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Subsurface and Biogeochemical Research Program
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ABSTRACT

This U.S. Department of Energy (DOE) Environmental Remediation Sciences Project (ERSP) was designed to test fundamental hypotheses concerning the existence and nature of indigenous microbial populations of Nevada Test Site subsurface nuclear test/detonation cavities. Now called Subsurface Biogeochemical Research (SBR), this program's Exploratory Research (ER) element, which funded this research, is designed to support high risk, high potential reward projects. Here, five cavities (GASCON, CHANCELLOR, NASH, ALEMAN, and ALMENDRO) and one tunnel (U12N) were sampled using bailers or pumps. Molecular and cultivation-based techniques revealed bacterial signatures at five sites (CHANCELLOR may be lifeless). SSU rRNA gene libraries contained diverse and divergent microbial sequences affiliated with known metal- and sulfur-cycling microorganisms, organic compound degraders, microorganisms from deep mines, and bacteria involved in selenate reduction and arsenite oxidation. Close relatives of *Desulforudis audaxviator*, a microorganism thought to subsist in the terrestrial deep subsurface on H_2 and SO_4^{2-} produced by radiochemical reactions, was detected in the tunnel waters. NTS-specific media formulations were used to culture and quantify nitrate-, sulfate-, iron-reducing, fermentative, and methanogenic microorganisms. Given that redox manipulations mediated by microorganisms can impact the mobility of DOE contaminants, our results should have implications for management strategies at this and other DOE sites.

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LIST OF ACRONYMS

AMS	Accelerator Mass Spectrometry
BER	Biological and Environmental Research
DNA	Deoxyribonucleic acid
DOE	Department of Energy
ERSP	Environmental Remediation Sciences Program
GC-MS	Gas Chromatography Mass Spectrometry
HRC	Harry Reid Center for Environmental Studies
ICP-MS	Ion Coupled Plasma Mass Spectrometry
LLNL	Lawrence Livermore National Laboratory
MEGA	Molecular Evolutionary Genetics Analysis
NTS	Nevada Test Site
PCR	Polymerase Chain Reaction
RFP	Request for Proposals
SBR	Subsurface Biogeochemical Research
SSU rRNA	Small Subunit of the Ribosomal RNA gene
UGTA	Underground Test Area Project
UNLV	University of Nevada Las Vegas

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EXECUTIVE SUMMARYAL

Background and Purpose

From 1951 to 1992, 828 underground nuclear detonations were conducted at the Nevada Test Site (NTS) (12). Approximately 145 km from Las Vegas, the NTS is located near Yucca Mountain and overlies regional and perched aquifers of growing potential importance. The juxtaposition of radionuclide-contaminated waste over a range of rock types, temperatures, redox chemistries, and hydrologic conditions represents a unique potential laboratory for the study of processes controlling the transport of legacy wastes and potential source of novel microorganisms for bioremediation.

Underground nuclear test locations are initially sterilized by the extremely high test-derived temperatures and pressures, sufficient to melt rock (59). Thus, these habitats, at a minimum, represent a study in the potential for the microbial re-colonization of a range of subsurface habitats. In reality however, the challenges for life are more extreme and include high-fluxes of ionizing radiation (up to 10^8 pCi/L, Table 1), persistent high temperatures (up to 95^{o}C, Table 2), and the presence of materials that may be toxic (e.g., Pb from vaporized instrument rack shielding), radioactive or both (e.g., Pu). As extreme as these conditions may be, numerous examples exist of habitats that had once been assumed sterile or nearly-so, but were later revealed to host significant microbial populations (e.g., polar rocks and ice, or deep sediments (10, 19, 39, 50)). Of greater relevance to this study, in the 1990s, for example, it was shown that up to ~10^4 culturable bacteria per g were present in highly radioactive vadose zone sediments at the Department of Energy (DOE) Hanford Site (16). Thus, the presence of living microorganisms in NTS test locations might not be entirely unexpected. Still, it was with these uncertainties in mind that we proposed an initial scoping study, under the ERSP science element of exploratory research, to determine if microbial communities presently occur in such cavities; and, if they were present, to explore their diversity and begin to develop a conceptual understanding of their potential impact upon radionuclide transport.

As detailed in the RFP to which we responded, the activity of subsurface microorganisms can profoundly impact the mobility of materials in the subsurface, facilitating the complexation, reduction, transformation, biomineralization and sequestration of DOE contaminants. Over the past decade or so our understanding of the ability of microorganisms to mediate redox transformations of radionuclides has significantly expanded. Microorganisms are currently known to reduce or sequester U (18, 35, 48, 55, 57), Tc (15, 31, 62), Pu (25, 43), Am (21), and even Np (52). Combinations of actinides, shorter lived radionuclides, organic chemicals and metal contaminants exist at many of the DOE facilities (51) and interest is keen in understanding the role microorganisms play in contaminant mobilization/immobilization and developing bioremediation strategies to control the mobility of these compounds in the subsurface. At DOE sites, the most common inorganic contaminants are U, Sr, Cs, Pu, Tc, Cr, Pb, and Hg (51). Among these, U, Pu, Tc, and Cr are less mobile when reduced, and all can be reduced and/or oxidized by microorganisms (6, 32-35, 53, 63). U, Pu, and Tc are found in significant concentrations in NTS nuclear test cavities (Table 1) and provide the major justification for this work.

Table 1. Radionuclide data from selected wells (provided by M. Zavarin).

Well name	Sample date	^{3}H	^{14}C	^{36}Cl	^{99}Tc	^{129}I	$^{239,240}Pu$
		---------- Half Life, yr^{-1} ----------					
		12.3	5730	301000	213000	15700000	-
		pCi/L					
GASCON U-4t PS#3a	03/19/08	3.0E+3	8.7E-1	<5E-3	3E-3	2.5E-4	<0.0015
CHANCELLOR U-19ad PS#1a	05/01/08	1.3E+7	1.6E+2	7.2E+0	2.5E+1	1.32	4.7E+1
NASH UE-2ce	07/02/08	2.7E+5	2.0E0	1.3E+0	2.3E-3	1.1E-2	<0.008
Tunnel Vent U12N.10	08/02/08	6.26E+6	1.3E+2	1.0E+2	1.9E-1	9.9E-1	8.0E-1
ALEMAN Ue3#4 P1	06/11/09	1.54E+6	ip	ip	ip	ip	ip
ALMENDRO U19v PS1ds	09/15/09	8.47E+7	ip	ip	ip	ip	ip

ip = in progress

Table 2. Water chemistry variables for sampled wells (provided by M. Zavarin).

Well name	Sample date	pH	T (°C)	Cond. (µS/cm)	DIC ppm C)	Cl^- (mg/L)	NO_3^- (mg/L)	SO_4^- (mg/L)	Na^+ (mg/L)	K^+ (mg/L)	Ca^{2+} (mg/L)	Mg^{2+} (mg/L)
GASCON U-4t PS#3a	03/19/08	8.5	20.3	558	24.8	73.6	<0.4	0.44	75.3	7.8	10.9	9.8
CHANCELLOR U-19ad PS#1a	05/01/08	8.9	65[a]	862	21.9	26.9	2.11	63.4	96.0	4.7	1.1	0.5
NASH UE-2ce	07/02/08	6.8	34.2	736	84.2	14.0	5.7	18.1	38	20.6	71.8	30.8
Tunnel Vent U12N.10	08/02/08	9.4	22.7	1110	25.6	35.5	1.9	288.6	221	13.6	14	0.19
ALEMAN Ue3#4 P1	06/11/09	11.4	24.3	1661	ip	82.6	ND	39.8	226	31.0	6.5	0.004
ALMENDRO U19v PS1ds	09/15/09	9.5	27.7	783	ip	73.1	0.5	1.1	162	13.2	0.48	0.009

ip = in progress
ND = not detected
[a] in-hole temperature measured at 95°C.

Although a number of prominent subsurface microbiology studies were conducted at the NTS by the U.S. DOE Subsurface Microbiology Program in the 1980s and 1990s (2, 22, 23, 28, 54, 56), none of these systematically targeted radioactive samples. Thus, the current study expands the scope of investigation to a new area; namely, post-detonation nuclear test cavity microbiology. In addition, the major approaches utilized here, e.g., SSU rRNA gene libraries were not available at the time of the previous investigations. For this reason, the present study has a greater probability of interrogating the true microbial diversity than was previously possible. The one site sampled by both projects (the U12N tunnel) illustrates the potential of the new study very well. Whereas, tuff from this site was shown to contain mostly strains of *Pseudomonas* in the previous work (3), the current study shows much greater and phylogenetically deeper diversity.

