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Diversity among bacteria isolated from the deep subsurface

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Culturable bacteria from the deep subsurface (179 m) at Cerro Negro, New Mexico were isolated and characterized. The average number of viable aerobic bacteria was estimated to be 5×10^5 g^{-1} of sediment, but only about 0.1% of these could be recovered on agar medium when incubated under aerobic conditions. Of 158 strains isolated from this depth, 92 were characterized by cellular fatty acid profiles (FAME), 36 by analysis of partial 16S rDNA sequences, and 44 by rep-PCR genome fingerprint analysis using three different sets of oligonucleotide primers (REP, BOX, or ERIC). These analyses showed the majority of isolates (67%) were Gram-positive bacteria and primarily members of genera with a high %G + C DNA. The remaining isolates were α-subdivision *Proteobacteria* (19%) and members of the flavobacteria group (14%). The diversity indices based on these different methods of characterization were very high suggesting this subsurface habitat harbors a highly diverse microbial community.

Keywords: heterotrophic bacteria; diversity; deep subsurface; 16S rDNA; FAME; rep-PCR

Introduction

The genotypic and phenotypic diversity within a microbial community may increase as the physical or chemical complexity of the environment increases [4]. This complexity would cause physical isolation of bacterial cells and the development of chemically varied and unique ecological niches that would promote specialization and speciation of the bacterial strains present as they adapt to the existing conditions. Deep subsurface sediments represent one such environment where the microbial diversity should be high because bacterial cells have persisted in a physically and chemically complex oligotrophic environment for at least thousands and perhaps millions of years. Previous investigators have shown there is a high level of phenotypic diversity among bacteria isolated from paleosols (buried ancient soils), lacustrines (lake sediments), fluvial sediments (river sediments), basalts and clay formations, belonging to geologically distinct regions, namely: Savannah River Site (S Carolina), Nevada Test Site, Hanford Site (Washington), Lula aquifer (Oklahoma), Parachute Creek Site (Colorado), and Mol (Belgium) [2,6,7,11–14,19,20,22,23,30,32,47].

The characterization of microbial communities and populations from the deep subsurface suggests these vary considerably from site to site and between various depths at any particular site. As a result, it is difficult to reach general conclusions about the microbial diversity of the subsurface. Nonetheless, it seems clear that deep subsurface bacterial populations differ taxonomically from those found in surface sediments at the study sites [2,12,26,48]. The density and diversity of bacterial populations do not decrease directly with the depth (or the age of deposition) of the sediment [12]. Although there are undoubtedly numerous factors that influence the bacterial diversity at any given site, sediments with higher moisture content and influenced by high water recharge usually have greater numbers of bacteria than adjacent sediments with lower moisture levels [29]. Gram-negative bacteria predominate in the former sediments, whereas Gram-positive bacteria are more common in the latter sediments [13]. The culturable bacteria from deep subsurface sediments include aerobic heterotrophs belonging to the α, β, γ-proteobacteria, Gram-positive bacteria of low and high %G + C content (including a group of *Streptomyces*) [2,6,7,11,13,14,19,22,24,32,39,47]; anaerobic bacteria (including methanogens and sulfate reducers) [7,30,32,37]; and chemolithotrophs [20,49]. While significant diversity has been found in culturable populations, it is clear that a large fraction of many subsurface microbial communities has not been cultured [13,24,26,30] and may represent bacterial populations that cannot be cultured by methods commonly used [44,52] or are nonculturable bacteria [27,29]. These studies indicate that the microbial diversity of many subsurface environments is quite high.

In this study we isolated and characterized aerobic heterotrophic bacteria from a deep subsurface sample obtained from Cerro Negro, New Mexico. The heterotrophic bacteria isolated on plate cultures were characterized using a combination of three different methods including the analysis of partial 16S rDNA gene sequences, profiles of total cellular fatty acids (FAME), and comparison of rep-PCR genomic fingerprints.

Materials and methods

Geology of the site and sampling procedures

Sediment cores were aseptically retrieved from a vertical bore hole located on the San Juan basin at Cerro Negro, New Mexico. These sediments are interbedded Mancos Shale and Dakota Sandstone that have an estimated age of 90–100 My [5]. The sediments were of near-shore marine origin that had been deposited during the Late Cretaceous period. The sample used in this study was from 178.9 m

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below the surface level at the boundary of Paguate Sandstone and Clay Mesa Shale. The core sample was collected using aseptic air rotary drilling methods [15]. The core sample was processed as described by Colwell *et al* [15], pulverized at site, maintained at 4°C during overnight shipment to the laboratory, and stored at 4°C.

