ISOLATION AND CHARACTERIZATION OF THERMOPHILIC, CALCIUM-PRECIPITATING BACTERIA FROM CALCITE DEPOSITS AT YUCCA MOUNTAIN

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Document ID: TR-03-004
Revision 0
July 30, 2003

Prepared for the U.S. DOE/UCCSN Cooperative Agreement
Number DE-FC08-98NV12081
Task 22

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ABSTRACT

Calcite deposits, composed of a mixture of calcium carbonate and silicon dioxide, were found in fractures and small cavities within the welded tuff of Yucca Mountain. This research investigation involves determining the presence of thermophilic, calcium-precipitating bacteria within these deposits. The possible existence of thermophilic bacteria may help to resolve the issue of whether these calcite deposits formed from precipitation of dissolved calcium carbonate in rain water transported from the overlying soil environment or as a result of upwelling of geothermally-heated waters transported from below the mountain. Evidence for microbially-influenced calcite precipitation in these deposits is indicated by the presence of moderately-thermophilic, calcium-precipitating bacteria.

Growth of bacteria enriched from crushed calcite and calcite/tuff mixed samples collected from tunnels within Yucca Mountain indicate a tendency for thermophiles to be found in calcite deposits and mixed rock samples compared to tuff samples (no calcite) which lacked bacterial growth at temperatures of 50° C and higher. Calcite isolates growing at 60 and 70° C were identified as thermophiles, the most common identification being *Bacillus stearothermophilus*. SEM and electron dispersion spectroscopy (EDS) results showed that bacteria, isolated from Yucca Mountain calcite and calcite/tuff, produced calcite (CaCO$_3$) when grown on calcium-enriched medium. This evidence indicates a possible warm water influence in the history of Yucca Mountain.
INTRODUCTION

Yucca Mountain, NV is the proposed site for a high-level nuclear waste repository. The suitability of Yucca Mountain has been challenged by the hypothesis that calcite deposits found in fractures and cavities within the welded tuff were formed as a result of upwelling hot waters from Paleozoic limestones underlying present day tuff. The currently accepted hypothesis is that the calcite deposits precipitated from rainwater recharge that percolated through overlying soil and into rock fractures located within the mountain.

Studies of fluid inclusions within the calcite indicate that a number of the calcite samples collected from Yucca Mountain were formed at temperatures ranging from 35°C to as high as 75°C (Dublyansky, et al. 1998, Dublyansky, 1998). Data have already been collected on the characterization of microbial communities indigenous to Yucca Mountain as part of a general site characterization (Kieft et al., 1997) and from Rainier Mesa at the Nevada Test Site (Haldeman and Amy, 1993, Haldeman, et al. 1994b, Haldeman, et al. 1995). Further characterization of the effects these microbial communities might have on the corrosion of structural materials such as metal, concrete, grout, and wood are also under investigation (Castro et al., 1996, See Chaps. 2 and 4). What has not been analyzed is the presence of thermophilic bacteria within or in close proximity to the calcite deposits. Concerns over the possibility that these calcite deposits are a result of hydrothermal activity make it necessary to determine if thermophiles can be isolated from these calcite deposits in comparison to the welded volcanic tuff that comprises Yucca Mountain. Upwelling of hot waters from the subsurface may have deposited thermophiles, therefore, the presence of thermophilic bacteria in the calcite
minerals may lend support for the hydrothermal origin of the calcite deposits within Yucca Mountain.

Individual bacteria, as well as larger bacterial groups, exhibit wide growth temperature ranges. In addition, they exhibit diverse optimal growth temperatures. The range of temperatures that a particular microorganism can tolerate will determine its ability to survive in a given ecosystem (Atlas and Bartha, 1998). Psychrophiles exhibit optimal growth temperatures from less than 0° C to approximately 15° C. Mesophiles grow optimally from about 20 to 40° C. Optimal growth temperatures for thermophiles range from 40 to 80° C with extreme thermophiles typically growing from above 80° C to as high as 110° C (Atlas and Bartha, 1998). The ability for thermophiles to grow at elevated temperatures lies in the presence of high proportions of saturated lipids in their membranes which prevent melting and the production of enzymes that are not readily denatured by high temperatures.

