International Journal of Astrobiology 1 (4): 357–363 (2003) Printed in the United Kingdom
DOI: 10.1017/S1473550403001319

Raman spectroscopy and light microscopy of a modern and sub-fossil microstromatolite: *Rivularia haematites* (cyanobacteria, Nostocales)

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Abstract: A study of *Rivularia* stromatolites demonstrated seasonal and non-seasonal banding patterns of calcification, 'sun-screen' scytonemin pigment and nitrogen-fixing heterocysts. Calcification was controlled by seasonal events with abiogenic 'winter' deposition and biogenic 'summer' deposition. Scytonemin was produced as a series of complex bands, probably as a response to summer Atlantic weather systems. Its production was also correlated in part with the appearance of heterocysts. The heterocysts were produced in bands, the pattern of which was probably controlled by an internal regulatory system. Raman spectra of modern and ancient (up to 4000 year old) *Rivularia* showed that scytonemin and carotenoid pigment can persist in dried material for >100 yr. The 4000 year old fossils did not reveal any useful biomarkers.

Accepted 10 October 2002

Key words: calcification, cyanobacteria, Raman, scytonemin.

Introduction

The cyanobacteria have a long fossil history and are widely believed to be the major organisms responsible for the initial formation of atmospheric oxygen (Schopf 2000). Their dominance in the late Precambrian is attested by the abundance of stromatolites, formed largely, if not entirely from members of this group. Today, the cyanobacteria remain important phototrophs, being among the most significant primary producers in the world's oceans (Paerl 2000). They are widespread microbes of the marine littoral, and have been found in numerous terrestrial and freshwater habitats. They are known from extreme environments such as hot springs (Brock 1978) and cold Antarctic wastes (Wynn-Williams 1990). Their ability to fossilize is largely related to their phototactic behaviour coupled with secretion of extracellular polymeric substances (EPS) that trap and bind sediment particles. The resulting structures (stromatolites) often undergo only moderate diagenesis and fossilize with characteristic biofabrics.

This study focuses on the genus *Rivularia*, colonies of which are common in shallow calcareous freshwater environments (Whitton 1987). Although not an extremophile, *Rivularia* is a useful research organism. It may be studied *in situ* over a period of years. Its colony structure is based on simple principles and it can be found as an almost 'pure' growth on rock surfaces. Several cyanobacteria are notable for their ability to form stromatolites. Stromatolites are defined as 'organosedimentary structures produced by sediment trapping, binding and/or precipitation as a result of growth and metabolic activity of microorganisms' (Walter 1976). Much of the interest in *Rivularia* has been stimulated by the fact that *R. haematites* is a model stromatolite-building organism. Fossils closely resembling *Rivularia* are known from the Proterozoic onwards (Bertrand-Sarfati 1976; Richter *et al.* 1979; Bertrand-Sarfati & Pentecost 1992; Golubic *et al.* 2000).

Rivularia is noteworthy for its concentrically laminated colonies, which often contain layers of calcite and sheath pigments. The colonies are exposed to high light intensities for long periods and the sheath pigments, consisting of scytonemin, probably protect the cells from UV damage. Although some cyanobacterium-like fossils appear earlier in the fossil record, they cannot be so clearly correlated with modern forms, making *Rivularia* a prime subject for studies of mineralization, taphonomy and carbonate diagenesis.

In this paper we investigate the interrelationships between the mineralization, growth and sheath pigment production. Since *Rivularia* frequently fossilizes we also look at the Raman spectra of recent and old material to determine whether biogeochemical markers could assist in the interpretation of ancient *Rivularia*-like fossils. Since the biogeochemistry of *Rivularia* is shared with other cyanobacteria, producing extracellular polysaccharides and scytonemin, the results are relevant to both cyanobacterium palaeontology and astrobiology.