Media and buffers

Three different types of agar media were used for isolation of bacteria: R2A (Difco Co, Detroit, MI, USA), and LF or HF media. The latter two were based on artificial freshwater (FDB) that mimics the composition of groundwater at the site. LF and HF were FDB supplemented with yeast extract (0.5 and 2.5 g L^{-1}), peptone (2.5 and 10.0 g L^{-1}), as well as Tween 40 and Tween 80 (15 and 500 mg L^{-1}), respectively (Balkwill DL, Florida State University, Tallahassee, FL, personal communication). FDB contains: 0.1 mM NaF, 5 μM KBr, 0.1 mM KCl, 1 mM $CaCl_2$, 5 mM Na_2SO_4, 0.35 mM $MgCl_2$, 3.5 mM NaCl, 0.8 mM Na_2SiO_3, 11 mM $NaHCO_3$ (Balkwill DL, personal communication). Phosphate buffer (PB) contained 10 mM KH_2PO_4 and 8 mM Na_2HPO_4 adjusted to pH 7. All chemicals were from Sigma (St Louis, MO, USA).

Enumeration of viable bacteria

Viable bacteria were estimated by counting red-colored cells using a microscope Axioscop (Carl Zeiss, Thornwood, NY, USA) after staining with *p*-iodonitrotetrazolium (INT) [13,51]. Usually 30–40 fields were counted to give statistically valid results.

Enumeration and isolation of culturable bacteria

Pulverized sample (0.1–0.3 g) was dispersed in 1–3 ml of PB and vortexed vigorously for 3 min prior to plating on appropriate agar medium. Aliquots of undiluted or serially diluted suspensions of sediment (0.1 ml) were plated in triplicate onto agar plates. All manipulations were done in a sterile Laminar-flow biosafety cabinet (Model NU-425-600, Nuaire, Plymouth, MN, USA). Plates were sealed with Parafilm and incubated at room temperature (RT) for 2–4 weeks. After that period of time, the plates were stored at 4°C for 2 more months. Colonies were counted after 1, 2, 4 and 8 weeks of incubation. Aerobic heterotrophic bacteria were purified by restreaking single colonies on R2A medium. This procedure was repeated in six independent experiments performed during a 12-month period.

Stock cultures were prepared by resuspending well-isolated colonies in 1.0 ml of PB that contained 10% DMSO and stored at –70°C.

Characterization of the bacterial populations

Total cellular fatty acids were extracted from 0.2–1.0 g of cells scraped from 3 to 10-day old R2A plates [21]. Methyl esters of the fatty acids were prepared as previously described [21] and the profiles of fatty acid methyl esters (FAME) were analyzed using the MIDI gas chromatograph-software system (Microbial Identification Systems, Inc, Newark, DE, USA). The most likely matches to the isolate among the library strains were expressed by a similarity index. Indices of 0.6–1.0 indicate an excellent match; however indices of less than 0.3 may indicate some relatedness to the matching strain in the database [41]. The data were used by the Microbial Identification Systems' cluster analysis package 'Dendogram' [41] to obtain a topological relatedness of pairs of entries expressed in Euclidean Distances (ED). According to the manufacturer's recommendation, ED of approximately 25, 10, and 6 represent relatedness of the entries at the genus, species and subspecies levels, respectively [41].

PCR amplification of 16S rDNA genes was done using as a template total genomic DNA that had been isolated from several large colonies of each strain using a previously published protocol [46]. The universal primers pA (8F, AGA GTT TGA TCC TGG CTC AG) and pH* (1542R, AAG GAG GTG ATC CAG CCG CA) [17] were used. Following PCR amplification [34] the resulting product was purified by electrophoresis using a 1% agarose gel and 1.4-kb fragment was cut from the gel and cleaned using a Geneclean II kit (Bio 101, Frederick, MD, USA), or Wizard PCR Preps #A7170 (Promega, Madison, WI, USA). Automated fluorescent sequencing was performed by the MSU-DOE-PRL Plant Biochemistry Facility at Michigan State University using the ABI Catalyst 800 for Taq cycle sequencing and the ABI 373A Sequencer for the analysis of products. The DNA sequence was determined using pA or pD* (536R, GTA TTA CCG CGG CTG CTG) oligonucleotide primers [17]. The 16S rDNA gene sequences were compared with those found in GenBank using the BLASTN algorithm [1], and visually aligned with sequences specific for eubacterial subdivisions found in the RDP Database. The sequences were analyzed for maximum parsimony phylogeny by PHYLIP 3.5 software [18]. The bootstrap support was estimated after 100 replications using the DNABOOT algorithm. The percentage of bootstrap resamplings that support the branching topology was 100%.

Rep-PCR genomic fingerprints were obtained using previously described methods [43]. The DNA fragments obtained by PCR amplification using REP, BOX-I, or ERIC primers [43] were separated by 1.75% agarose gel electrophoresis and stained with ethidium bromide. The gel images were scanned using a ScanJet 3C Scanner (Hewlett-Packard, Wilmington, DE, USA) and analyzed using a GelCompar version 3.1 software (Applied Maths, Kortrij, Belgium). A similarity matrix was generated using the Pearson product moment and cluster analysis was performed using the UPGMA method, as described by Schneider and de Bruijn [43].