Calcium plays a major role in many biological processes of both prokaryotes and eukaryotes. Intracellular calcium is intricately regulated due to the fact that its concentration mediates diverse physiological activities (Appanna et al. 1997). The cytoplasmic content of calcium returns to basal level, through different calcium transport systems, after the biochemical responses have been elicited (Norris et al., 1991). Elevated levels of free calcium can cause irreversible damage to cells by inhibiting these physiological responses. Therefore, many organisms have devised strategies to maintain cytoplasmic levels of calcium. One strategy, as demonstrated by *Pseudomonas fluorescens*, is to precipitate calcite, a crystalline calcium carbonate, in order to avoid the negative effects of excess free calcium. This is the first example of microbial calcite
precipitation as a detoxification strategy (Anderson and Appanna, 1994). In addition, strontianite, the crystalline SrCO$_3$, is also precipitated by *Pseudomonas fluorescens* when challenged with excess levels of strontium (Anderson and Appanna, 1994).

In order for calcite to precipitate, the mobilization of calcium and carbonate is necessary along with the presence of a matrix where nucleation of crystals can occur. Carbonate could be provided by the fixation of carbon dioxide into bicarbonate ion, HCO$_3^-$, which requires the use of carbonic anhydrase by the microorganisms. Under normal conditions, the regulation of HCO$_3^-$ allows for the decomposition of toxic cyanate into ammonia. It is also possible that this CO$_2$-fixing system may be involved in calcite precipitation. Similarly, in photosynthetic organisms, the fixation of CO$_2$ during photosynthesis can precipitate calcite as long as sufficient calcium levels exist and a matrix, e.g., the S-layer, is present for nucleation (Thompson et al., 1997, Schultze-Lam, 1992).

A second focus of this study will be to determine if calcium-precipitating bacteria isolated from the calcite deposits, including thermophiles, might be responsible, in part, for the calcite deposition. Mineralization of calcite by marine microorganisms is widely recognized as well by bacteria in lacustrine environments (Monger et al., 1991, Thompson and Ferris, 1990, Buczynski and Chafetz, 1991, Schultze-Lam et al., 1992, Robbins and Blackwelder, 1992, Hodell et al., 1998). Precipitation of calcium carbonate by microorganisms is significant in the development of a variety of geological formations, however, calcite deposition in arid soils has been viewed as an inorganic process. Recent evidence suggests that soil microorganisms may play a key role in the formation of calcite precipitation in modern soils as well as paleosols (Monger et al.,
The presence of calcite has become increasingly important in determining the paleoclimate and age of desert soils (Monger et al., 1991). The presence of calcium-precipitating bacteria in the calcite deposits of Yucca Mountain may provide additional evidence for the origin of these calcite deposits.

Bacteria, cyanobacteria, and some fungi deposit calcium carbonate extracellularly (Thompson and Ferris, 1990, Robbins and Blackwelder, 1992, Schultze-Lam et al., 1992, Thompson et al., 1997). One exception is *Achromatium oxaliferum* which has been reported to deposit calcium carbonate intracellularly (Ehrlich, 1996). Extracellular precipitation of calcium carbonate can occur during the removal of carbon dioxide from photosynthesis (Thompson et al., 1997). *Synechococcus* can alkalinize its surrounding microenvironment due to the exchange of HCO$_3^-$ into and OH$^-$ out of the cell. This exchange process results from fixation of HCO$_3^-$ during photosynthesis (Thompson and Ferris, 1990). Increased OH$^-$ levels will drive the bicarbonate/carbonate equilibrium reaction towards higher carbonate (CO$_3^{2-}$) levels. If calcium ion concentrations are high enough, calcium carbonate precipitation will occur. Filamentous cyanobacteria associated with stromatolites, cyanobacteria, and algae can precipitate calcium carbonate as a result of their photosynthetic activities (Ehrlich, 1996, Thompson et al., 1997).

To further investigate the origin of the calcite deposits in Yucca Mountain, microbial analysis of subsurface water from monitoring wells and warm springs located in neighboring locations around Yucca Mountain were conducted to see if these water sources might be connected to subsurface thermal waters located below paleozoic limestone layers beneath Yucca Mountain. Microbial analysis of water sampled from these sources may provide additional evidence for a connection between Yucca Mountain...
calcite deposits if similar populations of thermophilic bacteria are found.

MATERIALS AND METHODS

Calcite/ Tuff Collection and Preparation

Samples of calcite, tuff adjacent to calcite, and welded tuff from areas not associated with calcite deposition were removed from natural rock formations in the ESF and ECRB tunnels in Yucca Mountain (see Tables 1-4 for more specific locations) using tools cleaned with 10% bleach followed by 95% ethanol and air drying. The calcite and tuff specimens were placed in sterile, plastic sample bags and stored on ice. The samples were transported within 6 hours of collection and stored at -20° C (Kieft et al., 1997).

Rock surfaces were sterilized using serial washes of 10% bleach, sterile water, and 95% ethanol. After air drying, the calcite and tuff were then crushed aseptically using an alcohol flame-sterilized mortar and pestle.

