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Characterization of the Microbiology Within a 21 m^3 Section of Rock from the Deep Subsurface

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Abstract. The distribution of aerobic chemoheterotrophic microorganisms within a 21 m^3 section of deep subsurface rock was determined. Nineteen samples for microbiological analysis were aseptically taken by hand from the walls of a 400 m deep subsurface tunnel after an alpine miner created fresh rock faces 0.76, 1.52, 2.28, and 3.04 m into the tunnel wall. The direct counts were several orders of magnitude greater than viable counts in all samples. One of each morphologically distinct bacterial type from each sample was purified and analyzed for fatty acid methyl esters (FAME) using the Microbial Identification System (MIDI). Numbers of bacterial types, diversity, and equitability of recoverable microbial communities were the same or similar using either morphotype or FAME analyses as the basis for distinguishing between bacterial types. Twenty-nine genera (Euclidean distance of ≤ 25) were found within the rock section, while 28 of the 210 bacterial types isolated were nonculturable under the growth regime required for cluster analysis. Most isolates clustered at the genus level with *Arthrobacter, Gordona,* and *Acinetobacter*. Two genera, containing 16 isolates, were unmatched to known organisms within the MIDI data base and clustered with other isolates at a Euclidean distance greater than 50. While some species (Euclidean distance ≤ 10) were recovered from multiple sites within the rock section, most were found at 1–3 sites and usually without a definitive pattern of distribution.

Introduction

Researchers within the Deep Subsurface Microbiology Subprogram, funded by the US Department of Energy, are interested in fundamental research in subsurface microbial ecology including presence, abundance, distribution, diversity, and genetic/phylogenetic traits of subsurface microorganisms.

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Microbiological and geological analysis of drill hole cores has been initiated or completed in deep subsurface environments, including sediments [6, 14, 15, 24, 26], basalts, and interbeds [11, 12], and paleosols [10]. These reports include stratigraphic distribution of organisms within a borehole or between boreholes that may be quite distant from each other. At the Nevada Test Site (NTS), rock has been sampled from the walls of tunnel systems for microbiological analysis. Sampling points were from 1 to 10 kilometers apart and ranged in depth from 50 to 450 m from the surface in volcanic ashfall tuff [1, 16]. These results reveal heterogeneity in microbiological communities from deep subsurface environments but have not addressed the three-dimensional distribution of microorganisms on a small scale.

The distributions of organisms in various deep subsurface environments have been described by comparing characters of recovered isolates, such as colony and cellular morphology, physiological profiles provided by API-rapid-NFT strips and/or BIOLOG microtiter plates [7, 20], and antibiotic and metal resistances [1, 16]. These profiles have proven valuable in describing organisms in subsurface environments and for comparative purposes, analyzing organisms from different depths and individual boreholes or tunnels. Organism identification has proven a more complex task [1, 5]. API and BIOLOG systems, designed to identify clinically important bacteria, have a limited ability to identify environmental organisms.

The purpose of this project was to define the distribution of subsurface microbiota on a small, three-dimensional scale and to determine how related groups of culturable aerobic heterotrophs were distributed throughout a rock section. The Nevada Test Site provided an ideal opportunity for a study such as this, because samples could be obtained in a three-dimensional sampling pattern from the walls of existing tunnel systems at depths to 400 m. Identifications and relatedness of microorganisms were determined using a Microbial Identification System (MIDI; Microbial ID, Inc., Newark, Delaware). MIDI provides identification of environmental isolates by comparing methyl ester fatty acid profiles of unknown organisms to profiles of known organisms contained in a data base, and also defines relatedness groupings of microorganisms when an acceptable identification may not be possible.

Materials and Methods

Media and Solutions

R2A agar (Difco, Detroit, MI) was used for platings. Artificial pore water (APW), developed to simulate ambient rock water [1, 16], was used for dilution blanks, and APW containing 1% sodium pyrophosphate was used to make 1:10 w/v slurries of asceptically ground rock.

Sampling and Sample Location

Samples were taken from the U12n tunnel system at Rainier Mesa, Nevada Test Site. The section of rock appeared as geologically homogeneous friable tuff, but some samples were more welded than others. The section of rock was located approximately 1.8 km from the tunnel portal and was approximately 400 m beneath the caprock of the mesa. Nineteen samples were taken in a central

