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Diversity within a Colony Morphotype: Implications for Ecological Research

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Sets of bacterial isolates with the same colony morphologies were selected from spread plates of bacteria from deep subsurface rock samples; each set had a unique morphology. API-rapid-NFT analysis revealed that isolates within a set were the same. Fatty acid methyl ester analysis of one set of isolates clustered organisms within the same species, defining variation between isolates at the biotype (subspecies) and strain levels. Metal resistances consistently tracked with colony morphology, while antibiotic resistances were less reliable.

Random or representative colonies are usually selected for isolation and pure culture work in microbial ecology (4, 6, 7). Because accurate identification on the basis of morphotype is impossible, it is often necessary to make the assumption that colonies of the same morphotype represent the same species or biotype. Although researchers realize the limits of this assumption, much of the research of the deep subsurface has relied on selection and characterization of representative colonies with distinct morphotypes (1, 5, 10, 11). However, the extent of the variability within a colony morphotype is unknown. Of interest is the validity of using colony morphology as the selection criterion for calculating ecological indices of the diversity and equitability (evenness of distribution) of recoverable microbial communities.

Deep subsurface rock samples (ca. 400 m) were taken within the NO5 drift of U12n tunnel at Rainier Mesa, Nevada Test Site. The site and sampling techniques have been previously described (1). Ground rock from each sample was made into a slurry by dilution (1:10, wt/vol) with artificial pore water (1) containing 0.1% pyrophosphate. Rock slurries were diluted, plated, and incubated for 2 weeks at 24°C as previously described (1). Sets of isolates, each representing one morphologically distinct colony type, were chosen from spread plates of three separate rock samples designated SAM, RET, and AL. Isolates were purified on R2A agar (Difco) until two successive pure-culture plates were obtained. Isolates were profiled as previously described (1) in terms of colony appearance, cellular morphology, Gram stain, API-rapid-NFT test strip results, and resistance to nalidixic acid, ampicillin, tetracycline, and triple sulfa. Resistance to zinc and mercury was tested by placement of 0.64-cm-diameter analytical paper discs (Schleicher and Schuell) containing 150 and 25 μg of zinc chloride and mercuric chloride, respectively, onto bacterial lawns spread onto R2A agar. All incubations were at 24°C.

Colonies of the same morphotype were chosen from three rock samples, each of which had been treated in a different manner. Sixteen morphologically identical colonies were chosen from three replicate plates of the AL rock sample, which had been crushed and stored in the dark for 1 week at 4°C in artificial pore water containing 150 μg of rifampin per ml. Eighteen colonies were selected from a single plate of the RET rock sample, which had been crushed and stored for 24 h at 24°C. Twenty colonies were selected from three plates of the SAM rock sample, which had been stored at 4°C for 6 h in large pieces (approximately 5 to 10 g each) to minimize the effects of perturbation.

Microscopic observation showed all isolates within sets to be gram-negative rod-shaped bacteria. Sets were distinguishable from one another by microscopic observation of size and shape and by colony morphology on agar plates.

API-rapid-NFT results showed little difference between isolates within each of the three sets. One RET isolate (6% of the set) differed in β-galactosidase activity, while no differences in API-rapid-NFT results between isolates within the SAM and AL sets were observed. The API data base was not able to match any of the isolate profiles to known bacteria at acceptable or higher confidence levels (≥80% identity) (2). At 48 h of growth, AL isolates keyed to *Moraxella phenylpyruvica*, but when the strip was read at 72 h (because of slow growth), no match was found in the code book. SAM isolates keyed to *Flavobacterium indologenes*, but at a low level of confidence (69%).

