Geomicrobiology Journal
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/ugmb20

Factors Influencing Observed Variations in the Structure of Bacterial Communities On Calcite Formations in Kartchner Caverns, AZ, USA

A. Legatzki [a], M. Ortiz [a], J. W. Neilson [a], R. R. Casavant [b c], M. W. Palmer [d], C. Rasmussen [a], B. M. Pryor [e], L. S. Pierson III [f] & R. M. Maier [a]

[a] Department of Soil, Water, and Environmental Science, University of Arizona, Tucson, Arizona, USA

[b] Arizona State Parks, Phoenix, Arizona, USA

[c] Kartchner Caverns State Park, Benson, Arizona, USA

[d] Department of Botany, Oklahoma State University, Stillwater, Oklahoma, USA

[e] Division of Plant Pathology and Microbiology, School of Plant Sciences, University of Arizona, Tucson, Arizona, USA

[f] Department of Plant Pathology and Microbiology, Texas A&M University, College Station, Texas, USA

Version of record first published: 05 Mar 2012.

To cite this article: A. Legatzki , M. Ortiz , J. W. Neilson , R. R. Casavant , M. W. Palmer , C. Rasmussen , B. M. Pryor , L. S. Pierson III & R. M. Maier (2012): Factors Influencing Observed Variations in the Structure of Bacterial Communities On Calcite Formations in Kartchner Caverns, AZ, USA, Geomicrobiology Journal, 29:5, 422-434

To link to this article: http://dx.doi.org/10.1080/01490451.2011.581326

Geomicrobiology Journal, 29:422–434, 2012

ISSN: 0149-0451 print / 1521-0529 online
DOI: 10.1080/01490451.2011.581326

Taylor & Francis
Taylor & Francis Group

Factors Influencing Observed Variations in the Structure of Bacterial Communities On Calcite Formations in Kartchner Caverns, AZ, USA

A. Legatzki,[1] M. Ortiz,[1] J. W. Neilson,[1] R. R. Casavant,[2,3] M. W. Palmer,[4] C. Rasmussen,[1] B. M. Pryor,[5] L. S. Pierson III,[6] and R. M. Maier[1]

[1]*Department of Soil, Water, and Environmental Science, University of Arizona, Tucson, Arizona, USA*
[2]*Arizona State Parks, Phoenix, Arizona, USA*
[3]*Kartchner Caverns State Park, Benson, Arizona, USA*
[4]*Department of Botany, Oklahoma State University, Stillwater, Oklahoma, USA*
[5]*Division of Plant Pathology and Microbiology, School of Plant Sciences, University of Arizona, Tucson, Arizona, USA*
[6]*Department of Plant Pathology and Microbiology, Texas A&M University, College Station, Texas, USA*

Kartchner Caverns is an oligotrophic subterranean environment that hosts a wide diversity of actively growing calcite speleothems (secondary mineral deposits). In a previous study, we demonstrated that bacterial communities extracted from these surfaces are quite complex and vary between formations. In the current study, we evaluated the influence of several environmental variables on the superficial bacterial community structure of 10 active formations located in close proximity to one another in a small room of Kartchner Caverns State Park, Arizona, USA. Physical (color, dimensions) and chemical (elemental profile and organic carbon concentration) properties, as well as the DGGE-based bacterial community structure of the formations were analyzed. While elemental concentration was found to vary among the formations, the differences in the community structure could not be correlated with concentrations of either organic carbon or any of the elements evaluated. In contrast, the locations of formations within a distinct region of the cave as well as the relative location of specific formations within a single room were found to have a significant influence on the bacterial community structure of the formations evaluated. Interestingly, Canonical Correspondence Analysis suggests an association between the observed drip pathways (drip lines) feeding the formations (as determined by the patterns of soda straws and small stalactites that reveal water flow patterns) and the bacterial community structure of the respective formations. The results presented here indicate that a broad range of formations fed by a diversity of drip sources must be sampled to fully characterize the community composition of bacteria present on the surfaces of calcite formations in carbonate caves.

Keywords carbonate cave, speleotherm, bacterial community structure, oligotrophic, drip water

Received 14 October 2010; accepted 7 April 2011.

We appreciate the good partnership with the Kartchner Caverns State Park. Part of the park's philosophy is to use science-informed management and to advance scientific literacy of the general public. Knowledge and discoveries derived from this and other scientific studies are immediately integrated into park education and tour programs and thus made available to cave visitors. We want to thank cave manager Ginger Nolan in particular for her expertise and assistance in sample site selection. We thank Dr. Jay Quade from the University of Arizona, Department of Geosciences for the organic carbon analysis of the speleothem samples. Furthermore, we thank Dr. Mercer Meding from the Center for Environmental Physics and Mineralogy for the determination of the surface color of the speleothems and his assistance in data analysis. The research was supported by Microbial Observatories Grant MCB0604300 from the National Science Foundation.

Address correspondence to Raina M. Maier, Department Soil Water and Environmental Science, Shantz Bldg Room 429, 1177 E. 4th Street, University of Arizona, Tucson, AZ 85721-0038. E-mail: rmaier@ag.arizona.edu

INTRODUCTION

Despite the abundance of life on the terrestrial surface, most of the biomass is located beneath the surface and the majority of this biomass is microbial (Whitman et al. 1998). Caves represent habitats that provide valuable laboratories for studying such subterranean microbial communities along with the physicochemical and ecological conditions that may be controlling their structure. Compared to other subsurface habitats, caves are relatively accessible, while still representing ecosystems with a broad worldwide distribution. Caves are typical features of karst terrains that represent approximately 20% of the earth's dry ice-free surface (Ford and Williams 2007).

Over the past years, numerous studies have investigated the microbial communities in cave systems (reviewed in e.g., Northup and Lavoie 2001; Barton and Northup 2007; Engel

2010). One unique feature of these caves is the presence of a wide diversity of speleothems (secondary mineral deposits). Hill and Forti (1997) recognize 38 speleothem types, with numerous subtypes and varieties, and describe over 250 minerals found in caves all around the world. Factors contributing to speleothem formation and diversity include local geochemistry, temperature, and rates of CO_2 off-gassing and precipitation. In addition, current research suggests that microbial activity plays a critical role in speleothem formation (Banks et al. 2010; reviewed in Barton and Northup 2007). There is still no agreement on whether the microbial populations described in caves contribute to any type of conserved functional community structure that is necessary for speleothem development.

In a recent study of Kartchner Caverns, Arizona, (Legatzki et al. 2011), complex bacterial communities were characterized from the surfaces of two calcite speleothems using PCR denaturing gradient gel electrophoresis (PCR-DGGE) and PhyloChip (Brodie et al. 2007) analyses of 16S rRNA genes. These communities were of interest for several reasons: i) they were complex as indicated by the large number of phyla identified by PhyloChip analysis; ii) they included unique phylotypes as determined by the sequencing of DGGE bands; and because particularly iii) the structure of the microbial communities differed from speleothem to speleothem (referred to hereafter as speleothem-specific community structure). The study used PCR-DGGE to characterize the community structure of bacterial and archaeal populations present on two adjacent speleothems exposed to similar environmental conditions and hypothesized that a correlation might exist between substrate geochemistry and the observed differences in microbial community structure.

Kartchner Caverns State Park is approximately 13 km south of the town of Benson in southeast Arizona. The park is characterized by a series of exposed, low-lying karstic limestone hills and ridges that host the cavern system. The cavern's rooms and passageways developed within a massively bedded and intensely deformed block of a Lower Carboniferous-age Escabrosa limestone formation (Jagnow 1999). These rooms host a wide diversity of actively growing calcite speleothems, including diverse stalactites, soda straws, stalagmites, columns, draperies, shields, flowstone, popcorn, helictites and boxwork (Hill 1997). This subterranean ecosystem is sustained by ephemeral stream flow that infiltrates into the system at the intersection of faults and fractures, groundwater recharge derived from the highlands of the Whetstone Mountains to the west, and direct infiltration of local summer and winter precipitation events.