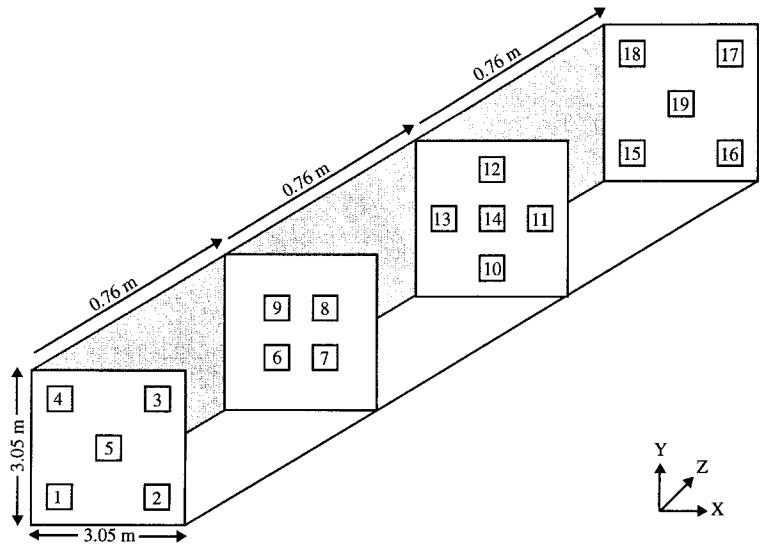

Fig. 1. Composite sampling design of Box et al. [8], showing the 19 sample sites within the 21 m^3 section of subsurface rock.

composite design [9], as shown in Fig. 1. The rock samples (ranging from 0.1 to 1 kg) were taken in a perched water zone located in volcanic ashfall tuff. The volumetric moisture of the samples ranged from 14 to 27%, and the ambient temperature of the rock was 18°C. Detailed geology and geochemistry of the rock samples will be described elsewhere.

An alpine miner was used to extend the existing tunnel to prescribed sample points 0.76, 1.52, 2.28, and 3.04 m into the tunnel wall. An alpine miner is capable of gouging into rock, excavating tunnel sections up to 4.57 m in diameter, without the use of drilling fluid. An initial 0.61 m section of rock was removed before beginning the composite sampling design. The miner was backed out and samples were taken by hand in less than 5 min. Before sample rock was taken, fresh rock faces were created with alcohol-flame sterilized tools, and rock pieces approximately 1–20 g were hand chipped into sterile containers [1, 16]. Samples were transported back to the laboratory on ice, and sample analysis was initiated in less than 8 h.

Sample Analysis

Rock samples were homogenized with sterile mortar and pestle, made into 1:10 w/v slurries with APW containing 0.1% pyrophosphate and shaken for 1 h at 24°C (125 rpm). Two portions of 9 ml from each sample were fixed with 1 ml of filtered (0.22 or 0.45 μm cellulose acetate) and sterile 1% noble agar, and 135 μl of filter-sterilized formaldehyde for 4'-6-diamidino-2-phenylindole (DAPI) direct counting. Portions of fixed slurry samples (50 μl) were stained with 0.3 ml of 0.01 $\mu g\ ml^{-1}$ DAPI for 15 min on 0.22 or 0.1 μm filters (Gelman, Ann Arbor, MI) in darkened filtration columns. Filters containing stained samples were rinsed with filtered (0.45 μm Gelman) distilled water, dryed, and placed onto a drop of immersion oil on ethanol cleaned slides. A drop of oil and a coverslip were added, and slides were viewed under oil immersion at ×1000 with a Nikon Fluor 100 lens (Nikon, Torrance, CA, USA), a 100 W mercury bulb, a Nikon Optiphot epifluorescence microscope fitted with a UV-B filter block. Twenty fields were counted from duplicate smears of each sample, and the average direct count was calculated.

Portions of slurries were concentrated by membrane filtration (Gelman, 0.45 μm), as well as serially diluted in APW for plating on R2A agar. After 2 weeks of incubation at 24°C, viable counts were determined by averaging counts of triplicate plates at the highest dilution that provided distinct colonies

for enumeration by morphotype. One of each morphologically distinct colony type from each sample was selected for isolation and purification [16, 17]. If two morphologically distinct colony types were separated from the initially picked colony, both colony types were purified for MIDI analysis. The proportion of each of the two subsequent morphotypes was considered to be the same proportion as the original colony type, i.e., all colonies enumerated as a distinct morphotype were assumed to have been mixtures of two morphotypes, subsequently separated from the originally chosen representative colony. Viable counts were then determined accordingly. After two successive pure colony streak plates were observed, purity was confirmed by Gram staining.

For MIDI analysis, fatty acids were extracted and esterified from bacterial cultures grown for 24 h on trypticase soy agar according to the specifications of the manufacturer (Microbial ID, Inc.). MIDI provided identifications of organisms when similarity indices of subsurface organisms matched profiles of known organisms in the data base (TSBA aerobe library version 3.6), and provided a dendrogram showing relatedness of all subsurface organisms based on a Euclidean distance (ED) scale. Although no universal acceptance of ED exists, EDs of <2, <6, <10, and <25 represent isolates within the same strain, subspecies or biotype, species, and genus, respectively, based on interpretations of dendrograms describing relatedness of known organisms. When interpreting dendrograms it is important to consider the confidence of identifications; a match ≥ 0.3 was considered acceptable for environmental organisms, and a match of ≥ 0.6 was considered acceptable for clinically important organisms.

Shannon-Weaver diversity and equitability [4] were determined by enumerating the proportion of each distinct colony type on each set of triplicate plates after first grouping isolates that clustered together with an ED of ≤ 10 (species level) on the relatedness dendrogram produced by MIDI.