Zones of inhibition around metal- and antibiotic-containing discs were measured to further characterize the sets of isolates. Isolates from a single set, displaying the same API physiological profile, showed variability in resistance to the metals and antibiotics tested (Table 1), especially with the antibiotics. Fredrickson et al. (12) have shown a significant relationship between plasmid-bearing deep subsurface isolates and resistance to ampicillin compared with nonplasmid-bearing isolates. However, multiple attempts to obtain plasmid DNA from selected isolates by using the method of Portnoy and White (9) failed to demonstrate the presence of plasmids. However, with this extraction method, we have demonstrated the presence of plasmids in other subsurface isolates and control strains (data not shown).

There may be many reasons why organisms within a set do not respond similarly to antibiotics. The variability may be due to minor genetic differences between isolates (3, 13), gene expression, glycocalyx production (8), inoculum size, or other unknown variables. Variability in the sensitivity patterns of isolates to antibiotics was observed when the initial inoculum was not uniform in age; i.e., an inoculum from the same plate tested on successive days demonstrated different zones of inhibition. Incubation time was also found to be critical for ampicillin resistance testing. Cultures that were resistant at 48 h of incubation demonstrated clear zones of inhibition at 24 h. In contrast, metal resistance data were quite uniform and did not vary with length of incubation or age of inoculum. There was little variance in the inhibition

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TABLE 1. Sizes of zones of inhibition[a] from metals and antibiotics[b]

Isolate	Zone of inhibition (mm) in response to:				
	Antibiotic			Metal	
	Nalidixic acid	Ampicillin[c]	Tetracycline	Zinc	Mercury
RET	51 (13)	0 (0)	66 (10)	21 (3)	19 (1)
AL	50 (3)	22 (21)	41 (3)	12 (1)	17 (3)
SAM	57 (7)	17 (12)	66 (6)	18 (4)	15 (2)

[a] Standard deviations are in parentheses.
[b] All isolates were resistant to triple sulfa.
[c] Organisms resistant to ampicillin were considered to have an inhibition zone of 0 mm.

zone around metal-containing discs, either within a set of isolates or when individual isolates were tested in triplicate.

Fatty acid methyl ester (FAME) analysis was performed on the 18 RET isolates by using the Microbial Identification System (MIDI; bacterial Trypticase soy broth agar REV 3.60) according to the method of Osterhout et al. (14). A dendrogram provided by the MIDI system clustered RET isolates into relatedness groups on the basis of a Euclidean distance scale. Although no formal interpretation of the Euclidean distance scale as it relates to microbial taxonomy exists, results obtained by others (15) suggest that samples linked below 2, 6, 10, and 25 Euclidean distance units are from the same strain, biotype (subspecies), species, and genus, respectively. FAME analysis demonstrated that all 18 RET cultures were within the same genus, 17 were within the same species, 13 were within the same biotype, and 2 were within the same strain (Fig. 1). It is interesting that the RET 1 isolate, which grouped the furthest from the others in the dendrogram, was the only organism within the set of 18 RET isolates to test negative for β-galactosidase activity.

The RET isolates were not identified within the MIDI data base, but were shown to be related to gram-positive bacteria by their total lipid profiles. Although Gram staining demonstrated all 18 RET cultures to be gram negative, this result was not completely unexpected. Other deep subsurface bacteria have been isolated which stain either gram negative or variable but, by sequencing the 16S genes coding for rRNA, have been found to be related to *Arthrobacter* spp. (1).

The AL isolates may have originated from a single progenitor, increasing in number during the week-long incubation in artificial pore water containing rifampin. Although refrigerated, viable counts increased (2.5-fold) in the AL sample during the week of storage. The AL colonies showed the most similarity to each other in terms of nalidixic acid, tetracycline, and zinc resistance on the basis of calculated standard deviations (Table 1).

Although no nutrients were added to the RET rock sample during storage for 24 h at 24°C, 2 of the 18 isolates represent the same strain by MIDI analysis and may have arisen from outgrowth of a single cell or could have been two surviving identical cells cultured separately. Viable plate counts increased (60-fold) in the RET rock sample during storage. Even if some of the similarity between isolates could be attributed to outgrowth of a few original rock organisms, most of the 18 RET isolates do not fall within the same strain, although they easily fall within the same genus; all but one fell within the same species by MIDI analysis.