Preparation of Bacterial Cultures from Calcite/ Tuff

Slurries composed of a 1:10 dilution of crushed calcite or tuff and 0.1% sodium-pyrophosphate were prepared and shaken at 100 rpm for 1 hr. Portions of the slurry preparations (3 mL) were filtered through 0.45 μm filters (Gelman, Ann Arbor, MI) and then the filters were placed onto culture plates containing either R2A minimal nutrient agar (Difco/ BD Diagnostics Systems, Sparks, MD) or B4-calcium enriched agar (Monger et al., 1991). The cultures were incubated at 23, 45, 60, and 70° C for 2 wk. Bacterial growth for each plate was then semiquantitatively scored using a scale of 0, 1, 2, 3, and 4 (0: no growth, 1: confluent growth up to approximately 25% of the filter area, 2: approximately 25-50% of the filter area, 3: approximately 50-75% of the filter area,
and 4: approximately 75-100% of the filter area). Isolates growing at 45, 60, and 70°C were cultured and identified.

**Water Sample Collection and Preparation**

Water collection from the monitoring wells began with QA-controlled sampling of water pumped from various depths in these wells. Collection of water began after open flow for 16 hours. Water was aseptically collected using autoclaved Nalgene (Rochester, NY) polypropylene bottles. Water samples from warm springs (Ash Meadows, northwest of Yucca Mountain and Ash Springs, northeast of Yucca Mountain, were collected using sterile 500 mL Nalgene polypropylene bottles attached to a 12-foot pole. Samples were collected from the center of the springs as deep and as close to the source of the springs as possible. The water samples were maintained at ambient air temperature and transported to the lab within 6 hr of collection.

The samples were processed on the same day by filtering the water through 0.2 μm Gelman filters and placed onto culture plates containing R2A minimal nutrient agar. Cultures were grown at 23, 45, 60, and 70°C for two weeks. Bacterial growth was semiquantitatively scored and those microorganisms growing at 45, 60, and 70°C were cultured and identified.

**pH Measurement**

The VWR sympHony SB301 pH meter and VWR sympHony flat surface pH electrode (VWR International, West Chester, PA) were used to take pH measurements of the agar surface of the bacterial cultures precipitating calcite on B4 medium. Measurements were taken of areas with bacterial growth and without bacterial growth for comparison.
SEM Microscopy and EDS Analysis

Bacterial isolates were grown on B4 calcium-enriched medium (Monger et al., 1991). Scanning electron microscopy was performed to visualize crystals formed by calcite isolates (EPMA/SEM Laboratory, Dept. of Geosciences, University of Nevada, Las Vegas). Electron Dispersion Spectroscopy (EPMA/SEM Laboratory, University of Nevada, Las Vegas) was used to analyze these crystals.

RESULTS

Bacterial growth data showed that thermophilic bacteria were found in the calcite and calcite/tuff samples (Tables 1-3) collected in the ESF tunnel. Mesophilic bacteria were also isolated at 25 and 45° C from calcite and calcite/tuff in the ESF and ECRB tunnels, as well as, the control tuff (no calcite) sampled from Alcove 5 (north end of ESF tunnel). Bacterial growth at 25° C was heavier and occurred with more frequency than bacterial growth at 45° C for most samples from all Yucca Mountain sites (Tables 1-3).

Bacteria were not isolated at temperatures 50° C and higher from the control Alcove 5 tuff (Table 2). Also, no bacteria were isolated at temperatures 60° C and higher from the ECRB tunnel specimens (Table 3).

Thermophilic bacteria were isolated from neighboring locations around Yucca Mountain (Table 4). Bacteria were isolated at 50° and 60° C from Ash Springs, Point of Rock Spring, and Warm Springs.

EDS analysis revealed that the crystals formed on and near the bacterial colonies on B4 medium is probably calcium carbonate (Fig. 3). The height of the peaks indicate relative amounts of each element present in the crystals. Peaks for calcium, oxygen, and
carbon can be identified in the graph. pH data, collected on thermophilic, calcium-precipitating bacteria grown on B4 medium, show elevated pH values in the colonies compared to the surrounding medium (Table 5), which would suggest how these calcite crystals precipitate (Thompson and Ferris, 1990). SEM microscopy illustrated the shapes of calcite crystals formed by thermophilic calcite isolates on B4 medium (Figs. 1, 2).