The absence of natural light in this cavern prevents the production of organic carbon by photosynthetic activity. As a result, microbial communities present on the surfaces of cave formations depend on trace organic and inorganic nutrients that enter the cave primarily through percolating water and support some combination of chemoheterotrophic and chemoautotrophic activities. The trace nutrients contained in this percolating water include materials sequestered and dissolved along the path from the surface through leaking faults and fractures, and across bedrock to the formations. In general, all speleothems in caves contain trace organic and inorganic constituents, regardless of their primary mineral composition (James 1997).

The motivation for the current work was to expand on our previous study and to investigate factors that may influence the superficial bacterial community structure, with special attention given to the substrate geochemistry of the formations. Ten distinct formations were randomly selected within an approximately 18 m^2 area of the cave for physical (dimension, color, occurrence of drip water) and chemical (organic carbon and elemental content) analysis. The superficial bacterial community structure of each formation was also analyzed by PCR-DGGE.

First, multiple samples were taken along the length of 3 of the 10 formations to determine whether the bacterial communities were speleothem-specific as shown previously for speleothems in a different region of the cave (Legatzki et al. 2011). Second, a single sample from each of the 10 formations was taken to investigate the effect of selected environmental variables on the bacterial community structure. Third, single samples from 8 of the 10 formations were compared to the bacterial community structures of two samples from Legatzki et al. (2011) to determine whether samples taken from multiple speleothems located in a single room of the cave are more similar to each other than to samples taken from speleothems in a different region of the cave.

MATERIALS AND METHODS

Study Sites

The primary location for this work was the Big Wall site (Figure 1a). This site is located along and within a major fault zone that defines the western boundary of the Rotunda-Throne Room complex. Exposure to human activity in this area is carefully controlled by permitting at most five visits per year from research scientists or park rangers. The sampling site is located approximately 10 m below and 15 m to the west of the tour trail. Nine stalactites, referred to as formations A, B, D, E, F, G, H, J, and W, and a bacon drapery, referred to as formation C, were selected for analysis from the high diversity of formations found at this site (Figure 1b). All sampled formations, except formation A and B, originated from different speleothems. Two previously sampled stalactites (formation C and S) in the Strawberry Alcove site were also included in this study to investigate the differences in bacterial community structure of speleothems located in two separate regions of the cave (Figure 1a) (Legatzki et al. 2011).

Sample Analysis

Determination of Bacterial Community Structure

Sampling and DNA extraction. Superficial microbial communities were sampled from the formation surfaces using sterile cotton swabs wetted with sterile distilled H_2O (dH_2O). Multiple samples were taken along the length of three speleothems from

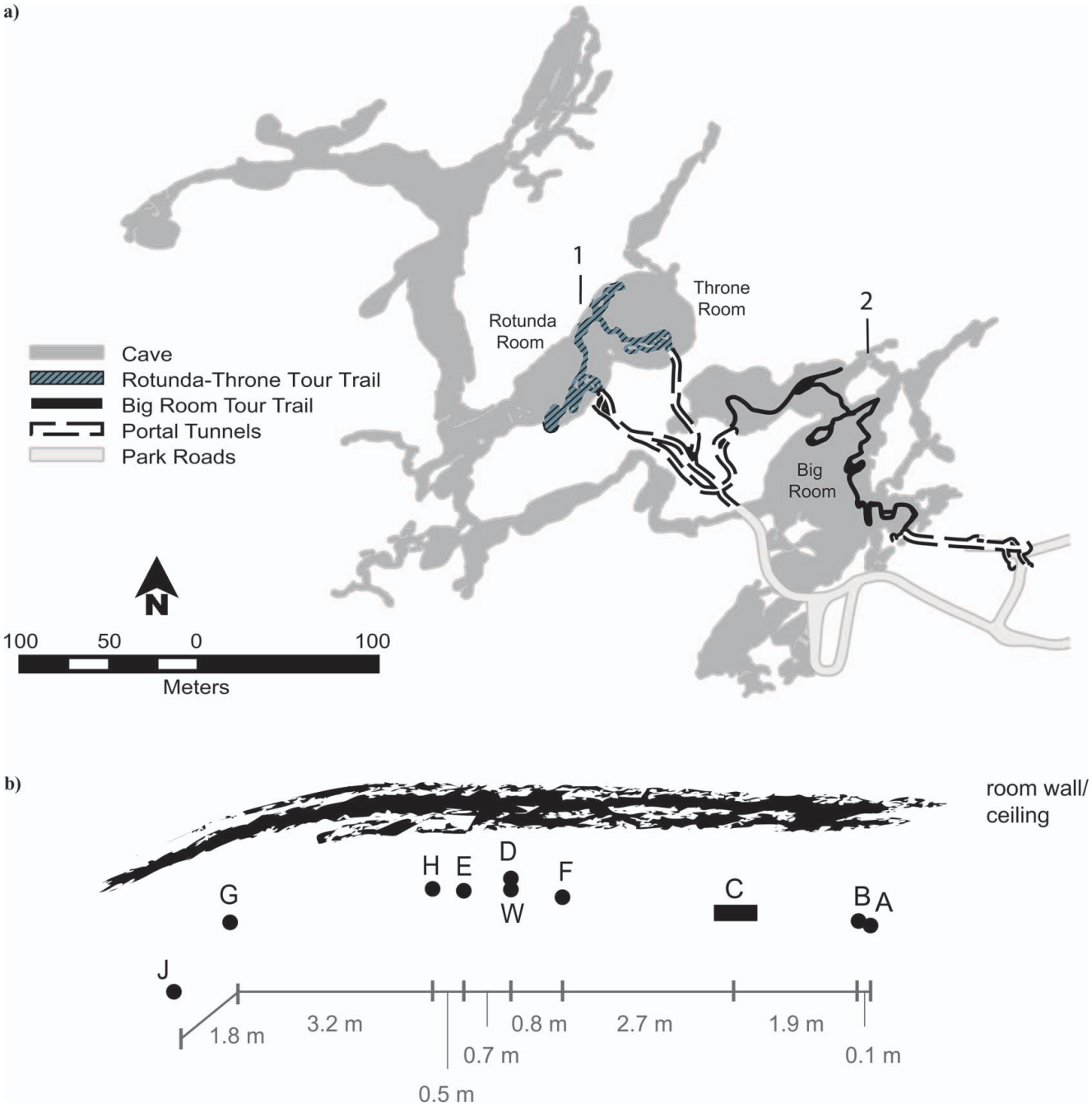

FIG. 1. a) Map of Kartchner Caverns. The map indicates the Big Wall site (1), the Strawberry Alcove site (2), and both tour trails with the Rotunda and the Throne Room as well as the Big Room. b) Shows the location of formations sampled in the Big Wall site and their horizontal distances in m. The stalactites (●) and the bacon drapery (▬) are marked with their alphabetical label (color figure available online).

the Big Wall site (June 2007) (Figure 2a) to evaluate differences in intra- vs inter-speleothem community structure. A total surface area of 27 cm^2 per sample was swabbed using three swabs. A similar procedure was followed to take single samples from all 10 formations in the Big Wall site (November 2007) and two stalactites in the Strawberry Alcove site (April 2007) (Legatzki et al. 2011). Immediately after sampling, swabs were immersed in sterile dH_2O and stored on ice for transport. In the laboratory samples were stored at 4°C and processed for DNA extraction within 24 h. DNA extractions were performed as described in Legatzki et al. (2011). Briefly, swabs were vortexed in sterile dH_2O and bacterial cells collected by centrifugation.

