Letter to the Editor

The Permian Bacterium that Isn't

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There is growing evidence for the presence of viable microorganisms in geological salt formations that are millions of years old. It is still not known, however, whether these bacteria are dormant organisms that are themselves millions of years old or whether the salt crystals merely provide a habitat in which contemporary microorganisms can grow, perhaps interspersed with relatively short periods of dormancy (McGenity et al. 2000). Vreeland, Rosenzweig and Powers (2000) have recently reported the isolation and growth of a halotolerant spore-forming *Bacillus* species from a brine inclusion within a 250-Myr-old salt crystal from the Permian Salado Formation in New Mexico. This bacterium, *Bacillus* strain 2-9-3, was informally christened *Bacillus permians*, and a 16S ribosomal RNA gene was sequenced and deposited in GenBank under the name *B. permians* (accession number AF166093). It has been claimed that *B. permians* was trapped inside the salt crystal 250 MYA and survived within the crystal until the present, most probably as a spore. Serious doubts have been raised concerning the possibility of spore survival for 250 Myr (Tomas Lindahl, personal communication), mostly because spores contain no active DNA repair enzymes, so the DNA is expected to decay into small fragments due to such factors as the natural radioactive radiation in the soil, and the bacterium is expected to lose its viability within at most several hundred years (Lindahl 1993). In this note, we apply the-proof-of-the-pudding-is-in-the-eating principle to test whether the newly reported *B. permians* 16S ribosomal RNA gene sequence is ancient or not.

There are several reasons to doubt the antiquity of *B. permians*. The first concerns the extraordinary similarity of its 16S rRNA gene sequence to that of *Bacillus marismortui*. *Bacillus marismortui* was described by Arahal et al. (1999) as a moderately halophilic species from the Dead Sea and was later renamed *Salibacillus marismortui* (Arahal et al. 2000). The *B. permians* sequence differs from that of *S. marismortui* by only one transition and one transversion out of the 1,555 aligned and unambiguously determined nucleotides. In comparison, the 16S rRNA gene from *Staphylococcus succinus*, which was claimed to be ''25–35 million years old'' (Lambert et al. 1998), differs from its homolog in its closest present-day relative (a urinary pathogen called *Staphylococcus saprophyticus*) by 19 substitutions out of 1,525 aligned nucleotides. Using Kimura's (1980) two-parameter model, the difference between the *B. permians* and *S. marismortui* sequences translates into 1.3 $\times 10^{-3}$ substitutions per site along the two lineages. The rate of substitution for 16S ribosomal DNA in prokaryotes is remarkably uniform among diverse lineages and ranges between 1×10^{-8} and 5×10^{-8} substitutions per site per year (Ochman and Wilson 1987; Hillis and Dixon 1991; Munson et al. 1991; Clark et al. 1992; Moran et al. 1993; Aksoy, Pourhosseini, and Chow 1995; Bandi et al. 1995; Clark, Moran, and Baumann 1999; Ochman, Elwin, and Moran 1999). Applying these rates, we estimate the time of divergence between *B. permians* and *S. marismortui* to be about 13,000–65,000 years. We note, moreover, that the degree of divergence between *B. permians* and *S. marismortui* is smaller than the degree of divergence between two strains of *S. marismortui* (Arahal et al. 1999).

The second reason to doubt the antiquity of *B. permians* concerns the rate of evolution of its 16S rRNA gene. We used the relative-rate test (Sarich and Wilson 1973) with the phylogenetic weighting scheme of Robinson et al. (1998) to test the number of substitutions on the *B. permians* branch against that on the *S. marismortui* branch, with *Virgibacillus proomii* as the outgroup (fig. 1). There was no significant difference in the numbers of nucleotide substitutions between the two lineages ($P = 0.48$). Were we to accept the antiquity of *B. permians*, we would have to conclude that the number of substitutions accumulated by *S. marismortui* during 250 Myr of evolution is equal to that accumulated by *B. permians* during the 3–7 days of active replication in the laboratory following its rescue from a 250-Myr evolutionary slumber.

Assuming, as Vreeland, Rosenzweig, and Power (2000) did, that *B. permians* did not evolve for 250 Myr, all the differences between the two sequences would have to be attributed to at least 250 Myr of evolution in *S. marismortui*. Consequently, we must conclude that the rate of substitution in the 16S rRNA gene of *S. marismortui* is about 5×10^{-12} substitutions per site per year, i.e., a reduction of four orders of magnitude in comparison with the typical prokaryotic rate. Such a low rate of nucleotide substitution has never been encountered in nature, least of all in bacteria. Under the assumption that *S. marismortui* evolves at a rate that is typical of eubacteria, we must conclude that the time of divergence between *B. permians* and *S. marismortui* is quite short.

