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Changes in Bacteria Recoverable from Subsurface Volcanic Rock Samples during Storage at 4°C

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The abundance of viable microorganisms recovered from deep subsurface volcanic rock samples increased after rock perturbation and storage for 1 week at 4°C, while the diversity and evenness of recoverable heterotrophic bacterial communities generally decreased. One sample of each morphologically distinct colony type, recovered both before and after storage of U12n rock samples, was purified and characterized by fatty acid methyl ester (MIDI) and API rapid NFT strips. As determined by MIDI cluster analysis, the composition of the recoverable microbial communities changed with storage of rock samples; some groups of organisms were recovered only before, only after, or at both sample times. In general, the isolates recovered only after storage of rock samples had a greater ability to utilize the carbohydrates included in API test strips and had faster generation times than isolates recovered only on initial plating. The nutritional versatility and faster growth rates of organisms recovered in higher proportions after sample storage provide evidence that some microbial community changes may be due to the proliferation of a few bacterial types. However, because some new genera are recovered only after storage, the possibility also exists that dormant bacterial types are resuscitated during sample perturbation and storage.

Claude Zobell first described the "bottle effect" in 1943, noting that the abundance of microorganisms increased during storage of water samples, especially on the surfaces of containers, where nutrients concentrated (30). Changes in microbial communities that occur during the storage and perturbation of microbiological samples has since been a concern of microbial ecologists, and special care is taken when designing experiments to alleviate these effects. Precautions include minimizing storage time before initiation of sample analysis, storing and transporting samples at temperatures that decrease the activity of microorganisms without causing cell death, flushing sample containers with inert gases, and procuring samples with as little disruption of the samples as possible (3, 28, 29).

An increase in viable counts and a decrease in recoverable microbial diversity were observed in deep subsurface volcanic rock samples that were analyzed 1 week after storage at 4°C (2). Others have reported similar changes in stored samples from deep subsurface environments: aquifers (17), volcanic rock (12), and paleosols (9). The purpose of this research was to quantify changes in microbial abundance, diversity, and evenness within deep subsurface volcanic rock samples and to characterize and compare microbiota recovered only before storage, only after storage, or at both sample times. Changes were documented in samples that were stored at a temperature that is commonly employed by microbiologists. Characterization of specific microbial types, including physiological testing and growth rate determinations, was initiated to determine mechanisms by which microbial types might change community composition. Hypotheses concerning the basis for microbial community change include (i) growth of a few bacterial types, (ii) resuscitation of dormant bacterial types, and (iii) a combination of both phenomena.

(Some of the results described here [from initial analysis of samples B, G1, G2, P1, P2, N9, and N19] have been previously presented by Haldeman and Amy [14], including direct and culturable counts.)

MATERIALS AND METHODS

Sample sites and collection. Samples were obtained from the walls of deep subsurface tunnels at Rainier Mesa, Nevada Test Site, as described by Haldeman and Amy (14). Rock was aseptically chipped into sterile containers after first creating fresh rock faces with alcohol flame-sterilized tools for samples B, G1, G2, and P1. For samples N2, N8, N9, N13, N16, N18, and N19, an alpine miner was used to excavate into the tunnel walls to distances of approximately 0.76 (N2), 1.52 (N8 and N9), 2.28 (N13), and 3.04 (N18 and N19) m, where fresh rock faces were created for sampling by hand (15). The physical characteristics of the sample sites are described elsewhere (14). Samples were transported to the laboratory in coolers containing ice blocks. The approximate ambient rock temperatures ranged from 16 to 24°C. Sample analysis was initiated less than 6 h after sample collection.

