Diversity of Microorganisms Isolated from Amber

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Abstract

Claims that organisms can be cultured from amber, if substantiated, would be significant contributions to our understanding of the evolution, tenacity, and potential spread of life. Three reports on the isolation of organisms from amber have been published. Cano and Borucki recently reported the isolation of *Bacillus sphaericus* and Lambert et al. have described a new species designated *Staphylococcus succinus* from 25–40 million year old Dominican amber. These characterized organisms were phylogenetically distant from extant relatives and the *Staphylococcus* sp. sufficiently far removed from other extant staphylococci to be considered a new species. Here we report the culture of bacteria from Dominican and previously untested 120 million year old Israeli (Lebanese lode) amber. Twenty-seven isolates from the amber matrix have been characterized by fatty-acid profiles (FAME) and/or 16S rRNA sequencing. We also performed a terminal restriction fragment pattern (TRF) analysis of the original amber before prolonged culture by consensus primer amplification of the 16S rRNA followed by restriction enzyme digestion of the amplicons. Sample TRFs were consistent with a sparse bacterial assemblage and included at least five of the isolated organisms. Finally, we microscopically mapped the internal topography of an amber slice.

Introduction

Galippe in 1920 [9] reported the isolation of several different microorganisms (moving forms, ovoid bacilli, and curved rods) from Baltic amber. On mechanical pulverization of the amber, he observed that most of the disrupted pieces gave positive cultures within 48 hours, but some only later. Cano and Borucki [5] used a very target-specific approach of sampling the abdomens of bees entombed in amber to revive or resuscitate symbiotic spore-forming bacilli. The evidence in support of their contention that these were ancient organisms was based on the nucleotide changes in 16S rRNA found when comparing their isolates of *Bacillus sphaericus* with present-day members of the same species. Very recently, Lambert et al. [12] isolated a bacterium, *Staphylococcus succinus* sp. nov., from Dominican amber, which is considered sufficiently novel to justify in characterization as a new species. Based on published substitution rates for 16S RNA of 0.01–0.02 substitutions per position per 50 million

years [15, 19], 10 substitutions between *S. succinus* and *S. xylosus* over a span of 1540 base pairs roughly translates to about 32 million years of evolution, the estimated geologic age of Dominican amber [13]. On a biochemical basis, the organism resembled *S. xylosus,* but fatty acid profiles differed from those of known staphylococci. In addition to these published reports, Ambergene, a company created to assay the possible uses of bacteria entombed in amber, kindly allowed us to examine their databank, which covered more than 1,600 isolates from various amber sources.

In the course of this study, we independently cultured amber from another source (Lebanese lode) as well as the same source (Dominican) utilized in the more recent studies [5, 12]. The organisms constituted a diverse sample of spore-forming bacilli, actinobacteria, and cocci, whose 16S rRNA and fatty acid profiles often did not correlate with those of extant organisms. Since it is difficult to rule out contamination from modern sources during culture and from external penetration of the outer amber surface, we performed two additional studies. A slurry of amber, incubated only a short period of time to activate any spores present, was directly amplified by PCR. The amplicons were then digested by restriction enzymes and the resulting fragments analyzed for evidence of microorganisms found in preceding culture studies. Lastly, an amber slice was examined by polarization microscopy to determine the extent of microfissures and stress as contributors to the entrance of external organisms.

Materials and Methods

Samples

Four samples of amber, each about 1 g, were utilized in this study. The Israeli amber, from earth corings of the southern slopes of Mt. Hermon, was obtained from Professor A. Nissenbaum at the Weizmann Institute of Science [16]. This area is considered an extension of the Lebanese amber lode dated approximately 120 million years. The Lebanese and Dominican ambers were gifts from the Ambergene Corporation (San Francisco, CA). Samples were examined for inclusions and surface defects under dissecting microscopes.

Surface Sterilization and Tests of Sterility

Following the initial inspection, all procedures were carried out under a laminar flow hood in a closed, dedicated area. Surface sterilization essentially were as described by Cano and Borucki [5] and Lambert et al. [12]. After separate submersions in glutaraldehyde and bleach, samples were placed in trypticase soy broth (TSB) for 5 to 7 days or until signs of bacterial growth were noted. If growth occurred, the amber was washed three times in sterile water and the sterilization process was repeated as above.

Pulverization and Culturing of Amber

When the TSB remained clear for 5–7 days, the amber was aseptically submerged in liquid nitrogen for 6 min. The amber was transferred to a sterile mini-Waring blender covered with sterile TSB (about 50 ml) and blended to pulverization (about 1 min). Approximately 0.5 ml aliquots of the amber flake-TSB suspension were spread with a glass rod on plates of six different culture media, including trypticase soy broth agar (TSBA), malt agar (MA), ISP Medium #2 (Difco, Sparks, MD) plus arginine (ISP2+) potato dextrose agar (PDA), or *Brucella* Laked Blood Kanamycin Vancomycin (Hardy Diagnostics, Santa Maria, CA) (LKV) agar. The last was used under anaerobic conditions. Environmental control plates for each medium used were left open under the hood during pulverization and plating (about 20 min). Additional control plates were overlaid with TSB from each batch used during blending and treated as the sample plates. All plates were incubated at 28°C until growth was observed or up to 1 month to confirm sterility.