Methods

Microscopy of modern Rivularia stromatolites

Modern microstromatolites of *Rivularia haematites* measuring up to 2 cm in thickness were collected from Gordale Beck, North Yorkshire (NGR 34/913645) and kept at 4 °C until examined. To investigate the relationship between calcification and scytonemin density, thin vertical sections were cut with a razor, floated on distilled water and examined with a light microscope. Transects were examined in sequential 50 μm sections and scored for four characters: (1) density of calcification (ranked on a scale of 1 to 5, where 1 represents negligible to 5 representing dense); (2) average calcite crystal size; (3) scytonemin pigment density (ranked on a scale of 0–10 ranging from negligible to high); and (4) heterocyst frequency (ranked on a scale of 0–2, from scarce/absent to abundant).

Raman spectroscopy of modern and ancient stromatolites

A modern sample of *R. haematites* from the Gordale site was examined along with *Rivularia* colonies preserved on herbarium sheets in the Natural History Museum, London (BM). Colonies were collected from Gotland in the Baltic Sea (1865) and the Pentland Hills, Scotland (1840). They appeared almost as fresh as the modern samples. A third collection of fossilized *Rivularia* was taken from the lower travertine mound of Gordale, dated to 4000–4500 yr BP (Thorpe 1981). This sample was heavily mineralized with calcite and had undergone considerable diagenesis.

Fourier-transform Raman spectra of the *R. haematites* samples were recorded with a Bruker IFS66 spectrometer and an FRA106 Raman module attachment using an Nd/YAG laser operating at 1064 nm. The sample 'footprint' in macroscopic mode was 100 μm and 2000 spectral scans over the 50–3800 cm^{-1} region were accumulated at 4 cm^{-1} spectral resolution to provide spectra with improved signal-to-noise ratios. The laser power was typically maintained at 20 mW at source to minimize biological degradation at the sample focus. Wavenumber shifts, calibrated internally against a helium-neon laser, are accurate to ± 1 cm^{-1}. Raman spectra were analysed from several replicates in each specimen; the modern and fossil stromatolite samples did not require any sample pre-treatment or detachment from the geological stratum or matrix.

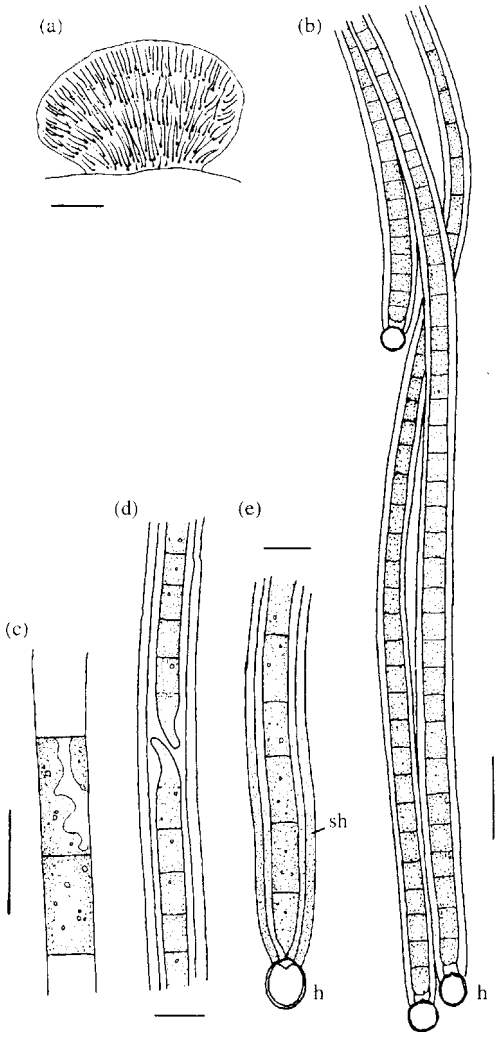

Fig. 1. Illustrations of *Rivularia haematites* from Gordale, North Yorkshire. (a) Vertical section through a decalcified colony showing concentric bands of heterocysts. Bar = 1 mm. (b) three trichomes showing tapering of cells and basal heterocysts (h). Bar = 10 μm. (c) detail of two cells, the upper with a liquid vacuole, the lower normal. Bar = 5 μm. (d) beginning of false branching of a trichome. The lower trichome tip will differentiate into a hair after breaking through the sheath. The upper trichome basal cell will differentiate into a heterocyst. Bar = 5 μm. (e) Detail of the basal part of a trichome with heterocyst (h) showing polar nodule and the sheath (sh) showing granular structure below caused by deposition of scytonemin pigment. Bar = 5 μm.

