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Fatty acid and DNA analyses of Permian bacteria isolated from ancient salt crystals reveal differences with their modern relatives

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Abstract The isolation of living microorganisms from primary 250-million-year-old (MYA) salt crystals has been questioned by several researchers. The most intense discussion has arisen from questions about the texture and age of the crystals used, the ability of organisms to survive 250 million years when exposed to environmental factors such as radiation and the close similarity between 16S rRNA sequences in the Permian and modern microbes. The data in this manuscript are not meant to provide support for the antiquity of the isolated bacterial strains. Rather, the data presents several comparisons between the Permian microbes and other isolates to which they appear related. The analyses include whole cell fatty acid profiling, DNA–DNA hybridizations, ribotyping, and random amplified polymorphic DNA amplification (RAPD). These data show that the Permian strains, studied here, differ significantly from their more modern relatives. These differences are accumulating in both phenotypic and molecular areas of the cells. At the fatty acid level the differences are approaching but have not reached separate species status. At the molecular level the variation appears to be distributed across the genome and within the gene regions flanking the highly conserved 16S rRNA itself. The data show that these bacteria are not identical and help to rule out questions of contamination by putatively modern strains.

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Introduction

A recent report of the isolation of live microorganisms from 250 million-year-old (MYA) Permian-aged salt crystals (Vreeland et al. 2000) was met with numerous scientific questions. Concerns were raised regarding the age of the crystals used, the ability of organisms to remain viable for hundreds of millions of years while exposed to environmental factors such as radiation, and on the lack of apparent molecular differences between the Permian isolate and their modern relatives. Hazen and Roedder (2001) contended that the particular crystal sampled (Vreeland et al. 2000) did not have the textural features normally associated with primary growth, and that in the absence of data showing crystallization at the Earth's surface, the exact age of the crystal would remain in doubt. Powers et al. (2001) addressed the question about the crystal age with geological evidence for the isolation of the Salado formation since the Permian. Recently, Satterfield et al. (2005) have provided additional evidence showing that the crystal did indeed form at the Earth's surface during the Permian period and that the chemistry of the inclusion fluid, evaporated Late Permian seawater, is consistent with the 250-MYA age of the crystal. They also demonstrated that crystals surrounding that used by Vreeland et al. (2000) were never exposed to life-destroying temperatures.

A second question regarded the potential cumulative effects of ^{40}K radiation on a DNA molecule (Kimenek and Bada, personal communication) over 250 million years. This question has been addressed by Nicastro et al. (2002) using potassium concentrations ten times higher than those found in the Salado formation where the crystal was obtained. These authors were able to provide theoretical evidence demonstrating that

radiation from ^{40}K (the only radioactive material present in this salt) would not have been sufficient to sterilize the salt. After analyzing the probability that radiation levels alone would cause enough DNA damage to kill the cells, Nicastro et al. (2002) showed that a significant proportion of the trapped bacterial population might be expected to survive as long as 1.5 billion years.

