Assessment of Human Impact on the Culturable Microbial Diversity of Kartchner Caverns

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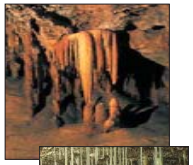
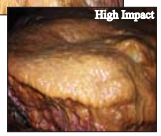
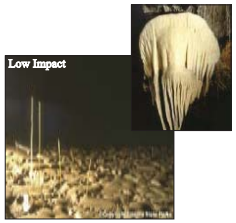
Abstract and Background

Kartchner Caverns, discovered in November 1974 in the Whetstone Mountains of southern Arizona, is a "living" cave in which carbonate features continue to grow due to percolating waters from the surface. In an effort to maintain Kartchner Caverns as a living cave system, it has been carefully developed and access to the cave, which was opened to the public in 1999, is restricted and closely supervised. Working with Arizona State Parks personnel, the cave was divided into areas characterized as: high impact (> 200,000 visitors per year), moderate impact (30-40 visitors per year), or low-impact (< 2 visitors per year). Bacteria in samples taken from these three areas were isolated on R2A agar, and Gram-stained to determine purity. Morphologically identical isolates were then grouped using Repetitive Extragenic Palindromic Sequence (REPS) PCR. Unique isolates were selected for PCR amplification of the entire 16s rDNA gene. The 1500 base pair PCR product was sequenced, and then identified using BLAST analysis. Results indicate that the high- and moderate-impact areas have higher culturable bacterial counts, but lower morphological diversity than low-impact areas. Specifically, culturable counts ranged from 12 to 5750 CFU/cm^2 in the moderate- and high-impact areas while they ranged from 11 to 58 CFU/cm^2 in the low-impact areas. Gross diversity was initially determined from a visual examination of morphologically distinct colonies, and showed that the moderate- and high-impact areas had 11 to 38 different colony types while the low-impact areas yielded 75 different colony types. Each of the unique colony morphs are undergoing identification based on their 16s rDNA sequences. Results completed thus far indicate a wide diversity of soil-borne bacteria. The culturable bacteria isolated from Kartchner caverns appear to be highly sensitive to the presence of humans as evidenced by the changes in population size and diversity observed in this study.







Objectives of the Current Study

- To compile a preliminary library of culturable bacteria from Kartchner Caverns based on the entire 1500 b.p. 16s-rDNA gene
- To assess whether anthropogenic pressure contributes to changes in the diversity of culturable heterotrophic bacterial populations on cave walls (flowstone)

Materials and Methods

Isolation, DNA extraction, and identification of culturable heterotrophs

1. Sample swabs were collected aseptically from three separate cave rock surfaces (flowstone) in each of the high, moderate, and low impact zones of the cave. Sterile water served as the medium for transport back to the laboratory for same-day analysis.

2. Sample tubes were vortexed for 5 minutes to allow for physical release of cells, and subsequent dilution plating from 10^{-1} to 10^{-5} using R2A medium. Plates were incubated in a humidified chamber at 25^{o}C. Unique colonies were isolated from each of the dilutions as they appeared, with a total incubation time of 12 weeks for slow-growing microbes. Isolates were re-streaked to obtain pure cultures and Gram-stained.

3. REPS-PCR was performed on isolates with similar morphologies, and a 3% agarose gel was run to differentiate the unique isolates.

4. The purified isolates were grown in sterile R2B until turbid growth was apparent, then subjected to freeze (liquid N_2)-thaw (boiling H_2O) for lysing. 16s rDNA PCR was performed on the lysates using the following protocol:

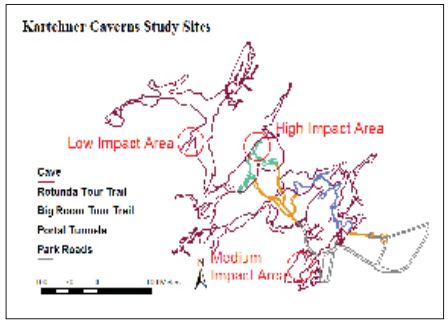
Reagent	Per 50 µL RxN (µL)
Molecular-grade H_2O	23.3
Buffer B	5.0
Primer mixture (27f and 1492r)	5.0
BSA	5.0
dNTP's	4.0
DMSO	2.5
Taq polymerase (reg)	0.2
Template DNA (lysate)	5.0

Cycling Times (min)	Temp (^{o}C)	Purpose
5	95	initial denaturation
1*	**94**	**denaturation**
1*	**60**	**annealing**
1.25*	**72**	**extension**
10	72	final extension

* 30 total cycles were run.*

5. 1.5 - 2% agarose gels were used to determine successful amplification of the 1500 base pair product.

6. Products were sent to the University of Arizona LMSE facility for purification, quantification, and sequencing. BLAST analysis via the NCBI database served to identify the isolates.