Results and discussion

The genus *Rivularia* belongs to the family Rivulariaceae, order Nostocales (Castenholz 2002). *Rivularia* is distinguished from other members of the family by its macroscopic hemispherical gelatinous colonies, usually found attached to rocks in littoral regions (Fig. 1a). It is characterized by the possession of tapered trichomes, a basal heterocyst (Fig. 1b), false-branching and a thick sheath, often coloured brown with scytonemin (Figs 1d and e). A few *Rivularia* species are further distinguished by the presence of calcium carbonate within the colonies. There are two reasonably well-defined freshwater species *R. biasolettiana* and *R. haematites*, and a marine form has been described by Golubic & Campbell (1981). In *R. biasolettiana* the crystals are disseminated while in *R. haematites* they occur in concentric zones and the calcification may be extensive. The material investigated here belonged to *R. haematites* and contained a series of fine and coarse calcite laminations (Fig. 2a). Regular bands of intense calcification occurred (Fig. 2a, arrows) and were identified as the 'winter bands', formed when the growth of the colonies is arrested and the *Rivularia* surface becomes encrusted in a dense, probably abiogenic crust of travertine. As the bands

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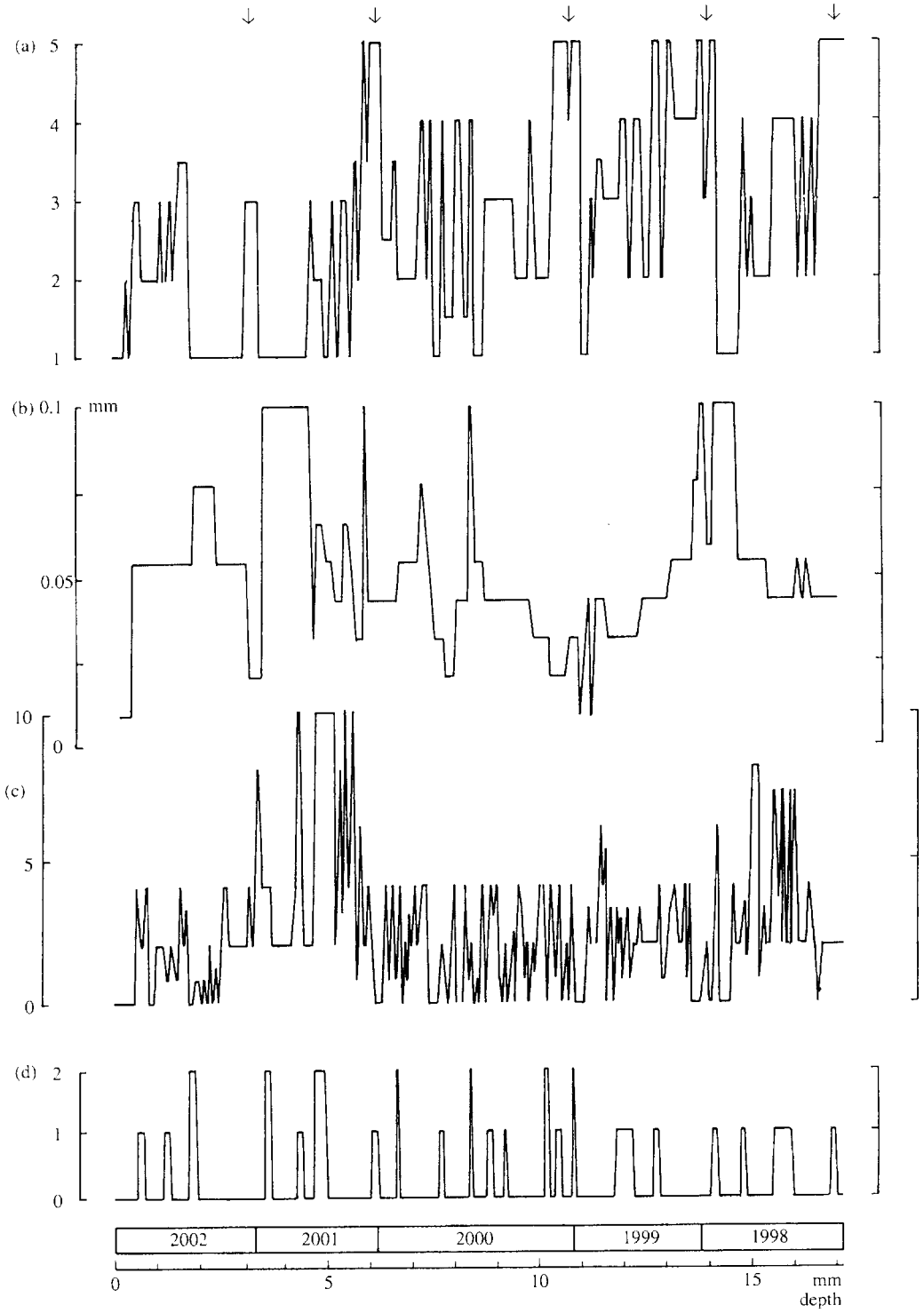