Perhaps the most vigorous questioning of the findings of Vreeland et al. (2000) arose from the lack of molecular differences between the Permian isolate and modern bacterial strains, especially *Virgibacillus marismortui* (Graur and Pupko 2001; Nickle et al. 2002). Both groups of authors utilized the original sequences of Vreeland et al. (2000) to perform a variety of statistical comparisons of the resulting phylogenetic trees (Graur and Pupko 2001; Nickle et al. 2002). They ultimately contested the conclusions of Vreeland et al. (2000) stating that the isolated strain could not be Permian in age because the 16S rDNA of a modern microbe, *V. marismortui*, had not sufficiently diverged from that of the Permian isolate. Vreeland and Rosenzweig (2002) pointed out that such conclusions are dependent upon acceptance of a single molecular clock where changes accumulated in the 16S rDNA gene sequence at a constant rate. They further suggested that this particular clock, calibrated using generation times of enteric bacteria and insect endosymbionts (Ochman et al. 1999), may not be appropriate, for some taxa, given the potentially long generation times recently detected in subsurface and marine microbial populations (Phelps et al. 1994; Parkes et al. 2000). Comparison with natural free-living populations may be more applicable to spore-forming bacteria (i.e. the four Permian strains versus the modern strains discussed here) that are able to remain dormant for long periods of time. Indeed, Maughan et al. (2002) discussed the implications of the lack of sequence divergence on reproductive rates and showed that the lack of divergence could be easily accounted for if the mean generation time of the modern Dead Sea isolate was around 875 years rather than the more rapid rates found in laboratories. While this time period would generally seem to be inordinately long, it was similar to those calculated for bacteria in numerous low nutrient environments (Phelps et al. 1994) and significantly faster than a marine population (Parkes et al. 2000). The data of Maughan et al. (2002), while being consistent with long generation times, still did not demonstrate differences between the Permian bacteria (Vreeland et al. 2000) and modern strains.

This manuscript, describes the results of comparative analyses conducted on four Permian-aged isolates [the original strain, 2-9-3 of Vreeland et al. (2000) and three additional Permian isolates designated 2-9-2, 2-10-1 and 2-10-2] and modern microbes whose 16S rRNA sequences are most similar to the Permian organisms. The data include whole cell fatty acid profiling, DNA–DNA hybridizations and two different molecular techniques, ribotyping and random amplified polymorphic DNA amplification (RAPD). Due to their high degree of discriminatory power, fatty acid profiling, DNA–DNA hybridizations, ribotyping, and RAPDs are all useful tools for differentiating between closely related bacterial strains. These techniques are proving to be most useful in microbial taxonomy where they are frequently recommended for the delineation of individual species (Priest and Austin 1986).

Materials and methods

Source of strains

Strain 2-9-3 was isolated from a fluid filled inclusion in a surface-sterilized Permian-aged salt crystal as described by Vreeland et al. (2000). This crystal was taken from a layer of bedded salt in the Air Intake Shaft of the US Waste Isolation Pilot Plant near Carlsbad New Mexico USA. Strain 2-9-2 was a second culture isolated from the same inclusion as 2-9-3. Three inclusions in this particular crystal were sampled; one yielded the two cultures listed above. A second inclusion proved to be sterile while a third yielded strain 2-10-2. Strain 2-10-1 was isolated in live form from a second primary crystal taken from the same geological layer but a different core. These strains have not yet been fully characterized and have not yet been deposited in a permanent collection. *V. marismortui* strain 123 was first isolated and described by one of the authors (AV). *V. pantothenticus* (=ATCC 14576) was obtained from the American Type Culture Collection. Due to its inability to grow on media with added salt, this strain was only used during the RAPD comparisons. *V. salexigens* (=DSMZ 11483) was purchased from the Deutsche Sammlung von Microorgaismen and Zellkulturen.

Lipid analyses

The lipid analyses described below were carried out at MIDI Laboratories (Wilmington Delaware, USA). Bacterial cultures for lipid analysis were grown on standard solidified Casamino acids medium supplemented with 8% (w/v) NaCl (CAS-8) (Vreeland et al. 1984). For comparison with library standards the strains were also grown on TSA medium; however all comparisons in this manuscript are based on the profiles from the CAS-8 medium. Approximately 40 mg of bacterial cells were removed from a petri plate generated from a single colony isolation. The cells were placed in a clean 13×100 mm culture tube. The fatty acids were extracted from the cells and saponified in a single step using 1.0 ml of a solution of 45 g of sodium hydroxide dissolved in 300 ml methanol/water (1:1 v/v). The extraction was carried out for 30 min in a boiling water bath. After the solution was cooled to room temperature, methylation was carried out by reducing the pH of the solution to 1.5 with 6.0 N hydrochloric acid in methanol then heating for

