ASSESSING HUMAN IMPACT ON HETEROTROPHIC BACTERIAL DIVERSITY IN KARTCHNER CAVERNS – A CULTURE-DEPENDENT STUDY

by

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ABSTRACT

Kartchner Caverns was opened to the public for viewing in November of 1999. Painted fiberglass surfaces installed within the cave during development to disguise piping and electrical wiring began to develop a wet, slimy biofilm. Efforts to remove the biofilm proved unsuccessful as the growth regenerated soon after sanitization. Samples aseptically collected of the biofilm in effort to identify the potentially responsible populations revealed nine unique bacteria, some exhibiting highly mucoid morphology. The paint was tested as a sole carbon source for the biofilm isolates. Seven were able to utilize the substrate, implicating the surface as a selective medium for the observed biofilm colonization and proliferation. Cave rock surfaces were also sampled along a human impact gradient: high (> 200,000 entrants/year), moderate (30 to 40 entrants/year), and low (2 to 3 entrants/year). Taxonomic and phylogenetic analyses revealed differences in heterotrophic populations according to the level of human impact.

INTRODUCTION

Kartchner Caverns is considered a "living" cave as a result of the ongoing formation of secondary cave mineral deposits termed *speleothems*. The mineralogy of Kartchner caverns is highly diverse due to the presence of six different crystal classes: carbonates, nitrates, oxides, phosphates, silicates, and sulfates (Hill and Forti 1997). Carved from a down-dropped block of Mississippian Escabrosa limestone, the caves are situated between the Whetstone Mountains and San Pedro Valley approximately thirteen kilometers south of Benson, Arizona. In 1974, the caverns were discovered by University of Arizona graduate students and spelunking enthusiasts Gary Tenen and Randy Tufts (Tufts and Tenen 1999), and kept a secret until 1978 when the Kartchner family was informed of its existence. To ensure preservation, it was decided that addition of the cave system to the American show cave circuit was the option ensuring restrictions and regulation of its usage. Following many years of continued secrecy and political wrangling at the state level, the property was officially handed over to Arizona State Parks and named the "James and Lois Kartchner Caverns State Park" in 1988. November of 1999 marked the opening of the Rotunda-Throne Room Complex for tours; 25 are given per day with a 20-person quota for each tour for up to 500 visitors per day (Toomey and Nolan 2002).

In early 2001, Raina Maier of the University of Arizona Department of Soil, Water, and Environmental Science received a phone call of concern from Rickard Toomey, Cave Resources Manager at Kartchner Caverns State Park. Cave Unit workers

had observed the presence of a slimy substance on various painted fiberglass surfaces installed during development of the Rotunda-Throne Room Complex. The painted fiberglass moldings were mounted to disguise electrical wiring and components, in addition to maintenance areas that might detract from the visitors' visual experience. In an effort to eliminate the slimy growths, the surfaces were removed from the cave and washed with a mild 10% bleach solution. Despite best efforts to eliminate them, the biofilm growths returned within one month of re-installation into the cave.

During spring of 2001, a sampling excursion was conducted by Julia Neilson, the course of which sample swabs were collected from various painted fiberglass pieces and cave rock surfaces. Microbial communities cultured from the painted fiberglass yielded several mucoid colony types that appeared to predominate. Cave rock cultures revealed a more diverse population of bacterial types in addition to some mucoid colonies, as well as actinomycetes and fungi (Photograph 1).

With such differences evident in the microbial populations between the two surface types, a second sampling investigation was initiated to determine the microbe(s) responsible for the slimy biofilm production on the painted fiberglass, as well as to compile a preliminary set of culturable microbes from cave rock surfaces in the high tourist-traffic area (>250,000 visitors per year) for comparison. The first objective was to determine whether the microbes were an aspect of the normal cave microflora, or transported into the cave by humans during development. This was of particular concern as conservation of the cave is a continuing goal. Once identities and possible origins of the painted fiberglass bacteria were verified, a second objective was investigated

involving the role of the painted fiberglass in selecting for the given microbial communities to establish the slimy biofilms. It was hypothesized that the paint was serving as a carbon source for the given consortium of microbes, allowing for colonization of the surface.

Results from the previous sampling expeditions, which indicated relatively increased morphological diversity of heterotrophic bacteria cultured from natural cave surfaces, prompted further studies concerning the impact human presence may have on the culturable heterotrophic bacterial communities of the caverns. During the summer of 2003, samples were collected from areas of the cave designated as moderate (30 to 40 visitors per year – Grand Central Station) and low impact (2 to 3 visitors per year – Subway Tunnel) to identify and compare microbial populations representative of the culturable microflora of the caverns (Figure 1, Table 1). Cave rock isolates cultured during the previous sampling runs in the Rotunda-Throne Room were representative of high impact zone (>250,000 visitors per year) bacterial populations. All isolates obtained were subjected to Repetitive Extragenic Palindromic- PCR (REP-PCR) analysis for differentiation and grouping of unique isolates, and followed by16s-rDNA PCR and sequencing for identification. Taxonomic and phylogenetic relationships of the bacterial communities, as well as associated environmental factors in each impact area, were then compared to determine whether increased human presence demonstrates an effect on the culturable heterotrophic bacterial diversity within the cave.

LITERATURE REVIEW

Types of Caves

Caves are defined as natural openings in rock at least 0.3 m in diameter (Gillieson 1997). They are carved from karstic terrain, where the topography is visually characterized by depressions due to dissolving of rock (i.e. limestone, gypsum, and sandstone) with relatively high solubility (Gillieson 1997). The most common type of karstic terrain is limestone, exemplified by Kartchner Caverns, Arizona and Mammoth Cave, Kentucky, the world's longest cave system. Natural dissolution of the limestone occurs as CO_2, released within the soil profile during heterotrophic oxidation of humus and other organic substrates, reacts with water to form carbonic acid (Gillieson 1997). Although weak relative to some of the more potent inorganic acids in nature including sulfuric acid (pK_a=1.92, 25^{O}C), carbonic acid (pK_a=10.25, 25^{O}C) easily promotes dissolution of the limestone mineral calcite ($CaCO_3$) into its component soluble ions (Equation 1).

carbon dioxide + water ↔ carbonic acid

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \quad (1)$$

carbonic acid + calcite ↔ calcium bicarbonate solution

$$H_2CO_3 + CaCO_3 \leftrightarrow Ca^{2+} + 2HCO_3^{-1} \quad (2)$$

Equation 1. Carbonic acid limestone dissolution

Sulfur, partnered with bacteria associated with ancient oil deposits, has been an important factor in the formation of many acid caves around the world including Carlsbad Caverns, New Mexico and Frasassi Gorge, Italy. Using methane as a carbon source and sulfur bound in the mineral gypsum as the terminal electron acceptor during respiration, these bacteria produce copious amounts of highly reduced hydrogen sulfide gas. Migration of the gas into the water table where it reacts with oxygen results in the formation of sulfuric acid. A stronger acid than carbonic in terms of dissolution power, diprotic sulfuric acid also reacts with calcite to form cavities in limestone as well as chemical by-products (Moore and Sullivan 1997) as seen in Equation 1. Note that the CO_2 produced during the process can also form carbonic acid to further promote dissolution within the limestone (Equation 2).

hydrogen sulfide + oxygen ↔ sulfuric acid

$$H_2S + 2O_2 \leftrightarrow H_2SO_4 \quad (1)$$

sulfuric acid + calcite ↔ calcium sulfate solution + carbonic acid

$$H_2SO_4 + CaCO_3 \leftrightarrow Ca^{2+} + SO_4^{2-} + CO_2 + H_2O \quad (2)$$

$CO_2 + H_2O \rightarrow H_2CO_3$ (carbonic acid)

Equation 2. Sulfuric acid limestone dissolution

Other types of subterranean landforms are sandstone caves, littoral (sea) caves, glacier and ice caves, and lava tubes. These caves are not only formed by way of solution chemistry, but also by physical processes such as wind erosion, tectonic movements and subjection to gravity. Designated as pseudokarsts, physical evidence of cave formation is often seen alongside features associated with chemical weathering, an example being the Proteozoic sandstones of Venezuela (Gillieson 1997). Wind and water erosion of sandstone has resulted in formation of shallow shelter caves that were preferred by early peoples for habitation, one famous example being the Mesa Verde Cliff dwellings of the southwest United States (Moore and Sullivan 1997). Wave erosion governs the formation of sea caves located at the base of coastal cliffs (Walthum 1976, Moore and Sullivan 1997). Numerous sea caves are located on the Pacific coast of the United States, such as Sea Lion Cave in Oregon.

Cave Life

As *trogloxenes*, or "cave guests", humans have utilized these enclosures through the ages for purposes of storage, residential shelter, burial of the dead, and spiritual odysseys. Ancient artists have left fantastic glimpses into the past upon the walls and ceilings of numerous caves throughout the world. Some of these wonders have become popular with naturists and hikers, in many cases leading to deterioration of the ancient paintings and/or a decline in overall cave health, one example being Altamira Cave in Spain. Other cave guests include a variety of animals that also usually live above ground. Bears, bats, skunks, moths and mosquitoes are known to seek refuge within caves from

the elements during some part of their individual life cycles (Moore and Sullivan 1997). The moist and dark environment of caves also allows for inhabitation of *troglophiles*. These "cave lovers" wander in and out of caves regularly and are adapted to life with little to no visible light, such as nocturnal crustaceans living under rocks and earthworms (Mohr and Poulson 1966).

Caves are considered extreme environments due to absence of light past the twilight zone, the area of the cave receiving light from the entrance sufficient to permit human vision (US Environmental Protection Agency 2002). As a result, plants are unable to thrive via photosynthesis and fulfill the role as a sink of organic material (Northup and Lavoie 2001). Full-time cave dwellers, termed *troglobites*, include numerous species of insects, fish, and salamanders characterized by reduced pigmentation and very small eyes or none at all (Moore and Sullivan 1997), in addition to low metabolic rates corresponding to the nutrient-poor environment (Mohr and Poulson 1966). The simplest troglobites are single-celled protozoans and the slightly more complex cave planarians that feed on the bodies of bats and other creatures that fall to the floor or into cave pools (Moore and Sullivan 1997). A biological survey conducted of Kartchner Caverns from 1989 to 1991 documented 38 invertebrate species including (11%) troglobites, (50%) troglophiles, and 1 trogloxene, in addition to 32% accidentals (animals that wander in or fall into the cave, but cannot survive there) (Wellbourn 1999).

Although considered *trogloxenes*, bats are vital to many cave ecosystems including that of Kartchner Caverns. Bat feces (guano) provide nutrients that allow for movement of energy from the invertebrate troglobites down to microbial heterotrophs. It

has been suggested that development of the Big Room Complex, opened October of 2003, may threaten a roost of about 1000-2000 insectivorous cave bats (*Myotis velifer*) that migrate to Kartchner Caverns each summer to give birth and nurture their young. Carbon-14 dating of the guano piles indicates the caverns have served as a maternity roost for the past 40,000 to 50,000 years (Buecher and Sidner 1999). If human presence becomes too intrusive the protective female bats could abandon the roost altogether, resulting in a marked decrease in food supply to the numerous obligate cave dwellers that depend upon the guano. Virtually most of the cave fauna, including 14 species of mites, exercise a strict dependence on the guano piles (Welbourn 1999).

The cave food pyramid sets forth a very delicate balance among the oligotrophic environment's life forms. Light penetrating into cave from the outside provides a gradient along which varying species of life can be found. The twilight zone marks the area of the cave where visible light sufficient for human vision penetrates, leading into the transition zone where no visible light is evident but external factors, such as seasonally fluctuating temperatures, are influential. A cave's dark zone is characterized by complete absence of light, high humidity, and relatively constant temperature dependent primarily on the annual mean surface temperature. Leafy plants are not inhabitants of caves past the twilight zone due to inability to photosynthesize (Moore and Sullivan 1997), while some species of green algae may be found only slightly further into the cave for the same reason (Mohr and Poulson 1966). Yet despite their visible absence within a cave's dark zone, plants provide nutrients in the form of dissolved carbon as debris can be transported via air or water flow (Mohr and Poulson 1966, Waltham 1976, Moore and Sullivan

1997). These nutrient reservoirs allow for proliferation of heterotrophic decomposers including fungi, actinomycetes, and bacteria in the nutrient-poor dark zone. Release of carbon dioxide to the cave environment by way of microbial respiration and degassing of water during mineralogical processes, accompanied with the lack of ventilation in the cave system, allows for CO_2 build-up similar to that seen in soils. In turn, the gas serves as the carbon source for populations of indigenous chemolithotrophs (Moore and Sullivan 1997).

Geomicrobial Investigations in Caves

Most cyanobacteria are classified as obligate photolithoautotrophs, depending upon the sun for energy and inorganic materials (usually CO_2) as a source of carbon. They are thought to participate in the constructive (building) processes of caves, particularly by way of calcite precipitation. Dense layers of exopolymeric substances (EPS) are produced by the microbes in effort to adhere to surfaces and establish communities. When permeating waters are supersaturated with respect to dissolved calcite ($CaCO_3$), the mineral infiltrates the porous EPS, leading eventually to calcification and encrustation of the microbial sheath (Davis and Rands 1981, Jones and Motyka 1987, Merz 1992, Leveille et al. 2000). The calcified microbes then serve as nucleation sites for further calcite precipitation (Jones 2001). Over 500 species of cave algae in 21 forms have been identified in caves around the world (Draganov 1977). Algal filaments also have been shown to promote the growth of speleothems in the caves of Grand Cayman Island (Jones and Kahle 1985, Jones and Motoyka 1987).

Fungi, actinomycetes, and heterotrophic bacteria are decomposers of organic material in both surface and subterranean environments. While fulfilling the role of scavenger, their metabolic byproducts and eventually their own decaying cell mass contributes to the cave food chain. Community fungal populations of caves have been studied in southern India (Koilraj et al. 1999), Lechugilla Cave of New Mexico (Northup et al.1994), and West Virginia where the similarity of cave fungal microflora was compared to that of desert and tropical soils (Rutherford and Huang 1994). In terms of geomicrobiological activity, the fungus *Cephalosporium lamellaecola* was found to participate in $CaCO_3$ precipitation within water droplets suspended from stalactite tips (Went 1969). Fungi have also been implicated in dissolution processes that eventuate in alteration or even breakdown of carbonate crystals in the presence of minimal amounts of water (Jones and Pemberton 1986). A diverse population of actinomycetes was documented in northern Spanish caves based on chemotaxonomic analyses (Groth et al. 1999), and isolates from the Grotto dei Cervi, Italy were found to precipitate calcite, indicating a possible role of actinomycetes in cave mineral deposition (Groth et al. 2001).

Calcium carbonate precipitation has also occurred via heterotrophic bacterial populations isolated from caves in southern Wales, in media containing calcium salts (Danielli and Edington 1983). Basic microbial respiration involving oxidation of a carbon source to CO_2 has been described as a cause of calcite precipitation in both aerobic and anaerobic conditions (Erlich 2002). Heterotrophic bacteria occupy the lower layers of microbial mats, mineralizing the organic remains of upper-layer cyanobacteria. Calcium carbonate precipitation transpires within the mats as depth increases, implicating

the underlying heterotrophic populations (Krumbein 1979, Chafetz and Buczynski 1992). Oligotrophic chemoheterotrophic bacteria thought to be introduced by way of water filtration through overlying rock, including *Seliberia* and *Caulobacter,* were isolated from the pools of Lechugilla Cave, New Mexico (Northup et al. 1994). These microbes were deemed indigenous due to their oligotrophy and residence in both well-traveled and remote areas of the cave.

Unlike heterotrophs who derive carbon from organic material, chemolithotrophs residing in caves synthesize nutrients from inorganic sources (usually cave minerals) in order to build new cell mass. Hydrogen sulfide caves have provided much of the known information concerning chemolithotrophic processes in such environs, particularly sulfuric acid production (Hose et al. 2000, Engel et al. 2001). In caves receiving virtually no allochthonous input of organic substrates, chemolithotrophs are indeed the only source of organic carbon for higher organisms including protozoans, demonstrating their importance in energy transfer through consumption in a cave environment (Langecker et al. 1996, Sarbu et al.1996, Moore and Sullivan 1997). The Movile Cave ecosystem of Romania is one of the earliest studied examples of such an environment. Chemolithotrophic sulfur-oxidizing bacteria including *Thiobacillus* and *Thiosphaera*, among others, were isolated from cave waters and microbial mats (Sarbu et al. 1994). Carbon and nitrogen isotopic analyses revealed that all organic carbon present in the system is derived from in situ chemoautotrophic production (Sarbu et al.1996). Lechuguilla Cave in New Mexico also receives limited organic and hydrologic input, and it was found that at least five varieties of chemolithotrophic bacteria were supporting an

extensive population of chemoheterotrophic bacteria as well as fungi (Cunningham 1995). The ecology of chemoautotrophic microbial mats and biofilms in acid caves has been researched, uncovering various species of sulfur-oxidizing bacteria (Mattison et al.1998, Thompson and Olson 1988, Vlasceanu et al. 2000). A study in the underwater Nullarbor Caves of Australia found mucoid mantles supported by novel nitrite-oxidizing, chemoautotrophic communities (Holmes et al. 2001).

Microbial Biofilms and Ancient Cave Paintings

Studies involving effects of microbial activity on cave paintings left by ancient peoples have been concentrated in Spain. Unmonitored, the caves were subject to curiosity-seekers who deposited garbage and disrupted the natural environment. The paintings of Altamira and Tito Bustillo caves have been inundated by biofilms comprised of phototrophic cyanobacteria and algae, as well as heterotrophic bacteria and actinomycetes (Groth et al. 1999, Canaveras et al. 2001). Cyanobacteria, capable of slow growth by way of oxidation of glucose and other simple sugars in the absence of sunlight, were also isolated from ancient paintings in Maltravieso Cave, Spain (Arroyo et al. 1997). The iron oxide paintings of Atlanterra rock shelter in southern Spain have also been exposed to anthropogenic pressure. Bacteria of the *Bacillus* genus, particularly *Bacillus megaterium*, were detected from the paintings using fatty acid methyl ester profiling (Gonzalez et al.1999).