Fatty Acid Methyl Ester Analysis

Isolates were grown on TSBA for fatty acid extraction for 24 hr or until substantial but nonconfluent growth was observed. Actinobacteria were grown at 28°C in TSBA for 7 days in order to ensure stabilization of fatty acid profiles prior to FAME analysis. Approximately 60 mg of wet cell weight was harvested and placed into 13 × 100 mm screwtop glass culture tubes with Teflon-lined caps (Corning, New York, NY). The cells were suspended by vortexing for 10 s in 1 ml saponification reagent consisting of 45 g and ACS-certified NaOH (Fisher Scientific, Fair Lawn, NJ), 150 ml HPLC-grade methanol, and 150 ml deionized distilled water (both from Sigma, St. Louis, MO). Phospholipids were then saponified by incubating samples at 100°C for 5 min, thoroughly mixing, and incubating at 100°C for an additional 25 min. After saponification, samples were placed in a room-temperature water bath to cool for 3 min, and mixed with 2 ml of methylation reagent (325 ml HCl (LabChem, Inc., Pittsburgh, PA) and 275 ml HPLC grade methanol (sigma)). Samples were then vortexed for 10 s and placed in an 80°C water bath for 10 min. After methylation, samples were cooled to room temperature and 0.625 ml each of HPLC-grade hexane (Fisher Scientific) and methyl-*tert*-butyl ether (Fisher Scientific) was added. Samples were then placed on an end-over-end tube rotator for 10 min to extract the fatty acid methyl esters into the organic phase. The aqueous phase was then discarded and the organic phase washed for 5 min on the rotator with 3 ml of a solution consisting of 10.8 g of certified ACS NaOH (Fisher Scientific) and 900 ml of deionized distilled water (Sigma). The organic layer was washed again and clarified with approximately 5 ml of deionized distilled water (Sigma) saturated with NaCl (EM Science, Gibbstown, NJ). This organic layer was removed and placed in a vial for GC analysis. The MIDI Microbial Identification System (MIDI, Inc., Newark, DE) protocol was used for separation, detec-

tion, and identification of FAMEs on a Hewlett-Packard 6890 Series gas chromatograph (Hewlett Packard, Palo Alto, CA). Isolate profiles obtained were compared to a standard library (MIDI Inc.) used with the Sherlock (MIDI Inc.) software system. The system compares sample profiles with a library of known profiles, generating similarity indices (SI) for each unknown, with SI values above 0.5 considered good matches. The retention time and peak area of each FAME were then exported into Excel and Minitab files. Taxonomic and species diversity was assessed by multivariate analysis, including principal components, discriminant, and cluster analysis.

Ribosomal DNA Sequencing

Ribosomal DNA sequencing was performed as described by Dodge et al. (Dodge DE, Sharaf M, Bost DA, Ellis N, Smith DH. 1996. Detection of polymorphisms in the 16S ribosomal gene of bacteria using fluorescent DNA sequencing. ASM General Meetings Abstract R-1, New Orleans, LA). Primers R 540 and F were used as described in the brochure for the Cycle Sequencing Core Kit, with dITP as the dye terminator. Sequencing was performed on the PE Applied Biosystems 377, using Factura software version 1.2 Or6 for analysis.

Terminal Restriction Fragment Pattern Analysis

General procedures followed Clement et al. [6]. Amber slurries were incubated at 45°C for 15 min in order to activate the spores and given a further 3-hr incubation at 37°C to promote spore germination. Slurries were then concentrated 10× in Microcon 100 filters (Amicon, Beverly MA). To extract DNA, 500 μl of the concentrated slurries were processed with the FastDNA Medium-Hard Tissue kit with a FastPrep bead beater (Bio 101, Vista, CA). Extracted DNA was further concentrated by ethanol precipitation; the final ratio (vol/vol) of slurry to DNA extract was approximately 500:1. Primers modified from Bruce et al. [4] and targeted to a ~500 base pair region of the 16S rRNA (8–536, *E. coli* numbering) were used to amplify extracted DNA. After restriction endonuclease digestion with *Sau* 3A and *Msp* II, the fluorescent-labeled terminal restriction fragments (TRFs) were separated by capillary gel electrophoresis and detected on an ABI Prism 310 Genetic Analyzer (Perkin Elmer, Foster City, CA). The same procedures were used to determine actual TRF lengths for seven individual isolates.

Phylogenetic Trees

Trees were constructed by the Maximum Likelihood method using the DNAML program of PHYLIP 3.5 and a least squares algorithm for fitting additive trees to proximity data. *Lactobacillus casei* was used as the outgroup, with 2,000 bootstrap replications, randomized data input and global rearrangement of data. A total of six independent runs were evaluated. All resulting trees were identical. Branch lengths were drawn to scale, using branch lengths obtained from maximum likelihood analysis.

Examination of Birefringence and Microscopic Examination of Amber

Through the kindness of Professor Harold Gascoigne of the Mechanical Engineering Department of California Polytechnic State University, a piece of Dominican amber similar in size and inclusion to those we had cultured was sectioned at 1 mm thickness. The surface was polished with fine abrasive paper and pumice. The sections were examined macroscopically between crossed polarizers. Under a 60× Olympus microscope the sections were examined under low power phase and polarization microscopy.

Results

Samples and Surface Sterilization

The Israeli and Lebanese pieces clarified on their first immersion in the sterilizing fluids and revealed small, nondescript inclusions and refractile internal partitions. The Israeli piece also contained well-preserved fungal elements from three genera in the class Phycomycetes. The first Dominican piece (Dom1) had an apparent line running through it, which separated it into a lighter and darker portion, inclusions that appeared as small rectangular plates, and what was probably a white termite head. The second Dominican piece (Dom2) had no obvious insect inclusions, but it had a considerable amount of debris.