10 ± 1 min at 80 ± 1°C. The saponified fatty acids were extracted with hexane methyl *tert*-butyl ether (1:1 v/v). If necessary, the sample was cleaned with a 1.0% (w/v) sodium hydroxide solution. Fatty acid separation was conducted using an Agilent 6850 gas chromatograph fitted with a 25 m×0.2 mm phenyl methyl silicone fused silica capillary. The gas chromatograph was set for a temperature program ramping from 170 to 270°C at 5°C per min with a flame ionization detector. The carrier gas was hydrogen with nitrogen as the "make-up" gas. Standard filtered air was used to support the detector flame. Fatty acid patterns and comparisons were conducted using the Sherlock System computer software configured for optimal analysis of fatty acid methyl esters by gas chromatography. The "Sherlock" analysis represents a principal component analysis based upon the fatty acid patterns of individual strains. The Euclidian distance expresses the straight-line similarity (and difference) between all of the strains included in the Principal Component Analysis. The larger the Euclidian distance the less similar the strains. Calibration for the software was conducted using an external calibration standard manufactured by Microbial ID Inc. The standard was a mixture of the straight-chained saturated fatty acids from 9 to 20 carbons in length (9:0–20:0) and five hydroxy acids. All compounds were added quantitatively in order to evaluate chromatographic performance. Retention time data obtained from the calibration mixture was converted to equivalent chain length (ECL) data for bacterial fatty acid naming and comparison.

Extraction of genomic DNA and determination of DNA base composition

cells from the four Permian strains and three modern isolates were harvested, washed, and suspended in 0.15 M NaCl–0.1 M EDTA buffer (pH 8.0). The DNA was extracted and purified by the method of Marmur (1961). The mol% guanine plus cytosine (G + C) content of the DNA was determined from the midpoint value of the thermal denaturation profile (Marmur and Doty 1962) obtained with a UV/Vis Lambda 20 spectrophotometer (Perkin-Elmer) at 260 nm. This instrument was programmed for a temperature increase of 1.0°C per min. The G + C content was calculated from the thermal denaturation temperature by using the equation of Owen and Hill (1979).

DNA–DNA hybridization experiments

The DNA was labeled by the multiprime system using (1′, 2′, 5−^{3}H) dCTP (Amersham). The labeled DNA was denatured before hybridization by heating at 100°C for 5 min and then placed on ice. The DNA–DNA hybridization studies were performed by the competition procedure described by Johnson (1994). The ratio of the concentration of competitor DNA to the concentration of labeled DNA was at least 150:1. After hybridization, radioactivity bound to the filters was measured with a liquid scintillation counter (Beckman Instruments) and the percentage of hybridization was calculated as described by Johnson (1994).

Ribotyping

Riboprint patterns were prepared and analyzed according to the instructions of the manufacturer. Cultures were grown on CAS medium (Vreeland et al. 1984) supplemented with 8% w/v NaCl for 24 h at 37°C. Samples were removed from the agar using sterile toothpicks and placed into 40 µl of sterile buffer. Thirty microliters were transferred to the riboprint cartridge and the cells were killed by heating at 90°C for 10 min. Following heating, the cells were lysed with 5 µl of two lysing agents (manufacturer designations A and B), after which the cartridge was placed into the DuPont Qualicon Riboprinter for automated Southern blot analysis.

