Isolation, characterization, and diversity of microorganisms from amber

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ABSTRACT

Retrieval of viable bacteria from fossil material dating from the Oligocene back to the Miocene opens the opportunity to study the evolution of prokaryotes through the evolution of their DNA, their physiology, and their ecology. This unique system in which ancient organisms and their genes may be compared directly, rather than by inference, with modern homologues is without precedent. Clearly, however, confirmation of the fossil origin of such isolates must be made in a manner that would allay reasonable skepticism. Present approaches of verification of authenticity of fossil DNA are either arbitrary or potentially unconvincing, because of their dependence on imprecise methodological observations. New approaches for verification must be developed at the same time as further research in fossil bacterial isolation and characterization proceeds. The discovery that viable bacterial spores from fossil materials opens the way to direct assessment of the evolution of complex biochemical systems in prokaryotic systems and to explore mechanisms for long term survival of microorganisms.

Keywords: amber, ancient microorganisms, endospores, ancient bacteria, long-term survival

1. INTRODUCTION

Amber is an amorphous polymeric glass with mechanical, dielectric, and thermal features common to other synthetic polymers¹. It originates from the resin of woody plants as an isoprenoid commonly recognized as sticky, odoriferous "pitch." Natural resins are complex mixtures of terpenoid compounds, acids, alcohols and saccharides secreted from parenchymal cells, some of which have preservative properties^{2, 3}. Resins are not restricted to the conifers but occur in a wide range of flowering plants⁴. The preservative properties of amber make it a suitable source of tissue with extractable DNA, from which genetic and molecular studies can be conducted^{5, 6, 7}.

Amber deposits occur throughout the world and range in age from more than 590 million years to less than a million years old. Amber is an excellent source of molecular diversity and ancient microorganisms because of its preservative properties. These deposits are time capsules of the earth's biological life and its metabolic and chemical diversity.

2. RECOVERY OF ANCIENT MICROORGANISMS FROM AMBER

Cano and Borucki^{8, 9} isolated a strain of *Bacillus sphaericus* from the abdominal contents of an extinct stingless bee (*Proplebeia dominicana*) in 25-40 million-year-old amber from the Dominican Republic. They showed that this bacterium was of ancient origin based upon methodological, phenetic (biochemical and enzymatic profiles), sequence comparison and molecular clock studies. These studies indicated that the ancient isolate, although it was morphologically similar to modern *B. sphaericus* isolates, exhibited unique enzymatic activities and had rDNA sequences that were ancestral to their modern counterparts as shown by phylogenetic analyses. DNA hybridization studies indicated that it had 80% homology with group III *B. sphaericus*. Since

the original report by Cano and Borucki this finding has been independently verified by DiTullio et al. (personal communication) and Lambert et al.¹⁰.

Figure 1. Four TEM views of *Staphylococcus succinus* sp. nov.

Lambert et al.¹⁰ reported the isolation and characterization of a novel *Staphylococcus*-like bacterium from amber. The bacterial isolate, designated AMG-D1, was isolated from 25-35 million year old Dominican amber (Figure 1). Biochemically, AMG-D1 most closely resembled *S. xylosus* physiologically, but differed significantly from the staphylococci in cell wall and fatty acid compositions. Phylogenetic analysis of 16S rRNA sequences indicated that AMG-D1 was most closely related to *S. xylosus*, *S. equorum*, and *S. saprophyticus*. DNA-DNA hybridizations under stringent conditions supported the phylogenetic analysis and revealed homologies of 38% with *S. equorum*, 23% with *S. xylosus*, and 6% with *S. saprophyticus*. These results suggested that isolate AMG-D1 was a novel organism, tentatively named *S. succinus* sp. nov. Lambert et al. (34) hypothesized that the physiological and phylogenetic characteristics suggest that AMG-D1 might be a branch-point organism between the corynebacteria and the staphylococci (Figure 2).

