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Using scanning electron microscopy to study microbial communities in speleothem samples collected from Iron Curtain Cave

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New microorganisms are continually sought after in the hopes of finding new bioactive compounds, so researchers are looking to extreme environments such as caves to find them. The scanning electron microscope (SEM) can be used to study environmental microorganism colony structure and morphology in a natural state. Sputter coated environmental samples can be used to characterize bacteria, but this method can be expensive and time consuming, making it cost-prohibitive for smaller labs. The aim of this study was to observe and compare cave bacterial isolates on Gold-Palladium (AuPd) coated and uncoated soda straw speleothem samples and from inoculated R2A agar using SEM. The resulting images were then used to describe colony structure and morphology of the isolated organisms and establish which specimen preparation treatment produced the most efficient means of characterization. The study revealed that the uncoated samples generally produced images of similar quality to the coated samples but the image quality was lower at higher magnifications. Although culturing bacteria from the speleothem and viewing them with the SEM proved to be faster than searching the entire speleothem, growth conditions could drastically change colony formation and potentially lead to missed species that are present.

Bacteria are present everywhere, including in soil, rocks, bodies of water, the atmosphere and even other living organisms. Bacteria that inhabit other organisms can positively or negatively affect the health of the host. Bacteria also play major roles in the environment by producing useful compounds, breaking down old material and reshaping the environment itself. This has led researchers to explore extreme environments in hopes of discovering novel microorganisms that may produce useful bioactive compounds. Volcanic caves have recently been shown to be an important research destination because they exhibit unique microbial life. The first volcanic cave microbiological study in Canada was done by Cheeptham et al. (2013) at the Helmcken Falls cave in British Columbia. Those researchers sampled various regions of the cave and determined that 80 out of the 400 bacterial isolates had antimicrobial effects against ß-lactamase producing *Escherichia coli*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae*. Also, when samples taken from the three mineral-rich rock, wall, and speleothem samples were viewed with the scanning electron microscope (SEM), a great abundance of bacterial life was revealed (1).

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Electron microscopy is one of the few techniques suitable for showing bacteria in their natural habitat. Scanning electron microscopy is especially useful because it is easy to perform and interpret because of the three-dimensional image it produces (2). Traditional SEM on coated samples results in higher-resolution micrographs, but the technique is relatively expensive and time consuming, making it impractical for smaller labs. The coating process may also introduce artifacts to the samples or otherwise alter them. Environmental SEM (ESEM) techniques, which may produce images with lower resolution than conventional SEM, are quicker because samples require little to no preparation time. Because ESEM samples are uncoated, the resulting images may be more "true-to-life". The large depth of field also allows for examining the surface structure of samples. This makes it useful for visualizing bacteria residing in complex colonies and even biofilms (3). SEM techniques have been used to determine relationships between hospital device surfaces and the bacteria that

colonize them (4, 5). These techniques have also been applied to environmental samples, such as mineral deposits in caves.

In caves, there are many types of secondary mineral deposits, which are simply referred to as decorations but are technically called speleothems. Cave soda straws are tubular speleothems (Fig 1) that form when water containing dissolved calcium carbonate or calcium sulfate drips from the tubes; as each drop hovers at the tip, it deposits a ring of precipitated mineral at the edge and the tube elongates. Soda straws are of great interest as they may form from the presence and activity of bacteria (6, 7). Many studies have been conducted using scanning electron microscopy to determine the relationship between mineralization and the responsible microorganisms (3, 7, 8, 9).

FIG 1 Speleothem in the Iron Curtain Cave system in Chilliwack, British Columbia, Canada. Picture courtesy of Dayon Traynor.