The Israeli amber required only one application of 2% glutaraldehyde for 18 hr before remaining sterile 7 days in TSB. Dominican sample #2 was treated for 3 days in 3% glutaraldehyde in PBS and 6 min sonication in 10% bleach, and another 30 min at 28°C, and thereafter remained sterile in TSB. The Lebanese sample and Dominican sample #1 remained contaminated through two recyclings with 3% glutaraldehyde (25 hr) and 10% bleach (1.5 min), but after a third treatment with 3% glutaraldehyde (96 hr) and 10% bleach (6 min), they remained sterile.

Bacterial Isolations

Three of the four amber specimens yielded microorganisms. Twenty organisms were isolated from the Israeli piece in two platings, with both resulting in a similar spectrum of organisms. Another 10 colonies were not characterized because of their morphological similarity to other isolates. Since one-fifth of the total homogenate was plated, this represented aboug 150 viable organisms per gram of amber. From the two Dominican samples, six additional organisms were cultured, or about 30 viable organisms per gram. The Lebanese sample yielded no organisms and therefore was considered

Table 1. Microorganisms isolated from amber

Designation and source	Closest extant organism match on FAME profile	Similarity index	Closest extant organism match on BLAST search	*E* value[a]
CG2—Israel	*B. megaterium*	0.362	*B. thuringiensis*	e-235
CG3—Israel	*B. sphaericus*	0.354	*Caryophanon tenue*	e-243
CG5—Israel	*B. megaterium*	0.812	*B. macroides*	e-237
CG6—Israel	*B. megaterium*	0.859	*B. megaterium*	e-235
CG7—Israel	*B. megaterium*	0.381	*B. macroides*	e-134
CG13—Dom 1	*B. coagulans*	0.405	Not done	—
CG14—Dom 2	*B. licheniformis*	0.639	*B. licheniformis*	e-232
CG17—Israel	*B. circulans*	0.026	*Bacillus sp.*	e-225
CG19—Israel	*B. licheniformis*	0.843	*B. licheniformis*	e-135
CG23—Israel	*B. pumilus*	0.879	*B. pumilus*	e-445
CG29—Dom 1	*B. pumilus*	0.885	*B. pumilus*	e-335
CG18—Israel	No match	—	*B. psychrosaccharolyticus*	e-32
CG9—Israel	*S. epidermidis*	0.241	*B. pumilus*	e-135
CG10—Israel	No match	profile-only, 89.5% peaks named	*Staphylococcus* sp.	e-35
CG11—Israel	No match	profile-only, 98.1% peaks named	*Staphylococcus* sp.	e-35
CG4—Israel	*Staphylococcus* sp.	0.103	Isolate IF 19	e-243
CG15—Dom 1	*S. epidermidis*	0.359	*S. caprae*	e-129
CG22A—Israel	*S. aureus*	0.045	Not done	—
CG22B—Israel	*S. aureus*	0.252	*Staphylococcus* sp.e-39	
CG21—Israel	*S. warneri*	0.815	*S. pasteuri*	e-235
CG24—Israel	*Micrococcus agilis*	0.185	*Arthrobacter* sp.	e-108
CG16—Israel	*Rathayibacter rathyi*	0.642	*Micrococcus kristinae*	e-235
CC8—Israel	No match	profile-only, 88.8% peaks named	*Streptomyces tendae*	e-119
CG25—Dom 2	No match	profile-only, 98.4% peaks named	*Streptomyces* sp.	e-235
CG30—Israel	*Paenibacillus curdanolyticus*	0.256	*Amycolatopsis sp.*	c-235
CG20A—Dom2	*Nocardiodes albus*	0.141	*Nocardiodes* OS4	e-235
CG20B—Dom 2	No match	profile-only 95.8% peaks named	*Micromonospora fulvoviolaceus*	e-235

[a] Results of BLAST search expressed as probability to the natural logarithm.

to have fewer than 10 microorganisms per gram. Control media, either as exposed plates or mock poured plates, yielded two contaminants, both of which were characterized by conventional methods, FAME, and 16S rRNA analysis as *Staphylococcus epidermidis.* These two isolates had 100% sequence similarity 16S rRNA with *S. epidermidis* ATCC 14990. In two individual harvests, there were no contaminants and six isolates from amber.

Isolations occurred on all the media, with TSBA being somewhat better than the others. Organisms appeared fairly quickly, with nearly half of the isolates (11 of 27) isolated by the second day, and 18 of the 25 by 1 week. The *Bacillus* spp. generally appeared early (6 of 7), while actinobacteria appeared later (on days 7 and 13). Two anaerobic cocci were also late to appear (on days 7 and 14). Most plates remained negative throughout the month of observation, and isolates tended to appear both in clusters and as single colonies. One plate each had 15, 6, and 4 colonies; two plates had 3 colonies, two developed 2 colonies, and 15 plates had only single colonies. Another 35 plates had no colonies.

Morphology

All organisms isolated were gram positive. Rod-shaped forms predominated (12 of 27, 44%), followed by cocci (8 of 27, 30%), and two actinobacteria (2 of 27, 7%).