Bacterial pigments were extracted from the whole cells using previously described methods [42] and analyzed spectroscopically using a diode-array spectrophotometer HP 8452A (Hewlett-Packard, Wilmington, DE, USA).

Calculation of diversity indices

The diversity found among the isolates from the subsurface was described using three indices of diversity. The Shannon–Weaver index of species diversity [3,4] was calculated using the following formula:

$$H = C/N(N \log N - \Sigma\, n \log n)$$

The species uniformity was calculated using the following formula:

$$U = \sqrt{\Sigma\ (m/n)^2}$$

And the equitability indices were calculated using the following formula:

$$J = H/(\mathrm{C}\ \log N)$$

Wherein N is the total number of isolates or subgroups in m groups; n is the number of isolates or subgroups within a particular group; and C is a constant (3.3219) [3,4]. All of the strains included in each analysis (cellular fatty acid profiles, partial 16S rDNA sequence homology, and rep-PCR genome fingerprint analysis) were included in the calculations and the final values for the Shannon and uniformity indices were normalized to the size of the set that contained the fewest strains. The equitability indices are independent of sample size [4].

Results

Density of bacterial population in the deep subsurface sediment

To determine the number of bacteria in sediment capable of growing in plate culture from 178.9 m beneath the surface at Cerro Negro, a suspension of pulverized rock in phosphate buffer was plated onto R2A agar or FDB-based agar media. A total of 158 aerobic heterotrophic isolates were obtained from 0.4 g of pulverized sediment on 62 R2A plates. The bacterial densities were calculated for each of the six independent experiments and did not vary significantly with the amount of time the pulverized sample had been stored at 4°C (Spearman's rank correlation; $P = 0.05$) [9]. The average number of colonies per R2A plate was three, and varied from 0 to 15. The average density of bacteria able to grow on R2A agar was 720 ± 200 CFU g^{-1} whereas on FDB-based agar the number was 50 ± 30 CFU g^{-1}, about one order of magnitude lower. Many of the isolates obtained required a long period of time to form colonies on agar plates: only 53% of the strains formed colonies after 1 week, whereas 18% required 2 weeks of incubation, 9% required 1 month and 20% were observed only after an additional 1–2 months incubation at 4°C.

All the isolates obtained from LF or HF agar plates could be regrown later on R2A agar. However, only about 46% of the strains isolated on R2A agar grew on FDB-based agar suggesting that R2A agar allowed for recovery of a larger proportion of the microbial community. Higher numbers of bacteria were obtained when more diluted suspensions of the sediment were plated onto R2A agar (Figure 1) suggesting the actual density of culturable bacteria in the sample might be higher. Extrapolation of the data from Figure 1 to very dilute suspensions (approximating 1–2 mg of the pulverized rock per plate), gives an estimated density of plateable bacteria on the order of 3000–4000 CFU g^{-1} of sample. The number of metabolically active bacteria in the same sediment was determined by direct optical microscopy using INT and was approximately 5×10^5 g^{-1} of sediment. Based on these data, it appears that only 0.1–1% of the total microbial community could be cultivated on R2A medium.

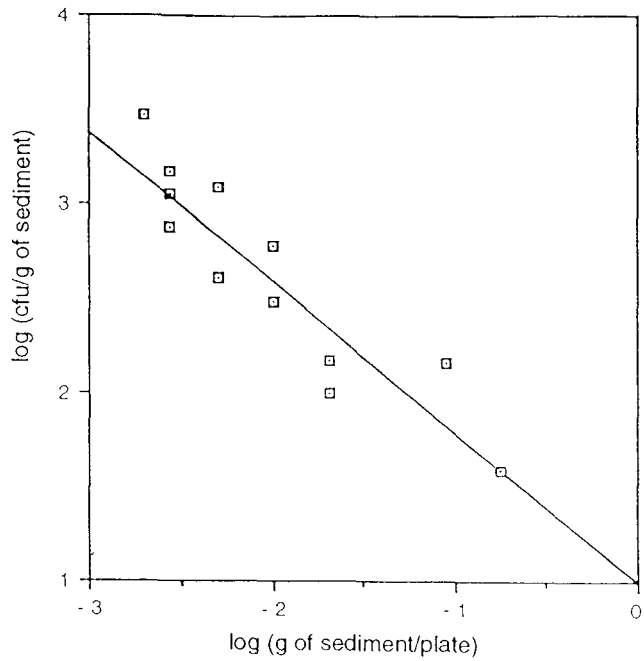

Figure 1 Relationship between the observed density of aerobic heterotrophic bacteria and the amount of sample (pulverized sediment) plated on R2A medium. The regression line was calculated by the least squares method and had a correlation coefficient (r^2) of 0.81.