Results

Table 1. Bacterial isolates from the high impact area of Kartchner Caverns (>200,000 visitors per year)

Isolate ID	Nearest Relative	Accession # of Nearest Relative	% Homology
B12-21	*Alcaligenes eutrophus*	M32021	99
F2	*Curtobacterium sp. SG041*	AF474329	99
	Curtobacterium flaccumfaciens	AJ312209	99
D4	*Erythromonas ursincola*	AB02489	99
	Sphingomonas natatoria	AB024288	99
M4	*Flavobacterium johnsoniae*	AB078043	98
A4a	*Kocuria erythromyxa*	Y11330	98
I1	*Phenanthrene-degrader*	AY117357	99
	*Sphingomonas sp. IFO 15917**	AB033950	98
B17	*Pseudomonas alcaligenes*	AF094721	99
J4	*Pseudomonas anguilliseptica BI*	AF439803	98
G1	*Pseudomonas sp. HR 26*	AY032726	99
B7, B15	*Pseudomonas sp. SMCC B0628*	AF501878	99
	Pseudomonas sp. SMCC B0361	AF500621	99
	Pseudomonas sp. DHA-51	AJ011507	99
B13	*Sinorhizobium sp. BK1*	AJ012210	100
B11, A3	*Sphingomonas sp. S37*	AF367204	99
C15	*Uncultured soil bacterium*	AF423261	99
O4	*Uncultured gamma proteobacterium*	AF324537	99
R4	*Variovorax sp. K6*	AF532867	99
I4	*Variovorax sp. TUT1027*	AB098595	99

*2nd highest match offering the genus description.

Table 3. Bacterial isolates currently sequenced from the low impact area of Kartchner Caverns (< 2 visitors per year)

Isolate ID	Nearest Relative	Accession # of Nearest Relative	% Homology
L18	*Bacillus cereus ATCC 14579*	AE017013	99
	Bacillus cereus strain BGSC	AE016998	99
L31	*Bacillus firmus*	AJ509007	98
L45	*Bacillis fusiformis*	AJ310083	99
L46	*Bacillus sp. ICSC-1*	AB159770	100
L101	*Bacillus indicus*	AJ583158	99
L66	*Bacillus sp. LMG 21002*	AJ316308	99
L105	*Bacillus luciferensis*	AJ419629	99
L41	*Bacillus sp. MK03*	AB062678	99
L33	*Lysobacter sp. Dae16*	AB166878	97
L48	*Mesorhizobium chacoense*	AJ278249	99
L47	*Mycobacterium elephantis*	AJ536100	97
L24	*Paenbacillus alvei*	AJ320491	95
L63	*Paenbacillus kobensis*	AB073363	98
L44	*Pseudomonas sp. HR 26*	AY032726	99
L53	*Streptomyces lipmanii*	AB045861	99
L49	*Streptomyces KN-1220*	AY029699	98
L49	*Streptomyces phaeochromogenes*	AF500071	98
L42	*Streptomyces sp. VTT E-99-1326*	AF429390	98
L16	*Taxeobacter sp. SAFR-033*	AY167829	95

Table 2. Bacterial isolates from the moderate impact area of Kartchner Caverns (30 - 40 visitors per year)

Isolate ID	Nearest Relative	Accession # of Nearest Relative	% Homology
37a	*Aminobacter amonivorans*	AJ011759	99
53a	*Bacillus asahii*	AB109209	98
	Bacillus sp. 19489	AJ315057	98
C1b, C1a1	*Bacillus cereus LRN*	AY138275	100
	Bacillus cereus 2000031498	AY138274	100
P3, S2	*Bacillus sp. LMG 21002*	AJ316308	100
Lc	*Bacillus sp. LMG 20241*	AJ316313	99
58a	*Bacillus pichinotyi RS2*	AF519464	97
B1	*Bacillus pumilus KL-052*	AY030327	100
72a	*Bacillus pumilus*	AB020208	100
Qb	*Bacillus sp. PAMU-1.13*	AB118223	99
22a1	*Bacillus sp.*	AJ276809	98
23a-a1	*Bacillus thuringiensis*	AF155954	99
	Bacillus cereus ATCC 53522	AF290551	99
65a	*Bacillus sp. 433-D9*	AY266991	100
CC1	*Bacillus sp.*	AB017587	99
8a	*Nocardiodes sp. A3*	AB087724	93
10a	*Phenanthrene-degrading bacterium*	AY177375	97
O2	*Pseudomonas sp. CA-10*	AB047273	99
44a	*Pseudomonas HR 26*	AY032726	99
57a	*Pseudomonas PCP2*	AF326380	98
Na, C1	*Ralstonia sp. AU3369*	AF500587	99
	Uncultured bacterium CCMC0	AY221074	99
	Ralstonia sp. BPC3	AF494541	99
C1b	*Ralstonia eutropha KT1*	AB015605	99
11a	*Rhodococcus equi DSM 2030*	AF490539	98
A3	*Sphingomonas sp. S37*	AF367204	99

Conclusions

- Isolates from the low impact zone exhibited slower growth rates, and proved more difficult to culture in R2B than those of the high and moderate impact zones.
- Isolates collected from each of the three population impact zones are microbes commonly found in soils.
- Bacterial isolates collected from flowstone rock surfaces in the high and moderate impact zones yielded substantially different identification profiles from one another, with *Pseudomonas, Sphingomonas*, and *Ralstonia* species being cultured from both areas.
- Isolates belonging to *Bacillus* appear to be more predominant in areas of the cave where human contact is less frequent. Although *B. thuringiensis* and *Brevibacillus* were previously isolated from painted fiberglass surfaces, no *Bacillus* isolates were isolated from the high impact, true rock surfaces.

Future Objectives

- Alignment of sequences and construction of phylogenetic trees for current data set(s)
- Continue routine monitoring of changes in the microbial diversity of Kartchner Caverns due to human impact by use of community DNA extraction, 16S rRNA PCR, and DGGE analysis

Acknowledgments

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