This project was responsive to the DOE Biological and Environmental Research (BER) Long Term Measure: "to provide sufficient scientific understanding to allow a significant fraction of DOE sites to incorporate coupled biological, chemical and physical processes into decision making for environmental remediation". In particular, our work endeavored to initiate a characterization of the biological component of this larger combined objective at a major DOE site. At the NTS, although a large amount of effort has been dedicated to characterizing the hydrology and geochemistry in subsurface systems (7, 8, 24, 26, 27, 44), prior to our study, microbiological characterizations have been neglected. As a result, the relationships between hydrology/geochemistry and biology in the subsurface of the NTS remain incompletely understood. The fact that at the beginning of this project, it was unknown whether or not microorganisms even existed in these potential habitats, represented a significant knowledge gap and speaks to the value of this work from the DOE perspective.

Another justification for this project was that, whereas a great deal of characterization of subsurface microbiological communities and their activities has been performed across the DOE complex, this body of work has focused almost exclusively on nuclear materials production sites (4, 17, 36, 60, 61). Although there are likely some similarities, the selective pressures, risks, and roles for microorganisms at nuclear detonation sites are probably different. At detonation sites, microorganisms will have been exposed to an extremely high, acute dose of ionizing radiation, followed by re-colonization of materials sterilized by high temperatures and in the presence of heavy metals (from the vaporized instrument rack). In contrast, microorganisms from disposal sites will have seen chronic, high-level exposure over decadal scales, often in the presence of co-contaminants such as nitrate. Whereas, it is conceivable that microorganisms may serve as agents of both solubilization and immobilization at both types of sites, they may serve an additional role at detonation sites as mediators of the dissolution of melt glass, for example.

An improved understanding of the diversity and capabilities of natural radioresistant and radionuclide-impacted bacteria from both production and detonation sites would lead to better-designed bioremediation strategies and a deeper understanding of the microbiological role in observed transport behavior. Once the preferred nutrient requirements and other stimulatory factors of bacteria which can, for example reduce actinides in the subsurface under specific NTS field conditions are determined, it would be possible to design environmental amendments that will enrich those populations or

augment their activity. The biodiversity contained within the NTS blast cavities will undoubtedly represent a significant source of genetic innovation of likely relevance to the management of other contaminated sites across the US and internationally. The results reported here are a first step towards these goals.

EXPERIMENTAL DESIGN, METHODS, AND PROCEDURES

As this activity was the first microbiological work to have been performed in coordination with major NTS monitoring activities for many years, we were aware that our early activities would set the tone for potential future studies. It was with this in mind that we endeavored to minimize the impact of our project on the environmental management activities of our UGTA (Underground Test Area Project) collaborators. The UGTA sub-project is an ongoing Environmental Management activity tasked with addressing groundwater contamination associated with underground nuclear testing at the NTS. The Department of Energy, National Nuclear Security Administration, Nevada Site Office has oversight responsibility for this project. The microbiological research was initiated with agreement from the UGTA sub-project director and in coordination with LLNL collaborators responsible for contaminated groundwater sampling and characterization for the UGTA project.

Sampling protocols were developed to coincide with ongoing groundwater sampling activities performed by the UGTA project and minimize our impact to ongoing EM activities. For example, it was noted very early that on-site filtration (our otherwise preferred method for accumulating biomass for DNA analysis) was of concern to site workers. For this reason, we adjusted our sampling protocol such that microbiological collections took the form of grab samples which were subsequently filtered in a containment hood at the LLNL laboratory in Mercury, NV. The result was that we obtained the required samples and, in the process, saved a great deal of time at the wellhead. On the downside, this compromise sometimes lead to the collection of more samples than were needed and ultimately to disposal concerns. This concern was partially alleviated in later sampling by coordinating our sample collection activities with the sample needs of our LLNL collaborator. In the end, however, our field protocols integrated well into the existing monitoring program and our group became an accepted part of the monitoring team engaged with UGTA hot well sampling.

Field Site

The NTS (Figure 1) is located in Nye County in southern Nevada; the southernmost point of the NTS is about 105 km northwest of Las Vegas. The site contains 3,500 km^2 of federally-owned land with restricted access and varies from 45 - 56 km in width (east-west) and 64 - 88 km in length (north-south). The most common method of underground nuclear testing at the NTS was to emplace the test device at the bottom of a vertically-drilled hole produced using so-called big hole drilling technology. Big holes ranged from 1 to 3 m in diameter and from 213 to 762 m deep. The nuclear device itself, the “package”, or “rack”, which was about 2 m in diameter, 30 m long, and contained all of the instrumentation required to receive data at the time of the explosion (37). Holes were stemmed to prevent the escape of radioactive materials during or after the experiment. A variety of stemming materials were used to backfill the holes including layers of magnetite, sand, concrete, and epoxy (37).

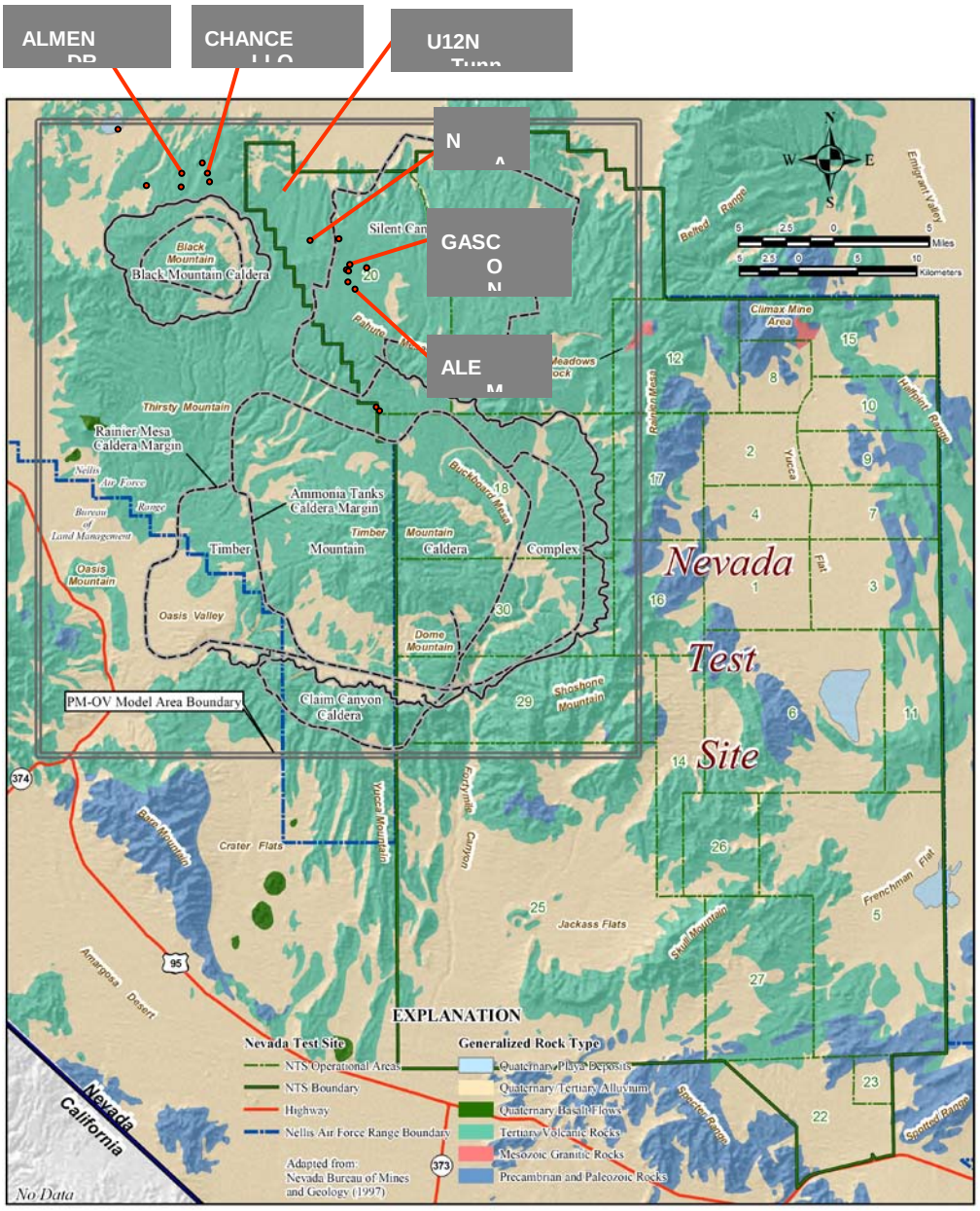


Figure 1. Approximate locations of the sites sampled during this work. Modified from (13).