**DISCUSSION**

The potential for subsurface, thermal waters to rise within Yucca Mountain is an important factor in assessing the suitability of Yucca Mountain as a high-level, nuclear waste repository and in the materials/design selected for the repository. From a microbial ecology perspective, entombed endolithic bacteria are of interest. Thermal water exposure has recently been determined to have last been present approximately 2 million years ago (Wilson and Cline, in press). The survival implications of these findings are interesting and in line with other subsurface findings (Amy et al., 1993, Amy, 1997, Lamber et al., 1998, Morita, 2000).

Compared to bacterial growth at 25 and 45° C, bacteria which grew at 60 and 70° C were found with less frequency and in smaller quantity (Tables 1-3). To date, only calcite and calcite/tuff samples obtained from the ESF tunnel have been shown to contain thermophilic bacteria. Bacteria growing at 25 and 45° C were found in the ESF and ECRB tunnel samples as well as in the control tuff (no calcite) samples of Alcove 5.

The calcite and calcite/tuff samples from the ESF were categorized by location; samples taken from locations at the northern half of the tunnel and samples from the southern half of the tunnel. Samples from the northern half supported more bacterial
growth at 25 and 45° C compared to samples from the southern half (Tables 1, 2). In contrast, there was a tendency to find more thermophilic bacteria in the southern half of the ESF compared with the northern half of the ESF.

The Alcove 5 tuff was chosen as a control due to the lack of calcite present in the welded tuff. It is important to note that there was no bacterial growth from this sample at temperatures 50° C and higher. Thermophiles growing at 60° C and higher were isolated only in samples containing calcite (Tables 1-3). Such a finding indicated that there was warm water influence in specific regions of Yucca Mountain.

A connection between outlying monitoring wells and warm springs and subsurface thermal waters beneath Yucca Mountain has yet to be determined. Thermophilic bacteria have been isolated from water collected at the Nye County monitoring Well NC-EWDP-1DX but have not been identified. Water samples collected from warm springs in Ash Meadows, and hot springs located in Ash Springs, NV, northeast of Yucca Mountain yielded bacteria capable of growth at 50° C and higher. If similar species of thermophilic bacteria found in Yucca Mountain calcite deposits are isolated in the surrounding warm and hot springs as well as monitoring wells, a physical connection between subsurface thermal waters beneath Yucca Mountain and these outlying thermal water sources would be suggested.

The biochemical evidence of microbial precipitation of calcite indicate that soil microorganisms may have played a significant role in pedogenic calcite precipitation in modern soils and paleosols (Monger et al., 1991). Further evidence that microorganisms are able to precipitate calcite is found in laboratory experiments which demonstrate bacterial cultures able to precipitate calcite in media containing calcium acetate.
Biomineralization of calcite has important implications, such as in microfossils, that may reflect paleoclimatic conditions and in the origin of calcite deposits in Yucca Mountain.

EDS analysis has shown that the thermophilic bacteria isolated from Yucca Mountain calcite deposits precipitate crystals probably composed of calcium carbonate when cultured on calcium-enriched B4 medium (Fig. 3). The mechanism used is not clear; however, increased pH values in the bacterial colonies, compared to the surrounding B4 medium (Table 5), suggest that increased OH⁻ concentrations drove the bicarbonate/carbonate equilibrium towards higher carbonate (CO₃⁻²) levels causing calcium carbonate to precipitate (Thompson and Ferris, 1990).

The morphologies of the calcite crystals formed (Figs. 1, 2) are similar to the crystal morphologies of calcite precipitated by *Bacillus pastueri* during a study of microbially-mediated calcite precipitation (Warren, et.al. 2001). Although the thermophiles isolated from Yucca Mountain, to date, have been capable of precipitating calcium carbonate, there is little evidence to suggest that these thermophiles may have had a significant influence on the formation of calcite deposits in Yucca Mountain. The thermophilic microorganisms isolated so far are too few in number to study in situ.

Based on bacterial growth data and identification of thermophilic bacterial isolates, there is evidence to suggest a warm water influence occurred in Yucca Mountain which led to bacterial deposition. Data continues to be collected regarding groundwater connections between outlying thermal springs and subsurface thermal waters beneath Yucca Mountain. The possibility of warm/hot water rising from the subsurface could compromise the structural integrity of the repository if it can be demonstrated that it has occurred in the last 65,000 years.
Whether bacterial isolates found in calcite deposits within Yucca Mountain had a significant influence on calcite formation is yet to be determined. There are numerous examples of microbially-mediated calcite precipitation in the environment: the whiting events of Fayetteville Green Lake, New York (Thompson, et. al. 1997) and in the Bahama Banks (Robbins and Blackwelder, 1992); a saltern pond (Rivadeneyra, et. al. 1994); the marine environment (Morita, 1980); hot springs (Jones and Renaut, 1996); and caves (Contos, et. al. 2001). It is possible that thermophiles isolated from Yucca Mountain calcite deposits had some influence on calcite formation within Yucca Mountain. Direct study of the Yucca Mountain calcite deposits to locate and examine bacteria and isotopic analysis of the calcite crystals may provide evidence needed to determine the origin of the Yucca Mountain calcite deposits.
REFERENCES


