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Comparison of Identification Systems for Classification of Bacteria Isolated from Water and Endolithic Habitats within the Deep Subsurface

P. S. AMY,[1]* D. L. HALDEMAN,[1] D. RINGELBERG,[2] D. H. HALL,[1] AND C. RUSSELL[3]

Department of Biological Sciences, University of Nevada, Las Vegas, Las Vegas, Nevada 89154[1]; Desert Research Institute, Las Vegas, Nevada 89120[3]; and Institute for Applied Microbiology, University of Tennessee, Knoxville, Tennessee 37932[2]

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One water and three rock samples were taken from a mined tunnel system, U12n, in Rainier Mesa at the Nevada Test Site. Endolithic microorganisms were cultured from ashfall tuff, which was crushed and made into slurries with a formulation of artificial pore water, on R2A agar plates. Microbial counts ranged from 10^2 to 10^4 viable cells per g (dry weight) of rock sampled. The cultured water sample yielded 10^2 viable cells per ml. Many of the isolates were very small (<1 μm) when viewed in the rock matrix and remained small even when cultured. Most were gram-negative rods. Individual isolates were profiled by API-NFT strip number, antibiotic and metal resistance patterns, and colony and cellular morphologies. Three identification systems, API-NFT strips, BIOLOG, and MIDI, were compared. Each system identified only a small percentage of the total isolates, and in only seven cases were the isolates identified the same way by more than one system. The same genus was identified in three of these cases, but different species were indicated. The genus *Pseudomonas* was the most commonly identified. The isolate profiles and the three identification systems demonstrated that water isolates were considerably different from endolithic isolates.

The Deep Subsurface Microbiology program, funded by the Department of Energy, has allowed many scientists access to environments deep below the surface of the earth. At the Savannah River Plant in Georgia, the Hanford Reservation in Washington, and the Idaho National Engineering Laboratory in Idaho, deep subsurface samples have been obtained by drilling rock cores (30). The most extensive research has come from samples taken about six years ago at the Savannah River Plant. In the last 2 years, researchers have begun to quantify and characterize the microbial communities of subsurface environments from various geographical locations and geological types. At the Nevada Test Site, Rainier Mesa contains several active and inactive mines (called tunnels) used for underground nuclear testing. These tunnels total over 25 km of mined passageways at depths between 350 and 450 m below the surface. Access to rock that has been freshly mined with hand tools or with the aid of an alpine miner has given the Deep Subsurface Program another subsurface environment to investigate. The samples taken at the Nevada Test Site were obtained not by coring or drilling but rather by hand chipping into existent tunnel walls after several centimeters of rock had been removed.

Of interest is the mechanism by which the indigenous microorganisms have colonized the rock matrix of subsurface environments. One mechanism may have been the colonization of rock between depositional events. Another is through movement with water from the surface or in lateral fluxes. In either case, these microorganisms may represent living cells that have survived for long periods of time (years to thousands of years) or perhaps since the diagenesis of the rock.

Also of interest to microbiologists is the identification of microbes that are capable of surviving for extended periods of time with little or no water and nutrients. There are now several systems for the identification of bacteria by specific metabolic types (API strips and BIOLOG), fatty acid methyl ester analysis (MIDI), and genetic comparisons (19). For identification of unknown microorganisms, each system has a bias toward organisms that are best characterized by the particular system, often based on data banks of pathogenic species that are of the most immediate importance to mankind. These systems often fail to identify isolates from the deep subsurface and environmental isolates (7, 9).

Of particular interest to the Department of Energy is the existence of individual microorganisms or consortia of microorganisms that can degrade contaminating substances from organic, metal, and radionuclide spills and leakage at Department of Energy sites (31, 32). If the indigenous microorganisms are extremely old, they may possess degradative capabilities that are lost to surface microbes.

The research reported here was initiated to determine whether viable microorganisms could be cultured from the tuff formations of Rainier Mesa and, if so, what the identity of such microorganisms might be. Of additional interest was the question of spatial variability within one tunnel system in Rainier Mesa and comparison of endolithic bacteria with those of rapid recharge water by using various identification techniques.

