SIMS analyses of the oldest known assemblage of microfossils document their taxon-correlated carbon isotope compositions

J. William Schopf, Kouki Kitajima, Michael J. Spicuzza, Anatoliy B. Kudryavtsev, and John W. Valley

Analyses by secondary ion mass spectroscopy (SIMS) of 11 specimens of five taxa of prokaryotic filamentous kerogenous cellular microfossils permineralized in a petrographic thin section of the ~3,465 Ma Apex chert of northwestern Western Australia, prepared from the same rock sample from which this earliest known assemblage of cellular fossils was described more than two decades ago, show their δ13C compositions to vary systematically taxon to taxon from −31‰ to −39‰. These morphospecies-correlated carbon isotope compositions confirm the biogenicity of the Apex fossils and validate their morphology-based taxonomic assignments. Perhaps most significantly, the δ13C values of each of the five taxa are lower than those of bulk samples of Apex kerogen (−27‰), of SIMS-measured fossil-associated dispersed particulate kerogen (−27.6‰), and those typical of modern prokaryotic phototrophs (−25 ± 10‰). The SIMS data for the two highest δ13C Apex taxa are consistent with those of extant phototrophic bacteria; those for a somewhat lower δ13C taxon, with nonbacterial methane-producing Archaea; and those for the two lowest δ13C taxa, with methane-metabolizing γ-proteobacteria. Although the existence of both methanogens and methanotrophs has been inferred from bulk analyses of the carbon isotope compositions of pre-2,500 Ma kerogens, these in situ SIMS analyses of individual microfossils present data interpretable as evidencing the cellular preservation of such microorganisms and are consistent with the near-basal position of the Archaea in rRNA phylogenies.

Apex chert | Archaea | Archean | methanogens | methanotrophs

Weakly regarded as among the oldest known evidence of life, the morphologically diverse cellular carbonaceous (kerogenous) microscopic fossils of the ~3,465 Ma Apex chert, systematically described more than two decades ago (1, 2), have been a focus of controversy. Perhaps spurred by a reluctance to affirm the veracity of “claims for life in the earliest 2.0–2.5 billion years of Earth’s history” (3), some workers have suggested the Apex fossils to be chert-embedded mineralic pseudofossils composed of “abiotic graphite” (4, 5), barium carbonate (6, 7), or hematite in secondary veinlets (8). Other studies implied that the fossils are nonindigenous clay mineral needle-like crystallites (9) or suggested them to be composed of “vermiculate-like” minerals produced via a “nontaxonomic formation model” involving the hydration and exfoliation of mica flakes followed by their superficial adsorption of later-introduced hydrocarbons (10).

Principal deficiencies of these suggestions are that carbonaceous (kerogenous) cellular microbe-like assemblages of nontaxonomic pseudofossils are evidently unknown in the geological record; abiologically produced kerogenous particulate organic matter is similarly unreported from the geological record; and virtually none of these studies is reported to have been based on examination of the scores of demonstrably kerogenous (4, 11, 12) morphometrically diverse well-characterized (1, 2) originally described Apex specimens archived at London’s Natural History Museum (NHM) (2).

Although the earlier disputed biogenicity of the Apex fossils seems largely to have been laid to rest (12), the biological affinities and physiological characteristics of these exceedingly ancient fossil microbes remain to be established. Initially formally described as “prokarystes Incertae Sedis”—nonnucleated microorganisms of uncertain and undefined systematic relations (ref. 2, p. 643)—the present study suggests a solution to this unresolved problem.

We here present results of in situ analyses of 11 specimens of five taxa of permineralized microscopic fossils embedded in Apex chert petrographic thin section 4 of 6/15/82-1H prepared from the same rock sample at the same time as the six holotype-and-paratype-containing sections previously archived at the NHM (sections 4 of 6/15/82-1B through 6/15/82-1G; refs. 1 and 2). The indigenousness and syngenicity of the permineralized fossils to their encompassing chert matrix is shown by optical microscopy supported by Raman spectroscopy which also establishes their kerogenous composition. The biogenicity and taxonomic relations of the analyzed fossils are documented by their demonstrably cellular cylindrical filamentous morphology; the taxon-defining size ranges of their medial cells and, where preserved, the morphology of their terminal cells; and their morphometric comparison with previously reported specimens from the same rock. Analyses of each of the 11 specimens by secondary ion mass spectroscopy (SIMS) document the carbon isotope compositions of the five taxa studied.