Geomicrobial Techniques

Laboratory cultivation has been the primary method of study for both chemolithotrophic (Sarbu et al. 1994, Andrejchuk and Klimchouk 2001, Engel 2001) and heterotrophic populations of cave bacteria (Danielli 1983, Arroyo et al. 1997, Canaveras et al. 1999, Groth et al. 1999, Koilraj et al. 1999, Laiz et al. 1999, Groth et al. 2001, Canaveras et al. 2001). Such studies have utilized media designed for growth of known oligotrophic terrestrial microbes such as R2A. It is believed however that the vast majority of microbes, particularly in environmental samples, have yet to be cultured (Amann et al. 1995, Siering 1998). Advances in nucleic acid technology, such as ribosomal RNA and denaturing gradient gel electrophoresis (DGGE), and molecular phylogeny have allowed for circumvention of cultivation setbacks and increasingly detailed studies of cave microbes. The majority of cave studies employing said techniques have sought to investigate chemoautotrophic populations of hypogenic caves (Angert et al. 1998, Hose et al. 2000, Vlasceanu et al. 2000, Engel 2001). Holmes et al. (2001) investigated mucoidal microbial mantles from aquatic caves (Australia) using 16S-rDNA PCR and restriction fragment length polymorphism (RFLP) analysis. Results pointed to a novel, chemoautotrophic community dependent on nitrite oxidation. The Paleolithic paintings of Altamira and Tito Bustillo Caves (Spain) were also analyzed using culture-independent techniques. Community DNA extraction, 16S rRNA amplification, DGGE fingerprinting, and phylotyping led to the finding of unknown bacterial communities that also had yet to be cultured in vitro (Schabereiter-Gurtner-Altamira Cave, 2002) Schabereiter-Gurtner-Tito Bustillo 2002). More recent culture-

independent studies in Wind Cave, South Dakota (Chelius and Moore 2004), Fairy Cave, Colorado (Barton et al. 2004), and Llonin and La Garma Caves in Spain (Schabereiter-Gurtner et al. 2004) have allowed for simultaneous detection of numerous species of both chemolithotrophic and heterotrophic populations of bacteria, many of which have yet to be cultured. As with culture-based studies, biases have been acknowledged concerning the efficacy of culture-independent techniques including inefficient cell lysis during DNA extraction, preferential amplification of 16s-rDNA sequences, and comigration of sequences during DGGE (Barton 2004, Chelius 2004, Schabereiter-Gurtner 2004). Although molecular phylogeny studies without prior cultivation have uncovered increased patterns of bacteriological diversity in caves, it is acknowledged that culture-based studies are vital for determination of valid physiological and metabolic frameworks in such oligotrophic environments (Barton 2004, Chelius 2004, Schaberieter-Gurtner, Siaz-Jimenez, Pinar 2002, and Schabereiter-Gurtner 2004). In addition, human impact on the presence of microbial communities in a hypogean environment has previously been demonstrated by way of culture-based techniques (Somavilla et al. 1978).

MATERIALS AND METHODS

Sampling Site Information

Phase One: Painted Fiberglass and High Impact Zone Culturable Cave Rock Populations / Utilization of 100% Acrylic Paint as Sole C Source by Painted Fiberglass Isolates

Painted fiberglass surfaces were installed during cave development for aesthetic purposes throughout the Rotunda – Throne Room Complex (24.5 m high x 44 m long), including door overhangs and box-like enclosures constructed to house electronic wiring for cave lighting. The facades began to exhibit growth of a visibly slimy biofilm within eight weeks of installation. In order to determine the microbial populations potentially responsible for the biofilm growth, two painted fiberglass moldings (2.1 m high x 1.4 m wide) were selected for sampling during August 2001 (Photograph 2). Six samples were collected from the painted fiberglass, encompassing approximately 8.6 m^2 of surface area. Located along the 293 m long visitors' trail, each of the barriers displayed a clear, slimy layer over the whole of their surfaces. Coated with a mixture of white, beige and brown paints presumed to be 100% acrylic-based upon expert consultation, the two pieces were visually similar in color and textural appearance to cave walls. Their original purpose was two-fold - to shield a small maintenance area just off of the visitors' path, and retain an element of surprise as the centerpiece formation of the complex, Kubla Khan, remains hidden behind the large fiberglass pieces until visitors reach a designated sitting area for a unique light show.

Collection and analysis of cave rock samples were performed for comparison of the culturable heterotrophic microbial populations between the two surfaces, and to carry out a preliminary assessment of cave microflora. Sample swabs were also gathered during the August 2001 sampling trip from four separate cave rock surfaces – three located on either side of the painted fiberglass slabs, and the fourth directly across the visitor's path. The total area of sample coverage was approximately 5.0 m^2.

Phase Two: Evaluation of Human Impact

Since initiating development of the cave for viewing by the public, the Arizona State Parks Service has aimed to maintain the cave environment as close to pre-human impact conditions as possible. Results from the first phase of the study demonstrated the effects of anthropogenic pressures via inadvertent selective growth of cave microflora, prompting further inquiry into the microbiological diversity of Kartchner Caverns. Of particular interest was the potential impact of human presence in effecting changes in the composition of culturable heterotrophic communities. For the third phase of the study, the cave was zoned according to the average number of human visitors gaining access per year (Figure 1, Table 1).

The Rotunda - Throne Room Complex was the first section of Kartchner Caverns opened to the public for tours in November of 1999. The area was designated as high impact (HI) due to the extensive number of visitors passing through it annually (Table 1). Isolates previously cultured from cave rock during August of 2001 served as the high impact community example for the second phase of the study.

Samples from the moderate and low impact areas for the third phase of the study were collected during June 2003. A large section of the Big Room Complex (122 m long x 73 m long) opened for tourist visitation in October of 2003. Tours are seasonally scheduled so as not to disrupt the summer maternity roost of approximately 1000-2000 *Myotis velifer* bats, which have frequented the cave for at least 40,000 years (Buecher and Sidner 1999). Designated as moderate impact with only 30 to 40 authorized persons entering each year, samples were collected in the Grand Central Station area located in the southwest corner of the Big Room (~ 100 m from the visitors' trail) that would maintain limited access (no visitors allowed) even after the Complex was opened. The low impact Subway Passage is an area of the cave rarely frequented by humans, seeing only 2 -3 human entrants each year for monitoring purposes. It is characterized by seasonal submersion during the rainy seasons. No bat roosts are located in the area – during sampling one lone bat was encountered.

Sample Collection and Processing

Samples of painted fiberglass and cave flowstone surfaces (30 cm^2 surface area per swab) were collected in triplicate to ensure representative populations were isolated. Aseptic conditions were maintained to as high a degree as possible by use of pre-sterilized materials. Cotton-tipped swabs were used to collect the samples, which were then immersed in propylene test tubes containing 3 ml of sterile tap water (pH = 7.2 to 7.8). Transport of the samples back to the University of Arizona on ice was followed by same-day processing of the samples.

Samples were prepared for dilution plating by vortexing the swabs for five minutes to ensure a high degree of microbial removal from the cotton fibers. Dilution plating on R2A medium was performed to 10^{-5} for the purposes of enumerating heterotrophs, and to allow for visual assessment of culturable morphological diversity. Incubation conditions ranged from 23 to 25^{O}C in a humidified chamber for a three-month time period.

Isolation and Grouping of Culturable Bacterial Isolates

During the three-month course of incubation, morphologically distinct colonies were selected for streak plating on R2A from each of the dilutions as they appeared. Visualization of the cells using the Gram's stain (Johnson 1998) served as confirmation of isolation. Selection of colonies from each dilution series (10^{-1} to 10^{-5} CFU/mL) coupled with multiple rounds of streak plating resulted in the isolation of morphologically similar colonies. Colony size, shape, color, and concavity served to characterize and group applicable isolates as morphologically similar. Confirmation of identical genotypic patterns among the morphologically similar isolates was performed using Repetitive Extragenic Palindromic- PCR (REP-PCR), a fingerprinting technique that utilizes a primer mix compatible with naturally repetitive sequences interspersed with known functional operons in the bacterial genome. The method allows for differentiation of bacterial isolates, with variances observed even at the strain level (Versalovic 1991).

In preparation of pure broth-based cultures, one or more colonies were inoculated into five ml of R2B, and agitated on a gyratory shaker (Labline Instruments, Inc., Melrose Park, R.I.) at 170 rpm until turbidity was observed. One ml quantities of each isolate were then transferred to heat-freeze tolerant 1.7 ml microfuge tubes, and centrifuged in an Eppendorf Centrifuge (Model 5415c) at 13,000 rpm for 10 minutes to pellet the cells. The supernatant was removed, and one ml of autoclaved and UV sterilized dd-H_2O was added to each tube; vortexing prompted resuspension of the cells into solution.

Lysing of the cells was then performed to obtain genomic DNA for further analysis. Three freeze-thaw cycles employing liquid nitrogen and boiling de-ionized water were followed by a 15-minute boil and final freeze in liquid nitrogen. Cell lysates were stored at -20^{O}C for the duration of the study.

Cell lysates of isolates exhibiting morphological similarity on R2A were thawed at room temperature (24 to 25^{O}C) and subjected to REP-PCR analysis according the reagent protocol and PCR conditions outlined in Table 2. UV-sterilized dd-H2O served as the negative control. To prepare the REP products for separation and visualization, 5.0 µl of each 25 µl REP-PCR product solution was mixed with 5.0 µl of 6X Loading Buffer, and separated using a 3% NuSieve 3:1 agarose gel (FMC Bioproducts, Rockland, ME) in 1xTBE Buffer. Morphologically similar isolates were run side-by-side on the gels when possible for ease of comparison. Voltage was set at 100 V, and the gel exposed for three hours to ensure optimal separation of the bands. The gel was then stained in 40 µg/µl ethidium bromide (EtBr) for 30 minutes, and rinsed for 10 minutes in dd-H_20 to remove

excess EtBr. Visualization of the gel was performed using the AlphaImager 2000 (Alpha Innotech Corporation, San Leandro, CA) at a UV wavelength of 302 nm. The fingerprints were then compared to determine whether the isolates were identical or different (Photograph 3).

Amplification of the 16s ribosomal-DNA Gene

To further the process of microbial identification, 16s ribosomal-DNA (rDNA) PCR was performed for amplification of the 1500 base pair gene on isolates deemed unique by REP-PCR. Universal primers 27f and 1492r were employed for attachment to the flanking conserved regions, allowing for initiation and amplification of the highly variable intra-regions for subsequent sequencing and identification. As an objective of the project involved obtaining reliable identities based upon the entire 1500 base pair region, internal primers were also used to ensure amplification of the mid-sequence area of the 1500 base pair gene.

Initially, four separate 50 µl 16s-rDNA PCR reactions were run for each bacterial isolate (primers 27f, 1492r) in effort to acquire the 1500 base pair product (Table 3). UV sterilized/dd-H20 served as the negative control, while *Escherichia coli* originally served as the positive control. As the study progressed *Variovorax sp. K6*, isolated from the Throne Complex, replaced *E. coli* as the positive control due to its ability to consistently amplify under the conditions of the 16s-rDNA PCR reaction protocol utilized in this study (Table 3).

Amplification success of the 1500 b.p. gene was determined by mixing 3.5 µl of each 50 µl PCR product with 5.0 µl of 6X Loading Buffer. Mixtures of both the positive and negative controls, the 16s-PCR products from the bacterial lysates, as well as 5.0 µl of a 100 b.p. ladder size standard, were loaded separately into the wells of a 1.5% agarose gel. The gel was run for one hour at an applied field of 120 Volts, stained in 40 µg/µl ethidium bromide (EtBr) for 20 minutes, and rinsed for one minute in dd-H_20 to rid excess EtBr. Visualization of the gel was performed once again using the Alphaimager 2000, at a UV wavelength of 302 nm (Photograph 4).

DNA Clean-up, Quantification, and Sequencing

Successfully amplified 16s-rDNA products were submitted to The University of Arizona Lab of Molecular Systematics and Evolution (LMSE) for purification, quantification, and sequencing under their 96-well plate program. An Excel file was created to diagram the location of the isolates and their respectively assigned primers on the 96-well plates. (Figure 4). Two sterile, thin-walled polypropylene plates covered with aluminum sealing tape were submitted for each sequencing trial - one to contain only the 1:10 primer dilutions (5 µl per well), and the other storing the DNA templates of interest (remaining 46.5 µl of PCR product, brought up to 100µl using autoclaved, UV-sterilized dd-H_20). Each 50-µl reaction (four per isolate) was assigned a well on the "Template" 96-well plate (example: well A6). The corresponding well on the primer plate (also well A6) allowed for amplification of the DNA template in Well A6 with that primer alone.

In order to achieve the best possible identification, primers were used that ensured amplification of the internal and external regions of the 1500 b.p. gene. During much of the course of the study for the 5' to 3' direction, primers 27f (5' AGA GTT TGA TCC TGG CTC AG 3') and 518f (5' CCA GCA GCC GCG GTA AT 3') were used, while the 3' to 5' strand saw amplification via primers 1492r (5' TAC GGT TAC CTT GTT ACG ACT T 3') and 1070r (5' AGC TGA CGA CAG CCA T 3'). At times, faulty sequences were generated for the 27f primer during sequencing, in which case the alternative primer 518r (5' ATT ACC GCG GCT GCT GG 3') was used.

With an average return time of three to four days, LMSE electronically returned the DNA quantification results for each well. Wells with a concentration of at least 1 ng/µl of DNA were then approved for sequencing by the facility. Sequence results were available within two to three days, and retrieved by accessing the LMSE website (http://3700.arl.arizona.edu/). The FAKtory program was used to form contiguous sequences (contigs) based upon the four selected primers run for each isolate. The National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST/) was accessed, and generated contigs were then uploaded into the Basic Local Alignment Search Tool (BLAST) to retrieve the top 10 nearest identity matches.

Phylogenetic Analysis of Contiguous Sequences

To initiate phylogenetic comparison of the cultured isolates, the contiguous sequences generated were aligned in GCG/Seqlab using Pileup software. Pileup served

to provide an initial grouping of the isolates according to similarity in the 16s-rDNA gene. File types generated in Pileup (.msf) were then transferred to ClustalX, a tree-building program. In ClustalX, the sequence groups were subjected to "complete" alignment, which allowed for a more detailed output file (.aln). Columns generated during complete alignment lacking nucleotides then were removed to eliminate potential bias during tree-building. Neighbor Joining-Bootstrap trees were constructed with 1000 repetitions for confidence. Generated trees (.phb) were viewed and formatted using Treeview software.

Compilation and Sourcing of Taxonomic and Physiological Descriptions

Taxonomic assignments were made for all isolates according to *Bergey's Manual of Systematic Bacteriology, Second Edition* (Garrity et al. 2001). Morphological and physiological descriptions were described according to *Bergey's Manual of Determinative Bacteriology*, Ninth Edition (Holt at al. 1994).

Utilization of 100% Acrylic Paint as Sole Carbon Source by PF Isolates

This study was conducted during the summer of 2002 to assess the role of the paint in stimulating proliferation of the observed slimy biofilms. It was hypothesized that the paint was serving as a carbon source to select for growth of bacterial communities able to colonize the surface.

Tests were run to determine whether the isolates cultured from the painted fiberglass biofilms were able to utilize the paint as a sole carbon source. A Mineral Salts

Medium (MSM) was employed using Noble Agar to prevent addition of organic impurities (Table 4). R2A and 10% R2A were also tested to assess toxicity effects the paint may have had on growth. An assumption was made that 100% acrylic paint (oil-based) was used for enhanced durability on the painted fiberglass according to expert consultation from Dunn Edwards Paints (Tucson, AZ), as the company that had originally constructed the surfaces was no longer in business at the time of the study. Paint overlay solutions were prepared by dissolving 100% acrylic paint (Dunn Edwards Corporation, Los Angeles, CA) in chloroform to one ml solution concentrations of 10,000 (1%), 1,000 (0.1%), and 100 (0.01%) mg/l. The MSM, R2A, and 10% R2A plates were then overlaid with one ml of the paint-solvent mixture, with a venting period of 0.5 hours under sterile conditions to allow for release of chloroform. Controls of each media type were also run without paint for comparison.

The painted fiberglass isolates were cultured overnight in 5 ml of R2B in preparation for the study. One to two ml aliquots were microfuged at 13,000 rpm and resuspended in one ml of UV-sterilized water. Serial dilutions were prepared for each isolate, and cells were inoculated onto the plates in duplicate sets at a density of ~ 1.0 x 10^{-6} CFU/ml. Incubation took place at 23^{O}C in a darkened, humidified chamber for one month.

RESULTS AND DISCUSSION

In Kartchner Caverns, visualization of a slimy biofilm growth was evident only on the painted fiberglass surfaces installed; no cave rock surfaces were found to exhibit such growth. Samples collected by Julia Neilson during Spring of 2001 from painted fiberglass biofilms and cave rock revealed markedly different microbial populations. A smaller number of primarily mucoid colony types were cultured from the painted fiberglass, while a greater and more morphologically diverse population was collected from nearby cave rock surfaces, including actinomycetes and fungi (Photograph 1). It was apparent that the painted fiberglass was selecting for the growth of a particular community of microbes, some exhibiting highly mucoid morphology.

Microbial counts appeared to demonstrate a positive relationship with human impact - the high impact zone yielded the greatest microbial counts for the cave at 5.6 to 5.7 x 10^3 CFU/cm^2. Increase in colony morphology diversity was observed, however, as CFU/cm^2 decreased (Table 5). Greater morphological diversity was evident in the moderate and low impact areas relative to the high impact zone cultures.

Phase One

Painted Fiberglass Culturable Populations – Taxonomy and Description

Nine unique bacteria were isolated from the two painted fiberglass surfaces (2.1 m x 1.4 m) sampled along the visitors' trail in the Throne Room of Kartchner Caverns. Each bacterium demonstrated markedly different colony morphologies on R2A medium

(Table 6), as well as unique REP-PCR fingerprints (Photograph 3). Amplification of the 16s-rDNA gene from each isolate using primers 27f, 518f, 1070r, and 1492r, followed by assembly of each of the primer fragments employing FAKtory software resulted in contiguous sequences averaging 1,437 base pairs in length. Submission of the contigs to NCBI for BLAST analysis revealed an assemblage of bacteria belonging to three distinct phyla within the domain *Eubacteria*: *Firmicutes*, *Bacteriodetes*, and *Proteobacteria* (Figure 5). Contigs initially aligned using GCG-Pileup, were completely aligned in ClustalX followed by generation of a Neighbor Joining-Bootstrap tree with 1000 repetitions to ensure confidence. *Bacteriodetes* was chosen as the outgroup due to increased numbers of base changes relative to the other two clades from the ancestral node.

Three of the nine painted fiberglass isolates are aerobes or facultative anaerobes belonging to phylum *Firmicutes*: PF-A (*Bacillus thuringiensis*, 99% similarity), PF-G (*Brevibacillus agri strain NCU1002*, 98% similarity), and PF-F (*Staphylococcus sp. es1,* 99% similarity). Bacteria belonging to *Firmicutes* are classified as low G+C gram positive, with DNA G+C base composition measuring less than 50 mol%. Each of the three bacteria shares the same class (*Bacilli*) and order (*Bacillales*), with taxonomic differentiation occurring at the family level (PF-A: *Bacillaceae*, PF-G: *Paenibacillaceae,* PF-F: *Staphylococcaceae*).

PF-A (*B. thuringiensis,* 99% similarity*)* is representative of the largest genus, *Bacillus*, in the order *Bacillales. B. thuringiensis* is a rod-shaped endospore former capable of producing a parasporal body or toxin crystal, which has been increasingly

investigated and utilized for its virulent insecticide properties and use in optimizing agricultural yields. Cultured isolate PF-A's closest relative match (*B. thuringiensis,* 99% similarity*)* was investigated in an effort to enhance technology at the micro-scale for differentiation of closely-related *Bacillus* species including *B. anthracis* (Bavykin et al. 1999). PF-G (*Brevibacillus agri strain NCU1002,* 98% similarity*),* also a rod, belongs to the recently recognized family of *Paenibacillaceae* [Latin *paene*, almost, and bacillus] and has in the past been synonymously associated with *Bacillus galactophilus*, *Bacillus agri,* and *Bacillus pulvefaciens,* with reclassification to *Brevibacillus agri* taking place in 1997 (Nakamura 1997). A Gram positive irregularly clustering cocci, isolate PF-F is most closely related (99% similarity) to *Staphylococcus sp. es1,* which was isolated from a deep-sea station located in the Pacific Nodule Province (Xu 2000).

