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Characterization of microbial populations in the subsurface

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TECHNICAL REPORT

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1.0. TABLE OF CONTENTS

1.1. List of Tables

2.0 PURPOSE

2.1 Purpose and Scope

This task is part of a cooperative agreement between the UNLV Research Foundation and the U.S. Department of Energy (#DE-FC28-04RW12237) titled "Yucca Mountain Groundwater Characterization". The work was conducted in the Harry Reid Center for Environmental Studies, Microbiology Division of the University of Nevada, Las Vegas from October 1, 2004 to September 30, 2006. The overall goal of this research was to investigate the phenomena that affect the fate and transport of radionuclides in the environment. The purpose of this task (ORD-RF-01), "Characterization of Microbial Activity", was to develop a molecular biological method for the characterization of the microbial population indigenous to the Yucca Mountain Project site, with emphasis in detection and measurement of species or groups of microorganisms that could be involved in actinide and/or metal reduction, and subsurface transport. To quantify and characterize the microbial populations, including microorganisms that may be viable but are not currently physiologically active, a molecular biological approach was utilized to amplify and detect microbial DNA present in the subsurface. This approach, termed polymerase chain reaction (PCR), results in the amplification of DNA sequences that are unique to the groups of microorganisms of interest. Quantitative PCR (QPCR) assays were developed and used for the measurement of subsurface microbial populations. The protocols were evaluated in laboratory tests involving representative microbial species and genera, and tested by assaying available subsurface samples previously collected from the Yucca Mountain Project site. Other subtasks included Quality Assurance (QA) planning and preparation, and a literature review. This work was subject to the Nevada System of Higher Education (NSHE) QA Program requirements.

2.2 Limitations of Use

Storage of cores for several years likely affected the microbial population concentration and composition and the results obtained in these experiments with QPCR analysis are for protocol evaluation purposes only. It is anticipated that the protocol developed will be used to confirm previously reported culturable bacteriological populations, and will expand the knowledge base of bacterial populations present to include those that were not detected due to the limitations of culture. This will provide a more complete determination of microbial populations that may affect the repository environment.

3.0 QUALITY ASSURANCE

This report was written in accordance with the NSHE Quality Assurance Program. No subtask status was changed to non-Q. All conclusions of this report were based on qualified data; no unqualified data were used to support any conclusions.

NSHE procedures referenced in this report:
IPLV-068, "Bacterial DNA Extraction and Purification," rev. 0
IPLV-069, "Electronic Cell Enumeration," rev. 0
IPLV-070, "DNA Amplification," rev. 1, DCN 2

4.0 INTRODUCTION

The fate and transport of radionuclides can be influenced by microorganisms in the subsurface repository environment. This influence can be by direct action (e.g., reduction or complexation by siderophores) and indirect action (e.g., incorporation into the microorganism or sorption to the microbial surface that can result in passive colloidal transport as the organism moves through the subsurface). Barton *et al*. (1992) compiled a list of bacteria from various sources capable of binding, precipitating, absorbing, depositing, reducing and transforming various toxic elements, including *Aeromonas*, *Bacillus megaterium*, *Citrobacter*, *Desulfovibrio*, *Escherichia coli*, *Flavobacterium*, *Micrococcus lysodeikticus*, *Pseudomonas aeruginosa*, *P. maltophilia*, *P. mesophilica*, and *Wolinella succinogenes*. Migration of microorganisms in groundwater is accomplished by diffusion, advection or convective transport, and/or active movement (Harvey and Garabedian, 1991). The extent of bacterial sorption might be influenced by the electrostatic charges of both bacterial and solid surfaces, the production of extracellular polysaccharides, and cell hydrophobicity. McCarthy and Zachara (1989) reported that functional groups on bacterial cell surfaces bind metals. Also, it has been reported that the cell wall of gram-positive bacteria at circumneutral pH contains electropositive amino groups that can react with soluble anions (e.g., SiO_3^{2-}) through heavy metal cation bridges (Urrutia-Mera and Beveridge, 1993). Microbial exudates may be a source of organic material involved in sequestering metals and other organic substances (Harvey and Garabedian, 1991). Hydrophobic contaminants can adsorb to these cell products reducing their hydrophobicity and making them more soluble in water (McCarthy and Zachara, 1989). Geesey and Jang (1989) have shown that bacterial polymers exhibit binding affinity for metal cations (e.g., Cd, Co, Ni, Mn, Zn, Pb, and Cu). From these studies it can be inferred that indigenous microorganisms have the potential to affect actinide speciation and migration at the Yucca Mountain Project site if the waste containers are breached.

Previously conducted research demonstrated that a diverse microbiological population exists in the area (Amy, 1997; Amy *et al*., 1992 and 1993; Davis *et al.*, 1998; Haldeman and Amy 1993a and 1993b; Haldeman *et al*., 1993 and 1994; Hersman, 1997; Horn *et al.*, 1998a and 1998b; Horne and Meike, 1995 and 1996; Kieft *et al*., 1997; West *et al*., 1985). Over 60 isolates have been cultured from the site-related subsurface samples in the laboratory. Isolates that have been cultured include sulfate-reducing bacteria and members of the genera *Bacillus, Arthrobacter*, *Cellulomonas, Corynebacterium, Pseudomonas, Staphylococcus, Xanthomonas,* and *Flavobacterium.* Collectively, these microorganisms represent diverse consortia that include facultative aerobes/anaerobes, gram-negative and gram-positive bacteria, endospore-formers, denitrifiers, and metal reducers. From the perspective of actinide speciation, the most important of these are the metal reducers that are also likely to reduce the actinides, as well as facultative bacteria that are important in transitioning to an anaerobic reducing system that will promote actinide immobilization. Existing data were derived from traditional culture, direct count microscopy, and phospholipid analysis methods. Culture-based techniques identify less than 1% of all microbial populations in an environmental sample (Amann *et al*., 1995; Borneman *et al*., 1996). Microscopy provides data on total cell concentrations, but cannot assess viability or discriminate between genera or species. Phospholipid analysis provides information on microbial biomass and can be used to classify organisms by physiological traits, but different organisms share common lipid composition resulting in grouping of microbial populations rather than specific identification. In previous research at a deep-subsurface clay environment, the usefulness of molecular detection for subsurface organisms was demonstrated (Boivin-Jahns *et al*., 1996) and molecular methods have been shown to detect low numbers of organisms (Tsai and Olson, 1992). To quantify and characterize the microbial populations, including microorganisms that may

be viable but are not currently physiologically active, a molecular biological approach was utilized in this study to amplify and detect microbial DNA present in the subsurface. This approach, termed polymerase chain reaction (PCR), results in the amplification of DNA sequences that are unique to the groups of microorganisms of interest. Quantitative PCR (QPCR) is a recently developed technology that allows sensitive, specific detection and enumeration of target microorganisms. Scientists in our laboratory have used this technology for the detection, enumeration, and identification of bacteria and fungi in environmental samples (Buttner *et al*., 2001; Buttner *et al*., 2004a; Cruz and Stetzenbach, 2004; Cruz-Perez *et al*., 2001a and 2001b).

The goal of the proposed microbiology research was to develop and utilize a QPCR method to characterize the microbial populations indigenous to the Yucca Mountain Project site, with emphasis in detection and measurement of species or groups of microorganisms that could be involved in actinide and/or metal reduction, and subsurface transport. The protocol was evaluated in laboratory tests involving representative microbial species and genera previously characterized from the subsurface. The protocols developed were also tested by assaying available subsurface samples from the Yucca Mountain Project site. It is anticipated that the protocol developed will be used to confirm previously reported culturable bacteriological populations, and will expand the knowledge base of bacterial populations present to include those that were not detected because of the limitations of culture. This will provide a more complete determination of microbial populations that may affect the repository environment.

5.0 METHODS AND MATERIALS

The research was conducted in 5 subtasks. Subtask 1 involved QA indoctrination, and equipment acquisition, installation and training. Subtask 2 consisted of an extensive review of the scientific literature. Subtask 3 involved laboratory studies and protocol development. Subtask 4 consisted of evaluating the protocols developed in the laboratory with available subsurface samples from the Yucca Mountain Project. The final subtask, Subtask 5, focused on QA reviews and preparation of the final report.

