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Earliest fossil record of bacterial–cyanobacterial mat consortia: the early Silurian Passage Creek biota (440 Ma, Virginia, USA)

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ABSTRACT

Cyanobacteria in terrestrial and aquatic habitats are frequently associated with heterotrophic bacteria, and such associations are most often metabolically interactive. Functionally, the members of such bacterial–cyanobacterial consortia benefit from diverse metabolic capabilities of their associates, thus exceeding the sum of their parts. Such associations may have been just as ubiquitous in the past, but the fossil record has not produced any direct evidence for such associations to date. In this paper, we document fossil bacteria associated with a macrophytic cyanobacterial mat in the early Silurian (Llandovery) Massanutten Sandstone of Virginia, USA. Both the bacterial and the cyanobacterial cells are preserved by mineral replacement (pyrite subsequently replaced by iron oxyhydroxides) within an amorphous carbonaceous matrix which represents the common exopolysaccharide investment of the cyanobacterial colony. The bacteria are rod-shaped, over 370 nm long and 100 nm in diameter, and occur both as isolated cells and as short filaments. This occurrence represents the oldest fossil evidence for bacterial–cyanobacterial associations, documenting that such consortia were present 440 Ma ago, and revealing the potential for them to be recognized deeper in the fossil record.

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INTRODUCTION

Heterotrophic bacteria are frequently present on or in the extracellular polysaccharide matrix (exopolysaccharides) of cyanobacteria and bacteria in both terrestrial and aquatic habitats (Paerl, 1982, 1992; Rai, 1990; Paerl *et al.*, 2000; Takeda *et al.*, 2002; Lopez-Garcia *et al.*, 2005). This extracellular investment, referred to as the sheath or slime depending on its degree of cohesion (Bazzichelli *et al.*, 1985), provides microhabitats highly favourable for bacterial colonization. Accumulating evidence indicates that associations of bacteria and cyanobacteria are most often mutualistic, i.e. metabolically interactive, forming self-sustaining consortia, which survive even in extreme environments (Paerl, 1992; Paerl *et al.*, 2000). Bacterial–cyanobacterial consortia may have been just as ubiquitous in the past, and it has been proposed that they were important in the early evolution of cyanobacteria, when O_2-consuming, heterotrophic bacteria could have buffered host cyanobacteria against the effects of the rise in atmospheric O_2 or of CO_2 depletion (Paerl, 1982; Paerl *et al.*, 2000). However, the fossil record has not produced any direct evidence for such consortia to date. Here we document fossil bacteria preserved by mineral replacement in the exopolysaccharide matrix of a fossil cyanobacterial colony from the early Silurian Passage Creek biota.

Fluvial deposits of the early Silurian (Llandovery, *c.* 440 million years old) Massanutten Sandstone host a rich fossil biota at Passage Creek, Virginia, USA (Pratt *et al.*, 1978; Tomescu & Rothwell, 2006). The Passage Creek biota has yielded a macrofossil compression described recently as a macrophytic cyanobacterial colony (Tomescu *et al.*, 2006). This fossil yielded the associated bacteria that we report here.

MATERIALS AND METHODS

Extensive assemblages of carbonaceous fossil compressions occur in fluvial deposits of the lower Massanutten Sandstone, at Passage Creek, Shenandoah County, Virginia (38°56′N, 78°18′W; for location map see Pratt *et al.*, 1978). The fossil assemblages are preserved in numerous siltstone and silty shale

