Age of Bacteria from Amber

The conclusion of Raúl J. Cano and Monica K. Borucki (*1*), that they have succeeded in culturing a bacterium which has lain dormant for the past 25 million years entombed in a fossilized bee, raises the thorny problem of establishing that the revived bacterium differs from all extant species.

Bacillus sphaericus, the species to which the bacterium at issue is most closely related, is a fine example of our ignorance of the diversity of bacterial life. Strains phenotypically resembling *B. sphaericus* (that is, differentiate into a spherical endospore and have a highly aerobic metabolism) have been classified into six distinct DNA hybridization groups (*2*) that are supported by different ribosomal RNA (rRNA) sequences (*3*) and more than 16 phenetic taxa (*4*). Each of these probably constitutes a separate species as currently defined by most bacterial systematists. Moreover, single strains represented an additional 12 distinct "species" of *B. sphaericus* in these studies. At a conservative estimate, known *B. sphaericus* strains could be divided into at least 20 species-ranked taxa, and new isolates from the environment would probably comprise many more.

Until we have isolated and characterized the biodiversity of today, it is premature to claim that a bacterium is ancient by virtue of its distinction from some strains of *B. sphaericus* selected from the rRNA sequence database.

F. G. Priest
Department of Biological Sciences, Heriot watt University, Edinburgh EH14 4AS, Scotland, United Kingdom

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Cano and Borucki (*1*) report revival of bacterial spores from 25 to 40 million-year-old amber. The sequence divergence they provide is the only direct evidence that the bacterial isolate is of ancient origin, but their DNA analysis is seriously flawed. Figure 2 of their report gives sequences of the putative ancient bacteria aligned with three sequences from the same species taken from the GenBank database, accession numbers D16280, L14010, and X60639. Careful comparison (Fig. 1) of the database sequences from GenBank with those shown in figure 2 of the report shows numerous errors evidently introduced by Cano and Borucki. The most serious of these are two gaps of six and five bases shown for "B.SPHAER1" (D16280). These mistakes are particularly problematic, as all other sequences are reported simply as comparisons to "B.SPHAER1." Where a six nucleotide gap is shown (positions 98 to 103), D16280 has the five nucleotide sequence, 'ACCTT'. A six nucleotide sequence shown at this position for "BSPH16SR" (X60639), 'TACCTT', is only five: 'ACCTT'. A five nucleotide gap shown from positions 190 to 194 is 'GGCGT' in the GenBank D16280. In addition, a two base gap shown in "B.SPHAER1" at positions 360 to 361 is 'AT' in the GenBank sequence. Numerous mono- and dinucleotide changes have been introduced throughout the sequences (Fig. 1).

Because there are so many mistakes in the sequences shown in figure 2 of the

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B.SPHAER1  GAAAGCCTGA TGGAGCAACG CCGCGTGAGT GAAGAAGGAT TTCGGTTCGT   50
D16280     .......... C......... .......... .TT....... ..........
B.SPHAER2  GAAAGCCTGA TGGAGCAACG CCGCGTGAGT GAAGAAGGAT TTCGGTTCGT
L14010     .......... .......... .......... .......... ..........
BSPH16SR   GAAAGCCTCA TGGAGCAACG CCGCGTGAGT GAAGAAGGAT NTCGGTTCGT
X60639     .......... .......... .......... .......... ..........

B.SPHAER1  AAAACTCTGT TGTAAGGGAA GAACAAGTAC -GTA-GTAAC TGGCTTT---  100
D16280     .......... .......... .......... A...-..... ....G.-AC
B.SPHAER2  AAAACTCTGT TGTAAGGGAA GAACAAGTAC -GTA-GTAAC TGGCTTT---
L14010     .......... .......... .......... -...-..... ....-.-AG
BSPH16SR   AAAACTCTGT TGTAAGGGAA GAACAAGTAC AGTA-GTAAC TGGCCTTTAC
X60639     .......... .......... .......... ....-..... .N..TN.-..

B.SPHAER1  ---GACGGTA CCTTATTAGA AAGCCACGGC TAACTACGTG CCAGCAGCCG  150
D16280     CTT....... .......... .......... .......... ..........
B.SPHAER2  ---GACGGTA CCTTATTAGA AAGCCACGGC TAACTACGTG CCAGCAGCCG
L14010     CTT....... .......... .......... .......... ..........
BSPH16SR   CTTGACGGTA CCTTATTAGA AAGCCACGGC TAACTACGTG CCAGCAGCCG
X60639     .......... .......... .......... .......... ..........