Figure 2. Phylogenetic position of AMG-D1 in Relation to other *Staphylococci*

The analysis of fatty acid methyl esters (FAME) has been used for the identification and classification of bacteria^{11, 12}. Using standardized growth and analytical conditions, reproducible fatty acid profiles suitable for multivariate statistical analysis can be obtained and thus identification systems such as Microbial ID, Inc. (MIDI, Newark, DE) have been established. Putative ancient isolates of *Bacillus thuringiensis*, ranging in age from 2 to 26 million years old, were characterized by fatty acid methyl ester (FAME) analysis, and matched using similarity indices to MIDI's library of 44 *Bacillus* species. Isolate AG4 and AG567 were identified as good matches to *B. thuringiensis*, while AG187 and AG262b were identified as atypical strains of *B. thuringiensis*¹³.

The FAME data obtained were used in Principal Component Analysis to assess species diversity among the various isolates of *B. thuringiensis* using Minitab 10.Xtra to analyze the data. The results suggested that although all isolates were identified by FAME analysis as *B. thuringiensis*, all modern isolates formed a well defined cluster while the four ancient isolates were clustered in separate groups outside the centroid for the modern isolates, but not forming a cluster among themselves. These results support the hypothesis that AG4, AG187, AG262b, and AG567 were isolates obtained from inclusion in amber and not modern laboratory contaminants (Figure 3).

Johnsonbaugh and Cano (personal communication) have recently isolated a new strain of *Bacillus sphaericus* from 40 Ma Dominican amber and coded it BCA17. The sequence analysis revealed that this isolate was most closely related to BCA16 and other extant strains of *B. sphaericus* isolated from both soils and insects. The phylogenetic tree based on 16S rRNA sequences and constructed using the maximum likelihood algorithm indicates that both BCA16 and BCA17 are ancestral to extant *B. sphaericus* when rooted by *Sporosarcina ureae*.

Figure 3. PCA Analysis of FAME Profile for Modern and Ancient *Bacillus thuringiensis* isolates

Ancient microbial isolates have been recovered from Tertiary ambers (20-40 Ma) and Colombian copal (≤ 2 Ma). All microorganisms recovered were Gram-positive, spore-formers, most of which were in the genus *Bacillus*, with the remaining microorganisms being other Gram positive bacteria (e.g., *Arthrobacter*, *Sporosarcina*, diphtheroids, and actinomycetes).

3. NATURAL HISTORY OF BACILLUS-BEE SYMBIOSIS AND POPULATION GENETICS

It is well established that there is a symbiotic relationship between *Bacillus* species and many species of bees^{14, 15, 16}. *Bacillus* spp. (*B. subtilis*, *B. megaterium*, *B. pumilus*, *B. sphaericus*, and *B. circulans*) have been consistently isolated from the abdominal cavity, glandular secretions, pollen, and larval provisions of stingless (e.g., *Trigona*, *Melipona*, *Plebeia*, *Centris*, and *Anthophora*) and honey bees (e.g., *Apis*). It is hypothesized that the larval provisions are inoculated with *Bacillus* endospores maintained in the crop of adult worker bees. The inoculated endospores germinate and have a fundamental role in the metabolic conversion, fermentation, and preservation of the food of perennial colonial insects that rely on stored food. Microscopic examination of abdominal contents from modern and amber-entombed bees reveal the presence of a large number of endospores, along with few cells, supporting the hypothesis that symbiotic *Bacillus* spp. are stored in the crop of workers primarily in the form of endospores, which could then be used to seed brood provisions.

It appears that certain strains of *Bacillus* spp. are selected by the bee colony based on their metabolic activity, growth in the presence of acid byproducts of fermentation, and production of enzymes that can digest the collected provisions. Gilliam et al.^{15, 16} found that the *Bacillus* spp. isolated from stingless bees produced a variety of enzymes including esterases, lipases, proteases, aminopeptidases, phosphatases, and glycosidases

that could convert food into more digestible products for storage. Gilliam et al.^{15, 16} also reported that the lipases most often associated with tropical bees are caprylate esterase lipase (CEL) and butyrate esterase and that CEL activity was detected in all bee isolates evaluated.