The goal of our study is to determine if bacteria could be observed on soda straw samples collected from the Iron Curtain Cave in Chilliwack, British Columbia, Canada. We wanted to compare minimally prepared (non-coated) and Gold-Palladium (AuPd) prepared (coated) techniques to assess the appropriateness of each. To achieve these objectives, we obtained a soda straw from the Iron Curtain Cave system located in Chilliwack, British Columbia in the summer of 2014. This soda straw was sectioned into two pieces: one section was sent to the University of British Columbia (UBC) Bioimaging Facility to be sputter coated in AuPd, whereas the other section was swabbed and plated in order to isolate and view the bacteria with the SEM. The swab was used to inoculate R2A agar (Difco ™ Pre-made dehydrated R2A agar) because this growth medium allows for the culturing of slower growing organisms. Once isolated, the coated, non-coated, and isolated bacteria from the soda straw were viewed on an SEM with ESEM capabilities (the Zeiss LS EVO SEM). The samples were viewed with SEM (coated) and ESEM (non-coated) conditions in order to determine the most efficient technique based on cost, preparation time and image quality.

MATERIALS AND METHODS

Culturing of Speleothem samples. Speleothem samples were collected from the Iron Curtain Cave system in Chilliwack, British Columbia, Canada on April 27, 2014 (Fig 1). The soda straws (Fig 2) were collected in pre-sterile plastic screw-cap tubes, kept at 4°C, and the tubes were wrapped in tinfoil to protect them from UV damage. In early May 2014, cotton swabs were used to inoculate R2A agar plates after swabbing the inside of the soda straw sample. The standard four-way streak method was used to streak for isolated colonies. The R2A agar plates were incubated at 4°C for approximately 140 days.

FIG 2 Soda straw speleothem from Iron Curtain Cave (units in mm).

Scanning Electron Microscopy of the Gold-Palladium Coated Soda Straw Samples. The soda straw samples were coated with 10 nm AuPd at 40 µA in a Crestington 208 HR sputter coater using a rotary-planetary-tilting Stage (RPT) and affixed to stubs (Specimen mount, pin type, slotted head: Canemco-Marivac, Quebec, Canada) with 12 mm diameter carbon conductive spectro tabs (Canemco-Marivac, Quebec, Canada) (Derrick Horne, personal communication, Nov 2014).

TABLE 1 SEM conditions used to view the AuPd coated soda straw, the uncoated soda straw, and the uncoated agar colony samples.

	Vacuum	Accelerating Voltage (kV)	Filament Amperage (A)	Spot Size (pA)	Detector	Working Distance (mm)
Gold-Palladium Coated	Full	20.00	1.676	100	#SE1	12-14
Uncoated Soda straw	Extended Pressure (44 Pa)	20.00	1.653	100	#VPSE	12-14
Uncoated Agar Colony	Extended Pressure (47 Pa)	20.00	1.684	100	#VPSE	12-14

#SPE1: Secondary Electron
#VPSE: Variable Pressure Secondary Electron

The stubs with the AuPd samples were loaded into the chamber of the Zeiss LS EVO SEM. The operating conditions used can be viewed in Table 1. The samples were viewed with various magnifications, but higher magnifications were used to view the bacteria on the sample. Images were adjusted for brightness and contrast with ImageJ, an image processing program.

Scanning Electron Microscopy of the Uncoated Soda Straw Samples. The soda straw sample was broken into smaller pieces to fit on a stub. Swabbed pieces may have disrupted colony structures and were avoided. Two shards (approximately 1 cm each) were used: one placed with the outside of the soda straw face up and one placed with the inside face up. The shards were attached to the stub with carbon tape and loaded into the chamber of the Zeiss LS EVO SEM. The operating conditions are listed in Table 1. Images were adjusted for brightness and contrast with ImageJ, image-processing program.

Scanning Electron Microscopy of the Uncoated Agar Colony Samples. The R2A agar was cut out and viewed with the Zeiss LS EVO SEM. Squares of approximately 0.5x0.5 cm size were aseptically cut out of the bacteria cultured agar plates using a sterile loop to prevent contamination. The agar squares contained isolated colonies of bacteria that were to be viewed. The squares were put on stubs with the use of carbon tape, such that the colonies were on the top side of the square. The stubs were then loaded into the Zeiss LS EVO SEM chamber (Table 1). The stubs were deliberately left in full vacuum for a few minutes to dry the agar, which allows for easier viewing of the bacterial colonies. Images were adjusted for brightness and contrast with ImageJ.

