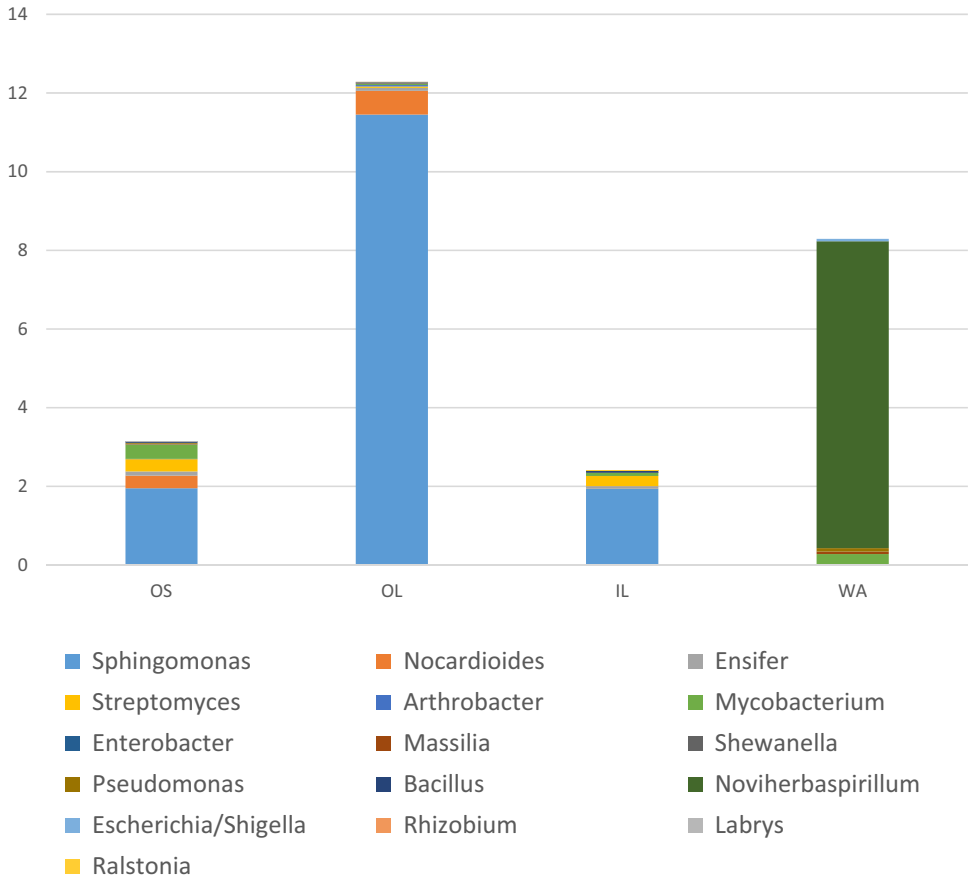

Fig. 4 The relative abundance of weathering-associated bacterial lineages in each habitat

Table 3 The taxonomy of OTUs and the bacterial references with the sequencing similarity higher than 95% to urease-producing bacteria

Classification	OTU	Reference of bacteria	Sequence ID	Identities	Taxonomy
Actinobacteria	karst1300	*Bacillus mycoides*	AB547222.1	432/432 (100%)	Bacillus
Firmicutes	karst791	*Bacillus megaterium*	JX893034.1	419/433 (97%)	Bacillus
	karst260	*Bacillus megaterium*	JX893034.1	416/431 (97%)	Bacillus
	karst2293	*Bacillus megaterium*	JX893034.1	411/430 (96%)	Bacillus
	karst1188	*Bacillus subtilis*	AB018487.1	417/434 (96%)	Bacillus
Gammaproteobacteria	karst189	*Halomonas denitrificans*	AM229317.1	418/432 (97%)	Halomonas
	karst2279	*Halomonas denitrificans*	AM229317.1	414/432 (96%)	Halomonas