Characterization by FAME and 16S rRNA Sequencing

Twenty-seven isolates from the amber matrix were characterized by FAME and/or 16S rRNA gene sequencing, along with three organisms recovered when the first surface sterilizations were ineffective. Table 1 summarizes the results of both methods. Results of the 16S rRNA gene sequencing demonstrate only a general correlation with the FAME characterization. All 27 isolates from amber were analyzed by FAME analysis. Two isolate sequences were not obtained, despite repeated attempts (CG13, CG22A). From Israeli amber, 10 *Bacillus* spp. were isolated, and 3 from Dominican sources. Six of the 12 *Bacillus* species analyzed by both systems were confirmed by both genotypic and phenotypic analysis. Two *B. licheniformis* had high SI values (0.843,

0.639), and this identification was confirmed by 16S rRNA analysis. Four were *B. megaterium* according to FAME, two of which had high SI values (0.812, 0.859), while 2 had lower SI values of 0.362 and 0.381. Sequence comparison software (BLAST) on the National Center for Biotechnology Information (NCBI) Web page was used to search the existing database of 16S rRNA sequences for known organisms with sequence similarity to a given isolate. BLAST searches of 16S rRNA sequences supported one *B. megaterium* FAME result (CG6); however, the other three sequences were more similar to *B. thuringiensis* (CG2) and *B. macroides* (CG5, CG7). One *Bacillus* sp. with a FAME SI of 0.026 (CG17, as *B. circulans*) was recovered from the Israeli amber, and 16S rRNA BLAST analysis also failed to place it at the species level. Another of the *Bacillus* sp., CG18, had no match on the FAME database, and 16S rRNA placed it among a group containing *B. saccharolyticus, B. macroides,* and *B. firmus.* Three organisms (CG10, 11, 25) returned no matches with FAME and were placed only at the genus level by sequence analysis. Figure 1 is a phylogenetic tree of the *Bacillus* isolates.

There were 8 cocci (7 from Isr, 1 from Dom1, and 2 from Dom2), 7 of which have both FAME and 16S rRNA results. Here there is almost no correlation between the two systems of analysis. Two of the Israeli isolates were facultative anaerobes. Species designations were *Staphylococcus aureus, S. epidermidis,* and *S. warneri.* Furthermore, there was on coccus designated *Rathayibacter ruthyi.* Figure 2 portrays the phylogenetic relationships of the cocci. Two actinobacteria-like isolates (by morphology) were isolated, one of which on 16S rRNA analysis was putatively identified as was *Streptomyces tendae.* The FAME data bank was too scanty to provide additional taxonomic information.

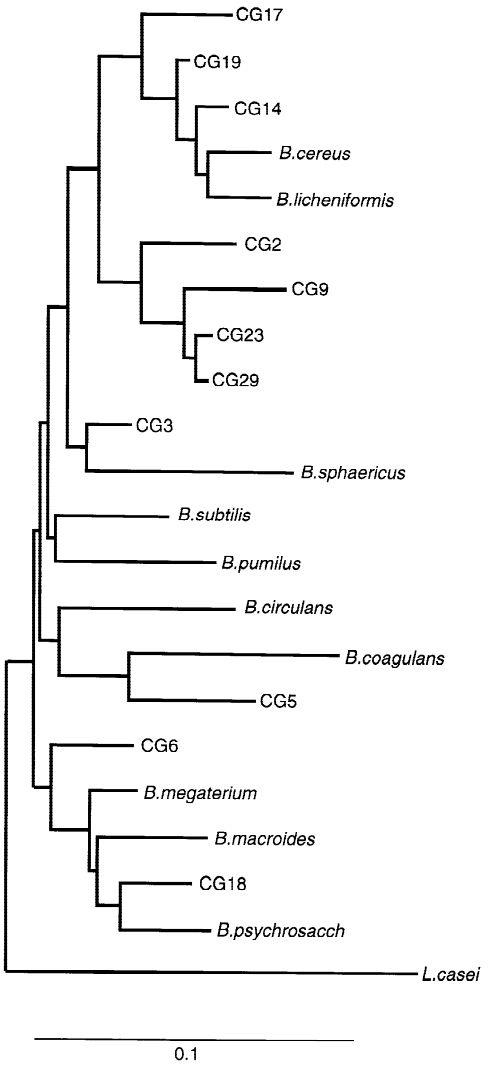

Fig. 1. Phylogenetic tree for *Bacillus* spp. and other rod-shaped amber isolates. Tree was constructed by the maximum likelihood method using the DNAML program of PHYLIP 3.5. *Lactobacillus casei* was used as the outgroup with randomized data input (J option) and global rearrangement of data (G option). A total of six independent runs were evaluated. All resulting trees were identical. Branch lengths were drawn to scale, using branch lengths obtained from maximum likelihood analysis and TreeTool.

Terminal Restriction Fragment Pattern Analysis

Only the Israeli sample extract produced PCR products. The two Dominican amber samples did not yield amplified DNA for TRF analysis, because of either low DNA yields or the presence of PCR inhibitors in the extracted DNA. Replicate TRFs showed great complexity below 60 nt (possibly due to degraded DNA in the medium) and the data were thus limited to fragments larger than 70 nt. Media controls and amber exterior washes also produced patterns (Fig. 3). However, 91% (31 of 34) of the *Sau*3A and 83% (10 of 12) of the *Msp*I amber TRFs were unique to the amber patterns. Among these, TRFs matching three isolates were observed in both enzyme digests (Table 2). Another two organisms were matched in one digest, with the expected TRF appearing in the other digest just below our detection limit.

Topography of Amber

We were interested in the nature of the inclusions and the possibility that they contributed to stresses and cracks in the amber. The sections showed strong birefringence at the edges of the inclusions when observed between crossed polarizers. On low power (100×) phase microscopy, microfissures could be traced extending out from the larger inclusions to smaller collections of debris. Upon closer examinations, these fissures ranged in width from 1 to 10 μm.