RESULTS

Culturing of Soda Straw Samples. The bacterial microbiome in caves could potentially house new bacterial species, so it is important that it be characterized. The soda straw cave speleothem was swabbed in order to inoculate R2A agar plates, which could then be examined for bacterial growth. The standard four-way streak method was used to isolate colonies, which subsequently produced four different bacterial colony types, each on a separate plate. The four isolated colonies were then designated as soda straw ground (SSG) B, C, E, and F to denote the different colony types.

Fig 3A shows the bacterial colony designated SSG-B. SSG-B produces large, grayish-white colonies that were shiny. SSG-C produces medium-sized, milky white colonies that were shiny (3B). SSG-E produces medium-sized, grey colonies that were opaque (3C; note that the brown mold-like colonies were contaminants as was confirmed with the SEM). 3D shows the bacterial colony designated SSG-F. SSG-F produced small, white colonies that were shiny. Taken together, the colonies isolated with R2A agar plates reveal a microbial community that inhabits the soda straw.

Scanning Electron Microscopy of the Gold-Palladium Coated Soda Straw Samples. The AuPd coated soda straw samples were viewed with the SEM at full vacuum. Traditionally, coated samples under conventional SEM operating conditions produce images with better resolution as compared to uncoated samples. This study

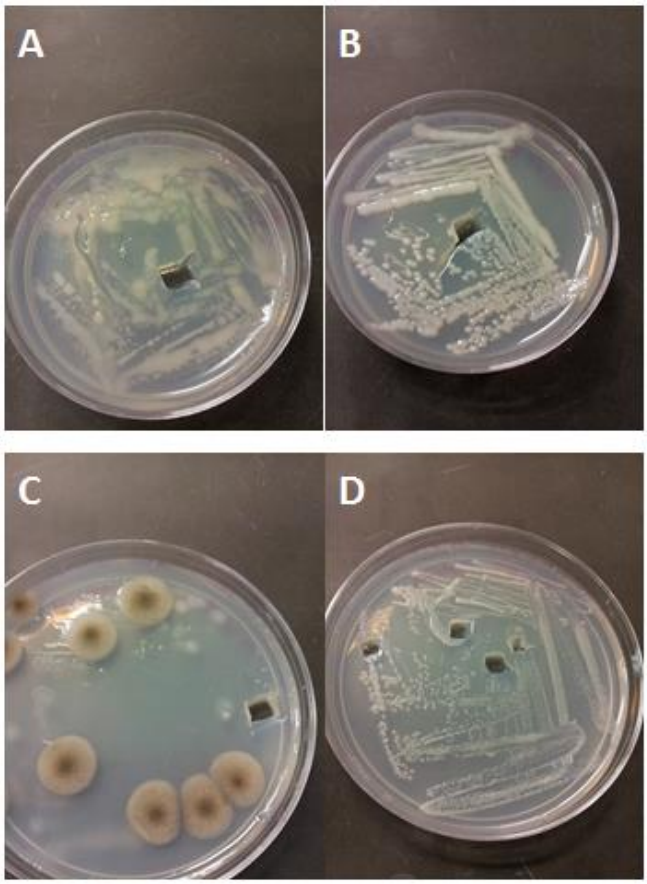

FIG 3 R2A plates resulting from swabbing a soda straw for isolated colonies of unidentified microorganisms found in Iron Curtain Cave, British Columbia. (A) SSG-B: large, shiny, greyish-white colonies. (B) SSG-C: medium-sized, shiny, milky white colonies. (C) SSG-E: medium-sized, opaque, grey colonies (brown mold-like colonies were contaminants). (D) SSG-F: small, shiny, white colonies.

supported this observation and showed that the coated samples had a good resolution at high magnifications. Fig 4 shows the results of viewing the coated sample on the SEM. Fig 4A shows a bacillary bacterium, Fig 4B shows a possible *Staphylococcus* species, and finally Fig 4C shows three coccal bacteria.