Upon detonation, the energy released vaporizes the nuclear device and surrounding rock within a fraction of a second and a generally spherical cavity forms at the emplacement position. As the hot gases cool, a lining of molten rock is known to puddle at the cavity bottom. After a period of minutes to hours, the overlying rock typically falls into the detonation cavity as the residual pressure from the test decays to below the lithostatic pressure of the overlying rock, producing a vertical, rubble-filled column known as a rubble chimney, which can extend to the land surface (59).

For this project, a number of “post-shot”, chimney, and near-field boreholes were selected from which microbiological samples were obtained. Post-shot boreholes are

wells drilled directly into the cavity created by the detonation of an underground nuclear test. Chimney boreholes are wells drilled above the detonation cavity into the rubbleized zone. Near-field wells are those wells that do not intersect the underground cavity, but are situated hydraulically to intersect contaminated groundwater associated with the underground nuclear tests. Groundwater samples are currently collected from some of the post-shot, chimney, and near-field boreholes periodically and were the basis for this study. Aquatic chemistry and radiological analyses were performed by our LLNL collaborator. Briefly, activation products ^{14}C and ^{36}Cl were analyzed by accelerator mass spectrometry (AMS) and fission products ^{99}Tc and ^{129}I were measured by ICP-MS and AMS, respectively.

Pumped or bailed water was obtained from subsurface radiological environments (Figure 2). The availability of these sites depended upon the sampling objectives of the Underground Test AREA (UGTA) hot-well sampling program during fiscal years FY2007, FY2008 and FY2009. All sampling procedures and safety requirements were tailored to coordinate with UGTA field operations. As a result, the health and safety requirements (e.g., Field Activity Work Packages and Radiological Work Permits) were integrated into ongoing operations of a major NTS project. UGTA radiochemical results, once cleared for public release, were used to augment the microbiological results.

Figure 2. DOE-cleared photograph of PI (white hat) and postdoctoral associate, Bruckner (brown hat) sampling at CHANCELLOR. Typical dress out is Shown here. Note pump manifold (silver wrapped) in middle ground with evaporation ponds in background.

Cultivation Experiments

Unfiltered water samples were collected in 50 cc disposable polypropylene centrifuge tubes or crimp-sealed glass serum vials when anaerobic technique was warranted. Samples were stored on wet ice in the field and at 4°C in the LLNL laboratory (Mercury, NV). Upon clearance from the NTS, samples were transported to the Radiochemistry Laboratory at UNLV's Harry Reid Center (HRC), where they were stored at 4°C. At the HRC, samples were decimally diluted (1 mL sample:9 mL diluent x 5 – 6 rounds each) into pre-made tubes containing a range of defined non-radioactive microbiological growth media targeted across a range of relevant microbial

physiotypes, including: aerobic heterotrophs, nitrate reducers, fermentative microbes, sulfate reducers iron reducers, and methanogens (Table 3). An NTS-specific basal medium was designed to approximate the average solute composition determined from prior samplings of NTS test cavities and pH adjusted for the individual sample locations. Dilutions were incubated at appropriate temperatures based upon data observed at the time of sampling. Dilutions were not done in triplicate as would be required for most probable number analysis in order to minimize the generation of radioactive waste.

Molecular Microbiological Diversity Assessment

One contingency envisioned in our proposal was that environmental DNA extracts from the NTS might be radioactive. A variety of methodologies involving processing and amplification onsite were thus considered in our original work plan. Fortunately, almost all of the radioactivity in our samples was present as tritium in the water itself. Samples for molecular analysis were collected in sterilized 1-L Nalgene® polypropylene carboys and planktonic cells were concentrated by filtration with 0.22 micron filters. Filtration was performed in the LLNL laboratory (Mercury, NV), with the exception of NASH, where tritium radioactivity levels below 400,000 pCi/L permitted on-site processing. Several filter types and filtration approaches were evaluated during the course of our project to minimize the risk of spills. Ultimately, Sterivex-GP (PES, Millipore) filter units were coupled to 60 cc syringes fitted with stainless steel three-way valves and siphon tubes (used to draw the sample from sample containers). This assembly minimized the risk of spills from hoses popping off due to excessive pressure which might otherwise be generated (e.g., with a peristaltic pump). After a sufficient volume had passed the filter (e.g., the filtration rate slowed due to plugging, 10 mL – 2 L throughput), filters were frozen at -20 °C onsite and transported on dry ice to the HRC upon clearance from the NTS.

Sample processing at the HRC consisted of nucleic acid extraction using commercial available kits (UltraClean Soil DNA Isolation Kits, MoBio) following manufacturer's protocols. Extracted nucleic acids were screened for radioactivity by scintillation (all of the samples were below detection limits post-processing) and then transferred to the Desert Research Institute (DRI) for subsequent molecular characterization. The polymerase chain reaction (PCR) was utilized in concert with universal and domain-specific 16S rRNA bacterial primers (27F-YM/1512Ur (14)) to screen for the presence of bacterial DNA in the extracted nucleic acids. To minimize the possibility of laboratory contamination, reagent blanks (negative controls) and stringent laboratory hygiene protocols were implemented throughout. Appropriate positive and negative controls were also employed during molecular reactions, which proved important as one of our sites failed to produce a bacterial amplicon (CHANCELLOR). No sites produced archaeal amplicons. Amplicons were purified (UltraClean GelSpin DNA Purification Kit, MoBio Laboratories, Inc.) and subjected to molecular cloning using TOPO-TA kits (Invitrogen). Inserts from selected clones were bi-directionally sequenced (Functional BioSciences) and sequence contigs generated using Sequencher 4.8 (Gene Codes). Contigs were aligned, matched with nearest neighbors (both isolates and uncultured organisms) and checked for chimeras using Greengenes (11). Alignments were refined and phylogenetic relationships determined using MEGA (58).

RESULTS AND DISCUSSION

Our first major objective was to determine if life exists and could be detected in a remotely-sampled extreme subsurface environment. Prior work by the PI (39, 40, 46) and that of others (49), focused on deep subsurface microbial communities, indicated that this would not be a simple or certain task. In particular, the likelihood of drilling- or other sampling-associated sources of microbial contamination were of concern throughout this project. All of the drill holes or other site access portals sampled were created years prior to our project; thus, the unavailability of contamination reference materials, such as drilling fluid negated the proven approach (45) of subtractively identifying indigenous microorganisms. When possible, however, inferences to this effect from available data were developed and are discussed below.

In our proposal, we asserted that either of two overarching eventualities (i.e., life exists in this habitat or it does not) would be of relevance to ERSP. The detection of microbial life would justify further examinations of its diversity and potential impact on radionuclide mobility; whereas, its absence would infer that some fundamental limit for life had been exceeded. Ultimately, the presence of microorganisms was confirmed using traditional cultivation- and molecular phylogeny-based methods for five of the six sites sampled: GASCON, NASH, U12N.10, ALEMAN (molecular-based confirmation only), and ALMENDRO. The exception was CHANCELLOR where very high temperatures (~95 °C) thirty five years after nuclear detonation suggested hydrologic isolation with a corresponding lack of opportunities for recolonization.

While not as inclusive or sensitive as molecular techniques, cultivation-based methods remain an important tool for evaluating the physiologic potential of microbial communities. The results of our cultivation studies indicated that, whereas, all physiotypes tested for were present, no one site possessed all of them (Table 3). No growth was observed for any medium inoculated with samples from ALEMAN or CHANCELLOR. However, it should be noted that lack of growth in defined culture media does not necessarily prove a lack of life. Rather, it only indicates that viable microorganisms capable of growth under the specific laboratory conditions employed were either not present or unable to take advantage of the media at the time of inoculation.

Table 3. Abundances of representative cultivable NTS physiotypes.