Sample analysis. Sample analyses, both before storage and after 1 week of storage at 4°C, were carried out identically. Samples designated with an asterisk represent stored samples; e.g., N2 and N2* were samples analyzed before and after rock storage, respectively. Sample workup is described by Haldeman and Amy (14). All materials were cleaned and sterilized before use. Rock was crushed with a mortar and pestle and stored in airtight containers at 4°C. Slurries were made at each sample time by diluting crushed rock 1:10 with artificial pore water, a formulation developed to mimic in situ pore water chemistry (2). Slurries were shaken for 1 h before viable count, direct count, and most-probable-number (MPN) analyses were initiated. Viable counts for all samples were determined by averaging colony counts from triplicate spread plates on R2A agar (Difco). Shannon indexes for diversity and evenness of microbial distribution (3) were determined from triplicate spread plates, where colonies could be easily differentiated.

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Artificial pore water was used as a diluent, and incubations were carried out for 2 weeks at approximately 24°C. Direct counts were determined with either acridine orange or 4′,6-diamidino-2-phenolindole (14). For MPN analysis, each of three serial dilutions of a rock sample were added to five tubes of nitrogen-fixing, denitrifying, nitrifying, sulfur-oxidizing, and iron-peptone media (14). Tubes were incubated for 6 weeks at 24°C.

Isolate analyses. One sample of each morphologically distinct colony type was selected from R2A spread plates of samples analyzed before and after storage (13). This has been shown to be a reliable means for selecting representative organisms at the species level (Euclidean distance, ≤10) from deep volcanic rock samples (13). Isolates were also recovered from turbid but negative MPN tubes of sulfur oxidation and nitrification media. Isolates were purified as previously described (14). Fatty acid methyl esters were prepared and analyzed by capillary gas chromatography (TSBA aerobe library version 3.6 [24]). The MIDI system generated dendrograms from fatty acid methyl ester profiles on the basis of a Euclidean distance scale (13, 15). Isolates were physiologically characterized by API rapid NFT test strips (Analytab) prepared as described by the manufacturer, except that artificial pore water was used as the diluent and incubations were carried out at 24°C. Overnight cultures of bacteria were inoculated into R2B broth (14), and culture optical density was determined in a Klett-Summerson colorimeter. The doubling times of specific isolates were determined from the logarithmic portions of replicate growth curves.

RESULTS

Microbial communities recovered from deep subsurface volcanic rock samples changed in abundance and composition after rock was stored for 1 week at 4°C (Table 1). Viable counts were several orders of magnitude greater in most samples, while Shannon diversity and evenness indexes generally decreased. The number of colony types recovered decreased in 8 (73%) of the 11 samples. In two of the samples in which diversity and evenness increased after storage (N16 and N18), more bacterial types were recovered after storage, while in a third sample (G2), the number of colony types remained constant. In sample N13-N13*, diversity remained the same even when the number of colony types increased during storage because a few colony types increased dramatically in dominance. Additionally, although 15 colony types were recovered after storage, 8 clustered into only three genera on the dendrogram (described below); thus, the increase was not as striking as if the new colonies had represented new genera. Direct counts, although difficult to determine on autofluorescing rock samples, did not appear to increase, certainly not several orders of magnitude, as observed in some culturable cell counts (data not shown).

MPN analyses demonstrated changes in bacterial communities of different metabolic types (Table 2). The numbers of nitrogen-fixing bacteria were greatly increased in the stored G2* and N19* samples compared with the initial analysis. The abundance of denitrifying bacterial populations was increased in samples P1 and N19. MPNs of H_2S-producing, sulfur-oxidizing, and nitrifying bacteria were below the limits of detection in all samples both before and after storage. Although testing negative for acid production, some sulfur-oxidizing and nitrifying MPN tubes became turbid within the 6-week incubation period. Although these media did not contain an organic carbon source, heterotrophic organisms from these tubes were isolated on R2A agar and found to be unique by colony and cellular morphology, MIDI identification, and/or API profiles compared with organisms originally isolated on R2A agar from the same rock samples. Some MPN isolates were identified by MIDI analysis as belonging to the genera *Arthrobacter*, *Xanthomonas*, *Micrococcus*, *Flavobacterium*, *Agrobacterium*, *Pseudomonas*, and *Acinetobacter*. Of the 60 MPN isolates, 5 (8%) were not matched to the MIDI database or any other organism cluster until a Euclidean distance of >25, the genus level (27).