Scanning Electron Microscopy of the Uncoated Soda Straw Samples. By viewing the uncoated soda straw sample in the SEM with partial vacuum, the images could be compared with the coated sample images and used to determine which method generated higher quality images. Fig 5 identifies two different types of bacteria observed on the non-coated sample. Fig 5A shows a biofilm-producing coccus, which was on the inside of the soda straw, whereas Fig 5B shows a rod-shaped bacterium on the outside of the soda straw.

Scanning Electron Microscopy of the Uncoated Agar Colony Samples. Agar samples were viewed under the SEM in order to observe the morphology of the cultured SSG bacteria. These agar samples were left in the vacuum intentionally in order to form cracks within the colonies,

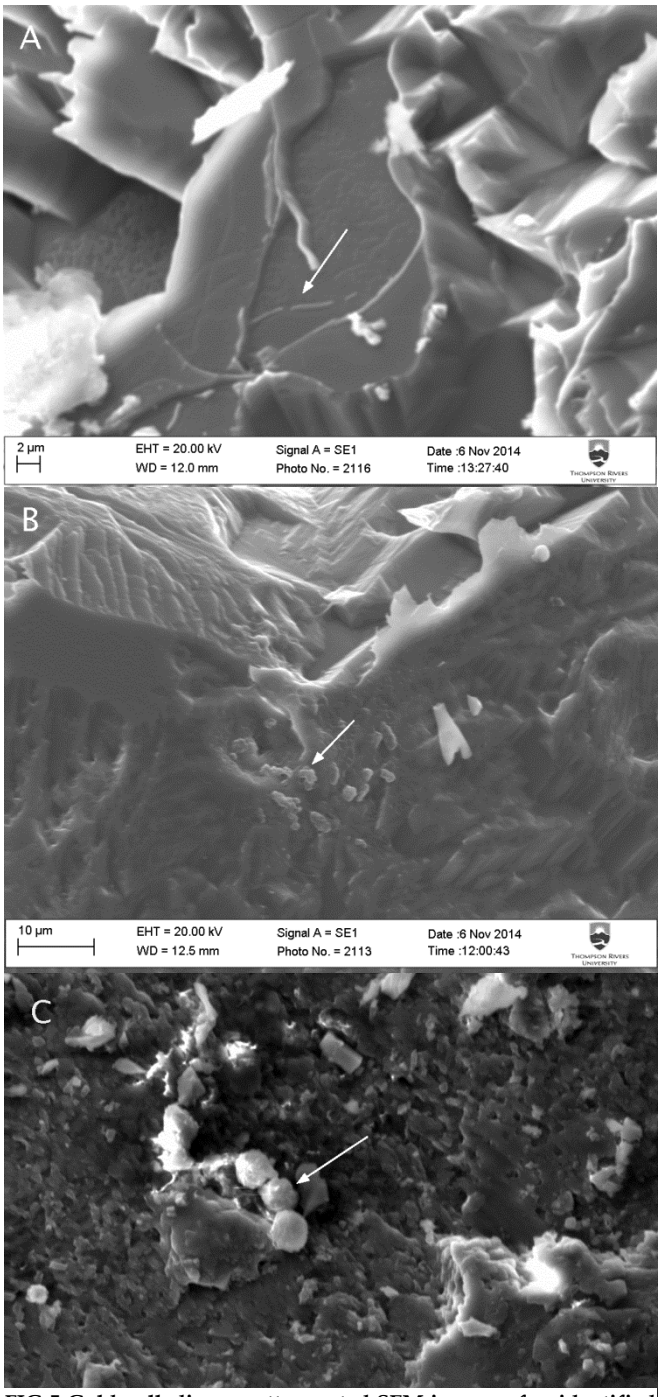

FIG 5 Gold-palladium sputter-coated SEM images of unidentified microorganisms observed in a soda straw found in Iron Curtain Cave, British Columbia, Canada. As indicated by arrows: (A) Rod-shaped bacteria. (B) Putative staphylococcal bacteria. (C) Putative streptococcal bacteria**.**

thus giving better detail of the bacterial colonies. Fig 6 shows the SEM micrographs of the four SSG samples on agar. SSG-B is a streptococcus-like species (Fig 6A). Fig 6B shows the morphology of SSG-C, which is a putative staphylococcal species. Fig 6C shows the morphology of SSG-E, which is a rod-shaped species. Finally, Fig 6D shows the morphology of SSG-F, which is a coccal species.