Painted fiberglass isolates PF-B (*Dyadobacter fermentens*, 98% similarity) and PF-H (*Benzo(a)pyrene-degrading bacterium BPC8*, 97% similarity) are classified as non-proteo, gram-negative members of the phylum *Bacteriodetes*. Taxonomic distinction begins at the class level with PF-B belonging to *Sphingobacteria* and PF-H to *Bacteriodetes*. PF-B's lineage continues with order *Sphingobacteriales* and family *Flexibacteraceae*, with *Dyadobacter fermentens* serving as the most closely related *(98%)* genus and species, respectively. As a class, *Sphingobacteria* are aerobic and exhibit only gliding motility, which requires a surface along which to move as they lack flagella (leaving a slime trail in the process); this trait renders them stationary while suspended in liquid medium. *D. fermentens* was initially isolated from surface-sterilized Zea Mays stems (Chelius and Triplett 2000). Classification of PF-H (*Benzo(a)pyrene-*

degrading bacterium BPC8, 97%) is limited to the class level of *Bacteriodetes,* with information as to the order, family, genus, and species unavailable due to lack of study. Preliminary assignment of PF-H to family *Bacteriodaceae* (order *Bacteriodales*) is unlikely however as members are obligately anaerobic; all bacteria were subjected to oxic growth conditions during the course of this study. Other possible family affiliations under order *Bacteriodales* for PF-H are *Rikenellaceae, Porphyromonadaceae,* and *Provotellaceae*. *Benzo(a)pyrene-degrading bacterium BPC8* was a member of a bacterial consortium studied for its capability to mineralize benzo(a)pyrene (Kanaly et al. 2002). Morphologically, PF-H was of particular interest in this study due to its highly mucoid appearance and predominance on each of the dilutions (10^{-1} to 10^{-5} CFU/mL) performed from the slimy painted fiberglass biofilm for isolation purposes (Table 6).

The remaining four painted fiberglass isolates belong to the large and morphologically, metabolically, and reproductively diverse phylum *Proteobacteria*: PF-M *(Rhizobium huatulense,* 98%), PF-I (*Sphingomonas sp. AVC6,* 99%*)*, PF-D (*Sphingomonas sp., strain B1, 100%), and PF-K (Sphingomonas sp. P2,* 98%*)*. *Proteobacteria* are mostly Gram negative and contain the purple phototrophic bacteria and their relatives (Prescott et al. 1999). Although highly diverse, 16rRNA sequence comparisons for proteobacteria demonstrate phylogenetic relatedness. Phylum *Proteobacteria* is divided into five classes designated by the Greek letters α (alpha), β (beta), γ (gamma), δ (delta), and ε (epsilon). The four isolates are α-proteobacteria encompassing a wide range of metabolic capabilities (chemoheterotrophic, photolithotrophic, chemolithotrophic, and methylotrophic). α-Proteobacteria are largely

oligotrophic, characterized by their typical residence in low nutrient level niches (i.e. soils) and ability to utilize a wide range of organic substrates. PF-M is gram-negative and pleomorphic under conditions of nutrient stress, belonging to order *Rhizobiales*, family *Rhizobiaceae*, with the genus and species names of *Rhizobium huatulense.* Members of genus *Rhizobium* are typically associated with root nodule cells of legumes as nitrogen-fixing symbionts. *R. huatalense* was differentiated taxonomically from *Rhizobium galagae* as both a novel species and symbiont of *Sesbania herbacia* (Wang et al.1998). Isolates PF-I (*Sphingomonas sp. AVC6,* 99%), PF-D (*Sphingomonas sp., strain B1, 100%),* and PF-K (*Sphingomonas sp. P2,* 98%) are each categorized in order *Sphingomonadales*, family *Sphingomonadaceae*, and genus *Sphingomonas*, with differentiation at the species level. The closest relatives for the isolated sphingomonads have been investigated for their ability to degrade aromatic compounds including hydroxylated and methoxylated monocyclics (PF-I: *Sphingomonas sp. AVC6,* 99%) (Di Gioia et al. 2002), chlorophenol (PF-D: *Sphingomonas sp., strain B1, 100%)* (Nohynek et al.1996), and phenanthrene (PF-K: *Sphingomonas sp. P2,* 98%) (Pinyakong et al 2003).

Assessment of Utilization of 100% Acrylic Paint as Sole Carbon Source by Painted Fiberglass Isolates

A number of studies have been previously conducted investigating biodeterioration of ancient wall paintings found in both churches and hypogean environments (Rolleke et al. 1996, Gonzalez et al. 1999, Groth et al. 1999, Gurtner et al., 2000, Schaebereiter-Gurtner et al. 2002, Schaebereiter-Gurtner et al. 2004). Findings

show that natural and artificial light sources stimulate primary production by phototrophic microbes (i.e. cyanobacteria), allowing for secondary metabolism by chemoheterotrophic populations while filamentous growth patterns of actinomycetes and fungi mechanically disrupts the painted surfaces. An investigation of biodegraded rock paintings in Atlanterra rock shelter (southern Spain) revealed the causative organisms as a heterotrophic population of bacteria belonging mostly to the *Bacillus* genus. These bacteria were found to reduce Fe (III)-(hydr)oxides from the mineral hematite for cellular uptake of Fe (II), a necessary metabolic component (Gonzalez et al. 1999). Results obtained via fatty acid methyl ester analysis (FAME) demonstrated that visual disruption of the paints took place along a redox gradient; the paint was not investigated as a possible source of carbon for the isolated *Bacilli*.

Knowledge of such studies led to formulation of the hypothesis that paint used to coat the fiberglass surfaces was serving as an organic substrate during respiration for a consortium of microbes able to colonize the surface. Establishment of this selective niche within the cave would allow for excessive proliferation of heterotrophic microbes normally held in check by competing fungi, actinomycetes, and bacteria found within cave rock communities. Each of the nine bacterial isolates cultured from the painted fiberglass surface were tested for their ability to utilize 100% acrylic paint as a sole source of carbon at three concentrations: 10, 000 mg/L, 1,000 mg/L, and 100mg/L. Of the nine isolates, two (PF-F: *Staphylococcus sp. es1*, 99% and PF-G: *Brevibacillus agri strain NCU1002,* 98%) did not produce any colony growth during the one-month incubation period. Isolates PF-K (*Sphingomonas sp. P2,* 98%*)* and PF-M *(Rhizobium*

huatulense, 98%) demonstrated growth only at the lowest concentration of paint (100 mg/L). The remaining five were able to utilize the paint as a sole source of carbon at each of the three tested concentrations (Table 7). All isolates displayed ample growth on both 10% and regular-strength R2A with paint overlay at all three concentrations. Therefore paint toxicity was eliminated as a factor of influence for the isolates studied. No growth was observed on MSM without paint, assuring aseptic conditions were maintained.

The slimy appearance of the painted fiberglass biofilm, in addition to the predominance of highly mucoid isolates from each dilution of the series of sample swabs relative to those collected from cave rock, indicates that these particular populations are a major component of the slimy growths. Both the mucoid PF-B and PF-H were able to utilize the paint as a substrate along with five other isolates, yet their phenotype appeared to prevail in both the biofilm and the dilution series from the cultured plates. Particulars of how the biofilm formed on the painted fiberglass in Kartchner Caverns were not tested in this study. The mucoid nature and physiological behaviors of isolates like PF-B and PF-H, in addition to the general knowledge of biofilm formation, do allow for assumptions to be made as to their possible role(s) in establishment and maintenance of the biofilm. As a sphingobacterium, PF-B lacks flagella, but is capable of movement via gliding motility when attached to a surface. A slime trail evident in its wake is thought to aid in both attachment to the surface and lubrication for more efficient movement. As PF-H has been classified only to the class level of *Bacteriodetes*, the trait of gliding

motility will not be assigned. Copious slime production, however, is a highly visible morphological trait of this organism.

Based upon the taxonomy of PF-B and PF-H and phenotypes resulting from membership in such a lineage, in addition to the presence of an optimal surface for colonization due to the presence of a readily mineralized food source, a possible mechanism can be offered as to the proliferation of the slimy biofilm. Although a number of other organisms isolated from the painted fiberglass may have genotypic and phenotypic traits favorable for biofilm formation not readily apparent during the course of this study, both PF-B and PF-H demonstrate morphological traits typified by the slimy biofilm. In addition, the *Sphingomonads* isolated from the painted fiberglass (PF-I, PF-D, and PF-K), although not visibly mucoid in their morphology, likely contributed to maintenance of the biofilm due to their ability to degrade aromatic hydrocarbons. Oxidation of residual aromatics within the paint would allow for these populations to proliferate, their decomposing matter serving as fodder for other heterotrophs within the biofilm matrix.

Proposed Mechanism of Biofilm Formation

1. "Clean" painted fiberglass surfaces (lacking evidence of biofilms slimy or otherwise) were installed in the Throne Room of Kartchner Caverns at various locations.

2. Dripping waters from overlying soil and rock that characterize Kartchner Caverns as a living cave, containing microbes and dissolved organic materials, fall upon the installed surfaces as well as cave rock and formations.
3. Surface conditioning of the painted fiberglass begins as dissolved organic materials in the waters bind to the paint. Microbes including *Sphingobacteria* (PF-B) also reach the surface and are able to exhibit gliding motility, leaving slime trails in their paths. *Bacteriodetes* (PF-H), exhibiting enhanced attachment capabilities due to its highly mucoid nature, also reaches the surface and begins colonization.
4. Enhanced proliferation of the highly mucoid bacteria (likely due to oxidation of the paint) establishes a protective matrix, allowing for infiltration and establishment of residency for other microbes. Some are able to use the paint as an organic substrate during respiration while others rely upon the dissolved organics that continue to percolate with waters from the overlying terrestrial strata, or also decomposing cellular material and slime trails from within the biofilm.

High Impact Cave Rock Culturable Populations – Taxonomy and Description

Twenty-one unique bacterial isolates were cultured from three cave rocks sampled in the Throne Room– two were located on either side of and adjacent to the painted fiberglass slabs that were sampled (Table 8). The third sample was collected from a rock surface directly across the visitor's path from the painted fiberglass pieces approximately

1.8 m away. These samples were originally collected in an effort to determine the culturable heterotrophic bacterial populations residing on natural surfaces for comparison to those inhabiting the slimy biofilms on the synthetic painted fiberglass. Contiguous sequences generated of the 16s-rDNA gene averaged ~1346 base pairs in length for submission to NCBI BLAST in effort to retrieve the closest relatives for the isolates. Taxonomic and phylogenetic analyses reveal four distinct phyla: *Proteobacteria, Actinobacteria. Firmicutes*, and *Bacteriodetes* (Figure 6).

Two of the twenty-two isolates were taxonomically classified in the phylum *Firmicutes,* class *Bacilli*, order *Bacillales*, family *Bacillaceae*, and genus *Bacillus*, comprised of low G+C Gram positives: HI-G2 (*Bacillus TB3-10-I* / *Bacillus thuringiensis 4Q281 / Bacillus thuringiensis ATCC 10792,* 100% similarity) and HI-D3 (*Bacillus macroides* / *Bacillus sp. LMG 21002,* 100%similarity). HI-G2 displayed 100% sequence similarity with painted fiberglass isolate PF-A (*B. thuringiensis,* 99% similarity) although the colony morphologies were visually different (Tables 5 and 6). *Bacilli* are renowned for high conservation of the 16s gene, making it difficult to differentiate members of the genus at the species level, and particularly at the strain level. Thus, it is highly probable that two closely related, yet morphologically different strains of *B. thuringiensis*, would generate identical taxonomy returns from BLAST. The contig generated for isolate HI-D3 returned two types of Bacilli for identification. *Bacillus macroides* was identified and characterized due to its association with the midgut of the Colorado Potato Beetle (Herndon and Spence 1999). *Bacillus LMG 21002* was one of a

consortium of bacteria implicated in the biodeterioration of mural paintings in St. Martin's Church, Germany (Gorbushina et al. 2004).

One non-proteo, Gram negative member of phylum *Bacteriodetes* was isolated from the cave rock surfaces in the Throne Room – isolate HI-M4 (*Flavobacterium johnsoniae*, 98%). The microbe is a member of class *Flavobacteria*, order *Flavobacteriales*, family *Flavobacteraceae*, with the genus and species names of *Flavobacterium johnsoniae*. Although both are classified in phylum *Bacteriodetes*, flavobacteria are mostly nonmotile unlike the gliding *Sphingobacteria* (e.g. PF-B, *D. fermentens*, 98%), and do not form endospores as do the *Bacilli* (PF-A, *B. thuringiensis,* 99% similarity*)*. Members of the genus *Flavobacterium* are denitrifiers, capable of using nitrate as a terminal electron acceptor, resulting in its reduction to N_2 or N_2O gas. Many of the bacteria are classified as psychrophiles, which have an optimal growth temperature of $15^{O}C$ or lower, are also members of the genus *Flavobacterium*.

Two high-G+C (> 50 mol% G+C), Gram positive bacteria belonging to phylum *Actinobacteria* were isolated: HI-F2 (*Curtobacterium sp. SG041/ Curtobacterium flaccumfaciens,* 99%) and HI-A4a (*Kocuria erythromyxa,* 98%). The two actinomycetes share class *Actinobacteria*, subclass *Actinobacteridae*, order Actinomycetales, and suborder *Micrococcineae*. Differentiation begins at the family level, with HI-F2 belonging to family *Microbacteriaceae*, genus *Curtobacterium*, and species *SG041/flaccumfaciens*. *Curtobacteria* begin morphologically as short rods in young cultures, and then become coccoid in old cultures. They are also obligate aerobes, motile by peritrichous flagella, and nonspore-forming. *Curtobacterium sp. SG041* and

Curtobacterium flaccumfaciens, (99%) have been isolated and characterized during studies assessing microbial diversity of prairie grass soils (Behrendt et al. 2002, Zinniel et al. 2002). HI-A4a diverges taxonomically from HI-F2, grouping with family *Micrococcaceae*, genus *Kocuria*, and species *erythromyxa*. The available studies citing *K. erythromxya* concern molecular systematics and classification of the organism from as early as 1981 (Brooks and Murray 1981).

The final seventeen of the twenty-one isolates cave rock isolates from the Throne Room are taxonomically classified in phylum *Proteobacteria*. At the class level there is further distinction: five of the seventeen are part of the largely oligotrophic α-proteobacteria (HI-CDE1: *Methylobacterium sp. strain F73*, 99%; HI-I1: *Phenanthrene-degrading bacterium M20 / Sphingomonas sp.IFO 15917*, 98%; HI-B13: *Sinorhizobium sp. strain BK1*, 99%; HI-K4: *Sphingopyxis chilensis, 100%*, and HI-D4: *Sphingomonas ursincola / Sphingomonas natatori*, 99%). The five isolates are then sub-divided into two orders. Order *Sphingomonadales*, family *Sphigomonadaceae*, and genus *Sphingomonas* classifies two of the isolates with differentiation at the species level. As previously mentioned, *Sphigomonads* have been investigated for their ability to degrade aromatic hydrocarbons, demonstrating their importance in the field of bioremediation. The closest relative match of isolate HI-I1 is no exception as it was investigated for its phenanthrene-degrading capabilities in a soil column system (Bodour et al. 2003). HI-D4 was reclassified from *Erythromonas ursincola* to *Sphingomonas ursincola* due to slight variations in the 16s gene (Hiraishi et al. 2000). Isolate HI-K4, while sharing the same order and family with HI-I1 and HI-D4, diverges at the genus level to *Sphingopyxis*, with

the species classification of *chilensis*. HI-K4 was found to degrade chlorophenol, as was painted fiberglass isolate PF-D (*Sphingomonas sp., strain B1, 100%)* in a separate study (Nohynek et al. 1996).

The second order in the α-class of *Proteobacteria* describing the Throne Room cave rock bacteria is *Rhizobiales*. Isolates HI-B13 (*Sinorhizobium sp. strain BK1,* 99%) and HI-CDE1 (*Methylobacterium* sp. strain F73, 99%) belong to this order, with variance occurring at the family level. HI-B13 is classified with family *Rhizobiaceae,* genus / species *Sinohizobium*, and *strain BK1*. Like the genus *Rhizobium*, *Sinorhizobium* are also root-nodule bacteria, fixing N_2 in a symbiotic partnership with legumous plants. *Sinorhizobium sp. strain BK1*, the nearest neighbor to isolate HI-B13, has been studied for its ability to uptake the Ti (tumor-inducing) plasmid via conjugal transfer, which can have implications for plant health (Teyssier-Cuvelle et al. 1999). HI-CDE1 is taxonomically grouped with family *Methylobactericeae*, genus/ species *Methylobacterium*, and strain F73. *Methylobacterium sp. strain F73* was one of many chlorine-resistant methylobacteria classified both phenotypically and genotypically from a variety of environments (Hiraishi et al. 1995).

Of the remaining eleven proteobacteria isolated from cave rocks in the Throne Room, six are *β-proteobacteria*. While *β-proteobacteria* overlap with *α-proteobacteria* in terms of metabolic habits – chemoheterotrophs, chemolithotrophs, methylotrophs, and photolithotrophs are members of both classes – they tend to utilize substances for energy that diffuse from the anaerobic zone of habitats such as hydrogen, ammonia, and volatile fatty acids. Each of the four isolates cultured are members of the order *Burkholderiales*,

with differentiation at the family level. HI-ABCE2 (*Alcaligenes eutrophus*, 99%) belongs to family *Alcaligenaceae*, with *Alcaligenes* and *eutrophus* classified as the genus and species names, respectively. An obligate aerobe, *Alcaligenes* is capable of chemolithotrophy as it can oxidize H_2 for energy production. In terms of heterotrophy, it can use the sugar D-fructose, but prefers organic acids for respiration.

β-proteo isolates HI-I4 (*Variovorax sp. TUT1027*, 99%) and HI-B9 (*Variovorax sp. K6*, 99%) under the order *Burkholderiales* are members of family *Comamondaceae* and genus *Variovorax*, with differentiation at the species level. Members of the genus *Variovorax* were classified with *Alcaligenes* until 1991 (Willems et al. 1991). Each genus has overlapping characteristics such as H_2 chemolithotrophy and ability to use most known organic acids for carbon, but differ as *Variovorax* is capable of growth on simple sugars (i.e. D-glucose and D-Xylose). Closest relative matches of both HI-I4 (*Variovorax sp. TUT1027*, 99%) and HI-B9 (*Variovorax sp. K6*, 99%) were recently studied as degraders of N-acylhomoserine lactones in relation to quorum-sensing applications of plant-pathogenic bacteria (Uroz et al. 2003).

Family *Oxalobacteraceae* (order Burkholderiales) contains both isolates HI-D2 (*Oxalobacter sp. p8E* , 98%) and HI-G4 (*Ultramicrobacterium strain ND5,* 99%). The genus *Oxalobacter* includes only one species, *formigenes*, but there are numerous genus-strain pairs including HI-D2 (*Oxalobacter sp. p8E*, 98%) *Oxalobacter* is both nonsporing and nonmotile, and can use the organic acid oxalate as a sole source of carbon and energy. *Oxalobacter sp. p8E* was studied as part of a consortium of protease-producing bacteria from an Antarctic soil. The genus *Ultramicrobacterium* is not listed

in the second edition of Bergey's Manual of Systematic Bacteriology (2001). The position of HI-G4 on the Bootstrap-Neighbor-Joining tree (1000 repetitions) relative to the other isolates shows that it shares the same common ancestor as the other *β-Proteobacteria*, particularly *Oxalobacter sp. p8E* (HI-D2, 98%). HI-G4's nearest match, *Ultramicrobacterium strain ND5,* was isolated during analysis of aerobic copiotrophic ultramicrobacteria from urban soils (Iizuka et al. 1998).