MATERIALS AND METHODS

Media and solutions. The formulation for artificial pore water was derived from hydrogeological data of pore water and free-flowing water in the tunnel system (10). It contained the following (in milligrams per liter of deionized water): $MgSO_4 \cdot 7H_2O$, 20; $Al_2(SO_4)_3$, 0.19; $Na_2B_4O_7$, 0.55; $CaSO_4$, 14; $Na_2SiO_3 \cdot 9H_2O$, 228; KNO_3, 13.5; $CaCl_2 \cdot 2H_2O$, 33; $NaHCO_3$, 170; and $FeSO_4 \cdot 7H_2O$, 0.05. The pH was adjusted to 8.0 before the sample was filtered and autoclaved.

* Corresponding author.

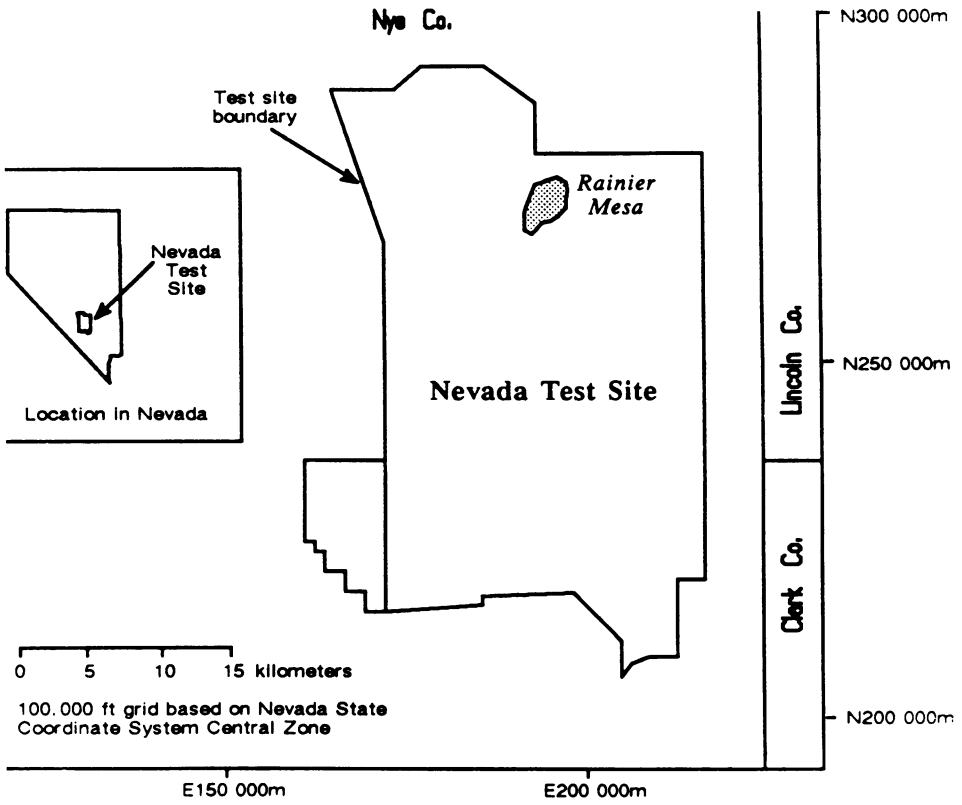

FIG. 1. Locations of Rainier Mesa and the Nevada Test Site.

The media used for viable cell plating were R2A (34) (Difco) and malt extract agar (diluted 1:10; Difco). R2B contained the following (in grams per liter of deionized water): yeast extract, 0.5; proteose peptone 3, 0.5; Casamino Acids, 0.5; dextrose, 0.5; soluble starch, 0.5; sodium pyruvate, 0.3; Na_2HPO_4, 0.3; and $MgSO_4$, 0.5. Antibiotic susceptibility was tested with spread plates of R2A and antibiotic discs containing ampicillin (10 μg; Difco), nalidixic acid (30 μg; Difco), tetracycline (30 μg; Difco), or triple sulfa (1.0 mg; BBL). Isolates were considered resistant if they grew closer than 12 mm (ampicillin), 13 mm (triple sulfa), 14 mm (nalidixic acid), or 15 mm (tetracycline) to the disc. Metal resistance media were prepared by adding filter-sterilized metal ion solutions to cooled R2A at the following concentrations: Cd^{+2}, 1.5 and 3.0 mM; Hg^{+2}, 0.025 and 0.05 mM; and Zn^{+2}, 5 and 10 mM. Isolates were scored from no growth (−) to growth as seen in the control (+++). Those receiving scores of ++ or +++ were considered resistant to the metal.