5.1 Subtask 1: Planning and Preparatory Activities

The first task of the project involved establishing and conducting procedures according to the NSHE QA Program. The research on this project was conducted according to the Scientific Investigation Plan (SIP), a document required by the NSHE QA Program prior to initiating work on Yucca Mountain-related projects. The SIP covers the scope and objectives of the research and includes information on the approach and methodologies planned, interface controls, procurements, hold points, and reviews. Personnel new to the QA program received scientific notebook training and other QA-required training. Implementing procedures were reviewed and updated, or new procedures were written. While QA documents and procedures were being developed, the necessary equipment was purchased and installed. The following activities were performed during this Subtask:

- Task Personnel were NSHE QA indoctrinated.
- Position descriptions (PDs) were prepared for all task personnel and submitted to QA staff.
- A training matrix (TM) showing the training required by all task personnel was prepared, updated as needed, and submitted to the QA Program.

- The Scientific Investigation Plan (SIP) was written, reviewed and approved by the Project Director (SIP-UNLV-046, Characterization of Microbial Activity).
- A qualified supplier of microorganisms (American Type Culture Collection) was located and QA approved for use on the task.
- Surveillance was performed by NSHE QA on the polymerase chain reaction (PCR) process that was used in the task.
- The PCR instrument required for this task, 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA), was ordered, received and installed by the supplier. Task personnel received training by Applied Biosystems' staff on the PCR instrument.
- A fire-rated safe for safekeeping of QA records pertinent to this task was obtained.
- Task personnel conducted Training by Reading as specified by the QA Program.
- Implementing procedures (IPLV-068, IPLV-069 and IPLV-070) were written, submitted to NSHE QA for review, and approved by the QA Manager.
- Calibration of M&TE by outside vendors was initiated and recalibrated as required.
- Laboratory notebooks were obtained from the QA Program, prepared for data entry and completed according to QA Program requirements.

5.2 Subtask 2: Literature Review

A literature review and a search of the genetic sequence databases was conducted to identify "universal" primer and probe sequences that amplify DNA from all bacterial genera. In addition, groups of interest, such as actinide-reducing bacteria, were identified and searches were conducted to determine PCR primers and probes that could potentially be used to amplify DNA from microorganisms within these groups. The references were obtained and entered into a database (EndNote, version 9.0.0, Thomson ResearchSoft, Stamford, CN). Task personnel attended the American Society for Microbiology annual meetings in 2005 and 2006 to gather information and methodologies available for soil characterization of microorganisms.

5.3 Subtask 3: Laboratory Studies and Protocol Development

5.3.1 Experimental Design

Candidate universal primer and probe sequences were identified in the literature review. Those which were compatible with the TaqMan® QPCR technology were selected, or designed, and tested in the laboratory. The selected universal primers and probe were those that most effectively amplified DNA from different bacterial groups spanning the spectrum of diversity within the prokaryotes. Cross-reactivity with DNA from nonbacterial sources was also tested. In addition, primers and probes that amplify specific bacterial groups of interest, such as actinide-reducing bacteria, were selected, or designed, and tested. PCR amplification conditions were optimized, and quantitation standards were prepared from a representative bacterial species by extracting the DNA from known concentrations of cells. A DNA extraction and concentration protocol previously developed in our laboratory was used (Buttner *et al*., 2001; IPLV-068).

5.3.2 Test microorganisms

Certified microorganisms (and DNA from certified microorganisms) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). At least one representative from all bacterial phyla was obtained, and several additional species were included from phyla of potential significance in actinide reduction, and subsurface transport. Non-bacterial DNA was included for cross-reactivity testing.

5.3.3 **Universal primers and probes**

To use the PCR technique, sequence information must be first identified for a specific target DNA segment. After an appropriate DNA sequence has been identified, oligonucleotide primers are selected, synthesized, and then tested for sensitivity, specificity and selectivity. Bacterial universal primers and probes will theoretically amplify regions of DNA that are common to all bacteria. However, this must be confirmed in the laboratory with DNA from a variety of bacterial and non-bacterial species. Several universal oligonucleotide probes and/or primers were identified from the literature for potential use with the TaqMan® QPCR technology used in our laboratory. Universal PCR primers and probes, existing or designed in our laboratory using Primer Express software (Applied Biosystems), were purchased from commercial sources (Operon Biotechnologies, Inc., Huntsville, AL, and Applied Biosystems, respectively), and evaluation tests were conducted on the candidate universal primer and probe sets obtained. The evaluation criteria used to determine the final universal primer and probe set were the amplification of the greatest number of target bacterial phyla, and the strength of the signal resulting from amplification of 10 ng of template DNA.

5.3.4 **Group-specific primers and probes**

Many of the microorganisms of concern are gram-positive bacterial genera. Within the gram-positive class of microorganisms, the phylum Actinobacteria contains genera of potential significance. Therefore, gram-positive microorganisms and also the phylum Actinobacteria were selected as targets for the design of two group-specific primers and probes. There are also gram-negative bacteria of concern belonging to the phylum Proteobacteria. However, group-specific primers and probes were not developed for this group due to the limitations in resources and of available cultures needed for thorough specificity testing.

A gram-positive group-specific oligonucleotide probe (RW03; Greisen *et al.*, 1994) was identified from the literature for potential use with the TaqMan® QPCR technology used in our laboratory. The RW03 probe sequence was checked for cross-hybridization by sequence comparison using the Basic Local Alignment Search Tool algorithm (BLAST, National Institutes of Health).

Twelve representative organisms of concern were identified from the scientific literature (i.e., *Desulfosporosinus sp. strain A10, Desulfosporosinus sp. strain STP12, Clostridium perfringens, C. lundense, Thermoterrabacterium ferrireducens, Bacillus subtilis, B. cereus, B. solfarensis, Cellulomonas fermentans, C. denverensis, Microbacterium flavescens,* and *M. arborescens*), and the sequences corresponding to the 16S rRNA gene of each organism were obtained from GenBank (National Center for Biotechnology Information, National Institutes of Health). The sequence of the oligonucleotide probe RW03 was located in the 16S rRNA gene of each representative organism, and the twelve sequences were aligned using the Brixoft SourceEdit (Version 4, Revision 3) software program (www.brixoft.net). These alignments were assessed visually and inspected for

regions of homology within the 16S rRNA sequence. Critical PCR primer design parameters, such as melting temperature, and guanine and cytosine (G+C) nucleotide base percentage values were verified for candidate primer sets using Primer Express software (Applied Biosystems).

Group-specific PCR primers and probes, existing or designed using Primer Express, were purchased from commercial sources (Operon Biotechnologies and Applied Biosystems, respectively), and evaluation tests were conducted on the candidate gram-positive and Actinobacteria-specific primer and probe sets obtained. The evaluation criteria used to determine the final group-specific primer and probe set were the amplification of the greatest number of target bacterial phyla and the strength of the signal resulting from amplification of 5 to 10 ng of template DNA.

5.3.5 **DNA extraction and quantitation**

DNA was extracted from certified microorganisms using the standard DNA extraction protocol (IPLV-068). The amount of DNA extracted from all certified microorganisms was measured spectrofluorometrically using the Quant-iT PicoGreen assay (Invitrogen, Carlsbad, CA) in a 96-well flat-bottom black polystyrene assay plate (Costar; Corning, Corning, NY), with a Flx800 Microplate Fluorescence Reader (BioTek, Winooski, VT). Standards containing known DNA concentrations were prepared according to the Pico Green assay instructions, and concentrations of samples were determined using a standard curve. Data analysis was done with the KCjunior software (BioTek).

5.3.6 **PCR DNA amplification**

The ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA) was used for QPCR analysis. Initially, default PCR amplification conditions supplied by Applied Biosystems for the TaqMan® technology were used for primer and probe testing. Two sets of default operating parameters are available for the TaqMan® assay chemistry, Standard Mode and FAST Mode. After screening experiments identified candidate primers and probes for further testing, several parameters were varied to optimize the sensitivity and specificity of the assay: primer concentrations, probe concentrations, the addition of bovine serum albumin as a PCR enhancer, DNase enzyme treatment of the Universal Master Mix, and Standard Mode vs. FAST Mode. While FAST Mode provides faster PCR amplification and reduces analysis time by approximately 1 hour, Standard Mode was used when sensitivity and/or specificity was greater than FAST Mode.