Genomic DNA from the cell pellet was extracted after cell lysis by repeated freeze-thaw cycles using a modification of

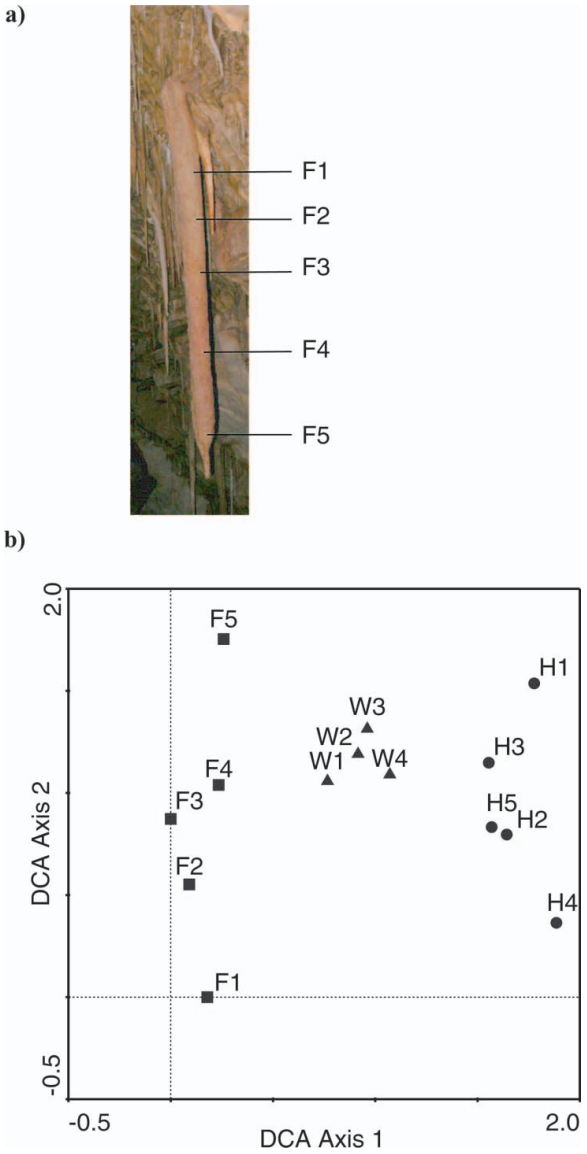

FIG. 2. a) The sampling locations of the five samples along the length (from top to the tip) of stalactite F (F1-F5) for Study 1. b) DCA for bacterial DGGE band profiles of four samples from stalactite W and five samples from stalactite F and H, respectively, from the Big Wall site (color figure available online).

the basic protocol for preparation of genomic DNA from bacteria (Ausubel et al. 1995). DNA extracts were quantified using a TBS-380 Fluorometer (Turner BioSystems, Sunnyvale, CA, USA) with PicoGreen dye (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's directions. Potential contamination from the sampling or extraction procedures was evaluated by opening sterile swabs in the cave and placing them in sterile dH_2O according to the sampling protocol. These negative controls were processed in parallel along with the sample swabs.

PCR-amplification and DGGE. Genomic DNA extracted from the swab samples was used as template for amplification of the bacterial 16S rRNA gene. A 352-bp fragment (including the primers) of the bacterial 16S rRNA gene [V7/V8 variable regions (Baker et al. 2003)] was amplified from the DNA extracts using the primers 1070F and 1406R-GC (Ferris et al. 1996). The PCR reaction was performed as described in Legatzki et al. (2011). PCR amplicons were visualized and evaluated by agarose gel electrophoresis and ethidium bromide staining.

DGGE analysis was performed using the Dcode Universal Mutation Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Acrylamide gels (7%) were prepared with a 45 to 65% urea–formamide denaturing gradient according to the manufacturer's protocol. Gel lanes were loaded with 5–25 μL PCR product according to quantification by agarose electrophoresis. The gels were run at a constant voltage of 50 V for 17 h at 60°C in 0.5 × TAE buffer (1 × TAE = 40 mM Tris acetate and 1 mM EDTA), followed by staining with 2 × SYBR Green I (Lonza, Rockland, ME, USA) for 40 min. The DNA was visualized with a UV transilluminator, and images were digitized with an Alpha Imager Digital Imaging System (Alpha Innotech Co., San Leandro, CA, USA).

The Bio-Rad Quantity One® 4.5.2 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to analyze the gel images and generate a data matrix based on the presence and absence of bands in individual profiles to be used for statistical analysis. DGGE profiles from the negative control DNA extracts were subtracted from the sample profiles when bands were detected. Contaminant bands are caused by the presence of DNA in *Taq* DNA polymerase and other PCR reagents. Typically five bands were detected in negative control profiles representing 19% of the total bands of an average sample profile.

Determination of Physical Properties

The dimensions (length, diameter) of the 10 formations in the Big Wall site were measured and the relative location of the formations along a horizontal line from formation J to formation A was recorded. The presence of drip water on each of the nine stalactites was observed at three time points (Nov. 7, 2007; Nov. 6, 2008; Dec. 9, 2009).

The surface color of the 10 formations was determined with a Minolta CR-200 Chroma Meter (Minolta Camera Co., Ltd., Osaka, Japan). Triplicate readings from one spot within the sampled area were taken for the stalactite formations and averaged. For the bacon drapery (formation C) three readings from one spot in each of the different color areas were taken and averaged. The readings were taken in the Munsell color system and the colors described by Hue, Value, and Chroma (Munsell 1976). Values were adjusted using a previously determined correction factor based on laboratory calibration using a wide range of soil samples from southern Arizona. The corrected values were documented as numerical values and color names corresponding to the Munsell soil book (ColorAccuracy.com, East Brunswick, NJ, USA).

Determination of Chemical Properties

Solid samples were removed from the formation surfaces in the Big Wall site for organic carbon and elemental analysis. An approximately 5 × 2 cm area (2 mm depth) of each formation surface was removed using a Dremel® MultiPro Model 770 (Dremel® Racine, WI, USA) with a tungsten carbide cutter. The powder collected was air dried for 72 h at room temperature prior to analysis.

The organic carbon concentration of 350–506 mg of surface material was determined manometrically after dissolving the sample material in 3N HCl. All measured concentrations of organic carbon were above the detection limit of 20 μg, representing 0.006% organic carbon in a 350 mg sample. Elemental analysis was performed on surface material (204 – 208 mg) from each formation following digestion and concentration in 70% HNO_3 as described previously (Legatzki et al. 2011). Analysis was done by the Arizona Laboratory for Emerging Contaminants (University of Arizona, Tucson, AZ, USA) using inductively coupled plasma mass spectrometry (ICP-MS). All evaluated element concentrations were above detection limit. The detection limits were 1.3×10^{-3} μg/g for Si and 1.5×10^{-3} μg/g for P. For all other evaluated elements the detection limits ranged from 0.3 to 31.5×10^{-6} μg/g.

Statistical Analysis

Elemental and organic carbon profiles from the 10 Big Wall site formations were compared using Principal Component Analysis (PCA). The data were log-transformed before performing PCA. Variables in PCA are represented by biplot arrows (Lepš and Šmilauer 2003).

A combination of multivariate analyses was used to describe variation in the structure of the bacterial communities (indirect gradient analysis) and to relate this variation to specific environmental variables (direct gradient analysis). Detrended Correspondence Analysis (DCA), an indirect gradient analysis, was used to gain a basic overview of the relative compositional gradient of the bacterial communities in the dataset and to obtain an estimate of the β-diversity. Canonical Correspondence Analysis (CCA), a direct gradient analysis widely used in community ecology (Legendre and Legendre 1998; Palmer 1993; ter Braak 1986), was used to test hypotheses correlating selected environmental variables with observed variations in bacterial community structure. CCA finds axes of variation in banding patterns that are maximally related to environmental (explanatory) variables.

CCA eigenvalues represent the strength of the relationship between DGGE profile bands and the environmental variables (ter Braak and Wiertz 1994). In order to investigate the statistical significance ($p < 0.05$) of the environmental variables a partial Monte Carlo permutation test was performed. The test was based on 1000 random permutations. The null hypothesis of this test presumed that species data (in this case DGGE band patterns) were unrelated to environmental variables. Both environmental variables, in the form of categorical variables (such as sample origin, or drip line group), and continuous variables (such as chemical element contents, organic carbon content and relative location) were tested.

All continuous variables were log-transformed, except for the relative location of the formations in the site. For CCA evaluations including more than one continuous environmental variable, the variable inflation factor (VIF) associated with each of the variables was analyzed. If the VIF of a variable is large (> 20), then the variable is almost perfectly correlated with the other variables being evaluated in the dataset (ter Braak and Šmilauer 2002) and can be excluded from the analysis. All CCA were performed with focus scaling on inter-sample distances. The multivariate statistical analyzes were computed using Canoco for Windows 4.5 (http://www.canoco.com; Biometris, Wageningen, Netherlands).