Putative physiotype	GASCON	NASH	U12N.10	ALEMAN	ALMENDRO
Nitrate reducer	10^2	10^4	---	---	---
Sulfate reducer	---	10^2	10^2	---	10^2
Iron reducer	---	---	10^3	---	---
Fermentative	10^2	---	10^1	---	10^1
Methanogen	n/a	n/a	10^2	---	---

--- none detected

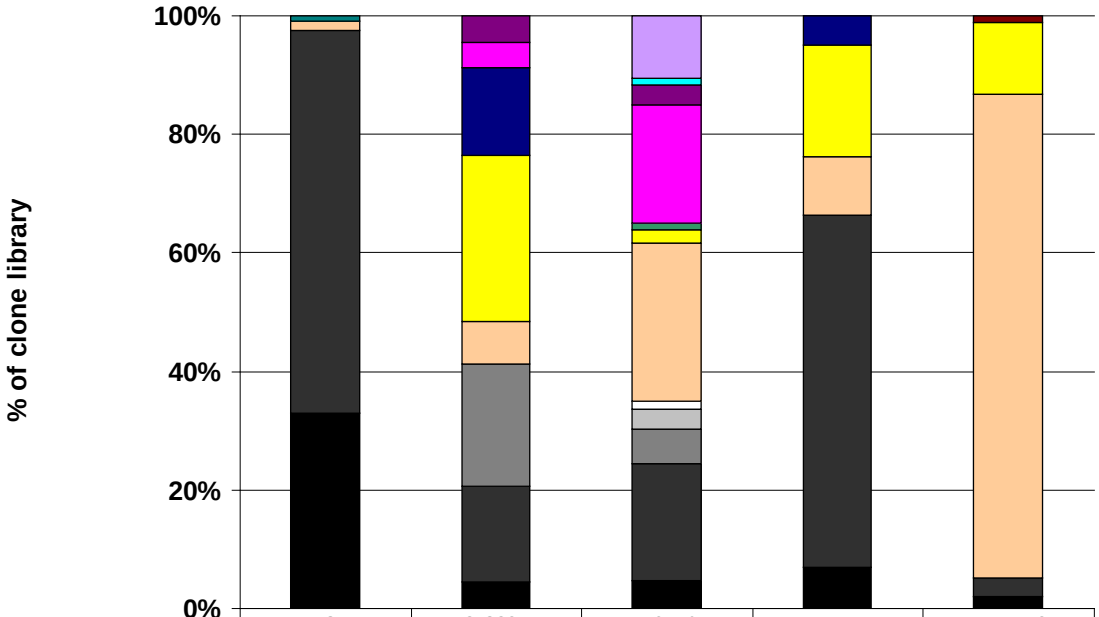
Molecular phylogenetic methods more accurately probe the presence and diversity of microbial life in samples (1). The techniques utilized for this study (PCR amplification of the SSU rRNA gene from environmental DNA extracts and molecular

cloning) were appropriate for a first pass survey of microbial diversity in a previously uncharacterized habitat. Samples from all sites, save CHANCELLOR, in our hands generated SSU rRNA gene amplicons, indicating the presence of bacterial life. Archaea were not detected by PCR amplification with appropriate primer sets from any of the samples. The significance of this observation remains unclear. Phylogenetic analysis of resulting 16S rRNA gene libraries revealed substantially differing microbial communities in fluids recovered from individual sites (Figure 3 and Figure 4). GASCON, the U12N tunnel vent, and ALEMAN possessed diverse communities, with clones distributed among 6, 9, and 5 bacterial phyla, respectively. Conversely, the NASH and ALMENDRO libraries contained overall less diversity at the phylum level. The NASH community was composed almost entirely of Proteobacteria. The ALMENDRO library, while presenting representatives from 5 phyla, was dominated by Firmicutes and Actinobacteria (81% and 12% of the total clones, respectively).

The Firmicutes and Proteobacteria represented the most ubiquitous phyla across the entire sample set. Classes within the Proteobacteria however, suggested a qualitative difference between the sites. The GASCON Proteobacteria-like sequences were comprised of primarily Betaproteobacteria (16% of total clones) and Deltaproteobacteria (20% of total clones) with a few representatives of the Alphaproteobacteria (4% of total clones). The U12N.10 Proteobacteria were the most diverse containing primarily Betaproteobacteria (20% of total clones) with Alpha-, Delta-, and Gammaproteobacteria comprising 5%, 6%, and 3%, respectively. ALEMAN proteobacterial clones were comprised heavily of Betaproteobacteria (60% of total clones) with a few Alphaproteobacteria sequences (7% of total clones). The NASH clones were comprised solely of Alpha- and Betaproteobacteria (34 and 65% of total clones, respectively). Lastly, ALMENDRO contained very few Proteobacteria-like sequences (Alpha- and Betaproteobacteria represented 2% and 3% of the total clones, respectively).

The proportion of other phyla within the clone libraries also suggested differences in bacterial community structure between sites. Whereas, very few or no clones grouped with the Actinobacteria from U12N.10 or NASH (2% and zero, respectively), these clones comprised substantial portions of the libraries for GASCON (28%), ALEMAN (19%), and ALMENDRO (12%). Members of the phylum, Spirochaetes were represented only in the GASCON (15%) and ALEMAN (4%) libraries; whereas, clones grouping within the Nitrospirae (20%), Bacteriodetes (10%), Acidobacteria (1%), Fusobacteria (1%) and Chlorobi (1%) were found only within the U12N.10 library. Lastly, the phylum Chloroflexi was represented only in the GASCON (4%) and U12N.10 (3%) clone libraries.

One of our original hypotheses was that the microbial communities of these unusual habitats would be dominated by Gram-positive bacteria. This was in partial deference to our expectation that, as was noted in radioactive sediments from the Hanford SX-108 tank farm (17), relatives of the well-known radioresistant Gram-positive bacterium, *Deinococcus radiodurans* (5, 20) would be present. It was thus somewhat of a surprise to us that in this dataset, despite the fact that hundreds of rRNA sequences were analyzed, no close relatives of *D. radiodurans* were detected. In fact, if anything, the opposite was true (e.g., that Gram-negative bacteria predominated).



	NASH	GASCON	U12N.10	ALEMAN*	ALMENDRO*
Unkown	1	0	0	0	0
Caldithrix	0	0	0	0	1
Bacteroidetes	0	0	10	0	0
Chlorobi	0	0	1	0	0
Chloroflexi	0	4	3	0	0
Nitrospirae	0	4	20	0	0
Spirochaetes	0	15	0	5	0
Acidobacteria	0	0	1	0	0
Actinobacteria	0	28	2	19	12
Firmicutes	1	7	27	10	81
Fusobacteria	0	0	1	0	0
Gammaproteobacteria	0	0	3	0	0
Deltaproteobacteria	0	21	6	0	0
Betaproteobacteria	64	16	20	60	3
Alphaproteobacteria	33	4	5	7	2

Figure 3. Phylum-level summary of bacterial distribution from 16S rRNA gene library data across the sample set. Note generally high diversity in most samples and very different community structures across the sample set. Classes of the phylum Proteobacteria are shown in shades of black and gray and vary from community dominance in NASH to representing a minor constituent in ALMENDRO. Firmicutes (peach color) were present in all samples, but most abundant phylum in U12N.10 and especially in ALMENDRO. Actinobacteria were common in most samples whereas most other groups varied from site to site.