The taxon-correlated SIMS carbon isotope data reported here reaffirm the carbonaceous, kerogenous (rather than mineralic)

Significance

Although the existence of the Archaea (one of three all-encompassing domains of life) in the Archean Eon (4,000 to 2,500 million years ago) has been inferred from carbon isotopes in bulk samples of ancient rocks, their cellular fossils have been unknown. We here present carbon isotope analyses of 11 microfossil fossils from the ~3,465-million-year-old Western Australian Apex chert from which we infer that two of the five species studied were primitive photosynthesizers, one was an Archaeal methane producer, and two others were methane consumers. This discovery of Archaea in the Archean is consistent with the RNA “tree of life,” confirms the earlier disputed biogenicity of the Apex fossils, and suggests that methane-cycling methanogen–methanotroph communities were a significant component of Earth’s early biosphere.

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composition of the exceedingly ancient Apex fossils; reinforce the widely assumed (but difficult to firmly establish) validity of the use of cellular and organismal morphology for the assignment of ancient microbes to biologically meaningful taxonomic categories; and provide insight into the physiology and biological affinities of the five Apex taxa examined.

Results and Discussion

Geologic Setting. Geologically initially mapped as a shallow marine facies (13, 14), the fossiliferous locality (3) of the ~3,465 Ma Apex chert (15) has more recently been reinterpreted to be a brecciated and altered hydrothermal vein deposit (16). The 11 specimens of five taxa of perminalized microfossils analyzed here are embedded in Apex chert petrographic thin section 4 of 6/15/82-1H prepared from a rock sample collected from outcrop in 1982 (cf. refs. 1 and 2).

Although a hydrothermal environment has been suggested to be unlikely for preservation of delicate fossil microbes (4, 5, 9), biota-prohibiting hydrothermal temperatures for the genesis of the Apex chert have not been demonstrated; microorganisms morphologically comparable to the Apex filaments are common in modern hydrothermal settings (17); filamentous microbes similar to *Primaevifilum amoenum*, the most abundant of the described Apex taxa (2), have long been known to occur at deepsea thermal vents (18); and chert-permineralized fossil filaments, including specimens so similar to those of the Apex chert that they have been assigned to two of the Apex taxa (19) are present in three other Palearchean hydrothermal units of the northwestern Australian Pilbara Craton (19–24).

Specimens Analyzed. The locations of the 11 SIMS-analyzed Apex microfossils in chert thin section 4 of 6/15/82-1H are shown in Fig. 1 A–N compared with three previously described Apex specimens (Fig. 1 P–Z) perminalized in NHM-archived thin section 4 of 6/15/82-1B (2).

As is shown (Fig. 1 A, E, H, and O), the Apex fossils typically occur in subrounded millimeter-sized carbonateous chert clasts in which they are embedded in flocculent organic matter. Within such clasts, the Apex fossils are commonly rather closely spaced, numerous specimens occurring within a given granular clast (Fig. 1 H–N and P–S). Optical photomicrographs (e.g., Fig. 1 D and O–S) and 3D (Fig. 17) and 2D (Fig. 1 U–Z) Raman images of the fossils show them to be cellular, exhibiting box-like cell lumina-enclosing kerogenous cell walls.

Like some of the microfossils perminalized in other Pale archaean hydrothermal units, it is possible that the Apex filaments represent remnants of thermophilic microbes preserved in situ. Given their clast-embedded mode of occurrence, however, it seems more likely that the fossils are allochthonous to the deposit, older than or penecontemporaneous with the Apex chert, fossilized microbes emplaced in the unit in reworked detrital carbonateous granules.