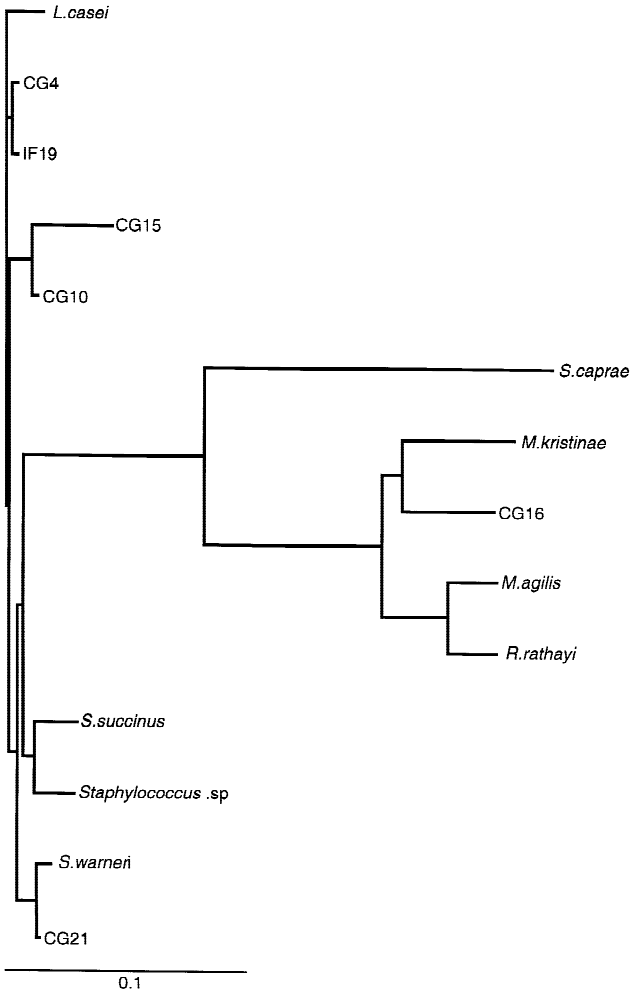

Fig. 2. Phylogenetic tree for *Staphylococcus* spp. and spherical amber isolates. Tree was constructed by the maximum likelihood method using the DNAML program of PHYLIP 3.5. *Lactobacillus casei* was used as the outgroup with randomized data input (J option) and global rearrangement of data (G option). A total of six independent runs were evaluated. All resulting trees were identical. Branch lengths were drawn to scale, using branch lengths obtained from maximum likelihood analysis and TreeTool.

Furthermore, under the surface of the amber, there were small lacunae occasionally emptying on the surface. In one case, a small thornlike object could be traced to the amber surface.

Discussion

When considering the origin of putative ancient organisms, sterility and several other points are noteworthy. In this study, microfissures and stressed areas (producing birefringence) around the inclusions in these amber samples make it difficult to argue that external organisms did not penetrate the amber over the last 120 million years. However, there are several factors indicating that if such invasions did occur, they took place in the distant past, and what we have cultured are amber inhabitants and/or survivors of a trapped microhabitat.

The stringency of our sterilization procedures would seem to rule out survival for any organisms exposed to the surface of the amber. This is demonstrated by the differences in pre- and postpulverization cultures. If contamination or surface organisms were isolated after the amber was pulverized the greatest quantity should have come from the Lebanese and Dominican 1 samples, as these samples proved difficult to surface-sterilize. Yet internally, these samples were nearly barren. In contrast, the Israeli sample was successfully surface-sterilized after one treatment, yet yielded a relatively large number of organisms after cracking. Furthermore, the organisms were isolated both in single colonies and "packaged" in small groups on a select group of plates. Such a nonrandom distribution suggests that survival occurred in discrete sections of the samples that were exposed during the crack procedure.

Comparing the yield and nature of our isolates to those of the Ambergene collection, some general similarities were noted. Using amber samples and sterilization methods similar to ours, the combined Ambergene studies produced 1644 isolates from 572 grams of amber, a yield of 2.87 isolates per gram (Robin Steele, personal communication). In our case, the yield from the Israeli sample was higher, at approximately 15.0 isolates per gram. Yields from the two Dominican samples were similar to Ambergene at about 3.0 isolates per gram, whereas the Lebanese sample seemed devoid of microorganisms. Morphologies from the larger Ambergene collection give some idea of the nature of the organisms isolated from various amber samples. These organisms consisted of bacilli, 49.8%; cocci (including "coccobacilli"), 21.45%; fungi 20.1%; and actinobacteria, 6.9%. These values are also consistent with those of our study.

Throughout this study, phenotypic (FAME) and genotypic (16S rRNA) characterizations rarely matched, as might be expected from common lab contaminants or clinical isolates. Instead, the characterizations were consistent for a group of unknown and/or poorly described organisms.