RESULTS AND DISCUSSION

Evaluation of Speleothem-Specific Bacterial Community Structure of Three Speleothems

The variability in bacterial community structure of multiple samples taken from three speleothems located in the Big Wall room was evaluated by PCR-DGGE. All three speleothems were located within a distance of 2 m of each other at the Big Wall site. The DGGE band profiles of four samples from speleothem W and five samples from speleothems F and H taken along the length of the respective stalactites were compared to each other using DCA (Figure 2b). Samples from each formation were numbered sequentially with the first sample taken closest to the ceiling and the last closest to the tip (Figure 2a). DCA revealed that the β-diversity among the samples was low. Nevertheless, samples from the same stalactite clustered together along the first axis, indicating that differences in community structure between samples from the same stalactite were smaller than those between samples from one of the other two stalactites. Further, there is more variation in the speleothem F and H communities than within the speleothem W community as indicated by the second axis. This pattern may correspond to the fact that the former speleothems are more than twice as long as speleothem W (Table 1). No consistent correlation was observed for the three stalactities between sample position on the speleothem and divergence in relative bacterial community structure. This observation was confirmed by analysis of Dice coefficients between each of the 14 bacterial communities evaluated (data not shown).

In light of the observed intra-speleothem community variation, further analysis using CCA was performed to test the hypothesis that sample origin (meaning stalactite H, W, or F) has a significant influence on the bacterial community structure of the 14 samples analyzed. The CCA confirmed that the structure of the bacterial communities characterized by PCR-DGGE was significantly affected ($p = 0.001$) by the specific stalactite from which the sample was taken. Thus, the bacterial communities sampled from the same stalactite were structurally more similar

TABLE 1
Physical characterization of the formations

Formation	Type	Relative location[a] (m)	Dimension		Color in Munsell system		Presence of drip water		
			Length (cm)	Diameter[b] (cm)	Color name	Color notation (H V/Chr)[c]	Nov/7/2007	Nov/6/2008	Dec/9/2009
A	Stalactite	11.7	26.7	3.8[d]–1.3	dark gray	3.8GY 4.3/0.2	+	+	+
B	Stalactite	11.6	26.7	3.8[d]–1.3	dark greenish gray	1.8GY 3.9/0.5	+	+	+
C	Bacon drapery	9.7	88.9		greenish gray light brownish gray dark gray	0.1GY 5.2/0.5 3.8Y 6.2/1.8 9.7YR 4.2/1.3	n.d.	n.d.	n.d
F	Stalactite	7	119.4	6.4–1.7	reddish brown to yellowish red	6.0YR 5.1/5.1	−	+	+
W	Stalactite	6.2	40.6	3.8–1.3	very pale brown	1.6Y 7.3/2.7	n.d.	+	+
D	Stalactite	6.2	62.2	1.9–1.3	brown	8.3YR 4.6/2.5	+	+	+
E	Stalactite	5.5	45.7	2.5–1.3	very pale brown to light yellowish brown	0.6Y 6.5/3.9	+	+	+
H	Stalactite	5	116.8	3.2–1.9	pale brown	0.3Y 5.7/3.2	+	+	+
G	Stalactite	1.8	36.8	5.1–1.3	brown	0.1Y 4.0/2.6	+	+	+
J	Stalactite	0	63.5	6.4–1.3	brown	9.2YR 4.7/3.8	+	+	+

n.d. = no data.
[a]Relative location refers to the location of the formations along a horizontal line between stalactite J and stalactite A.
[b]Diameter was measured at the top and tip of each stalactite.
[c]H = hue; V = value; Chr = chroma.
[d]Stalactites A and B descend from the same shield formation and have a top combined width of 11.4 cm.

to each other than to the communities from one of the other two stalactites. These results support the conclusions presented in our previous work investigating intra- vs inter-speleothem variability in bacterial community structure using a more limited sample size in a different part of Kartchner Caverns (Legatzki et al. 2011).

Characterization of Speleothem Physicochemical Properties and the Influence of These Properties on the Observed Variability in Bacterial Community Structure

The speleothem-specific structure of bacterial communities on the surface of formations in Kartchner Caverns was investigated further by exploring potential physical and chemical properties of speleothems that could explain the differences observed between the bacterial communities of different speleothems. In our previous study (Legatzki et al. 2011), we hypothesized that differences in substrate geochemistry influenced the structure of microbial communities on the surface of formations based on observed differences in the elemental profiles of the two speleothems evaluated. Previous studies have shown that variations in geochemistry can have an important affect on the structure of microbial communities colonizing rock surfaces (Barton et al. 2007; Hutchens et al. 2010).

Ten distinct formations, nine stalactites (A, B, D, E, F, G, H, J, W) and a bacon drapery (C), all located within the Big Wall site of the cavern (Figure 1a, b), were selected to evaluate the influence of a range of physical and chemical properties on the bacterial community structure of speleothems. A single sample was taken from the central area of each speleothem; however, modifications were made in the sampling protocol to obtain a greater DNA yield and cover a larger surface area than was obtained when sampling the three speleothems previously described. The surface area swabbed for each sample was increased from 27 cm^2 to 120 cm^2 by increasing the surface area per swab and the number of swabs to six.

The 10 formations evaluated were located on a transect along the strike of the Big Wall site, covering a horizontal distance of 11.7 m (Table 1). Due to their close proximity to one another and location within the same small room of the cave, it is assumed that the 10 formations were subjected to the same microclimate parameters such as temperature, relative humidity, and CO_2 content. In addition, several smoke tests were conducted (data not shown) to determine the potential influence of air currents on the transport of microbes or nutrients through this room of the cave. Small amounts of smoke were released as a vertical column from a source on the floor of the cave to detect lateral air movement through the chamber. No lateral movement of air could be detected. The site is located at the bottom of a break down pile, effectively blocking air along the trajectory typically observed in this portion of the cave. Thus, it was assumed that air currents in this Big Wall section of the cave would not differentially influence microbial communities present on the 10 formations being studied.

Physical and Chemical Characterization of the Selected Cave Formations

Each of the 10 formations evaluated in this study was distinct in dimensions and color (Table 1). The length of the stalactites ranged from 26.7 cm to 119.4 cm, each tapering from top to tip with the widest point located near the ceiling. Stalactites F and J (6.4 cm) had the largest diameter near the ceiling and stalactites F and H were the longest measuring 119.4 cm and 116.8 cm, respectively. Speleothem C was a bacon drapery that measured 88.9 cm in length horizontally. The colors of the formations varied from gray to brown shades for the selected formations. The striations in color of the bacon drapery were indicated by three measurements of color taken along the vertical axis of the feature.

The presence of drip water on each of the speleothems (except for the bacon drapery C) was recorded on three separate dates in November 2007 and 2008 and December 2009 (Table 1). We did not observe formation W for November 2007. All stalactites were found to be dripping when monitored with the exception of F during our site visit in November 2007. This observation is interesting because stalactites monitored in other regions of the cave ceased dripping during this time period.

The concentrations of organic carbon and selected major and minor elements were determined in surface material taken from each formation (Table 2). As expected the organic carbon concentration was extremely low, ranging from 0.016% for formation F and B to 0.033% for formation A, highlighting the oligotrophic nature of this ecosystem. This range corresponds to a 2.1-fold maximum difference among the 10 samples (Table 2). The analysis of speleothem organic matter in a study conducted by van Beynen et al. (2001) suggests comparable organic carbon concentrations in other cave systems. Elemental ICP-MS analysis revealed calcium to be a major component of all 10 formations occupying 39–52% of the total weight. Determination of crystalline mineral composition could not be performed on these samples due to the small sample size, but previous analysis of stalactite material from this cave revealed the only detectable mineral was a polymorph form of calcite, ($CaCO_3$) (Legatzki et al. 2011). The remaining elements were present in trace concentrations. The variation in minor elemental content among the 10 formations ranged from a 1.2- to 250-fold difference in concentration (Table 2). Elements showing a greater than 50-fold increase from lowest to highest concentration among the 10 formations were Mn, Al, Fe, Si, P, Cd, Pb, K, Ti, and Cr.