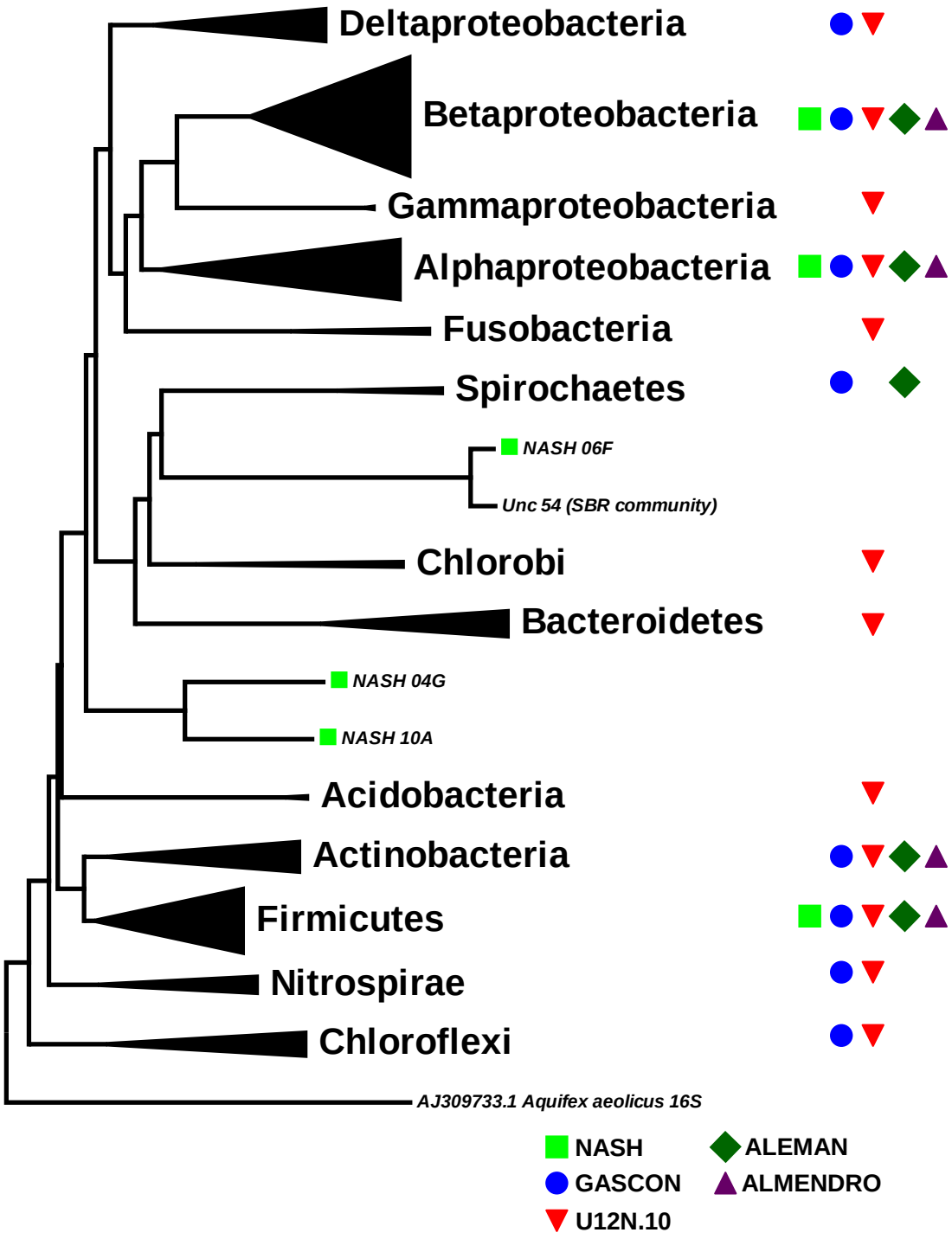


Figure 4. Neighbor-joining tree showing phylum-level abundance across the sample set. Site specific symbols illustrate occurrence of at least one clone from a given phylum present in site clone library. Wedge areas are proportional to the relative abundance of clones from each phylum. *Aquifex aeolicus* is used as an outgroup. With the exception of a small number of clones from NASH, all could be assigned to recognized phyla.

One possible explanation for the abundance of Proteobacteria (Gram-negative) in many of these samples may be sampling- or well-related contamination. It is interesting to note, for example, that several of the closest rRNA gene sequence matches for Alpha- and Betaproteobacteria clones (Figures 5 and 6 (41, 47)) were sequences from mine service water and drilling fluid detected in other studies. The presence of related sequences here, then, might indicate at least a partial overprint of cavity microbial populations by drilling remnants or microorganisms suited to growth on steel well casings. Whereas, the inference of physiology from phylogeny must always be pursued with caution, nearest neighbor analysis of NTS-clones may be instructive. A number of sequences detected, for example, suggest the presence of microorganisms capable of growth on well casings (or, alternatively, potential important redox transformations in the subsurface itself). Three sequences closely related (>97% identity) to the characterized iron-oxidizer, *Siderooxidans ghiorsii* LD-1 (accession # DQ386859; Figure 6) were identified in GASCON (4% of the total clones), while sequences with >98 identity to the dissimilatory Fe(III)-reducing bacteria *Pelobacter venetianus* (accession # U41562; Figure 6) were present in the GASCON and U12N.10 libraries, where they constituted 1% of total clones for each.

Clones with sequence identities >97% to known sulfur cycling microorganisms (Figures 7 and 8) were also identified: 3% of the U12N.10 library (3 clones) was comprised of sequences most similar to sulfur-oxidizing *Thioverga sulfuroxydans* (accession # AB118236); whereas, 10% of GASCON's library (7 clones) was comprised of sequences most similar to *Desulfovibrio* sp. zt31 (accession # AF109470), a sulfate-reducer. Additionally, two unique clones from U12N.10 were most closely related to (~96%) to *Dethiobacter alkaliphilus* AHT1 (accession # EF422412), a halophilic bacterium involved in the reductive sulfur cycle of soda lakes; and *Desulfobacterium aniline* AK1 (accession # EU020016), a sulfate-reducing microorganism capable of phenol degradation and carboxylation. Other sequences identified (Figure 6) included those most related (99%) to the selenate/selenite reducer *Azospira oryzae* N1 (accession # DQ863512) and arsenic-oxidizer *Ralstonia* sp. 22 (accession # EU304284), comprising 10% and 4% of the NASH clone library.

Especially noteworthy were a group of Firmicutes (Figure 8) whose only neighbors are a group of uncultured microbes from ultradeep South African mines, where candidatus *Desulforudis audaxviator* (9, 42) was described (including one sequence with 99% identity to *D. audaxviator*). *D. audaxviator* represents the only known organism to comprise its own ecosystem but, more importantly, appears to employ a sulfate-reducing lifestyle relying upon products from the radiolytic decay of water. The occurrence of clones most similar to these deep subsurface microorganisms (probably the same species in one case, Figure 8), hints at the potential for another microbial community utilizing radiolytically derived products. Its appearance at the NTS suggests a role for this novel microbial lifestyle at DOE sites, although currently there is no physiologic data to support this. Consistent with this hypothesis, however, were dissolved gas values obtained by GC-MS for our group by Brian Spalding of Oak Ridge National Laboratory from the most recent hot well sample obtained by the project, ALMENDRO. This sample contained 4133 and 13.2 ppmv of methane and hydrogen, respectively (Spalding, pers. comm.).

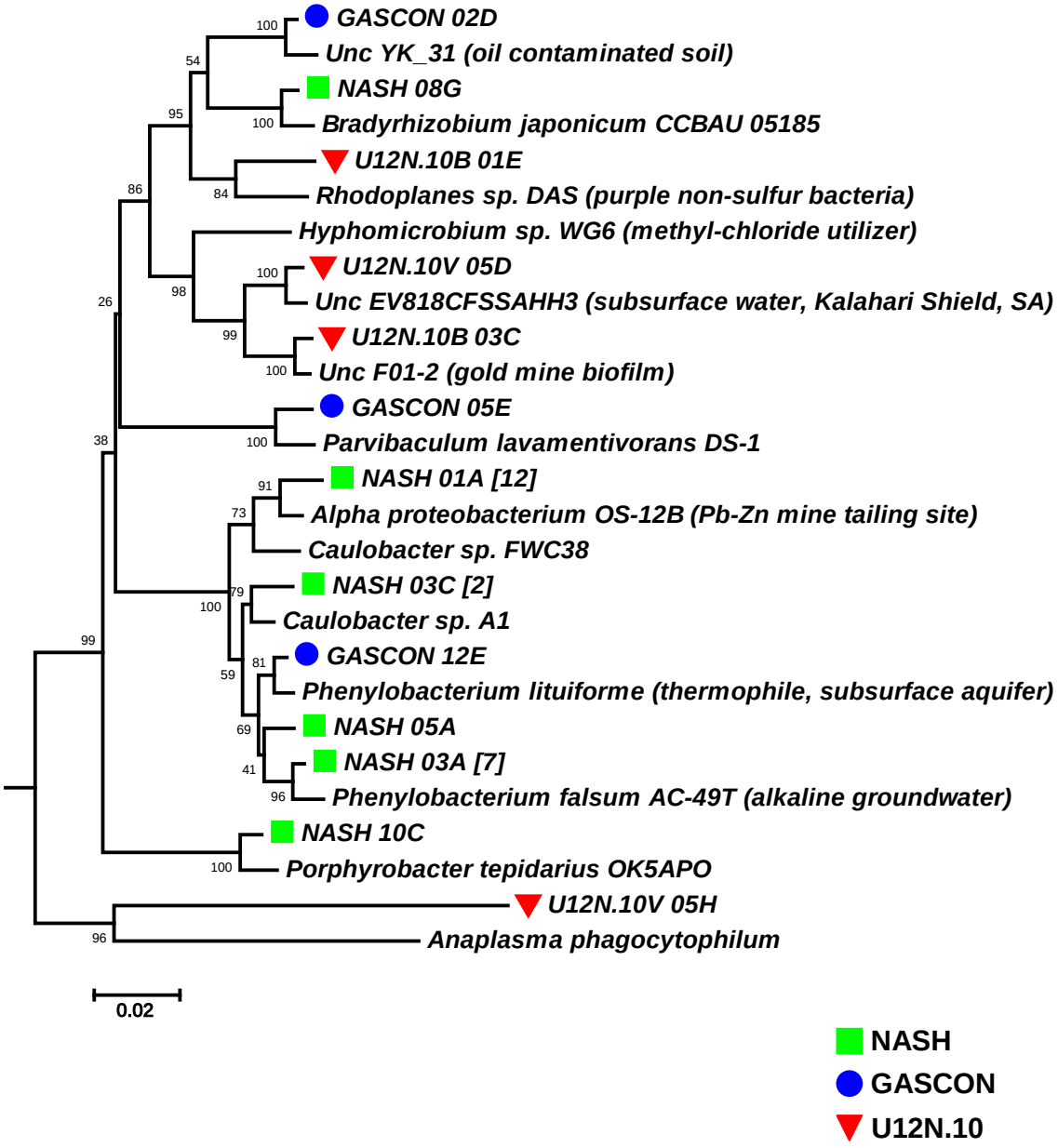


Figure 5. Alphaproteobacteria subtree. Note ubiquitous nature of this phylum in all sampled wells. ALEMAN and ALMENDRO sequence data not included on tree although initial phylogenetic data has indicated the presence of Alphaproteobacteria at both sites.

