COMMENT

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Vreeland et al. (2000) reported the successful isolation of a spore-forming bacterium, Bacillus strain 2-9-3, from a brine inclusion within a halite crystal recovered from a presumably 250 Ma Permian Salado Formation in Carlsbad, New Mexico. The authors claim the bacterium isolate to be as old as the formation itself, thereby nominating it to be the oldest viable organism ever recovered. Since then, both the age of the brine inclusion and the bacterium isolate itself have been questioned (Hazen and Roedder, 2001; Graur and Pupko, 2001; Nickle et al., 2002; Willerslev et al., 2004a; Hebsgaard et al., 2005). Recently, Satterfield et al. (2005) presented evidence that brine inclusions from the same layer of salt that housed Bacillus strain 2-9-3 are composed of evaporated Late Permian seawater. The authors find these results to be in strong support of the old age of strain 2-9-3. We strongly disagree with this conclusion. Although the age of the brine from where strain 2-9-3 was isolated might, indeed, be 250 Ma, it does not necessarily mean that Bacillus strain 2-9-3 is of similar age. In fact, there are a number of both theoretical and empirical reasons to suggest otherwise.

1. The use of nonspecific media for the culturing of Bacillus strain 2-9-3 makes the risk of false positive results extremely high. Although Satterfield et al. (2005, p. 265) state that Bacillus strain 2-9-3 “has received significant publicity because of the extreme sterilization techniques used to avoid contamination by modern microorganisms,” Vreeland et al. (2000) did not follow some of the most basic authentication criteria, such as replication of their result in an independent laboratory. This criterion is of great importance if laboratory based contamination is to be excluded (i.e., all types of contamination related to the laboratory), as it’s unlikely that different laboratories would obtain the same result due to a common lab contaminant (Willerslev and Cooper, 2005). Intriguingly, no claims of geologically ancient cultures or DNA sequences published to date (i.e., claims >1 Ma) have followed this simple criterion of authentication (Hebsgaard et al., 2005).

2. DNA is a relatively unstable molecule compared to other cellular components such as lignin and cutine, and will degrade with time if not repaired. The rate of degradation is known to be highly dependent on the environment, particularly the temperature (Smith et al., 2001; Willerslev et al., 2004b; Willerslev and Cooper, 2005). For example, calculations have shown that free DNA experiencing depurination damage will break down to <100 base-pair (bp) fragments in less than 10 k.y. under warm and humid conditions, and in less than 100 k.y. under cold conditions (Poinar et al., 1996; Smith et al., 2001; Willerslev et al., 2004b). No metabolic activity has yet been measured from endospores like Bacillus strain 2-9-3, excluding the possibility of active DNA repair prior to germination. Although endospores have special adaptations such as DNA binding σ/β-type small acid soluble proteins to reduce the rate of genomic modification, it still remains unlikely that they should be able to germinate after hundreds of millions of years of dormancy, particular under nonfrozen conditions. In support of this, a recent study of bacterial DNA in permafrost—an environment considered the most promising for long-term DNA survival—has shown that DNA from endospore-forming bacteria >600 bp in size cannot be obtained from samples older than 0.5 Ma, and not even 120-bp DNA fragments can be reproducibly obtained from samples ≥2 Ma (Willerslev et al., 2004a).

3. Relative rate tests conducted on 16S ribosomal DNA and protein coding gene (recA and splB) sequences obtained from Bacillus strain 2-9-3 strongly suggest that it is not geologically ancient (Graur and Pupko, 2001; Nickle et al., 2002; Maughan et al., 2002; Hebsgaard et al., 2005). This test investigates the relative genetic distance from an outgroup to the postulated ancient organism and its closest contemporary relatives. One assumption of the relative rate test is that the mutation rate is similar and constant in the DNA sequences for both the ancient organism and its contemporary relatives. If Bacillus strain 2-9-3 is ancient, then a significantly shorter genetic distance to the outgroup is expected as compared to its contemporary relatives, as the latter should have an additional 250 m.y. to accumulate substitutions. Although mutation rates are not always similar across organisms, it is striking that not a single claim of geologically ancient DNA, including that of Bacillus strain 2-9-3, has passed the rate test so far, strongly suggesting that contamination is involved (Hebsgaard et al., 2005).

Therefore, despite the recent results of Satterfield et al. (2005), it still remains highly controversial whether the Bacillus strain 2-9-3 is, indeed, millions of years old.