DISCUSSION

The ability to visualize bacteria on both the uncoated soda straws and agar samples shows that coating the speleothem is not necessary but can improve the resolution. We have demonstrated that the SEM micrographs can be used to characterize the bacteria found on uncoated soda straw and cultured agar samples.

This study used different SEM parameters to obtain the best possible resolution. The parameters used were vacuum strength, accelerating voltage (kV), filament amperage (A), spot size (pA), detector type and working distance. The SEM conditions selected for optimal viewing of the coated and uncoated samples

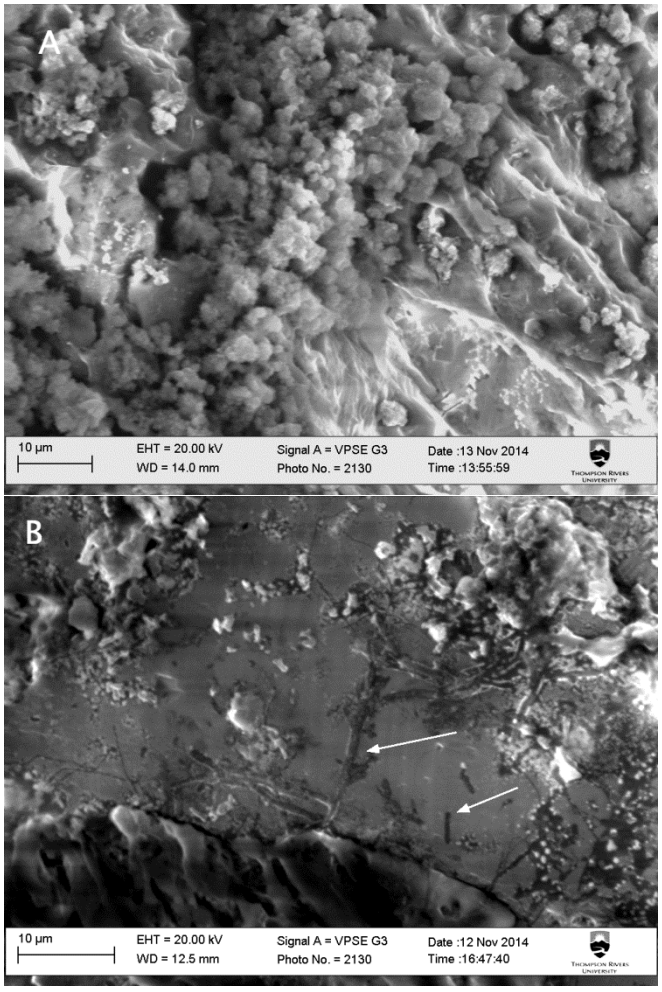

FIG 4 SEM images of unidentified microorganisms observed on a soda straw from Iron Curtain Cave, British Columbia, Canada. (A) Biofilm found inside of a soda straw. (B) Rod-shaped bacteria found outside of a soda straw as indicated by arrows.