From the characterization our isolates, some details are notable. Isolate CG4, which was a gram-positive coccus [2] occurring in clumps, had a FAME SI of 0.103 with *Staphylococcus* spp. However, 16S rRNA analysis showed that its

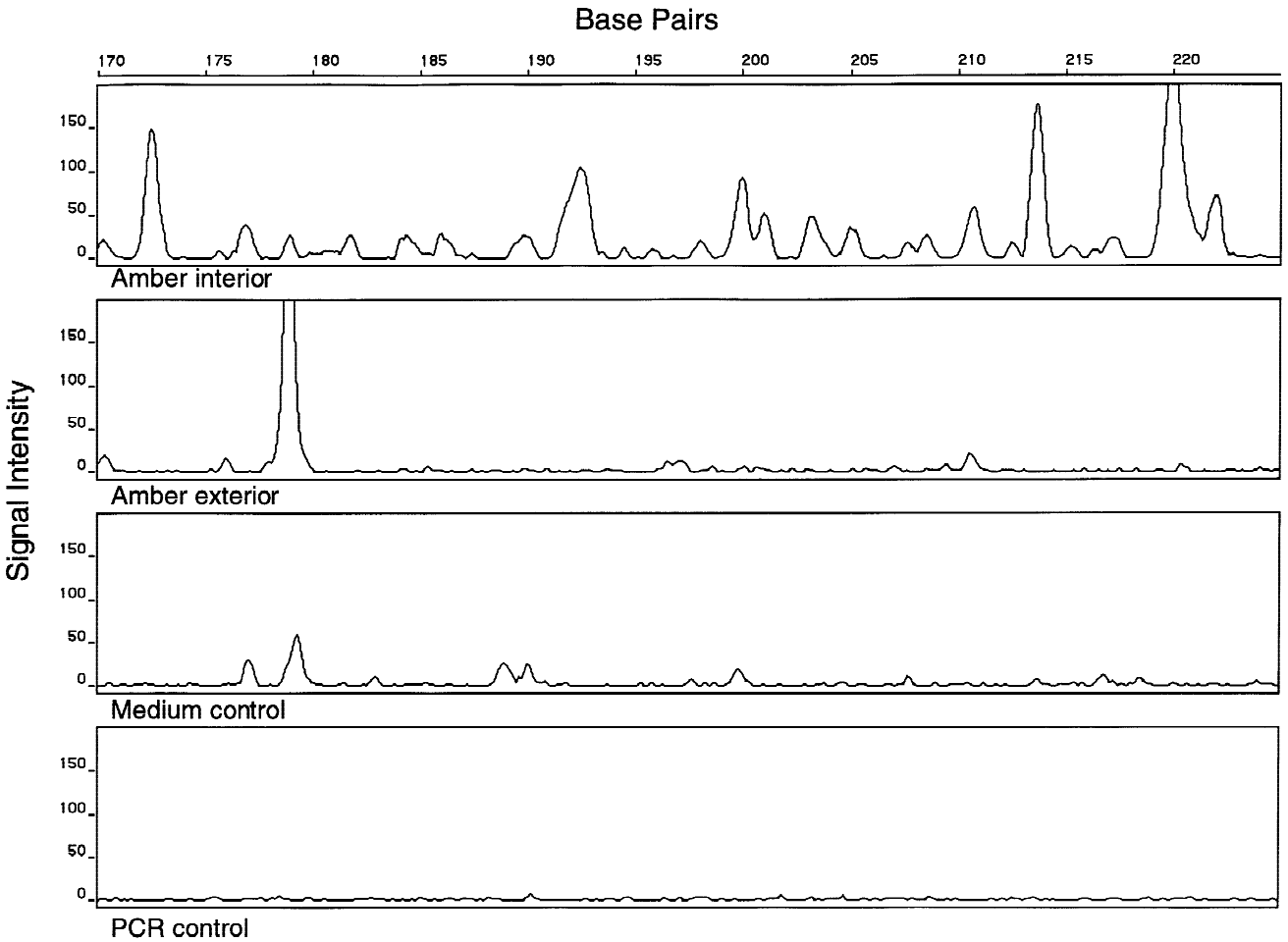

Fig. 3. Terminal restriction fragment (TRF) analysis of 16S 16S rRNA from amber community. TRF patterns of total 16S rDNA isolated from amber interior, amber exterior, reagents, and culture media were performed as described by Clement et al. [6]. DNA fragments 70–320 nucleotides (nt) in length resulting from *SauIIIa* digestion of amplified community 16S 16S rRNA are shown for one sample.

greatest homology was with isolate IF19 of Boivin-Jahn et al. [3] from a "deep subsurface environment." IF19 was found in a sterile horizontal coring 18 m into clay from a mine gallery 224 m deep. Isolate CG3 by FAME was identified as *B. sphaericus* (SI = 0.54) but by 16S rRNA sequence comparisons it found to be closest to *Caryophanon tenue* [8]. Recent taxonomic studies place this asporogenous group closer to *B. sphaericus* than to *Bacillus subtilis,* the type species for the genus *Bacillus.* Isolate CG16 was classified by FAME as *Rathayibacter,* one of a group of phytopathogenic coryneform bacteria [14]. By 16S rRNA sequence homology, however, it was placed closest to *Micrococcus kristinae.* Isolate CG30 was identified by 16S rRNA as *Paenibacillus curdanolyticus* and classified by FAME as an actinobacterium. It is interesting to note that it has recently been proposed to remove the *paenibacilli* from the genus *Bacillus* and to reclassify them as a new genus [22]. Whether CG30 is an actinobacterium or a *Paenibacillus* or something in between, these groups are associated with the communities of intestinal flora found in termites, an insect often found in amber [21].

Table 2. Detection of amber isolates in TRF patterns

	*Msp*I		*Sau*3a	
Isolate	TRF length (base pairs)	Presence in community TRF pattern	TRF length (base pairs)	Presence in community TRF pattern
CG2	151	+	306	+
CG11	155	+	232	+
CG16	155	+	232	+
CG7	153	—[a]	306	+
CG4	155	+	233	–[a]

[a] Corresponding peak appears on electropherogram but below the set threshold.