Diversity among the isolates

The primary isolates obtained were initially characterized by colony and cell morphology. Many isolates (43%) formed small colonies on R2A plates. Microscopic examination showed that the cellular morphology of the strains varied and could be placed in five groups: coccoids (35%), rod-shaped bacteria (28%), budding bacteria (25%), filamentous cells (5%) and cells of unusual morphology (7%). Only about 4% of the isolates were found to form spores, and about 9% were motile. Most of the isolates obtained (70%) were pigmented (usually shades of red and yellow). Spectrophotometric analysis of the lipid fraction for some of the pigmented isolates showed 2–4 major peaks in the region from 390 to 540 nm, suggesting the presence of various carotenoids [42].

A subgroup of the isolates was characterized by determining FAME profiles, partial sequencing of 16S rDNA genes and rep-PCR genomic fingerprints. The FAME profiles of 92 isolates which grew well on R2A medium were determined (Figure 2) and the taxonomic relatedness was determined by cluster analysis. The isolates were found to be in three large clusters designated I, II, and III (Figure 2) which contained 48, 30, and 22% of the isolates, respectively. A majority of the isolates characterized in this study (designated as clusters I, IA and II in Figure 2) contained high proportions of branched fatty acids which are characteristic for many Gram-positive bacteria [28]. The remainder of the strains (cluster III in Figure 2) had two major fatty acids (16:0 and 18:1). Based on this, along with closest matches with the MIDI database, they were assumed to be Gram-negative bacteria. Based on Euclidean distances between strains, the 92 strains were classified into 76 distinct species belonging to 28 different