The remaining six bacterial isolates cultured from the cave rock surfaces in the Throne Room belong to the γ class of *Proteobacteria,* the largest and most diverse in terms of physiology. DNA-rRNA hybridization studies have divided the γ-Proteobacteria into several deeply branching groups represented by RNA superfamilies. (The Ribosomal Database Project). γ-Proteobacteria isolated from Kartchner Caverns are part of the Ribosomal RNA superfamily II, composed of aerobes that use the Entner-Doudoroff and pentose phosphate pathways to catabolize a variety of organic substrates.

HI-F1 (*Lysobacter brunescens,* 100%) is classified under order *Xanthomonadales* and family *Xanthomonadaceae*. Genus *Lysobacter* exhibits gliding motility and a highly mucoid morphology, as well as strong proteolytic capabilities. These microbes are capable of lysing both Gram negative and Gram positive organisms, including actinomycetes, blue-green algae, yeasts, and filamentous fungi. *Lysobacter brunescens,* (100%) was investigated for its ability to lyse cyanobacteria that interfered with the flavor of a species of the commercial catfish, *Ictalarus punctatus* (Walker and Higginbotham 2000).

The five remaining γ-*Proteobacteria* belong to order *Pseudomondales* with differentiation at the family level. HI-O4 (*Uncultured gamma proteobacterium clone MTAG9 / Acinetobacter johnsonii*, 99%) is a member of family *Moraxellaceae* and occurs naturally in soils and water. During log and early exponential growth, cells are rod-shaped, but become spherical during stationary phase. They do not form spores, and exhibit "twitching" motility due to their polar fimbriae. *Uncultured gamma proteobacterium, clone MTAG9* was isolated from biofilms proliferating machinery in a beer bottling plant (Timke 2003).

Isolates HI-B17 (*Pseudomonas alcaligenes,* 99%), HI-B10 (*Pseudomonas anguilliseptica BI,* 98%), HI-G1 (*Pseudomonas sp. HR 26*, 99%), and HI-B7 (*Pseudomonas sp. SMCC B0628 / Pseudomonas sp. SMCC B0361 / Pseudomonas sp. strain DhA-51*, 99%) are taxonomically associated with family *Pseudomonadaceae* and genus *Pseudomonas* with separation at the species level. Genus *Pseudomonas* is widely distributed in nature and the most well studied in the order *Pseudomonadales*. They are aerobic, but nitrate can be used as a terminal electron acceptor under increasingly anoxic conditions. Motility is provided by way of one to a few polar flagella. Pseudomonads are capable of degrading a variety of organics including lipids and proteins, and are often implicated in the spoilage of foods such as milk and eggs as a result. Some species are also known pathogens of humans, animals, and plants. HI-B17's closet relative, *Pseudomonas alcaligenes* (99%) is a non-fluorescing, denitrifying bacterium capable of upper-range mesophilic growth at 41^{O}C; some strains are also saprophytic or opportunistic animal pathogens. Species *anguilliseptica* (HI-B10: *Pseudomonas*

anguilliseptica BI, 98%) are motile rods that rend to become filamentous with age. Animal-associated, *P. anguilliseptica* has been isolated from diseased pond-cultured eels (*Anguilla japonica*) (Holt et al. 1994). Isolate HI-G1 (*Pseudomonas sp. HR 26*, 99%), a *Pseudomonad* defined at the strain level as *HR-26*, was investigated for its association as a "helper bacterium" with the ectomycorhizae *Pisolithis albus*, which forms a symbiotic relationship with the roots of *Acacia holosericea* in West Africa (Founoune et al. 2001). Three separate strains returned identical score bits upon submission of isolate HI-B7's contig sequence to NCBI BLAST: *Pseudomonas sp. SMCC B0628, Pseudomonas sp. SMCC B0361, and Pseudomonas sp. strain DhA-51* (99%). Strains *SMCC B0628* and *SMCC B0361* were isolated from a deep subsurface environment (Vepritskiy et al. 2002), and *DhA-51* (Mohn et al. 1999) was investigated as part of a consortium of bacteria growing on resin acids.

Taxonomic and Phylogenetic Comparison of Painted Fiberglass and High Impact Cave Rock Populations

Samples collected from cave rock surfaces in the Throne Room yielded heterotrophic bacteria from four separate phyla – *Bacteriodetes, Firmicutes, Acinetobacteria,* including three classes of *Proteobacteria (α, β, and γ.)* (Figure 7). Cultured isolates from the painted fiberglass biofilms also stemmed from a diverse lineage although to a lesser degree, containing the same phyla as the cave rock: *Bacteriodetes, Firmicutes*, and class *α-Proteobacteria*. Thus, the heterotrophs inhabiting the biofilm that initially worried Cave Unit workers do not appear to be foreign, or a

variety of microbes unusual to the microflora of surrounding cave rock. They were oligotrophs innately capable of catabolizing a wide variety of substrates, happening onto a readily assimilated carbon source located upon a surface relatively free of competitors. Washing of the surfaces with a 10% bleach solution only temporarily alleviated the slimy biofilm growth. As Kartchner Caverns continued to drip waters per its nature as a living cave from overlying soils and rock, re-inoculation of the painted fiberglass continued to allow for the biofilm to return on more than one occasion.

As previously discussed, the majority of the heterotrophic bacterial isolates cultured from both surface types in the high-impact-Throne Room have been previously isolated and/or investigated in relation to functions in the soil environment. The taxonomy of isolates cultured from soils was compiled and described as range percentages of total viable counts (Alexander 1971). Comparisons of these ranges with those cultured in Kartchner Caverns are found in Table 9. Percentages of *Actinobacteria* and *Firmicutes* cultured from Kartchner Caverns were in the lower end of the ranges provided by Alexander. Isolates from phylum *Bacteriodetes* measured slightly higher, while each class of the *Proteobacteria* displayed markedly higher percentages relative to the ranges found in soils, particularly the *α-Proteobacteria*. This may be attributed to the increased number of nutrient niches in the high impact zone provided by human entrants, such as skin cell debris, hair, and oils. As oligotrophs, *α-Proteobacteria* are capable of degrading a wide variety of carbon sources that are normally considered recalcitrant. Greater ease in catabolizing such substrates not normally encountered by cave microflora

may have allowed for selective advantage for the *α-Proteobacteria*, resulting in population increase relative to the other microbial classes isolated.

Phase Two: Evaluation of Human Impact

The Throne Room was designated as a "high impact" zone due to the entrance of over 250,000 persons per year (Table 1). The presence of humans had allowed for selective growth of specific populations of heterotrophic bacteria in the Throne Room of Kartchner Caverns on painted fiberglass surfaces installed for aesthetic purposes. Two other areas of the cave were also defined according to the human impact gradient. The Grand Central Station area is located in the southwest corner of the Big Room Complex approximately 100 m away from a tourist trail that opened in October of 2003 (Figure 1). It was designated as a "moderate impact" zone as thirty to forty persons enter each year for environmental monitoring purposes only and there are no plans to develop this area. The "low impact" description was applied to the Subway Tunnel (Figure 1). This area is relatively difficult to reach, requiring navigation through mud flats measuring 0.6 to 0.9 m deep along some areas of the trail. It is rarely accessed by humans – only two to three people enter the Subway Tunnel each year for environmental monitoring. Identification of culturable heterotrophic populations from these areas allowed for assessment of their unique community structure and physiological function(s). In addition, comparison of these groups to those cultured from the high impact zone serve to indicate whether anthropogenic pressures are may have impacted the cave at the microbial level.

Moderate Impact Cave Rock Culturable Populations – Taxonomy and Description

The three sample swabs collected in the Grand Central Station area of Kartchner Caverns, designated as a *moderate impact zone*, yielded twenty-six unique heterotrophic bacteria (Table 10). A contiguous sequence of each isolate's 16s rDNA gene was generated averaging 1,296 base pairs, which was then submitted to NCBI BLAST for analysis and identification. Taxonomic and phylogenetic analyses revealed that the twenty-six cultured heterotrophs were classified under three distinct phyla: *Proteobacteria, Actinobacteria*, and *Firmicutes* (Figure 8).

Six of the twenty-six bacteria isolated from the moderate impact zone belong to phylum *Proteobacteria*. MI-p2a (*Aminobacter aminovorans,* 99%) was the only α-proteobacterium cultured from Grand Central Station, and is further classified under family *Rhizobiales*. The *Aminobacter* genus was first created in 1992 as a separate entity from *Pseudomonas* (Urakami et al. 1992), is motile by subpolar flagella, and reproduces by budding. In 2002 *Chelatobacter heintzii,* which had been studied for its utilization of nitrilotriacetate (Auling et al. 1993), was described as a later subjective synonym of *Aminobacter aminovorans* (Kampfer et al. 2002).

Two β-proteobacteria were isolated from the moderate impact zone: MI-na (*Ralstonia sp. AU3369, Uncultured bacterium CCMC0,* and *Ralstonia sp. BPC3*, 99%) and MI-10a (*Phenanthrene-degrading bacterium, 97%, Uncultured bacterium clone O-CF-10, 97%,* and *Janthinobacterium sp. J3*, 96%). The two β-proteobacteria are members of order *Burkholderiales*, with differentiation at the family level. MI-na

belongs to family *Ralstoniaceae* and genus *Ralstonia*. The 1,139 base pair long contig submitted to BLAST for the nearest relatives generated three matches with identical score bits. *Ralstonia sp. AU3369* is synonymous with *Wautersia respiraculi*, which was isolated from the respiratory tract of cystic fibrosis patients (Coenye et al. 2003). *Uncultured bacterium CCMC0* was detected during a molecular survey of bacteria from Mammoth Cave, Kentucky using the 16s rRNA gene (Fowler et al. 2003). *Ralstonia sp. BPC3* was studied as a member of a bacterial consortium capable of mineralizing benzo(a)pyrene (Kanaly et al. 2002). Isolate MI-10a was also closely related to several submitted sequences in the BLAST database, one being a *phenanthrene-degrading bacterium* studied in a soil column system at long-term exposure rates (Bodour et al. 2003). MI-10a was also closely matched to *Uncultured bacterium clone O-CF-10*, studied as part of phylogenetic characterization of bacteria from a semi-arid soil (Rutz and Kieft 2004). *Janthinobacterium sp. J3,* which exhibits slightly less sequence similarity at 96% but offers a genus description, was studied at the genetic level for its ability to degrade carbazole (Widada et al. 2002).

Three of the proteobacteria isolated from Grand Central Station were designated under class γ, order *Pseudomonadales*, family *Pseudomonadaceae*, and genus *Pseudomonas*: MI-o1 (99%), MI-45a (*Pseudomonas sp. HR 26*, 99%), and MI-57a (*Pseudomonas sp. PCP2*, 98%). The closest relative of MI-o1was identified as a carbazole-degrading pseudomonad whose *car* gene cluster was studied for genetic and evolutionary characterization (Nojiri et al. 2001). MI-45a's closest match was also isolated from the high impact zone of Kartchner Caverns from the cave rock surface (HI-

G1). It was investigated for its association as a "helper bacterium" with the ectomycorrhizae *Pisolithis albus*, which forms a symbiotic relationship with the roots of *Acacia holosericea* in West Africa (Founoune et al. 2001). *Pseudomonas sp. PCP2* (MI-57a) was researched for its Mn (II)-oxidizing capabilities at the genetic level, namely its *cumA* multi-copper oxidase genes (Francis and Tebo 2001).

Six heterotrophs were cultured from the moderate impact zone belonging to phylum *Actinobacteria*, class *Actinobacteria*, subclass *Actinobacteridae*, and order *Actinomycetales*, with differentiation at the suborder level. Isolates MI-26ac1 (*Aeromicrobium erythreum*, 96%) and MI-11a (*Rhodococcus equi DSM 2030*, 99%) are members of suborder *Cornyebacterineae* and family *Nocardiaceae*, with divergence at the genus level. *Aeromicrobium erythreum*, and erythromycin-producing bacterium, was described as a new genus and species in 1991 by Miller et al. The 16s genes of *Aeromicrobium erythreum* and *Rhodococcus equi DSM 2030* were studied during separate phylogenetic analyses in effort to further characterize genus *Nocardiodes*, which shares the same family *Nocardiaceae* (Yoon et al. 1998, Andrees et al. 2002). MI-69a (*Actinomycetaceae SR272,* 99%) is classified under suborder *Actinomycineae*, but no further information was provided by BLAST for the closest relative past the family level, *Actinomycetaceae*, which contains numerous parasitic and pathogenic forms. Isolate MI-46a (*Kocuria erythromyxa*, 98%) fits taxonomically under suborder *Micrococcineae* and family *Micrococcaceae*; it was also cultured from cave rock surfaces in the Throne Room of Kartchner Caverns (HI-A4a). Studies of this microbe have been primarily systematic and phylogenetic in nature (Brooks and Murray 1981). MI-59a is classified in suborder

Propionibacterineae, family *Nocardioidaceae*, with the genus and strain assignments of *Nocardiodes* and *A3*, respectively. Genus *Nocardioides* occur in both motile and non-motile forms, and are found in soils worldwide. Strain *A3* has been isolated and characterized as a degrader of dibenzofuran (Hiraishi 2002). Lastly, isolate MI-71a (*Streptomyces peucetius*, 99%) is a member of suborder *Streptomyceneae* and family *Streptomycetaceae*. Genus *Streptomyces* forms non-motile spores and is widely distributed and abundant in soils. Species level differentiation is difficult due to overlapping of physiological capabilities and morphological traits.

More than half of the twenty-six isolates cultured from the moderate impact zone (Grand Central Station) belong to phylum *Firmicutes*, class *Bacilli*, and order *Bacillales*. Thirteen of the fourteen isolates in this category are categorized under family Bacillaceae and genus Bacilli. One isolate, MI-61a (*Paenibacillus glycanilyticus*, 99%), does deviate from the others at the family level, belonging to the recently recognized family *Paenibacillaceae* which are motile by peritrichous flagella and have ellipsoidal endospores. *Paenibacillus glycanilyticus* was found to degrade a heteropolysaccharide synthesized by *Nostoc commune*, a cyanobacterium (Dasman-Kajiyama et al. 2002).

Genus *Bacillus* consists of a great variety of Gram positive, straight rods that are motile by peritrichous flagella. Characteristic of soil bacteria, *Bacilli* form endospores – they are also strictly heterotrophic but can vary in oxygen requirement as either aerobes or facultative anaerobes. Diversity reaches past the genus to the species level, with several hundred strains identified and characterized as well from a wealth of niches and environs.

MI-65a was most closely related to *Bacillus sp. 433-D9* (100%), a psychrotropic (can grow at 0^{O}C with optimal growth range of 20 to 30^{O}C), green-pigmented *Bacillus* isolated from soil in Wisconsin (Albert et al. 2003). MI-ab (*Bacillus amyloliquefaciens*), was first classified in 1987 (Priest et al. 1987) and is used industrially for the amylase enzyme it produces, which converts starches to simpler sugar forms. MI-33a1 is 97% related to *Bacillus aquaemaris,* a species first isolated from a tidal flat of the Yellow Sea in Korea (Yoon et al. 2003). *Bacillus cereus biovar toyoi* was 99% identical with the contig generated for MI-32a2. It was studied in effort to optimize rapid identification procedures of closely related probiotic *Bacillus cereus* strains, as well as differentiation from wild type strains (Pecoraro and Bucher 2002). *Bacillus cereus LRN* (MI-c1a1) is very closely related to *Bacillus anthracis*, a causative disease agent in both cattle and humans, thus allowing for use of its 16s rRNA gene in developing a rapid identification method for the pathogen (Sacchi 2002). Isolate MI-3a is most closely related (98%) to *Bacillus sp. IDA4917,* which has been employed for optimization of a selective PCR DGGE protocol designed for detection of a predominant group of *Bacilli* found in soil (Tzeneva et al. 2003).

MI-22a2 was most closely related to *Bacillus sp. LMG-19415* (98%), which was implicated in the biodeterioration of mural wall paintings in a culture-independent study (Gurtner et al. 2000). MI-qb2's closest matches, *Bacillus macroides* and *Bacillus sp. LMG 21002* (99%), were also cultured from the high impact zone cave rock. *Bacillus macroides* was identified and characterized due to its association with the midgut of the Colorado Potato Beetle (Herdon and Spence 1999), and *Bacillus LMG 21002* was one of

a consortium of bacteria implicated in the biodeterioration of mural paintings in St. Martin's Church, Germany (Gorbushina et al. 2004). *Bacillus pichinotyi, strain RS2* demonstrated 97% similarity to isolate MI-58a; it was isolated from tropical rice soils and characterized as a denitrifying *Bacillus* (Garcia et al. 2002). Two seperate strains of *Bacillus pumilus* (99%) were retrieved from BLAST for contig MI-33a2. *Strain KL-052* was employed during a study involving the use of ATP as a biomarker for detection of viable microbes in clean-room research facilities (Venkateswaran et al. 2003). The second strain was employed in attempt to determine the efficacy of polyphasic approaches in identifying and classifying *Bacillus pumilus* spores (Dickinson 2004). MI-72a was most closely matched (100%) to a strain of *Bacillus pumilus* studied in Japan and submitted to NCBI through the Yokohama Plant Protection Service (Tsukamato 1998). MI-23a1 (*Bacillus thuringiensis 4Q281 / Bacillus cereus ATCC 53522*, 99%) displayed 100% sequence similarity to isolates from both the painted fiberglass biofilm (PF-A: *B. thuringiensis,* 99%) and cave rock from the high impact zone (HI-G2: *Bacillus TB3-10-I* / *Bacillus thuringiensis 4Q281 / Bacillus thuringiensis ATCC 10792,* 100%). *Bacillus sp. YY* was isolated during characterization of filamentous bacteria from paper mill slimes, and was the closest match (99%) retrieved for MI-67a2 (Oppong et al. 2003).

Low Impact Cave Rock Culturable Populations – Taxonomy and Description

Three sample swabs were collected approximately 0.9 m from each other along a continuous cave rock surface in the Subway Tunnel area of Kartchner Caverns, designated as *low impact* as only two to three people enter each year for environmental

monitoring purposes. Thirty-two unique heterotrophic bacteria were isolated from the area. Contigs generated in FAKtory for BLAST identity retrieval averaged 1,381 base pairs in length. As with the moderate impact zone (Grand Central Station), three distinct phyla were represented by the cultured bacteria: *Proteobacteria, Actinobacteria*, and *Firmicutes* (Figure 9).