Table 1 Major fatty acids present in four Permian bacteria and two closely related *Virgibacillus* species

Fatty acid	2-9-2	2-9-3	2-10-1	2-10-2	1-77-5	1-77-6
14:0 iso	2.81	2.78	2.10	2.19	3.01	2.03
15:0 iso	21.13	21.93	23.48	23.41	29.95	27.13
15:0 Anteiso	39.36	38.82	39.43	40.31	39.55	36.11
16:1 ω7c alcohol	2.34	2.18	2.17	2.23	1.37	1.40
16:0 iso	6.75	6.30	4.24	4.42	4.85	4.89
16:1 ω11c	1.55	1.71	1.81	1.41	0.95	0.89
17:0 iso	4.09	4.18	4.40	4.10	4.24	5.31
17:0 Anteiso	17.03	16.39	16.12	16.34	11.52	16.99
Odd carbon	81.61	81.32	83.43	84.16	85.26	85.54
Even carbon	13.45	12.97	10.32	10.25	10.18	9.21
Total	95.06	94.29	93.59	94.41	95.44	94.75

All values are given in percent of the total amount detected. Except for two instances fatty acids comprising less than 1% of the total have been omitted from this table. Strains 2-9-2, 2-9-3, 2-10-1, and 2-10-2 represent organisms isolated from surface sterilized Permian-aged primary salt crystals. Strain 1-77-5 = *V. marismortui*; Strain 1-77-6 = *V. salexigens*

Random amplified polymorphic DNA amplification (RAPD)

The RAPDs were done the same for both primer sets using 25 µl reactions containing normal Taq polymerase buffer (Promega), 2.5 mM $MgCl_2$ (Promega), 0.2 mM of each dNTP, 50 pmol of each primer, 1 U Taq polymerase (Promega), and 10 ng of chromosomal DNA. The PCR reactions were run with 94°C 1 min, 92°C 40 s, 25°C 1 min, 75°C 2 min (repeat last three steps 30 times), and finished with a 75°C 5 min clean-up extension. Reaction products were run on 0.7 or 1.5% agarose gels for resolution of different band sizes.

Results and discussion

The various analytical techniques applied here do not (and cannot) provide evidence for the age differences between these various strains. That has been done using other data (Vreeland et al. 2000; Satterfield et al. 2005). The data presented here provides comparative biology between the four Permian strains studied and some putatively modern relatives.

Fatty acid profiling of microorganisms involves gas chromatographic comparison of the various fatty acids that comprise the lipids of the cell membranes of individual bacteria. Such profiles identify each fatty acid by size (number of carbons) and the number, type and location of double bonds. A comparison (Table 1) of the fatty acid profiles of the Permian isolates and their modern relatives shows that fatty acids typical of those present today, were being used to construct the membranes of microbes 250 million years ago. Figure 1 shows that while the Permian strains produce fatty acids similar to those of other microorganisms, a computer analysis of the various strains, using the "Sherlock" similarity program, clearly differentiates the Permian organisms from their closest known modern relatives. These data indicate that at the fatty acid level, the four Permian strains represent two different biotypes. Compared to more modern species, the data clearly show that these are different microbes. The data in Fig. 1 show that the Permian microbes can probably best be considered as subspecies of *V. marismortui* (Garabito et al. 1997; Heyndrickx et al. 1998; Arahal et al. 1999). Based upon the data in Table 1, the Permian microbes have less 14:0; and 15:0 iso fatty acid but more 16:1 ω7c alcohol and 16:1 ω11c fatty acid than do the modern organisms. The data presented here were based on lipid profiles from cells grown on CAS with 8% salt, which is the optimum for these organisms. However, a tree calculated using lipid profiles from the same organisms grown on TSA (data not shown) had an identical topology. The most interesting aspect of these profiles is the fact that four Permian strains group together.

There are several microbial characterization methods that involve analysis of cellular chromosomal DNA. The data in Table 2 compare the DNA of these microbes at two different levels. While the content of guanine plus cytosine (G + C) in the DNA of the Permian microbes is consistently lower than that of *Virgibacillus* species it is within the limits of that found in other members of the genus *Virgibacillus*. The data from DNA re-association assays can be used to detect large amounts of DNA sequence homology within two bacterial strains. The data in Table 2 show that the type strain of *V. marismortui* is related to Permian strain 2-9-3 at only 74%. *V. salexigens* (Garabito et al. 1997) and *V. pantothenticus* (Heyndrickx et al. 1998) show an even lower level of relatedness to strain 2-9-3. The DNA–DNA re-association data are most useful for discriminating microorganisms at species and subspecies levels. The level normally considered to indicate that two organisms represent strains of the same species falls somewhere between 70 and 75% (Priest and Austin 1986). Once again the data indicate that strain 2-9-3 and *V. marismortui* are close to being considered separate bacterial species. The data also indicate some degree of separation between strain 2-9-3 and the other three Permian isolates.