Data documenting the carbonaceous (kerogenous) composition of each of the 11 SIMS-analyzed Apex specimens are shown in Fig. 2. 2D Raman images acquired at the kerogen “G” band (~1,605 cm⁻¹) accompanied by optical photomicrographs of the specimens studied. Such data, acquired routinely to differentiate bona fide microfossils from mineralic “fossil-like” microscopical objects, provided the biogenic targets for subsequent SIMS analyses. Fig. 3 and Dataset S1 present pre- and post-SIMS images of the Apex fossils discussed here, showing that the SIMS-analyzed pits spatially correspond to the specimens exposed at the surface of the polished thin section. Table 1 summarizes the SIMS-measured δ¹³C values of the 11 specimens of the five taxa analyzed. Details regarding SIMS analyses of these specimens, measurements that we regard as among the most reliable reported for microfossils in chert, are documented in Supporting Information.

Biological Affinities of the Apex Fossils. SIMS δ¹³C data for the 11 Apex fossils analyzed (Figs. 1–3 and Table 1) provide a means to assess the physiology and biological affinities of the five taxa studied. The δ¹³C values of the individual fossils reported here range from −29.8‰ to −44.1‰ (Table 1), the data showing that each of the five morphometrically defined taxa has a characteristic carbon isotope composition that varies taxon to taxon from the highest in δ¹³C (an unnamed unciell, −30.9‰; and *Primaevifilum minutum*, −31.6‰) to the lowest (*Archaeocillatoriopsis disciformis*, −39.2‰, and *P. amoenum*, −39.4‰). The SIMS-determined δ¹³C values of the fossils thus differ from those of bulk samples of Apex kerogen (~27‰; ref. 25) and those of SIMS measurements acquired away from the fossils to determine the δ¹³C values of particulate kerogen dispersed in the chert matrix (~27.6‰; Table S1 and Dataset S2).

As discussed below, the microfossil δ¹³C values are similar to those of extant prokaryotic phototrophs, methanogenic Archaeaens, and γ-proteobacterial methanotrophs.

Direct evidence of the biological affinities of the Apex fossils is limited to their (i) cellular and organismal morphologies and (ii) SIMS-measured carbon isotope ratios, the plausibility of the resulting interpretations being evaluated by (iii) the similarity of their morphologies and δ¹³C values to extant microorganisms and their con consonance with inferences based on (iv) available geochemical and fossil data from comparably ancient sediments and (v) the position of the inferred biological lineages on the phylogenetic RNA “tree of life.” As noted above, for these evidently allochthonous clast-embedded specimens (Fig. 1), a sixth typically relevant criterion, their original ecological setting, cannot currently be accurately assessed.

Below, we evaluate the biological affinities of the Apex taxa in three categories ordered by their SIMS-determined highest to lowest average δ¹³C values (Table 1).

i) Specimens exhibiting the highest δ¹³C values measured include an unnamed unciell (δ¹³C −30.9‰; Figs. 1V, 2U and V, and 3I and J) and three specimens of *P. minutum* (δ¹³C −31.6‰; Figs. 1F, G, and K, 2O–T, and 3E, F, and S–V). Extant prokaryotic phototrophs have δ¹³C values reported to range, for cyanobacteria, from −8‰ to −31‰, and, for non- photoautotrophic bacteria, from −19‰ to −6‰ (26). The ranges of δ¹³C values of the three analyzed specimens of the unnamed unciell and *P. minutum* overlap with the δ¹³C values of such extant phototrophs. Their morphologies also resemble those of diverse modern (27) and Precambrian mat-building (stromatolitic) microbial phototrophs, both Archaeaens (e.g., refs. 24 and 28) and Palaeoproteorzoic (e.g., ref. 29), in which, as we infer for the organic-rich clasts of the Apex chert, microbes of diverse physiological cooccur on a millimetric scale.