Five of the thirty-two isolates cultured from the low impact zone belong to phylum *Proteobacteria*, with divergence at the class level. One α-proteobacterium (L-48) was isolated with 99% similarity to *Mesorhizobium chacoense*. A member of order *Rhizobiales* and family *Phyllobacteriaceae*, *Mesorhizobium chacoense* was first isolated from the Chaco Arido Region of Argentina as a symbiotic nodulating bacterium of *Prosopis alba* (Velazquez et al. 2001). The remaining four proteobacteria isolated from the Subway Tunnel are classified as γ: LI-60 (*Xanthomonas sp. TB8-9-II*, 97%), LI-40 (*Lysobacter sp. Dae16*, 97%), LI-13 (*Pseudomonas sp. HR 26,* 99%), and LI-70 (*Moraxella osloensis*, 99%). Differentiation begins at the order level - LI-60 and LI-10 both belong to order *Xanthomonadales* and family *Xanthomonadaceae*, and differ at the genus level. The genus *Xanthomonas* is characterized by strictly aerobic oxygen requirement and the ability to use a variety of carbohydrates and salts of organic acids as sole sources of carbon. *Xanthomonas sp. TB8-9-II* was part of a bacterial community phylogenetically studied for its association with the fruiting body of an ectomycorrhizal fungus (Barbieri et al. 2004). Genus *Lysobacter* displays gliding motility and is capable of lysing cells ranging from Gram negative to some nematodes. Another member of genus *Lysobacter* was isolated from the high impact area of Kartchner Caverns, HI-F1

(*Lysobacter brunescens,* 100%). *Lysobacter sp. Dae16* was isolated during a cultivation-based study of environmental samples (Im 2004). LI-13 and LI-70 are grouped into order *Pseudomonadales*, then diverge at the family level once again. LI-13 *(Pseudomonas sp. HR 26)* belongs to family *Pseudomonadaceae* and genus *Pseudomonas*, and was isolated from all three impact areas of Kartchner Caverns (HI-G1 and MI-45a). LI-70 belongs to family *Moraxellaceae* and genus *Moraxella*, members of which are pleomorphic facultative anaerobes and lack flagella (twitching motility). Members of genus *Moraxella* are also highly sensitive to penicillin, and react parasitically with mucous membranes cells of humans and other warm-blooded creatures. *Moraxella osloensis* was isolated during a study measuring anaerobic mineralization of pivalic acid by denitrifying bacteria (Harder 2003).

Six isolates were cultured belonging to phylum *Actinobacteria*, class *Actinobacteria*, subclass *Actinobacteridae*, and order *Actinomycetales* – divergence occurred at the suborder level. LI-5 (*Kocuria erythromyxa*, 98%) is a member of suborder *Micrococcaceae* and family *Micrococcaceae*. It was also isolated from the high (HI-A4a) and moderate impact (MI-46a) zones of the caverns. LI-47 (*Mycobacterium elephantis*, 97%) is grouped with suborder *Corynebacteriaceae* and family *Mycobacteriaceae*. Constituents of genus *Mycobacterium* are aerobic, usually slow-growing, and may be either free-living or pathogens of vertebrates (i.e. *Mycobacterium tuberculosis*). *Mycobacterium elephantis*, a rapid-growing mycobacterium, was first isolated from a lung abcess of an elephant that died from a chronic respiratory illness (Shoejaei 2000). The remaining four actinomycetes cultured from the low impact zone

belong to suborder *Streptomycineae*, family *Streptomycetaceae*, and genus *Streptomyces*. Members of genus *Streptomyces* form nonmotile spores and produce a variety of pigments, some diffusible in solid medium. *Streptomyces sp. KN-1220* (LI-49, 98%) was studied for its ability to eliminate pests (Liu et al. 2001). *Streptomyces phaeochromogenes* is capable of producing the halogenated metabolite bromoperoxidase when given monochlorodimedine as a carbon source (van Pée and Lingens 1985). LI-42's nearest relative, *Streptomyces sp. VTT E-99-1326* (98%) was isolated from water-damaged buildings and characterized (Suutari et al. 2002). *Streptomyces lipmanii* (LI-53, 99%) was employed during a study assessing the phylogenetic relationship between isopenicillin N-synthase and the 16s rRNA gene (Palaniappan and Seki 2000).

Approximately sixty-five per cent of the isolates cultured from the low impact zone of Kartchner Caverns were classified under phylum *Firmicutes*, which is subdivided into two classes: *Bacilli* and *Lactobacilli*, the latter producing lactic acid as its primary fermentation product. Each of the twenty-one isolates cultured of phylum *Firmicutes* are grouped under class *Bacilli* and order *Bacillales*, with differentiation at the family level. LI-10 (*Staphylococcus saprophyticus*, 99%) belongs to family *Staphylococcaceae* and genus Staphylococcus, which displays Gram positive cocci. Species *saprophyticus* is non-hemolytic on blood agar, and demonstrated 99% similarity to an Arctic Sea ice bacterium, *ARK9973,* isolated during a study comparing microbial diversity and community in Arctic versus Antarctic pack ice (Brinkmeyer et al. 2003). High impact isolate PF-F expressed 99% similarity to *Staphylococcus sp. es1,* also a marine isolate.

Seven of the twenty-one bacilli cultured from the Subway Tunnel belong to family *Paenibacillaceae* – divergence was evident at the genus level. LI-67's closest identification match, *Brevibacillus laterosporus* (99%), was characterized as an aerobic, spore-forming non-pathogenic bacterium prior to its official naming (Shida 1996). The remaining six isolates are grouped under genus *Paenibacillus* as varying species and strains. Two strains of *Paenibacillus alvei* were isolated, LI-23 and LI-55. *Paenibacillus alvei DSM 29T* (LI-23, 95%) exhibited a lawn morphology unique relative to all other isolates cultured from the caverns (Table 10). It was previously cultured during a study characterizing a novel cyclodextrin-producing species of *Paenibacillus* isolated from paperboard material (Suominen et al. 2001). The 16s genes of *Paenibacillus alvei* (LI-55, 98%), *Paenibacillus apiarus* (LI-32, 99%), and *Paenibacillus kobensis* (LI-63, 98%) were employed in effort to devise a rapid identification technique for genus *Paenibacillus* (Goto 2001). The closest relative match for isolate LI-54, *Paenibacillus honkongensis* (97%) was implicated as the causative agent in a case of pseudobactearemia characterized by neutropenic fever (Teng et al. 2003). *Paenibacillus O-3b* (LI-9, 99%) was isolated from the rhizosphere of spinach and characterized based upon the ability to degrade agar (Hosoda et al. 2002).

Thirteen of the thirty two isolates cultured from the low impact zone, ~ 40 % overall, belong to family *Bacillaceae* and genus *Bacillus*. *Bacillus* being the ubiquitous heterotroph, the closest relatives for the cultured *Bacilli* from Kartchner Caverns stem from a variety of environments, exercising diverse physiologies. *Bacillus sp. 19489* (LI-17, 99%) was one of many bacteria isolated from biodeteriorated mural paintings in the

Servilia tomb located in Seville, Spain (Heyrman and Swings 2001). LI-83's closest relative match, *Bacillus amyloliquefaciens* (99%), was also cultured from the moderate impact zone (Subway Tunnel) of Kartchner Caverns (MI-ab). *Bacillus catenulatus* (LI-80, 99%) was first isolated from shrimp-pond slurry located near the coast of Fujian, China (Wang et al. 2004). Isolate LI-18 is 99% similar to *Bacillus cereus ATCC 14579*, studied in conjunction with *Bacillus anthracis* for genomic comparison (Ivanova et al. 2003). LI-64 (*Bacillus sp. TB3-10*-I, 99%) was also isolated from the high impact zone (HI-G2), and was 99% similar to MI-32a2 from the moderate impact area (*Bacillus biovar toyoi*). Isolate LI-2 generated two matches at 99% matching similarity – *Bacillus sp. Fa29*, an epiphyllic bacterium living on the leaves of strawberry plants (Krimm et al. 2002), and a halotolerant strain of *Bacillus megaterium* isolated from Oklahoma's Great Salt Plains (Caton 2004). *Bacillus firmus* (LI-31, 99%) was analyzed at the molecular level with other culturable soil bacteria using a polyphasic approach (Muscillo et al. 2002).

Bacillus fusiformis (LI-45, 99%) is one of seventy-seven species of *Bacillus* under study for probiotic capabilities (Sanders 2003). *Bacillus sp. IDA4789* (LI-14, 98%) was classified at the strain level during the same study as moderate impact isolate MI-3a *(Bacillus sp. IDA4917)*, in effort to optimize a selective DGGE approach for soil bacilli. The nearest relative for LI-66 (*Bacillus sp. LMG 21002*, 99%) was also identified from cave rock surfaces in both the high and moderate impact areas, and has been isolated from mural paintings in St. Martin's Church, Germany (Gorbushina et al. 2004). *Bacillus luciferensis* (LI-105, 99%) was initially isolated from volcanic soils in Candlemas Island

(Logan et al. 2002), and *Bacillus sp. MK03* was studied extensively for its ability to produce an extracelluar α-neoagarooligosaccharide hydrolase (Suzuki et al. 2002). Lastly, the closest relative match for isolate LI-15, *Bacillus sphaericus S33* (99%), was one of a group of bacteria cultured from compost (Ohnishi 2003).

Taxonomic and Phylogenetic Comparison of Moderate and Low Impact Cave Rock Populations

The taxonomic designations of cultured cave rock populations from the moderate impact zone (30 to 40 human entrants per year-Grand Central Station) are homogenous with isolates from the low impact area (2 to 3 human entrants per year-Subway Tunnel) at the phylum level (Tables 9 and 10, Figures 8 and 9). Cultured heterotrophs from each area are classified under phyla *Firmicutes*, *Actinobacteria*, and *Proteobacteria*. The predominant phylum in both the moderate and low impact zones was *Firmicutes*, accounting for ~54% and ~65% of the isolates cultured, respectively. More diversity was evident within the phylum in the low impact zone, with isolation of a multitude of *Paenibacilli* species and strains, as well as a *Staphylococcus sp.* . Phylum *Actinobacteria* saw greater diversity in the moderate impact zone than the low impact area – approximately 67% of the actinomycetes cultured from the Subway Tunnel were classified under genus *Streptomyces*. Phylum *Proteobacteria* was also represented in each zone, with six isolates cultured from the moderate area and five from the low. Classes α, β, and γ were cultured from the moderate area, while only α and γ were isolated from the low impact zone.

Overlapping of isolates was evident between the moderate and low impact zones. Isolates demonstrating high percentage of homology to *Bacillus amyloliquefaciens*, *Bacillus cereus*, *Bacillus sp. LMG 21001*, and *Kocuria erythromyxa* were cultured from both zones.

Taxonomic and Phylogenetic Comparison of High, Moderate, and Low Impact Populations

Four phyla were represented in each of the high, moderate, and low impact zones: *Bacteriodetes*, *Proteobacteria*, *Actinobacteria*, and *Firmicutes* (Figure 10). The non-proteo, Gram negative isolates representing phylum *Bacteriodetes* were chosen as the outgroup from which the tree was rooted due to the greater distance exhibited of these isolates relative to the other phyla from the ancestral node.

Visualization of Figure 10 reveals clustering of the cultured isolates according to the designated human impact zones. The majority of the low and moderate impact isolates are found amongst the clades representing phyla *Firmicutes* and *Actinobacteria*, while most of the high impact cave rock and painted fiberglass isolates fall into the *Proteobacteria* groupings of α, β, and γ. Isolates representing the distantly related outgroup - PF-H, PF-B, and HI-M4, were each cultured from the high impact zone.

Marked differences between the high impact zone (Throne Room), and moderate-low zones prompted inquiry as to the potential causes of such a trend. Natural organic matter inputs vary for each site, transported primarily by percolating waters and/or bats. The Throne Room (high impact zone) receives the least amount of natural organic input –

no bats roost in this area during the maternity season, and the sampled areas located near the tourist area are not subject to seasonal floods. The Big Room area of the moderate impact zone is the chosen location of the annual maternity roost for *Myotis velifer*, which have been shown as invaluable in maintaining the heterotrophic food chain in Kartchner Caverns (Buecher and Sidner 1999, Welbourn 1999). The area of the Big Room sampled, Grand Central Station, does receive a greater influx of natural organic materials than the Throne Room, though bat guano is not the primary source. The main source of organic input in Grand Central Station is percolating water, which although low in flow relative to the other two zones, carries with it more dissolved organic carbon from the outside. The low impact zone is believed to receive the greatest input of organic carbon in addition to almost constant percolation of water. During the winter and summer-monsoon rainy seasons that characterize the climate of the Sonoran Desert terrestrial environment, the Subway Tunnel floods with waters rich in dissolved organics from the outside. Although seasonal, these floods are vital to the food chain of lower organisms residing in the area. Increased levels of natural organic materials may select for growth of microbial groups less oligotrophic in nature, such as *Bacillus*, while microbes more adapted to low nutrient conditions (i.e. *Proteobacteria*) are able to establish a niche in the high impact zone.

The high impact zone, however, has received organic input from new sources over the past half-decade more anthropogenic in origin. Humans entering the cave on a daily basis (Table 1) bring a variety of foreign substances such as hair, sloughed skin cells, sweat, skin oils, and lint from clothing that could potentially provide nutrient-

niches for metabolically-diverse oligotrophs. Misters were installed into the cave for the purpose of lint control, as algae and other microbes are capable of utilizing the substance for energy. Algal growth has been observed on a number of natural rock surfaces in the cave where it was not previously evident, prompting both the misters and enhanced light control in the tourist areas.

The presence of light in the high impact region is a factor of interest in the changes evident in the culturable heterotrophic populations. Very low numbers of bacilli and actinomycetes were cultured from the area, while high numbers of proteobacteria were isolated. The α, β, and γ classes of the phylum *Proteobacteria* contain the purple photosynthetic bacteria. Non-photosynthetic members of these classes are believed to have derived from a photosynthetic ancestor as each group retains strong phylogenetic and taxonomic relations with the phototrophic purple sulfur bacteria (Prescott et al. 1999). Nutrient stress from natural sources may have more of an adverse effect on the predominance of *Bacilli* and related heterotrophs evident elsewhere in the cave. Coupled with increased exposure to light, it may have allowed for selection of the oligotrophic proteobacteria as the increasingly dominant culturable species in the high impact zone relative to its recessive status in the moderate and low impact areas.

The impact of humans on the culturable microbial communities of Kartchner Caverns appears to be rather indirect. While alternative groups of microbes unique to the microflora of humans (i.e. fecal coliforms) may be transported into the cave on an almost daily basis, these organisms do not appear to be components of the cave rock populations. The painted fiberglass and natural cave rock surfaces are colonized by microbes

ubiquitous in soils and bodies of water around the world. Similar to subsoils, caves are considered stable environments characterized by relatively constant temperatures and humidity, with perturbations due primarily to changes in the terrestrial environment above. Development of Kartchner Caverns as a show cave altered the physico-chemical (i.e. average temperature and relative humidity) patterns of the cave system that had been relatively constant for a considerable period of time. Microbial communities are particularly susceptible to such changes that are more plainly visible at the micro-scale. While it cannot be definitively stated due to lack of data that microbial populations have changed in the high impact zone (Throne Room) relative to pre-show cave conditions, the population ratios are markedly different than those found in areas of the cave less traversed, and less impacted physically (i.e. lighting) and chemically (i.e. sloughed skin cells and oils) by people. Continued monitoring of the recently opened Big Room, as well as implementation of a polyphasic approach employing both culturable and molecular techniques, may allow for more definitive assertions as to the possible effects human presence has on the microbial communities of Kartchner Caverns.

Appendix 1: Figures

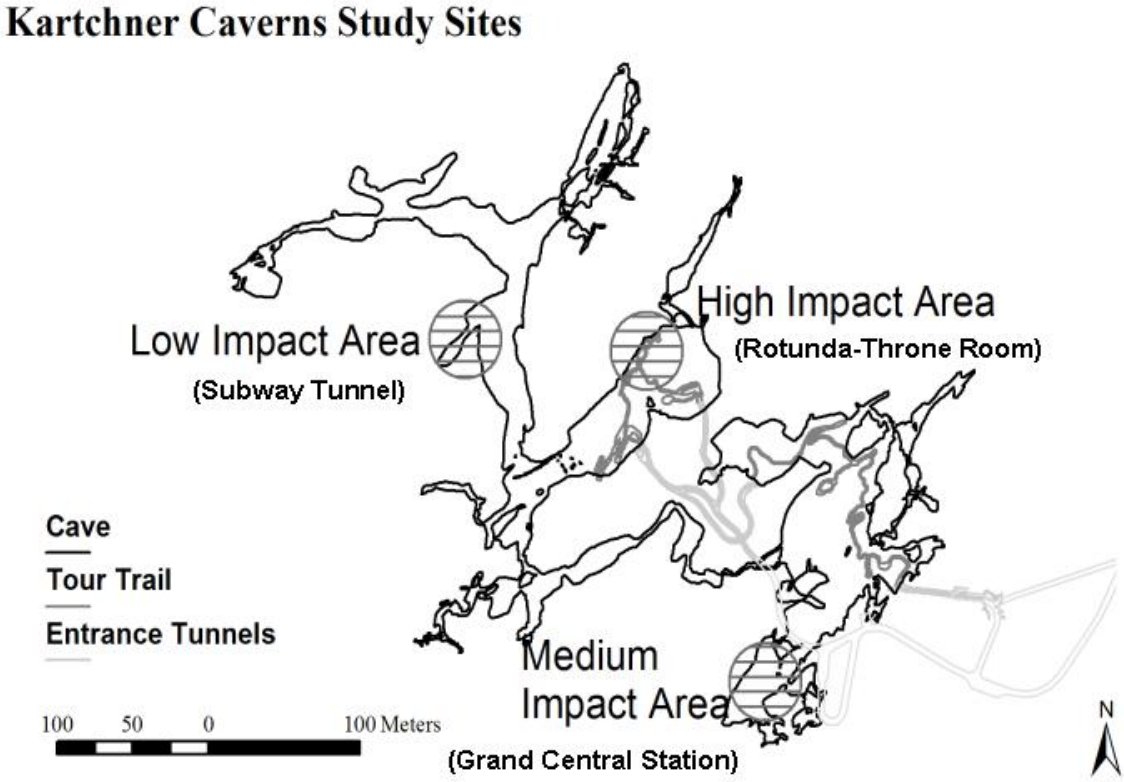


Figure 1. Human impact designations and sample collection points

DNA TEMPLATE MAP

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1a	61	60	A1a	61	60	A1a	61	60	A1a	61	60
B	CDE1	70a	36	CDE1	70a	36	CDE1	70a	36	CDE1	70a	36
C	30	Qb2*	55	30	qb2*	55	30	qb2*	55	30	qb2*	55
D	34	42	D2	34	42	D2	34	42	D2	34	42	D2
E	27	54	B19	27	54	B19	27	54	B19	27	54	B19
F	70	67	H	70	67	H	70	67	H	70	67	H
G	80	104	G2	80	104	G2	80	104	G2	80	104	G2
H	52	65	BR1-1	52	65	BR1-1	52	65	BR1-1	52	65	BR1-1

PRIMER MAP

	1	2	3	4	5	6	7	8	9	10	11	12
A	27f	27f	27f	518f	518f	518f	1070r	1070r	1070r	1492r	1492r	1492r
B	27f	27f	27f	518f	518f	518f	1070r	1070r	1070r	1492r	1492r	1492r
C	27f	27f	27f	518f	518f	518f	1070r	1070r	1070r	1492r	1492r	1492r
D	27f	27f	27f	518f	518f	518f	1070r	1070r	1070r	1492r	1492r	1492r
E	27f	27f	27f	518f	518f	518f	1070r	1070r	1070r	1492r	1492r	1492r
F	27f	27f	27f	518f	518f	518f	1070r	1070r	1070r	1492r	1492r	1492r
G	27f	27f	27f	518f	518f	518f	1070r	1070r	1070r	1492r	1492r	1492r
H	27f	27f	27f	518f	518f	518f	1070r	1070r	1070r	1492r	1492r	1492r