We note, however, that an alternative explanation to the modernity of *B. permians* may be raised. According to one such explanation, put forward by one of the co-authors of the Vreeland, Rosenzweig, and Powers (2000) report in a *New York Times* interview from October 19, 2000, *S. marismortui* may have also been trapped in salt for millions of years. In other words, the similarity between the two bacteria is explained by a lack of accumulation of nucleotide substitutions in both

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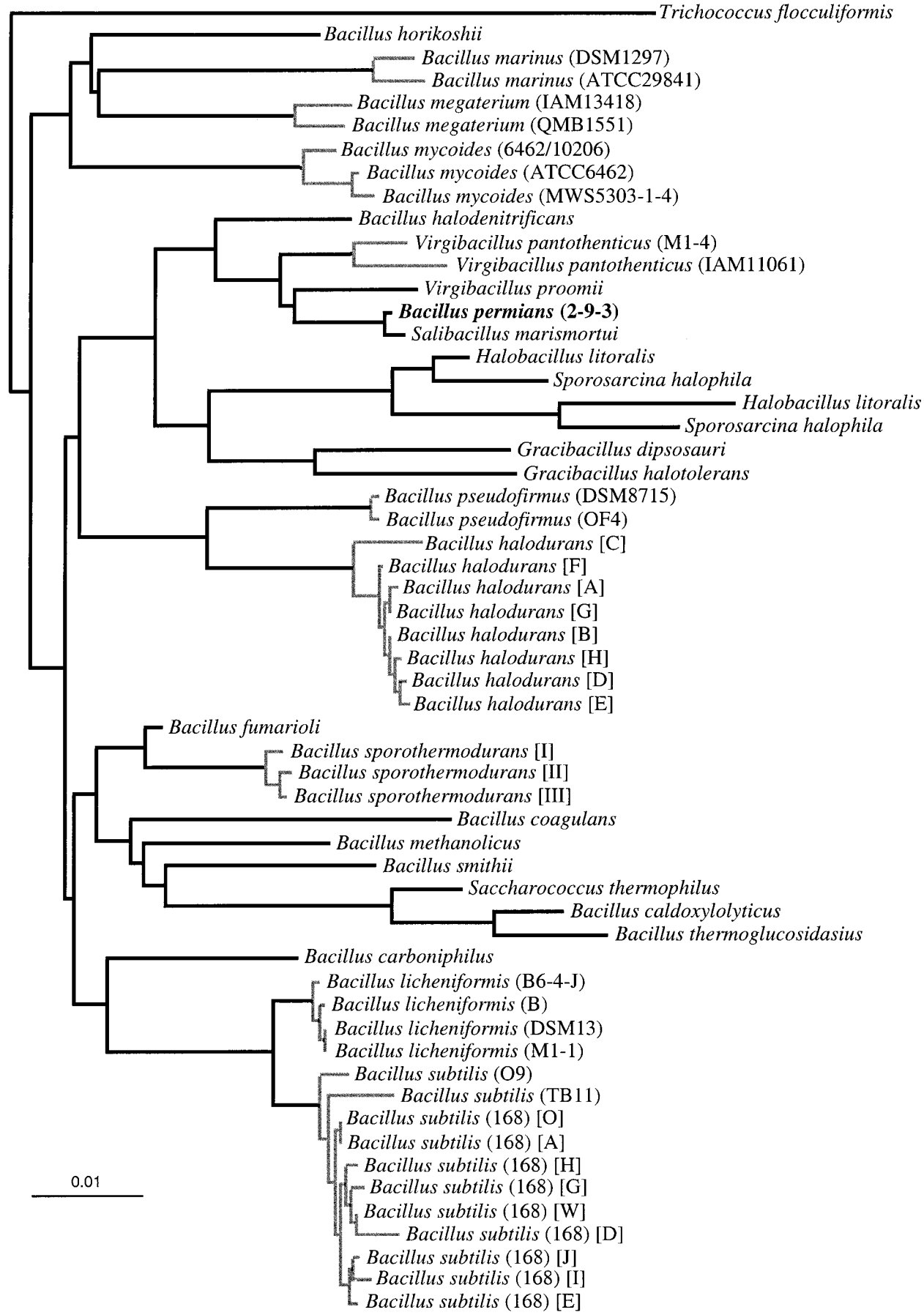
Trichococcus flocculiformis
Bacillus horikoshii
Bacillus marinus (DSM1297)
Bacillus marinus (ATCC29841)
Bacillus megaterium (IAM13418)
Bacillus megaterium (QMB1551)
Bacillus mycoides (6462/10206)
Bacillus mycoides (ATCC6462)
Bacillus mycoides (MWS5303-1-4)
Bacillus halodenitrificans
Virgibacillus pantothenticus (M1-4)
Virgibacillus pantothenticus (IAM11061)
Virgibacillus proomii
Bacillus permians (2-9-3)
Salibacillus marismortui
Halobacillus litoralis
Sporosarcina halophila
Halobacillus litoralis
Sporosarcina halophila
Gracibacillus dipsosauri
Gracibacillus halotolerans
Bacillus pseudofirmus (DSM8715)
Bacillus pseudofirmus (OF4)
Bacillus halodurans [C]
Bacillus halodurans [F]
Bacillus halodurans [A]
Bacillus halodurans [G]
Bacillus halodurans [B]
Bacillus halodurans [H]
Bacillus halodurans [D]
Bacillus halodurans [E]
Bacillus fumarioli
Bacillus sporothermodurans [I]
Bacillus sporothermodurans [II]
Bacillus sporothermodurans [III]
Bacillus coagulans
Bacillus methanolicus
Bacillus smithii
Saccharococcus thermophilus
Bacillus caldoxylolyticus
Bacillus thermoglucosidasius
Bacillus carboniphilus
Bacillus licheniformis (B6-4-J)
Bacillus licheniformis (B)
Bacillus licheniformis (DSM13)
Bacillus licheniformis (M1-1)
Bacillus subtilis (O9)
Bacillus subtilis (TB11)
Bacillus subtilis (168) [O]
Bacillus subtilis (168) [A]
Bacillus subtilis (168) [H]
Bacillus subtilis (168) [G]
Bacillus subtilis (168) [W]
Bacillus subtilis (168) [D]
Bacillus subtilis (168) [J]
Bacillus subtilis (168) [I]
Bacillus subtilis (168) [E]
0.01