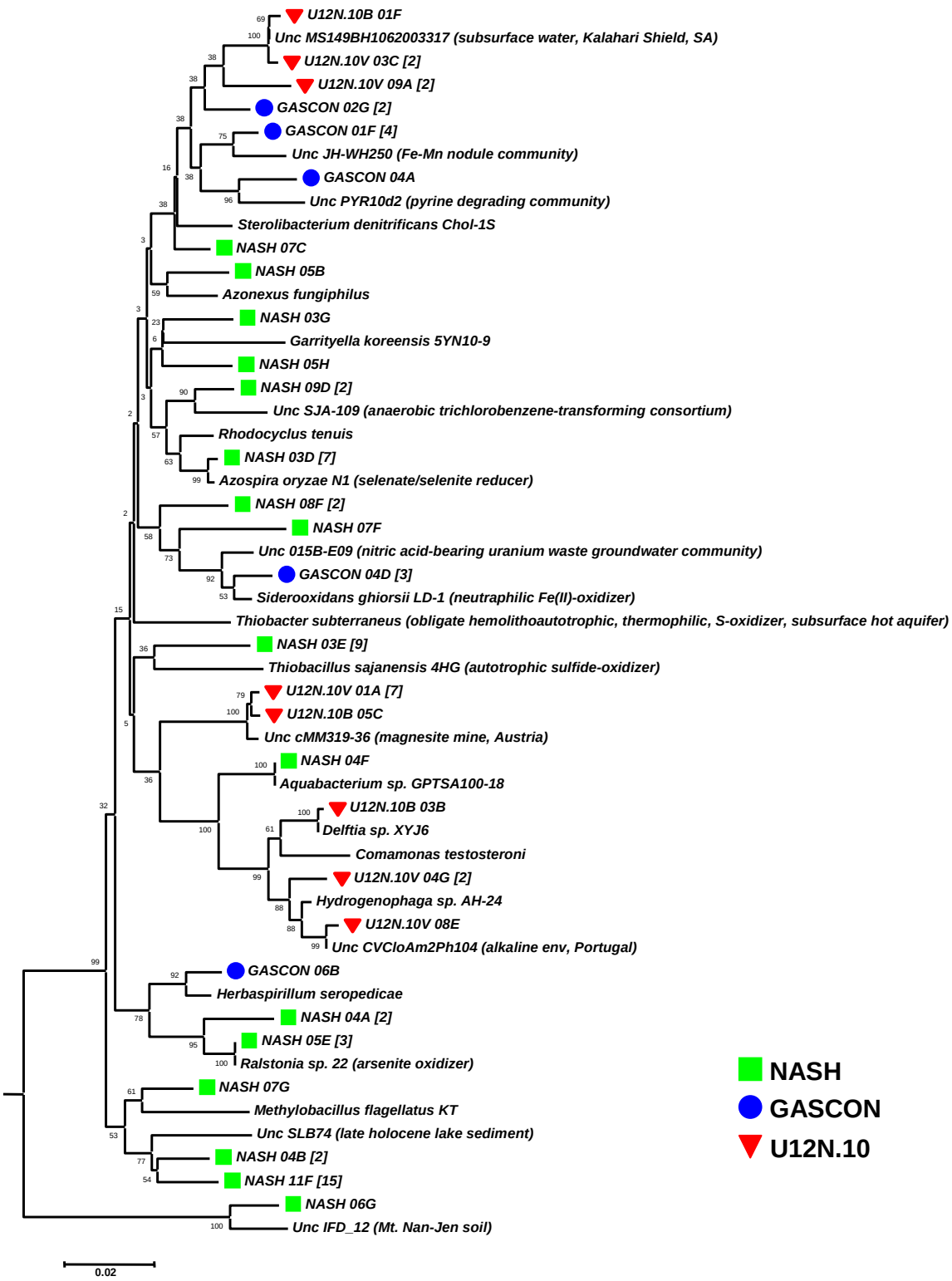


Figure 6. Betaproteobacteria subtree. Note ubiquitous nature of this phylum in all sampled wells. ALEMAN and ALMENDRO sequence data not included on tree although initial phylogenetic data has indicated the presence of Betaproteobacteria at both sites.

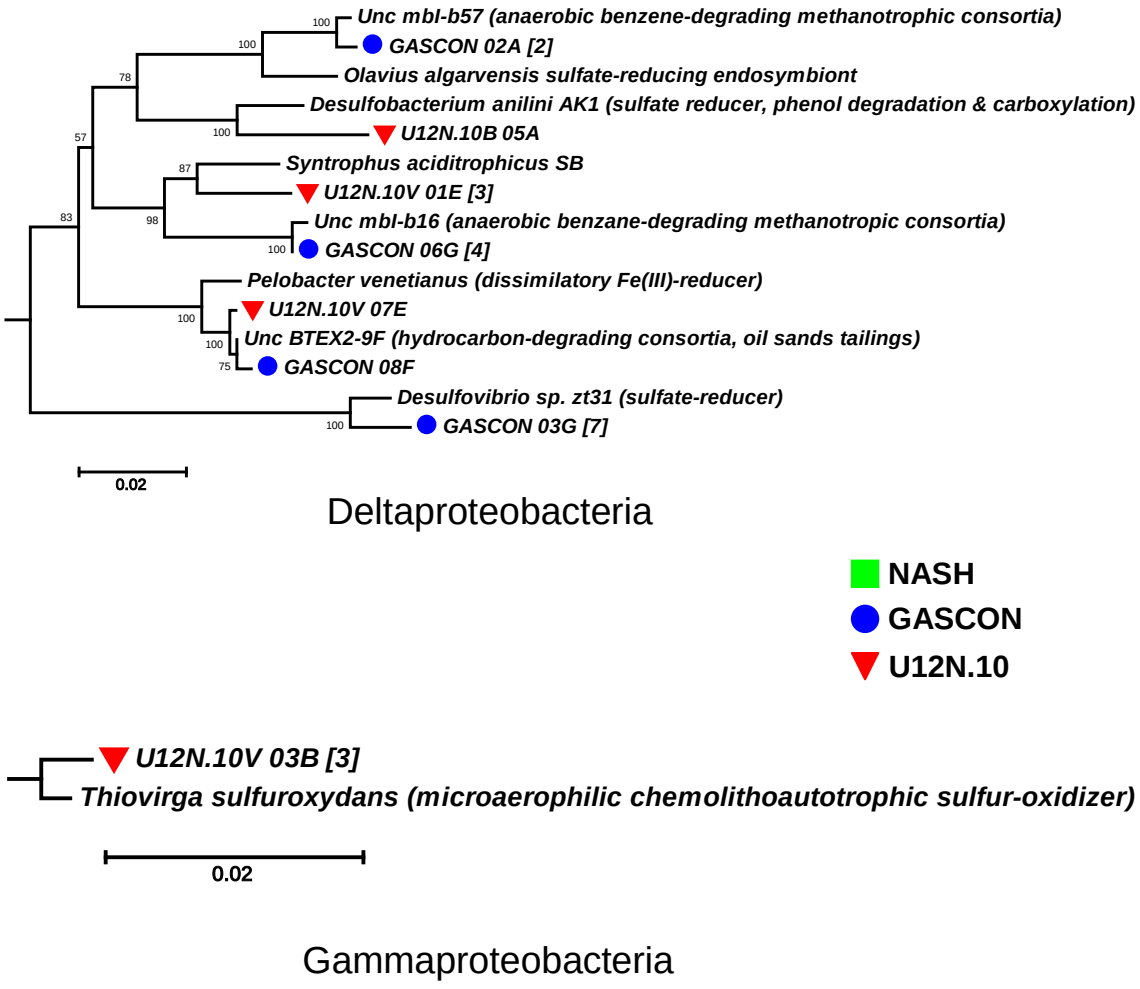


Figure 7. Gamma- and Deltaproteobacteria subtrees. Note lack of NASH clones from the Deltaproteobacteria and occurrence of one clone type from U12N.10 on the Gammaproteobacteria tree. No clones from either phyla have been detected in the ALEMAN or ALMENDRO clone libraries.