For Universal PCR, Standard Mode was utilized and final amplification concentrations and conditions for a 25 µl reaction volume were as follows: 5 µl DNA template, 1X Universal Master Mix (Applied Biosystems), 0.1 % BSA (Sigma, St Louis, MO), 0.2 µM forward primer, 0.5 µM reverse primer, 0.15 µM probe, and sterile nuclease-free water. Cycling conditions were: 50°C x 2 min, 95°C x 10 min, followed by 40 cycles of 95°C x 15 sec and 60°C x 1 min.

For gram-positive PCR, FAST Mode was utilized and final amplification concentrations and conditions for a 25 µl reaction volume were as follows: 5 µl DNA template, 1X DNase-treated FAST Universal Master Mix (Applied Biosystems), 0.1 % BSA (Sigma), 0.9 µM each of forward and reverse primers, 0.2 µM probe, and sterile nuclease-free water. Cycling conditions were: 95°C x 20 sec, followed by 40 cycles of 95°C x 1 sec and 60°C x 20 sec. Final amplification concentrations and conditions for Actinobacteria PCR were the same as for the gram-positive PCR

with one exception, BSA was not used for the Actinobacteria assay. The DNase enzyme treatment of the master mix was performed due to positive PCR results obtained in negative control samples with the gram-positive primers and probe. The purpose of the DNase treatment was to enzymatically digest residual contaminant DNA present in the FAST Universal Master Mix as a result of the manufacturing process. A 10-fold dilution of Turbo DNase (Ambion, Inc., Foster City, CA) and accompanying 10X Turbo DNase Buffer was added to the 2x FAST Universal Master Mix (Applied Biosystems) in a ratio of 1 µl: 1.5 µl: 12.5 µl, respectively. The mixture was incubated in a 37°C water bath for 3 hrs with shaking at 50 rpm. Residual DNase was deactivated in two-step process: first, by treatment for 30 min. at 75°C with vortexing and pulse centrifugation every 10 min., and second, by a second treatment as described above immediately prior to performing QPCR analysis.

PCR quantitation standards were prepared from a purified suspension of *Bacillus atrophaeus* spores (U.S. Army Dugway Proving Ground, Dugway, UT) enumerated electronically with a Coulter Multisizer II (Beckman Coulter, Inc., Miami, FL) using the same DNA extraction and purification methods used to process samples. Quantitation was achieved by amplification of standards containing DNA extracted from suspensions of known concentration (10^0 to 10^5 templates per reaction) of *B. atrophaeus*. Extraction of standards in the same manner as samples provides absolute quantitation of *B. atrophaeus* templates and can be used to estimate the total concentration of DNA templates in samples. Standards were amplified in duplicate at the same time and under the same conditions as the replicate unknown samples. After amplification, the data were analyzed using the software provided with the ABI Prism 7900 HT SDS. Using the concentrations assigned to each standard, the software constructed a standard curve of *Ct* value versus concentration. *Ct* refers to the PCR cycle at which fluorescence (i.e., amplification product) is first detected; and is inversely proportional to the log of the initial DNA template concentration. Concentration values for the unknown samples were extrapolated from the standard curve by the software and reported as the mean of two replicates.

5.3.7 **Yucca Mountain Core Samples**

A total of 18 core samples, representing the six geological subzones of Yucca Mountain, were obtained from the Yucca Mountain Sample Management Facility and stored at 4°C until needed. Two of the samples were used for development of a core processing protocol and the remaining 16 samples were processed and analyzed. The core processing protocol was used to obtain finely crushed material for DNA extraction and QPCR analysis. A core sample container was removed from storage at 4°C and placed in a Class II Biological Safety Cabinet that had been disinfected with a 10% bleach solution followed by exposure to UV light. All instruments and materials for handling cores were similarly disinfected or flame-sterilized. The core sample was opened and a section of the core was removed and secured in a vice. With a sterile chisel and a hammer, a section of the sample was removed, uncovering an interior face of the core. The chisel was flame-sterilized and used to chip away small pieces from the freshly exposed face of the core. The sample was collected in a sterile 150 x 15mm Petri dish placed under the core. With a sterile forceps, core sample pieces were placed into a sterile steel mortar and pestle, and crushed. The crushed sample was sieved using a sterile #35 screen and 3 g of sample was collected in a sterile 15 ml centrifuge tube. The tube was labeled with a sample identification number and stored at approximately -70°C until processing.

5.4 **Subtask 4: Protocol Validation**

The three QPCR protocols that were developed were evaluated in laboratory tests with an extensive array of microbial species and genera, both target and non-target microorganisms. The protocol was then tested with available core samples from the repository environment. Two of the core samples were used to determine the DNA extraction and purification protocols to be used with the QPCR assays. Several commercially-available DNA extraction/purification kits were compared to IPLV-068, "Bacterial DNA Extraction and Purification", for their effectiveness in DNA extraction from core samples seeded with known amounts of bacteria. Kits tested consisted of the Ultra Clean Soil DNA Kit (Mo Bio Laboratories, Inc., Carlsbad, CA), Power Soil DNA Isolation Kit (Mo Bio Laboratories), Ultra Clean Mega Prep Soil DNA Kit (Mo Bio Laboratories), Power Max Soil DNA Isolation Kit (Mo Bio Laboratories), and IT 1-2-3 Scoop Sample Purification Kit (Idaho Technologies, Salt Lake City, UT). Core dust samples (0.5 g) were autoclaved to destroy any DNA present, and seeded with a purified suspension of 1.15×10^8 *Bacillus atrophaeus* spores enumerated electronically with a Coulter Multisizer II (Beckman Coulter). After seeding of the cores, DNA was extracted following the manufacturers' protocols with minor modifications as indicated in the scientific notebook (SN # UCCSN-UNLV-089, vols. 1-3), and the purified DNA was eluted in Tris-EDTA (TE) buffer. Positive controls were included with candidate protocols (i.e., Ultra Clean Soil DNA Kit, Power Soil DNA Isolation Kit, and IPLV-068) and consisted of DNA extracted from *B. atrophaeus* spores (1.15×10^8), but without core material. DNA extracts were stored at approximately -70°C until PCR was performed. Samples were analyzed by *B. atrophaeus*-specific (Buttner *et al.*, 2004b) and/or Universal bacterial QPCR.

6.0 ASSUMPTIONS

The following assumption was made in conducting this research:

While one of the goals of this project was to test the protocols developed in our laboratory with core samples from Yucca Mountain, we assume that storage of cores for several years affected the microbial population concentration and composition and the results obtained in these experiments with QPCR analysis are for protocol evaluation purposes only.

7.0 RESULTS, DISCUSSION AND CONCLUSIONS

7.1 RESULTS AND CONCLUSIONS BASED ONLY ON Q DATA

7.1.1 **Subtask 2: Literature Review**

Microorganisms indigenous to the Yucca Mountain site that were previously identified from core samples are presented in Table 1 (Amy, 1997; Amy *et al*., 1992 and 1993; Davis, 1998; Haldeman and Amy 1993a and 1993b; Haldeman *et al*., 1993 and 1994; Hersman, 1997; Horn *et al.*, 1998a and 1998b; Horne and Meike, 1995 and 1996; Kieft *et al*., 1997; West *et al*., 1985). Microorganisms that are potentially involved in actinide reduction or subsurface transport were determined from published research and are also shown in Table 1 (Anderson *et al.*, 2003; Frederickson *et al.*, 2000; Holmes *et al.*, 2002; Jeon *et al.*, 2004; Liu *et al.*, 2002; Lovley *et al.*, 1991; Lovley *et al.*, 1993; Merroun *et al.*, 2005; North *et al.*, 2004; Rusin *et al.*, 1994; Rittmann and Reed, 2002; Sani *et al.*,

2002; Suzuki *et al.*, 2003 and 2005; Wackett *et al.*, 2004; Vrionis *et al.*, 2005; Wu *et al.*, 2006; Wall and Krumholz, 2006). There was no overlap between the two lists of microorganisms, with the exception of the genus *Bacillus*. This indicates that genera of potential concern in actinide transport have not been previously detected in core samples, with the exception noted. However, three phyla contain microorganisms present on both lists, indicating that microorganisms previously isolated are closely related to those of concern. A list of all microbial phyla was prepared and available representative microorganisms were obtained from each phylum (ATCC) for use in development and testing of universal and group-specific PCR primers and probes (Table 2). Multiple representative microorganisms of phyla of potential concern were obtained, where possible. A total of 39 bacterial species were obtained, as well as fungal and human DNA for cross-reactivity testing (Table 2). Several universal and group-specific oligonucleotide probes and/or primers were identified from the literature (Dionisi *et al.*, 2003; Greisen *et al.*, 1994; Horz *et al.*, 2005; Ji *et al.*, 2004; Fierer *et al.*, 2005; Lyons *et al.*, 2000; Marchesi *et al.*, 1998; Nadkarni *et al.*, 2002; Okano *et al.*, 2004; Ott *et al.*, 2004; Takai and Horikoshi, 2000; Rivas *et al.*, 2004; Suzuki *et al.*, 2000; Blackwood *et al.*, 2005). The primer and probe sequences potentially compatible with the TaqMan® QPCR technology used in our laboratory were determined.

