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Curiously Modern DNA for a "250 Million-Year-Old" Bacterium

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Abstract. Studies of ancient DNA have attracted considerable attention in scientific journals and the popular press. Several of the more extreme claims for ancient DNA have been questioned on biochemical grounds (i.e., DNA surviving longer than expected) and evolutionary grounds (i.e., nucleotide substitution patterns not matching theoretical expectations for ancient DNA). A recent letter to *Nature* from Vreeland et al. (2000), however, tops all others with respect to age and condition of the specimen. These researchers extracted and cultured a bacterium from an inclusion body from what they claim is a 250 million-year (Myr)-old salt crystal. If substantiated, this observation could fundamentally alter views about bacterial physiology, ecology and evolution. Here we report on molecular evolutionary analyses of the 16S rDNA from this specimen. We find that 2-9-3 differs from a modern halophile, *Salibacillus marismortui,* by just 3 unambiguous bp in 16S rDNA, versus the ~59 bp that would be expected if these bacteria evolved at the same rate as other bacteria. We show, using a Poisson distribution, that unless it can be shown that *S. marismortui* evolves 5 to 10 times more slowly than other bacteria for which 16S rDNA substitution rates have been established, Vreeland et al.'s claim would be rejected at the 0.05 level. Also, a molecular clock test and a relative rates test fail to substantiate Vreeland et al.'s claim that strain 2-9-3 is a 250-Myr-old bacterium. The report of Vreeland et al. thus falls into a long series of suspect ancient DNA studies.

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Several studies of ancient DNA from a diverse array of organisms have appeared in the molecular phylogenetic literature over the past decade (Cano et al. 1993; DeSalle et al. 1992; Golenberg et al. 1990; Soltis et al. 1992). The DeSalle group worked with a fossil termite (*Mastotermes electrodominicus*) preserved in Oligo–Miocene amber estimated to be 25 to 30 million years (Myr) old. The Golenberg and Soltis groups independently isolated and sequenced *rbc*L DNA from two fossils from the plant genera *Magnolia* and *Taxodium* excavated from the Pliocene Clarkia clay deposit (17–20 Myr old). Other groups have sequenced DNA from the frozen corpse of an Ice Age human (Handt et al. 1994) and from the remains of Pleistocene mammals trapped in tar pits (Janczewski et al. 1992). While some of these studies have achieved acceptance, others have been attacked on experimental or theoretical grounds. Cano et al.'s study of ancient weevil DNA, for example, has been criticized for having failed to rule out PCR contamination and for a lack of reproducibility (Austin et al. 1997; Walden and Robertson 1997). Cano et al.'s study has also been criticized on molecular evolutionary grounds by Gutierrez and Marin (1998). They demonstrated that the 18S rDNA from Cano et al.'s study was not significantly less diverged from the most recent common ancestor than a closely related modern weevil. All of these studies focused on deceased organisms and thus DNA degradation was a major hurdle to overcome. Some of the workers who succeeded in obtaining ancient DNA samples (Handt et al. 1994; Janczewski et al. 1992) had extreme

difficulty obtaining large fragments due to DNA degradation.

It was with great interest (and some skepticism) that we read a recent paper by Vreeland et al. reporting to have extracted a bacterium (strain 2-9-3) from an inclusion body in a salt crystal obtained from the 250-Myr-old Salado formation near Carlsbad, New Mexico. These researchers thus overcame what would seem to be insurmountable problems of DNA degradation, smashing the record for the oldest 16S rDNA fragment. Survival in this case, however, went well beyond DNA preservation: they were actually able to grow this bacterium in the laboratory. The thing that makes this so remarkable is that Vreeland et al. took what appears to have been the utmost precautions in their sample preparations including, among other measures, dipping this salt crystal in 10 *M* HCl and 10 *M* NaOH to sterilize the surface before drilling into the crystal. Notably, they found no bacteria in the salt fines (bits of salt released during drilling) until the drill penetrated the inclusion body. They also found no contaminating bacteria in plates of media that had been left open at random locations in the laminar flow hood.

Although these contamination measures and controls are impressive, their claim that the salt crystal that contained this bacterium was 250 Myr old (i.e., as old as the Salado formation itself) is not as convincing (Hazen and Roedder 2001). It is not hard to imagine that water seeped into this formation (e.g., during a recent glacial maximum within the last 100,000 years), resulting in the formation of new salt crystals in an otherwise old geological formation. In contrast to their elaborate controls for contamination, they did not present any data to verify the age of the crystal from which they extracted the bacteria. Hazen and Roedder (2001) have argued that the clarity of the crystal from which they extracted strain 2-9-3 is consistent with this crystal being of a more recent origin. Hazen and Roedder also pointed out that the fluids in the Delaware Basin (the geological region from which Vreeland et al. obtained their sample) are extremely heterogeneous with respect to the "absolute concentration as well as the ratios of halogen, alkali and alkaline-earth ions," suggesting that this region contains a mixture of ancient and modern waters, though Powers et al. (2001) have countered that such heterogeneity does not necessarily imply that these fluids are from different ages.