Distribution of urease-producing bacteria in the karst gulch

Table 3 shows the OTUs of habitats with sequences that had > 95% similarity to reference sequences of urease-producing bacteria, which by definition are microbes that can synthesize enzymes for urea hydrolysis, resulting in subsequent biocalcification in the presence of calcium ions. The reference table of urease-producing bacteria is shown in supplementary Table 2. The relative abundance of urease-producing bacteria in each habitat is shown in Fig. 5. Urease-producing bacterial lineages in the inner karst wall were related to *Bacillus megaterium*, *B. subtilis*, and *B. mycoides*. In water samples, urease-producing bacterial lineages were closely related to *B. megaterium* and *Halomonas denitrificans*. Urease-producing bacteria in both the inner karst wall and dripping water can contribute to the progress of biocalcification on the inner karst wall. Although the total organic carbon content on the outer karst wall was two times higher than on the inner karst wall, the numbers of urease-producing bacterial lineages might only exist in marginal amounts on the outer karst wall due to the extremely low portion of relative abundance of the bacteria (0.003%, Fig. 5). The relative abundance of urease-producing bacterial lineages on the inner wall (both in water and the karst wall) was approximately 200 times higher than that on the outer karst wall. The high portion in relative abundance of urease-producing bacteria on the inner wall indicated that a persistent stalactite formation occurs on the habitat, which is consistent with its ecological landscape.

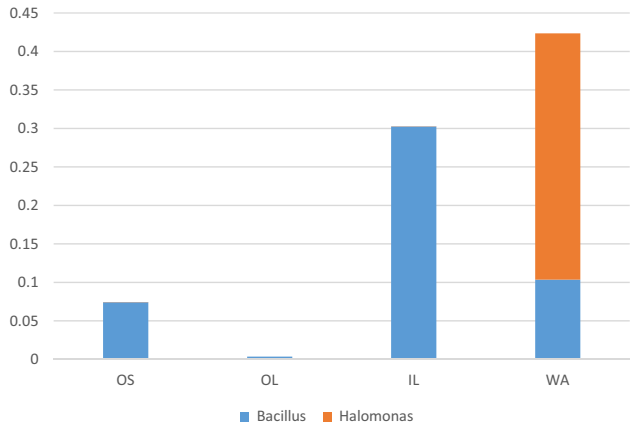

Fig. 5 The relative abundance of urease-producing bacterial lineages in each habitat

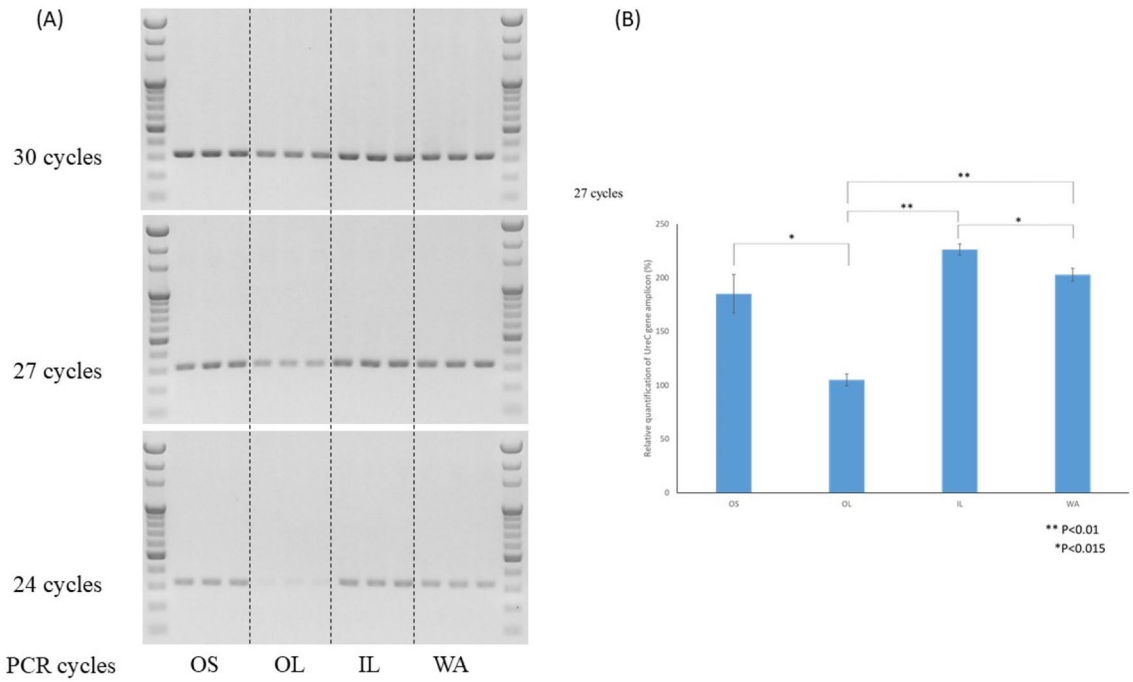

Fig. 6 Semi-quantitative PCR of *ure*C gene results from samples of four habitats in karst topography. **A** Agarose electrophoresis of *ure*C genes in different amplified cycles. Standard markers were loaded at both sides of samples. The samples from different habitats were separated by dash line. **B** The statistic results of semi-quantitative PCR after amplifying 27 cycles. The *ure*C gene levels were significantly higher in the IL habitat than in the OL habitats (*$p < 0.05$; **$p < 0.01$)