B.SPHAER1  CGGTAATACG TAGGTGGCAA GCGTTGTCCG GAATTATTG- ----TAAGCG  200
D16280     .......... .......... .......... .........G GCGTA.....
B.SPHAER2  CGGTAATACG TAGGTGGCAA GCGTTGTCCG GAATTATTG- ----TAAGCG
L14010     .......... .......... .......... .........G GCGT-.....
BSPH16SR   CGGTAATACG TAGGTGGCAA GCGTTATCCG GAATTATTGG GCGTNAAGCG
X60639     .......... .......... .....N.... ........N. ..........

B.SPHAER1  CGCGCAGGTG GTTTCTTAAG TCTGATGTGA AAGCC-ACGG CTCAACCGTG  250
D16280     .......... .......... .......... .....C.... ..........
B.SPHAER2  CGCGCAGGTG GTTTCTTAAG TCTGATGTGA AAGCC-ACGG CTCAACCGTG
L14010     .......... .......... .......... .......... ..........
BSPH16SR   CGCGCAGGTG GTTTCTTAAG TCTGATGTGA AAGCCCACGG CTCAACCGTG
X60639     .......... .......... ...N...... .......... ..........

B.SPHAER1  GAGGGTCATT GGAAACTGGG AGACTTGAGT GCAGAAGAGG ATAGTGGAAT  300
D16280     .......... .......... .......... .......... ..........
B.SPHAER2  GAGGGTCATT GGAAACTGGG AGACTTGAGT GCAGAAGAGG ATAGTGGAAT
L14010     .......... .......... .......... .......... ..........
BSPH16SR   GAGGGTCATT GGAAACTGGG AGACTTGAGT GCAGAAGAGG ATAGTGGAAT
X60639     .........N .......... .......... .......... ..N.......

B.SPHAER1  TCCAAGTGTA GCGGTGAAAT GCGTAGAGAT TTGGAGGAAC ACCAGTGGCG  350
D16280     .......... .......... .......... .......... ..........
B.SPHAER2  TCCAAGTGTA GCGGTGAAAT GCGTAGAGAT TTGGAGGAAC ACCAGTGGCG
L14010     .......... .......... .......... .......... ..........
BSPH16SR   TCCAAGTGTA GCGGTGAAAT GCGTAGAGAT TTGGAGGAAC ACCAGTGGCG
X60639     .........N .....N.... .......... .......... N.........

B.SPHAER1  AAGGCGACT- -CTGGTCTGT AACTGACACT GAGGCGCGAA AGCGTGGGGA  400
D16280     .........A T......... .......... .......... ..........
B.SPHAER2  AAGGCGACT- -CTGGTCTGT AACTGACACT GAGGCGCGAA AGCGTGGGGA
L14010     .........A T......... .......... .......... ..........
BSPH16SR   AAGGCGACTA TCTGGTCTGT AACTGACACT GAGGCGCGAA AGCGTGGGGA
X60639     .......... ...N...... .......N.. .......... .......N..

B.SPHAER1  GCAAACAGGA TTAGATACCC TGGTAGTCCA CGCCGTAAAC GATGAGTGCT  450
D16280     .......... .......... .......... .......... ..........
B.SPHAER2  GCAAACAGGA TTAGATACCC TGGTAGTCCA CGCCGTAAAC GATGAGTGCT
L14010     .......... .......... .......... .......... ..........
BSPH16SR   GCAAACAGGA TTAGATACCC TGGTAGTCCA CGCCGTAAAC GATGAGTGCT
X60639     .......... .......... .N........ .......... ..........

B.SPHAER1  AAGTGT
D16280     ......
B.SPHAER2  AAGTGT
L14010     ......
BSPH16SR   AAGTGT
X60639     N.....
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Fig. 1. Comparison of the sequences shown in figure 2 of the report by Cano and Borucki (*1*) with the GenBank sequences. In each pair of sequences, the lower sequence, identified by accession number, is compared to the corresponding sequence provided by Cano and Borucki. Dots indicate identical nucleotides. A one base "conserved gap" is included at position 85 to retain alignment with figure 2 in their report.

report, it is not possible to unambiguously reconstruct the sequence for the putative ancient bacterial strain (BCA16). I have produced an alignment (Fig. 2) with the use of the GenBank sequence D16280, along with my best guess for the sequence of BCA16. Because figure 3 of the report purportedly shows the secondary structure of the "BCA16 amplicon," I attempted to resolve the ambiguities using it, but there are more mistakes in this figure than in the figure 2 sequences. (I include this sequence in my Fig. 2, as sequence "Fig. 3.") One region of 23 bases, from positions 74 to 97, shows only eight nucleotides corresponding to those of the sequences given in their figure 2. The asterisks placed on figure 3 are particularly puzzling, as it suggests that they actually did a nucleotide-by-nucleotide comparison. The positioning of their asterisks is also given by me (Fig. 2).