MIDI dendrograms based on fatty acid methyl ester profiles of bacterial isolates recovered from samples N and N* were used to confirm observations of changes in the composition of culturable communities. Two representative dendrograms of isolates recovered from samples N2-N2* and N16-N16* are

TABLE 1. Changes in total counts, viable counts, diversity, evenness, and kinds of microorganisms recovered both before and after sample storage[a]

Sample or parameter	Total count (log cell/g of dry wt)	Viable count (log CFU/g of dry wt)	Shannon diversity index	Evenness index	No. of colony kinds
N2	7.06	4.45	1.83	0.71	14
N2*	ND[b]	7.05	1.51	0.61	12
Net change[c]	ND	+7.05	−0.32	−0.10	−2
N8	6.60	1.49	1.73	0.83	6
N8*	ND	>2.00	ND	ND	4
Net change	ND	>+1.84	ND	ND	−2
N9	7.25	1.73	1.94	0.88	9
N9*	5.80	2.86	1.37	0.77	6
Net change	−7.23	+2.83	−0.57	−0.11	−3
N13	7.58	1.08	1.82	0.87	8
N13*	ND	2.94	1.82	0.67	15
Net change	ND	+2.93	0	−0.20	+7
N16	6.52	2.92	1.33	0.61	10
N16*	ND	4.83	1.81	0.69	14
Net change	ND	+4.82	+0.48	+0.08	+4
N18	7.24	3.99	ND	ND	4
N18*	ND	4.04	1.58	0.69	10
Net change	ND	+3.08	ND	ND	+6
N19	7.37	4.26	1.93	0.66	19
N19*	6.35	5.39	1.00	0.42	11
Net change	−7.32	+5.36	−0.93	−0.24	−8
B1	5.61	<1.02	0.57	0.83	2
B1*	ND	<1.02	ND	ND	1
Net change	ND	ND	ND	ND	−1
P1	5.99	4.94	1.36	0.55	12
P1*	ND	4.43	1.08	0.61	6
Net change	ND	+4.78	−0.28	+0.06	−6
G1	6.23	3.43	2.02	0.75	15
G1*	ND	4.51	1.62	0.67	11
Net change	ND	+4.48	−0.40	−0.08	−4
G2	6.16	5.37	1.47	0.76	7
G2*	ND	6.17	1.74	0.90	7
Net change	ND	+6.10	+0.27	+0.51	0

[a] Samples were stored for 1 week at 4°C in airtight containers.
[b] ND, not determined.
[c] Net change is the difference between counts and was determined before log transformation.

TABLE 2. MPN enrichment data showing estimates of numbers of cells per gram of wet weight

Sample	N_2 fixation	Denitrification (nitrite formation)	Denitrification (N_2 formation)
G1	BDL[a]	ND	ND
G1*[b]	BDL	ND	ND
G2	780	ND	ND
G2*	>160,000	ND	ND
P1	BDL	32	2
P1*	BDL	180	2
N9	23	13	BDL
N9*	BDL	ND	ND
N19	BDL	>1,600	280
N19*	>160,000	>1,600	1,600

[a] BDL, below the detection limit.
[b] An asterisk indicates that the sample was stored for 1 week at 4°C before analysis.

depicted in Fig. 1A and B, respectively. Microorganism clusters at a Euclidean distance of <25 (the genus level) were used to determine whether organisms were recovered only upon initial plating, only after sample storage for 1 week, or at both sample times. Clusters were numbered and labeled B (before) or A (after) to reflect the time points at which the isolates included in each cluster were recovered. Clusters containing isolates recovered at both time points were designated T (throughout). Community proportions were determined as the frequency of occurrence of a particular isolate compared with the total number of colonies on original R2A plates.