Sample collection. The U12n tunnel system was mined into zeolitized alkaline to peralkaline ashfall tuff (11) in the upper portion of a perched groundwater lens hundreds of meters thick. The tunnel depth is approximately 400 m below the surface but hundreds of meters above the regional water table. Occasionally, water flows freely from the tunnel walls where they intercept faults or water-bearing fractures; sample SD was collected from a flowing fault or fracture. Samples were collected for microbial and geological analyses from four separate locations within Tunnel U12n, Rainier Mesa, Nevada Test Site (Fig. 1). Brief descriptions of the three rock samples, North Drift (ND), N05, and N21 reentry (N21), are given in Table 1. The mean porosities and saturations of all three rock samples were 36 and 96%, respectively (11). A substantial data base, maintained by Sandia National Laboratory and the Department of Defense, was used to determine the physical properties of the sample locations. The geology was determined from existing U.S. Geological Survey publications (Table 1). Atmospherically exposed surface rock was swab sampled with R2B as a wetting agent. The swabs were placed in sterile screw-cap tubes containing R2B and transported on ice to the laboratory for spread plating on R2A. Surface rock was removed to approximately 10 cm with a geologic hammer and/or an impact hammer; the implements were alcohol sterilized and

TABLE 1. Physical properties of rock sample locations in Rainier Mesa

Sample point	Sample name	Geologic unit[a] and description
12n.21 re-entry drift	N21	Tunnel bed 3d: massive, red, fine-grained, zeolitized calkalkaline ash-fall tuff with moderately abundant pumice fragments
12n.05 drift	N05	Tunnel bed 4k: thick-bedded, green-brown, fine- to coarse-grained, zeolitized calkalkaline ash fall tuff with moderately abundant pumice fragments
12n extension north	ND	Tunnel bed 4k

[a] Inferred from Dickey and Emerick (13) and an unpublished U.S. Geological Survey report (37a).

then used to aseptically expose fresh rock faces. The surface was swabbed again with a sterile cotton swab wetted with R2B before sampling. The rock was chipped into sterile glass jars; the jars were sealed and placed on ice until they were returned to the laboratory in Las Vegas (<6 h). During sampling, exposed rock surfaces were periodically swab sampled as described above to determine whether surface contamination had occurred. Fungi, which were abundant at the surface but absent from the sample rock, served as a surface contamination marker.

One water sample (sample SD) was collected at U12n South Drift from a free-flowing seep (5 gallons [ca. 18.9 liters]/min) emanating from a fault that cross cuts the tunnel wall at that location. Parameters such as dissolved oxygen, pH, and electrical conductivity were measured in the field. The ionic geochemistry data of SD water at 16.4°C are as follows: electrical conductivity, 238 μS; dissolved O_2, 12.8 ppm; SIO_2, 65 mg/liter; pH, 8.31; HCO_3^-, 132 mg/liter; CO_3^{-2}, 0.37 mg/liter; Cl^-, 6.0 mg/liter; SO_4^{-2}, 7.9 mg/liter; NO_3^-, 1.51 mg/liter; Na^+, 33.7 mg/liter; K^+ 6.54 mg/liter; Ca^{+2}, 13.9 mg/liter; Mg^{+2}, 2.37 mg/liter; NO_2^-, 0.020 mg/liter; NH_4^+, <0.01 mg/liter; total Kjeldahl nitrogen, 0.2 mg/liter; total phosphate, 0.07 mg/liter; organic phosphate, 0.06 mg/liter; Fe, 0.03 mg/liter. Free-flowing water is thought to be recharged by atmospheric precipitation.

Sample workup. All equipment used to process rock samples was sterilized. Fifteen grams of each rock sample was ground with a mortar and pestle, suspended in 150 ml of filtered artificial pore water, and shaken at 100 rpm for 1 h at 23°C. The slurry was used after brief settling for spread plating onto duplicate R2A plates. Plates were incubated at 23°C for 2 weeks.