If the credibility of the findings of Cano and Borucki rests on the DNA sequence analysis, their report fails that test. I would suggest that a better test would be an accurate DNA sequence comparison of the putative ancient strain with the modern strains they apparently have growing in their laboratory (table 1 in the report), not with database sequences from evidently unrelated strains.

Andrew T. Beckenbach
Institute of Molecular Biology and Biochemistry,
Department of Biological Sciences,
Simon Fraser University,
Burnaby, B.C. V5A 1S6, Canada

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D16280      GAAAGCCTGA CGGAGCAACG CCGCGTGAGT GTTGAAGGAT TTCGGTTCGT   50
B.SPHAER1   .......... T......... .......... .AA....... ..........
B.SPHAER2   .......... T......... .......... .AA....... ..........
BCA16       ........C. T......... ........AC .AA.....C. ..........
Fig. 3      .......... T......... .......... .AA....... ....A.....
                                                *     *       *
BSPH16SR    ........C. T......... .......... .AA....... N.........

D16280      AAAACTCTGT TGTAAGGGAA GAACAAGTAC AGTAGTAACT GGCTGT-ACC  100
B.SPHAER1   .......... .......... .......... ---------- ....T.----
B.SPHAER2   .......... .......... .......... ---------- ....T.----
BCA16       ...G...... .......... .......... C.G....... .C.GT.C-..
Fig. 3      ...G...... ...T.....G ...GGGTAC. G.G..A..G. CC.GC-C...
                                              * * * * *  *  *  *
BSPH16SR    .......... .......... .......... .......... ...CT.T...

D16280      TTGACGGTAC CTTATTAGAA AGCCACGGCT AACTACGTGC CAGCAGCCGC  150
B.SPHAER1   --........ .......... .......... .......... ..........
B.SPHAER2   --........ .......... .......... .......... ..........
BCA16       .......... ....CC.... .......... .......... ..........
Fig. 3      .......... ..A.CAGA.. .......... .......... ..........
                        *
BSPH16SR    .......... .......... .......... .......... ..........

D16280      GGTAATACGT AGGTGGCAAG CGTTGTCCGG AATTATTGGG CGTAAA-GCG  200
B.SPHAER1   .......... .......... .......... ........-- ---T......
B.SPHAER2   .......... .......... .......... ........-- ---T......
BCA16       .......... .......... ....A..... .........- -..T......
Fig. 3      .......... .......... ....A..... T.A.TA.T.. GTGT..A.G.

BSPH16SR    .......... .......... ....A..... .......... ...N......

D16280      CGCGCAGGTG GTTTCTTAAG TCTGATGTGA AAGCCCACGG CTCAACCGTG  250
B.SPHAER1   .......... .......... .......... .....-.... ..........
B.SPHAER2   .......... .......... .......... .....-.... ..........
BCA16       .T........ ..C....... .......... .......... ..........
Fig. 3      .T........ ..C....... .......... .......... ..........
             *      *    *                       *            *
BSPH16SR    .......... .......... .......... .......... ..........

D16280      GAGGGTCATT GGAAACTGGG AGACTTGAGT GCAGAAGAGG ATAGTGGAA-  300
B.SPHAER1   .......... .......... .......... .......... .........-
B.SPHAER2   .......... .......... .......... .......... .........-
BCA16       .......... .......... .......... .......... .G.......-
Fig. 3      .......... .......... .......... .......... .G.......A
                                  **
BSPH16SR    .......... .......... .......... .......... .........-

D16280      TTCCAAGTGT AGCGGTGAAA TGCGTAGAGA TTTGGAGG-A ACACCAGTGG  350
B.SPHAER1   .......... .......... .......... ........-. ..........
B.SPHAER2   .......... .......... .......... ........-. ..........
BCA16       .....C.... .......... .......... .G......-. ..........
Fig. 3      .....C.... .......... .......... .G......G. ..........

BSPH16SR    .......... .......... .......... ........-. ..........

D16280      CGAAGGCGAC TATCTGGTCT GTAACTGACA CTGAGGCGCG AAAGCGTGGG  400
B.SPHAER1   .......... .--...... .......... .......... ..........
B.SPHAER2   .......... .--...... .......... .......... ..........
BCA16       .......... .......... .........G ......A... ..........
Fig. 3      .......... .......... .........G ......A... ..........
                       *                              *
BSPH16SR    .......... .......... .......... .......... ..........