Organisms from some clusters, such as those in 1, 4, 6, 7, and 8 (Fig. 1A), were no longer recovered after 1 week of sample storage. Often, these organisms represented a small proportion of the original recovered bacterial community; e.g., clusters 1, 4, and 6 from the N2/N2* dendrogram contained <1, 5, and 1% of the initial recoverable community, respectively.

Some genera were recovered at both sample times, as demonstrated by clusters 3, 5, 9, and 11 of Fig. 1A. However, the isolates within the clusters were not always recovered in the same proportions at both sample times. For example, organisms within the ninth cluster (*Acinetobacter* spp.) were recovered at both sample times, 32% of the initial community and 2% of the after-storage community. In cluster 11 (*Hydrogenophaga* spp.), the opposite trend occurred, i.e., organisms making up <1% of the initial community and approximately 8% of the after-storage community were recovered. Not all changes in bacterial communities were associated with gram-negative genera. In the N16-N16* dendrogram, cluster 3 (Fig. 1B), approximately 40% of the initial *Arthrobacter* community (three isolates) was decreased to a single isolate comprising 19% of the community after storage.

Some genera recovered only after storage made up significant proportions of the recoverable bacterial communities, exemplified by cluster 2 of the N2-N2* dendrogram (*Pseudomonas* spp.) and cluster 5 of the N16-N16* dendrogram (*Arthrobacter* spp.). Isolates from these clusters comprised 55 and 43%, respectively, of the total recoverable community yet were undetected upon initial plating. Interestingly, some of the bacteria recovered only after sample storage were only distantly related to organisms recovered before storage; i.e., clusters 6, 7, and 8 in Fig. 1B were not related to before-storage organisms until a Euclidean distance of >50.