Culture isolation. Colonies were examined visually and chosen for their dominance from duplicate R2A plates. In addition, several unique colonies were chosen for their unusual pigmentation or peculiar colony morphology. Individual colonies were repeatedly transferred to R2A and incubated at 23°C until pure cultures were obtained on three successive streak plates. Gram-stained samples were viewed to confirm purity.

Tentative identification of some isolates was determined by API test strips for nonfermenting gram-negative bacteria (API-NFT). These were prepared according to the specifications of the manufacturer, except that the incubation temperature was 23°C for 48 h and artificial pore water was the diluent. Freshly grown cells were harvested from R2A plates. For fatty acid methyl ester analysis with the MIDI system, pure cultures were grown for 24 h on Trypticase soy agar plates as previously described (29). Isolates tested in the BIOLOG system were grown according to the specifications of the manufacturer and as described by Mauchline and Keevil (24). Microtiter plates were scored visually. A portion of the 16S rRNA gene was amplified by the polymerase chain reaction and sequenced in the laboratory of R. H. Reeves (Florida State University) with a model 373A automated DNA sequencer (Applied Biosystems, Inc., Foster City, Calif.) as specified by the manufacturer (5).

RESULTS

Individual microbial isolates were cultured from rock grains or a slurry made from rock and artificial pore water (1:10, wt/vol). By this method, unattached cells or cells attached to rock particles were exposed to supportive growth media (17). Viable cell counts ranged from 10^2 per ml in the SD water sample to 10^4 per g at N21. The average count was in the range of 10^2 to 10^3 CFU/g of rock. After 1 week, an identical treatment of rock samples N05 and ND yielded 100-fold higher viable counts than those from the original platings, with much less diversity. Large mucoid colonies dominated each agar plate. No colonies that could not grow on R2A grew on 1:10 malt extract agar, and the number of colony types was much greater on R2A than on malt extract agar. Plates made from surface swabs contained microbial types that were very different from those on plates with samples from only a few centimeters below the surface. The surface microbial community was dominated by bacterial colonies overgrown by fungi, whereas once the surface rock was removed, the colonies that developed from swab samples of the fresh rock surface were few in number and often pigmented, and the plates showed no fungi.

TABLE 2. Cell characteristics of deep subsurface isolates

Sample site	No. of isolates	% of isolates with the following properties:					
		Pigmented	Gram negative	Cocco-bacilli	Rods	Cocci	Oxidase positive
N21	9	56	78	44	44	11	67
N05	13	54	85	46	46	8	92
ND	8	100	100	12	88	0	100
SD	16	63	81	56	44	0	88

Many isolates were small (<1 μm) when viewed under the microscope and remained small even during growth, changing very little when held without nutrients (data not shown). The majority of isolates from rock were gram negative (89%), coccobacilli or rods (95%), and pigmented (65%). Most isolates were oxidase positive and nonfermentative (Table 2). Gram-positive isolates came primarily from site N21, a sample taken from overhead with water dripping from its surface.

In an effort to compare isolates that might not be readily identified to species, a profile that included an API-NFT strip seven-digit number and resistances to various antibiotics and metal ions was made of each isolate. The antibiotic resistance data are displayed in Table 3. All isolates from N05 were resistant to nalidixic acid, ampicillin, and triple sulfa. Additionally, 92% of the N05 isolates were resistant to tetracycline. No other site provided isolates showing such a high percentage of resistance to these four antibiotics. Isolates from N21 demonstrated appreciable resistance to the antibiotics used, this was similar to the resistance of ND isolates to nalidixic acid, ampicillin, and tetracycline. The SD water isolates demonstrated the most sensitivity to antibiotics, with only 12% resistance to tetracycline. Tetracycline resistance clearly provided the best discriminator for profiling these organisms.