7.1.2 **Subtask 3: Laboratory Studies and Protocol Development**

7.1.2.1 **Universal primers and probes**

Candidate universal primers and probes were identified from the literature or designed for use with a QPCR assay and purchased from commercial sources (Table 3). Primers and probes underwent preliminary testing by amplifying positive and negative control samples and DNA from selected microorganisms. Experiments performed with selected certified microorganisms indicated that 10 ng of template DNA per PCR reaction produced optimal amplification results. Therefore, 10 ng of template DNA was used in subsequent experiments. The protocol of Suzuki *et al.*, 2000 was rejected due to positive results obtained with no template controls (NTCs). Positive NTCs have been reported previously as a potential problem with universal PCR assays (Greisen *et al.*, 1994; Nadkarni *et al.*, 2002; Vliegen *et al.*, 2006). It is believed to be because of nonspecific amplification of residual contaminant DNA present in the Taq polymerase during the production and purification of the enzyme (Corless *et al.*, 2000). Alternate primers were designed using the probe of Suzuki *et al.*, 2000, to better match the criteria for optimal amplification according to the Primer Express software (Table 3). These primers were rejected due to poor sensitivity of amplification of the test microorganisms. Several forward and reverse primers were designed using the probe of Nadkarni *et al.*, 2002, modified by using the complementary DNA sequence to better fit the Primer Express criteria (NadPc). All combinations of forward and reverse primers were tested using standard mode, FastChem, and with and without DNase treatment of the master mix to remove residual DNA contamination. The forward primer UnivF3 and reverse primer UnivR1 demonstrated the greatest amplification of DNA and were selected for further testing. However, positive NTCs remained a problem and only 13 of 25 test organisms were amplified; therefore, this primer and probe set was rejected. Next, the forward primer NadF and the reverse primer NadR (Nadkarni, et al., 2002) were tested with the modified Nadkarni probe, NadPc. Results showed that NTCs were negative but sensitivity was poor. Finally, the primers (NadF/NadR), probe (UnivP) and amplification conditions of Nadkarni *et al.*, 2002 were tested (Table 3), despite numerous violations of Primer Express

criteria for optimal amplification. NTCs were negative and sensitivity was improved in that 24 of 37 organisms were detected by PCR.

Optimization experiments were conducted to determine the concentrations of the universal primers and probe to use in the PCR assay. Results indicated that a final concentration of 200nM forward primer (NadF), 500nM reverse primer (NadR), and 150nM probe (UnivP) produced optimal PCR results. PCR sensitivity results using these conditions showed an increase in sensitivity of detection of 1-2 PCR cycles or up to 0.5 log of cell concentration. Specificity testing results showed that 26 of 37 certified microorganisms were detected by PCR using the new, optimized universal primers and probe. The amplification was further improved by the addition of 0.1% final concentration bovine serum albumin (BSA), a known PCR enhancer (Wilson, 1997). The final universal PCR protocol was then evaluated for amplification of DNA from test microorganisms from all phyla. A total of 33 of 39 microorganisms representing all microbial phyla were amplified using the universal PCR protocol (Table 4). All phyla of potential significance in actinide transport were amplified, and the universal PCR did not cross-react with non-bacterial groups, such as the fungi *S. chartarum* and *A. fumigatus*, and human DNA.

7.1.2.2 **Group-specific primers and probes**

Sequence alignment results for the 12 organisms of concern demonstrated that the RW03 probe was located in a highly conserved region of the 16S rRNA gene. Therefore, primers were designed in relation to probe RW03 using the *Desulfosporosinus* sp. strain A10 16S rRNA gene as the template. Candidate group-specific primers and probes were designed for use with a QPCR assay and purchased from commercial sources (Table 5). Both gram-positive and Actinobacteria group-specific primers were designed using a modified probe of the sequence reported by Greisen *et al*., 1994. The probe was modified by shortening the length by one nucleotide (excluding the 5' guanosine base) to comply with the guidelines for probe design specified by Primer Express software, and renamed Gram+P. BLAST search results showed that the probe region is specific for gram-positive bacteria. Because Actinobacteria are a subset of gram-positive bacteria, the same probe was used for both groups, and primer design was focused on specific amplification of each of the two groups. Only one of the primers designed, GR1, fully complied with primer design criteria specified by the Primer Express software. Primers and probes underwent preliminary testing by amplifying positive and negative control samples and DNA from selected microorganisms. All combinations of forward (GF1, GF2, GF3, AF1, AF2, Fi1) and reverse (GR1, AR1) primers were evaluated. Forward primers GF1, GF2 and Fi1 were rejected due to poor amplification efficiency, regardless of the reverse primer used. The primer combinations GF3/GR1 and GF3/AR1 demonstrated the greatest amplification and specificity for the gram-positive and Actinobacteria groups, respectively, and were selected for further testing.

DNase treatment of the master mix improved the specificity of the assays by eliminating cross-reactivity, that is, the amplification of non-target microorganisms. Preliminary results showed that sensitivity of amplification of target DNA was negatively affected by treatment of the master mix with DNase, presumably due to residual DNase digestion of the target DNA or inhibition of the PCR assay. Therefore, the DNase treatment was modified to produce optimal sensitivity and specificity of the assays. The addition of BSA was found to further improve amplification efficiency; therefore, the final PCR protocol for the gram-positive assay included the use of BSA in the reaction (0.1%

final concentration). The use of BSA as a PCR enhancer was not tested with the Actinobacteria-specific PCR assay.

The gram-positive PCR protocol was evaluated for amplification of DNA from test microorganisms from all phyla. Results showed that 11 of the 13 gram-positive microorganisms tested were amplified with the protocol using the GF3/GR1 primers with the Gram+P probe (Table 6). One of the two microorganisms that were not amplified, *Deinococcus radiodurans* was not amplified by any of the primers and probes tested. The genus *Deinococcus* has been previously shown to not hybridize with a gram-positive specific probe (Greisen, et al., 1994). Only one of the 26 non-target gram-negative organisms tested, *Borrelia burgdorferi*, was amplified with this protocol. There was no cross-reactivity observed with fungal and human DNA.

The Actinobacteria PCR protocol was evaluated for amplification of DNA from test microorganisms from all phyla. All of the six Actinobacteria tested were amplified with the protocol using the GF3/AR1 primers with the Gram+P probe (Table 7). However, cross-reactivity was observed with 3 non-target species, two gram-negative bacteria and a closely related gram-positive bacterium. There was no cross-reactivity observed with fungal and human DNA.

7.1.3 **Subtask 4: Protocol Validation**

Seeding experiments with known concentrations of microorganisms added to selected sterilized core material were performed to determine the DNA extraction/purification protocol to be used with the QPCR protocols that were developed. The Power Soil DNA Isolation Kit (Power Soil) and the Ultra Clean Soil DNA (Ultra Clean) Kit consistently produced greater amplification results than the other commercially-available kits tested. Therefore, these two kits were selected for further testing and compared to the protocol developed in our laboratory, IPLV-068. Results obtained from seeding experiments showed that the Power Soil kit was slightly better than the Ultra Clean Kit. Both of these kits were considerably better than the samples extracted using the boil/Pellet Paint method (IPLV-068). Inhibition of the PCR reaction was observed with the boil/Pellet Paint samples, but not with any of the Power Soil or Ultra Clean samples. The Power Soil kit was selected for inclusion into the QPCR sample processing protocols.