The SAM rock sample was not treated, to minimize changes in the microbial community; it was left unaltered until sample analysis. The standard deviations for metal and antibiotic testing were not different from those of the other isolate sets that had been treated in different manners (Table 1). It is doubtful that the similarity between isolates was due to outgrowth of individual cells in this case.

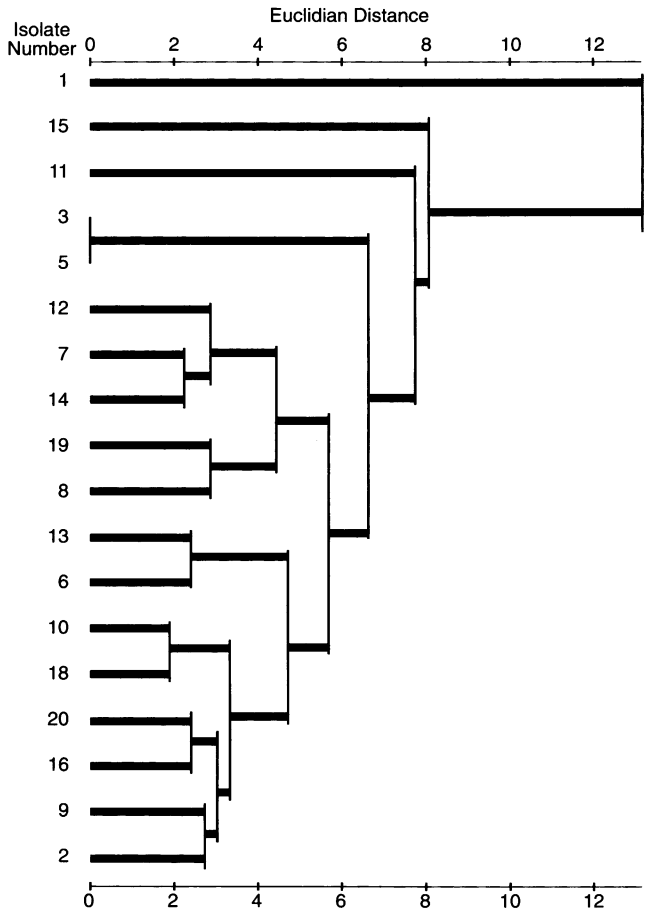

FIG. 1. Dendrogram showing relatedness of RET isolates by FAME analysis. Organisms paired at Euclidian distances less than 2, 6, 10, and 25 imply that isolates are the same strain, biotype (subspecies), species, and genus, respectively (15).

In this study, isolates within sets were very similar, especially in terms of colony morphology, microscopic appearance, resistance to metals, and response to API-rapid-NFT tests. Resistance to antibiotics proved variable within sets of isolates, even when the same strains (by MIDI analysis) were compared. Therefore, antibiotic resistance data are not as reliable as the previously mentioned tests for describing a representative of a population. MIDI analysis has been shown to correlate very well with rRNA and genes coding for rRNA homologies in *Pseudomonas* sp. (16) and therefore provides a fast and reliable means of comparing the relatedness of isolates.

Selection of a representative colony by morphotype for pure culture work did not account for all of the diversity within a population, as evidenced by FAME analysis of RET isolates. It does reflect organisms of a similar type, organisms shown to be identical by API-rapid-NFT analysis, and organisms shown to be the same species by FAME. Al-

though taxonomically the organisms appear the same, the differences in lipid profiles may reflect the long-term isolation of bacteria in microniches.

FAME analysis can prove useful for distinguishing between isolates of the same species or biotype; however, isolates belonging to the same biotype can be selected by morphotype, and this level of similarity is used in most diversity indices. Therefore, colony morphology can provide an accurate basis on which to define recoverable diversity.

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