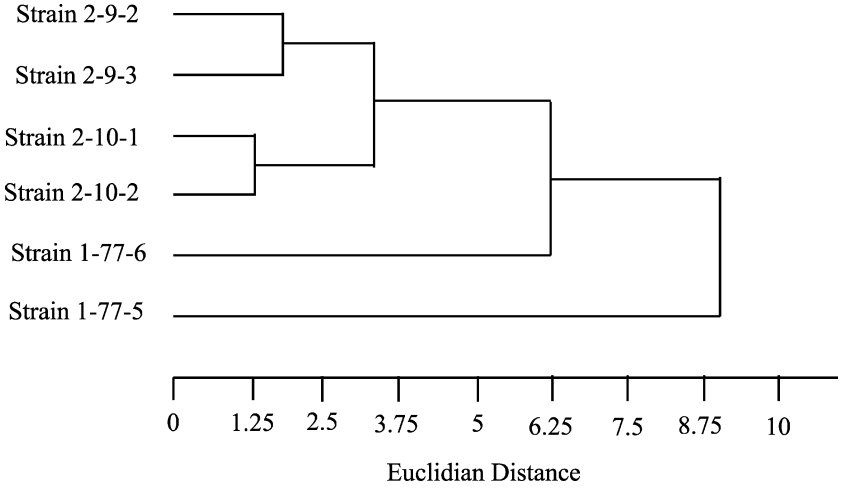

Fig. 1 Graphic comparison of fatty acid profiles of four Permian isolates with their closest known relatives in the genus *Virgibacillus*. 1-77-5 = *V. marismortui* . 1-77-6 = *V. salexigens*. 2-9-3, 2-9-2, 2-10-1, 2-10-2 = strains isolated from Permian-aged salt crystals

Table 2 Comparison of the DNA of four Permian microbes and several putatively modern *Virgibacillus* species

Source of unlabeled DNA	G + C content (mol %)	Relatedness (%) with ^{3}H labeled DNA from Permian strain 2-9-3
Strain 2-9-3	36.3	100
Strain 2-9-2	36.8	74
Strain 2-10-1	35.6	47
Strain 2-10-2	37.0	70
V. marismortui DSM 12325^{T}	40.7[a]	74
V. salexigens DSM 11483^{T}	39.5[b]	31
V. pantothenticus DSM 26^{T}	36.9[c]	46

[a]Arahal et al. (1999)
[b]Garabito et al. (1997)
[c]Heyndrickx et al. (1998)