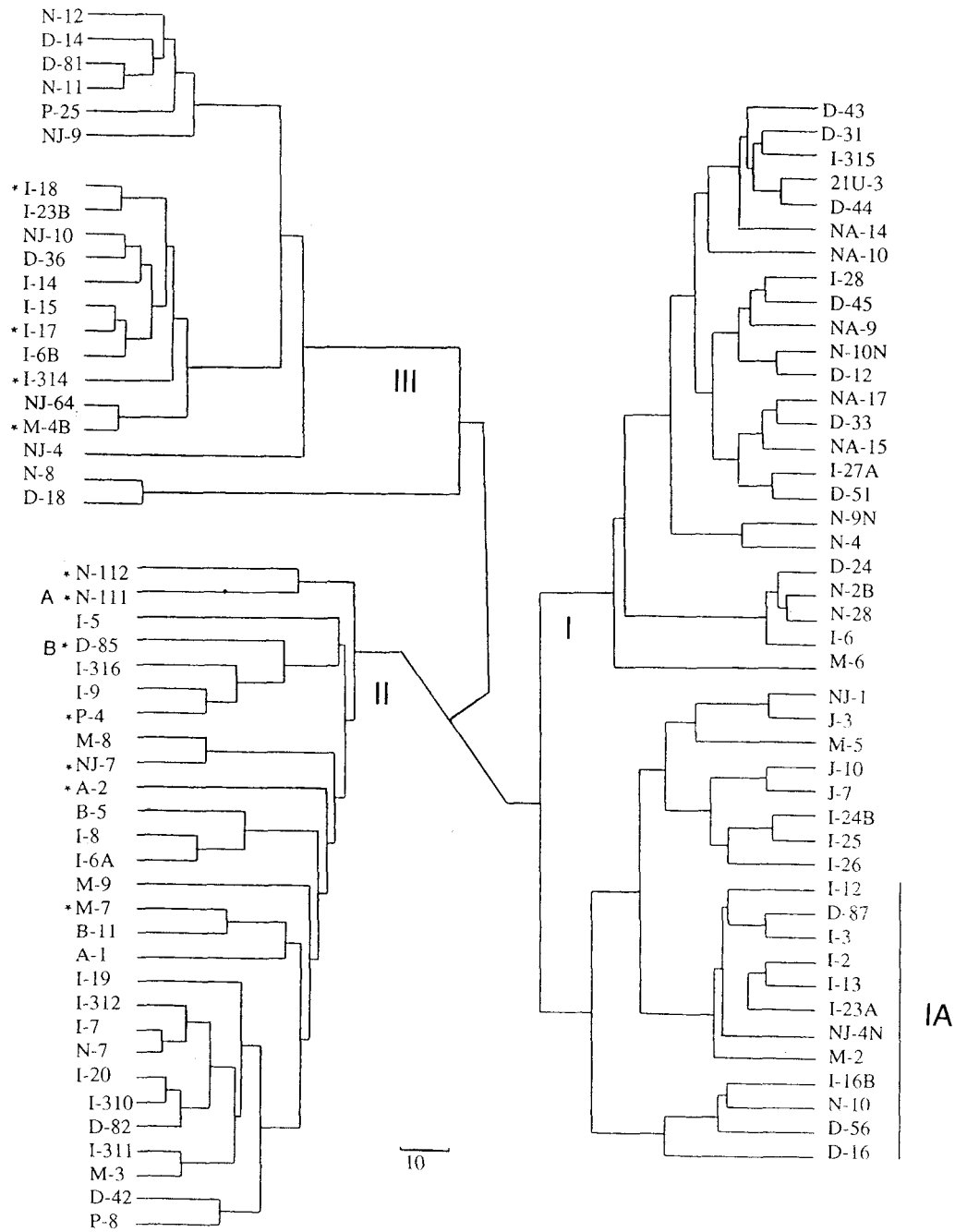

Figure 2 Dendrogram showing the relationship of 92 bacterial isolates based on their FAME profiles. Three major clusters and one subcluster are designated by Roman numerals. The bar represents 10 Euclidean distance units. Isolates with profiles that matched strains in the MIDI database with a similarity index of greater than 0.3 are designated with an asterisk. Only two strains, designated A and B, matched strains in the MIDI library with a similarity index of more than 0.6.

genera. Only two strains belonged to the same subspecies, and the remainder could be differentiated at the subspecies level. The profiles of most strains did not match those of bacteria in the MIDI reference library, and only 34 isolates (or about 37%) matched reference strains in the MIDI database to any degree at all. However, even for these 34 isolates, the probability of correct identification was generally low; only 11 isolates matched strains in the database with a probability of greater than 0.3, and only two of these with a probability of more than 0.6. None of the strains within the largest group (group I, Figure 2) corresponded to any of the strains in the database. Thus, few isolates could be classified using this procedure and most were previously uncharacterized strains.

The partial (400 bp) sequences of 16S rDNA genes amplified from 36 isolates were determined and compared against the GenBank library by using BLASTN. According to Bianchi and Bianchi [10], analysis of this number of strains is sufficient to estimate the extent of diversity among the culturable aerobic heterotrophic organisms. The

majority of the isolates appeared to be Gram-positive bacteria (67%), with most of these being bacteria with high %G + C content (53%). The remaining isolates were α-subdivision *Proteobacteria* (19%) or belonged to the flavobacteria group (14%). Only 10 isolates (28%) matched with at least 95% identity the 16S rDNA gene sequences of strains that had been previously deposited in GenBank suggesting that a large percentage of the isolates characterized were novel.

The classification of strains based on partial 16S rDNA gene sequences was compared to the classification based on FAME profiles (Table 1). Only five (14%) of the isolates were classified in the same genus by these two methods. In a few cases (strains I-5, I-14, and P-4) there was a large discrepancy between the two methods and the strains were classified in different divisions of the eubacteria.

The information gained from the analysis of 16S rDNA sequences provided more insight to the groupings found through FAME profile analysis. The sequences obtained from selected isolates from cluster I (Figure 2) showed that it was not homogeneous and contained a subgroup of Gram-negative flavobacteria (cluster IA), in which five of six members were characterized by 16S rDNA sequence analysis. Eight of nine strains from the remainder of cluster I (exclusive of cluster IA) were Gram-positive bacteria with high %G + C and the remaining strain was also Gram-positive but with low %G + C. Sequences obtained from strains within cluster II suggest this cluster contains Gram-positive bacteria with both high and low %G + C (11/14 and 2/14 strains sequenced, respectively). One of the 14 strains sequenced from this group was found to be a Gram-negative bacterium (strain M-9). From cluster III, most of the strains were found to be α-subdivision *Proteobacteria* (six of seven strains), while the remaining strain (NJ-10) was Gram-positive with low %G + C. Thus, all of the clusters contained both Gram-positive and Gram-negative bacteria, although each was predominantly a particular type.

The rep-PCR genomic fingerprints of 44 isolates were obtained using three different sets of oligonucleotide primers: BOX (Figure 3) and ERIC, or REP (data not shown). The genotypic relatedness of the strains using the fingerprints obtained using BOX oligonucleotide primers was

Table 1 Comparison of taxonomic position for some of the deep subsurface isolates as determined on the basis of partial sequence of 16S rDNA and FAME-MIDI analysis