Empirical testing of *ure*C gene expression

To substantiate the prediction of the 16S metagenomics for bacteria carrying the urease genes, semi-quantitative PCR was conducted to determine the relative bacterial urease gene levels for each sample. A triplicate study of amplicons obtained from the strains from the four habitat samples was optimized using three different PCR cycle numbers, as shown in Fig. 6A. The band intensities were low with 24 cycles of amplification. A marginal enhancement in these intensities was observed upon increasing the cycle number to 27. The quantification of the intensities showed significantly lower bacterial *ure*C gene expression in the outer limestone (OL) sample with respect to those in the other three habitat samples (Fig. 6B). While comparing the two limestone samples (OL and IL), the abundance of urease-producing bacteria communities in the lightless habitat samples was also confirmed using NGS analysis.

Discussion

Understanding the microbial diversity in the karst landscape provides insights into how bacteria survive in extreme environments and the consequence of geological evolution after their interaction. Many studies focusing on the abundance of microorganisms in karst caves have showed a large microbial diversity in limestone landscapes (Barton et al. 2010; Ortiz et al. 2013, 2014; Tomczyk-Żak and Zielenkiewicz 2015; Zepeda Mendoza et al. 2016). Most of these studies have confirmed that Actinobacteria and Proteobacteria were the dominant species in karst samples. Our study results revealed that total OTUs distributed in the phyla of Actinobacteria, Proteobacteria, Cyanobacteria, and Acidobacteria in karst habitats were approximately 3500, suggesting the extreme diversity of microorganisms in karst landscapes in our studied site. The bacterial communities from different geological areas exhibited regional difference. For example, the majority of bacterial phyla in karst soil in Guizhou China were Proteobacteria, Actinobacteria, Acidobacteria, and Planctomycetes (Zhou et al. 2009). Our data of karst soil revealed that this habitat exhibited the highest microbial diversity. We posit that weathering bacteria present in the outer karst wall and soil contribute to the nutrient level of the soil, causing a higher total organic carbon content and Shannon index of the soil habitat. Our study indicated that light penetration, together with other physical parameters, specify the development of particular microbial communities as showed in Fig. 3. In the long run, the subtle changes of the composition of microbial communities alter the geochemical reactions, rendering the variation of karst landscapes.

With the application of the NGS platform for acquiring 16S-metagenomic data in various karst habitats, we could examine the effects of physical parameters on the evolution of microbial communities and the consequential changes in the microenvironment. To make the most of the 16S-metagenomic data, we used the sequence similarity tool, BLAST, to determine the likeness of representative DNA sequences of OTUs compared with the functional bacteria available in the NCBI database. Although the relative abundance in the phylum levels of karst habitats was similar, the compositions of functional bacteria tested in each habitat were substantially different. We set the cutting point of similarity at 95% to compare functional bacteria in various habitats, which is approximately the level of the genus. However, it is still under debate whether the 95% cutoff in the DNA sequence similarity is a proper setpoint to cluster a category of functional bacteria. Based on this calculus, our data revealed a large difference in the final results, as shown in Figs. 4 and 5, suggesting that a considerable difference exists in the relative abundance of functional bacteria in different habitats. Further confirmation of specific functional bacteria in various habitats can be achieved through molecular cytogenetic techniques, such as fluorescence in situ hybridization.

We hypothesized that the two primary activities of karst landscapes, namely weathering and stalactite formation, might affect the dynamic changes and geographic evolution of karst walls. Functional bacteria associated with these activities were analyzed based on the NGS platform. Our data revealed that a drastic shift in key microorganisms, weathering bacteria and urease-producing bacteria, occurred in the habitats of various physical parameters, suggesting that these parameters play a role in the initiation of different paths in geological evolution. Previously, pH has shown to play a major role driving the specific microbial community shift in karst area as Verrucomicrobia, Acidobacteria, Gemmatimonadetes, Firmicutes are abundant in lower pH, whereas Gammaproteobacteria, Alphaproteobacteria are abundant in higher pH (Yun et al. 2016). Present study also corroborated same while demonstrating that TOC amount might be linked to pH values. With a decrease in pH, the organic matter (OM) decomposition rate by native heterotopic bacteria was enhanced, and such OM concentration is interconnected with TOC amount. The present also suggested similar trend, showing the existence of firmicutes in the inner gulch samples where pH and TOC are low. Further, Gammaproteobacteria, Alphaproteobacteria were found in outer gulch samples having high pH and TOC concentrations, relatively. Some of the previous studies described that in alkaline environment (pH = 7–8.7) the efficiency of urease-producing bacteria to form the stalactite was shown to be maximum, and the most favourable temperature range for optimum urase enzyme activity is 20–37 °C (Omoregie