Fig. 2. Vertical section of a large *Rivularia haematites* colony about 5 years old from Gordale Beck, Yorkshire showing pattern of mineralization, scytonemin pigmentation and heterocyst frequency. (a) Calcite density on a relative scale of 1 (low) to 5 (high). Arrows indicate positions of the calcite 'winter bands'. (b) Average calcite crystal size. (c) Scytonemin density on a relative scale of 0 (low) to 10 (high). (d) Heterocyst density on a relative scale: 0 absent or scarce; 1 frequent; 2 abundant.

are seasonal, they can be used to date the stromatolites and to estimate their growth rate. The investigated sample was estimated to be about 5 years old, with a mean annual growth rate of 3.45 mm. This is only slightly more than previous records for this species using direct measurement at a nearby site (2.14 ± 0.77 mm, Pentecost 1987). In between the coarse mineral bands are numerous fine bands of calcite. They are produced as a response to episodes of low water flow and high photosynthesis, leading to the biogenic deposition of calcite within the colonies. Calcite crystal size ranged from

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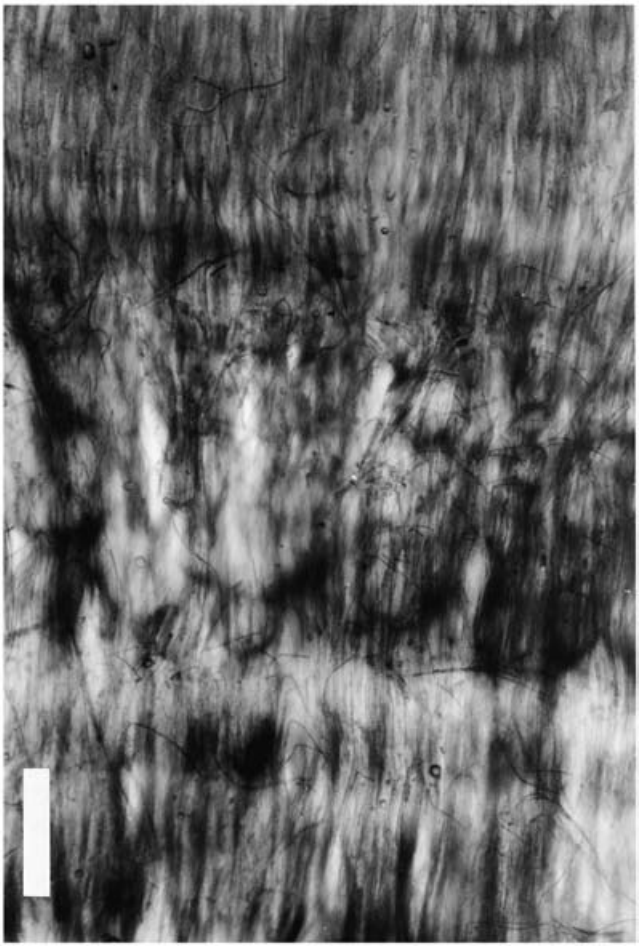