TABLE 3. Antibiotic resistances of bacterial isolates

Sample site	No. of distinct isolates	% of isolates resistant to:			
		Nalidixic acid	Ampicillin	Triple sulfa	Tetra-cycline
N21	9	89	78	89	33
N05	13	100	100	100	92
ND	8	86	75	75	38
SD	17	56	65	59	12
Total	47	80	79	79	43

TABLE 4. Metal resistances of bacterial isolates

Sample site	No. of isolates	% of isolates resistant to:			
		Mercury		Zinc	
		0.25 mM	0.5 mM	5 mM	10 mM
N21	9	44	44	44	11
N05	13	77	62	62	31
ND	8	63	13	38	13
SD	17	65	35	24	13

Differences between rock and water isolates were also demonstrated with respect to resistance to mercury and zinc ions (Table 4). The highest percentage resistance to both metals was found with the N05 isolates, 73% of which were resistant to 0.05 mM mercury. Isolates from N21, ND, and SD were only 13 to 44% resistant to 0.05 mM mercury. Likewise, 62 and 31% of the N05 isolates were resistant to 5 and 10 mM zinc, respectively, whereas only 25 to 44% and 11 to 13% of the remaining isolates were resistant. No isolate grew on either concentration of cadmium, although some environmental isolates have this ability (25).

The 21 metabolic tests in the API-NFT strips were used to compare isolates from one site to another. In six tests, the percentage of positive reaction was lower in the water isolates (SD) than in the endolithic isolates. These tests included esculin hydrolysis; *para*-nitrophenyl-β-D-galactopyranoside cleavage; and *N*-acetylglucosamine, maltose, malate, or glucose oxidation (data not shown). Sample N21 isolates, influenced by water, were most similar to SD water isolates when API-NFT results were compared. The isolates from SD and N21 showed a high percentage of positive reactions for arginine dihydrolase, urease, gluconate, and citrate utilization and a low percentage of positive reactions for mannose and oxidase (data not shown).

Two identification systems, API-NFT strips and MIDI, were used to identify all 47 isolates obtained from rock and water samples. Analysis with the API-NFT strips yielded identifications for 14 of 47 isolates tested (Table 5). MIDI fatty acid methyl ester analysis identified 11 of the 47 isolates (Table 6). The 18 isolates identified by either API-NFT strips or MIDI were subsequently analyzed with the BIOLOG metabolic identification system (Table 7).

TABLE 5. API-NFT identification of deep subsurface isolates

Site	Isolate no.	Profile no.	Identification	Level of certainty[a]
ND	R2	0462344	*Pseudomonas paucimoblis*	Good
	R7	1020004[b]	*Pasteurella haemolytica*	Good
SD	R3	1200004	*Moraxella phenylpyruvica*	Good
	R6	1347557	*Pseudomonas fluorescens*	Excellent
	R11	1000000	*Pasteurella* sp.	Good
	R14	1020004[b]	*P. haemolytica*	Good
	M1	1410044	*Pseudomonas* sp.	Acceptable
N21	R1	1157557	*P. fluorescens*	Very good
	R9	0470344	*Pseudomonas vesicularis*	Good
N05	R4	1420204	*P. vesicularis*	Good
	R5a	1463304[c]	*P. paucimoblis*	Acceptable
	R5b	1147551	*P. fluorescens*	Very good
	M2	1463304[c]	*P. paucimoblis*	Acceptable
	M3	1463344	*P. paucimoblis*	Good

[a] Excellent (≥99.9%), very good (99%), good (90%), or acceptable (80%) certainty (4).
[b] Profile identical to that of *P. haemolytica*.
[c] Profile identical to that of *P. paucimoblis*.

TABLE 6. MIDI system identifications of deep subsurface isolates

Site	Isolate no.	Identification	Similarity ratio[a]	Cluster position
ND	R2			Pseudomonad[b]
	R4			Pseudomonad[c]
	R6			Pseudomonad[b]
SD	R1	*Pseudomonas syringae* or *P. putida*	0.491 or 0.472	Pseudomonad
	R2	*Pseudomonas facilus*	0.266	Pseudomonad
	R4	*Bacillus coagulans*	0.249	Gram positive
	R6	*P. putida*	0.357	Pseudomonad
	R7	*P. putida*	0.289	Pseudomonad
	R8	*P. syringae* or *P. putida*	0.443 or 0.437	Pseudomonad
	R9	*Hydrogenophaga pseudoflava*	0.247	Pseudomonad
	R10	*H. pseudoflava*	0.332	Pseudomonad
	M1	*P. putida*	0.449	Pseudomonad
	M2			Pseudomonad
N21	R1	*P. syringae* or *P. putida*	0.556 or 0.446	Pseudomonad
	R3	*Micrococcus luteus*	0.171	Gram positive
	R4			Gram positive[d]
	R5	*A. saperdae*	0.220	Gram positive
	R9			Unknown[e]
N05	R4	*Sphingobacterium spiritovorum*	0.115	Unknown
	R5a	*S. spiritovorum*	0.114	Unknown
	R6			Gram positive
	R9			Gram positive
	M2			Unknown

[a] A similarity ratio of ≥0.3 is considered a strong match to the data base.
[b] Euclidean distance indicates that R2 and R6 are same species.
[c] Similar to isolate M2.
[d] Similar to isolate R5.
[e] Clustered isolates with no identified member.