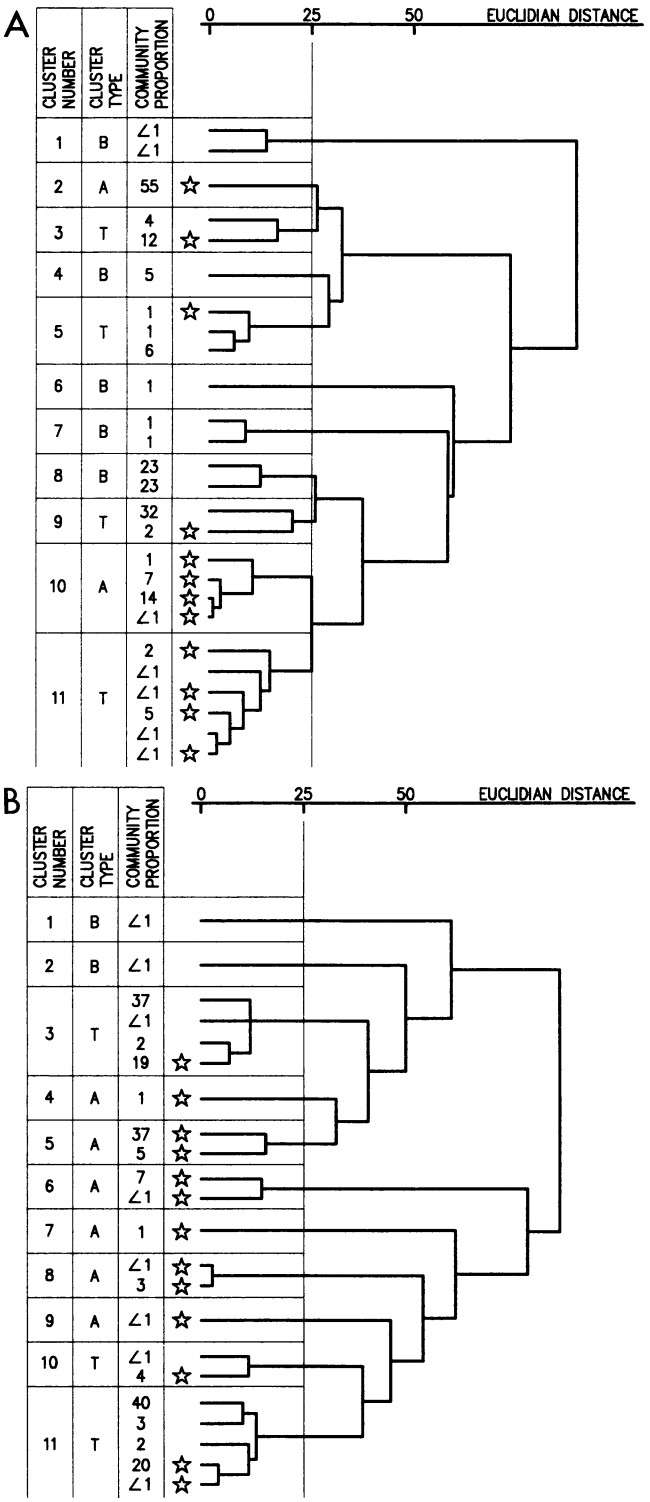

FIG. 1. Dendrograms A and B depict the relatedness of groups of isolates from samples N2-N2* and N16-N16*, respectively, at the genus level (Euclidean distance, ≤25, [27]). The stars represent organisms recovered after 1 week of sample storage. Cluster type designations describe whether organisms were isolated only before (B), only after (A), or at both time points (T) of sample storage.

TABLE 3. Genera of specific isolates recovered before, after, or throughout sample storage

Samples	Before storage		Throughout (both before and after) storage		After storage	
	% of community[a]	Identification	% of community (B, A)	Identification	% of community	Identification
N2-N2*	1	*Methylobacterium* sp.	4, 12	*Arthrobacter* sp.	55	*Arthrobacter* sp.
	5	*Brevibacterium*[b] sp.	7, 1	*Arthrobacter*[b] sp.	23	*Pseudomonas* sp.
	1	Unknown A	32, 2	*Acinetobacter* sp.		
	1	*Nocardioides*[b] sp.	1, 8	*Variovax*, *Hydrogenophaga*, *Pseudomonas* spp.		
	46	*Nocardioides*[b] sp.				
N8-N8*	13	*Micrococcus*[b] sp.			100[c]	Unknown C, *Pseudomonas*[b] sp.
	13	*Arthrobacter*[b] sp.				
	25	Unknown A				
	13	*Gordona*[b] sp.				
	25	Unknown B				
N9-N9*	8	Unknown A	15, 66	*Arthrobacter* sp.	30	*Micrococcus*[b] sp.
	38	*Micrococcus*[b] sp.	23, 3	*Arthrobacter* sp.		
			16,1	Unknown B		
N13-N13*			11, 1	*Kurthia* sp.	19	*Micrococcus* sp.
			11, 1	*Aureobacterium* sp.	1	Unknown B
			7,1	Unknown A	1	*Acinetobacter* sp.
			38, 64	*Gordona*[b] sp.		
N16-N16*	<1	*Micrococcus* sp.	40, 19	*Arthrobacter* sp.	43	*Micrococcus*[b] sp.
	<1	*Micrococcus* sp.	<1, 4	Unknown A	8	*Methylobacterium* sp.
			45, 21	*Gordona*[b] sp.	5	*Pseudomonas* sp.
					<1	Unknown B
N19-N19*	1	Unknown A	1, 6	Unknown F	48	*Acinetobacter*[b] sp.
	1	Unknown B				
	1	Unknown C	42, 2	*Acidovorax*[b] sp.	3	Unknown G
	4	*Pseudomonas* sp.	6, 1	*Curtobacterium*[b] sp.	3	Unknown H
	1	Unknown D	5, 3	*Hydrogenophaga* sp.		
	1	Unknown E				

[a] The percentages of B and A isolates may not sum to 100% because if the isolates were found at both time points they were listed in the throughout column. Each numerical value describes by percentage the proportion of all isolates within a cluster that contributed to the total community composition.
[b] Genera were identified with similarity indices of less than 0.300.
[c] Proportions of both isolate types were not determined.