Fig. 3. Vertical section through a sample of *Rivularia* decalcified with EDTA showing the scytonemin banding pattern. Surface of colony to the top. Bar = 0.2 mm.

about 0.05 to 0.2 mm. Although it is not evident from Fig. 2(b), a Spearman rank correlation indicated a significant decline in crystal size as one proceeded down through the stromatolite from the surface ($r = -0.20$, $p < 0.01$). Also, the densest layers of calcite, forming the winter bands, had significantly smaller crystals than the less dense layers (Spearman $r = -0.35$, $p < 0.01$).

The scytonemin pigment distribution varied continuously down through the colony as a series of well-marked zones of varying thickness and intensity (Figs 2c and 3). A particularly dense set of zones was found about 5 mm below the surface followed by another dense region at about 15 mm. No detailed temporal analysis was undertaken since the dates of the zones are subject to error, but in all cases the mid-winter estimate corresponded to areas containing the least dense pigment zones. The more dense layers of pigment therefore corresponded to warmer and brighter parts of the year, where one might expect greater pigment production to protect the surface layer of cells from ultraviolet (UV) light. However, there is not a clear seasonal pattern as there is with calcite, and there was no significant correlation between pigment density and either the degree of mineralization or the crystal size.

The relative abundance of *Rivularia* heterocysts within the section is shown in Fig. 2(d). Heterocysts occurred in well-defined bands throughout the section and a simple variance to mean ratio test (Diggle 1983) demonstrated that the pattern was regular ($\chi^2 = 4.18$, df = 20, $p < 0.05$). The mean distance between the heterocyst bands was 0.64 mm, representing an average growth period of about 70 days between one band and the next. No seasonal relationship was apparent but there was a significant positive correlation between heterocyst abundance and scytonemin density (Spearman $r = 0.18$, $p < 0.01$, not adjusted for ties).

Raman spectroscopy of the modern *Rivularia* sample from the same location (Fig. 4a) revealed strong bands at 1085/6, 712, 281, 154 and 118 cm^{-1} from the calcium carbonate. Weaker bands indicated CH stretching (2943), carotenoid C═C, C—C stretching and CCH deformation (1518, 1156, 1003) and CH_2 deformation (1436 cm^{-1}). Bands at 1592/1550 cm^{-1} indicated scytonemin. The sample from Gotland, kept dry for 136 years showed clear evidence of calcium carbonate and scytonemin and new, unidentified features at 574, 1350 and 1630 cm^{-1} (Fig. 4b). There was no evidence of carotenoid. Another slightly older (161 years) dried specimen from Scotland showed no evidence of scytonemin but again showed calcium carbonate and traces of organic matter. The subfossil *Rivularia* from Gordale provided no evidence of organic matter and only calcium carbonate, but it did contain about 1% by weight of unidentifiable organic material as determined by gravimetric analysis.

The striking appearance of *R. haematites* has often been remarked upon, and has been the subject of several investigations. Wallner (1935) considered the concentric mineralization to be the product of periodic precipitation. Golubic & Campbell (1981) observed that calcite was formed in freshwater forms and aragonite in marine forms. Field investigations of *R. haematites* demonstrated a seasonal pattern in both the growth rate of colonies and their calcification rate and also revealed at least two patterns of mineralization. One resulted from seasonal changes in water chemistry and the other to photosynthesis within the colonies (Pentecost 1987, 1990), with both processes being influenced by the catchment meteorology (Caudwell *et al.* 2001). The observations made in the present study support previous work and show new relationships between the deposited calcite and crystal size. If one assumes that the calcite crystals are formed close to the colony surface in the vicinity of the growing trichomes, they should first show an increase in size. An overall decrease in size may be the result of biological oxidation and respiration below the upper photosynthetic zone, where the decay of *Rivularia* would release CO_2, dissolving some of the crystal surface. While plausible, further study is required since the size decrease was irregular and of small magnitude.