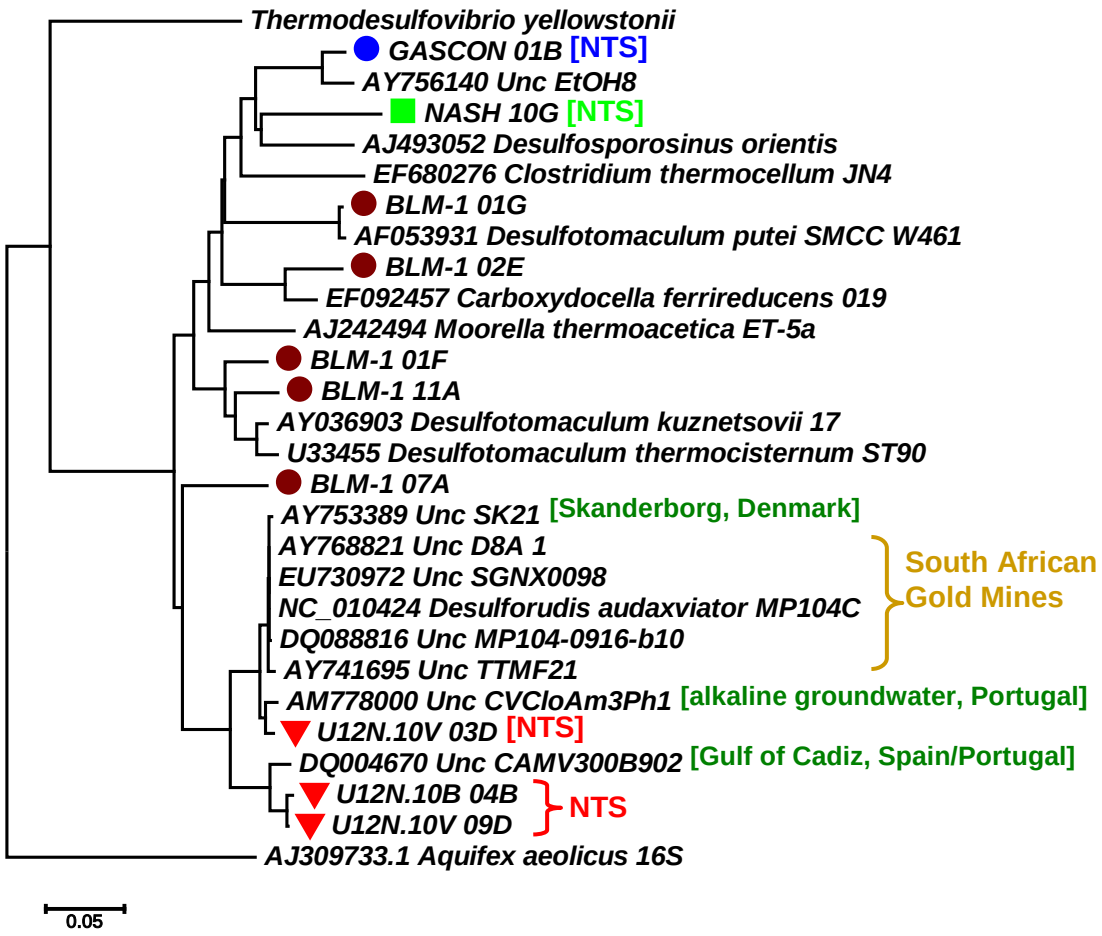


Figure 8. Portion of the Firmicutes subtree indicating known database clones similar to candidatus *Desulforudis audaxviator*. *Thermodesulfovibrio yellowstonii* and sequences obtained from Inyo County (CA) well BLM-1 included for reference. Tree rooted to *Aquifex aeolicus*.

DELIVERABLES AND OUTCOMES

In our original proposal, five hypotheses were delineated. An assessment of our ability to address these hypotheses is detailed below.

Hypotheses

1) **Hot well sampling for microbiology can be effectively incorporated into existing sampling programs with minimal cost and impact.**

   This hypothesis could actually be thought of as a central goal/objective of our proposal. Our successful sampling of fluids from six NTS nuclear test locations indicates that this goal was met and the corresponding hypothesis was confirmed.

2) **Microbial communities, probably dominated by Gram-positive bacteria, will be detectable in the blast cavities.**

   This hypothesis was partially confirmed. Certainly, with the exception of CHANCELLOR, microbial communities were detected. The nature of these communities was, however, more variable and greater diversity was present than we anticipated. At least several of the more prominent groups detected are considered Gram-positive. These include the Firmicutes or 'low G+C Gram-positives" and the Actinobacteria (Figure 4). Conversely, the high proportion of proteobacteria (Gram-negative) and other mostly Gram-negative groups, such as the Spirochaetes and Bacteroides, contradicts the hypothesis. Given that Alpha-, Beta, and Gammaproteobacteria related to examples detected here are all considered evidence for mine-induced contamination in deep subsurface research (38, 47), the possibility that these microorganisms were only introduced through well drilling and sampling must be acknowledged.

   A new project soon to be undertaken by this group (DOE ERSP DE-PS02-09ER09-07), will likely enable us to better address this question as several of the wells we have already sampled (ER-EC-7) or will sample will be drilled during the course of our study. Thus, drilling fluid was or will be available, enabling us to develop an understanding of the importance of introduced microorganisms in the final communities sampled.

3) **"Blast cavity" microbial communities will be distinct from those from wells within geologically-similar, unimpacted strata.**

   This hypothesis was only incompletely addressed during the course of our study, mostly due to the lack of availability of non-contaminated reference samples. In one case, however, somewhat similar microorganisms from within the Firmicutes (Figure 8) were noted between a deep well sample (BLM1) collected from another study in the nearby Amargosa Valley (Figure 8). This result appears to contradict the hypothesis however, because the supporting lithology is very different between these two sites: BLM1 is within a deep, warm, ancient carbonate aquifer and U12N is located in relatively modern meteoric water emanating from volcanic tuff. A new project soon to be undertaken by this group (DOE ERSP DE-PS02-09ER09-07), will likely enable us to better address this question. In particular, two holes recently drilled or being drilled at this time

(ER-EC-7 and EC-20-7) will be particularly informative as these holes penetrate similar strata within (EC-20-7) and down-gradient (ER-EC-7) from what is evidently the same advancing radionuclide-containing plume. Thus, the impacts of radionuclides and other attendant detonation effects on microbial community structure will be apparent. Further, these two samples have been or will be extensively pumped immediately prior to our sampling, presumably negating the effects of well-borne contamination.

4) Cavity microorganisms will affect the mobility of DOE-relevant radionuclide and metal contaminants.

This hypothesis appears to be at least circumstantially confirmed. In favor of accepting the hypothesis is the presence of Fe- and potentially U-reducing microorganisms obtained in the development of our NTS culture collection. In addition, a number of the closest matches to some of our 16S rRNA clones are noted as metal reducers (e.g., Figure 7).

5) Some cavity microorganisms will be radioresistant.

This task was not completed during the course of our study.

Project Objectives

Project objectives were clearly delineated in our original proposal and thus will serve as the basic structure around which our results will be presented here. In addition, several sections detailing other deliverables such as presentations and personnel supported will be included.

Objective 1. Establish the technical, regulatory, and professional infrastructure required to permit sampling of NTS drill backs for microbiology.

Since all other tasks associated with this project were dependent upon objective 1, a significant amount of project resources were devoted to it. Throughout the length of the project, Mr. Bill Wilborn, the DOE UGTA FSPD was apprised of our efforts and all of our activities were coordinated through his office. Mr. Wilborn remains in routine contact and has indicated a continuing interest in our research.

Objective 2. Verify the presence or absence of microorganisms in fluids from below the zone of root influence from water-containing nuclear test cavities.

Addressing this question was the central justification for the entire project and a negative result at this stage would have negated all remaining objectives. This task was achieved by a combination of cultivation-based research as well as culture-free techniques such as PCR amplification of ribosomal RNA genes from environmental DNA extracts.