Division	Strain	Best match in GenBank	S%[a]	Best match in MIDI library	SI[b]
α-Subdivision	D-14	*Rhizomonas suberifaciens*	97	NM[c]	
Proteobacteria	N-8	*Rhizomonas suberifaciens*	94	NM	
	I-6B	*Methylobacterium* sp	78	NM	
	I-14	*Rhodoplanes elegans*	92	*Enterococcus casseliflavus*	0.164
	M-4B	*Sphingomonas yanoikuyae*	96	*Sphingomonas capsulata*	0.334
	M-9	*Blastobacter natatorius*	88	NM	
	N-12	*Erytrobacter longus*	86	NM	
Flavobacteria group	D-87	*Microscilla sericea*	81	NM	
	I-2	*Microscilla sericea*	82	NM	
	I-12	*Microscilla sericea*	80	NM	
	NJ-4N	*Flavobacterium heparinum*	82	NM	
	I-16B	*Flavobacterium thalpophilum*	75	NM	
Deinococcus-Thermus group	M-2	*Deinococcus radiodurans*	92	NM	
High % G + C	I-5	*Arthrobacter globiformis*	92	*Bacillus macerans*	0.162
Gram-positive bacteria	I-7	*Arthrobacter globiformis*	91	NM	
	M-3	*Arthrobacter globiformis*	93	NM	
	M-6	*Arthrobacter simplex*	90	NM	
	21U-3	*Arthrobacter simplex*	86	NM	
	D-85	*Aureobacterium testaceum*	94	*Aureobacterium barkeri*	0.612
	I-24B	*Blastococcus aggregatus*	95	NM	
	I-27A	*Blastococcus aggregatus*	93	NM	
	N-9N	*Blastococcus aggregatus*	96	NM	
	I-19	*Micrococcus agilis*	94	NM	
	M-8	*Micrococcus agilis*	92	NM	
	N-111	*Micrococcus luteus*	97	*Arthrobacter ramosus*	0.867
	N-112	*Micrococcus luteus*	96	*Micrococcus luteus*	0.372
	A-1	*Micrococcus lylae*	94	*Micrococcus kristinae*	0.170
	J-3	*Micrococcus sedentarius*	97	NM	
	I-9	*Microbacterium lacticum*	92	NM	
	P-4	*Microbacterium lacticum*	96	*Rathayibacter tritici*	0.307
	M-5	*Streptomyces tnedae*	90	NM	
	I-6	*Nocardioides luteus*	95	NM	
Low % G + C	M-7	*Staphylococcus epidermidis*	90	*Rathayibacter rathayi*	0.483
Gram-positive bacteria	NJ-10	*Bacillus brevis*	89	NM	
	I-25	*Bacillus benzeovorans*	94	NM	
	B-5	*Bacillus licheniformis*	78	*Bacillus brevis*	0.201

[a]Percentage of similarity between the sequence and that from GenBank.
[b]Similarity index for the best match in the MIDI library [41].
[c]NM: Isolate had no match with isolates in the MIDI library >0.1.