Figure 2. EXCEL file sample, 96-well program for 16s-rDNA sequencing

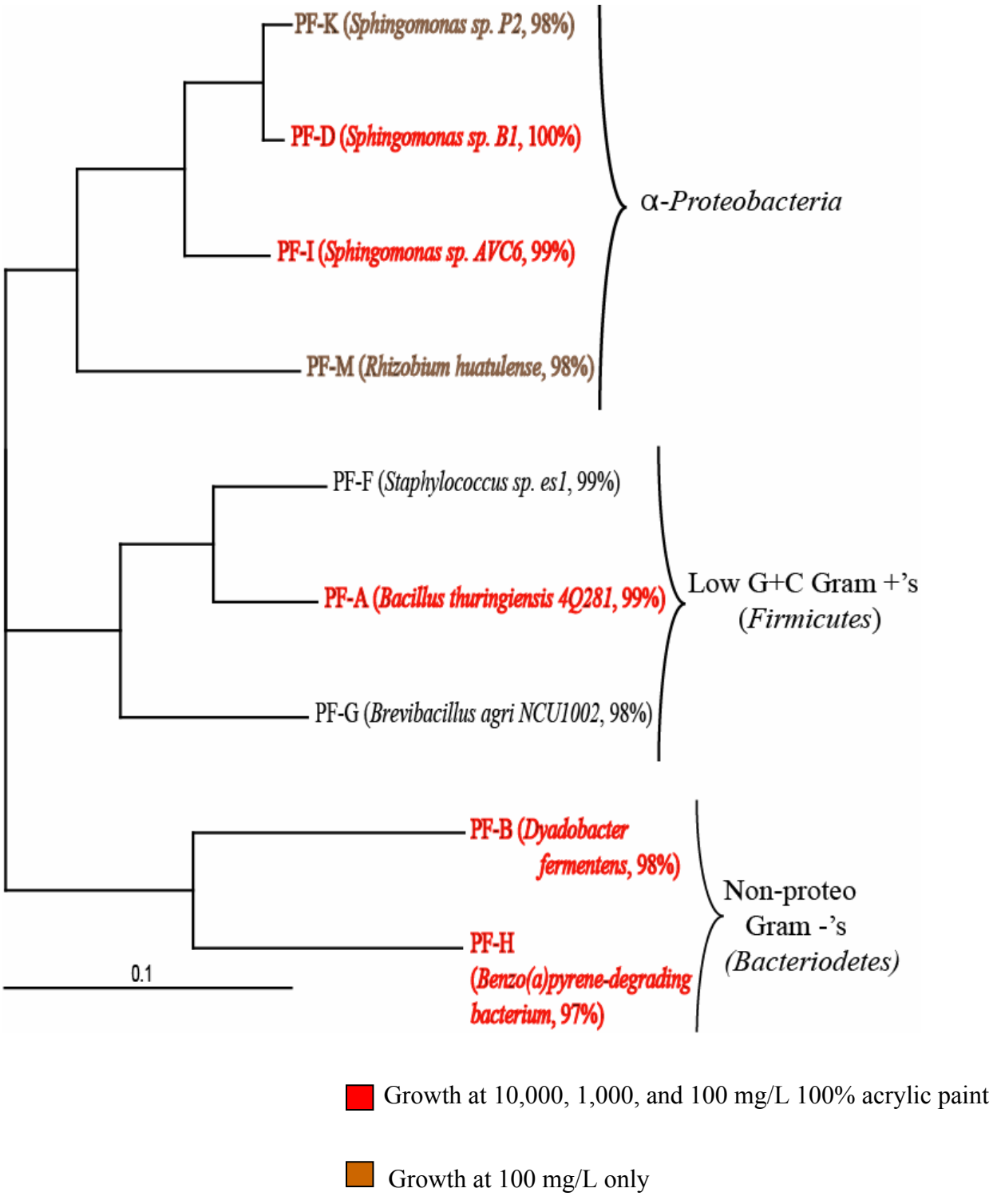


Figure 3. Neighbor Joining-Bootstrap Tree of Painted Fiberglass Isolates

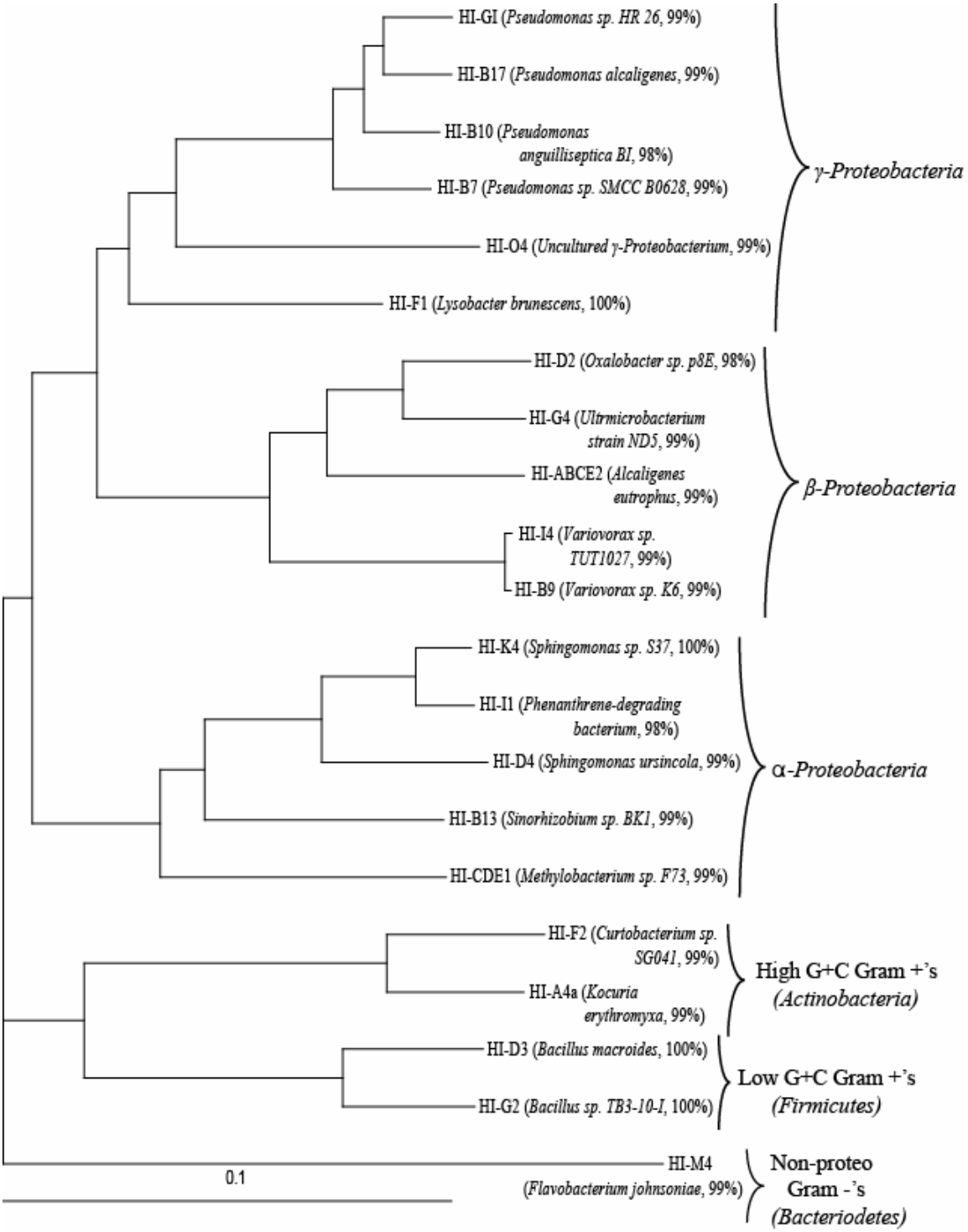


Figure 4. Neighbor Joining-Bootstrap Tree of High Impact Cave Rock Isolates

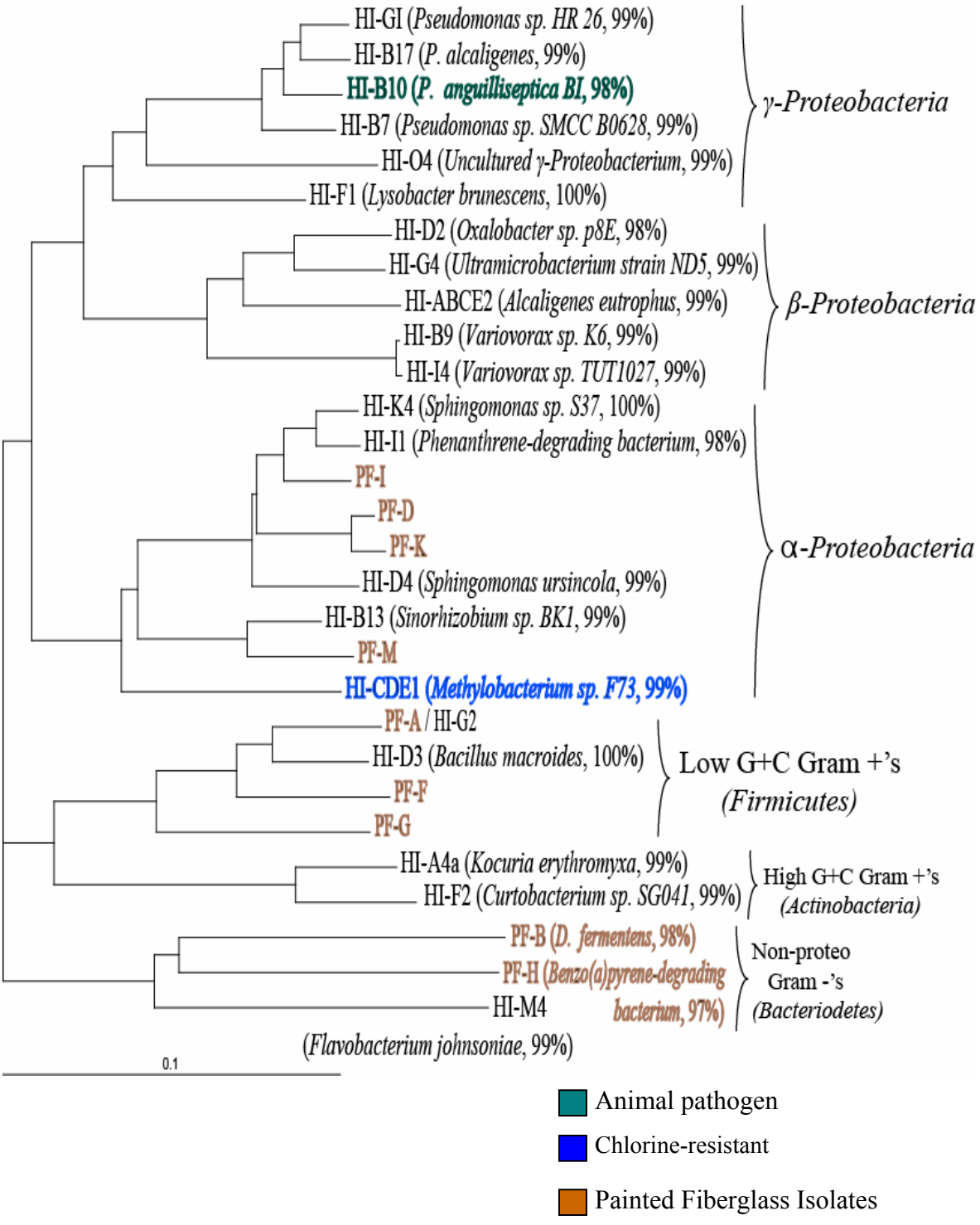


Figure 5. Neighbor Joining-Bootstrap Tree of Painted Fiberglass and High Impact Cave Rock Isolates