No isolates were taken from sample N18, and only one plate was available for determining the viable count. Although sample N18 was subsequently replated, the data were not included because recoverable microorganisms from subsurface samples have been shown to undergo changes in viable count and diversity after storage [10].

Results

Isolates were originally selected on the basis of morphotype, but when grouped together by MIDI relatedness, clusters at an Euclidean distance of ≤ 10 (species level), diversity, and the number of bacterial types in each sample either decreased, indicating fewer microbial types, or stayed the same (Fig. 2). Equitability remained relatively constant in all but sample 1, where an increase from 0.66 to 0.87 was observed. Organisms that were nontransferrable after initial isolation or were nonculturable under the MIDI growth regime were considered as distinct microbial types in diversity and equitability calculations.

Differences were observed between bacterial communities from the individual samples within the rock section (Table 1). Viable counts ranged over 3 orders of magnitude, from 2.68×10^1 to 4.14×10^4, while total counts ranged 2 orders of magnitude, from 6.92×10^5 to 4.79×10^7. The number of distinct colony types (based on FAME analysis) isolated from each sample ranged from 5 to 15. Shannon diversity and equitability (also based on FAME analysis) ranged from 1.28 to 2.40, and 0.59 to 0.95, respectively. No spatial trend within the rock section or correlation between parameters of diversity, equitability, number of colony types, total counts or viable counts was observed.

Fig. 2. Bacterial types were selected on the basis of morphotype (solid bars), or by FAME (MIDI) analysis at an Euclidean distance of ≤ 10 (2A). Shannon-Weaver diversity (2B) and equitability (2C) indices were compared using the two selection criteria.

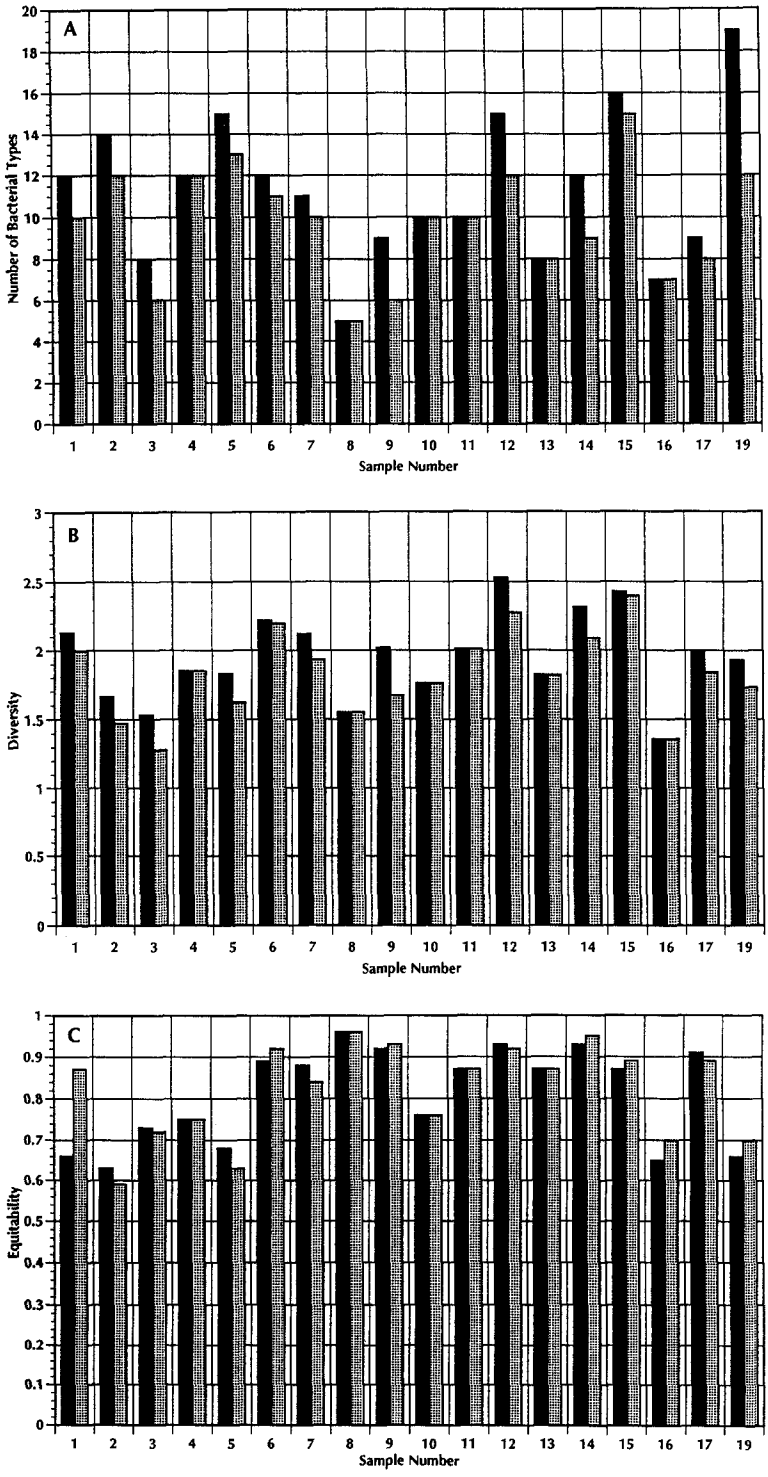
A
Number of Bacterial Types
Sample Number
B
Diversity
Sample Number
C
Equitability
Sample Number

