SCREENING FOR ANTIBIOTICS IN NEW MEXICO CAVE MICROBIAL COMMUNITIES

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Abstract

Caves conjure up images of darkness and an overall absence of life. In truth, caves are one of nature's remaining terrestrial frontiers. The characteristically musty smell associated with caves is the byproduct of one group of bacteria, the actinomycetes, which are the source of the majority of the world's antibiotics. My research focuses on developing media to culture these organisms, screening for novel antimicrobials produced by these microbes, and determining the environmental factors involved in producing antimicrobials. Cave formations, roots, and soil were aseptically sampled, streak-plated onto solid media, and incubated in the cave for 24 hours to seven days. These were then grown in a 15°C incubator for 5 days and subcultured onto R2A plates. To screen for antibiotic production, subcultures were spotted onto either *Escherichia coli* (Gram-negative) or *Staphylococcus saprophyticus* (Gram-positive) lawns, and monitored for the development of zones of inhibition. Preliminary data from Fort Stanton Cave, NM suggests that some cave bacteria do produce antibiotics. Surprisingly, "hits" came from areas of high human visitation. Three Fort Stanton organisms produced large zones of inhibition on both lawn types. Two other organisms were effective against the Gram-positive analog, while another was effective against the Gram-negative. These preliminary results contradict one of our hypotheses that novel antibiotics are more frequent in low-human impacted, remote cave locations and tell us that cave microbes produce antibiotic secondary metabolites. Similar testing is currently underway for samples obtained in Carlsbad Cavern. To date, Pahoehoe results are pending on 2 of 7 sampling sites. Continuing to look to nature for next generation medications, or skeletons for revolutionary synthetics remains important.

Research Questions

- How can media be adapted to culture the "unculturable" from caves?
- Are microbes in cave environments producing antibiotics?
- What abiotic/biotic factors affect the propensity for antimicrobial expression? (ie: cave zone, levels of organic carbon, humidity etc.)

Results

Fig 1. Media array from a single sample site at Pahoehoe cave in El Malpais Nat. Park illustrating the varying results in both culture diversity and growth rates. These plates are the original parent plates innoculated on site.

Growth pattern/rate differences exhibited in subcultures. Shown above are two pairs of isolates subcultured under the same conditions for the same amount of time. The media supplimented with rock flour appears to dramatically increase the rate of overgrowth.

Cave Name	Number of cultures screened	"Hits" vs Gram - (*Escherichia coli*)	"Hits" vs Gram+ (*Staphylococcus saprophyticus*)
Fort Stanton	18 each on 2 lawns 36 total	3	6
Carlsbad Cavern	20 each on 2 lawns 40 total	1	
Pahoehoe	43 each on 2 lawns 86 total	4 in progress	3

Pictures by Jessica Snider

< (Fig. 2) Variations on a familiar theme: Left is the original method used to screen the Fort Stanton Samples. It was found that multiple specimens could be screened at once on a single lawn and so the Carlsbad Caverns sample on the right, with two specimens became the standard method for our study

Methods

- Rock flour was obtained by pulverizing bassalt lava rock from El Malpais National momument by means of a shatter box machine. The powder was further screened using a 65µm mesh screen. The fine powder was then autoclaved for 30min to ensure sterility. 5g of Rock Flour was added per Liter of media to be modified.
- Samples were collected aseptically from cave surfaces, water pools and soil in Carlsbad Cavern, Fort Stanton Cave, and Pahoehoe cave in El Malpais Nat. Park, NM. Each collection site in Pahoehoe was sampled using an array of media, including R2A, ½ R2A, RASS, and PDA, with and without rock flour (see fig 1). R2A is a low nutrient media, RASS is formulated to encourage the growth of actinomycetes, and Potato Dextrose Agar (PDA) is used to culture fungi.
- Cultures were left on site in PVC cylinders for 24 hours to a week to allow cultures to incubate under cave climate conditions.
- Cultures are then subcultured into pure cultures on R2A media in the lab. Subcultures are incubated in a 15°C incubator. Any observations of note concerning growth patterns, are monitored. Contaminated subcultures are reisolated and subcultured on new media. Subcultures that fail to grow on R2A are resubcultured onto the parent plates' media type (PDA, RASS, or +Rock Flour)
- Each half of a fresh lawn of *E. coli* and *S. saprophyticus* are then dotted with a loop full of bacteria four times- each side containing a different specimen. These plates are then incubated at 25°C for 24 hours and monitored over the course of a week for the formation of zones of inhibition. (See Fig. 2)

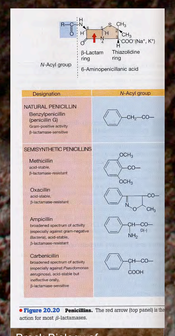
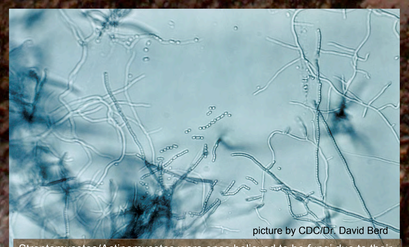


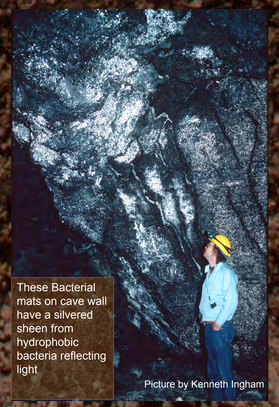
Figure 20.20 **Penicillins.** The red arrow (top panel) is the site of action for most β-lactamases.

Brock Biology of Microorganisms 11th ed figure showing natural penicillin and its synthetic derivatives



picture by CDC/Dr. David Bard

Streptomycetes/Actinomycetes were once believed to be fungi due to their hyphae-like structures and sporing bodies.



Picture by Kenneth Ingham

These Bacterial mats on cave wall have a silvered sheen from hydrophobic bacteria reflecting light

What does this all mean?

- Antibiotics are produced by microorganisms present in Caves
- Results of testing specimens from Ft. Stanton contradicted the hypothesis that low-impact areas result in more "hits"
- The "four dot" technique appears to be more efficient and effective. It reduces cost, and provides more results per unit than other methods. The Streak plate method left more to interpretation regarding zones of clearing
- The use of a media array per sample site as well as simple modifications to media composition can have dramatic results. The addition of rock flour alone seemed to affect organism growth patterns as well as what types of organisms will culture.

Future Studies

- Further sampling at El Malpais Nat. Park; testing of Roots Galore, Pahoehoe, and Four Windows
- Further sampling at Carlsbad Cavern
  Comparison to Ft. Stanton
- Identification of microorganisms producing antimicrobials using DNA sequencing.
- Collaboration with pharmacologist work on cave microorganisms to isolate and identify antimicrobials
- Testing for antifungal compounds
- Further experimentation with media

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Background picture by Kenninth Ingham: Inside of Pahoehoe cave lava tube. Each stack of plates represents an entire media array and was inoculated from the same site.