2016; Zhu and Dittrich 2016). Furthermore, weathering bacteria can dissolve the calcium and magnesium ions from carbonate rock surface by secreting acidic metabolites and these solubilized ions might help the stalactite formation by niche specific urease-producing bacteria in karstification process within a geologic landscape (Lian et al. 2011; Mitchell and Santamarina 2005). The differences in functional bacterial compositions in various habitats supported the fact that the speleothem formation occurred primarily in the inner karst wall in the gulch, suggesting physical conditions in the inner karst wall favor the growth of urease-producing bacteria and promote calcite precipitation. Studies on Cyanobacteria and calcium precipitation have shown that microorganisms may highly enhance the precipitation of $CaCO_3$ minerals in hot spring water (Kaźmierczak et al. 1996; Kawano and Obokata 2007). In this study, the relative abundance of Cyanobacteria in the inner karst wall was twice as large as the relative abundance of Cyanobacteria in the outer karst wall, suggesting that the environment of the inner karst is favorable for the development of Cyanobacteria and the consequential mineral precipitation.

Biocalcification has been widely applied in the ecosystem for many purposes, including land consolidation, groundwater control, crack remediation, and immobilization of toxic metals (Abo-El-Enein et al. 2012; Anbu et al. 2016; Animesh and Ramkrishnan 2016; Kumari et al. 2016; Uroz et al. 2007). Various bacteria, shown in Supplementary Table 2, effectively produce urease, resulting in the precipitation of calcite. Although many environmental factors that affect the growth conditions of urease-producing bacteria have been tested, none of the previous studies have investigated the effects of sunlight penetration on the natural selection of bacterial development. In the study of calcifying bacteria in the Stiffe cave, *Bacillus* and *Arthrobacter* were isolated from natural habitats, which might have contributed to speleothem formation. In this study, several distinct features were found from the data of the NGS platform and the analysis of total organic carbon. First, we found that *B. megaterium* and *H. denitrificans* were the predominant species among calcifying bacteria. Second, urease-producing bacteria were dominant in the inner karst wall. Finally, urease-producing bacterial lineages were also present in the dripping water of the inner wall, which possessed different species of urease-producing bacteria. Most importantly, the interface between water dripping and the inner karst wall was subjected to the biocalcification effects of both urease-producing bacteria. Our data suggests that bacteria in the water drips of the inner karst wall play an important role in facilitating speleothem formation.

Upon evaluation of *ure*C gene levels using semi-quantitative PCR along with the bioinformatics data analysis for the OL sample, lower *ure*C gene expression and low urease-producing bacterial abundance were recorded. High bacterial amount was observed in the OS and IL soil samples wherein the *ure*C gene levels did not present significant differences. Some studies indicate that various urease-producing bacteria would show different performance in ureolytic microbial $CaCO_3$ precipitation and a closely related species of bacteria would show similar urease-producing efficiency (Hammes et al. 2003; Xu et al. 2016). In contrast to the relatively higher urease-producing bacterial abundance seen in the water sample (WA) with respect to that in the rock sample (IL) in the 16S metagenomic analysis, a low abundance was observed in the WA through semi-quantitative PCR. This contradiction may have arisen due to the differences in the urease-producing bacterial genera in the IL and WA samples. For instance, there was only one *ure*C gene-harboring bacterial genus (Bacillus) in the IL sample but two *ure*C gene-harboring genera (Bacillus and Halomonas) in the WA sample, wherein the *Bacillus* spp. were considered as powerful strains for ureolytic microbial $CaCO_3$ precipitation. Indeed, *Bacillus sphaericus* has proved its significant urease activity in the past (Hammes et al. 2003). In summary, the ureolytic precipitation of microbial $CaCO_3$ by urease-producing bacteria could play a crucial role in landscape formation in lightless limestone habitat.