Table 1. Bacterial communities, including viable count, total count, bacterial types, Shannon-Weaver diversity, and equitability based on FAME (MIDI) analysis

Sample number	Log viable count[a]	Log total count[b]	Bacterial types	Shannon diversity	Equitability
1	3.00	7.10	10	2.00	0.87
2	4.62	7.06	12	1.47	0.59
3	2.41	7.03	6	1.28	0.72
4	2.43	6.77	12	1.85	0.75
5	3.30	6.55	13	1.62	0.63
6	2.37	7.29	11	2.20	0.92
7	2.70	7.17	10	1.93	0.84
8	1.43	6.60	5	1.55	0.93
9	1.67	7.25	6	1.67	0.76
10	2.90	7.01	10	1.76	0.87
11	2.39	7.44	10	2.01	0.92
12	2.01	6.75	12	2.28	0.92
13	2.56	7.58	8	1.82	0.87
14	3.00	7.68	9	2.09	0.95
15	2.50	7.41	15	2.40	0.89
16	2.93	6.52	7	1.36	0.70
17	1.99	5.84	8	1.84	0.89
18	4.24	7.24	ND[c]	ND	ND
19	4.24	7.37	12	1.73	0.70

[a] Viable count is expressed as log CFU/g dry wt
[b] Total count is expressed as log cells/g dry wt
[c] ND, not determined

Of the isolates successfully identified or clustered by FAME (fatty acid methyl ester) analysis, 76.5% were Gram positive and 23.5% were Gram negative, (as determined from data in Table 2, discussed below). Only samples 1, 2, and 19 had relatively abundant numbers of Gram negative isolates. All isolates that were nonculturable under the standardized MIDI growth regime, or had no match within their cluster to the data base, were not considered in the Gram reaction analysis (61 of 210 isolates).

Every unique bacterial colony type was originally selected from 18 samples within the rock section, and only 2 colony types were nontransferrable with subsequent platings on R2A agar. MIDI analysis of each isolate was attempted, including each colony type that was subsequently separated into two morphologically distinct isolates during purification. Twenty-eight isolates could not be analyzed because they were nonculturable under the standard conditions required to match organisms within the MIDI data base (plating on trypticase soy agar and incubation for 24 h at 28°C). MIDI analysis provided a large comprehensive dendrogram, clustering organisms by an unweighted pair matching method. The dendrogram was interpreted at 3 Euclidean distances, representing broad relationships between isolates (ED 45), genus level clustering of isolates (ED $\leq$25), and clustering at the species level (ED $\leq$10).

At very broad Euclidean distances ($>$ 45), the subsurface organisms clustered

Table 2. Dendrogram Groups at an Euclidean distance ≤25

Group number	Genus[a]	Number of isolates	Sample site(s)	Similarity value
1	*Methylobacterium*	2	2	(0.402)
2	*Micrococcus*	1	4	(0.289)
3	*Staphylococcus*	1	11	(0.150)
4	*Micrococcus*	3	7, 14, 17	(0.528)
5	*Arthrobacter*	21	2–10, 12, 15–17	(0.817)
6	*Micrococcus*	3	5,15	(0.189)
7	Mixed Gram positive	6	2–4, 7, 10, 19	
	Corynebacterium			(0.571)
	Aureobacterium			(0.553)
	Clavibacter			(0.398)
	Curtobacterium			(0.345)
8	Mixed Gram positive	29	1, 2, 4–15, 17	
	Arthrobacter			(0.662)
	Micrococcus			(0.636)
	Staphylococcus			(0.631)
	Aureobacterium			(0.464)
9	*Micrococcus*	1	16	(0.437)
10	*Arthrobacter*	3	1, 14	(0.701)
11	No match[b]	1	13	
12	*Bacillus*	2	1, 5	(0.517)
13	No match	1	5	
14	*Xanthomonas*	2	4, 5, 19	(0.836)
15	No match	3	3, 10, 14	
16	*Nocardoiodes*	4	4, 10, 17	(0.539)
17	No match	11	2, 6–9, 11–13, 15	
18	No match	1	2	
19	No match	1	4	
20	*Acinetobacter*	14	1–3, 10, 14, 15, 19	(0.867)
21	No match	2	5, 15	
22	*Corynebacterium*	4	2, 4, 6, 10	(0.692)
23	*Gordona*	36	4, 6–8, 10–19	(0.495)
24	No match	1	7	
25	No match	4	9, 19	
26	No match	10	9, 17, 19	
27	Mixed Gram negative	16	1, 2, 5–7, 12, 15, 19	
	Acidovorax			(0.926)
	Hydrogenophaga			(0.774)
	Pseudomonas			(0.342)
28	*Acidovorax*	1	19	(0.207)
29	No match	4	3–5, 14	
30	No growth[c]	22	1, 3–6, 8, 11–19	
	Total	210		