lineages due to the fact that both *B. permians* and *S. marismortui* are ancient. To test this possibility, we conducted a relative-rate test of *B. permians* and *S. marismortui* versus *V. proomii,* with two strains of *Virgibacillus pantothenticus* as outgroup (fig. 1). Again, we found no significant difference in the number of nucleotide substitutions between the two ingroup lineages ($P = 0.74$), and under the assumption of the molecular clock, we must conclude that *B. permians, S. marismortui,* and *V. proomii* are contemporaneous organisms. Of course, the claim may be raised that *V. pantothenticus* is also ancient, so we also performed the relative-rate test on *B. permians, S. marismortui,* and *V. proomii* against *V. pantothenticus,* with *Bacillus halodenitrificans* as the outgroup. Again, we found no significant difference in the mean numbers of nucleotide substitutions between the two ingroup lineages ($P = 0.51$). Interestingly, the results were robust to changes in the topology of the phylogenetic tree. We note, however, that this line of reasoning, i.e., assuming that the organisms with which we compare *B. permians* are also ancient, may be carried on *ad infinitum.*

Finally, we conducted an extensive phylogenetic study of *B. permians* and closely related species. From this study, we excluded genes whose sequenced lengths were shorter than that of the *B. permians* sequence (e.g., *Bacillus circulans*). We also excluded sequences that contained more than 1% ambiguously determined nucleotides (e.g., *Salibacillus salexigens*), as well as sequences that could not be easily aligned to the rest of the sequences (e.g., the 16S rRNA B paralog from *B. subtilis* strain 168). As an outgroup, we chose a bacterium from the Lactobacillaceae whose branch length was the shortest to the ingroup. Thus, we chose *Trichococcus flocculiformis* instead of *Lactobacillus casei* as in Vreeland, Rosenzweig, and Powers (2000). In the end, we were left with 57 closely related 16S rRNA gene sequences from 29 species. As is evident from figure 1, *B. permians* does not occupy an ancestral position in the tree, and the degree of variation between *B. permians* and *S. marismortui* is much smaller than either the degree of variation among conspecific paralogous genes or the degree of variation among alleles from conspecific strains. In the analysis presented in figure 1, many paralogous 16S rDNA sequences were excluded because they were only distantly related to the bulk of the sequences in the analysis. Thus, the relative degree of variation between *B. permians* and *S. marismortui* is even smaller than that shown in the figure.

Notwithstanding the heroic contamination checks by Vreeland, Rosenzweig, and Powers (2000), which included microscopical examinations, alkali and acid sterilizations, and UV radiations, the pudding just tastes too fresh to be Permian, and *B. permians* is most probably destined to join the growing list of such purportedly Methuselan specimens as the Miocene magnolia (Golenberg et al. 1990), the Cretaceous weevil (Cano et al. 1993), and the would-be dinosaur (Woodward, Weyland, and Bunnell 1994), which turned out to be contemporary artifacts (Austin, Smith, and Thomas 1997; Walden and Robertson 1997; Gutiérrez and Marín 1998).

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FIG. 1.—Scaled neighbor-joining phylogenetic tree (Saitou and Nei 1987) from 16S rDNA sequences of *Bacillus permians* and closely related species. The alignment is available at http://kimura.tau.ac.il/~tal. Gray lines mark branches leading to nine groups of species (*Bacillus marinus, Bacillus megaterium, Bacillus mycoides, Bacillus pseudofirmus, Bacillus halodurans, Bacillus sporothermodurans, Bacillus licheniformis, Bacillus subtilis,* and *Virgibacillus panthotenticus*) for which two or more unambiguously alignable homologous sequences are available in the literature. Strains are shown in parentheses. Paralogous sequences are shown in brackets.

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