Objective 3. Develop a culture collection.

Positive enrichment cultures from a variety of physiologies are noted in Table 3. To date, three aerobic heterotrophs closely related (99 and 100% over most of the SSU rRNA molecule, respectively) to *Sphingomonas faeni* and *Brevundimonas mediterranea* are in culture and being utilized for sorption-desorption studies

being conducted on other funding by project postdoc, Jen Fisher. Additional efforts leading towards obtaining pure cultures of iron reducers, manganese reducers, elemental sulfur reducers, and aerobic heterotrophs using archived samples from the U12N tunnel in particular are still underway. This work is in support of a new Laboratory Science Focus Area project (DE-AC52-07NA27344) with LLNL being led by our co-I, Mavrik Zavarin.

Objective 4. Begin to characterize radiation resistance from test cavity microorganisms.

An early objective of our project was to engage in the testing of radiation resistance of NTS isolates in the laboratory of collaborator Michael Daly. As pure cultures of what we judged to be interesting or relevant microorganisms only became available fairly late in the project, this objective was not pursued. No funding was requested for this task, however, and this remains a priority future direction assuming continued interest from Dr. Daly.

Objective 5. Characterize the metal and radionuclide reduction capabilities of test cavity microorganisms.

Microorganisms obtained in the performance of objective 3 were screened for the ability to directly reduce metals of interest (e.g., Fe(III) as well as selected radionuclides (e.g., U(VI). Their characterization continues with our ongoing research, especially in collaboration with LLNL.

NEW PROPOSALS, PROJECTS AND PROFESSIONAL RELATIONSHIPS

As we understand it, an overarching goal of the DOE ERSP Exploratory program was the development of new capacity for DOE. This new capacity could include full proposals which hopefully would lead to fundable research for ERSP (now SBR) or new collaborations. In the case of this project, both are true.

One noteworthy result of our project was the detection of 16S rRNA gene sequences at one site (U12N) with about 99% full-length sequence identity to the previously reported microorganism, candidatas *Desulforudis audaxviator* (Figure 8, (9)). As *D. audaxviator* is reported to utilize radiochemically-produced substrates in the very deep continental subsurface (9, 29, 30, 42), the appearance of a close relative at radioactive, subsurface habitats at the NTS was used in part to justify a full proposal led by the PI (D.P.M.) to the ERSP DE-PS02-09ER09-07–Full call in 2009. This proposal for $1,178,957 entitled *Radiochemically-Supported Microbial Communities: A Potential Mechanism for Biocolloid Production of Importance to Actinide Transport* was co-authored by a combined university and national laboratories team (Moser, D.P., C. Russell, T.C. Onstott, K. Czerwinski, B. Sherwood Lollar, M. Zavarin, G. Anderson, J. Zhou, Z. He) and was ultimately recommended for funding.

Additionally, results from this project and our relationship with Mavrik Zavarin and others from the UGTA program supported our involvement at the co-I level in a full proposal to the ERSP National Laboratories Science Focus Areas call (DE-PS02-09ER09-07), *Geochemical Processes at Femtomolar Concentrations and Nanometer Scales*. This was ultimately approved for funding at near its $6,000,000 request and

commenced in March of 2010. Thus, this Exploratory project substantially contributed to the development of new research in ERSP (now SBR).

The unusual appearance of *D. audaxviator* and the implication that radiation-supported subsurface ecosystems may exist at DOE sites led the PI to seek input from Dr. Tullis Onstott of Princeton University, the original PI on NSF and NASA-funded research that led to this discovery in U-rich mine ores (9, 29, 30), for the above-mentioned ERSP full proposal. Onstott is a very highly regarded geochemist from Princeton University and was featured as one of Time Magazine's 100 most influential people in the world in 2007. His addition to the SBR team must certainly be regarded as a benefit for the program. Further, the new team includes Dr. Barbara Sherwood Lollar of the University of Toronto, a noted authority in subsurface gas production, again, representing new capabilities for the program. Additional new collaborations between the PI with established SBR researchers, which have resulted from this project, include work with Drs. Eoin Brodie, Gary Anderson, and Joe Zhou.

PATENTS, PRESENTATIONS, AND PUBLICATIONS

No patents have resulted from this work. At least one manuscript for submission to a peer-reviewed microbiology journal is now in preparation by the team. This manuscript is focused on the microbial diversity noted across this sample set and will emphasize the apparent discovery of *D. audaxviator* in a new subsurface habitat (previously only noted from South Africa), with a target submission to Applied and Environmental Microbiology or Proceedings of the National Academy of Sciences.

Relevant Presentations

Moser, D.P., J. Bruckner, K. Czerwinski, M.J. Daly, R.E. Lindvall, M. Marshall, C. Russell, and M. Zavarin. 2009. Poster. Characterization of microbial communities in subsurface nuclear blast cavities of the Nevada Test Site. Poster. Annual PI Meeting Environmental Remediation Sciences, Lansdowne, VA, April 7th, 2008.

Bruckner J.C., J.C. Fisher, R. Lindvall, M. Zavarin, K. Czerwinski, C. Russell, and D.P. Moser. 2009. Poster. Microbial communities of underground nuclear blast cavities. N-124. 109th General Meeting of the American Society for Microbiology, Philadelphia, PA. May 17-21, 2009.

Bruckner J.C., J.C. Fisher, M. Zavarin, K. Czerwinski, M.J. Daly, R.E. Lindvall, M. Marshall, C. Russell, and D.P. Moser. 2009. Poster. Characterization of microbial communities in subsurface nuclear blast cavities of the Nevada Test Site. DOE-Environmental Remediation Sciences Program (ERSP) 4th Annual PI meeting. Lansdowne, VA. April 20-23, 2009.

Moser. D.P. Deep Earth life and the Nevada Test Site connection. Talk. Community Environmental Monitoring Program (CEMP) workshop, Ely, NV. July 29th, 2009

Fisher, J.C., S.A. Faye, K.R. Czerwinski, D.P. Moser. 2009. Poster. Microbial effects on the sorption of uranium: kinetics and distribution ratios for tuff samples from the Nevada Test Site. Migration 2009, Richland, WA. August 9th, 2009

Bruckner J.C., J.C. Fisher, M. Zavarin, K. Czerwinski, M.J. Daly, R.E. Lindvall, M. Marshall, C. Russell, and D.P. Moser. 2010. Poster. Characterization of microbial communities in subsurface nuclear blast cavities of the Nevada Test Site. DOE-Environmental Remediation Sciences Program (ERSP) 4th Annual PI meeting. Washington, D.C. March 27th – 29th, 2010.

Moser, D.P. 2010. Earth's Deep Continental Biosphere: A Previously Unrecognized Rock-Hosted Biome?. Origins Lecture. McMaster University, Hamilton, ON. April 5th, 2010.

Moser, D.P. Edge of the abyss: new windows into the deep continental biosphere. 2010. Talk. Arizona-Nevada Branch American Society for Microbiology. Las Vegas, NV. April 22nd, 2010.

Moser, D.P., Bruckner, J.C., Ehrsam, M., Fisher, J.C., Gihring, T., Lin, L.H., Newburn, J., Onstott, T.C. 2010. Poster. Globally-distributed lineages of putative sulfate reducing firmicutes from varied deep subsurface habitats. Submitted. International Society for Microbial Ecology (ISME). Seattle, WA. August, 2010.

CONCLUSIONS AND FUTURE DIRECTIONS

The work described here revealed the presence of microbial communities in five of six nuclear detonation altered subsurface habitats at the Nevada Test Site. The one cavity for which DNA-based evidence for life could not be generated appears to have remained sterile or nearly so as a result of some combination of high temperature and hydrologic isolation. Overall, the diversity of microorganisms was quite high for subsurface habitats and variable between sites. With the exception of a small group (n = 5) of actinobacterial rRNA clones from GASCON and the U12N tunnel most closely related to sequences previously detected in a flushing borehole in a South African mine (Figure 4, not shown), none of the types were present in more than one sample, indicating that communities were distinct between cavities. As of this writing, full phylogenetic analysis from three of the five sites providing positive PCR products are complete. The final two libraries are finished and final data analyses are in progress. The close relationship between previously reported sulfate reducers which utilize radiogenic substrates in the South African deep subsurface and the U12N tunnels is consistent with the hypothesis that microorganisms at DOE sites may be able to indirectly utilize energy substrates from radiochemical reactions (e.g., H_2). Collectively, the results of this study hint at the presence of a novel, important, and fascinating biosphere that may exist in nuclear explosion altered habitats.

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