A summary of all dendrograms from pairs of N-N* isolates is presented in Table 3. Although similarity levels of <0.300 were included in Table 3 for ease of discussion, values of ≥0.300 were considered acceptable for the environmental isolates in this study (15). Organisms no longer recovered after initial platings belonged to the genera *Methylobacterium* (N2-N2*), *Micrococcus* (N16-N16*), and *Pseudomonas* (N19-N19*) and genera unmatched to the MIDI database or matched with low similarity indices. Often, the genera lost comprised only a small component of the bacterial community, but not in all cases. Organisms within the *Norcardioides* (sample N2) and *Micrococcus* (sample N9) clusters were dominant at the time of initial plating. Organisms recovered throughout sample storage that demonstrated large changes (>10% difference in community composition between initial and after-storage values) included members of the genera *Arthrobacter* (N2-N2*, N9-N9*, and N16-N16*) and *Acinetobacter* (N2-N2*) and genera unmatched to the MIDI database or distantly related to the genus *Gordona* (N13-N13* and N16-N16*). Organisms recovered only after sample storage belonged to the genera *Arthrobacter* (N2-N2*), *Pseudomonas* (N2-N2* and N16-N16*), *Micrococcus* (N13-N13*), *Acinetobacter* (N13-N13*), and *Methylobacterium* (N16-N16*) and genera unmatched to the MIDI database. Often, the isolates found in clusters recovered only after sample storage were dominant in those communities.

Analysis of specific isolates from U12n tunnel samples by carbohydrate utilization tests (API rapid NFT test strips) revealed changes in the nutritional versatility of isolates recovered before (B), after (A), or throughout (T) sample storage (Table 4). In all samples except N9, a higher percentage of the

TABLE 4. Percentages of isolates capable of utilizing two or more substrates as a sole carbon source

Samples	% of isolates capable of utilizing ≥2 substrates		
	Before storage	Throughout[a] storage	After storage
N2-N2*	83	86	100
N8-N8*	60	0	100
N9-N9*	25	60	0
N13-N13*	0	50	100
N16-N16*	0	38	89
N19-N19*	40	38	100

[a] Throughout-storage isolates were recovered both before and after 1 week of storage at 4°C.

TABLE 5. Doubling times for isolates obtained from samples N2-N2* and N16-N16*

Time of isolation	No. of isolates tested	Mean doubling time (h) ± SD	Range of doubling times (h)
Before storage	7	4.79 ± 2.8	1.23–9.18
Throughout storage[a]	25	3.20 ± 2.6	0.98–9.90
After storage	11	2.21 ± 0.93	1.09–3.77

[a] Throughout-storage isolates were recovered both before and after 1 week of storage at 4°C.

isolates recovered after storage had the ability to utilize two or more carbon substrates compared with isolates recovered only before storage. The percentages of isolates recovered at both sample times that were able to utilize two or more of the compounds tested as a sole carbon source were intermediate values in most cases. Interestingly, 80% (48 of 60 isolates) of the organisms recovered on R2A plates from turbid but negative sulfur-oxidizing and nitrifying MPN tubes were capable of utilizing at least two of the API test strip carbon sources.

Bacteria isolated from rock samples before, after, and throughout storage were selected from N2-N2* and N16-N16* samples, and their generation times were determined (Table 5). The doubling times of isolates recovered before sample storage were significantly longer than those of after-storage isolates ($P = 0.10$), with mean doubling times of 4.79 and 2.21 h, respectively. Isolates recovered throughout sample storage demonstrated a wide range of doubling times, from 0.98 to 9.90 h.

