Letter to the Editor

The Paradox of the "Ancient" Bacterium Which Contains "Modern" Protein-Coding Genes

Heather Maughan, C. William Birky Jr.,*† Wayne L. Nicholson,*‡ William D. Rosenzweig,§ and Russell H. Vreeland§*

*Graduate Interdisciplinary Program in Genetics, †Department of Ecology and Evolutionary Biology, and ‡Department of Veterinary Science and Microbiology, University of Arizona; and §Department of Biology, West Chester University

The isolation of microorganisms from ancient materials and the verification that they are as old as the materials from which they were isolated continue to be areas of controversy. Almost without exception, bacteria isolated from ancient material have proven to closely resemble modern bacteria at both morphological and molecular levels. This fact has historically been used by critics to argue that these isolates are not ancient but are modern contaminants introduced either naturally after formation of the surrounding material (for further details, see Hazen and Roeder 2001 and the reply by Powers, Vreeland, and Rosenzweig 2001) or because of flaws in the methodology of sample isolation (reviewed recently in Vreeland and Rosenzweig 2002). Such criticism has been addressed experimentally by the development of highly rigorous protocols for sample selection, surface sterilization, and contamination detection and control procedures. Using the most scrupulous and well-documented sampling procedures and contamination-protection techniques reported to date, Vreeland, Rosenzweig, and Powers (2000) reported the isolation of a sporeforming bacterium, *Bacillus* strain 2-9-3, from a brine inclusion within a halite crystal recovered from the 250-Myr-old Permian Salado Formation in Carlsbad, NM.

As had been noted in earlier studies, a striking observation by Vreeland, Rosenzweig, and Powers (2000) was that the 16S rDNA of isolate 2-9-3 is 99% identical to that of *Salibacillus marismortui*, a bacterium isolated from the Dead Sea in 1936 (Arahal et al. 1999). In fact, Arahal et al. (1999) identified as *S. marismortui* three strains with 16S rDNA sequences differing by 0.01%, suggesting that isolate 2-9-3 might also be classified as *S. marismortui*.

Two groups have since used phylogenetic analyses of 16S rDNA sequences to argue that isolate 2-9-3 is unlikely to be 250 Myr old. Graur and Pupko (2001) used a relative rate test to compare evolutionary rates of 16S rDNA on the branches leading to isolate 2-9-3 and *S. marismortui* and found no differences between the evolutionary rates. Considering the possibility that *S. marismortui* may also be ancient (Arahal et al. 1999; Vreeland, Rosenzweig, and Powers 2000), they also compared the evolutionary rates of isolate 2-9-3, *S. marismortui* and *Virgibacillus proomi*, a close relative of *S. marismortui*, and again found similar rates of evolution (Graur and Pupko 2001). More recently, Nickle et al. (2002) also performed relative rate tests using 16S rDNA with the same result; the branch leading to isolate 2-9-3 is not extraordinarily short, as would be expected of an organism that has not been evolving for millions of years. Nickle et al. (2002) used evolutionary rates derived from enteric bacteria to argue that if isolate 2-9-3 has not been evolving for 250 Myr, then *S. marismortui* must itself have been evolving 5–10 times more slowly than did aphid endosymbionts on which the rate calculations were based. We note that although the evolutionary rates calculated from enterics and endosymbionts are the best estimates we currently possess, it is entirely likely that rates of sporeformer evolution may indeed be slower by several orders of magnitude. Sporeformers have been shown to remain in the metabolically dormant spore state, thus not replicating their DNA, for conservative estimates of anywhere from 10^2 to 10^4 years between times of growth (Kennedy, Reader, and Swierczynski 1994; Nicholson et al. 2000).

As the analyses discussed above used 16S rDNA genes, the evolution of which may not be representative of the organism as a whole, we wanted to know if the similarities between isolate 2-9-3 and *S. marismortui* are seen with protein-coding genes as well as with 16S rDNA genes. We therefore analyzed phylogenetic relationships between strain 2-9-3 and *S. marismortui*, using the spore-forming bacteria as our comparison group. The rationale for this design was that the evolutionary rate among the sporeformers would more closely approximate that of 2-9-3. We used amino acid data from two genes, *recA* and *splB*. The *recA* gene is found throughout all bacteria, and its product is required for homologous recombination and DNA repair. Because of the functional constraints on *recA* evolution, it can be used to resolve the older evolutionary relationships. The *splB* gene, on the other hand, has to date only been reported in gram-positive spore-forming bacteria and is important in the repair of spore-specific DNA damage resulting from UV radiation during spore dormancy (Nicholson et al. 2000). Because *splB* is only found in gram-positive spore-forming bacteria, it can be assumed to have a more recent origin than *recA* has and might be useful in resolving closer evolutionary relationships.