API-NFT analysis identified 30% of the isolates to confidence levels that were acceptable or better (4) (Table 5). Only one isolate showed an excellent match, i.e., 99.9% certainty that the isolate was *Pseudomonas fluorescens*. Approximately 70% of the isolates were scored as low discrimination, doubtful, or not indexed in the data base (4). Genera identified to acceptable or higher confidence levels include *Pseudomonas*, *Pasteurella*, and *Moraxella*. Additionally, several isolates were identified to the species level. Many of these genera have been previously identified by API-NFT analysis from drilling programs at the Savannah River DOE site (14).

The MIDI system provided identification for 23% of the isolates with similarity ratios of 0.22 or higher. Additionally, this system clustered the poorly matched isolates into a dendogram with three major relatedness groups (Table 6). The groups were pseudomonads and related genera, a group of gram-positive bacteria, including *Bacillus*, *Micrococcus*, and *Aureobacterium* spp., and a group of unknown identity.

The 18 isolates identified by either API-NFT or MIDI were tested with BIOLOG. This system identified 8 of 18

TABLE 7. BIOLOG identification of deep subsurface isolates

Site	Isolate no.	Identification	Time of incubation (h)	Similarity index[a]
ND	R2[b]	*Acinetobacter haemolyticus*	4	0.90
SD	R3[b]	*A. haemolyticus*	4	0.77
	R6	*Pseudomonas fluorescens*	24	0.50
	R9	*Moraxella bovis*	24	0.72
	R10	*Moraxella lincolnii*	24	0.61
	M1	*Xanthomonas campestris*	24	0.69
N21	R1	*P. fluorescens*	24	0.47
	R5	*Clavibacter michiganense*	24	0.64
	R9	*Pseudomonas vesicularis*	24	0.41
N05	R4	*Sphingobacterium multivorum*-like	24	0.66
	R5a	*S. multivorum*-like	24	0.42
	M2	*S. multivorum*-like	24	0.45
	M3	*M. bovis*	24	0.42
Controls		*P. fluorescens*	24	0.57
		Pseudomonas syringae	24	0.54

[a] BIOLOG designations: 0.77 to 0.90, excellent; 0.50 to 0.72, good; 0.41 to 0.45, poor.
[b] No identification with 24 h of incubation.

TABLE 8. Comparison of identified bacterial isolates

Site	Isolate no.	Identification at the genus level by:		
		API-NFT[a]	BIOLOG[b]	MIDI[c]
ND	R2	*Pseudomonas*	*Acinetobacter*	Pseudomonad
	R4			Pseudomonad
	R6			Pseudomonad
	R7	*Pasteurella*		
SD	R1			*Pseudomonas*
	R2			Pseudomonad
	R3	*Moraxella*	*Acinetobacter*	
	R4			Gram positive
	R6	*Pseudomonas*		*Pseudomonas*
	R7			Pseudomonad
	R8			*Pseudomonas*
	R9		*Moraxella*	Pseudomonad
	R10		*Moraxella*	*Hydrogenophaga*
	R11	*Pasteurella*		
	R14	*Pasteurella*		
	M1	*Pseudomonas*	*Xanthomonas*	*Pseudomonas*
	M2			Pseudomonad
N21	R1	*Pseudomonas*		*Pseudomonas*
	R3			Gram positive
	R4			Gram positive
	R5		*Clavibacter*	Gram positive
	R9	*Pseudomonas*		Unknown
N05	R4	*Pseudomonas*	*Sphingobacterium*	Unknown
	R5a	*Pseudomonas*		Unknown
	R5b	*Pseudomonas*		
	R6			Gram positive
	R9			Gram positive
	M2	*Pseudomonas*		Unknown
	M3	*Pseudomonas*		

[a] Identified to a confidence level of acceptable, good, very good, or excellent.
[b] Identified to a confidence level of good or excellent.
[c] Identified to a confidence level of ≥0.300 or cluster position (indicated as pseudomonad, gram positive, or unknown).

tested isolates (44%) to a similarity index value of >0.5 (Table 7). Five other isolates were identified with poor certainty; these were assigned to the genera *Pseudomonas*, *Acinetobacter*, *Moraxella*, *Xanthomonas*, and *Sphingobacterium*. No identifications with similarity ratios below 0.40 were considered.