Remarkably, habitats with a lower relative abundance of urease-producing bacteria showed a higher value in relative abundance of weathering bacteria. Meanwhile, the TOC was higher in samples at the gulch opening compared with the sample in the inner wall. We concluded that sunlight and nutrient levels may be two factors affecting TOC in these habitats. Sunlight is an important source providing energy for the accumulation of biomass. Light penetration provided a discriminatory growth condition to heterotrophic microorganisms on habitats in inner and outer walls. In the gulch, more than 90% of the luminance from sunlight was filtered out by the vegetation coverage at the opening of the gulch, and the karst structure of the steep wall further filtered off 0–85% of light penetration inside the gulch, depending on the angle of the sun and the horizon, which affects the photosynthesis reaction in these areas. We also noticed that the effective number of species in the soil increased drastically, suggesting that an elevation of mineral nutrients, one important consequence of weathering effect on rocks, caused by the weathering process could provide a favorable growth condition for many other bacteria in the soil. Previous studies have documented how the dissolution of calcite can be enhanced in the presence of heterotrophic microorganisms (Jacobson and Wu 2009). Our data revealed that the composition of Acidobacteria increased in the habitat of soil, which is consistent with that of a previous study (Zhou et al. 2009). We propose that light penetration plays a pivotal role in natural selection to promote the growth of weathering-associated bacteria, which in turn increase the nutrient level in situ and favor the development of microorganisms.

Conclusion

Given an example of the karst landscape, we provided evidence regarding how physical parameters change the microbial community and the consequential landscape evolution. Furthermore, we showed that light penetration regulates the microbial population, leading to the breakdown of calcite, whereas the chemical composition of limestone might deliver certain conditions that limit the growth of bacterial species. These factors, namely light penetration, water dripping, moisture, the chemical composition of karst, and selected bacteria that are intertwined, shape the weathering process and stalactite formation. We also provided the empirical evidence for the highest levels of urease gene expression in the lightless limestone sample using semi-quantitative PCR. The natural selections of bacteria were achieved by the preferential growth of two bacterial groups: urease-producing bacteria in the inner karst wall and weathering bacteria in the outer karst wall. Our data reveals a causal relationship between environmental factors that contribute to the remodeling of the topography and partly are mediated by microorganisms (Supplementary Figure 1). To the best of our knowledge, this study is the first to address the distinct role of bacteria in the water dripping of karst in biocalcification and the effect of light penetration in the microenvironment on the colony selection of microbial communities.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12665-021-09969-w.

Acknowledgements This research was supported by the Ministry of Science and Technology of Taiwan (MOST 109-2116-M-194-013 and 109-2116-M-194-012). This research was also supported by Ditmanson Medical Foundation Chia-Yi Christian Hospital (RCN007) and the Center for Innovative Research on Aging Society (CIRAS) from The Featured Areas Research Center Program within the framework of the Higher Education Sprout Project by the Ministry of Education (MOE) in Taiwan.

Author contributions Conceptualization, F.-C.Y, B.-M.H, J.-S.C and C.-W.F; Methodology, T.-Y.H, H.-C.T and J.-S.C; Software, H.-C.T and J.-S.C; Validation, C.-W.F, J.-S.C and S.-W.H; Formal Analysis, B.-M.H, J.-S.C and C.-W.F; Investigation, J.-S.C, S.-W.H, H.-C.T and C.-Y.T; Resources, C.-W.F; Data Curation, J.-S.C, H.-C.T and T.-Y.H; Writing-Original Draft Preparation, J.-S.C, F.-C.Y and C.-W.F; Writing-Review and Editing, J.-S.C, B.-M.H, T.-Y.H, V.N and C.-W.F; Visualization, B.-M.H and F.-C.Y; Supervision, S.-W.H, B.-M.H, T.-Y.H, V.N and J.-S.C; Project Administration, J.-S.C, B.-M.H, T.-Y.H and C.-W.F; Funding Acquisition, B.-M.H, F.-C.Y and C.-W.F.

Funding This study was funded by Ministry of Science and Technology, Taiwan (MOST 108-2811-M-194 -507 -, MOST 108-2116-M-194 -005 -), Buddhist Tzu Chi Medical Foundation (TCRD 108-39) and Ditmanson Medical Foundation Chia-Yi Christian Hospital-National Chung Cheng University Joint Research Program, CYCH-CCU Joint Research Program (CYCH-CCU-2021).

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The manuscript does not contain clinical studies or patient data.

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