Data Sources and Electronic Data Control

Data Identification Number: 022DT.001 for all data, graphs, and tables was submitted to the Data Management Database, Start/End Period: 12/16/2000 - 06/30/2003. The source of the data is the Scientific Notebook UCCSN-UNLV-020 and are qualified as the notebook has completed the technical and QA review process. These data supersede data previously identified by DID: 022PA.010. This data set extends the data observation/measurement period through 06/30/2003. Although 022DT.001 supersedes 022PA.007, file 022PA.007 contains the data for sample 00529592. File MO0307UCC000TE.001 contains unqualified data listed in Table 2.

Data was entered electronically into Microsoft Excel 2000 (version 9.03821 SK-1) according to implementing procedure IPLV-020. The data was zipped and recorded on a zip disk in the following files: 1) TASK 22 GROWTH AND DATA.xls 2) TASK 22 pH DATA (CALCITE).xls 3) WATER SAMPLE AND pH DATA.xls 4) WATER SAMPLE.xls.

Data was also printed from the Excel file and pasted into the Scientific Notebook, UCCSN-UNLV-020. After printing and before inserting into the notebook, it was visually inspected for accuracy and the result of the inspection was also recorded in the Scientific Notebook.

This method for managing electronic data QAP - 3.1, “Control of Electronic Data”, is described/recorded in the Scientific Notebook on pp ii-iii.
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<th>60 °C</th>
<th>70 °C</th>
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Yucca Mountain (the growth score for each plate was obtained as explained in Materials and Methods.

Table 1. Bacterial Growth Data of Calcite/Tuff Samples Collected from the Northern Half of the ESF Tunnel.
Table 2. Bacterial Growth Data of Calcite/Tuff Samples Collected from the Southern Half of the ESF Tunnel (the growth score for each plate was obtained as explained in Materials and Methods)

<table>
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<tr>
<th>Sample Identification</th>
<th>Growth</th>
<th>Room temperature</th>
<th>45 deg C</th>
<th>60 deg C</th>
<th>70 deg C</th>
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* For corroborative use only

1) All samples collected on 06/23/1999 and processed on 10/09/1999.
2) Numbers under columns labeled Room temperature, 45 deg C, 60 deg C, and 70 deg C correspond to the number of isolated colonies per plate, (for example: 3,0,0 corresponds to 3 colonies on 1st plate and 0 colonies on 2nd and 3rd plate).
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Table 3. Bacterial Growth Data of Calcite/Tuff Samples Collected from Southern Half of the ESF Tunnel and Alcove 5 (00529592) Tuff from Yucca Mountain (the growth score for each plate was obtained as explained in Materials and Methods)
Table 4. Bacterial Growth Data of Calcite/Tuff Samples Collected from the ECRB Tunnel, Yucca Mountain (the growth score for each plate was obtained as explained in Materials and Methods)

<table>
<thead>
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<th>Sample Identification</th>
<th>Growth</th>
<th>Room temperature</th>
<th>45 deg C</th>
<th>60 deg C</th>
<th>70 deg C</th>
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Table 5. Water Sample Growth Data on R2A Medium (the growth score for each plate was obtained as explained in Materials and Methods)

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<th>Growth Scores at 50°C</th>
<th>Growth Scores at 60°C</th>
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<td>50 mL/ 80mL volumes</td>
<td>50 mL/ 80mL volumes</td>
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Table 6. pH Measurements of Calcite-precipitating Bacteria Isolated from Calcite/Tuff Samples

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Table 7. pH Measurements of Calcite-precipitating Bacteria Isolated from Water Samples

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Figure 1. Calcite Crystal formed from Thermophile isolated from Point of Rock Warm Spring (SPC01019703) - Ash Meadows
Figure 2. Calcite Crystals formed from Thermophile isolated from Point of Rock Warm Spring (SPC01019703) - Ash Meadows
Figure 3. EDS Analysis of calcite crystal formed from Thermophile isolated from Point of Rock Warm Spring (SPC01019703) - Ash Meadows. The height of the peaks indicate relative amounts of each element present in the crystals. Peaks for calcium, oxygen, and carbon can be identified in the graph.