When the identifications of isolates with the three systems were compared, several organisms appeared to be very similar or identical. By API-NFT profiles, NDR7 and SDR14 were identical, as were N05R5a and N05M2. Pairs of isolates were so closely related by fatty acid methyl ester cluster analysis that they were considered to be the same species. N05R5a and N05M2 clustered within the unknown group, NDR2 and NDR6 clustered with NDR4 and SDM2 within the pseudomonad group, and N21R4 and N05R6 clustered near the *Aureobacterium* group (gram-positive bacteria) but were not matched to a specific genus. These pairs may be examples of the same bacterium isolated more than once, but in most cases some characteristic of the isolate, e.g., antibiotic or metal resistance patterns, indicated that the isolates were different. BIOLOG also recognized the close relatedness of N05R5a and N05M2, naming them *Sphingobacterium multivorum*-like. Interestingly, MIDI grouped these two isolates together, along with N05R4, and named them *Sphingobacterium spiritovorum*, but the similarity ratios were very low (Table 6).

Each identification system named some of the isolates, but seldom was a single isolate identified by more than one system (Table 8). In the case where the same isolate was identified by more than one system, the identifications were not identical. With both the API-NFT and MIDI systems, most of the isolates (17 of them) were placed in the genus *Pseudomonas*. However, of these 17 identifications, in only 7 cases did both systems match the same isolates to the same genus, and in no case were the identifications identical at the species level. With the BIOLOG system, only 3 of 18 isolates were classified as *Pseudomonas* spp., but many of the other genera indicated were closely related. In three cases, the MIDI system identified an isolate as *Pseudomonas putida* or *P. syringae*, whereas the API-NFT system matched the same isolates to *Pseudomonas fluorescens* or *Pseudomonas* sp., and BIOLOG named them *P. fluorescens*. The API-NFT system classified NDR2 and the N05R5a and N05M2 pair as *Pseudomonas paucimoblis*. The MIDI system also recognized the relatedness between N05R5a and N05M2 but identified them with low confidence as *S. spiritovorum*, a placement within the *Cytophagales* (22). NDR2 was identified by two systems as a pseudomonad (*Pseudomonas* sp. [MIDI] or *P. paucimoblis* [API-NFT]) and by BIOLOG as *Acinetobacter* sp. The remaining eight isolates were identified differently by each system.

With MIDI analysis, few endolithic bacteria were identified, whereas 9 of the 15 SD water isolates matched the data base. Only two N21 sample isolates and no ND or N05 isolates were matched to the data bank; however, many of the N05 isolates clustered into the unknown bacterial group. BIOLOG identified approximately equal numbers of isolates from each of three sample sites, SD, N21, and N05, but the similarity ratios for water isolates were consistently high, whereas those for endolithic isolates were lower. API-NFT identified equal numbers of isolates from SD and N05 but only two isolates each from N21 and ND.

The API test bank, designed to identify pathogenic or

common species of gram-negative nonfermenting bacteria, sometimes gave erroneous identifications of subsurface isolates. For example, the data bank matched four isolates showing very little metabolic activity to *Pasteurella* sp. Other isolates also keyed to *Pasteurella* sp. but were not considered to match the API-NFT data base because they stained gram positive or variable. One isolate identified as *Pasteurella* sp. was an actinomycete; isolate SDR11, listed in Table 6 as *Pasteurella* sp., was analyzed by 16S rDNA sequencing and shown to be either an *Arthrobacter* sp. or a bacterium closely related to this genus (34a). Many subsurface microorganisms stain gram negative or variable, but by rDNA sequencing they key to *Arthrobacter* and related gram-positive genera.