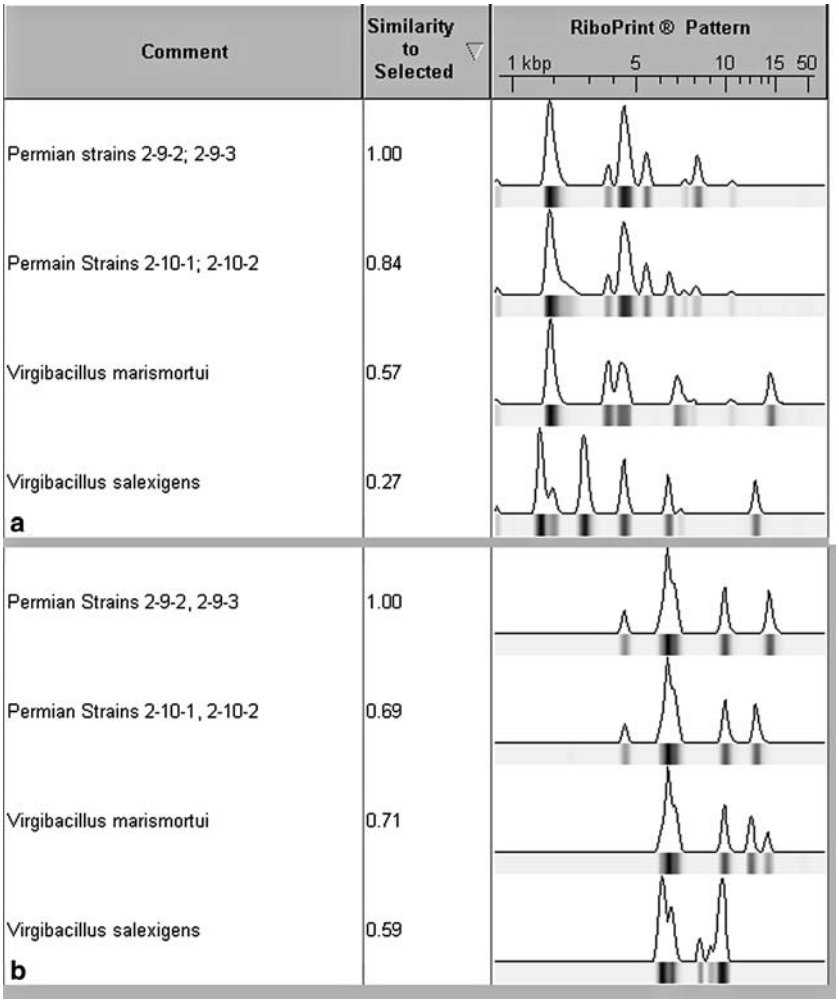

Fig. 2 Riboprint comparison of four Permian strains relative to *V. marismortui* and *V. salexigens*. **a** *Eco*R1 pattern; **b** *Pvu*II pattern

All four Permian microbes also show other significant molecular differences from the modern isolates. The data presented in Fig. 2 show the patterns generated through the use of restriction fragment (RiboPrint DuPont-Qualicon Corp.) comparisons of the entire rRNA gene of the four Permian strains relative to the *Virgibacillus* species. The riboprints arose from two different enzymes (*Eco*R1 and *Pvu*II) and were produced using an automated RiboPrinter provided by DuPont-Qualicon Corp. Each pattern is the average from at least four different runs for each strain. The *Eco*R1 target sequence is GAATTC with the cut occurring between the G and the first A. The *Pvu*II enzyme cuts the DNA anywhere the sequence of bases corresponds to CAGCTG with the cut occurring between the G and the C. The bioluminescent probes are complementary to DNA regions that flank the 16S rRNA gene. Binding of these probes to the Southern blot membrane, therefore, highlights sequence differences in gene regions surrounding the 16S rRNA structural gene. Consequently, the patterns reflect sequence differences in the larger ribosomal RNA gene of the organisms and not simply the small subunit rRNA. Figure 2a shows that the *Eco*R1 sequence appears at least seven times in the rRNA genes of strains 2-9-2 and