The occurrence and ecological significance of the sheath pigment scytonemin has been investigated in depth in other cyanobacteria (Garcia-Pichel & Castenholz 1991). The

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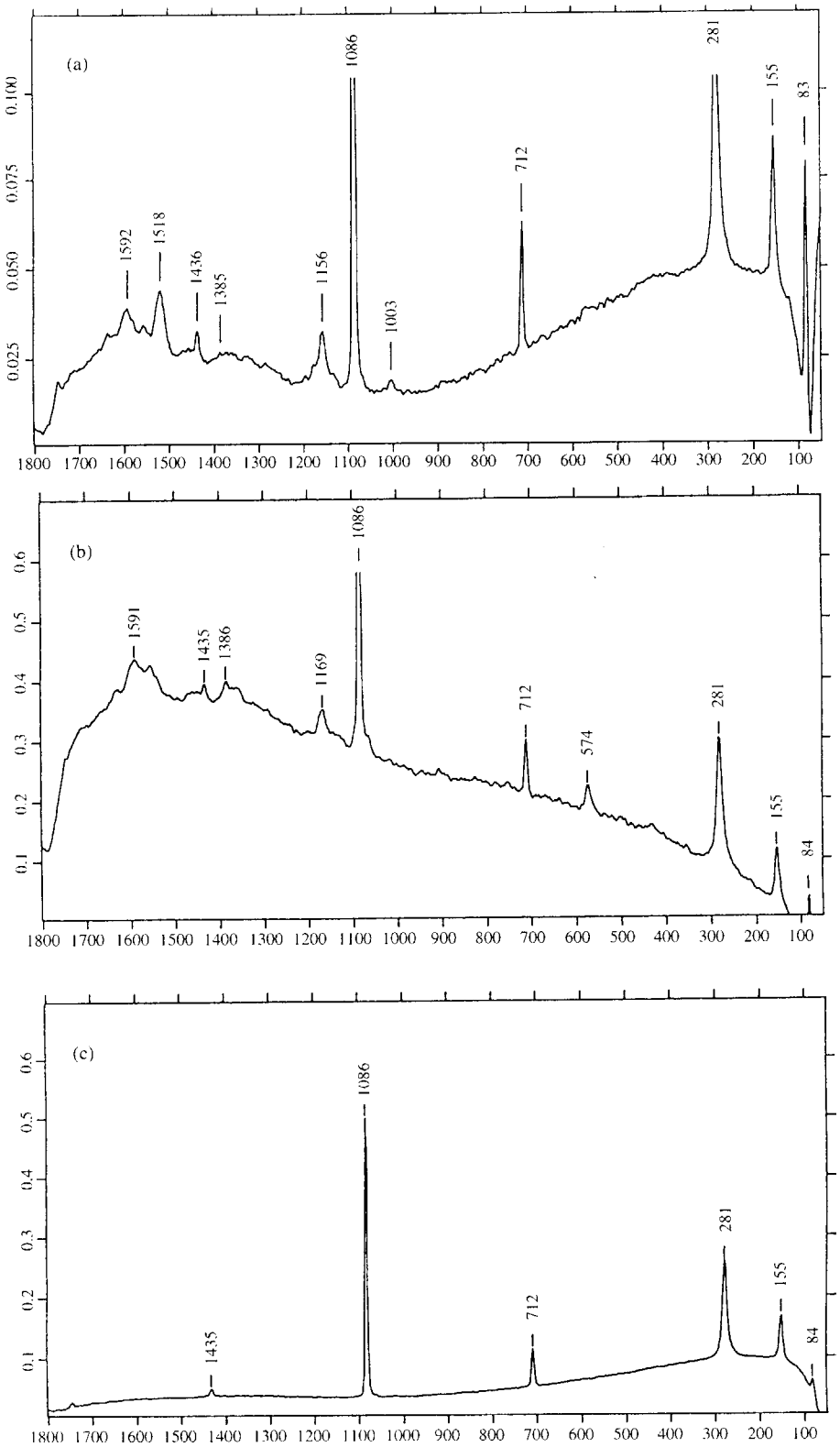