A PCA biplot was generated to compare the elemental and organic carbon contents of the 10 formations (Figure 3). The first two axes of the PCA together explained 70.1% of the total variation. The first axis explained 57.3% of the total variation and was positively correlated with a number of elements including As, Cr, Pb, Mn, Ti, Fe, Al, K, Si, Cu, Cd, P, Ba, Zn, and Mg; and negatively correlated with Ni. The second axis, which explained 12.8% of the total variation, was positively correlated with Na, Se, Co and Sr concentrations and negatively correlated

TABLE 2
Chemical characterization of surface material of the 10 formations

Chemical data	Formation										x-fold difference in concentration[a]
	A	B	C	F	W	D	E	H	G	J	
Organic carbon (%)	0.033	0.019	0.026	0.016	0.019	0.026	0.028	0.024	0.026	0.026	2.1
Element (μg/g)											
Ca	406 037.49	440 858.64	393 176.70	407 306.82	390 273.68	420 921.68	421 481.71	443 573.29	520 445.07	406 474.46	1.3
Mg	1 417.19	1 445.40	2 041.51	1 733.94	1 932.20	1 865.25	1 522.56	2 620.62	1 322.84	1 470.56	2.0
Al	13.83	24.07	278.22	635.86	18.51	160.56	100.75	3 234.93	844.16	788.34	233.9
Fe	8.62	15.11	177.76	579.80	20.02	122.60	60.87	2 000.62	625.76	658.64	232.1
P	11.96	23.83	307.45	2 070.74	25.68	94.27	100.10	203.82	268.75	722.76	173.1
Mn	0.23	0.65	9.17	30.49	0.61	5.62	2.34	36.71	57.13	29.81	249.9
Co	11.71	13.53	14.49	12.91	12.90	13.60	13.98	11.93	13.31	12.42	1.2
K	6.02	9.05	52.34	110.98	9.10	28.81	23.22	385.84	130.16	124.03	64.0
Zn	1.02	1.14	2.64	8.08	2.08	2.56	6.04	5.03	2.81	4.98	7.9
Cu	0.42	0.66	1.42	3.05	0.85	1.48	1.78	2.95	2.12	2.21	7.2
Na	13.82	18.90	23.03	26.06	10.34	11.86	15.38	14.99	13.58	13.86	2.5
Si	3.02	5.98	13.11	176.85	11.85	24.73	15.36	552.95	163.97	213.60	183.2
Ti	0.47	0.55	3.43	13.87	0.51	3.68	1.86	28.88	11.31	12.44	61.5
Cr	0.04	0.16	0.82	0.97	0.04	0.28	0.13	2.06	1.03	1.77	55.9
Ni	15.89	16.99	15.80	14.36	12.70	16.76	16.41	14.80	14.84	14.01	1.3
As	0.22	0.31	0.35	0.83	0.14	0.21	0.27	0.76	0.48	0.63	6.1
Se	0.66	0.80	0.74	0.70	0.71	0.76	0.82	0.53	0.69	0.59	1.5
Sr	21.98	18.35	25.54	31.21	19.90	15.94	25.93	19.89	16.99	15.09	2.1
Cd (10^{-3})	0.67	1.02	9.02	51.72	21.45	15.16	86.62	51.62	9.50	52.21	130.2
Ba	6.38	5.10	9.51	12.78	7.34	6.96	9.23	12.76	9.68	6.84	2.5
Pb	0.02	0.03	0.39	1.28	0.08	0.25	0.27	2.46	1.19	1.40	102.5

[a]The x-fold difference in concentration is calculated dividing maximum by minimum concentration.

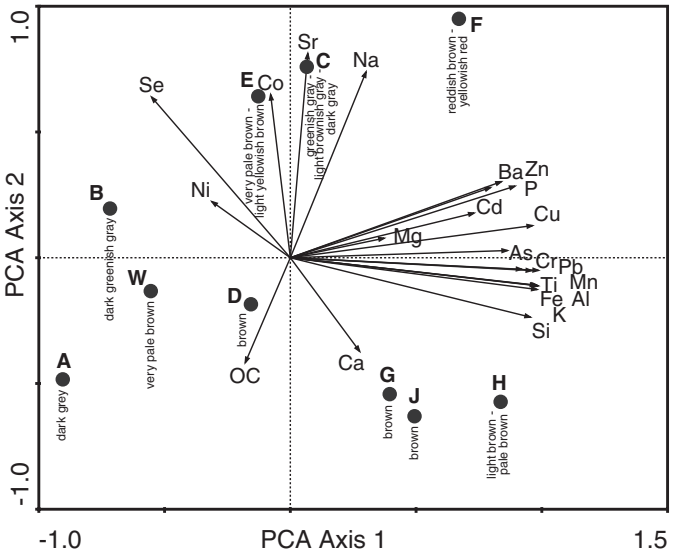

FIG. 3. PCA of elemental and organic carbon content for surface samples of ten formations from the Big Wall site. Formations (●) are marked with their alphabetical label and additionally by the color name using the Munsell color system. Arrows represent the relationship (direction and strength) of each element or organic carbon (OC) content to each formation.

with Ca and organic carbon content. The direction of a biplot arrow in PCA indicates an increase in that variable.

The position of a formation sample along the length of a biplot arrow indicates the concentration of the element represented by the arrow in that formation relative to the other formations. For example, formations H and F had higher concentrations of species that were positively correlated with the first axis than formations A and B (Figure 3). The widely spaced distribution of the 10 speleothems across the PCA plot clearly indicated the variability in elemental content of the different speleothems. Simultaneous plotting of the formation colors on the PCA biplot showed no clear association between speleothem color and elemental content (Figure 3). Speleothem color can be influenced by factors other than those analyzed here, such as the relative concentration of humic and fulvic acids, and inorganic pigments (White 1997).

The Influence of Physical and Chemical Parameters on Bacterial Community Structure

The hypothesis that variations in bacterial community structure are correlated with substrate geochemistry, was evaluated using nine of the elements (Fe, Mn, P, K, Mg, Ca, Co, Cu, and Zn) reported in Table 2. These specific elements were selected for analysis because they were detected in each of the 10 formations and are known to be involved in microbial metabolic processes (Madigan et al. 2006). In addition, organic carbon content was included in our analysis based on the hypothesis that even the trace amounts of organic carbon present may have a significant influence on microbial community structure. Similarly, Fe and Mn were of particular interest due to the potential source of energy for chemolithoautotrophs provided by reduced forms of these elements (Fe^{2+} or Mn^{2+}). Microorganisms that couple oxidation of these ions with CO_2 fixation have been found in various caves (as summarized in Engel 2005). Finally, the relative location of each speleothem along the Big Wall site was also analyzed. The location of each feature was defined in terms of its relative position along a horizontal transect from formation J to A (Table 1, Figure 1b). The bacterial community structure of each formation was characterized by 16S rRNA gene PCR-DGGE. Between 21 and 30 DGGE bands were detected for each of the 10 speleothems analyzed. CCA was used to test the influence of the environmental variables described here on the relative bacterial community structure of each formation.

The first hypothesis tested was that calcium, the dominant element present in all formations (Table 2), might significantly influence the bacterial community structure of the 10 formations. The CCA Monte Carlo permutation test showed no significant effect of calcium on the bacterial community structure of the 10 formations ($p = 0.39$), and so this element was eliminated from further consideration. A second hypothesis tested the influence of the remaining eight trace elements (P, K, Mg, Fe, Mn, Co, Cu, Zn), the organic carbon content, and the relative location of the formations in the Big Wall site on the structure of the bacterial communities.

As explained previously, environmental variables displaying a variable inflation factor (VIF) greater than 20 were excluded from CCA, assuming collinearity of the respective variable with other variables in the examined dataset. The CCA revealed VIF values >20 for Cu, Mn and K, thus these variables were excluded from the hypothesis test. As a result, the final analysis tested the influence of the following variables on the structure of the bacterial communities present on each of the 10 formations: Fe, Mg, P, Co, Zn, organic carbon content and the relative location of each formation in the Big Wall site (Figure 4).