A preliminary study conducted in our laboratory compared the production of caprylate esterase lipase by *Bacillus megaterium* isolates from the stingless bees (*Plebeia frontalis* and *Proplebeia dominicana*) and *B. megaterium* from soils (both modern and in amber inclusions). The results, summarized in Table 1, indicate that CEL was produced by all the *B. megaterium* symbionts tested but only in 2/13 (15%) of the soils isolates and that the levels of CEL produced (measured in nanomoles) was greater in the symbionts (3.8 ± 0.79) than in the free living strains (0.3 ± 0.68).

TABLE 1. PRODUCTION OF CAPRYLATE ESTERASE LIPASE (CEL) BY *BACILLUS MEGATERIUM*

Sources of Isolates	Number of Isolates		NMOLES
	CEL +	CEL -	
Modern sources			
Bee Isolates	14	0	3.8 ± 0.79
Soil Isolates	2	11	0.3 ± 0.68
Amber sources			
Bee Isolates	4	0	3.5 ± 0.96
Soil Isolates	1	2	-0.25 ± 0.5

These results support those published by Gilliam et al.^{15, 16}. Thus, the bee-derived *B. megaterium* can be discriminated from those of soil origin by the level of expression of the CEL gene. It is not clear from the results, however, whether the increased level of activity is due to an increased copy number of the gene in bee symbionts or as a result of mutational events affecting the regulation or primary structure of the CEL gene, leading to a more efficiently-expressed gene.

Both solitary and social bee species have physical, physiological, behavioral, and chemical adaptations which control spoilage of food stores rich in protein, lipid, and carbohydrate content. These adaptations, in addition to potential mutualistic relationships with microbes and other organisms, may be particularly important in perennial bee species that rely on stored food for survival.

The Bee-*Bacillus* association is well described and the bacterial spores appear to be ubiquitous in the abdominal cavity of worker bees. Additionally, fossil bees are commonly found as inclusions in Dominican amber, thus a readily-available and abundant source of specimens for study.

There is evidence that the symbiotic relationship between *Bacillus* species and many species of bees relationship dates back millions of years, since *Bacillus* DNA has been amplified from the abdominal tissue of 25-40 million year old bees that were preserved in amber. Furthermore, it is possible that the tissue of these amber-entombed bees may harbor viable *Bacillus* endospores preserved in a desiccated state for millions of years. The successful germination and culture of these ancient endospores would allow the study of the physiology and may provide insights into ancient symbiotic relationships.

Bacillus spp. have been consistently isolated from the abdominal cavity, glandular secretions, pollen, and larval provisions of stingless bees (e.g., *Trigona*, *Melipona*, *Plebeia*, *Centris*, and *Anthophora*) and honey bees (e.g., *Apis*) and appear to play a vital role in the production, metabolic conversion, and/or preservation of

larval provisions. In particular, *B. subtilis*, *B. megaterium*, *B. pumilus*, *B. sphaericus*, and *B. circulans* are frequently associated with bees. Unlike honeybees, stingless bees use mass provisioning, and each larva is provided with the total amount of food required for adult development before the cell is sealed. It is hypothesized that the provisions are "seeded" with *Bacillus* endospores maintained in the crop of adult worker bees. The inoculated endospores germinate and have a fundamental role in the metabolic conversion, fermentation, and preservation of the food of perennial colonial insects that rely on stored food.

Studies by Gilliam et al.¹⁵⁻¹⁷ reported a striking similarity between the microbial contents of different types of food from two social bees. They hypothesized that this similarity may reflect similar metabolic roles of *Bacillus* species that have evolved in the nutrition of bees. The results of a study by Machado¹⁸ supported this hypothesis. His study uncovered an association between the pollen of *Melipona quadrifasciata*, a stingless bee, and a *Bacillus* species resembling *B. pumilus*. Machado reported that the elimination of this *Bacillus* species from pollen stores caused eventual death of the colony. Microscopic examination of abdominal contents from modern and amber-entombed stingless reveal the presence of a large number of endospores, along with few cells. These results support the hypothesis that symbiotic *Bacillus* spp. are stored in the crop of workers primarily in the form of endospores, which could then be used to seed brood provisions.