[a] Sometimes more than one genus was indicated

[b] No match, indicates that the organism does not match any profile in the database

[c] No growth, indicates that the organism would not grow under MIDI required conditions

into 9 separate groups (Fig. 3). Importantly, 1 cluster (cluster 8) contained 15 organisms that were not related to other subsurface isolates until an ED of approximately 50, and none of the 15 isolates was successfully matched to a known

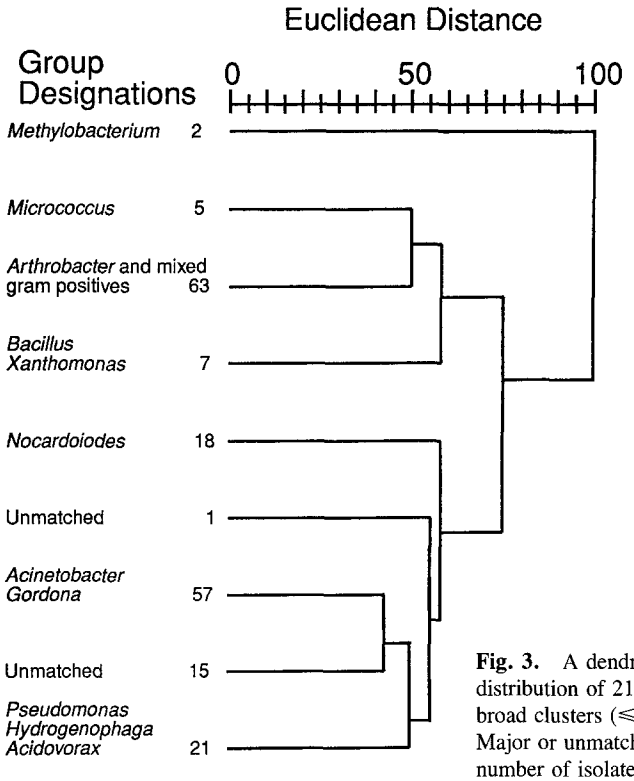

Fig. 3. A dendrogram showing the distribution of 210 subsurface isolates into broad clusters ($\leq$ 45 Euclidean distance). Major or unmatched groups are listed with the number of isolates contained in each.

organism within the MIDI data base. Another cluster (cluster 6) was also unique in this respect but contained only one organism.

The isolates fell into 29 broad groups at an ED$\cong$25 (genus level) (Table 2). Forty-eight (23%) of the isolates were matched to known organisms in the MIDI data base with similarity indices over 0.3, and an additional 123 (59%) of the isolates were clustered with one or more organisms identified to a similarity index ≥ 0.1, providing insight into the classification of these isolates. FAME profiles of 39 isolates (19%) did not match the data base and did not cluster with any isolate that was identified at a similarity index above 0.1. Three samples had relatively high proportions of their communities that could not be analyzed by MIDI. For example, samples 13, 16, and 19 contained approximately 32, 22, and 26%, respectively, of their recoverable community that did not grow under the MIDI regime.

An ED $\leq$ 25 should group all organisms of the same genus together into one cluster. However, there are four *Micrococcus,* and two *Arthrobacter* clusters listed in Table 2. These results may be indicative of the diversity of FAME found within a large genus such as *Arthrobacter*. It is also interesting to note that only 2 of the *Micrococcus* groups contain isolates identified at a similarity index above 0.3, a level we considered acceptable for environmental organisms. Groups 7, 8 and 27 contained isolates that were identified as belonging to several different genera. For

example, group 7 contains 6 isolates, 4 of which are identified above the 0.3 similarity index (*Corynebacterium, Clavibacter, Aureobacterium,* and *Curtobacterium*). Foremost, the organisms within a cluster were more closely related by FAME to one another than to other groups containing the same genus (i.e., *Corynebacterium,* group 22).

Thirty-three species were recovered from multiple samples within the rock section (Table 3). Table 3 divides genera (listed in Table 2) into species at an ED ≤ 10. Also listed are species names, the highest similarity index value within the species cluster, and the sites from which the species were recovered. In five instances, an *Arthrobacter* sp. was found in more than one sample. Likewise, two *Staphylococcus* sp., three *Acinetobacter* sp., four *Gordona* sp., two *Hydrogenophaga* sp., and 1 each of 7 other genera were found at 2 or more sites. Groups 7 and 8g contain 2 different genera, identified at high confidence levels, that cluster to the same species. Organisms with similar FAME compositions may result in matches to the library with similarities > 0.5. Not surprisingly, in Table 2 the genera with the largest number of isolates contained the greatest number of species found at multiple sites (Table 3), i.e., groups 5, 8, and 23, containing 21, 29, and 36 isolates, respectively. Many of the species were found at only 2 sites (groups 5c, 7, 8b, c, e, f, 15, 16, 17, 22, 23e, 25, 26, 27c, and 29), while one species was recovered from 8 samples (group 5a).