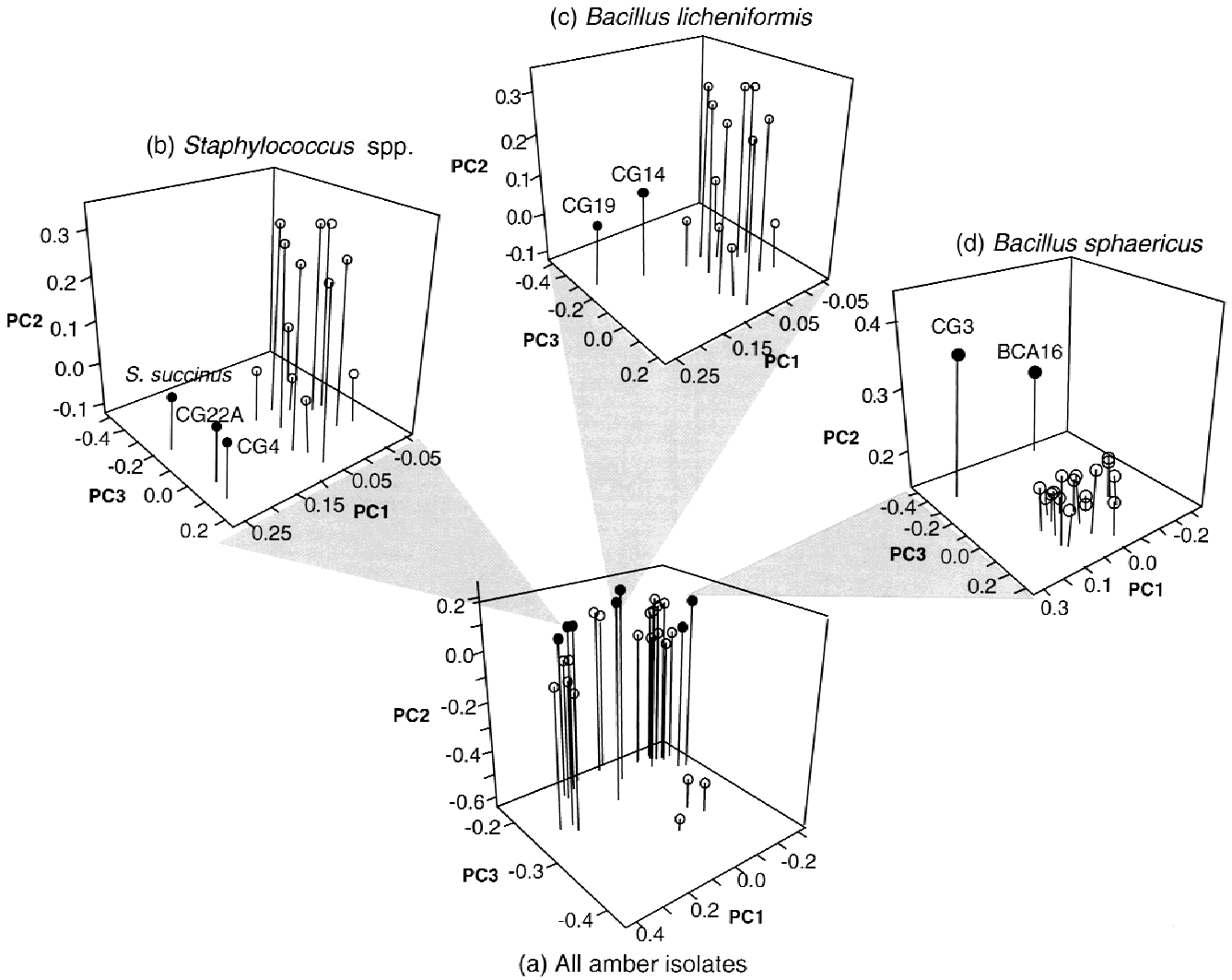

Fig. 4. Principal components analysis of FAME profiles of amber isolates and extant strains. Three principal components were calculated and plotted. Closed circles indicate amber isolates under study. Open circles in graphs b, c, and d represent extant bacterial strains.

Thus, these organisms, while often of indeterminate characterization, seem to be related to deep soil, plants and insects, with a few adapted to an anaerobic life style—all fitting the expectations of an amber habitat [3, 20].

Further evidence of ancient origin can be found in the distances between our isolates and their nearest relatives as determined by FAME and DNA sequencing. Figures 1 and 2 show the phylogenetic relationships of the *Bacillus* spp. and the cocci isolated in this study. Maximum likelihood analysis of aligned 16S rRNA sequences indicated that amber isolates were generally closer to the branch node than to its closest extant counterpart. In a similar fashion, principal components analysis (PCA) of the FAME profiles placed many of the amber isolates as outliers from the centroid within the extant taxon (Fig. 4). It has been well established that the total fatty acid composition of a microorganism is an important taxonomic character [17] and that fatty acid data can be analyzed quantitatively to provide useful taxonomic information at both the species and subspecies levels [7, 10, 18]. Because phenotypic characters such as fatty acids are both less robust for taxonomy and more likely to change than 16S rRNA sequences, comparison of an ancient organism with extant relatives might be expected to show a wildly divergent phenotypic pattern combined with a similar 16S rRNA sequence. Given that these organisms were isolated from amber, the data support an ancient origin.