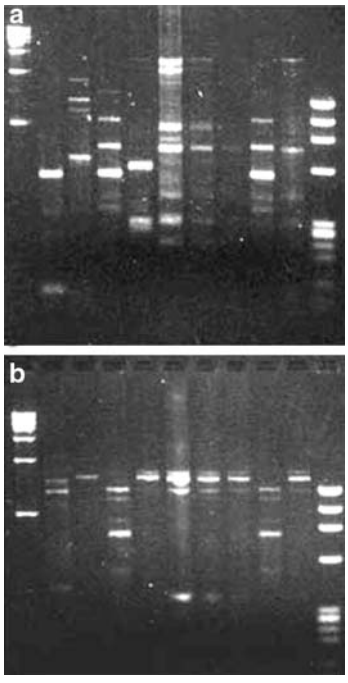

Fig. 3 Random Amplified Polymorphic DNA patterns. The sample order is: left to right *Lane 1* 1 kb step ladder 10 kb at the top, 0.5 kb at the bottom; *Lane 2 Bacillus subtilis* strain 168; *Lane 3 V. pantothenticus*; *Lane 4 V. salexigens*; *Lane 5 V. marismortui*; *Lane 6* Permian strain 2-9-3; *Lane 7* Permian strain 2-9-2; *Lane 8* Permian strain 2-10-1; *Lane 9 V. salexigens*; *Lane 10* Permian strain 2-10-2; *Lane 11* phiX174 HaeIII low molecular weight marker—top band equals 1.3 kb, bottom band equals 72 bp. **a** Primers R1 and S1; **b** Primer S2

2-9-3 and six times in the rRNA genes of isolates 2-10-1, 2-10-2, *V. marismortui* and *V. salexigens*. The patterns in Fig. 2b show that there are probably six *Pvu*II sites in the rRNA gene of all the microbes except *V. salexigens* which had five of the target sites. However, since the band sizes correspond to the number of bases separating the target sequence the patterns show that these restriction sites are not distributed in the same way within the gene of these organisms. Strains 2-9-2, 2-9-3, 2-10-1, 2-10-2, and *V. marismortui* share at least one and possibly two, common *Eco*R1 restriction sites which are not present for *V. salexigens* (Fig. 2a), but all of the other sites are obviously distributed at different positions within the rRNA gene. The Riboprint output grouped the four Permian isolates into two different *Eco*R1 ribogroups consisting of strains 2-9-2 and 2-9-3 in one group and 2-10-1 and 2-10-2 in a second (Fig. 2a). A similar grouping occurred with the *Pvu*II data (Fig. 2b). Both *V. marismortui* and *V. salexigens*were excluded from grouping with the Permian strains under both conditions.

Examination of the riboprints shows that *V. marismortui*, while sharing some common bands, is clearly different from the Permian isolates. These ribogroups reflect the overall similarity of the patterns produced during the analyses and show that the *V. marismortui* *Eco*RI restriction pattern is only 57% similar to the Ribogroup containing its 16S rDNA relative 2-9-3 while that produced by *Pvu*II has only 70% similarity with the 2-9-3 ribogroup. In addition, the Permian isolates are more similar to each other than they are to the putatively modern organisms. The low level of *Pvu*II similarity between the two Permian Ribogroups is more likely a reflection of the slight shift in the 4 and 10 kb bands (caused by the averaging calculations) than it is reflective of real differences between these organisms. The slight shift seen in these two bands in the figure were not as prevalent in the individual patterns produced by a single run. At the same time the degree of difference seen between *V. marismortui* and the Permian ribogroups is too extensive to be the result of any calculation problem. These patterns show clearly that despite having nearly identical 16S rDNA sequences there is a significant difference in the rest of the rDNA genes of these microbes. Whether these differences are localized within the 5S and 23S genes remains to be determined.

A second molecular technique that can compare even more of the genome is RAPD. The RAPD comparisons differ from RiboPrints by using larger primers, which bind to complementary sequences anywhere in the genome thus allowing amplification of larger and more diverse DNA regions. In the specific case of the Permian organisms, three different 10 base primers were used. The data in Fig. 3a used a two primer set designated R1 and S1 whose sequences were 5′-GAAGCAGCGTGG-3′ (R1) and 5′-CCGCAGCCAA-3′ (S1). The RAPD data shown in Fig. 3b were developed using a single primer (R2) with a sequence of 5′-GGTTTCCGCC-3′. These data also show differences in the genomes of the four Permian microbes relative to those of the modern strains. As with the earlier Riboprint data, strains 2-9-2 and 2-9-3 are closely related to each other while 2-10-1 can be grouped with 2-10-2. The data also show that neither group is closely related to *V. marismortui* or any of the other *Virgibacillus* sp. included on the gel. Primer R2 did provide indication that there is some degree of relatedness between *V. marismortui* and the isolates from Permian salts, since all five reactions produced two bands at approximately 1.5–1.6 kb. However, even this primer revealed differences in the genomes of the organisms, with each of the Permian isolates also producing smaller bands at about 1.3 kb and with 2-9-2 and 2-9-3 producing RAPD products at about 0.5 kb. While both primers yielded significant PCR products, primers R1 and S1 clearly produced a more extensive amplification of the genomes of strains 2-9-2 and 2-9-3.