DISCUSSION

When volcanic rock samples were stored at 4°C for 1 week, the abundance of recoverable microbiota increased while the diversity and evenness of microbial communities changed, usually decreasing. Other researchers have described similar phenomena in stored subsurface samples (paleosols [8, 9], sediments [11], and aquifers [17]). Analysis of specific isolates throughout storage was not determined in those studies, and thus, results of that research cannot be compared to those of the isolate analyses discussed here. This is the first study in which specific microbial isolates were analyzed in conjunction with microbial changes that occur in stored samples.

Direct counts were difficult to estimate because of autofluorescing rock particles, but they did not appear to increase in those samples analyzed, certainly not by orders of magnitude, as observed with culturable cell counts. Other subsurface researchers have reported constant direct counts during storage of subsurface samples for various time periods, i.e., over 6 weeks (11) and over 100 days (9). It would be valuable to have a more reliable technique to estimate total cell counts that would definitively prove whether or not total cell counts increase in correlation to culturable cells. However, even if total counts could be accurately determined, it would not necessarily lend insight into which microbial types change in abundance and/or are culturable. For example, some cell types may undergo lysis, providing growth substrates for other organisms (6), thereby leaving a sample with the same total count but perhaps with a quite divergent microbial composition.

The total viable biomass could be determined from the phospholipid ester-linked fatty acids recovered directly from the samples (5). This technique could have been used to determine if the total viable biomass increased and, from a determination of the diglyceride content, the changes in degree of lysis with storage. But, as with direct counts, examination of the changes in phospholipid ester-linked fatty acid patterns might have indicated changes in cell types with storage; however, these changes would still not have provided definitive evidence for changes in the status of viable bacteria, i.e., culturable or viable but nonculturable (VBNC).

A particular focus in this study was the change which occurred in the composition of the recoverable microbial communities; some bacterial types were isolated on R2A agar only upon initial sample plating, some only after sample storage for 1 week at 4°C, and some at both sample times. The isolates recovered at both sample times often comprised different proportions of the total recoverable community at the two storage times. There did not appear to be a pattern in the types of bacteria that underwent changes in abundance during sample storage. For example, both gram-negative and gram-positive organisms were recoverable at all time points and there was not a predominant change in community composition from gram-negative to gram-positive organisms or vice versa. However, within individual samples, the types of organisms recovered during sample storage often changed dramatically. For example, in sample N2-N2*, five clusters (eight organisms), including an actinomycete that represented 46% of the before-storage community, were lost during storage and were replaced by two clusters (*Arthrobacter* and *Pseudomonas* spp.) which made up 78% of the after-storage community.

The changes observed were not restricted to those organisms culturable on R2A agar. Although the results of tests for chemoautotrophic bacteria were below the level of detection in both before- and after-storage samples, the numbers of nitrogen-fixing and denitrifying bacteria exhibited changes (usually increases) in abundance with sample storage similar to that seen with culturable heterotrophs.

Several hypothetical causes can be proposed to explain the observed increase in culturable cell count and decreased diversity, including (i) the proliferation of specific microbial types encouraged by sample perturbation or storage conditions, (ii) previously nonculturable cell resuscitation due to sample handling, and (iii) a combination of the two phenomena.

Although the inherent spatial variability of microbiota within the rock could have impacted the results, it is unlikely that heterogeneity accounted for all of the changes observed during storage. It is statistically unlikely that all 11 samples would have demonstrated increased culturable counts after storage. Likewise, diversity and evenness decreased in most samples. Samples analyzed subsequent to this study with replicates of crushed and homogenized rock have shown that heterogeneity between replicates consistently decreased during storage (unpublished data).