The results of our analyses are consistent with the phylogenetic relationships shown by Graur and Pupko (2001) and Nickle et al. (2002). At the nucleotide level, isolate 2-9-3 and *S. marismortui* differed by two nucleotides out of the 404 *recA* nucleotides examined. Both

Key words: 2-9-3, ancient, evolution, relative rate test, *Salibacillus marismortui*.

Address for correspondence and reprints: Heather Maughan, Graduate Interdisciplinary Program in Genetics, Building 90 Room 112, 1117 E. Lowell Street, University of Arizona, Tucson, Arizona 85721. E-mail: hmaughan@u.arizona.edu.

Mol. Biol. Evol. 19(9):1637–1639. 2002

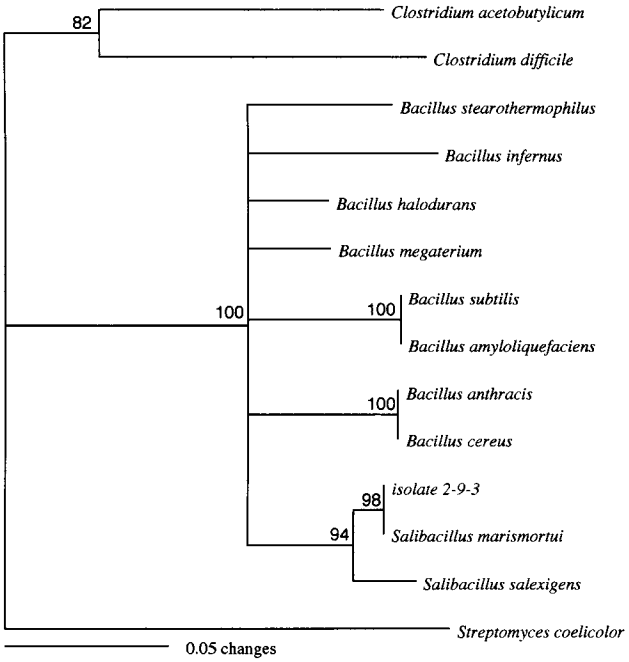

FIG. 1.—Neighbor-joining tree from *recA* amino acid data (139 characters). One thousand bootstrap replicates were performed using *Streptomyces coelicolor* as the outgroup.

of these substitutions are synonymous, making these two taxa identical at the amino acid level. The phylogenetic reconstruction (Swofford 1998) using amino acid sequences of *recA* (amino acids were used because of site saturation at the nucleotide level across distantly related taxa) places 2-9-3 and *S. marismortui* in a more recent clade, instead of their occupying a more basal position as one would predict if the clade had not been evolving for 250 Myr (fig. 1).

Similar results were obtained for *splB* (fig. 2). Out of the 619 nucleotides examined, only one synonymous substitution was observed between isolate 2-9-3 and *S. marismortui*, again making these two taxa identical at the amino acid level. Consistent with the *recA* data (fig. 1), the *splB* data also support the hypothesis that isolate 2-9-3 and *S. marismortui* diverged from each other more recently than their divergence from the other *Bacillus* species (fig. 2).

We performed relative rates tests (Robinson et al. 1998) using amino acid data from both *recA* and *splB*. *Salibacillus marismortui* could not be used in testing for relative rates against isolate 2-9-3 because of their 100% identity at the amino acid level. We found similar rates of evolution comparing isolate 2-9-3 with *S. salexigens*, the closest relative of isolate 2-9-3 after *S. marismortui*, with either *Clostridium acetobutylicum* or *B. subtilis* as