The abundance of a particular species in a sample was estimated by multiplying the viable count of the sample by the proportion of the recovered community that the isolate originally represented [17] (data not shown). When examining the abundance of a species found at more than one site, four patterns were observed: (1) species that were recovered from only two or three samples, (2) species with no apparent pattern of distribution throughout the rock section, (3) a vertical distribution of a species in either the XY or YZ plane, or (4) a horizontal distribution (XZ plane). Pattern 1 was the most common; many isolates were recovered from just 2 or 3 samples (22/32 or 69%). Pattern 2 was demonstrated by 5 out of 9 cases where a species was found at more than 3 sites, and is exemplified by *Arthrobacter protophormiae/ramosus* (group 5a). This species was recovered from many samples (8) within the cube, but showed no trend of distribution (Fig. 4A). Pattern 3 was displayed by *Rhodococcus maris* (group 23a), which was recovered from 6 samples, all within the two inner faces of the cube, and by *Gordona bronchialis* (23f), recovered from 3 sites in the last face of the cube (Fig. 4B). An *Acinetobacter* species (group 20c) and an *Arthrobacter* sp. (group 5b) were recovered primarily from sites at the bottom of the cube (Fig. 4C), demonstrating a layered distribution.

Discussion

Viable bacteria were recovered from 19 samples within a 21 m^3 block of subsurface rock at 400 m depth. Sampling by hand from the tunnel walls provided uncompromised subsurface material for microbiological analysis [1, 16]. Kieft et al. [19], observed a drying effect with samples taken into the wall of tunnel U12b at the Nevada Test Site. However, the U12b tunnel system, in place since the 1950s, is located in the vadose zone at a depth of just 50 m from the surface and approxi-

Table 3. Bacterial species recovered from more than one sample within the rock section

Genus group[a]	Organism identification[b] (similarity value)	Samples with indicated species[c]
5a	*Arthrobacter* (0.609) *protophormiae/ramosus*	2, 5, 6(2), 8, 9, 12, 16(2), 17(2)
5b	*Arthrobacter* (0.817) *protophormiae/ramosus*	3(3), 7, 10, 15, 16
5c	*Arthrobacter* (0.602) *crystallopoites*	4, 3
7	*Aureobacterium* (0.553) *liquefaciens* *Clavibacterium* (0.398) *michiganense*	4, 12
8a	*Arthrobacter* (0.190) *oxydans*	2(2), 8, 10
8b	*Staphylococcus* (0.391) *aureus*	1, 6
8c	*Arthrobacter* (0.105) *oxydans*	10, 15
8d	*Staphylococcus* (0.631) *kloosii*	5(3), 9, 11, 15
8e	*Bacillus* (0.057) *psychrophilus*	7, 11
8f	*Micrococcus* (0.636) *kristinae*	7, 17
8g	*Aureobacterium* (0.464) *liquefaciens* *Micrococcus* (0.393) *luteus*	6, 12, 13, 15, 17
15	No match	10, 14
16	*Nocardioides* (0.539) *luteus*	4, 10
17a	No match	7, 9
17b	No match	2, 6, 8, 12(2)
20a	*Acinetobacter* (0.867) *johnsonii*	1, 2, 14, 19
20b	*Acinetobacter* (0.420) *calcoaceticus*	14, 15(2)
20c	*Acinetobacter* (0.230) *johnsonii*	1, 2(2), 10, 15, 19
22	*Corynebacterium* (0.692) *renale*	4, 6
23a	*Rhodococcus* (0.261) *maris*	6, 7, 8, 10, 12, 14(4)
23b	*Gordona* (0.079) *bronchialis*	6, 10, 11
23c	*Gordona* (0.292) *bronchialis*	4, 7, 8, 11, 12(2)
23d	No match	6, 13, 16
23e	*Gordona* (0.246) *bronchialis*	7, 15
23f	*Gordona* (0.495) *bronchialis*	15, 17, 19

Table 3. *(Continued)*

Genus group[a]	Organism identification[b] (similarity value)	Samples with indicated species[c]
25	No match	9(2), 19(2)
26	No match	9(3), 19(6)
27a	*Pseudomonas*[d] (0.167) *stutzeri*	1(2), 10, 12(3)
27b	*Hydrogenophaga* (0.774) *pseudoflava*	2, 7(2), 5, 19
27c	*Hydrogenophaga* (0.228) *pseudoflava*	2, 6
27d	*Acidovorax* (0.926) *facilis*	15, 19(2)
29	No match	3, 5

[a] Genus group number from Table 2

[b] The genus species identified with the highest confidence within a cluster

[c] Values in parenthesis indicate the number of isolates from a particular sample that were identified as the same species

[d] A *Staphylococcus* (0.391) was identified within this cluster, but was not included in the table

mately 350 m above the perched water zone where the rock section of this study was mined. No evidence of drying was observed in the nearly saturated rock section (unpublished data). Further, to minimize any possible external influence, a section of rock (0.61 m thick) was removed before the first rock face in the composite design was sampled.