4. THE ENDOSPORE: A TIME CAPSULE FOR SURVIVING EXTENDED DORMANCY

Bacteria of the genus *Bacillus* can initiate the process of sporulation, generally when one or more nutrients becomes limiting for growth. Spores are much more resistant than their growing cell counterparts to a variety of environmental stresses, including heat, radiation and chemicals, and can survive in a metabolically dormant state for extremely long periods of time¹⁹.

An early event in sporulation is an unequal division of the cytoplasm, giving rise to small and large progeny, each with a complete genome. The small compartment, termed the forespore, is destined to become the mature spore; the large compartment, termed the mother cell engulfs the forespore resulting in a cell (forespore) within a cell (the mother cell). Eventually, after a series of further morphological and biochemical changes, the mother cell lyses, releasing the mature spore into the environment²⁰. From the outside in, the layers of the spore include the exosporium, which has not been well characterized, the proteinaceous spore coat, the cortex composed of peptidoglycan with a structure similar but not identical to that of cell wall peptidoglycan, and finally the central core, the site of most spore enzymes, ribosomes, and of course the DNA²¹. The spore coat and cortex are relatively impermeable, thus restricting access of potentially toxic molecules into the spore core²¹.

A major difference between the environments within a growing cell and a dormant spore is the amount of water present. Growing cells are 75-80% water, or 3-4 g water per gram dry weight. Values for the water content of the spore coat and cortex are similar to those for intact growing cells. However, the spore core has much less water per gram dry weight; from 0.5-1 g water per gram dry weight depending on the species examined. Thus the spore core has much less free water than cells, and consequently mature spores have no detectable metabolism (they are dormant). The reduced amount of water in the cell is also largely responsible for enzyme inhibition which is required for a spore's enzymatic dormancy and vital to the long term survival of the spore. In their state of dormancy, spores neither contain nor make ATP.

Ultimately, spore DNA must be protected so that germination can occur without error. One of the early steps in germination is the initiation of hydrolysis of the spore cortex: the breakdown of the spore cortex allows a tremendous influx of water into the spore, increasing the volume of the spore core more than twofold, thus rehydrating the spore and restoring its metabolic activity.

The major factor preventing damage to spore DNA is the saturation of this DNA with a novel group of small, acid-soluble proteins (SASP) of the a/b-type whose binding greatly alters DNA's chemical and enzymatic reactivity as well as its UV photochemistry. Binding of these proteins is thus a key factor in spore DNA resistance to UV radiation, depurination, and oxidizing agents^{21, 22}.

The other two mechanisms protecting spore DNA from oxidative damage are decreased spore permeability to oxidizing agents and decreased spore water content. Hydrogen peroxide can cleave the DNA backbone, but due to the decreased permeability of the spore cortex to hydrogen peroxide, less hydrogen peroxide can get into the spore and cause oxidative damage. With the decreased water content of a spore there are fewer hydroxyls to cause oxidative damage in the spore²³. Finally, SASP DNA binding proteins protect spore DNA from hydroxyl radical cleavage. These proteins saturate the spore chromosome and protect the DNA backbone against hydroxyl-radical cleavage²⁰. Protection of DNA from free radical damage by a/b-type SASP could be a major component of spore longevity²⁰. Therefore, while one might infer that a dormant cell, with no means of DNA repair would incur significant DNA damage, there are several unique mechanisms that spores have for protecting their DNA.

The bacterial endospore is a biological strategy that overcomes the intrinsic instability of DNA²³. Additionally, amber entombed endospores also benefit from the unique preservative qualities of the amber. It has thus been noted that spores and amber provide an environment of partial dehydration. In addition, spores are largely impermeable to oxygen (oxidizing agents), while amber achieves the same end by completely sealing off the inclusions from oxygen². Amber inclusions are not exposed to microbial contamination, have high-osmolality which reduces depurination 10-fold, and a neutral-pH environment, all of which enhance the resistance of biomolecules to denaturation and hydrolysis. Furthermore, Dominican amber mines located 50-100 feet underground, provide an environment for fossilized tissues of moderate stable temperatures and protect them from DNA-damaging UV irradiation. These five factors, dehydration, high osmolality, oxygen-free environments, protection from UV radiation, and stable ambient temperatures, make endospores preserved in amber a source of microorganisms for evolutionary and long-term survival studies.

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