One independent check would be to conduct molecular evolutionary analyses of the 16S rDNA sequence from this organism. We expect a DNA sample obtained from an organism that has been entombed for 250 Myr in a salt crystal to differ considerably from extant organisms that have had many additional years to evolve new mutations. Vreeland et al. pointed out that strain 2-9-3 differs from the modern halophilic bacterium *Virgibacillus pantothenticus* at 45 positions in the 16S rDNA gene and suggested that this corroborated their proposition of 250 Myr of evolutionary separation. However, this is not the most appropriate comparison since *V. pantothenticus* is not the nearest modern sister taxon of strain 2-9-3. We were struck, instead, by Vreeland et al.'s observation that the 16S rDNA nucleotide sequence of strain 2-9-3 shows "99% similarity" to another modern bacterium, *Salibacillus marismortui* [previously called *Bacillus marismortui* (Arahal et al. 2000)]. Using the same data, we observed just three unequivocal differences out of 1559 exactly homologous nucleotides in 16S rDNA, corresponding to an actual level of similarity of 99.8%. This level of similarity suggests a substitution rate of 0.2% per 250 Myr in *S. marismortui,* or an effective divergence rate for these species of 0.4% per 250 Myr. Other bacterial 16S rDNA sequences, by contrast, have been estimated to diverge at a rate of ~5–10% per 250 Myr (Moran et al. 1993; Ochman et al. 1999). (We also observed three ambiguous differences between 2-9-3 and *S. marismortui,* but in each case one of the assignments in 2-9-3 matched the corresponding base pair in *S. marismortui,* indicating that these ambiguous differences are likely to reflect sequence artifacts rather than actual differences.) Plugging different divergence rates into a Poisson model for nucleotide substitution, we find that the probability of observing three nucleotide changes after 250 Myr is less than 5% for divergence rates above 1% per 250 Myr (Fig. 1). In other words, unless it can be shown that *S. marismortui* evolves ~5–10 times more slowly than the bacteria studied by Moran et al. (1993), Vreeland et al.'s claim would be rejected at the 0.05 level.

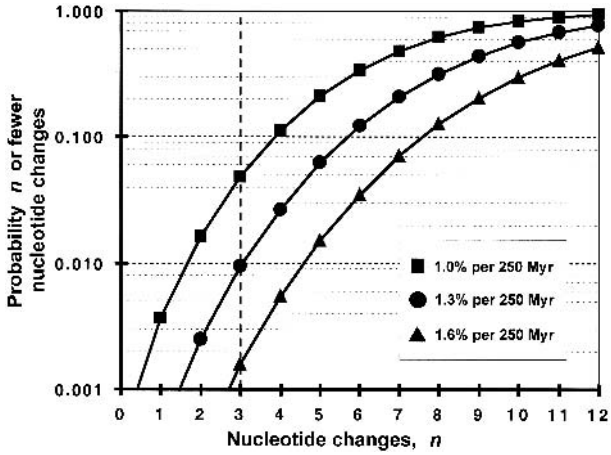

Fig. 1. Probability of observing *n* or fewer nucleotide changes after 250 Myr of separation assuming that strain 2-9-3 did not evolve inside the salt crystal. Lines marked with *squares, circles,* and *triangles,* respectively, correspond to divergence rates of 1.0, 1.3, and 1.6% per 250 Myr in the *S. marismortui* clade (versus 5–10% per 250 Myr reported for other bacteria). The *dashed vertical line* at $n = 3$ corresponds to the number of unambiguous nucleotide differences between strain 2-9-3 and its closest moder relative, *S. marismortui.* We note that if strain 2-9-3 did evolve during the time it was trapped in the salt crystal, the corresponding *p* values would be even lower.

We note that this conclusion is not as strong as a similarly skeptical commentary on this same subject by Graur and Pupko (2001), who argued that the apparent rate of substitution in 16S rDNA in *S. marismortui* suggests "a reduction of four orders of magnitude in comparison with the typical prokaryotic rate." Graur and Pupko, however, used the wrong divergence rate: "1×10^{-8} and 5×10^{-9} substitutions per site per year" instead of the 2×10^{-10} and 4×10^{-10} substitutions per site per year that one would expect from Moran et al.'s estimate of 1–2% per 50 Myr in 16S rDNA. By our calculations the apparent substitution rate in *S. marismortui* is 12–24 times lower than the typical 16S rDNA substitution rate—still a big enough difference to cast doubt on Vreeland et al.'s conclusions.