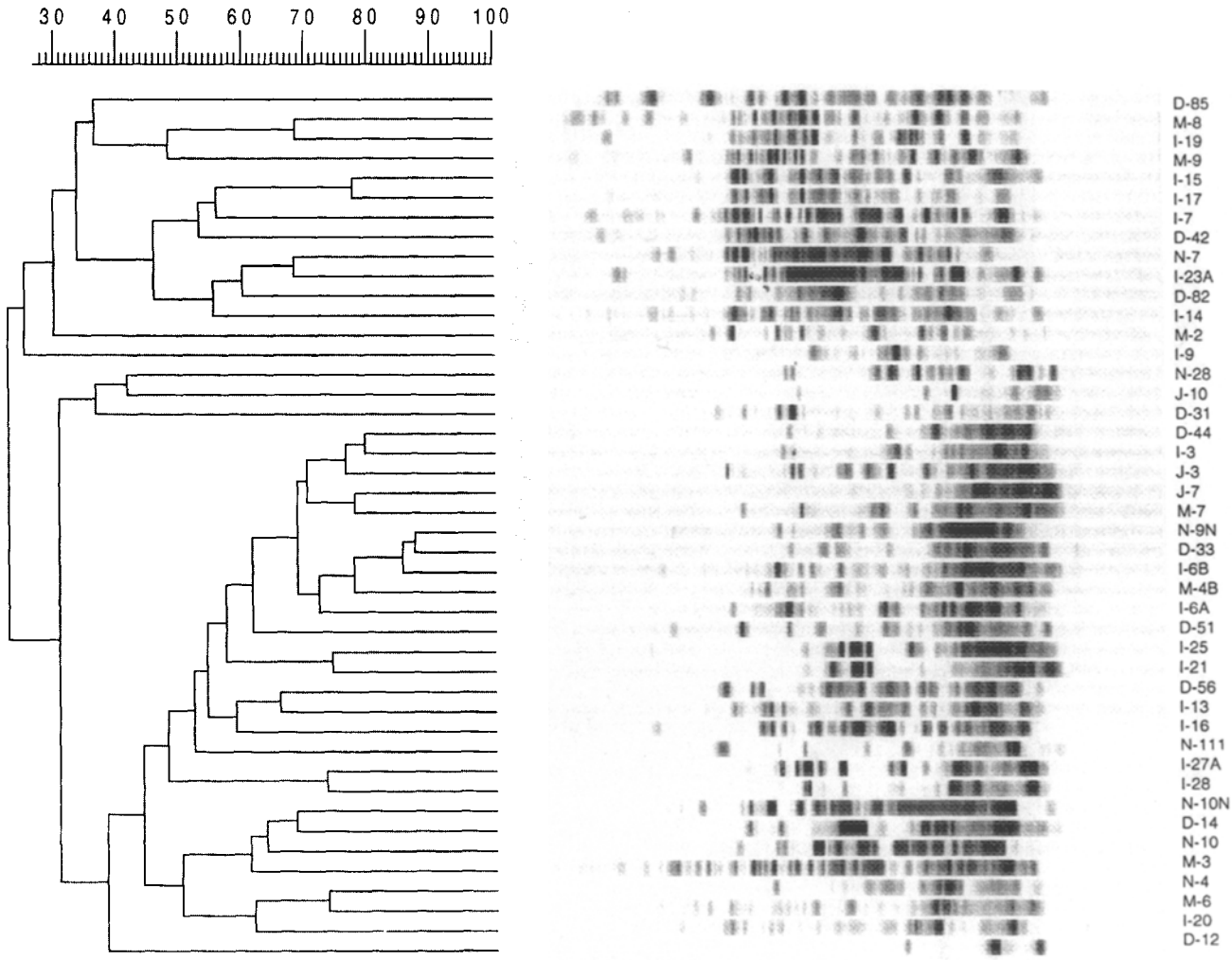

Figure 3 Similarity for rep-PCR genome fingerprints of 44 isolates produced using BOX oligonucleotide primers.

assessed using GelCompar version 3.1 software. These data show that the fingerprints of all the isolates were unique from one another suggesting that the genetic diversity of the deep subsurface sediment was very high.

The Shannon–Weaver, equitability, and uniformity indices were calculated to describe the diversity of plateable, aerobic heterotrophic bacteria in the sediment. The Shannon–Weaver Index (H) is a commonly used measure of diversity that is sensitive to both species richness and relative species abundance [3]. The equitability index (J) is derived from the Shannon–Weaver index and reflects how the observed diversity varies from the maximal diversity [3]. In contrast to uniformity and Shannon Index, equitability is independent of sample size. The uniformity index (U) describes the population on the basis of species evenness within the community [3]. Data for FAME profiles for 92 isolates as well as 16S rDNA sequence and rep-PCR genomic fingerprint data (containing 36 and 44 isolates, respectively) were used in these calculations. Where possible, the indices were calculated for various taxonomic levels that included subdivision, genus, species, subspecies, and strain (Table 2). The Shannon and equitability indices for diversity increased and the uniformity index decreased with increasing taxonomic resolution. All three indices reached the theoretical maximum for diversity at the strain level since no two isolates were identical to one another. The diversity indices for species calculated using data obtained with various taxonomic methods were in close agreement (Table 2), suggesting that each method was capable of distinguishing the isolates.

Discussion

Density of bacteria in the deep subsurface sediment

There is no single medium that allows one to isolate all culturable bacteria from an environmental sample and there can be significant differences between the kinds of strains cultivated using different media [23]. In this study, we used three kinds of synthetic media for isolation of bacteria from Paguate sandstone sediment from Cerro Negro. Thus, it was not surprising to find differences in the numbers of bacteria cultivated using R2A agar [38], and two other (LF and HF media) based on FDB. R2A agar provided for the recovery of the greatest number and diversity of bacteria from the sediment since all the isolates from LF or HF agar media could be regrown on R2A agar, but only about 46% of the

Table 2 Indices of microbial diversity among bacteria isolated from Paguate sandstone at various taxonomic levels as determined by three independent methods

Taxonomic level	Shannon–Weaver (*H*)			Uniformity (*U*)			Equitability (*J*)		
	16S[a]	FAME[b]	rep[c]	16S	FAME	rep	16S	FAME	rep
Subdivision	1.84	2.20	–[d]	0.59	–	–	0.36	0.43	–
Genera	3.96	3.43	–	0.29	0.42	–	0.77	0.66	–
Species	4.50	4.86	4.50	0.22	0.20	0.26	0.87	0.94	0.85
Subspecies	–	5.15	–	–	0.17	–	–	0.99	–
Strain	–	–	5.17	–	–	0.17	–	–	1.00

[a]16S: Sequences of 16S rDNA genes.
[b]FAME: Profiles of cellular fatty acid methyl esters.
[c]rep: Genomic fingerprints obtained by rep-PCR.
[d]–: Not calculated.

strains isolated on R2A agar could grow on FDB-based agar. The reasons for the lower number of bacteria on the FDB-based agars are unknown, but may relate to differences in the mineral composition and carbon sources found in the different media used. For example, R2A contains carbohydrates and organic acids, whereas both LF and HF agar media contain Tween 40/80 as carbon sources (all three media contain amino acids and yeast extract). Moreover, FDB-based media contain fluoride which may be toxic for some bacteria [33].

The fraction of the total microbial community that could be cultivated on R2A agar was rather low. There may be several reasons for this. First, we found that the recovery of bacteria was increased if diluted samples were plated suggesting there are components of the sediment that are toxic, perhaps only ones solubilized through the addition of dilution buffer. Second, we analyzed only aerobic heterotrophic bacteria which grew on solid medium. Thus, other physiological groups of bacteria (for example, anaerobic and autotrophic bacteria) would not have been recovered, and organisms with special nutritional requirements would have been excluded. Finally, the microbial community may well contain nonculturable bacteria or bacteria that are culturable but which do not grow on solid media [44,52].