The distribution (or lack thereof) of microorganisms within the rock section provides further evidence for the pristine nature of the rock samples taken within the U12n tunnel system. Total and viable counts varied by 2 and 3 orders of magnitude, respectively, and did not show a trend of distribution within the rock section. Diversity, equitability, and numbers of distinct isolates from each sample did not display a spatial pattern of distribution within the rock section, nor was any correlation found between these microbial parameters. Specific genera or species did not show a trend in location within the rock section. Most species, recovered from multiple sites, were found in only a few samples, and many species were unique to a single sample site. Although geologists use the term "representative elemental volume" to define the largest volume of substrate that results in uniform characterization, it is doubtful that microbiologists will be able to define this same principle for microbial communities. Without the ability to sample microniches and at the same time have enough material to comprehensively characterize microbiological, physical, and chemical parameters, we may never be able to determine spatial heterogeneity on a small enough scale.

Numbers of distinct isolates, and thus diversity and equitability, did not change appreciably when isolates were grouped by FAME profiles (to the species level) rather than by morphotype. Selecting a representative colony by morphotype has been shown to be a reliable basis for the examination of subsurface microbial community composition; i.e., all colony types that appear the same morphologically are the same, to the species, subspecies, or strain level [17]. The data in Fig. 2

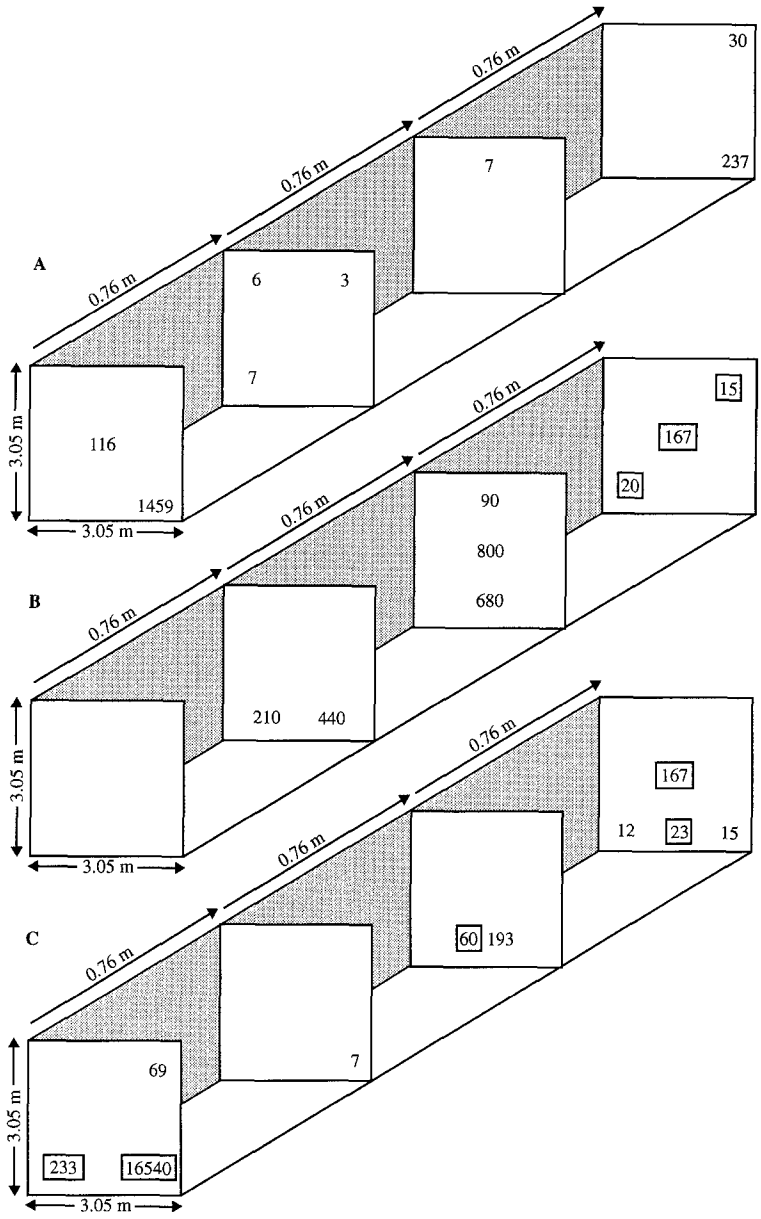

Fig. 4. Patterns of species distribution found at more than one sample site through the rock section. *Arthrobacter protophormiae/ramosus* demonstrates wide distribution throughout the rock section (4A); *Rhodococcus maris,* and *Gordona bronchialis* (abundances outlined in boxes) demonstrate species found only in the center or back faces, respectively (4B); *Arthrobacter protophormiae/ramosus,* and *Acinetobacter johnsonii* (abundances outlined in boxes) demonstrate species recovered from samples in the bottom layers of the rock section (4C).

further support selection of colonies by morphotype because most of the colonies selected as morphologically distinct colony types were unique by FAME analysis.