Fig. 4. Raman spectra of whole *Rivularia* samples. The abscissa shows the wavenumber in cm^{-1}. The ordinate is intensity in arbitrary units. (a) *R. haematites* modern sample from Gordale, UK. (b) Dried specimen of *R. haematites* from Gotland, collected 1865. (c) Subfossil *Rivularia* travertine from Gordale, UK.

pigment occurs in a wide range of cyanobacteria but is not produced by all species and is most apparent in aerophilic species. It is widely thought to protect cyanobacterium cells from UV radiation and often occurs in slow-growing species where light intensities although low, are experienced over long periods (Pentecost 1993). Its occurrence in *Rivularia* is to

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be expected as the colonies grow in shallow exposed waters and persist for several years. The banding of the pigment is not so easy to explain. The patterns show a range of periodicities throughout the colonies and the pigment is laid down in the mucilaginous EPS. The EPS is formed continuously as the trichomes grow, and the pigment variation must reflect environmental change, presumably the intensity and duration of light. The bands observed however are too broad to reflect diel patterns and most probably relate to longer periods of bright weather, perhaps those separating sequences of Atlantic low-pressure systems during summer. The overall higher pigmentation pattern during the summer months may reflect this, and also the greater exposure of the trichomes to direct sunlight. In winter, the dense 'winter band' calcite might provide sufficient protection on its own, thus accounting for the lower levels of scytonemin observed at this time. The lack of correlation between the band patterns in summer between calcification and scytonemin production was unexpected as it was assumed that rapid photosynthesis, leading to summer calcification bands would be associated with bright weather and scytonemin production. Since the summer bands are much thinner and weaker than the winter band, they would be unlikely to provide much of a radiation shield during summer and the precise timing of these bands requires more investigation.

In the non-branched heterocystous cyanobacteria, the production of nitrogen-fixing heterocysts at regular intervals is believed to be controlled by concentration gradients of inhibitory metabolites (discussed by Fay 1973). The same phenomenon, accompanied by cell breakage and false branching presumably occurs in *Rivularia*, but the synchronicity of heterocyst production to form bands of these cells is remarkable and may reflect inter-trichome regulation of heterocyst formation. Control by an external environmental factor cannot be excluded, but it is difficult to see how this could be the case given the regularity of heterocyst production irrespective of season. The association of increased scytonemin production with the heterocysts has not been reported previously.

Raman spectrosocopy has provided useful information from modern *Rivularia* colonies and shown that scytonemin and carotenoid are detectable in material dried for over 100 years. Nothing is known concerning the process of organic decay and diagenesis in calcified *Rivularia*, but is seems apparent that if the organic decay products are to provide diagnostic spectra, then the calcite will need to be removed to provide a more concentrated sample. Since *Rivularia*-like fossils are found in carbonates dating to the Upper Proterozoic (*ca.* 800 Ma) there is potential to look for biomarkers derived from scytonemin or its precursors.

The application of Raman spectroscopy to the analysis of diagenetic material in ancient stromatolites is highly topical for the detection of biomarkers using a remote-sensing instrument suite for planetary surface and sub-surface exploration. (Edwards & Newton 1999). Although scytonemin, identified in cyanobacterial sheaths exposed to intense UV insolation on exposed Antarctic lacustrine beaches has been well characterized in modern samples using Raman-spectroscopic techniques (Garcia-Pichel & Castenholz 1991), it is perhaps surprising that this material cannot be diagnosed spectroscopically in older specimens. Future spectroscopic studies relating to the extension of the Raman spectroscopic database for key biomolecular markers will need to address the question of survival or organic molecules in rocks, ancient sediments and fossils.

Acknowledgement

We are grateful to Dr Dave John for permission to sample *Rivularia* from the BM algae collection.

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