The cumulative species-environment relation for CCA axis 1 (28.5%) and 2 (19.4%) together was 47.9%. After manual forward selection of environmental variables, and testing using Monte Carlo permutation tests, CCA revealed that the relative location of the formations in the site was the only environmental variable with a significant influence on the bacterial community structure ($p = 0.005$). Thus, while the observed differences in substrate geochemistry may influence the presence or absence of a specific bacterial population in a community, none of the six elements evaluated here, nor the organic carbon content was identified as an explanatory variable influencing the speleothem-specific differences in bacterial community structure observed on the formations present in the Big Wall site of Kartchner Caverns.

The conclusion that relative location was the only significant factor found to influence the structure of bacterial communities on the surfaces of speleothems led us to reevaluate the physical

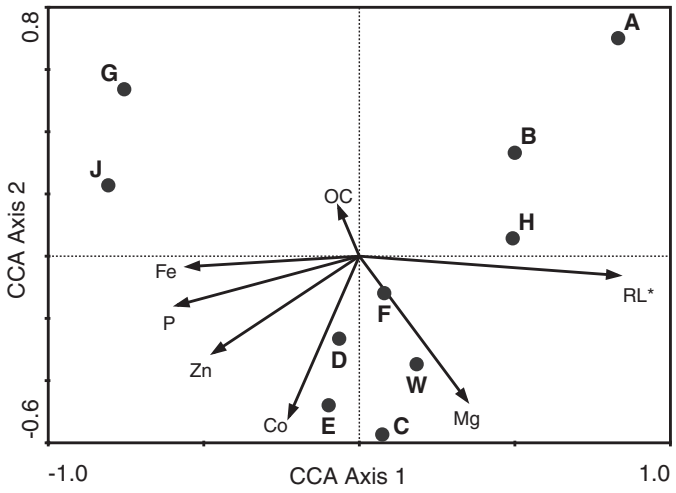

FIG. 4. CCA for bacterial DGGE band profiles for samples of 10 formations (●) from the Big Wall site. The arrows represent the seven environmental variables Fe, P, Zn, Co, Mg, organic carbon (OC) and the relative location (RL). The eigenvalues of the CCA axis 1 and axis 2 are 0.253 and 0.173, respectively. Only RL was found to be significant (*), (RL, $p = 0.005$; Fe, $p = 0.16$; P, $p = 0.20$; Zn, $p = 0.20$; Mg, $p = 0.25$; Co, $p = 0.48$; OC, $p = 0.98$) using forward selection.

structure of the Big Wall sampling site. The objective of the characterization was to identify specific physical and hydrological factors, other than the physical proximity of one formation to another, that could be tested to explain the relationship between formation location and superficial bacterial community structure. For this characterization, we recorded and mapped physical attributes that were specific to the formations sampled, as well the general geologic setting of the sampling site. We observed that all 10 formations were exposed to water entering the cave from along the same fault zone. This fault zone exceeds 10 m in width and exhibits a complex architecture with various fault splays that interconnect and host intervals characterized by varying amounts of fault gouge, clay, breccia, and a number of other geologic features including flowstone and various speleothem types.

West-dipping curvilinear and sometimes branching patterns and zones of active soda straws and small stalactites marked narrow zones that we termed "drip lines." In this section of Kartchner Caverns, drip lines most often originate from leaky faults, fractures and bedding planes in the overhead layered limestone. The drip lines near the Big Wall area were mapped extending downslope and westward of the fault zone, traversing the west-sloping portions of the cave ceiling and flowing down the west wall of the Rotunda-Throne Room to support speleothem growth within our sampling site. These drip lines were characterized by various levels of mineral-staining and the presence of narrow zones of abundant cave formation growth, dominated by soda straws (Figure 5a).

Narrow zones of formations, also dominated by soda straws with occassional turnips, small shields, young stalactites, etc. could be traced or mapped down along the ceiling surface to the region where the drip water sourced the 10 "live" or wet formations that were sampled for this study (Figure 5b). The soda straw patterns or routes suggested that distinct drip lines (or drip water paths) were the primary drip sources for each of the individual formations. Five drip lines connected to the 10 formations were identified; one drip line sourced formations A and B, a second drip line sourced formation C, a third sourced D, W, and F, a fourth sourced H and E, and the fifth drip line sourced J and G.

Dripping water was observed over the last three years on all nine stalactites included among the 10 formations sampled (Table 1), suggesting each of these formations was being actively fed. CCA was used to test the hypothesis that the suggested drip lines influence the bacterial community structure. The 10 formations were assigned to drip line groups according to the observed drip lines leading to the formations. The permutation test indicated a significant influence of the suggested drip lines ($p = 0.004$) (Figure 5c).

These results suggest that variations in bacterial community structure are correlated with individual or interconnected drip lines identified above the formations and throughout the room. One explanation for this observed association could be that drip water not only supplies the moisture facilitating speleothem growth, but also nutrients serving as potential substrates for microbial activity. Drip water analysis from Altamira Cave, a cave in northern Spain, revealed potential nutrient sources such as SO_4^{2-}, Mg^{2+}, K^+ and NO_3^- in the drip water along with the high concentrations of Ca^{2+} and HCO_3^- (Cañaveras et al. 1999; Laiz et al. 1999). The dissolved organic carbon concentration in the drip water was highly variable from less than 5 mg C L^{-1} in winter to about 2200 mg C L^{-1} in late spring (Laiz et al. 1999). The total organic carbon values detected in drip water samples from Lechuguilla Cave, a cave located in the arid northern Chihuahuan desert of New Mexico, were much lower ranging from < 1.0 to 1.3 mg L^{-1} (Levy 2007).

In addition, Mn^{2+} and inorganic nitrogen in the form of NH_4^+ and NO_3^- were also detected. In general, the drip water flow rates are variable and the metal ions, minerals, and organic carbon transported both in solution and in suspension by the subsurface water source are influenced by the path traveled from the surface. The amounts of bacterial cells transported with drip water can also vary between different sampling points (Laiz et al. 1999). Thus, we hypothesize that components of the drip water, e.g., the consistency and rate of flow over time, the concentration of elements or organic carbon transported or the actual bacterial populations transported with the water over time, have influenced the evolution of the unique microbial community present on the surface of each of the speleothems. Further investigation of this hypothesis would require investigation of the structure of these communities over time to evaluate