Characteristics of isolates

It was difficult to determine the FAME profile of many strains isolated from the subsurface either because they grew poorly on R2A plates, or because the lipids were not efficiently extracted. The former might reflect the very slow or 'pseudo-stationary' growth of these strains [36], or the 'stable starvation-survival' [50] growth of many oligotrophic bacteria. Analysis of FAME profiles of 92 isolates showed that many of the strains have a high percentage of fatty acids (like *cis*-vaccenic acid or cyclopropyl fatty acids), which have been previously observed in bacteria adapted to stress conditions [21,31,36]. Most of the bacteria analyzed fall into groups of unknown bacteria, having no matches in the MIDI library, which supports the conclusion that most of these represent novel bacterial strains.

Brockman *et al* [13] suggested that Gram-positive bacteria may be predominant in sediments with low moisture content or that experience infrequent water recharge events. A majority of the isolates characterized in this study had cellular fatty acid profiles and 16S rDNA sequences that are characteristic of Gram-positive bacteria and the flavobacteria. The predominance of Gram-positive strains among the aerobic heterotrophic isolates obtained in this study is similar to other deep subsurface sediments with low moisture content [6,13,25,47]. Among the isolates obtained from this subsurface sediment were Gram-positive spore-forming bacteria (at least 4% of the isolates) and strains of *Deinococcus* (about 5% of the isolates), both of which are bacterial groups known to have specialized mechanisms to survive long-term desiccation [35,45]. The means used by other bacteria to survive the presumably adverse conditions are unknown and worthy of further study.

Diversity among the bacterial populations cultured

Analysis of the distribution pattern of the bands obtained following electrophoretic separation of three different rep-PCR amplified fragments showed that no one isolate from the set was exactly the same for all of the primers used. Although a small portion of the isolates did show a rather good similarity level based on REP primer (generally giving a smaller number of amplified fragments), using another primer from the set (BOX or ERIC, which yield a more complicated pattern of amplified fragments) revealed much bigger differences between the isolates. As an example, the distribution pattern of the bands obtained after separation of the BOX-PCR amplified fragments (Figure 3) shows that no two isolates from the set were exactly the same. The taxonomic tree based on rep-PCR data differed significantly from those based on FAME profiles or 16S rDNA sequences which is consistent with previous studies that have shown the method is most useful for distinguishing strains of the same species and is of limited use in the classification of bacterial species [43].

There was rather good agreement between classification of strains based on 16S rDNA sequences and FAME profiles, at least at a coarse level, since both methods placed Gram-positive and Gram-negative bacterial strains into coherent groups. There was also reasonable agreement between the methods in their classification of bacteria within divisions of the eubacteria and various bacterial genera. Moreover, the methods cluster pairs of strains that

appear to be closely related based on colony and cell morphology such as I-27A and N-9N; M-8 and I-19; as well as I-6B and I-14. There were occasional discrepancies between the two methods. For example, strain M-9 was grouped with Gram-positive bacteria based on its FAME profile, but found to be phylogenetically related to *Blastobacter* (a Gram-negative Proteobacterium) based on the sequence of the 16S rDNA gene. This discrepancy suggests this strain has an unusual lipid composition for a Gram-negative bacterium, and instead has one that more closely resembles those found in Gram-positive bacteria.

The data obtained suggest the microbial community of the subsurface sediment was exceedingly diverse. Based on FAME profiles, the 92 strains were classified into 76 distinct species belonging to 28 different genera and based on rep-PCR genomic fingerprints, no two strains were identical to one another. The diversity of aerobic heterotrophic bacteria isolated from the subsurface was clearly evident from the diversity indices calculated from the various data sets. It is unknown what processes or factors lead to the maintenance of such high diversity with this ecosystem.

Bacteria of possible marine origin from the deep subsurface

The sample used in the investigation was retrieved from the depth of 178.9 m below the surface and was deposited more than 90 million years ago in a shallow marine environment [5]. Therefore, it was of particular interest to determine if any of the bacteria cultivated from these sediments were phylogenetically related to those found in contemporary marine environments, and might potentially be descendants of those originally deposited with the sediments. This approach has been recently applied for analysis of bacteria from a continental oil reservoir [25]. Only a few bacterial genera have been described that are thought to exist solely in marine habitats [40]. Some of these genera are culturable but do not form colonies on agar media [44] and thus would not have been recovered by the methods used here. Analyses of partial 16S rDNA gene sequences showed that at least three strains were phylogenetically related to the genus *Microscilla* (Table 2). This genus is thought to contain bacterial species that are exclusively found in marine environments [8] and are among the most abundant microorganisms found in microaggregates within oceans [16]. This observation raises the possibility that at least some of the bacteria isolated from these sediments, are indeed of marine origin and could be descendants of the microbial community that inhabited ancient oceans.

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