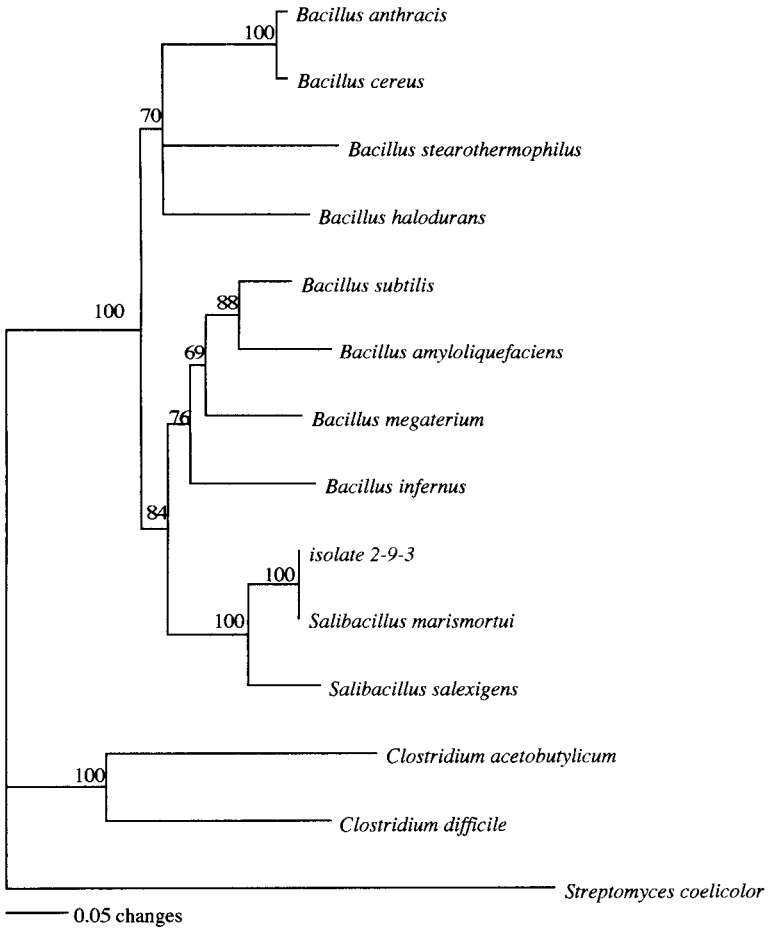

FIG. 2.—Neighbor-joining tree constructed from *splB* amino acid data (190 characters). One thousand bootstrap replicates were performed using *Streptomyces coelicolor* as the outgroup.

the outgroup (data not shown). Using nucleotide data from isolate 2-9-3, *S. marismortui*, *B. subtilis*, and *B. cereus*, we related the distance between 2-9-3 and *S. marismortui* to the distance between *B. subtilis* and *B. cereus* using the following logic. Because the substitutions between 2-9-3 and *S. marismortui* are all synonymous, they can be used to reflect the mutation rate. If three synonymous substitutions out of the 1,023 total nucleotides examined (1/619 from *recA* and 2/404 from *splB*), thus 0.2% divergence, are representative of the mutation rate since the divergence of 2-9-3 and *S. marismortui* 250 MYA, then the 121 synonymous substitutions (12% divergence) between *B. subtilis* and *B. cereus* would place their last common ancestor at 15 BYA, much longer than the age of the earth.

One could argue that isolate 2-9-3 and *S. marismortui* spores were both dormant for 250 Myr and, hence, were not evolving. This hypothesis is easily dismissed by looking at the branching patterns of the *recA* and *splB* trees. If 2-9-3 and *S. marismortui* were both dormant for 250 Myr, then all other taxa on the tree would have experienced even greater lengths of dormancy because they have more primitive ancestry. In other words, every taxon on the tree would have had to be metabolically and evolutionarily dormant for at least 250 Myr sometime in their past, a hypothesis which is impossible to test.