DISCUSSION

Nevada Test Site samples were retrieved easily with a sterile chisel and hammer. The indigenous microbiota was recovered from samples taken approximately 10 cm below the tunnel wall surface. One advantage of this sampling method is that large quantities of rock can be obtained without the expense and concern for contamination associated with drilling procedures (8, 16, 30).

Recovered bacteria were aerobic heterotrophs capable of growing on complex media. Aerobic heterotrophs were also found by others in the Savannah River Plant subsurface drilling operations (8, 14). Most of the bacteria were gram negative, although several isolates (6 of 15 identified by MIDI), especially isolates from site N21, were gram positive. Others have found gram-positive and pigmented bacteria in subsurface environments as well (17a).

Water flowing relatively rapidly through faults in the rock sections, for example, the SD sample, yielded bacteria that were either more easily identified or identified to a higher confidence level by the API-NFT, BIOLOG, and MIDI systems. In contrast, the endolithic isolates were seldom identified by any of the three systems. The endolithic and water isolates did not appear to be closely related to one another. A few exceptions in which ND and SD isolates appeared to be related were noted. Antibiotic resistance data also support the differences observed between endolithic and the water-borne isolates. All of the endolithic isolates, particularly those from NO5, were more resistant to four antibiotics than were the water-borne isolates. The NO5 isolates were also unique in their high percentage of resistance to the metals zinc and mercury. It is not known why these isolates demonstrated such high resistance to environmental stressors, but many of the isolates do contain plasmids (data not shown).

There is mounting evidence that subsurface endolithic microorganisms in Rainier Mesa may be very old. There are two hypotheses that account for the transport of microorganisms to the subsurface depths studied. They may be the original rock colonizers after each volcanic ash fall, or they may have been transported to this depth at a later time by water. Water from the surface can reach the subsurface sites via three methods: fracture flow, matrix flow, or a combination of the two. In Rainier Mesa, the travel time for fracture flow recharge water (35) has been estimated at between 6 and 30 years by ^{3}H dating (12). Calculations based on data presented by Thordarson (36) indicate that travel times for pure matrix flow are orders of magnitude greater (potentially as long as 250,000 years). This sets the minimum age of these microbes at 250,000 years if they were deposited by matrix flow from the surface. The actual age is probably greater, since others have shown that bacteria do not travel well through soils of low matric potential (38, 39). This is significant, since a large vadose zone is present above the U12n tunnel, the source of the samples in this study. Although the SD water sample and the N21 site contained fracture flow water at the tunnel level, water travel to the ND and N05 samples would have required mostly matrix flow and thus longer travel times. Lateral water flow is essentially negligible in the sample area (36), because the water is perched in a bowl-shaped area with impermeable layers below and air-rock interfaces at the lateral boundaries of the mountain. Lateral water movement, therefore, does not account for the presence of endolithic bacterial cells at this depth in a shorter time than that needed for overhead recharge. Researchers at the Defense Nuclear Agency at the Nevada Test Site believe that the pore waters may be close to the age of the diagenic zeolitization. This process altered the intergrain porosities so that they do not allow appreciable intergrain migration of water (34b). Further research into the origins of these endolithic bacteria and comparisons of genetic and structural components of endolithic subsurface bacteria to modern bacteria might prove interesting.

The age of the rock that was sampled and the lack of water movement in nonfractured rock of Rainier Mesa imply that endolithic microbiota have been in place for a long time and therefore would have had to endure an environment of little or no nutrients. Environmental bacteria often survive periods of starvation in nature (1, 2, 33). Pseudomonads were the most common microorganisms isolated from these rock samples and are known to survive for long periods of time (months to decades) (11a, 18, 21). Pseudomonads fill the ecological niche that is equivalent to that of the marine vibrios, on which most starvation studies have been based (1, 23, 28). Mechanisms for long-term survival of bacteria include fragmentation or division without growth, miniaturization of cells (2, 23, 27, 28, 37), and changes in nutrient uptake kinetics (15, 20, 26). Many of the isolates of this study were very small as seen by epifluorescence; unlike many bacteria from soil and marine waters (3, 6, 37), most remained very small even during growth.

Although we do not know the true age of these endolithic bacteria, an effort is being made to age the pore water to confirm the minimal survival time of the bacteria.

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