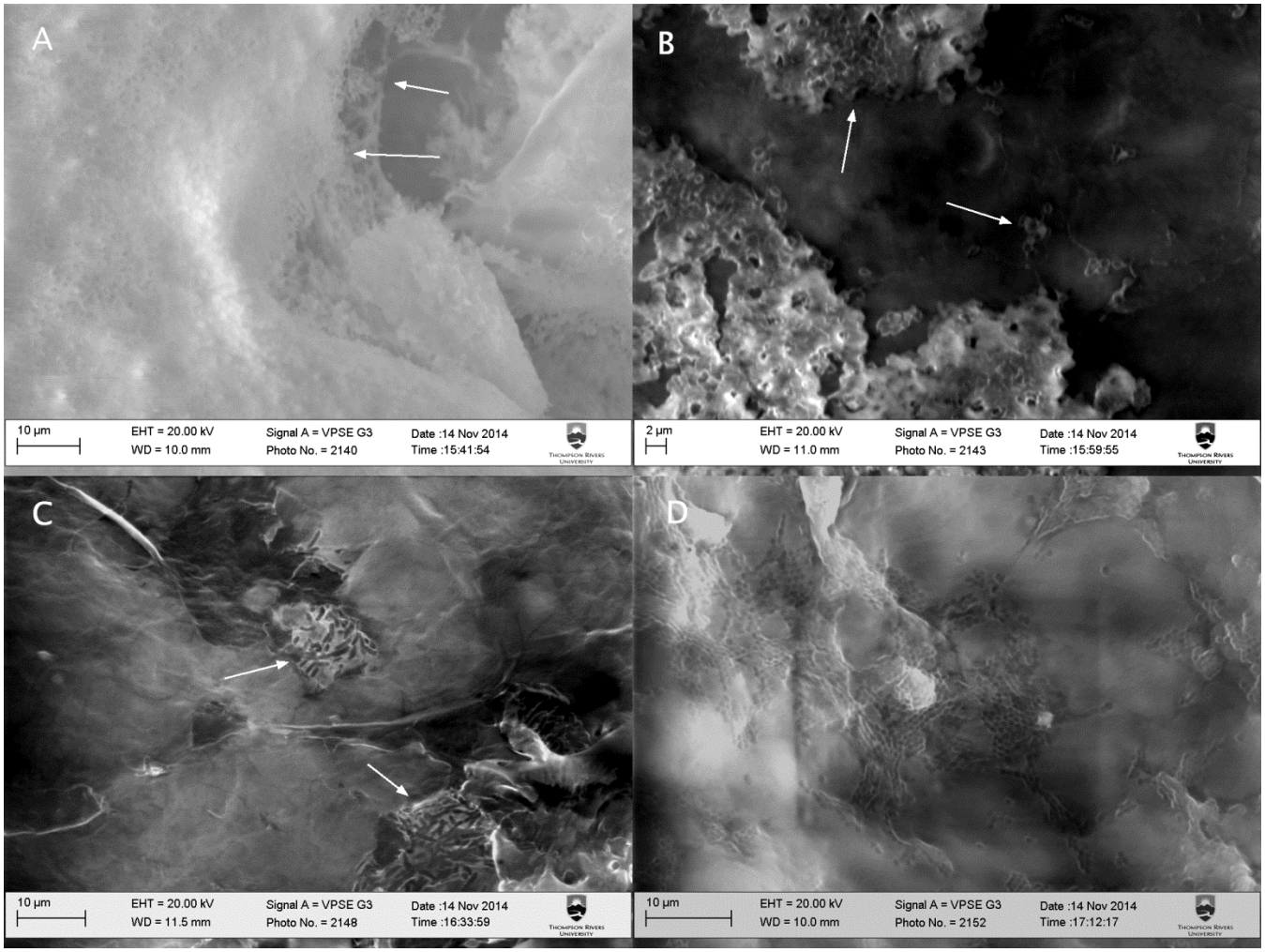

FIG 6 SEM images of unidentified microorganisms cultured from a soda straw found in Iron Curtain Cave, British Columbia, Canada. As indicated by arrows: (A) SSG-B: putative streptococcal bacteria. (B) SSG-C: putative staphylococcal bacteria. (C) SSG-E: rod-shaped bacteria. (D) SSG-F: coccal bacteria.

only differed in the vacuum strength (full versus partial) and detector type (SE1 versus VPSE) (Table 1). The uncoated soda straw and agar colony samples were examined with very similar parameters. Both experiments used an extended pressure vacuum at ~45 Pa and the "VPSE" detector. Additionally, all three samples were examined with the same acceleration voltage, filament amperage and working distance. The uncoated samples were viewed under partial vacuums to prevent charge build up on the specimen, allowing for a clearer image. Overall, though, the SEM conditions for both the uncoated and coated samples resulted in images of similar quality.