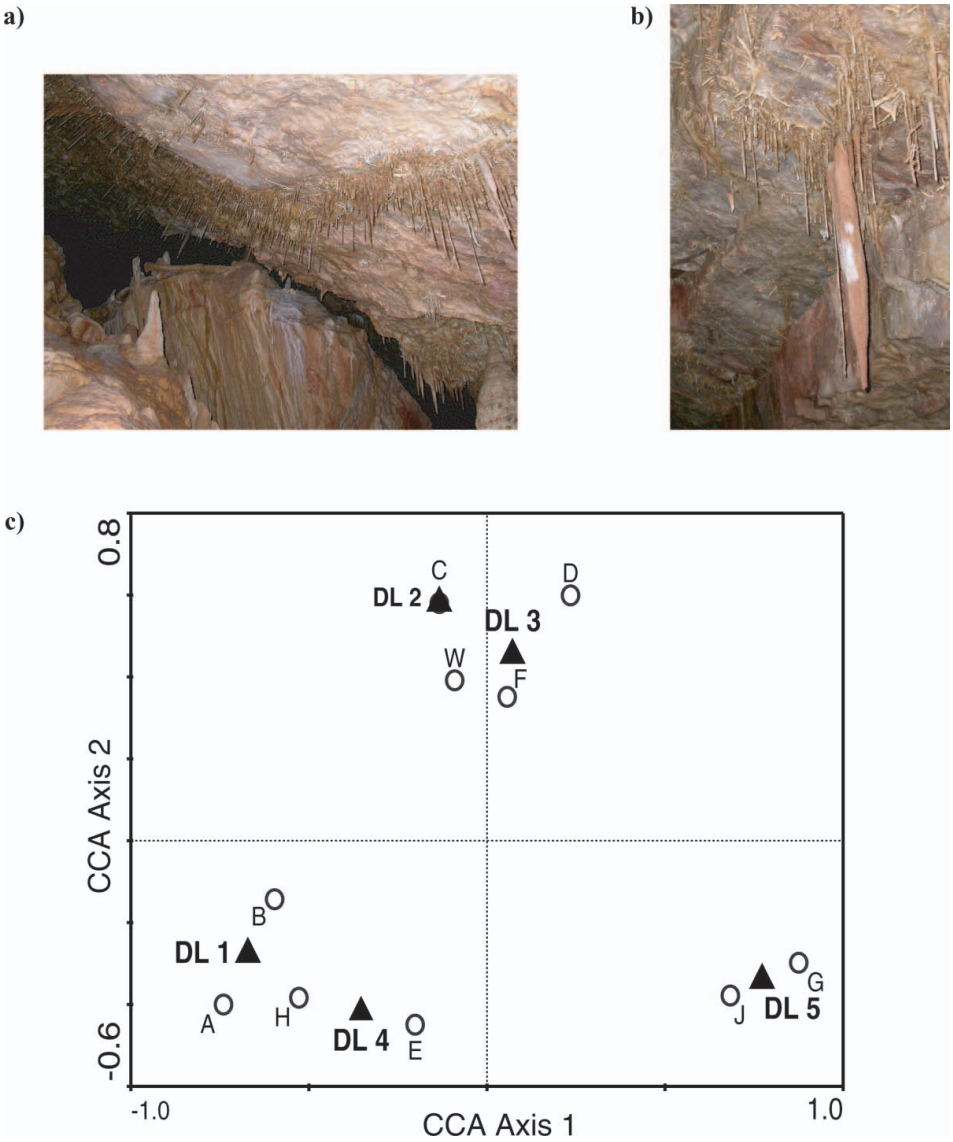

FIG. 5. a) An example of the accumulation of soda straws and small stalactites on the ceiling of the Big Wall fault zone referred to as a drip line. b) Formation J with adjacent soda straws and young stalactites indicating the drip water source. c) CCA for bacterial DGGE band profiles evaluating the potential effect of the suggested drip lines on the bacterial community structure. The eigenvalues of the axis 1 and axis 2 are 0.244 and 0.177, respectively. The formations (◯) are shown. The centroids (▲) for each drip line (DL) group are represented. The centroids represent the weighted mean of the specific drip line multivariate data set along all axes (color figure available online).

whether the populations present at this time represent a stable or evolving consortium.

A Comparison of the Bacterial Community Structure on Formations in the Big Wall Room to That of Formations in the Strawberry Alcove Area

PCR-DGGE bacterial community profiles from eight (A, B, C, D, E, F, H, W) of the 10 formations from the Big Wall site were compared to the two samples analyzed in a previous study from two stalactites located (Cb and Sb) in the Strawberry Alcove site (Figure 1a) (Legatzki et al. 2011) using DCA (Figure 6). This comparison was conducted to evaluate whether the differences in bacterial communities within a single room were similar in magnitude to differences observed between those communities and communities from speleothem surfaces in a different region of the cave.

The DCA plot revealed that the bacterial communities of formations from the same room clustered more closely than those of formations from a separate room along the first axis. However, the β-diversity between the samples measured by DGGE was still quite low. Thus, CCA was used to test the hypothesis that sample origin, referring to the location within

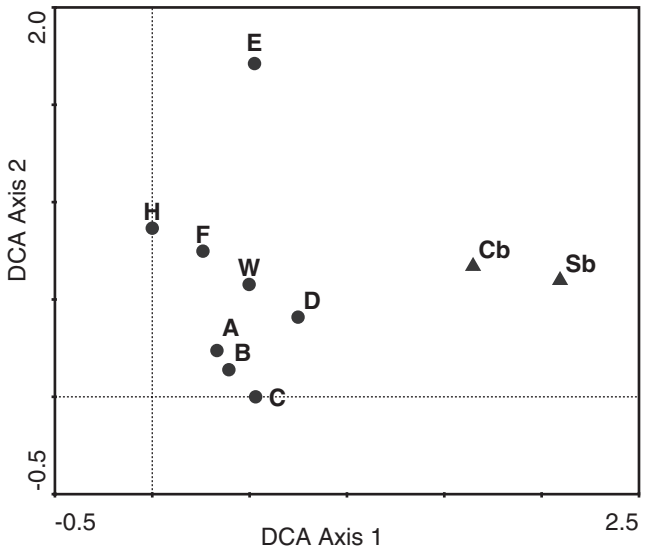

FIG. 6. DCA for bacterial DGGE band profiles of eight formation samples (●) from the Big Wall site and two formation samples (▲) from the Strawberry Alcove site. The samples are marked with their alphabetical label.

either the Big Wall site or the Strawberry Alcove site, had an effect on the bacterial community structure. The CCA results indicated that the bacterial community structure of a speleothem was affected significantly by its association with a particular cave room ($p = 0.015$).

Various factors can explain the observation that bacterial communities on formations from the same room are more similar to each other than to those found on formations from a second room. The two rooms are located in distinct portions of the cavern and are exposed to different environmental factors. In addition, the Strawberry Alcove site is located in a portion of the caverns that houses an annual colony of *Myotis velifer* bats that roosts in the central part of the Big Room from late April until mid September (Buecher and Sidner 1999), while the Big Wall site is not exposed to these bats. Theoretically, this could lead to differences in nutrient sources or microorganisms that might be transported by the cave atmosphere or the atmospheric condensate (Cunningham et al. 1995).

Furthermore, the Big Wall analysis indicated that the drip water associated with each formation influences the bacterial community structure of specific speleothems. The flow rate of drip water is variable depending on seasonal rainfall and local hydrology and concentrations of nutrients may ebb and flow within this variable water supply depending on the subsurface flow path traveled by the water source. Different regions of the cave are exposed to water traveling distinct paths through alternate fault lines and the depth of the two rooms related to the ground surface is quite different, 65.8 m for the Big Wall site and 46 m for the Strawberry Alcove site. These are all possible differences between the two rooms that could influence the microbial community structure of the respective speleothem surfaces.

CONCLUSIONS AND BROADER CONTEXT

In summary, the results presented in this paper indicate that the structure of bacterial communities colonizing the surfaces of calcite speleothems is speleothem-specific, and influenced by the location of the individual formations within the cave. In previous work (Legatzki et al. 2011), we speculated that the speleothem specificity of the superficial microbial community structure was correlated with the substrate geochemistry of the formation surfaces. While the elemental composition was similar among the 10 formations, elemental concentrations were unique for each formation.

However, the differences in the community structure of the 10 speleothems in the Big Wall site could not be correlated with concentrations of either organic carbon or any of the specific elements evaluated, despite the fact that there was a order of two in magnitude difference in concentration between some of the elements analyzed (including Fe and P). Emphasis must be placed on the fact that these PCR-DGGE structural profiles are designed to reflect the relative presence of dominant populations in a broad range of bacterial communities (community members with $> 1\%$ abundance) and cannot be used as measures of quantitative community diversity (Muyzer et al. 1993; Gelsomino et al. 1999).

Broad ecological surveys of microbial communities that include large numbers of samples, must rely on community profiling strategies because the work involved in generating large numbers of Sanger sequenced clone libraries for diversity analysis is prohibitive. PCR-DGGE was selected as the profiling strategy used to compare the 26 samples analyzed for this report because we have found this technique to give consistent representation of microbial community structure (Drees et al. 2006; Grandlic et al. 2009; Maier et al. 2010). Thus, the conclusions presented in this paper do not imply that the presence, absence or relative activity of individual bacterial populations is not influenced by differences in substrate concentrations. Rather we find that the observed differences in the overall community structure cannot be explained by these variables. In contrast, the relative location of the formations in the cave, and specifically their location with respect to drip water sources, was found to be an environmental variable imposing a significant influence on the superficial bacterial community structure, implying that drip water source may influence the bacterial community present on the surface of a specific formation.

Future work will further investigate the patterns presented in this work. First, a five year study is being conducted evaluating the stability of bacterial communities on speleothem surfaces over time to determine whether the formation specific differences observed here are transient or stable. Preliminary observations presented by Legatzki et al. (2011) of samples taken over a one year period from two formations suggest that community structure is relatively stable. The bacterial communities evaluated from multiple samples taken over time from a single speleothem remained more similar to each other than to similar samples taken from a different speleothem. Second, a concurrent study is focused on characterizing the

diversity of the bacterial communities present on the formations in the Big Wall site in order to further explore the structural patterns observed in this study.