The data presented here indicate that despite having three key genes (Vreeland et al. 2000; Maughan et al. 2002) with nearly identical nucleotide sequences, strain 2-9-3, isolated from Permian salt (Vreeland et al. 2000), is different from other halotolerant spore-forming microbes found in modern environments. There are also differences between the three other Permian isolates (2-9-2, 2-10-1, and 2-10-2) and modern isolates as well as differences between the Permian isolates themselves.

Given the geologically supported age of strain 2-9-3 (Satterfield et al. 2005), the most likely scenario is that *V. marismortui* is the divergent microbe. The fact that bacteria from the Permian are biologically similar, but not identical, to microbes found in modern areas, is not at all surprising (Vreeland and Rosenzweig 2002). The molecular data arising from the RiboPrint and RAPD patterns suggest that much of the difference between the Permian and modern microbes is found outside of the three housekeeping genes that have already been sequenced (Vreeland et al. 2000; Maughan et al. 2002). One possible explanation for these findings is that bacterial evolution is occurring at different rates in different DNA regions. Recent studies using whole genome comparisons suggest that genome organization, and even some genes in bacteria; evolve more quickly than critical gene sequences (Huynen and Bork 1998; Cournoye and Lavire 1999). This explanation is supported by recent genome sequencing efforts that have uncovered large discrepancies in gene content among bacteria with nearly identical 16S rDNA sequences. For example, two strains of *Prochlorococcus* show less than 3% divergence in their 16S rDNA but only share 1350 genes (out of 1716 and 2275 genes in each of the two genomes) and have genome sizes that differ by almost a megabase (Rocap et al. 2003). Because of the dynamic nature of the bacterial chromosome, with gene content being balanced by gene loss and the acquisition of new genes, (Ochman et al. 2000; Mira et al. 2001) this variation in genome content among strains of bacteria, rather than nucleotide substitutions, represents the majority of differences between two closely related genomes (Ausubel et al. 2002; Snel et al. 2002).

The differences between 2-9-3 and *V. marismortui* could certainly be the result of differences in gene content, a hypothesis readily testable with modern genomic technology. In fact, based upon the data being obtained during numerous comparisons of the four Permian organisms and *V. marismortui* sequencing the entire genome of these microbes may be the only way to determine the exact degree of relatedness between these organisms. Another possibility might be that free-living microbes in low nutrient, hypersaline environments may be evolving at rates that are slower than those calculated from other cultures. There is certainly no doubt that bacteria possess the capacity for rapid evolution and differentiation. One need only look to such things as antibiotic resistance and biodegradation for confirmation. However, having the capacity to change rapidly does not necessarily require that one do so.

In the months since their initial publication, the data and claims about Permian microbes (Vreeland et al. 2000) have been intensely questioned and scrutinized as has been described above. The data presented here show unequivocal differences between four Permian strains and microbes isolated from present day environments. In addition, the geological evidence (Vreeland et al. 2000; Powers et al. 2001; Satterfield et al. 2005) supports the 250-million year age of the salt crystals. The data show that these bacteria are not identical. They are most likely related at the subspecies level. These data do help to rule out questions of contamination by putatively modern strains during the original isolation.

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