Core samples were processed and the DNA present in 0.5 g core samples was extracted and purified. A total of 16 of 18 core samples were analyzed by QPCR using the protocols developed in Subtask 3. Results of QPCR amplification with the universal primers and probe showed that microbial DNA was present in all samples analyzed, with mean DNA concentrations ranging from 8.32×10^3 to 7.69×10^6 DNA templates/g of sample (Table 8). Results are expressed as the DNA template equivalents equal to concentrations of *Bacillus atrophaeus* cells, the microorganism used as the quantitation standard. The measured DNA concentrations were greatest, on average, for the core samples obtained from the crystal poor member, upper lithophysal geologic zone (mean = 2.66×10^6 templates/g) and lowest for the crystal poor member, lower nonlithophysal geologic zone (mean = 1.27×10^4 templates/g). Previous microorganism concentration estimates using culture assay methods indicated core sample concentrations ranging from 10^1 to 10^5 CFU/g (Amy *et al.*, 1992; Haldeman and Amy, 1993a; Haldeman *et al.*, 1993). Previous total count estimates ranged from 10^4 to 10^7 cells/g (Haldeman and Amy, 1993a; Haldeman *et al.*, 1993; Kieft *et al.*, 1997). Total count methods are more directly comparable to QPCR because these assays do not distinguish between

viable and non-viable cells. While the measurements obtained in this study are consistent with those previously obtained using other total count assays, it must be emphasized that this analysis was performed with core samples that were 8-13 years old. The cores were obtained and stored under optimal conditions; however, it has been observed that storage effects can alter the concentration and composition of microbial populations in samples (Haldeman *et al.*, 1994). This is thought to be due to growth of a few bacterial types or resuscitation of dormant types and is likely due to changes in the available amount of water during storage. Another potential source of variability is that the amplification efficiency with universal primers varies between microorganisms (Table 4). The concentrations measured in this study are relative to the concentrations obtained by amplification of known concentrations of *B. atrophaeus*, the microorganism used in the quantitation standards. Therefore, the measurements are estimations of microbial concentrations based on similar amplification efficiencies of sample populations with *B. atrophaeus*. As the focus of the QPCR assay narrows to groups or sub-groups, and the number of different target microorganisms in a QPCR assay decreases, it is expected that the problems and uncertainty in measured concentrations will decrease (Blackwood *et al.*, 2005).

Results of QPCR amplification with the gram-positive primers and probe showed that gram-positive microorganisms were detected (lower detection limit = 21 templates/g) in only one of the core samples (Table 9). Similar results were obtained for the Actinobacteria, which are a subset of the gram-positive bacteria. QPCR amplification with the Actinobacteria primers and probe were also positive for only one of the core samples, the same core sample that was positive with the gram-positive QPCR protocol (Table 10). These data indicate that, at the time of sample analysis, predominantly gram-negative bacteria were present in the core samples. This is inferred because the difference between total bacterial concentrations measured with the universal QPCR protocol and gram-positive concentrations should be due to the presence of gram-negative bacteria. It is unknown whether this observation is due to the effects of storage of the core samples for several years (Haldeman *et al.*, 1994). Previous research with culturable isolates has shown that the ratio of viable gram-positive isolates to gram-negative isolates varies widely from one extreme to the other, depending on the core sample that was analyzed (Haldeman and Amy, 1993a; Haldeman *et al.*, 1993).

The QPCR approach has several advantages, as well as limitations, for the characterization of environmental samples. The advantages are speed, sensitivity and the potential for high throughput. In theory, the DNA from several core samples could be extracted and the purified DNA could be amplified in a single work day, compared with weeks or months for culture analysis. The method that is currently most commonly used in microbial population research is to amplify the 16S rDNA gene sequences in a sample, followed by cloning and sequencing of the DNA, and then identifying the microorganisms by comparing the sequences with genetic databases. The limitations of the cloning and sequencing approach are the labor intensive, time-consuming effort required and the fact that, even if hundreds of clones representing individual microorganisms are sequenced, only a very small percentage (<<0.1%) of the population is identified. Another advantage of the QPCR is sensitivity of the assay (10^1 cells/gram) that no other currently available methods can match. Lastly, the entire process can be automated and amplification can be performed with either a 96-well or 384-well plate format, allowing the analysis of hundreds or thousands of samples in a relatively short time. Along with the advantages of QPCR are limitations of the method (Janssen, 2006; Kirk *et al.*, 2004). Validated protocols for environmental microorganisms or groups of microorganisms

are lacking. Furthermore, a problem with many existing QPCR protocols is that the primers and probes have been designed based on theory by using existing sequence information in the genetic databases and have not been subjected to rigorous empirical testing (Marchesi *et al.*, 1998). The results of this project demonstrate that systematic laboratory testing is essential to validate primer and probe specificity before analysis of environmental samples. Another limitation of QPCR applied to groups of microorganisms is unequal efficiency of amplification of target microorganisms, as previously mentioned. One of the reasons for this inequality is the variation in the number of rDNA gene copies in a given species. Not knowing the exact number of copies of 16S rDNA genes in any given species at the time of analysis represents the main limitation to the absolute determination of bacterial numbers by real-time PCR based on 16S rDNA (Nadkarni *et al.*, 2002). However, for characterization of environmental microbial populations, other methods are likely to be far less sensitive or precise. Lastly, inhibition of the PCR assay by environmental or biological compounds can reduce assay sensitivity or result in false negatives. DNA extraction methods and kits have demonstrated varying degrees of the effectiveness in DNA purification and recovery. Environmental inhibition was not observed with the DNA extraction protocol used for processing seeded core samples tested in these experiments; however, internal positive controls (IPC) are available from Applied Biosystems that can be incorporated into the reaction mix and used to detect inhibition of the PCR assay.

7.1.4 CONCLUSIONS BASED ONLY ON Q DATA

Three QPCR protocols were developed that have potential for use in characterization of the microbial populations in the Yucca Mountain subsurface environment as well as other environments. A "top-to-bottom" approach was used beginning with a broad analysis of the total microbial population using universal QPCR primers and probe, and progressively narrowing the focus by developing a QPCR assay for gram-positive microorganisms and then an assay for the phylum Actinobacteria, a subset of the gram-positive group. Amplification efficiency varies between microorganisms amplified with multi-species PCR primers and probes, therefore, the uncertainty in quantitative measurements decreases as the assays become more focused on smaller groups of microorganisms. Sample processing and DNA extraction and purifications protocols were developed using archived Yucca Mountain core samples. Results of the testing and evaluation of the three QPCR protocols showed that:

- For the universal QPCR protocol
  - A total of 33 of 39 microorganisms representing all bacterial phyla amplified
  - All phyla of significance in actinide transport amplified
  - Core DNA concentrations ranged from 10^3 to 10^6 templates/g of sample; consistent with previous total count estimates
- For the gram-positive QPCR protocol
  - A total of 11 of the 13 target microorganisms amplified
  - One non-target microorganism amplified
  - Target microorganisms were detected in only one of the core samples

- For the Actinobacteria QPCR protocol
  - All of the six Actinobacteria species tested amplified
  - Two non-target microorganisms amplified

– Target microorganisms were detected in only one of the core samples

It should be emphasized that the effects of storage of the core samples for several years likely affected the microbial species composition and concentration. Because sample storage is known to affect microbial populations, it is important that fresh, aseptically collected samples are obtained for microbiological characterization. The approach developed in this research project focused on measuring key groups of microorganisms that could play a role in actinide transport in the subsurface, but other important species and groups of microorganisms may also be important in actinide transformation. Additional group- and species-specific QPCR assays should be developed in future work to provide a comprehensive molecular microbiological approach to measuring microorganisms in environmental samples.

7.2 CORROBORATION INCLUDING UQ DATA

Not applicable.