REFERENCES CITED


REPLY

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Willerslev and Hebsgaard’s comment on the evidence for the 250 Ma age of the halite, brine inclusion, and Virgibacillus strain 2-9-3 reported in Satterfield et al. (2005) comes at a time when earth scientists are busily searching for signs of microscopic life in ancient samples of permafrost, ice, deep-sea sediments, amber, salt, and chert. In the not too distant future, sedimentary rocks may be returned from Mars. It is critically important that the scientific community agree on the methods used for study of ancient microbes and ancient DNA and that issues of sample age, contamination, sterilization, and replication of results be addressed now, not later. In this regard, Willerslev and Hebsgaard here question the age and origin of Virgibacillus strain 2-9-3 cultured from a brine inclusion in halite from the Permian Salado salts. They do not take issue with the evidence for the Permian age of the brine inclusions discussed in Satterfield et al. (2005), but they imply that Virgibacillus strain 2-9-3, reported by Vreeland et al. (2000), is a modern organism, presumably a laboratory contaminant. That conclusion is based on (1) lack of replication of results in an independent laboratory, (2) degradation of relatively unstable DNA molecules over geological time, and (3) “relative rate tests,” which suggest that Virgibacillus strain 2-9-3 is not geologically ancient.

1. Willerslev and Cooper (2005) and Hebsgaard et al. (2005) reviewed issues of contamination and proposed important guidelines for geobiological studies by offering criteria for the authentication of results for the study of ancient DNA and viable microbial cells. Vreeland et al. (2000) described their laboratory procedures and the sterilization techniques used to avoid contamination by modern organisms. They meet the guidelines of Hebsgaard et al. (2005). Willerslev and Hebsgaard are correct that Vreeland et al. (2000) did not obtain replication of their results by an independent laboratory before publication. Such verification is important because it is not likely that separate laboratories would have common microbial contaminants. We welcomed any geobiologists interested in studying the Salado halites and fluid inclusions in the years since 2000. None have so far taken up the offer.

Satterfield et al. (2005) showed that halites of the Permian Salado salts trapped surface brines as fluid inclusions. This halite is ideal for the study of ancient DNA and microbial cells because samples of brine, once trapped inside crystals as fluid inclusions, can remain completely sealed and isolated from the environment for periods of more than 500 m.y. Such preservation of fluid inclusions has allowed samples of ancient evaporated seawater to be analyzed from halites going back to the late Precambrian (Lowenstein et al., 2001). Careful surface sterilization of similar halite crystals should also allow study of uncontaminated samples of Earth’s ancient biosphere in fluid inclusions.

2. Willerslev and Hebsgaard state, “DNA is a relatively unstable molecule compared to other cellular components… and will degrade with time if not repaired.” Twelve years ago, it was thought that DNA, as short fragments, may survive 10³ years (Lindahl, 1993). Now it is reported that, under certain conditions such as in permafrost, preservation of DNA may approach 10⁶ years (Hebsgaard et al., 2005). Clearly, the upper limit of DNA survival has changed and should not therefore be used as evidence against the Permian age of Virgibacillus strain 2-9-3. Willerslev and Hebsgaard state, “The rate of degradation (of DNA) is known to be highly dependent on the environment.” In this regard, fluid inclusions in halite from shallow burial environments (<1 km, <30 °C) offer a “friendly” setting for long-term preservation of microbial cells and DNA. They are low in oxygen and their salty waters are known from experiments to slow down the breakup of DNA by depurination (Lindahl and Nyberg, 1972). There has been no systematic research on DNA preservation in such oxygen-poor, saline systems at low temperatures, although that is changing. We are currently studying ancient microorganisms and DNA in fluid inclusions in halite from Death Valley and Saline Valley salt cores, 10⁴ to 10⁶ years old.

3. Relative rate tests, which suggest that Virgibacillus strain 2-9-3 is not geologically ancient, are based on the assumption that evolution follows a predictable mutation rate and that the rate is known. The rates being used by the researchers cited are based on nucleotide substitutions in laboratory-grown bacteria, which may not be realistic for all organisms. Furthermore, growth rates of microorganisms in nature may, in some cases, be measured on time scales of centuries or longer, as illustrated by Parkes et al. (2000) for bacteria isolated from subseafloor sediments. Such long generation times might explain the similarities in 16S ribosomal DNA of Virgibacillus strain 2-9-3 and its contemporary relatives (Maughan et al., 2002).

As we continue to study microorganisms preserved in ancient halite, perhaps others will participate or conduct the additional independent work that Willerslev and Hebsgaard want to see. We agree that extreme care is required when analyzing small numbers of ancient cells or small amounts of ancient DNA. As the field of geobiology expands, the need for discipline-wide laboratory standards is greater than ever.

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