The relationships between strain 2-9-3 and its closet relatives can also be investigated using phylogenetic methods. Based on molecular evolutionary theory we would predict that a phylogenetic tree containing a 250 Myr-old strain and its modern cousins would fail a molecular clock test (Felsenstein 1981), since this test assumes that all taxa evolved for equal durations. Although the branch connecting 2-9-3 and *B. marismortui* is relatively short, we could not reject a molecular clock for a tree including strain 2-9-3 and six closely related species presented by Vreeland et al. (*B. subtilis, B. coagulans, V. pantothenticus, V. proomii,* and *B. marismortui*) ($p = 0.18$). We have also found, in agreement with Graur and Pupko (2001), that a relative rate test (Muse and Weir 1992) among strain 2-9-3, *B. marismortui,* and the next most closely related taxon, *V. proomii,* provides no support for the hypothesis that strain 2-9-3 is less diverged from *V. proomii* than modern *B marismortui* ($p = 0.48$). We have found by simulation that, if 2-9-3 were 250 Myr old and if *S. marismortui* had evolved at the same rate as the bacteria considered by Moran et al. (1993), a relative rates test would have sufficient power to reject the null hypothesis that 2-9-3 is less diverged from *V. proomii* than *M. marismortui* ($p < .001$). The molecular data, therefore, do not support the hypothesis that strain 2-9-3 stopped evolving 250 Myr before the others.

To be sure, neither of these tests explicitly rejects the hypothesis that strain 2-9-3 is ancient. These tests do, however, reject the hypothesis of rate heterogeneity. If we assume for the sake of argument that Vreeland et al. are correct in their assertion that strain 2-9-3 is 250 Myr old and suppose that the apparent rate homogeneity is due to a remarkably slow molecular clock, then we can use the rate determined by the divergence between strain 2-9-3 and *B. marismortui* to date the age of other nodes in the tree. Based on three unambiguous nucleotide changes, we obtain an expected divergence time for *B. subtilis* and *B. marismortui* of 5.8 billion years (Fig. 2). Assuming that substitution rates are Poisson distributed and that the mean of this distribution is the observed number of unequivocal nucleotide differences between

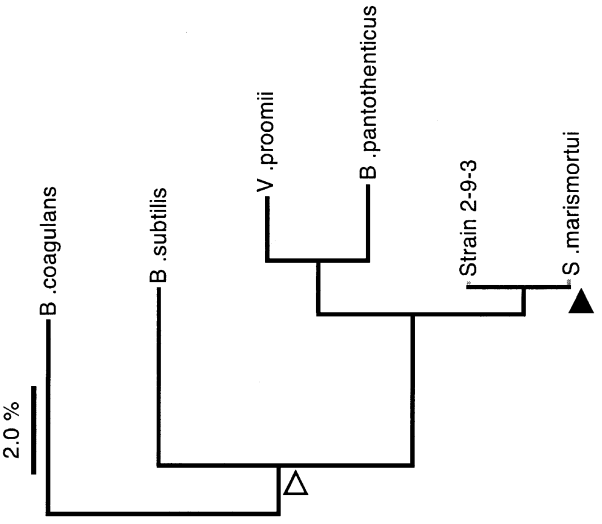

Fig. 2. The maximum-likelihood tree (under an HKY + Γ model of evolution) for strain 2-9-3 and its five close sister taxa. We would expect the branch leading to *B. marismortui* to be 12 to 24 times longer if that species had evolved for 250 Myr longer than strain 2-9-3. Our analysis shows that we cannot reject a molecular clock for this tree, indicating that the tree depicted here is not significantly more likely than one with all the branches ending at the same time. Assuming that Vreeland et al. are correct in their claim of antiquity, the rate homogeneity gives us the ability to date the divergences in the tree based on a rate determined by 2-9-3. This rate, for example, would predict *B. subtilis* and *S. marismortui* to have diverged from each other 5.8 billion years ago.

B. marismortui and strain 2-9-3, we obtain a lower 5% bound for the divergence time for *B. subtilis* and *B. marismortui* of 3.2 billion years, barely 600 Myr since the estimated origin of life on earth. Given how close these species are to each other on the overall tree of life, we are positioned to reject Vreeland et al.'s claims of antiquity.

We suggest two experiments that Vreeland et al. could do to test further their claims of antiquity for strain 2-9-3. First, long-term efforts should be made to estimate the survival rate of strain 2-9-3 under conditions similar to those which existed in the salt crystal from which this strain was extracted. From Arahal et al. (1999), we know that *S. marismortui* can survive for 57 years in 500 ml of salt water supplemented with peptone, but this qualitative observation of survival tells us nothing about the half-life of strain 2-9-3. If long-term experiments show that the half-life of strain 2-9-3 in salt water is but a few months or years, Vreeland et al.'s claim would be severely undercut. Second, Vreeland et al. should work hard to perform carbon-14 dating (or other radioactive dating techniques) on samples from inclusion bodies in other crystals from the same location of the Salado for-

mation. If these crystals are more than a million years old, there should be essentially no ^{14}C left in these samples. While this idea about using ^{14}C can be criticized for requiring large samples, the ability to date these crystals independently is critical (Hazen and Roedder, 2001). Until these or comparable experiments are completed, Vreeland et al.'s claim that strain 2-9-3 is a 250 Myr-old bacterium must be viewed with serious skepticism.

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