The TRF patterns indicate that some organisms within the Israeli sample were not cultured as is typical of comparisons between culture and PCR-based investigations of complex microbial communities [1, 24]. The presence of a large

number of TRFs in the media and extraction control is cause for some concern, in terms of sterility (Fig. 3). However, the patterns appear to be contaminated primarily with short segments of DNA (<60 bp). This may be the result of extracting sample DNA from a concentrated medium that, while sterile, still harbored degraded DNA after autoclaving. Evidence for this lies in the fact that control TRFs had the same percent of electropherogram peak area below the 70 nt cutoff with both restriction enzymes (data not shown). In contrast, the total area of sample TRFs < 70 nt observed in the sample patterns varied significantly with restriction enzyme, as expected with patterns derived from microbial communities [6]. Another concern was the inability to amplify sequences from the Dominican sample. The number of Dominican isolates may indicate a lower quantity of organisms that did not possess enough DNA for analysis, or the Dominican samples may harbor PCR inhibitors.

The observation of TRFs consistent with five isolates in the amber slurry seems to preclude external origins, since those TRFs were not present in the controls. More interesting is the lack of further correlation between the isolates and the TRFs. If amber harbors only a sparse group of survivors and/or contaminating (or colonizing) organisms, one would expect few TRFs and a large correlation between isolates and TRFs. However, if amber provides a type of stasis from which few bacteria can return to viability, one would expect to retrieve TRFs primarily from nonviable species, as they would represent the majority of available sequences. The latter scenario is consistent with our data, and thus amber may provide a window into ancient bacterial communities.

The few isolates characterized in this article, when combined with the Ambergene collection, represent a cross-section of ancient bacteria found in amber. This can be compared to other bacterial assemblages in three interesting studies as an example of culturing bacteria from sources where survival is unexpected. Sneath published a study in 1962 [23] specifically designed to determine the longevity of bacteria, a central concern in the isolation of bacteria from amber. Sneath cultured soil clinging to the roots of herbarium samples varying from 50 to 300 years in age. He quantified the recovery of bacterial colonies (mostly *Bacillus* spp.) and calculated a survival curve, from which he estimated that bacilli could survive thousands of years (although his data showed nearly infinite survival beyond 150 years—see Ref. [23], Fig. 1). Another such assemblage is described by Boivin-Jahn et al. [3]. These authors described 74 isolates from a deep subsurface environment, one of which, IF 19, seemed very close to our isolate CG 4 on 16S rRNA analysis. In this collection bacilli represented only 5.4% of the organisms and cocci 36%. The actinobacteria were 6.8% of the total Boivin-Jahn et al. [3] noted, as we have, that a great number of discrepancies occurred between the phenotypic and genotypic classification (42 disagreements in their collection of 74 isolates). Additionally, from the work of Waite and co-workers (Waite KJ, Hugghins K, Battista JR, Ward-Rainey NL, Rainey, FA 1998. Eighth Int. Symp. Microbial. Ecol., Halifax, Nova Scotia, Canada, p 342), it appears that certain bacteria, including *Deinococcus,* endospore formers, and actinobacteria, are resistant to high radiation doses (100,000–1,500,00 rads) in a desiccated state and therefore could withstand the inevitable bombardment of ionizing radiation during their cryptobiosis in amber or other preserved samples.

It would appear from the profile of organisms contained in amber without major insect inclusions that this resin is a repository of soil microorganisms that is interesting in its diversity, much like that of Boivin-Jahn et al. [3], and contains organisms as durable and tenacious as the those described by Sneath [23] and Waite et al. (op. cit.). Amber containing viable bacteria may have ecological implications, in terms of how genetic material is stored and reissued. Just as geologic shifts expose old formations to new environments, bacteria enclosed in old deposits and surviving with slow or no metabolism may eventually be exposed to new ecosystems. This may represent a new, albeit small, source of migration and genetic diversity in certain microbial habitats.

Kennedy et al. [11] discussed many aspects of "palaeomicrobiology" including the terminology (are we speaking of anabiosis or cryptobiosis?) and cited some 62 examples of the culture of palaeo-organisms by 37 independent groups from diverse sources. These have included geological deposits, earth cores, salt mines, oil, coal, and numerous other sources of cultivatable microorganisms. More recently, even granite and deep-sea sediments have yielded an amazing diversity of microorganisms [20]. Kennedy et al. also discussed the "rationale for claiming preservation" when culturing microbes from ancient sources [11]. They identified seven categories, four of which we agree with and have addressed here. The (i) inaccessability of the amber to the environment (particularly water) and successful (ii) contamination precautions are demonstrated by both the general nature of amber and our viable cultures following external, liquid sterilization. We have (iii) shown a significant difference from contaminants (*Staphylococcus epidermidis*) and similarity to replicate isolations (between Israeli and Dominican isolate genera) in this study. Furthermore, we

have seen that our (iv) rRNA sequences do not match any known sequences exactly, although we have strong phenotypic (FAME) matches for some isolates. Kennedy et al. also identified rationales for disputing preservation claims, most of which remain unanswerable yet reasonable doubts, including biological decay rates, radiation damage, and transport between samples and the environment. However, Kennedy's fourth rationale for disputing our claims, that of in situ reproduction, may actually support them. We claim here only to have isolated organisms from amber; it is impossible to determine the metabolic state of our collection prior to culture. The hypothesis that these organisms exist in a state of slow metabolism agrees with our assertion that amber inclusions are a type of microhabitat that, over time, can release ancestral bacteria into the environment.

Our study contributes to the development of palaeomicrobiology and what it can teach us of bacterial diversity. It may be as Poinar [21] has stated: "Reconstructing from amber inclusions the ecosystem that existed during the actual period of resin flow is definitely a challenge." This challenge is worthy of study, as it can help define the types of organisms, their ecology, and preferred ecosystems that might provide biological exchange over large gaps of time, as seen here, and distance, as postulated by Sneath in his discussions of space travel [23].

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