8.0 INPUTS AND REFERENCES

8.1 Inputs

Table	Data ID Number (DID)	File Name
4	R01PC.001	TDA ATCCuniv ORD-RF-01.xls
6	R01PC.002	TDA ATCC Gram+ ORD-RF-01.xls
7	R01PC.003	TDA ATCC Actino ORD-RF-01.xls
8	R01PC.004	TDA CoresUniv ORD-RF-01.xls
9	R01PC.005	TDA CoresGram+ ORD-RF-01.xls
10	R01PC.006	TDA CoresActino ORD-RF-01.xls

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9.0 SOFTWARE

Not applicable

10.0 ATTACHMENTS

Table 1. Microorganisms identified from the literature review (light grey = microorganisms indigenous to the Yucca Mountain site cultured previously; dark grey = microorganisms of concern for actinide reduction or subsurface transport).

Phylum	Class	Organism
Deinococcus-Thermus	Deinococci	*Deinococcus radiodurans*
Proteobacteria	Alphaproteobacteria	*Agrobacterium*
		Methylobacterium
	Betaproteobacteria	*Acidovorax facilis*
		Hydrogenophaga pseudoflava
	Gammaproteobacteria	*Acinetobacter calcoaceticus*
		Acinetobacter haemolyticus
		Acinetobacter johnsonii
		Moraxella bovis
		Moraxella lincolnii
		Moraxella phenylpyruvica
		Pasteurella haemolytica
		Pseudomonas facilus
		Pseudomonas fluorescens
		Pseudomonas paucimoblis
		Pseudomonas putida
		Pseudomonas stutzeri
		Pseudomonas syringae
		Pseudomonas vesicularis
		Shewanella sp.
		Xanthomonas campestris
	Deltaproteobacteria	*Anaeromyxobacter dehalogenans*
		Desulfovibrio desulfuricans
		Desulfovibrio vulgaris
		Geobacter metallireducens
		Geobacter sulfurreducens
Firmicutes	Bacilli	*Bacillus* sp.
		Bacillus coagulans
		Bacillus psychrophilus
		Staphylococcus kloosii
	Clostridia	*Clostridium* sp.
		Desulfosporosinus sp.
		Thermoterrabacterium ferrireducens
Actinobacteria High GC	Actinobacteria	*Arthrobacter oxydans*
		Arthrobacter crystallopoites
		Arthrobacter protophormiae
		Arthrobacter ramosus
		Aureobacterium liquefaciens
		Cellulomonas sp.
		Clavibacter michiganense
		Corynebacterium renale
		Gordonia bronchialis
		Microbacterium flavescens
		Micrococcus kristinae
		Micrococcus luteus
		Nocardioides luteus
		Rhodococcus maris
Bacteroidetes	Flavobacteria	*Flavobacterium indologenes*
	Sphingobacteria	*Sphingobacterium spiritovorum*
		Sphingobacterium multivorum-like

Table 2. Microorganisms used in the development and testing of universal and group-specific PCR primers and probes (ATCC = American Type Culture Collection; Neg = gram-negative; Pos = gram-positive; N/A = not applicable).

Phylum	Class	Organism (Genus and species)	Gram Reaction	ATCC #
Crenarchaeota	Thermoprotei	*Aeropyrum pernix (Archaea)*	Neg	700893D
Euryarchaeota	Methanococci	*Methanococcus maripaludis (Archaea)*	Neg	43000D
Aquificae	Aquificae	*Hydrogenothermus marinus*	Neg	BAA-483
Thermotogae	Thermotogae	*Thermotoga maritima*	Neg	43589D
Thermodesulfobacteria	Thermodesulfobacteria	*Thermodesulfobacterium commune*	Neg	33708
Deinococcus-Thermus	Deinococci	*Deinococcus radiodurans*	Pos	13939D
Chrysiogenetes	Chrysiogenetes	*Chrysiogenes arsenatis*	Neg	700172
Chloroflexi	Chloroflexi	*Chloroflexus aurantiacus*	Neg	29366
Thermomicrobia	Thermomicrobia	*Thermomicrobium roseum*	Neg	27502
Nitrospirae	Nitrospira	*Thermodesulfovibrio yellowstonii*	Neg	51303
Deferribacteres	Deferribacteres	*Geovibrio thiophilus*	Neg	BAA-311
Cyanobacteria	Cyanobacteria	*Nostoc* sp.	Neg	29133D
Chlorobi	Chlorobi	*Chlorobium tepidum*	Neg	49652D
Proteobacteria	Alphaproteobacteria	*Methylobacterium extorquens*	Neg	14718D
	Betaproteobacteria	*Hydrogenophaga pseudoflava*	Neg	700892
	Gammaproteobacteria	*Shewanella oneidensis*	Neg	700550D
	Deltaproteobacteria	*Desulfovibrio vulgaris* subsp. *vulgaris*	Neg	29579D
		Geobacter sulfurreducens	Neg	51573D
Firmicutes	Bacilli	*Bacillus cereus*	Pos	14579D
		Bacillus halodurans Nielsen et al.	Pos	BAA-125D
		Bacillus subtilis	Pos	82D
	Clostridia	*Clostridium difficile*	Pos	9689D
		Clostridium perfringens	Pos	13124D
		Desulfosporosinus meridiei	Pos	BAA-275
Actinobacteria	Actinobacteria	*Arthrobacter crystallopoietes*	Pos	15481
		Cellulomonas fimi	Pos	484
		Gordonia rubripertincta	Pos	14352
		Microbacterium flavescens	Pos	13348
		Micrococcus luteus	Pos	53598D
		Rhodococcus erythropolis	Pos	27854
Planctomycetes	Planctomycetacia	*Planctomyces maris*	Neg	29201
Chlamydiae	Chlamydiae	*Parachlamydia acanthamoebae*	Neg	VR-1476
Spirochaetes	Spirochaetes	*Borrelia burgdorferi*	Neg	35210D
Fibrobacteres	Fibrobacteres	*Fibrobacter intestinalis*	Neg	43854
Acidobacteria	Acidobacteria	*Geothrix fermentans*	Neg	700665
Bacteroidetes	Bacteroides	*Bacteroides fragilis*	Neg	25285D
Fusobacteria	Fusobacteria	*Ilyobacter insuetus*	Neg	BAA-291
Verrucomicrobia	Verrucomicrobia	*Verrucomicrobium spinosum*	Neg	43997
Dictyoglomi	Dictyoglomi	*Dictyoglomus thermophilum*	Neg	35947
Ascomycota	Ascomycetes	*Stachybotrys chartarum*	N/A	MYA-3310
Ascomycota	Ascomycetes	*Aspergillus fumigatus*	N/A	36607
Chordata	Mammalia	Human DNA	N/A	N/A

Table 3. Candidate universal bacterial TaqMan® primer and probe sequences designed in the laboratory or previously published (* = UNLV-modified).

Primer name	Probe name	Sequence	Reference
SuzF		cggtgaatacgttcycgg	Suzuki *et al*., 2000
SuzR*		gghtaccttgttacgactt	Suzuki *et al*., 2000
	SuzP	6-FAM-cttgtacacaccgcccgtc-TAMRA	Suzuki *et al*., 2000
UnivF		tgaatacgttcccggc	UNLV designed
UnivR		ggtgttacaaactctcgtgg	UNLV designed
	SuzP	6-FAM-cttgtacacaccgcccgtc-TAMRA	Suzuki *et al*., 2000
UnivF1		cagaagaagcaccggctaactc	UNLV designed
UnivF2		gcagaagaagcaccggctaa	UNLV designed
UnivF3		gcagaagaagcaccggctaac	UNLV designed
UnivR1		tttacgcccagtaattccgatt	UNLV designed
UnivR2		ctttacgcccagtaattccgatt	UNLV designed
	NadPc*	6-FAM-gtgccagcagccgcggtaatacg-TAMRA	Nadkarni *et al*., 2002
UnivF3		gcagaagaagcaccggctaac	UNLV designed
UnivR1		tttacgcccagtaattccgatt	UNLV designed
	NadPc*	6-FAM-gtgccagcagccgcggtaatacg-TAMRA	Nadkarni *et al*., 2002
NadF		tcctacgggaggcagcagt	Nadkarni *et al*., 2002
NadR		ggactaccagggtatctaatcctgtt	Nadkarni *et al*., 2002
	NadPc*	6-FAM-gtgccagcagccgcggtaatacg-TAMRA	Nadkarni *et al*., 2002
NadF		tcctacgggaggcagcagt	Nadkarni *et al*., 2002
NadR		ggactaccagggtatctaatcctgtt	Nadkarni *et al*., 2002
	UnivP	6-FAM-cgtattaccgcggctgctggcac-TAMRA	Nadkarni *et al*., 2002

Table 4. QPCR results obtained for 27 phyla using universal bacterial primers and probe. Ten nanograms of bacterial DNA and 15 nanograms of human DNA were used per PCR assay. Fungal DNA consisted of 2.77 x 10^4 *S. chartarum* templates and 3.97 x 10^5 *A. fumigatus* templates. Four replicates were amplified for each DNA sample with two exceptions *T. yellowstonii* and *P. maris* (n=2). Ct value is inversely proportional to the concentration of DNA measured, and a Ct value of 40 represents a negative result (QPCR = quantitative polymerase chain reaction; Neg = gram-negative; Pos = gram-positive; N/A = not applicable) (DID R01PC.001, TDA ATCCuniv ORD-RF-01.xls).