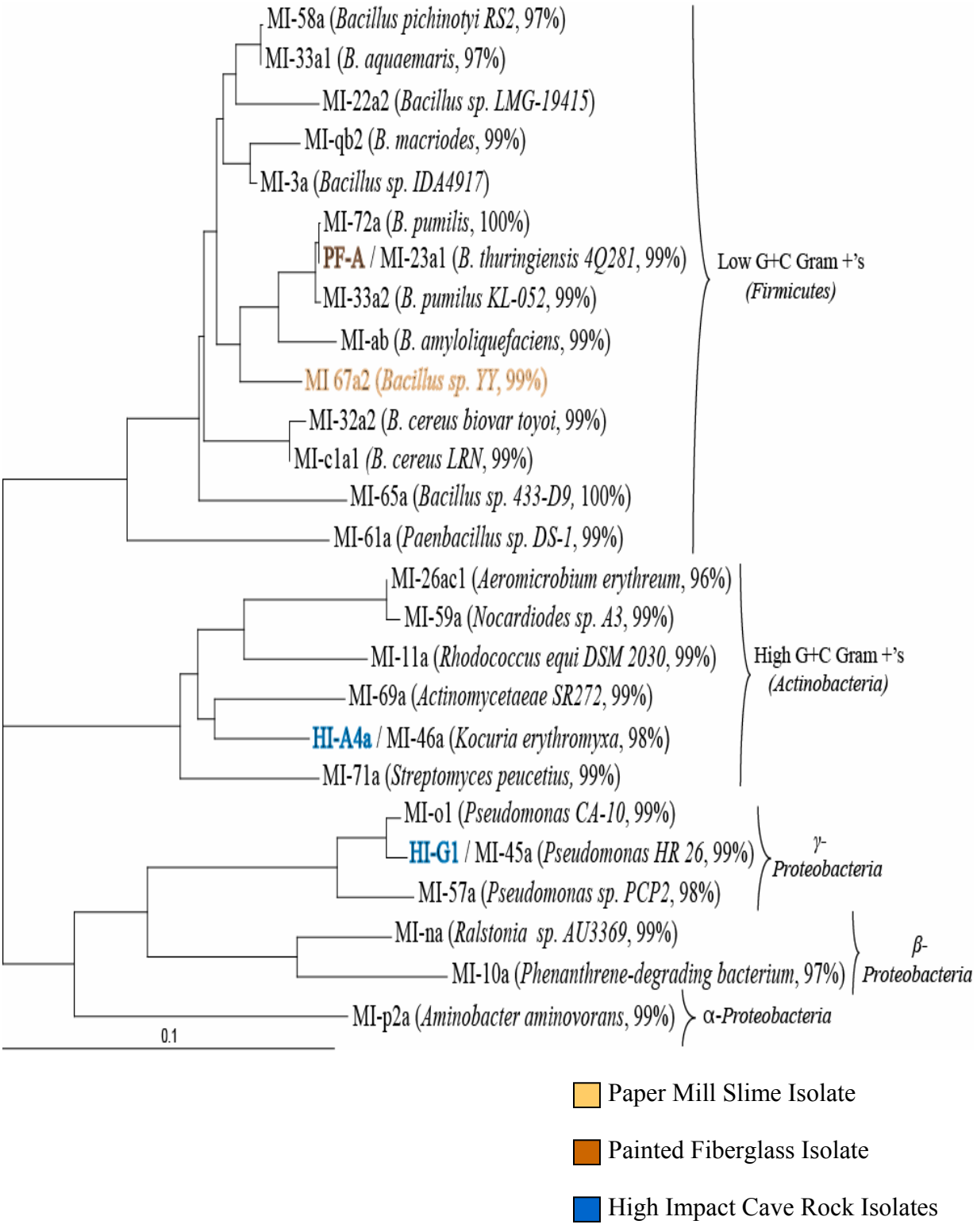


Figure 6. Neighbor Joining-Bootstrap Tree of Moderate Impact Cave Rock Isolates

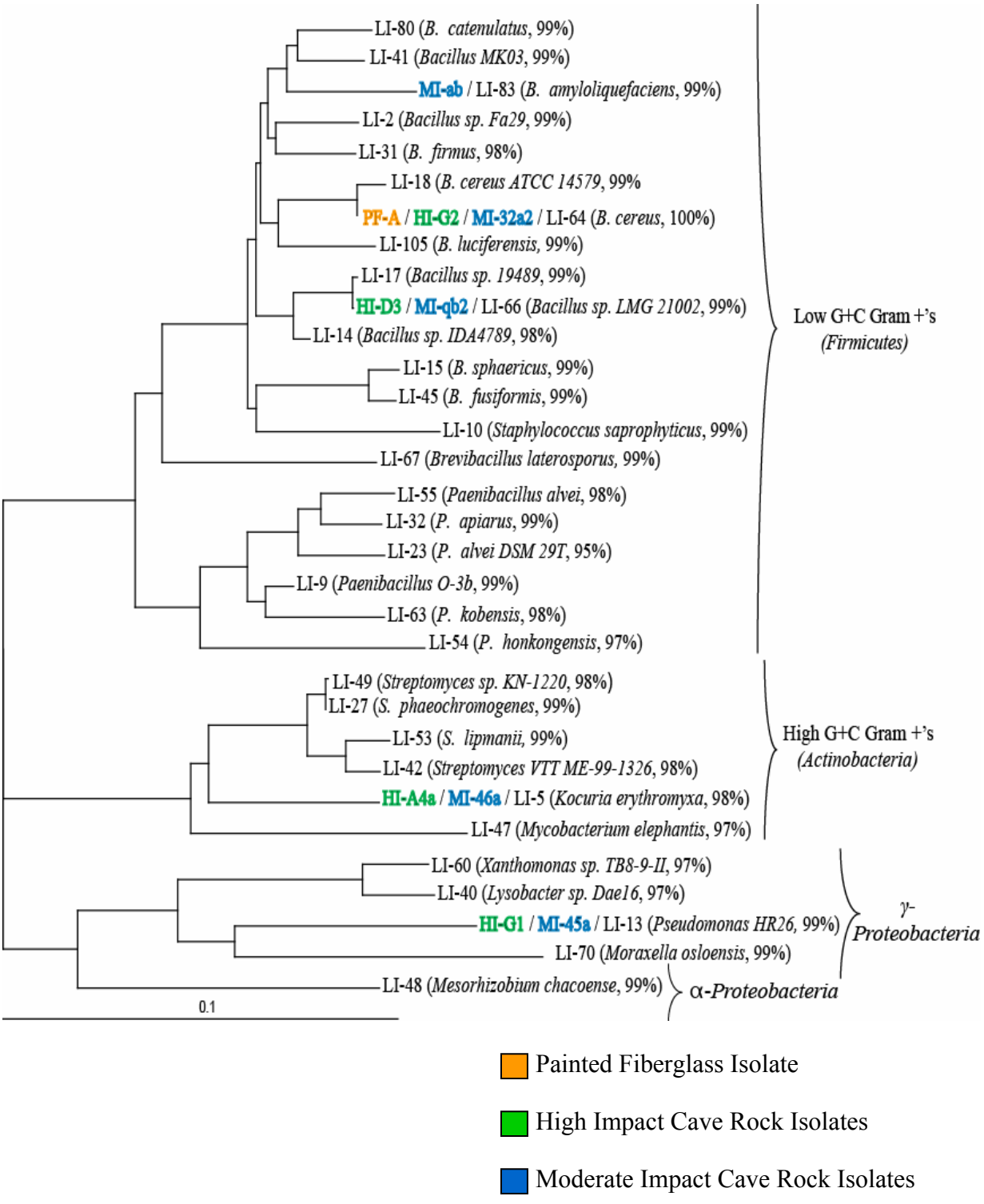


Figure 7. Neighbor Joining-Bootstrap Tree of Low Impact Cave Rock Isolates

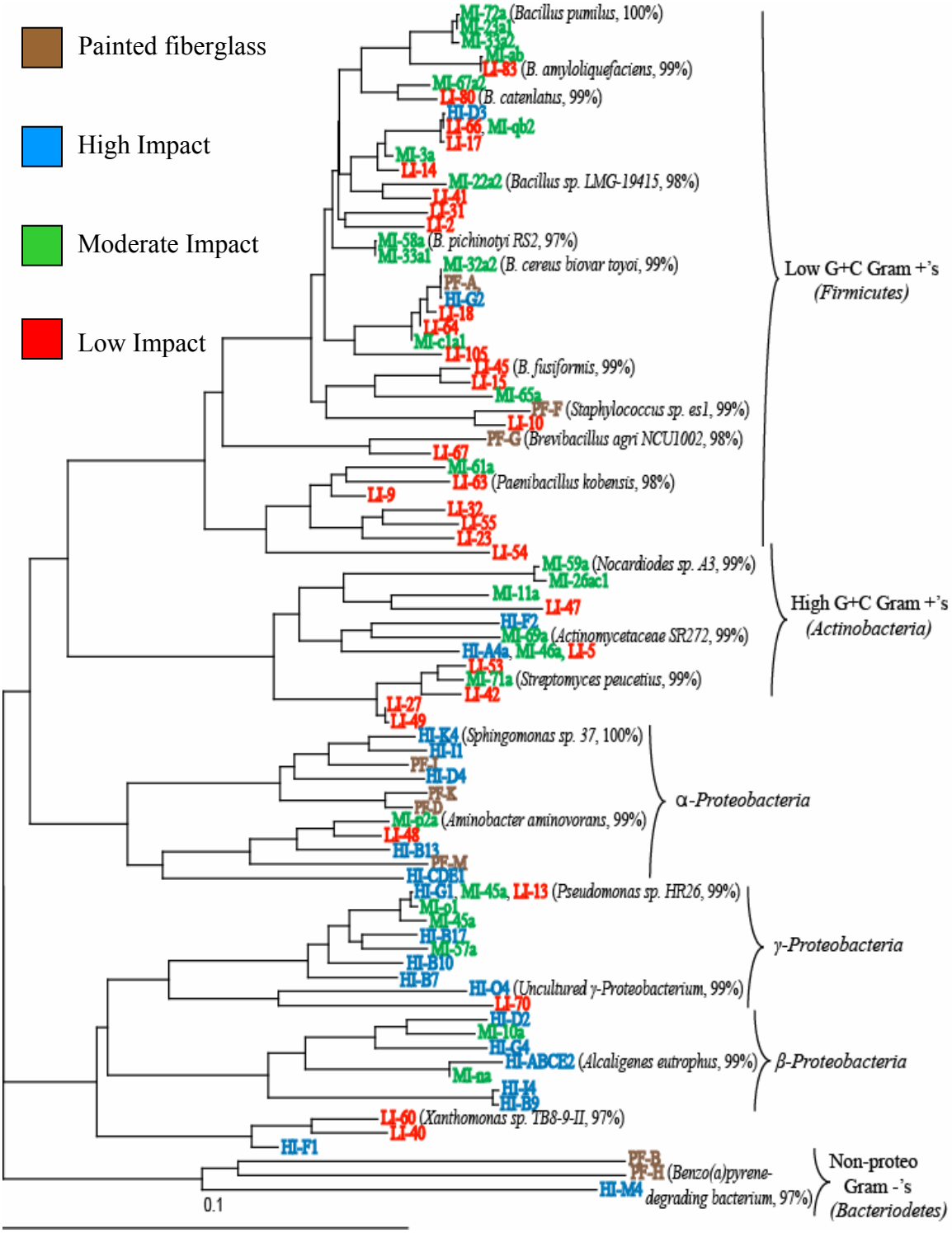
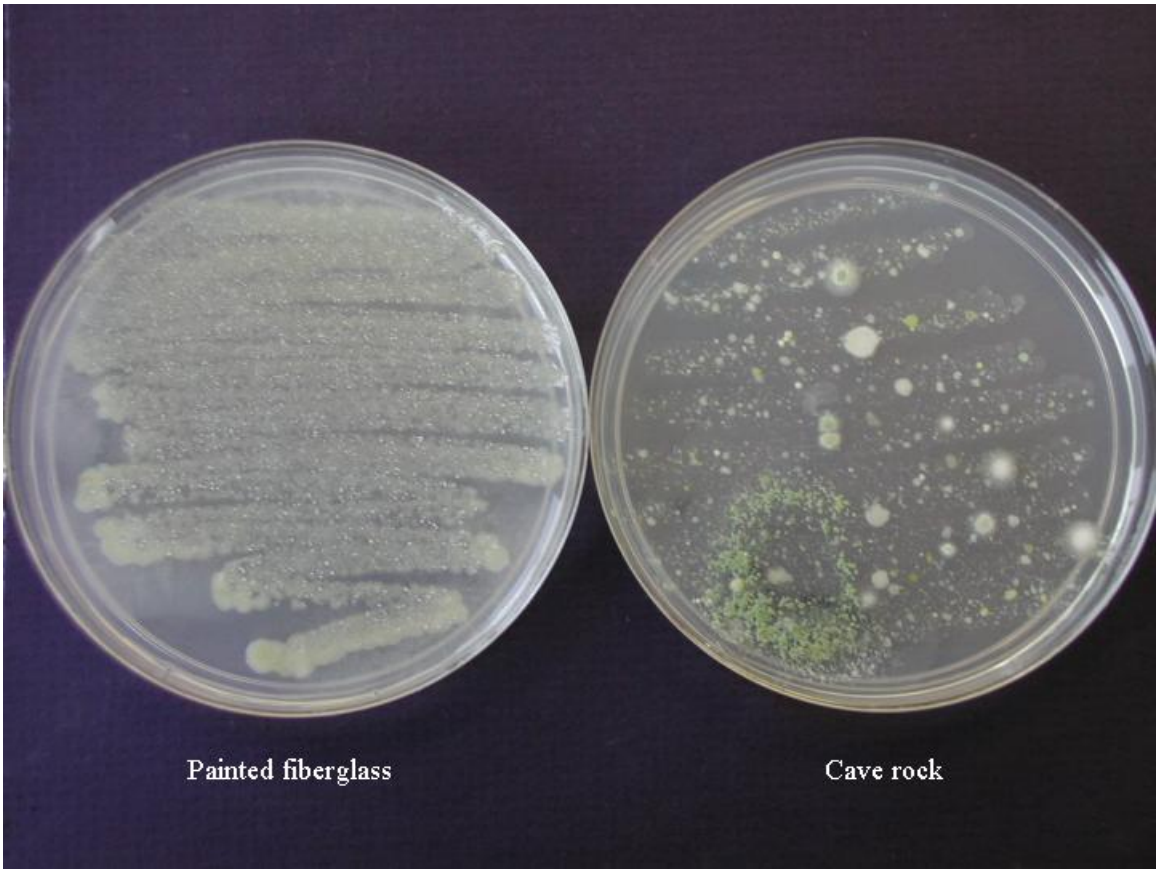
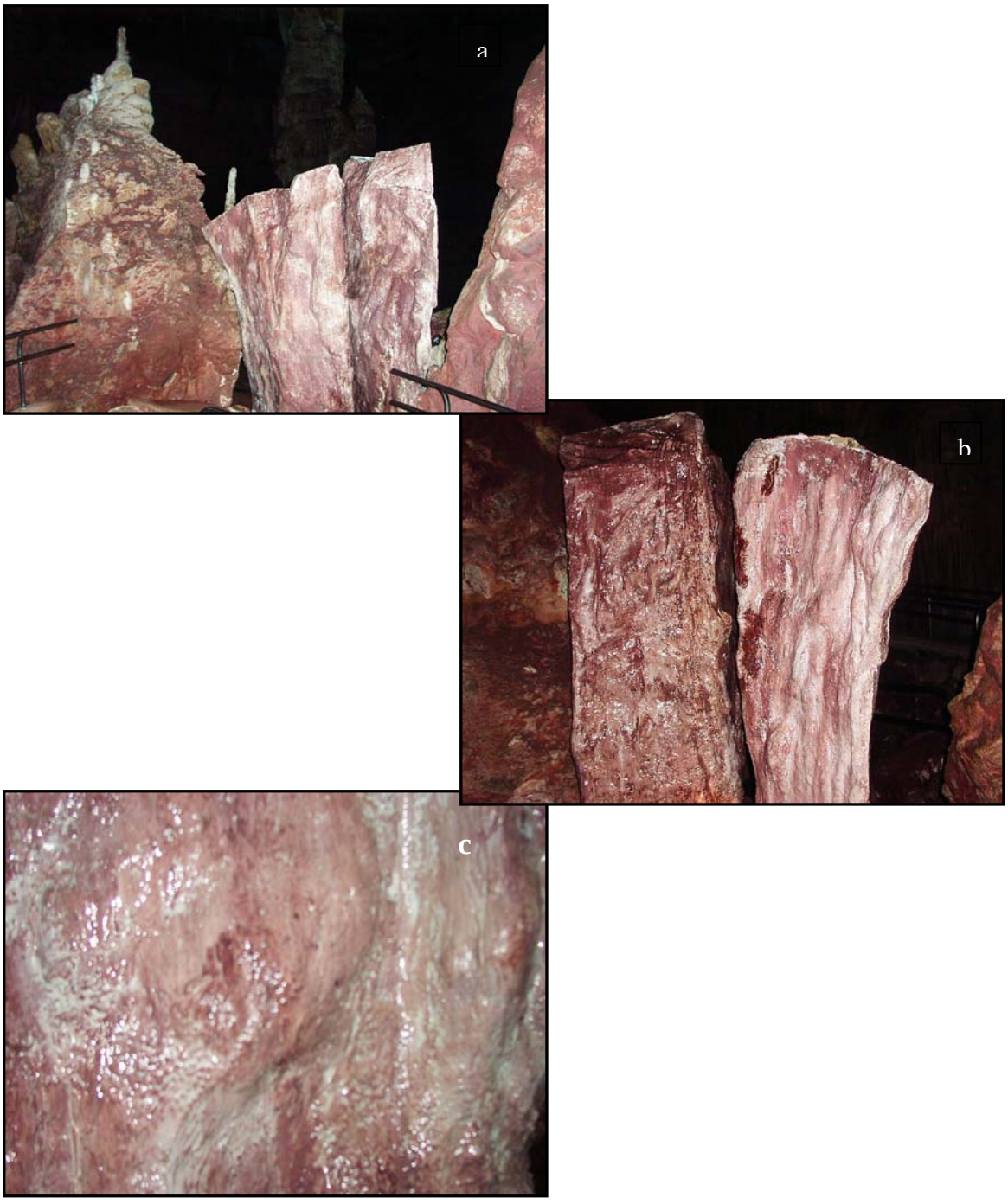


Figure 8. Neighbor Joining-Bootstrap Tree of Cultured Heterotrophic Bacteria from Painted Fiberglass and Cave Rock (High, Moderate, and Low Impact Zones)

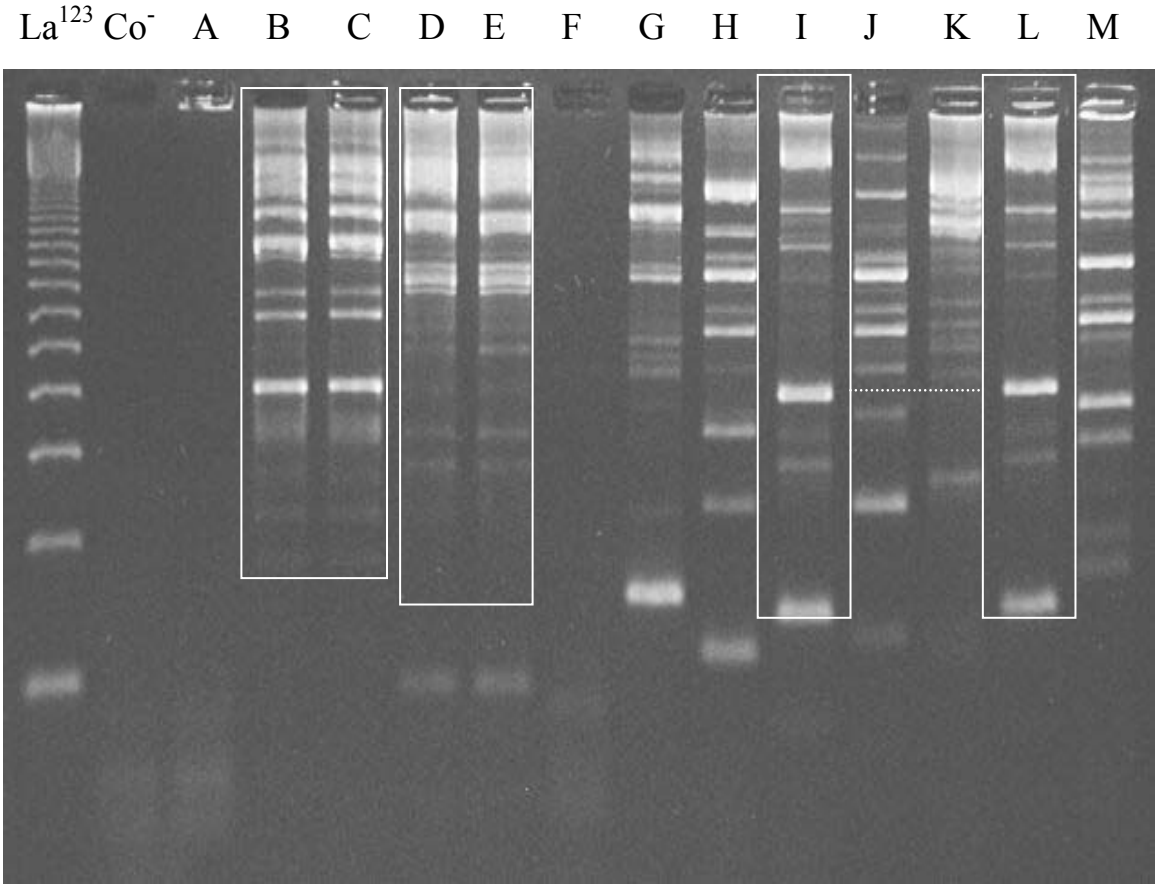
Appendix 2: Photographs



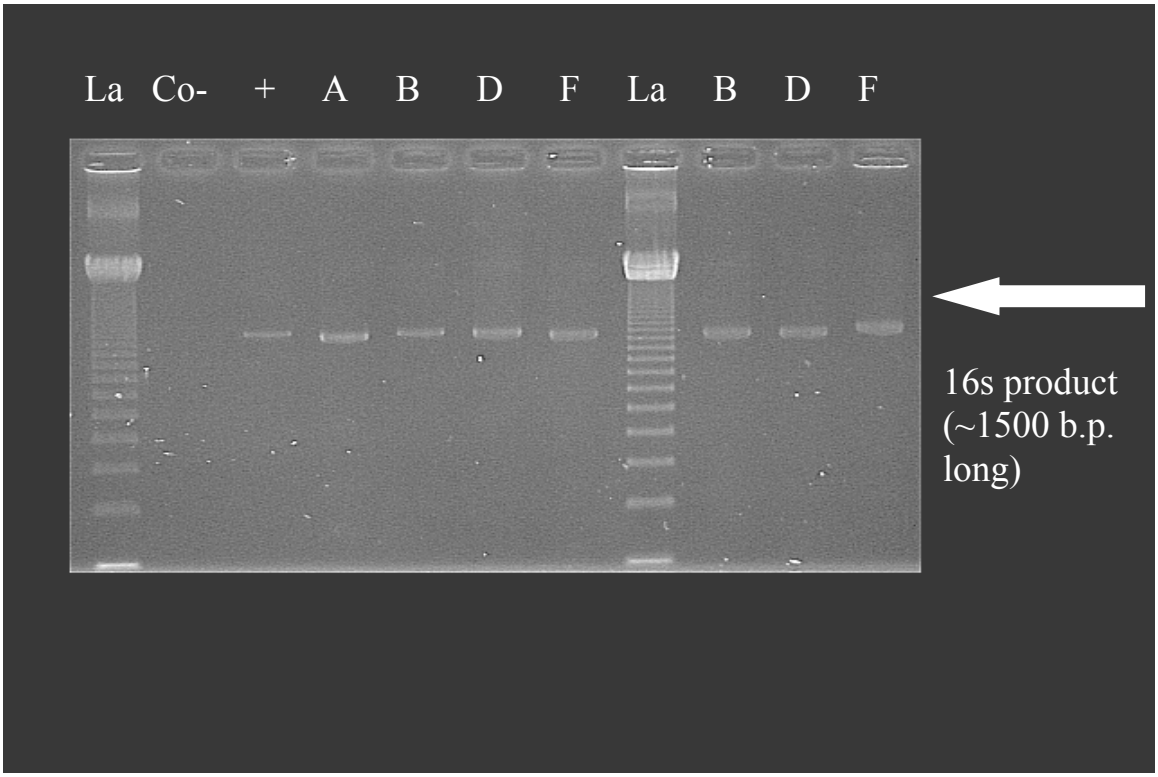
Photograph 1. Sample swabs of painted fiberglass versus cave rock (High Impact Zone, > 200,000 human entrants per year). Photograph courtesy of Julia Neilson.



Photograph 2. Painted fiberglass pieces sampled along Visitors' Trail (Throne Room – High Impact Zone), a) view from Visitor's Trail, b) view from maintenance area, and c) close-up of painted fiberglass surface



Photograph 3. REP-PCR fingerprint visualization, painted fiberglass isolates shown. Genetically identical isolates are boxed.



Photograph 4. Visualization of 16s-rDNA PCR product amplification, painted fiberglass isolates shown.

APPENDIX 3: TABLES

Table 1. Phase Two: Sample Site Classification

Official Cave Nomenclature	Impact Designation	Number of human entrants per year
Rotunda-Throne Room Complex	High	250,000 to 300,000
Grand Central Station	Moderate	30 to 40
Subway Passage	Low	2 to 3

Table 2. REP-PCR Protocol (Reaction size = 25 µl)

Reagent	Per Reaction (µl)
dd-H_2O	12.65
Buffer L*	2.50
Rep Primer Mix** (1µM conc.)	2.50
dNTP (1.25 mM)	3.10
DMSO (5.0 %)	1.25
Taq Polymerase***	0.50
DNA Template (cell lysate)	2.50

* Buffer L Recipe (1%): 10 mM Tris-HCl, 50mM KCl, 2.5 mM $MgCl_2$ (pH 8.9)

** REP1R-1 5'III ICG ICG ICA TCI GGC 3';

REP2-1 5'ICG ICT TAT CIG GCC TAC 3'

*** Roche, Indianapolis, Indiana.

REP-PCR Conditions:

The amplification program run for REP-PCR was 95^{O}C for five minutes, followed by 35 cycles of 95^{O}C for 0.5 minutes, 43^{O}C for 0.5 minutes, and 72^{O}C for 4 minutes, and a final extension at 72^{O}C for 16 minutes. PCR products were stored at –20^{O}C.