D16280      GAGCAAACAG GATTAGATAC CCTGGTAGTC CACGCCGTAA ACGATGAGTG  450
B.SPHAER1   .......... .......... .......... .......... ..........
B.SPHAER2   .......... .......... .......... .......... ..........
BCA16       .......... .......... .......... .......... ..........
Fig. 3      .......... .......... .......... .....-.... ..........
             *
BSPH16SR    .......... .......... .......... .......... ..........

D16280      CTAAGTGT
B.SPHAER1   ........
B.SPHAER2   ........
BCA16       ........
Fig. 3      ........

BSPH16SR    ........
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Fig. 2. Alignment of sequences from figures 2 and 3 of the report by Cano and Borucki with the GenBank accession number D16280. "Fig. 3" is taken from the secondary structure depiction of "BCA16 amplicon." Sixth line in each group gives the positions of the asterisks in figure 3 of the report, which are supposed to indicate the locations of nucleotide substitutions.

Response: Critical evaluation of our work is both necessary and welcome. Priest indicates that *B. sphaericus* is a heterogeneous taxon and may include at least 16 phenetic taxa. We compared (*1*) the 16S rRNA sequence from the purported ancient strain BCA16 with eight different extant *B. sphaericus* sequences (GenBank accession numbers L14010 to L14016 and X60639). The comparisons were initially made with approximately one-third of the 16S rRNA sequence of each isolate, and this analysis has now been extended to the entire 16S rRNA sequence. The results (Fig. 1) are essentially the same as stated in our report; BCA16 is still most closely related to the round-spored *Bacillus* and, in particular, *B. sphaericus*.

Priest's comment, in essence, requires negative evidence (that the query sequence is unlike any extant sequence) to support the hypothesis that BCA16 is of ancient origin. Such hypothesis testing would require impossible experimental designs (*2*). In the absence of extensive population genetic data, which could be used to test for aberrant rates of substitution that would support the antiquity of the sample, we tested the hypothesis that the isolate BCA16 is a laboratory contaminant. As *B. sphaericus* can be readily cultured from natural environments (*3*), it should not prove difficult to isolate it from the laboratory environment if it is present therein. As an integral part of our experiments, we monitor the concentration and nature of microbial contamination in and around the laboratory environment in an ever-increasing geographical radius, with the assumption that contamination will have the greatest chance of occurring from the sample, then from the safety

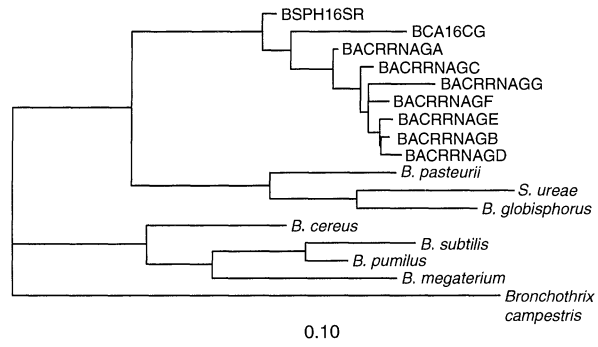

Fig. 1. Phylogenetic tree for *B. sphaericus*. GenBank accession numbers for *B. sphaericus* are as follows: BACRRNAGF (L14015); BACRRNAGD (L14013); BACRRNAGE (L14014); BACRRNAGB (L14011); BACRRNAGC (L14012); BACRRNAGA (L14010); BACRRNAGG (L14016); BSPH16SR (X60639); *S. ureae* (L38654); *B. pasteurii* (X60631); *B. globisporus* (X60644); *B. cereus* (X55063); *B. subtilis* (X60646); *B. pumilus* (X60637); *B. megaterium* (X60629); and BCA16CG, the putatively ancient *B. sphaericus* isolate, BCA16 (L38654). Sequences were aligned manually with the use of Genetic Data Environment (GDE 2.1) text editor. Trees were constructed by the maximum likelihood method with the use of the DNAML program of PHYLIP 3.5 (*4*) and a least squares algorithm for fitting additive trees to proximity data (*5*). *Bronchothrix campestris* (X56156) was used as the outgroup, with 2000 bootstrap replications, randomized data input, and global rearrangement of data. Six independent runs were evaluated. All resulting trees were identical. Branch lengths were drawn to scale, with the use of branch lengths obtained from maximum likelihood analysis and TreeTool (*6*).