Organism	Gram Classification	PCR Analysis (Mean Ct value)	Standard Deviation
Aeropyrum pernix (Archaea)	Neg	36.46	0.06
Methanococcus maripaludis (Archaea)	Neg	40.00	0.00
Hydrogenothermus marinus	Neg	40.00	0.00
Thermotoga maritima	Neg	24.43	0.40
Thermodesulfobacterium commune	Neg	35.88	0.31
Deinococcus radiodurans	Pos	21.98	0.61
Chrysiogenes arsenatis	Neg	39.46	0.66
Chloroflexus aurantiacus	Neg	27.71	0.67
Thermomicrobium roseum	Neg	40.00	0.00
Thermodesulfovibrio yellowstonii	Neg	29.00	0.03
Geovibrio thiophilus	Neg	24.58	0.34
Nostoc sp.	Neg	40.00	0.00
Chlorobium tepidum	Neg	21.41	0.23
Methylobacterium extorquens	Neg	20.36	0.29
Hydrogenophaga pseudoflava	Neg	24.30	1.39
Shewanella oneidensis	Neg	19.10	0.72
Desulfovibrio vulgaris subsp. *vulgaris*	Neg	18.30	0.81
Geobacter sulfurreducens	Neg	27.02	0.37
Bacillus cereus	Pos	17.34	0.26
Bacillus halodurans Nielsen et al.	Pos	16.59	0.19
Bacillus subtilis	Pos	18.05	0.39
Clostridium difficile	Pos	16.51	0.61
Clostridium perfringens	Pos	15.56	2.53
Desulfosporosinus meridiei	Pos	40.00	0.00
Arthrobacter crystallopoietes	Pos	20.66	1.07
Cellulomonas fimi	Pos	28.33	5.60
Gordonia rubripertincta	Pos	38.92	1.22
Microbacterium flavescens	Pos	40.00	0.00
Micrococcus luteus	Pos	20.04	0.16
Rhodococcus erythropolis	Pos	34.04	2.46
Planctomyces maris	Neg	35.31	0.04
Parachlamydia acanthamoebae	Neg	33.46	1.58
Borrelia burgdorferi	Neg	24.33	1.08
Fibrobacter intestinalis	Neg	30.53	0.86
Geothrix fermentans	Neg	34.51	1.65
Bacteroides fragilis	Neg	20.60	0.76
Ilyobacter insuetus	Neg	33.34	1.20
Verrucomicrobium spinosum	Neg	39.57	0.31
Dictyoglomus thermophilum	Neg	28.48	1.47
Stachybotrys chartarum	N/A	40.00	0.00
Aspergillus fumigatus	N/A	40.00	0.00
Human DNA	N/A	40.00	0.00

Table 5. Candidate gram-positive bacterial TaqMan® primer and probe sequences designed in the laboratory or previously published. Wobble (interchangeable) bases are indicated by the letters s, m, r, y, and k (s = cytosine or guanine; m = adenine or cytosine; r = adenine or guanine; y = cytosine or thymine; k = guanine or thymine; * = UNLV-modified).

Primer name	Probe name	Sequence (Name)	Reference
GF1		actctagacagrstgccggtgataa	UNLV designed
GR1		ggccattgtagcacgtgtgt	UNLV designed
Fi1		actctarrgagactgccggtgataa	UNLV designed
AF1		actcatrggakactgccggggtcaa	UNLV designed
AF2		actgccggggtcaactcgga	UNLV designed
GF2		actcttrrgagactgccggtgacaa	UNLV designed
GF3		aamycggaggaaggtggggatg	UNLV designed
AR1		ggccattgtagcatgcgtga	UNLV designed
	Gram+P	6-FAM-acgtcaaatcatcatgccccttatgtc-TAMRA	Greisen *et al*., 1994*

Table 6. QPCR results obtained for 27 Phyla using gram-positive bacterial primers and probe. Ten nanograms of bacterial DNA were used per PCR assay with one exception, *C. perfringens* (5 ng). Human DNA consisted of 15 nanograms, and fungal DNA consisted of 2.77×10^4 *S. chartarum* templates and 3.97×10^5 *A. fumigatus* templates. Two replicates were amplified for each DNA sample with one exception *D. meridiei* (n=4). Ct value is inversely proportional to the concentration of DNA measured, and a Ct value of 40 represents a negative result (QPCR = quantitative polymerase chain reaction; Neg = gram-negative; Pos = gram-positive; N/A = not applicable) (DID R01PC.002, TDA ATCC Gram+ ORD-RF-01.xls).

Organism	Gram Classification	Mean Ct value (n=2)	Standard Deviation
Aeropyrum pernix (Archaea)	Neg	40.00	---
Methanococcus maripaludis (Archaea)	Neg	40.00	---
Hydrogenothermus marinus	Neg	40.00	---
Thermotoga maritima	Neg	40.00	---
Thermodesulfobacterium commune	Neg	40.00	---
Deinococcus radiodurans	**Pos**	40.00	---
Chrysiogenes arsenatis	Neg	40.00	---
Chloroflexus aurantiacus	Neg	40.00	---
Thermomicrobium roseum	Neg	40.00	---
Thermodesulfovibrio yellowstonii	Neg	40.00	---
Geovibrio thiophilus	Neg	40.00	---
Nostoc sp.	Neg	40.00	---
Chlorobium tepidum	Neg	40.00	---
Methylobacterium extorquens	Neg	40.00	---
Hydrogenophaga pseudoflava	Neg	40.00	---
Shewanella oneidensis	Neg	40.00	---
Desulfovibrio vulgaris subsp. vulgaris	Neg	40.00	---
Geobacter sulfurreducens	Neg	40.00	---
Bacillus cereus	**Pos**	17.04	0.09
Bacillus halodurans Nielsen et al.	**Pos**	15.43	0.06
Bacillus subtilis	**Pos**	18.35	0.50
Clostridium difficile	**Pos**	15.43	0.03
Clostridium perfringens	**Pos**	18.63	0.00
Desulfosporosinus meridiei	**Pos**	18.97	2.18
Arthrobacter crystallopoietes	**Pos**	26.96	0.19
Cellulomonas fimi	**Pos**	26.60	0.06
Gordonia rubripertincta	**Pos**	26.63	0.18
Microbacterium flavescens	**Pos**	28.27	0.07
Micrococcus luteus	**Pos**	40.00	---
Rhodococcus erythropolis	**Pos**	24.72	0.10
Planctomyces maris	Neg	40.00	---
Parachlamydia acanthamoebae	Neg	40.00	---
Borrelia burgdorferi	Neg	20.47	0.18
Fibrobacter intestinalis	Neg	40.00	---
Geothrix fermentans	Neg	40.00	---
Bacteroides fragilis	Neg	40.00	---
Ilyobacter insuetus	Neg	40.00	---
Verrucomicrobium spinosum	Neg	40.00	---
Dictyoglomus thermophilum	Neg	40.00	---
Stachybotrys chartarum	N/A	40.00	---
Aspergillus fumigatus	N/A	40.00	---
Human DNA	N/A	40.00	---

Table 7. QPCR results obtained for 26 Phyla using Actinobacteria-specific primers and probe. Five nanograms of bacterial DNA were used per PCR assay. Human DNA consisted of 15 nanograms, and *A. fumigatus* DNA consisted of 3.97 x 10^5 templates. Two replicates were amplified for each DNA sample with one exception *P. acanthamoebae* (n=1). Ct value is inversely proportional to the concentration of DNA measured, and a Ct value of 40 represents a negative result (QPCR = quantitative polymerase chain reaction; Neg = gram-negative; Pos = gram-positive; N/A = not applicable; nd = not determined) (DID R01PC.003, TDA ATCC Actino ORD-RF-01.xls).