A recent review by Parkes, Cragg, and Wellsbury (2000) shows that generation times of some bacteria isolated from subseafloor sediments are of the order of thousands of centuries. If a generation time of 100,000 years is applicable to isolate 2-9-3 after it diverged from *S. marismortui* 250 MYA, then isolate 2-9-3 went through approximately 2,500 generations during that time. If, on average, there are 10^{-8} mutations per site per generation, then this would give isolate 2-9-3 and *S. marismortui* 2.5×10^{-5} differences per site. The analysis reported here looked at 1,023 sites, and thus one would have expected to see 0.026 differences between isolate 2-9-3 and *S. marismortui* if they had generation times of the order of 100,000 years. By applying the same mutation rate, the observed 3/1,023 differences between isolate 2-9-3 and *S. marismortui* imply a mean generation time of 850 years, a rate similar to that found by Phelps et al. (1994) for subsurface bacteria. This analysis assumes that isolate 2-9-3 was able to grow inside the salt crystal and was not present as a dormant spore, a scenario which is extremely unlikely because the salt concentration inside brine inclusions is well above the upper limit of salinity at which isolate 2-9-3 can grow (Vreeland, Rosenzweig, and Powers 2000).

The evidence presented here clearly indicates that isolate 2-9-3 should be considered a strain of *S. marismortui* under the established standards of 16S rRNA systematics, which state that isolates sharing >97% identity should be considered as the same species (Stackebrandt and Goebel 1994). But does such a close relationship to modern bacteria mean that isolate 2-9-3 is itself modern? The answer to this question must be sought by resolving what appears to be an increasingly common paradox. We have a large set of rigorous geological and microbiological data which can be interpreted in favor of the antiquity of these organisms, and an equally large set of rigorously obtained molecular data which can be interpreted in favor of their modernity. As it stands, our present molecular work can neither confirm nor disprove the age of isolate 2-9-3.

Acknowledgment

This work was supported by a research-training grant from the Department of Ecology and Evolutionary Biology to H.M.

LITERATURE CITED

ARAHAL, D. R., M. C. MARQUEZ, B. E. VOLCANI, K. H. SCHLEIFER, and A. VENTOSA. 1999. *Bacillus marismortui* sp. nov., a new moderately halophilic species from the Dead Sea. Int. J. Syst. Bacteriol. **49**:521–530.

GRAUR, D., and T. PUPKO. 2001. The permian bacterium that isn't. Mol. Biol. Evol. **18**:1143–1146.

HAZEN, R. M., and E. ROEDDER. 2001. How old are the bacteria from the Permian age? Nature **411**:155.

KENNEDY, M. J., S. L. READER, and L. M. SWIERCZYNSKI. 1994. Preservation records of micro-organisms: evidence of the tenacity of life. Microbiology **140**:2513–2529.

NICHOLSON, W. L., N. MUNAKATA, G. HORNECK, H. J. MELOSH, and P. SETLOW. 2000. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbiol. Mol. Biol. Rev. **64**:548–572.

NICKLE, D. C., G. H. LEARN, M. W. RAIN, J. I. MULLINS, and J. E. MITTLER. 2002. Curiously modern DNA for a ''250 Million-Year-Old'' bacterium. J. Mol. Evol. **54**:134–137.

PARKES, R. J., B. A. CRAGG, and P. WELLSBURY. 2000. Recent studies on bacterial populations and processes in subseafloor sediments: a review. Hydrogeology **8**:11–28.

PHELPS, T. J., E. MURPHY, M. PFIFFNER, and D. WHITE. 1994. Comparison between geochemical and biological estimates of subsurface microbial activity. Microb. Ecol. **28**:335.

POWERS, D. W., R. H. VREELAND, and W. D. ROSENZWEIG. 2001. How old are bacteria from the Permian age? [reply to Hazen and Roedder]. Nature **411**:155.

ROBINSON M., M. GOUY, C. GAUTIER, and D. MOUCHIROUD. 1998. Sensitivity of the relative-rate-test to taxonomic sampling. Mol. Biol. Evol. **15**:1091–1098.

STACKEBRANDT, E., and B. M. GOEBEL. 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. **44**:846–849.

SWOFFORD, D. L. 1998. PAUP*: phylogenetic analysis using parsimony and other methods. Sinauer Associates, Sunderland, Mass.

VREELAND, R. H., and W. D. ROSENZWEIG. 2002. The question of uniqueness of ancient bacteria. J. Ind. Microbiol. Biotechnol. **28**:32–41.

VREELAND, R. H., W. D. ROSENZWEIG, and D. W. POWERS. 2000. Isolation of a 250 million-year-old halotolerant bacterium from a primary salt crystal. Nature **407**:897–900.

KEN WOLFE, reviewing editor

Accepted May 10, 2002