ii) One taxon, *P. delicatulum*, represented by four analyzed specimens, has a lower average δ¹³C value of −35.4‰ (Figs. 1I–M, 2K–N, and 3E–H and K–N). Although the average δ¹³C value of the four specimens of this taxon analyzed marginally overlaps with the lowest values recorded for extant anoxygenic phototrophs (~36‰; ref. 26), their range of carbon isotope values (32.7 to −38.2‰; Table 1) is particularly similar to that of modern methanogenic Archaeaens (~27‰ to −38‰; ref. 26). Nevertheless, the cellular 1.8- to 3.2-μm-broad formations of *P. delicatulum* (2) differ markedly in morphology from described methanogens, almost all such modern taxa being composed of spherical, rod-shaped, or rectangular single cells. The sole filamentous methanogen yet reported, *Thermofilum*, is characterized by thin straight or curved filaments 0.1 to 0.3 μm broad (30, 31) and, thus, is unlike the much broader filaments of the Apex taxon. This apparent absence of a modern morphological analog of *P. delicatulum* may reflect the relatively recent, 1990 recognition of the Archaea Domain (32) and a resulting lack of comprehensive surveys of extant members of the group or, perhaps, the possibility that the ~3,465 Ma Apex taxon represents an early-evolved but now extinct Archaeal lineage.
iii) The lowest δ^{13}C values measured are exhibited by two specimens of *A. disciformis* (δ^{13}C = −39.2‰; Figs. 1 B and C, 2 A–D, and 3 K–P) and one specimen of *P. amoenum* (δ^{13}C = −39.4‰; Figs. 1D, 2 I and J, and 3 Q and R). Relative to extant prokaryotic phototrophs and methanogens, the SIMS-measured carbon isotope values of the three specimens comprising this grouping are appreciably lower. Characteristically low δ^{13}C Archaeal methanogen-produced methane, having values of −50 to −110‰ (33), is a logical candidate for the source of such carbon, a supposition used to explain the occurrence both of the comparably low δ^{13}C values of carbonaceous matter in pre-2,500 Ma sediments (34, 35) and of modern microbial communities in which, as we infer for the Apex assemblage, anaerobic methane producers and consumers intimately coexist (e.g., refs. 33 and 36).

Incorporation of low δ^{13}C methane into potentially fossilizable biomass is carried out by γ-proteobacterial methanotrophs, members of the largest class of the Gram-negative proteobacteria (37) among which they are unique in using methane as their sole carbon and energy source (38). However, virtually all such methanotrophs are small single-celled rods, coccoids, or ellipsoids (38, 39) that differ distinctly in cellular morphology from much larger-diameter filamentous specimens of *A. disciformis* and *P. amoenum*. The single exception to this generalization known to us is *Crenothrix polyspora* Cohn 1870, a filamentous bacterium now studied for 150 y (27, 40–42) but only recently shown to be a γ-proteobacterial methanotroph (43, 44), a modern taxon characterized as unbranched filaments composed of cylindrical to disk-shaped cells ∼1–6 µm broad (27) and thus similar to the 1.8- to 5-µm-diameter quadrate to disk-shaped cell-containing filaments of *A. disciformis* and *P. amoenum* (2).

Regardless of whether *C. polyspora* represents a modern analog of Paleoproterozoic *A. disciformis* and/or *P. amoenum*—there being insufficient data at present to establish such relationships—the low δ^{13}C SIMS-documented compositions of these ∼3,465 Ma fossil taxa are most plausibly interpreted as evidencing methanotrophy. This, in turn, requires the presence of biogenic methane produced by Archaeal methanogens, inferred from the SIMS data to have perhaps
been generated by taxa such as *P. delicatulum*. Although the SIMS data do not exclude the possible affinity of two of the Apex taxa (an unnamed unicell and *P. minutum*) to phototrophic cyanobacteria and/or photosynthetic bacteria, those of three of the taxa are more plausibly interpreted as evidencing an early-evolved methanogen–methanotroph biocoenose, physiological characteristics compatible with the near-basal position of methane-generating Archaea in rRNA phylogenies (32) and, given the obligate anaerobic metabolism of methanogenic Archaea and the oxygen-deficient setting inhabited by extant methanogen–methanotroph communities (33, 36), consonant also with an anoxic early environment (45, 46).