Organism	Gram Classification	Mean Ct value (n=2)	Standard Deviation
Aeropyrum pernix (Archaea)	Neg	40.00	---
Methanococcus maripaludis (Archaea)	Neg	40.00	---
Hydrogenothermus marinus	Neg	40.00	---
Thermotoga maritima	Neg	40.00	---
Thermodesulfobacterium commune	Neg	40.00	---
Deinococcus radiodurans	Pos	40.00	---
Chrysiogenes arsenatis	Neg	40.00	---
Chloroflexus aurantiacus	Neg	40.00	---
Thermomicrobium roseum	Neg	40.00	---
Thermodesulfovibrio yellowstonii	Neg	nd	---
Geovibrio thiophilus	Neg	40.00	---
Nostoc sp.	Neg	32.22	0.48
Chlorobium tepidum	Neg	40.00	---
Methylobacterium extorquens	Neg	40.00	---
Hydrogenophaga pseudoflava	Neg	40.00	---
Shewanella oneidensis	Neg	40.00	---
Desulfovibrio vulgaris subsp. *vulgaris*	Neg	40.00	---
Geobacter sulfurreducens	Neg	40.00	---
Bacillus cereus	Pos	40.00	---
Bacillus halodurans Nielsen et al.	Pos	40.00	---
Bacillus subtilis	Pos	40.00	---
Clostridium difficile	Pos	40.00	---
Clostridium perfringens	Pos	40.00	---
Desulfosporosinus meridiei	Pos	27.60	1.60
Arthrobacter crystallopoietes	**Pos**	19.05	0.11
Cellulomonas fimi	**Pos**	18.99	0.12
Gordonia rubripertincta	**Pos**	27.13	0.12
Microbacterium flavescens	**Pos**	20.75	0.24
Micrococcus luteus	**Pos**	27.99	0.37
Rhodococcus erythropolis	**Pos**	25.62	0.14
Planctomyces maris	Neg	40.00	---
Parachlamydia acanthamoebae	Neg	39.96	---
Borrelia burgdorferi	Neg	40.00	---
Fibrobacter intestinalis	Neg	40.00	---
Geothrix fermentans	Neg	40.00	---
Bacteroides fragilis	Neg	40.00	---
Ilyobacter insuetus	Neg	40.00	---
Verrucomicrobium spinosum	Neg	40.00	---
Dictyoglomus thermophilum	Neg	40.00	---
Stachybotrys chartarum	N/A	nd	---
Aspergillus fumigatus	N/A	40.00	---
Human DNA	N/A	40.00	---

Table 8. QPCR results obtained for Yucca Mountain core samples amplified with universal bacterial primers and probe. DNA was extracted from 0.5 gram of dust from each core sample using the Power Soil DNA Isolation Kit. PCR quantitation standards consisted of *Bacillus atrophaeus* spore suspensions of known concentration extracted using the same protocol used with core samples. Four replicates were analyzed for each DNA core sample with some exceptions, core 6 (n=2) and cores 8, 9, 12, and 18 (n=3). The lower detection limit (LDL) was 22 templates per gram (QPCR = quantitative polymerase chain reaction; HRC = Harry Reid Center for Environmental Studies; nd = not determined; Std. Dev. = Standard deviation) (DID R01PC.004, TDA CoresUniv ORD-RF-01.xls).

Specimen Id. Chain of custody	HRC Id.	Mean Templates per gram (n=4)	Std. Dev.
2041278[a]	1	1.38E+05	1.16E+04
2041283	2	9.14E+04	1.40E+04
2041286	3	8.73E+05	1.08E+05
2041279[b]	4	1.65E+04	3.76E+03
2041288	5	nd	---
2041284	6	8.92E+03	4.89E+02
2041289[c]	7	3.74E+04	1.45E+04
2041292	8	1.08E+04	1.73E+03
2041282	9	8.32E+03	7.43E+02
2041277[d]	10	7.69E+06	2.60E+06
2041287	11	2.65E+05	6.80E+04
2041281	12	2.45E+04	6.16E+03
2041276[e]	13	1.88E+05	3.85E+04
2041290	14	6.13E+05	7.89E+04
2041291	15	nd	---
2041280[f]	16	8.02E+04	4.41E+04
2041285	17	4.68E+05	3.28E+05
2041293	18	1.44E+04	3.28E+03

[a]Topopah Spring Tuff, crystal poor member, lower lithophysal zone
[b]Topopah Spring Tuff, crystal poor member, lower nonlithophysal zone
[c]Topopah Spring Tuff, crystal poor member, middle lithophysal zone
[d]Topopah Spring Tuff, crystal poor member, upper lithophysal zone
[e]Topopah Spring Tuff, crystal rich member, lithophysal zone
[f]Topopah Spring Tuff, crystal rich member, nonlithophysal zone

Table 9. QPCR results obtained for Yucca Mountain core samples amplified with gram-positive bacterial primers and probe. DNA was extracted from 0.5 gram of dust from each core sample using the Power Soil DNA Isolation Kit. PCR quantitation standards consisted of *Bacillus atrophaeus* spore suspensions of known concentration extracted using the same protocol used with core samples. Two replicates were analyzed for each DNA core sample. See Table 8 for zone designation of each core (QPCR = quantitative polymerase chain reaction; HRC = Harry Reid Center for Environmental Studies; LDL = 21 templates per gram; nd = not determined; Std. Dev. = Standard deviation) (DID R01PC.005, TDA CoresGram+ ORD-RF-01.xls).

Specimen Id. Chain of custody	HRC Id.	Mean Templates per gram (n=2)	Std. Dev.
2041278	1	LDL	---
2041283	2	LDL	---
2041286	3	LDL	---
2041279	4	LDL	---
2041288	5	nd	---
2041284	6	LDL	---
2041289	7	LDL	---
2041292	8	LDL	---
2041282	9	LDL	---
2041277	10	4.54E+03	2.70E+03
2041287	11	LDL	---
2041281	12	LDL	---
2041276	13	LDL	---
2041290	14	LDL	---
2041291	15	nd	---
2041280	16	LDL	---
2041285	17	LDL	---
2041293	18	LDL	---

Table 10. QPCR results obtained for Yucca Mountain core samples amplified with Actinobacteria-specific primers and probe. DNA was extracted from 0.5 gram of dust from each core sample using the Power Soil DNA Isolation Kit. PCR quantitation standards consisted of serial dilutions of *Cellulomonas fimi* DNA extracted using IPLV-068 and quantitated spectrofluorometrically (89436.5 ng/µl). DNA templates per PCR assay were calculated based on the approximate genome size of *C. fimi* (4000 kbp). Two replicates were analyzed for each DNA core sample. See Table 8 for zone designation of each core (QPCR = quantitative polymerase chain reaction; HRC = Harry Reid Center for Environmental Studies; LDL = 40 templates per gram; nd = not determined; Std. Dev. = Standard deviation) (DID R01PC.006, TDA CoresActino ORD-RF-01.xls).

Specimen Id. Chain of custody	HRC Id.	Mean Templates per gram (n=2)	Std. Dev.
2041278	1	LDL	---
2041283	2	LDL	---
2041286	3	LDL	---
2041279	4	LDL	---
2041288	5	ND	---
2041284	6	LDL	---
2041289	7	LDL	---
2041292	8	LDL	---
2041282	9	LDL	---
2041277	10	9.89E+01	5.72E+01
2041287	11	LDL	---
2041281	12	LDL	---
2041276	13	LDL	---
2041290	14	LDL	---
2041291	15	ND	---
2041280	16	LDL	---
2041285	17	LDL	---
2041293	18	LDL	---