Table 3. 16s-rDNA PCR Protocol (Reaction size = 50 µl)

Reagent	Per Reaction (µl)
dd-H2O	23.3
Buffer B*	5.0
Primer Mix** (1.0 µM)	5.0
BSA (4 µg/ul)	5.0
dNTP (0.2 mM)	4.0
DMSO (5.0 %)	2.5
Taq Regular***	0.2
DNA template (lysate)	5.0

* Buffer B Recipe (1%): 10 mM Tris-HCl, 50mM KCl, 2.0 mM $MgCl_2$ (pH 8.3)

** 27f (5' AGA GTT TGA TCC TGG CTC AG 3')

1492r (5' TAC GGT TAC CTT GTT ACG ACT T 3')

*** Roche, Indianapolis, Indiana.

16s-rDNA PCR Conditions:

The amplification program run for 16s-rDNA PCR was 95^{O}C for five minutes, followed by 30 cycles of 94^{O}C for 1.0 minute, 60^{O}C for 1.0 minute, and 72^{O}C for 1.25 minutes, and a final extension at 72^{O}C for 10 minutes. PCR products were stored at -20^{O}C.

Table 4. Mineral Salts Medium (MSM) Recipe

Component	**Quantity (g/l)**
KH_2PO_4	1.0
Na_2HPO_4	1.0
NH_4NO_3	0.5
$(NH_4)_2SO_4$	0.5
$MgSO_4 \cdot 7H_2O$	0.2
$CaCl_2 \cdot 2H_2O$*	0.02
$FeCl_3$**	0.002
$MnSO_4 \cdot 2H_2O$**	0.002

*Prepared as a 10% (w:v) stock solution, added at 0.2 ml/l to produce 0.02 g/l.

** Prepared as 1% (w:v) stock solutions, added at 0.2 ml/l to produce 0.02 g/l.

Each component was dissolved in Nanopure water in the order listed, each being added after the last completely dissolved. Noble agar was added at 15 g/L, and the pH adjusted to 7.2 with 0.1 M NaOH.

Table 5. Microbial Counts (CFU/cm^2)

Official Cave Nomenclature	Human Impact Designation	Number of human entrants per year	Culturable Counts (CFU/cm^2)
Painted Fiberglass	High	>200,000	15-20
Rotunda-Throne Room Complex	High	> 200,000	5600-5750
Grand Central Station	Moderate	30 to 40	50-60
Subway Passage	Low	2 to 3	12-17

Table 6. Painted fiberglass isolates, morphology and description (High Impact Zone, >200,000 people per year)

Isolate ID	Nearest Relative	Accession # of Nearest Relative	% homology	Colony Morphology Description
PF-A	*Bacillus thuringiensis, strain 4Q281*	AF155954	99	0.9 mm, irregular w/ undulate margin, raised and ringed, opaque and white
PF-H	*Benzo[a]pyrene-degrading bacterium BPC8*	AF494539	97	0.6 mm., slightly irregular w/ entire margin, raised and smooth, mucoid, opaque and white
PF-G	*Brevibacillus agri strain NCU1002*	AY319301	98	0.2 mm, round w/ entire margin, raised and smooth , opaque and white
PF-B	*Dyadobacter fermentens*	AF137029	98	0.6 mm, round w/ entire margin, raised and smooth, translucent and yellow
PF-M	*Rhizobium huatalense* *Sinorhizobium sp. S009*	AF025852 AF285965	98 98	0.3 mm, round w/ entire margin, raised and smooth, slightly opaque and off-white
PF-I	*Sphingomonas sp. AVC6*	AF434172	99	0.4 mm, slightly irregular w/ entire margin, raised and smooth, mucoid, translucent and yellow
PF-D	*Sphingomonas sp., strain B1*	X94099	100	0.1 mm, round w/ entire margin, raised and smooth, slightly opaque and yellow
PF-K	*Sphingomonas sp. P2*	AB091683	98	0.3 mm, round w/ entire margin, raised and smooth, opaque and dark yellow
PF-F	*Staphylococcus sp. es1*	AJ704792	99	< 0.1 mm, round w/ entire margin, raised and smooth, opaque and orange

Table 7. Utilization of 100% acrylic paint as a sole carbon source by painted fiberglass isolates

Isolate Code	Nearest Relative/ Accession #	10,000 mg/L	1,000 mg/L	100 mg/L
PF-A	*Bacillus thuringiensis, strain 4Q281* / AF155954	+	+	+
PF-H	*Benzo[a]pyrene-degrading bacterium BPC8* / AF494539	+	+	+
PF-G	*Brevibacillus agri strain NCU1002* / AY319301	-	-	-
PF-B	*Dyadobacter fermentens*	+	+	+
PF-M	*Rhizobium huatulense*/ AF025852 *Sinorhizobium sp. S009/* AF285965	-	-	+
PF-I	*Sphingomonas sp. AVC6*	+	+	+
PF-D	*Sphingomonas sp., strain B1*/ X94099	+	+	+
PF-K	*Sphingomonas sp. P2* / AB091683	-	-	+
PF-F	*Staphylococcus sp. es1*/ AJ704792	-	-	-

Table 8. High impact cave rock isolates, morphology and description (> 200,000 visitors per year)

Isolate ID	Nearest Relative	Accession # of Nearest Relative	% homology	Colony Morphology Description
HI-ABCE2	*Alcaligenes eutrophus*	M32021	99	< 0.1 mm, round with entire margin, smooth and convex, translucent and off-white
HI-D3	*Bacillus macroides* *Bacillus sp. LMG 21002*	AF157696 AJ316308	100 100	0.7 mm, irregular w/ undulate margin, ringed and flat, opaque and white
HI-G2	*Bacillus sp. TB3-10-I* *Bacillus thuringiensis 4Q281* *Bacillus thuringiensis ATCC 10792*	AY599744 AF155954 AF290545	100 100 100	2.0 mm, round with entire margin, smooth and flat, translucent w/ yellow tinge
HI-F1	*Bacterium SG-3* *Lysobacter brunescens**	AF548381 AF548381	100 100	0.2 mm, round w/ entire margin, smooth and raised, translucent w/ dark yellow tinge
HI-F2	*Curtobacterium sp. SG041* *Curtobacterium flaccumfaciens*	AF474329 AJ312209	99 99	0.5 mm, round w/ slight undulate margin, smooth and flat, outer 0.1 mm translucent w/ yellow opaque centers
HI-M4	*Flavobacterium johnsoniae*	AB078043	99	0.7 mm, irregular with undulate margin, smooth convex, translucent w/ pale yellow tinge
HI-A4a	*Kocuria erythromyxa*	Y11330	99	0.1 mm, round w/ entire margin, smooth and convex, opaque and yellow, opaque and white

HI-CDE1	*Methylobacterium sp. strain F73*	D32237	99	0.1 mm, round w/ entire margin, smooth and raised, opaque and pink
HI-D2	*Oxalobacter sp. p8E*	AJ496038	98	< 0.1 mm, round w/ entire margin, smooth and raised, translucent w/ yellow tinge
HI-I1	*Phenanthrene-degrading bacterium* *Sphingomonas sp.IFO 15917**	AY117357 AB033950	98 98	0.2 mm, round with entire margin, smooth and convex, translucent w/ orange tinge
HI-B17	*Pseudomonas alcaligenes*	AF094721	99	0.2 mm, round w/ entire margin, smooth and raised, translucent and off white
HI-B10	*Pseudomonas anguilliseptica BI*	AF439803	98	0.8 mm, irregular w/ undulate margin, smooth and flat, translucent and off-white
HI-B7	*Pseudomonas sp. SMCC B0628* *Pseudomonas sp. SMCC B0361* *Pseudomonas sp. strain DhA-51*	AF501878 AF500621 AJ011507	99 99 99	1.1 mm, slightly irregular with entire margin, smooth and flat, mucoid, translucent w/ yellowish tinge
HI-G1	*Pseudomonas sp. HR 26*	AY032726	99	1.0 mm, irregular w/ undulate margin, smooth and raised, outer 0.2 mm translucent, yellow-tinged opaque centers
HI-B13	*Sinorhizobium sp. strain BK1*	AJ012210	99	0.3 mm, round w/ entire margin, smooth and raised, slightly opaque and off-white
HI-K4	*Sphingomonas sp. S37*	AF367204	100	0.2 mm, round w/ entire margin, smooth and convex, opaque and yellow

HI-D4	*Sphingomonas ursincola* *Sphingomonas natatori*	AB024289 AB024288	99 99	0.4 mm, round w/ slightly undulate margin, smooth and raised, translucent and dark orange
HI-G4	*Ultramicrobacterium strain ND5*	AB008506	99	0.1 mm, round w/ entire margin, smooth and convex, opaque and off-white
HI-O4	*Uncultured gamma proteobacterium* *Acinetobacter johnsonii**	AF324537 AB099655	99 99	0.1 mm, round w/ entire margin, smooth and convex, opaque and white
HI-B9	*Variovorax sp. K6*	AF532867	99	0.2 mm, round w/ undulated margin, smooth and convex, translucent w/ yellow tinge
HI-I4	*Variovorax sp. TUT1027*	AB098595	99	0.8 mm, round w/undulated margin, smooth and flat, translucent and off-white

* Offers the genus description.

Table 9. High impact culturable heterotrophs versus viable heterotrophic range %'s from soils

Cultured phylum	Viable Range %'s in Soil (Alexander 1971)	Kartchner Isolate Ranges (PF and HI Isolates)
Actinobacteria	5 – 60 %	6 – 7 %
Bacteriodetes	2 – 10 %	10 – 11 %
Firmicutes	7 – 67 %	17 – 18 %
α-Proteobacteria	Up to 20 %	31 – 32 %
β-Proteobacteria	2 – 12 %	17 – 18 %
γ-Proteobacteria	3 – 15 %	17 – 18 %

Table 10. Moderate Impact Cave Rock Isolates, Morphology and Descriptions (30 to 40 visitors per year)

Isolate ID	Nearest Relative	Accession # of nearest relative	% homology	Colony Morphology Description
MI-69a	*Actinomycetaceae SR272*	X87310	99	0.6 mm, irregular w/ undulate margin, smooth and umbonate, opaque and bright yellow
MI-26ac1	*Aeromicrobacterium erythreum*	AF005021	96	<0.1 mm, round w/ undulate margin, smooth and raised, opaque and white
MI-p2a	*Aminobacter aminovorans*	AJ011759	99	0.6 mm, round w/ entire margin, smooth and flat, mucoid, translucent w/ brownish tinge
MI-65a	*Bacillus sp. 433-D9*	AY266991	100	0.5 mm, irregular w/ lobate margin, smooth and flat, translucent and colorless
MI-ab	*Bacillus amyloliquefaciens*	AY620954	99	0.8 mm, slightly irregular w/ moderately undulate margin, ringed flat, opaque and white
MI-33a1	*Bacillus aquaemaris*	AF483625	97	0.3 mm, round w/ undulate margin, smooth and convex, opaque and off-white
MI-32a2	*Bacillus cereus biovar toyoi*	AJ310100	99	0.7 mm, round w/ filiform margin, smooth and flat, opaque and white
MI-c1a1	*Bacillus cereus LRN*	AY138275	99	1.5 mm, irregular w/ filiform margin, smooth and raised, opaque and white
MI-3a	*Bacillus sp. IDA4917*	AY289504	98	0.2 mm, irregular w/ undulate margin, smooth and flat, opaque and white

MI-22a2	*Bacillus sp. LMG-19415*	AJ276809	98	0.3 mm, irregular w/ undulate margin, slightly wrinkled and flat, opaque and white
MI-qb2	*Bacillus macroides* *Bacillus simplex* *Bacillus sp. LMG 21002*	AF157696 AJ628747 AJ316308	99 99 99	0.9 mm, irregular w/ undulate margin, ringed and flat, opaque and off-white
MI-58a	*Bacillus pichinotyi RS2*	AF519464	97	0.3 mm, round w/ slight undulate margin, smooth and flat, translucent and off-white
MI-33a2	*Bacillus pumilus KL-052*	AY030327	99	0.1 mm, round w/ entire margin, smooth and convex, mucoid, opaque and white
MI-72a	*Bacillus pumilus*	AB020208	100	0.2 mm, round w/ slightly undulate margin, smooth and convex, translucent and off-white
MI-23a1	*Bacillus thuringiensis 4Q281* *Bacillus cereus ATCC 53522*	AF155954 AF290551	99 99	0.9 mm, irregular w/ undulate margin, ringed and flat, mucoid, opaque and white
MI-67a2	*Bacillus sp. YY*	AF414443	99	0.8 mm, round w/ slightly undulated margin, smooth and convex, translucent w/ yellow tinge
MI-46a	*Kocuria erythromyxa*	Y11330	98	0.1 mm, slightly irregular w/ entire margin, smooth and convex, translucent and colorless
MI-59a	*Nocardioides sp. A-3*	AB087724	99	0.1 mm, round w/ filiform margin, smooth and raised, opaque and white
MI-61a	*Paenbacillus sp. DS-1*	AB042938	99	0.2 mm, round w/entire margin, smooth and flat, opaque and white

MI-10a	*Phenanthrene-degrading bacterium* *Uncultured bacterium clone O-CF--10* *Janthinobacterium sp. J3**	AY177375 AF443565 AB097172	97 97 96	0.3 mm, round w/ entire margin, smooth and convex, opaque and orange
MI-o1	*Pseudomonas sp. CA-10*	AB047273	99	0.1 mm, irregular w/ undulate margin, smooth and umbonate, outer 0.3 mm translucent w/ off-white, opaque centers
MI-45a	*Pseudomonas sp. HR 26*	AY032726	99	0.3 mm, irregular w/ undulate margin, smooth and umbonate, translucent and off-white
MI-57a	*Pseudomonas sp. PCP2*	AF326380	98	0.4 mm, round w/ entire margin, smooth and flat, translucent and colorless
MI-na	*Ralstonia sp. AU3369* *Uncultured bacterium CCMC0* *Ralstonia sp. BPC3*	AF500587 AY221074 AF494541	99 99 99	0.5 mm, round w/ undulate margin, smooth and flat, translucent w/ brown tinge
MI-11a	*Rhodococcus equi DSM 2030*	AF490539	99	0.1 mm, round w/ entire margin, smooth and raised, opaque and off-white w/ brown center
MI-71a	*Streptomyces peucetius*	AB045887	99	0.6 mm, round w/ curled margin, powdery (white) and umbonate, opaque and purple

* Offers the genus description.

Table 11. Low impact cave rock isolates, morphology and description (2 to 3 visitors per year)

Isolate ID	Nearest Relative	Accession # of nearest relative	% homology	Colony Morphology Description
L17	*Bacillus sp. 19489*	AJ315057	99	1.2 mm, round w/ lobate margin, smooth and flat, opaque and off-white
L83	*Bacillus amyloliquefaciens* *Bacillus so. Bch1* *Bacillus sp. PP19-H3*	AY620954 AF411118 AB050667	99 99 99	0.7 mm, round w/ undulate margin, smooth and flat, opaque and off-white
L80	*Bacillus catenulatus*	AY523411	99	0.2 mm, irregular w/ undulate margin, smooth and flat, opaque and white
L18	*Bacillus cereus ATCC 14579*	AE017013	99	0.5 mm, filamentous w/ filiform margin, flat, opaque and off-white
L64	*Bacillus cereus* *Bacillus sp. TB3-10-I*	AJ629413 AY599744	100 100	0.3 mm, round w/ undulate margin, smooth and raised, opaque and white
L2	*Bacillus sp. Fa29* *Bacillus megaterium strain MO31*	AY131222 AY553118	99 99	1.4 mm, irregular w/ undulate margin, mucoid, smooth and raised, opaque and white
L31	*Bacillus firmus*	AJ509007	98	0.1 mm, round w/ slight undulate margin, smooth and flat, translucent and colorless
L45	*Bacillus sp. fusiformis*	AJ310083	99	<0.1 mm, round w/ entire margin, smooth and convex, opaque and white
L14	*Bacillus sp. IDA4789*	AY289499	98	0.8 mm, irregular w/ undulate margin, smooth and flat, opaque and white

L66	*Bacillus sp. LMG 21002*	AJ316308	99	0.7 mm, irregular w/ undulate margin, ringed and flat, opaque and white
L105	*Bacillus luciferensis*	AJ419629	99	0.7 mm, round w/ undulated margin, smooth and raised, opaque and off-white
L41	*Bacillus MK03*	AB062678	99	0.1 mm, round w/ undulated margin, smooth and flat, opaque and white
L15	*Bacillus sphaericus*	AB116123	99	0.4 mm, irregular w/ undulated margin, smooth and raised, opaque and white
L67	*Brevibacillus laterosporus*	AB112720	99	0.1 mm, round w/ entire margin, smooth and raised, opaque and white
L5	*Kocuria erythromyxa*	Y11330	98	0.1 mm, slightly irregular w/ entire margin, smooth and convex, translucent and colorless
L40	*Lysobacter sp. Dae16*	AB166878	97	0.5 mm, round w/ entire margin, smooth and convex, slightly opaque and orange
L48	*Mesorhizobium chacoense*	AJ278249	99	0.6 mm, round w/ entire margin, mucoid, smooth and convex, translucent and off-white
L70	*Moraxella osloensis*	AJ505859	99	0.1 mm, round w/ entire margin, smooth and convex, translucent and off-white
L47	*Mycobacterium elephantis*	AJ536100	97	0.2 mm, round w/ entire margin, smooth and crateriform, opaque and off-white
L23	*Paenbacillus alvei DSM 29T*	AJ320491	95	diffuse, smooth, opaque lawn over the entire agar surface, off-white

L55	*Paenbacillus alvei*	AB073200	98	0.1 mm, round w/ entire margin, smooth and flat, translucent and colorless, colonies grow separately and in clusters over the entire plate
L32	*Paenbacillus apiarus*	AB073201	99	0.1 mm, round w/ entire margin, smooth and convex, opaque and white
L54	*Paenbacillus honkongensis*	AF433165	97	0.2 mm, round w/ entire margin, smooth and raised, translucent and colorless
L63	*Paenbacillus kobensis*	AB073363	98	growth appears as mucoid and raised, opaque and off-white swirls w/ entire margin
L9	*Paenbacillus sp. O-3b*	AB089251	99	0.7 mm, slightly irregular w/ entire margin, ringed and umbonate, opaque and off-white
L13	*Pseudomonas sp. HR 26*	AY032726	99	0.5 mm, irregular w/ undulate margin, smooth and raised, translucent and off-white
L10	*Staphylococcus saprophyticus* *Arctic Sea ice bacterium*	L37596 AF468443	99 99	0.1 mm, round w/ entire margin, smooth and raised, opaque and white
L42	*Streptomyces sp. AS 4.1182*	AY114179	98	0.2 mm, round w/ slightly undulate margin, ringed and crateriform, opaque, dark brown perimeter w/ off-white center, diffusible pigment
L49	*Streptomyces sp. KN-1220*	AY029699	98	0.8 mm, round w/ undulate margin, wrinkled and crateriform, opaque and dark orange w/ red centers
L53	*Streptomyces lipmanii*	AB045861	99	<0.2 mm, round w/ entire margin, powdery (white) and convex, opaque and dark brown

L27	*Streptomyces phaeochromogenes*	AF500071	99	0.1 mm, round w/ slightly undulate margin, powdery (white) and convex, opaque and pink
L60	*Xanthomonas sp. TB8-9-II*	AY599706	97	0.1 mm, round w/entire margin, smooth and raised, translucent and colorless

*Offers the genus description

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