We have demonstrated that bacteria not only successfully colonize the calcite surfaces of carbonate caves, but their communities vary among different speleothems. Extensive characterization of cave drip water collected over time may provide insights into the specific nutrient sources driving the diversity of these bacterial communities and evidence for whether the community structure responds to fluxes in nutrient concentrations. The influence of drip water source on bacterial community structure indicates that a thorough sampling of multiple speleothems fed by drip water from a variety of fault zones must be conducted to fully characterize the bacterial diversity present on speleothems in such karst ecosystems. Concurrently, the study underlines the importance of intensive geochemical and physical characterization of sampling sites and their settings to be able to compare microbial communities between different cave systems or even between different regions of the same cave.

REFERENCES

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K. 1995. Preparation of genomic DNA from bacteria. In: Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K, editors. Current Protocols in Molecular Biology. New York: John Wiley & Sons, Inc., p. 2.4.1–2.4.2.

Baker GC, Smith JJ, Cowan DA. 2003. Review and re-analysis of domain-specific 16S primers. J Microbiol Meth 55:541–555.

Banks ED, Taylor NM, Gulley J, Lubbers BR, Giarrizo JG, Bullen HA, Hoehler TM, Barton HA. 2010. Bacterial calcium carbonate precipitation in cave environments: A function of calcium homeostasis. Geomicrobiol J 27:444–454.

Barton HA, Northup DE. 2007. Geomicrobiology in cave environments: Past, current and future perspectives. J Cave Karst Stud 69:163–178.

Barton HA, Taylor NM, Kreate MP, Springer AC, Oehrle SA, Bertog JL. 2007. The impact of host rock geochemistry on bacterial community structure in oligotrophic cave environments. Int J Speleol 36:93–104.

Brodie EL, DeSantis TZ, Parker JP, Zubietta IX, Piceno YM, Andersen GL. 2007. Urban aerosols harbor diverse and dynamic bacterial populations. Proc Natl Acad Sci USA 104:299–304.

Buecher DC, Sidner RM. 1999. Bats of Kartchner Caverns State Park, Arizona. J Cave Karst Stud 61:102–107.

Cañaveras JC, Hoyos M, Sanchez-Moral S, Sanz-Rubio E, Bedoya J, Soler V, Groth I, Schumann P, Laiz L, Gonzalez I, Sainz-Jimenez C. 1999. Microbial communities associated with hydromagnesite and needle-fiber aragonite deposits in a karstic cave (Altamira, northern Spain). Geomicrobiol J 16:9–25.

Cunningham KI, Northup DE, Pollastro RM, Wright WG, Larock EJ. 1995. Bacteria, fungi and biokarst in Lechuguilla Cave, Carlsbad-Caverns-National-Park, New-Mexico. Environ Geol 25:2–8.

Drees KP, Neilson JW, Betancourt JL, Quade J, Henderson DA, Pryor BM, Maier RM. 2006. Bacterial community structure in the hyperarid core of the Atacama Desert, Chile. Appl Environ Microbiol 72:7902–7908.

Engel AS. 2005. Chemoautotrophy. In: Culver DC, White WB, editors. Encyclopedia of Caves. New York: Elsevier/Academic Press. p. 90–102.

Engel AS. 2010. Microbial diversity of cave ecosystems. In: Barton L, Mandl M, Loy A, editors. Geomicrobiology: Molecular and Environmental Perspectives. New York: Springer. p. 219–238.

Ferris MJ, Muyzer G, Ward DM. 1996. Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. Appl Environ Microbiol 62:340–346.

Ford D, Williams P. 2007. Karst Hydrogeology and Geomorphology. West Sussex, UK: John Wiley & Sons Ltd.

Gelsomino A, Keijzer-Wolters AC, Cacco G, van Elsas JD. 1999. Assessment of bacterial community structure in soil by polymerase chain reaction and denaturing gradient gel electrophoresis. J Microbiol Meth 38:1–15.

Grandlic CJ, Palmer MW, Maier RM. 2009. Optimization of plant growth-promoting bacteria-assisted phytostabilization of mine tailings. Soil Biol Biochem 41:1734–1740.

Hill CA. 1997. Kartchner Caverns, Arizona. In: Hill CA, Forti P, editors. Cave Minerals of the World. Huntsville, AL: National Speleological Society, p. 350–354.

Hill CA, Forti P. 1997. Cave minerals of the world. In: Hill CA, Forti P (Eds.)Cave Minerals of the World. Huntsville, AL: National Speleological Society. p. 117–216.

Hutchens E, Gleeson D, McDermott F, Miranda-CasoLuengo R, Clipson N. 2010. Meter-scale diversity of microbial communities on a weathered pegmatite granite outcrop in the Wicklow Mountains, Ireland; evidence for mineral induced selection? Geomicrobiol J 27:1–14.

Jagnow DH. 1999. Geology of Kartchner Caverns State Park, Arizona. J Cave Karst Stud 61:49–58.

James JM. 1997. Minor, trace, and ultra trace constituents of speleothems. In: Hill CA, Forti P (Eds.)Cave Minerals of the World. Huntsville, AL: National Speleological Society, p. 236–237.

Laiz L, Groth I, Gonzalez I, Saiz-Jimenez C. 1999. Microbiological study of the dripping waters in Altamira cave (Santillana del Mar, Spain). J Microbiol Meth 36:129–138.

Legatzki A, Ortiz M, Neilson JW, Dominguez S, Andersen GL, Toomey RS, Pryor BM, Pierson LS, Maier RM. 2011. Bacterial and archaeal community structure of two adjacent calcite speleothems in Kartchner Caverns, Arizona, USA. Geomicrobiol J 28:99–117.

Legendre P, Legendre L. 1998. Numerical Ecology. Amsterdam: Elsevier. 853 p.

Lepš J, Šmilauer P. 2003. Multivariate analysis of ecological data using CANOCO. Cambridge, UK/New York: Cambridge University Press.

Levy DB. 2007. Oxidation-reduction chemistry of Lechuguilla Cave seepage. J Cave Karst Stud 69:351–358.

Madigan MT, Martinko JM, Brock TD. 2006. Brock Biology of Microorganisms. Upper Saddle River, NJ: Pearson Prentice Hall.

Maier RM, Palmer MW, Andersen GL, Halonen MJ, Josephson KC, Maier RS, Martinez FD, Neilson JW, Stern DA, Vercelli D, Wright AL. 2010. Environmental determinants of and impact on childhood asthma by the bacterial community in household dust. Appl Environ Microbiol 76:2663–2667.

Munsell C. 1976 Munsell Book of Color, Glossy Finish Collection. Baltimore, MD: Kollmorgen Corporation.

Muyzer G, Dewaal EC, Uitterlinden AG. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction amplified genes coding for 16S ribosomal RNA. Appl Environ Microbiol 59:695–700.

Northup DE, Lavoie KH. 2001. Geomicrobiology of caves: A review. Geomicrobiol J 18:199–222.

Palmer MW. 1993. Putting things in even better order—The advantages of Canonical Correspondence-Analysis. Ecology 74:2215–2230.

ter Braak CJF. 1986. Canonical Correspondence-Analysis—A new eigenvector technique for multivariate direct gradient analysis. Ecology 67:1167–1179.

ter Braak CJF, Šmilauer P. 2002. CANOCO Reference manual and CanoDraw for Windows User's Guide: Software for Canonical Community Ordination (version 4.5) Ithaca, NY, USA: Microcomputer Power.

ter Braak CJF, Wiertz J. 1994. On the statistical-analysis of vegetation change—A wetland affected by water extraction and soil acidification. J Veg Sci 5:361–372.

van Beynen P, Bourbonniere R, Ford D, Schwarcz H. 2001. Causes of colour and fluorescence in speleothems. Chem Geol 175:319–341.

White WB. 1997. Color of speleothems. In: Hill CA, Forti P, editors. Cave Minerals of the World. Huntsville, AL: National Speleological Society. p. 239–244.

Whitman WB, Coleman DC, Wiebe WJ. 1998. Prokaryotes: The unseen majority. Proc Natl Acad Sci USA 95:6578–6583.