Nonculturable and dead organisms could not be included in diversity measurements because they were not recovered on R2A plates. Underestimation of diversity is a concern of microbial ecologists [3], but it has been suggested that if samples are treated in a similar manner, then comparisons between samples are valid [27]. In addition, recoverable microorganisms may represent the most important portion of a microbial community. Viable cells often make up the large size classes of bacteria within samples, and thus represent a major portion of the microbial biomass [23]. Additionally, the larger cells in an environment often have higher metabolic activities, and thus may be most important when considering energy flow through an environment [23].

Some isolates (28 out of 210) could not be analyzed by MIDI because they demonstrated either limited growth or no growth on trypticase soy agar. Trypticase soy agar is a richer medium than the R2A agar used for the original isolation of subsurface isolates, and it is doubtful that it was missing a cofactor required for growth. Inhibition of growth could be indicative of the oligotrophic nature of these microorganisms. It has been shown that high concentrations of nutrients can be inhibitory to oligotrophic microorganisms [21, 24, 25]. The environment from which the subsurface microorganisms were isolated is believed to be oligotrophic due to limited water and nutrient flux [1].

Most isolates that were successfully matched or tightly clustered with known organisms in the MIDI data base were identified as Gram positive. Only samples 1, 2, and 19 contained relatively abundant numbers of Gram negative isolates. Interestingly, these samples yielded some of the highest viable counts, although no direct correlation was observed. Gram positive organisms were predominantly recovered from other subsurface sites (vadose zone volcanic tuffs in New Mexico, vadose zone basalt/sediment interface and intersedimentary samples, Idaho National Engineering Laboratory [11], and a shallow aquifer in Oklahoma [7]. However, at other deep subsurface sites large numbers of Gram negative isolates have been recovered (saturated sediments in Savannah River [5], unsaturated palesols at Hanford, Washington [10] and an aquifer in Germany [20]. Other studies at the Nevada Test Site in tunnel systems U12b, U12g, U12p [16] and other drifts within tunnel system U12n [1] have also yielded predominantly Gram negative bacteria. There does not appear to be a definitive correlation between moisture and the types of organisms recovered (Gram negative/Gram positive ratios).

A higher proportion of Gram negative isolates was indicated by Gram-staining than by MIDI cluster analysis (data not shown). *Arthrobacter,* the most commonly identified genus in this study, is known to decolorize readily [18] and has been mistakenly identified from deep subsurface sites because they stained Gram negative or variable. In these instances, some isolates were subsequently keyed to *Pasteurella* by API-rapid NFT strips but were found by 16S ribosomal DNA sequencing to be *Arthrobacter* sp. [1, 6]. The clustering information provided by MIDI analysis is believed to be less subjective and thus more reliable.

The microorganisms appear to belong to a very few broad groups. A majority of the isolates clustered with *Arthrobacter,* but several *Micrococcus, Bacillus, Corynebacterum, Gordona, Acinetobacter, Acidovorax, Hydrogenophaga,* and *Pseudomonas* clusters were identified with high similarity values. Other subsurface

studies have used API-rapid NFT strips and have identified bacteria belonging to the genera *Pseudomonas, Agrobacterium,* and *Acinetobacter* [6, 16]. API-rapid NFT strips, BIOLOG, and MIDI analysis were compared in a study by Amy et al. [1], which identified predominately *Pseudomonas,* but also identified some Gram positive organisms. The MIDI dendrogram proved especially useful in this study to determine and compare bacterial diversity among subsurface samples.

Two distinct clusters of organisms were unmatched to others within the MIDI data base at ED > 50. These isolates may comprise unnamed bacterial genera because they may have been isolated from the surface for a long time. No appreciable water movement is believed to occur within the zeolitic tuff in the perched-water zone from which the samples were taken, and matrix flow of bacteria with water through the vadose (unsaturated) zone would take approximately 250,000 years to transport bacteria to this depth [1]. Although nearly saturated, the low permeability and poor interconnection of fractures in the tuff would allow negligible bacterial or nutrient transport via water either horizontally or vertically in the saturated rock strata [1]. In fact, geologists at the NTS believe that pore water in the rock matrix may be several million years old [2]. Two species in this study were found to display a layered distribution, possibly entrained when rock stratifications were being developed by water movement and before the formation of zeolites that subsequently restricted water flow. Perhaps the isolates recovered from samples within the rock section are not interacting bacterial populations, evidenced by the fact that most species were found at only one or a few sites. Many of these organisms were not readily identified by the MIDI data base, however, genetic analysis may enhance the elucidation of organism identities and relationships.

Arthrobacter sp., the predominant organism recovered in this study, has been shown to inhabit oligotrophic environments and to survive well under conditions of low or no nutrients [8, 13, 25]. The microorganisms we recovered may represent a small number of good survivors, isolated from microniches, that have been dormant in the subsurface for long periods of time.

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