Both the coated (Fig 4) and the uncoated (Fig 5) cave straw samples yielded images of bacteria residing on the sample, although the coated sample appears to have slightly betterr resolution. The AuPd coated sample seems to show small groups of rod shaped and coccal bacteria. The uncoated sample mainly shows coccal bacteria aggregated into a biofilm structure (Fig 5A). This difference in bacterial clustering is not surprising as the coating process dehydrates the sample. This means that the water containing matrix of biofilm bacterial colonies could be disfigured or at least changed in the coating process (3). While the images may be of similar quality, the uncoated samples seem to yield images of microbes in a more natural state. However, it was difficult to obtain images of the uncoated sample at magnifications similar to previous studies involving SEM imaging of biofilms (3). The coated soda straw sample may produce images of higher resolution, but the bacterial formations may not be indicative of how the bacteria colonized in the natural environment.

Fig 4A and Fig 5B depict bacteria that are similar in morphology and both images show singular, rod-

shaped bacteria. In the uncoated sample (Fig 5B), the rod-shaped bacteria were initially difficult to see. After decreasing the brightness and increasing the contrast, the rod shape became apparent. Fig 4B and Fig 4C both show coccal morphologies similar to those Fig 5A, which was taken from the uncoated, inner side of the soda straw and appears to show the bacteria in an alginate biofilm formation. The coccal bacteria in Fig 4 do not appear to have the alginate biofilm, but could still be the same bacterial species.

The cave straw samples were also cultured on agar and observed with the SEM to visualize the microbes that may be present. These samples were also uncoated and not treated in anyway. Fig 6 shows images from the four isolates with all having relatively good image quality. These bacteria seem to grow in colonies but do not appear to produce an extracellular matrix or biofilm as seen in the uncoated soda straw samples (Fig 5). While the bacteria cultured on agar would most likely be the same isolates from the cave straw, it is possible that the different structures observed are due to the different growth conditions. Bacterial growth on different surfaces and the formation of biofilms depend greatly on factors such as surface topography, liquid medium and pH (4, 10, 11). Of course, the identity of these bacteria can only be hypothesized and comparisons that are more concrete would be needed, such as DNA sequencing or biochemical tests.

While some bacteria were cultured from the soda straw, these bacteria may only make up a small fraction of the total bacteria found on the soda straw. The reason for this is that only about or less than 1.0% of bacteria are able to be cultured (12). It is well known that culture-dependent methods only allow for the detection of the small fraction of microorganisms which are able to grow on specific culture media (13). Furthermore, the bacterial communities in these caves could be scarce and there may be only a few species present. While comparisons could be made with regards to bacteria from the coated, uncoated, and agar samples, these bacteria might not be identical species. Future research would include 16S sequencing of the microbial life on the soda straw to determine species that could not be cultured (8). Using 16S rRNA sequencing, all of the bacterial species present on the soda straw can be characterized, and any new bacterial species present will be evident. Finally, for future research, more time could be used to exhaustively explore the different samples in order to find more microbial life on the soda straw. A grid method would be useful for this additional search because it would allow for the areas that have been already searched to be noted, thus eliminating any overlap that could otherwise occur.

We have found that viewing uncoated samples using the SEM with certain parameters produced images of similar quality to the SEM images of coated samples. Using uncoated samples is advantageous because the bacteria are in a more natural state and are not altered by the coating process. From these images of the coated and uncoated samples, we were equally able to determine the morphology of the bacteria. Furthermore, we were able to use these morphological characteristics to compare the uncoated and coated speleothem isolates to R2A cultured bacterial samples. We found that culturing the samples from soda straws and viewing them with the SEM was easier and more efficient than trying to search the entire soda straw sample for bacteria. Overall, this study evaluated the quality of SEM images produced using different sample preparation methods. SEM images coupled with sequencing technology may help further characterize newly discovered bacteria isolated from caves.

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