**Conclusions**

From the data summarized above (and presented in Supporting Information), we interpret these SIMS-based carbon isotope analyses of 11 specimens of five taxa of the ∼3,465 Ma Apex chert of northwestern Western Australia to indicate that (i) their taxon-correlated SIMS δ¹³C values reinforce both their biogenicity and the widely held assumptions that the organismal and cellular morphology of ancient microbes can be used to establish biologically meaningful taxonomic categories and provide insight into the physiology and biological affinities of the specimens analyzed; (ii) two of the taxa exhibit δ¹³C compositions not inconsistent with phototrophic metabolism; (iii) SIMS data for the other three Apex taxa studied are more compatible with affinities to Archaeal methanogens and γ-proteobacterial methanotrophs, physiological characteristics consonant with the near-basal position of the Archaea in rRNA phylogenies; and (iv) the preservation in this ∼3,465 Ma deposit of such Archaea and γ-proteobacteria suggests that methane cycling methanogen–methanotroph communities were a significant component of the Paleoarchean biosphere.
Table 1. SIMS-determined carbon isotope values of the five taxa of Apex microfossils discussed here

<table>
<thead>
<tr>
<th>Taxon (specimen)</th>
<th>Figures</th>
<th>Number of measurements</th>
<th>δ13C vs. VPDB*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Unnamed unicell</strong> (Clast #2)</td>
<td>Figs. 1G, 2E, and 3P</td>
<td>(n = 3)</td>
<td>–31.6</td>
</tr>
<tr>
<td><strong>P. minutum</strong> (Clast #3)</td>
<td>Figs. 1G, 2F, and 3R</td>
<td>1</td>
<td>–30.9</td>
</tr>
<tr>
<td><strong>P. delicatulum</strong> (Clast #4)</td>
<td>Figs. 1H, 2M, and 3A</td>
<td>1</td>
<td>–32.7</td>
</tr>
<tr>
<td><strong>A. disciformis</strong> (Specimen #H)</td>
<td>Figs. 1I, 2L, and 3A</td>
<td>1</td>
<td>–34.1†</td>
</tr>
<tr>
<td><strong>P. amoenum</strong> (Specimen #I)</td>
<td>Figs. 1J, 2M, and 3A</td>
<td>1</td>
<td>–32.6†</td>
</tr>
</tbody>
</table>

For detailed summaries of the data, see Supporting Information.

†Calibrated vs. VPDB, the “Vienna PDB” standard.
‡Repeatability.
§Excluding high-δ13C outlier (−9.7%).

SIMS. At the University of Wisconsin-Madison WiscSIMS Laboratory, analyses of the carbon isotope compositions of the optically and Raman-identified micrometer-sized permineralized fossils in petrographic thin section 4 of 6/15/82-1H were carried out using a SIMS CAMECA IMS 1280. Fossil-containing areas were excised by use of a water-cooled diamond saw, cleaned in ethanol, mounted in epoxy together with two grains of the Bolot scapolite standard (Bolt, Mesops. ref. 47), and ground and polished using a water-lubricated diamond paste to expose the target fossils at their surface. Calibration of δ13C was performed using a separate 25-mm-diameter epoxy mount containing the Bolt standard and carbon isotope standard PPRG215 (48, 49). Two types of PPRG215 mounts were used: grain and chip mounts (Figs. S1 and S2). The Bolt standard was calibrated based on PPRG215 and analyzed as a running standard. All mounts were cleaned with ethanol and gold-coated before analyses. SIMS data were collected in two analytical sessions (Session-1, 5/9/2016 to 5/12/2016; and Session-2, 5/12/2016 to 5/20/2016). Following analysis of each specimen, SIMS pits were imaged by scanning electron microscopy (SEM), and the epoxy mounts were reground and repolished to expose new target fossils at their surface (Fig. S2). After subsequent repolishing and before SIMS analyses, the newly exposed specimens were imaged by optical microscope and SEM.

Analyses of carbon isotope ratios were acquired using a 133Cs+ primary ion beam typically having a ~12-μm-diameter spot size, an intensity of 2.7 nA to 2.9 nA, and a secondary ion accelerating voltage of 10 kV. Details of the analytical conditions used are described by Morag et al. (48). Measurements of the carbon standard mount (Bolt and PPRG215) were performed using the same analytical conditions. To assure the reliability of the results obtained, during the course of the two analytical sessions, the carbon isotope standard was analyzed 190 times (130 spots in Session-1, 60 spots in Session-2) (Figs. S3–S5).

For use of this 12-μm-diameter spot size, external precision was 1.3 to 2.6‰ (μ – δS5).

The specimens analyzed here have been archived by J.W.V. in the collections of the Geology Museum of the Department of Geoscience, University of Wisconsin-Madison.

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