The appearance of, or increase in, bacterial populations after sample storage adds evidence to support the growth hypothesis. Results of dendrogram analyses indicated that some microbial populations were recovered exclusively, or comprised increased proportions of the total recoverable community, after storage. Colony types appearing only after storage may have been rare types, below the level of detection upon initial plating but detectable after growth. Organisms recovered only after sample storage (or in increased proportion) would be expected to be successful competitors for resources. Results presented here support this assertion, because isolates recovered only after sample storage often exhibited an ability to use a wider array of carbon sources in the API rapid NFT strips and had shorter generation times than isolates recovered only before sample storage. Mason and

Hamer (23) have suggested that microbes capable of utilization of a wide variety of carbon sources may be capable of cryptic growth, i.e., growth without added carbon sources. No carbon was intentionally added to the stored rock samples or the MPN tubes, but increases in cell abundance were observed in both instances.

Results from this study also support the resuscitation hypothesis. Results from this and other studies suggest that VBNC organisms may represent a large portion of natural microbial communities and that they may be resuscitated by physical and chemical means (10, 18, 20, 25). Evidence pointing to the presence of VBNC cells before sample storage includes the recovery of new bacterial types found only after storage that were often unrelated (large Euclidean distances) to any isolates recovered before storage. Further, it is improbable that an organism, such as that seen in cluster 5 of Fig. 1B, which was not detectable upon initial plating came to represent 37% of the after-storage community by proliferation in an environment with no nutrient addition and a restrictive growth temperature (4°C). Byrd et al. (10) noted increases in the numbers of culturable organisms in aqueous microcosms without addition of exogenous nutrients, demonstrating that nutrient availability for growth may not be the only factor responsible for the increase in the culturable count. Low temperature may not only have inhibited growth but may have facilitated the resuscitation of dormant bacteria stored in rock samples. Nilsson et al. (25) have shown temperature to be important in the resusitation of VBNC organisms. Other physical factors may also be necessary for microbial resuscitation, including unavoidable perturbations caused by sampling and sample handling (19). Although new bacterial types may have been initially rare (below the level of detection) and were subsequently detected after growth, it cannot be definitively proven that this is the case and the supporting evidence described above suggests that a second phenomenon occurred.

The recovery of MPN isolates further supports the resuscitation hypothesis. The MPN isolates were determined to be different from those recovered on R2A from the same samples by comparison of colony and cellular characteristics, API rapid NFT profiles, and MIDI identifications. Even though these isolates were capable of growing on R2A, they may have required resuscitation in carbon-free media. Lopez and Vela (20) demonstrated that nonculturable bacteria could be cultured only after resuscitation in a soil extract medium. Others have demonstrated that, like organisms in this study isolated in carbon-free media, some bacteria can be recovered only on low-nutrient or nonselective media and these organisms can be transferred to high-nutrient (21) or selective (1, 7) media after initial resuscitation. For the MPN isolates, R2A would represent the high-nutrient (selective) medium on which only those organisms that were resuscitated could be recovered.

In light of evidence for both the growth and resuscitation hypotheses, it is likely that the combination hypothesis (growth and resuscitation) is needed to explain the phenomena that occur during storage of rock. Some microbial populations increase significantly in number during sample storage, likely because of proliferation, while some microbial types, especially those with slow growth rates that only appear after sample storage, may represent VBNC organisms that were resuscitated by some process of sampling or sample handling. In any case, microbial communities change with storage and thus it is imperative that samples be immediately analyzed, especially if comparisons between samples are to be made. This may be especially important when monitoring environmental change, such as during bioremediation.

Microbial ecologists have frequently reported the recovery of different portions of microbial communities on various media (4, 16, 22, 26). Perhaps the different groups of organisms seen on specific media represent not only those whose growth requirements are being met but also those which have been resuscitated under the handling and growth conditions employed. Different bacterial types were recovered in this study not only because of the media used (R2A agar or carbon-free MPN media with subsequent transfer to R2A) but also because of the storage process. To realize full community potential, resuscitation conditions, as well as media and growth conditions, must considered.

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