FEMS MICROBIOLOGY LETTERS

LETTER TO THE EDITOR

Reply to Santiago-Rodriguez *et al.*: Was *luxS* really isolated from 25- to 40-million-year-old bacteria?

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Although Santiago-Rodriguez *et al.* (2014) attempt to reconstruct the evolutionary history of bacterial quorum sensing, the authors fail to address previous criticisms of this work and do not provide the appropriate experimental controls and analyses required to make the claims put forth in this article.

In the recent publication, '*luxS* in bacteria isolated from 25- to 45-million-year-old amber' published in FEMS Microbiology Letters (January, 2014), Santiago-Rodriguez *et al.* claim to have obtained ancient *luxS* sequences from 25- to 40-million-year-old bacteria isolated from amber, which are used to reconstruct the evolutionary history of quorum sensing. However, the authors have completely ignored previous responses and publications criticizing the isolation of the actual ancient bacterial isolates used in this study, as well as fail to demonstrate an understanding of the rigorous controls and experimental methodologies required to achieve such results.

The putative 'ancient' bacteria obtained for this study were previously isolated in 1995 by Cano and Borucki, as part of a study that has been heavily criticized (Fischman, 1995) and disproven using phylogenetic analysis (Yousten & Rippere, 1997). It is highly likely that modern microorganisms contaminated reagents or tools used during the initial isolation of bacterial species trapped within ancient amber (Cano & Borucki, 1995), which the authors cannot verify and fail to discuss in their current manuscript. In addition, the authors state that amber possesses 'preservative properties' that allow DNA (not living microorganisms) to be isolated and extracted from amber, citing only the highly criticized study by Cano and Borucki (1995); however, they fail to mention the wealth of publications demonstrating the opposite – that DNA alone could not be obtained from copal (unfossilized amber) only 10 000 years old (Austin *et al.*, 1998) or that identifiable DNA fragments, let alone nonreplicating microorganisms, do not persist beyond 1.5 million years (Allentoft *et al.*, 2012).

Ancient DNA studies typically apply phylogenetic metrics to identify ancient species, as well as rule out modern contamination. Santiago-Rodriguez *et al.* (2014) interpret a phylogenetic analysis of 16S rRNA sequences [fig. 1b in (1)] to demonstrate that they have obtained *luxS* sequences from several different ancient species. However, this finding directly contradicts the findings by Cano and Borucki, who identified the isolates in 1995 to be closely related to *Bacillus sphaericus* (Cano & Borucki, 1995). Cano and Borucki suggested that ancient bacteria capable of surviving for millions of years can only do so in a sporulated form, as is well documented for *Bacillus* species. Our interpretation of the phylogenetic tree constructed from 16S rRNA sequences (fig. 1b and SI table 3) suggests that isolates 41_AG11AC7 and 46_AG11AC9a are likely *Staphylococcus* species, which are not known to sporulate and are also common microorganisms on the human skin. Surprisingly, the authors never comment on this specific identification of bacterial species within the main text of this study or discuss how nonsporulating microorganisms that are common on human skin may have survived millions of years.

The authors also fail to present negative control sequences to confirm that the DNA sequences presented within this study are not the results of laboratory or reagent contamination, rather than contamination that likely occurred in 1995. The primers designed to amplify 16S rRNA target a wide range of microorganisms present within laboratory reagents. Previous publications from the authors demonstrate that they are aware of modern DNA contaminants within PCR laboratory reagents. However, modern contamination is only mentioned when discussing cross-contamination between samples. 'Cross-contamination can also be discarded due to the differing 16S rRNA gene sequences among the isolates that were positive for *luxS*', suggesting that *luxS* came from numerous different species. This clearly does not rule out contamination from multiple bacterial species within laboratory reagents. Typically, modern contamination is investigated by sequencing negative control samples or by sampling the laboratory environment.

Regardless of previous criticisms and without appropriately examining laboratory contamination, the authors still attempt to reconstruct the evolutionary history of the quorum sensing locus. They conclude that all of the ancient *luxS* sequences are from the Firmicutes (fig. 1a), i.e. *Bacillus*, even though the 16S rRNA phylogenetic tree did not agree with this finding. The authors reconcile this discrepancy by suggesting that quorum sensing originated

in *Bacillus* species and was then transferred across known bacterial species. However, for this to occur, the ancient *luxS* fragments would have to fall basal to known, modern sequences on the phylogenetic tree, which is not the case (fig. 1a). The *luxS* sequences only fall basal when the sequences are presented in a cluster dendogram, which is not a sufficient or recognized method for establishing the evolutionary history of a species or gene family.

The authors fail to demonstrate an appropriate knowledge of past literature in the area, ignoring the limitations and methodologies of ancient organismal and DNA research that were intensely argued in the 1990s. This would appear simply as an embarrassing oversight if it were not for the fact that one of the co-authors was featured prominently in several widely discredited studies of that period and is clearly aware of these issues. Disregarding published scientific debate without any attempt to clarify, justify or confirm previous findings sets a dangerous precedent for future work and should not be taken lightly.

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