cabinet in which the amber is processed, then from the laboratory at large, then from the building, and finally from the grounds surrounding the building. We have processed more than 80 amber specimens, both before and after the recovery of BCA16. During those recoveries we have sampled the sterilized amber itself before processing as well as representative sites of the safety cabinet, the laboratory, and the building itself (including the filters on air ducts). We have cataloged potential contaminants on the surface of Dominican and Mexican ambers; these include coliforms, diphtheroids, pseudomonads, endospore-forming rods, and other Gram-positive bacteria, but not *B. sphaericus*. Similarly, we have not recovered *B. sphaericus* from any of the sites tested to date. On the basis of these results, the only plausible source of BCA16, we have concluded, is from within the amber inclusion (*Proplebeia dominicana*) itself. We continue to sample the laboratory environment for *B. sphaericus*.

We and other colleagues have noted the sequence discrepancies pointed out by Beckenbach. The sequence published in figure 2 of our report is the correct sequence. We have updated our initial GenBank submission (GenBank accession number L38654) to reflect the actual sequence data, which now includes 1482 bases of the 16S rRNA gene of BCA16 for further scientific scrutiny. The discrepancies noted by Beckenbach between the sequences in figures 2 and 3 in our report, although disturbing, do not affect, in essence, the secondary structure of the rRNA molecule illustrated in figure 3 of our report. This is supported by the reconstruction of the secondary structure of the entire 16S rRNA molecule obtained from BCA16.

Beckenbach suggests that a better test of antiquity would be to compare the putatively ancient strain (BCA16) with the modern strains growing in our laboratory, presumably to eliminate the possibility that BCA16 was a modern contaminant from our laboratory. We had no strains of *B. sphaericus* growing in our laboratory before the recovery of BCA16 (*B. sphaericus*). It was not until much after we recovered and identified BCA16 as *B. sphaericus* that we endeavored to isolate *B. sphaericus* from bees (NM13, PJ23, and PJ18 in table 1 in our report) or obtain them from the American Type Culture Collection (ATCC 13805 and ATCC 17932). Even then, the cultivation and characterization of all six *B. sphaericus* isolates were conducted in another laboratory (in another floor of the building), eliminating the likelihood that one of them could serve as a source of contamination from which BCA16 could originate. We performed DNA sequence comparisons with sequences published in nucleic acid databases, primarily because we wished to introduce no extant DNA into our laboratory.

Our conclusion that BCA16 was an ancient isolate is not based solely on sequence comparisons, but on several lines of evidence that include phenetic and phylogenetic analysis. Furthermore, a compelling case can be made by comparing the putatively ancient *B. sphaericus* isolate (BCA16) with the sequence of the amplicon PD_Ex6 (GenBank accession number L38655) obtained from the abdominal tissue of bees in the same amber specimen. These sequences were essentially identical (figures 1 and 2 of our report), which suggests that the BCA16 DNA (or that of a similar organism) was present in the amber inclusion before the organism was cultivated in the laboratory.

Although further tests must be done, when all the evidence gathered thus far are evaluated and weighed, they appear to support our claim that BCA16 is indeed ancient.

R. J. Cano
Biological Sciences Department, California Polytechnic State University, San Luis Obispo, CA 93407, USA

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The Effect of Sodium Salicylate and Aspirin on NF-κB

Elizabeth Kopp and Sankar Ghosh find that activation of the transcription factor nuclear factor–κB (NF-κB) is inhibited by aspirin and salicylate, which suggests an explanation for the anti-inflammatory nature of these drugs (*1*). Because the conclusion has significant implications for the development of novel anti-inflammatory agents, we explored the phenomenon further. We found that at concentrations required for inhibition of NF-κB–dependent transcription, sodium salicylate inhibits activation of a variety of transcription factors. This appears to result from the ability of salicylate to nonspecifically inhibit cellular kinases.

Consistent with the previous report (*1*), we found that salicylate inhibited phorbol 12-myristate 13-acetate (PMA)/ionomycin-dependent induction of NF-κB DNA binding activity (not shown) and similarly induced transcription from an NF-κB–dependent enhancer (Fig. 1A). To ascertain the nature of this event, we examined the specificity of salicylate. Salicylate inhibited transcription from an AP-1–dependent enhancer induced by PMA/ionomycin (Fig. 1B). This effect is not secondary to the inhibition of NF-κB, as in these cells the immunosuppressive drug FK-506 also inhibits induction of NF-κB–dependent promoter activity by PMA/ionomycin, but has no inhibitory effect on induction of AP-1–dependent activity (*2*). As activation of NF-κB and AP-1 share the same stimuli,