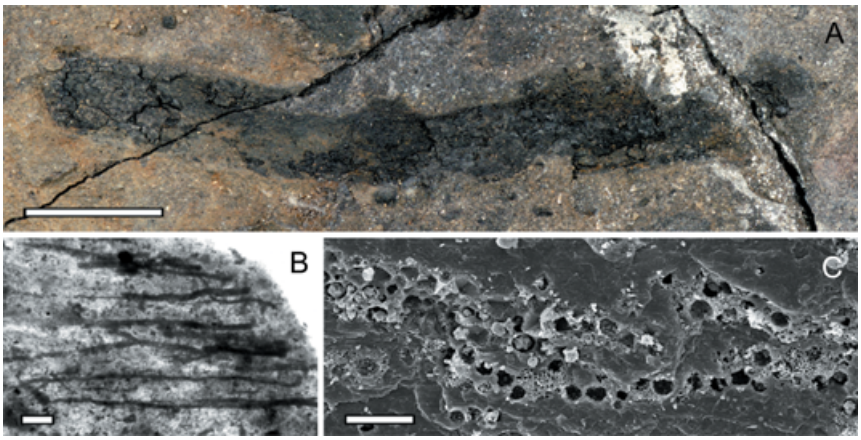

Fig. 1 Cyanobacterial colony preserved as carbonaceous compression (Massanutten Sandstone, Llandovery, Virginia, USA). (A) macroscopic view of colony. Scale 10 mm. (B) Cyanobacterial filaments in a common slime matrix as seen in a fossil fragment cleared in sodium hypochlorite. Scale 100 μm. (C) Scanning electron microscopy of fresh break in the fossil showing filaments (mostly molds of mineral-replaced cells that fell off during preparation) in the amorphous slime matrix. Scale 30 μm. (B) and (C) published with permission from Taylor & Francis.

layers that form discontinuous fine-grained partings at multiple levels between the thick sandstone and fine conglomerate beds of the Massanutten. These fossiliferous layers represent sedimentation in overbank settings of the river systems that deposited the lower Massanutten Sandstone (for details on the sedimentology of the unit and a discussion of the depositional environments of fossiliferous layers see Tomescu & Rothwell, 2006). The age of the lower Massanutten Sandstone is early to middle Llandovery (Pratt *et al.*, 1978).

Fossil compressions in the Passage Creek biota can exceed 10 cm in greatest dimension and are preserved in abundance on multiple bedding planes within each fossiliferous layer. The majority of compressions exhibit thalloid morphologies of different sizes, but more extensive crustose forms, as well as strap-shaped fossils, are also present (Tomescu & Rothwell, 2006). The cyanobacterial colony (Fig. 1A) that contains the bacterial fossils described here is one of the largest compressions discovered to date at Passage Creek: 61 mm long, 7–9 mm wide and 250–300 μm thick. A detailed description of these cyanobacterial fossils, as well as discussions of their taphonomy, mode of fossil preservation and systematic affinities, is provided in Tomescu *et al.* (2006).

Small carbonaceous fragments (1–4 mm) were sampled from the organic compression of the cyanobacterial colony and were prepared for investigation in light and electron microscopy. (1) Some of the fragments were prepared for light microscopy by clearing in sodium hypochlorite. This entailed soaking in household-grade bleach (sodium hypochlorite) for 9 days, and then rinsing in distilled water by progressive dilution of the sodium hypochlorite solution until complete removal in a 4 h interval. The material was then mounted on microscope slides after dehydration in graded ethanol and xylene series. (2) Other carbonaceous fragments of the cyanobacterial colony were examined in scanning electron microscopy (SEM). The carbonaceous fragments were mounted on aluminium stubs without any prior treatment and subsequently sputter-coated in gold. SEM observations were made on fresh breaks in the fossil fragments using a Hitachi S4000 field emission microscope. (3) Fossil fragments for transmission electron microscopy (TEM) were cleaned of mineral inclusions in 30% hydrofluoric acid (1 h) and 40% hydrochloric acid (0.5 h). Fragments were then rinsed in distilled water, dehydrated in a graded ethanol series and embedded in Epon-type resin (Electron Microscopy Sciences, Fort Washington, PA, USA) for ultra-thin sectioning, without prior chemical fixation. Ultra-thin sections were cut on Reichert Ultracut microtomes using glass and diamond knives. No staining was performed on the ultra-thin sections. Ultra-thin sections were investigated and imaged using a Hitachi H7000 transmission electron microscope (Hitachi High-Technologies Corporation, Tokyo, Japan). Specimens are deposited in the Ohio University Palaeobotanical Herbarium as numbers 15988, 16001, and 16002.

RESULTS AND DISCUSSION

Description of fossils

The cyanobacterial colony (Fig. 1A) that hosts the bacterial fossils exhibits a particular type of fossil preservation. The amorphous carbonaceous material of the fossil represents the common exopolysaccharide matrix containing the cyanobacterial cells. The latter form multitrichomous filaments and are preserved by mineral replacement (Fig. 1B,C). The cyanobacterial cells were initially filled with pyrite, which was subsequently replaced by iron oxyhydroxides during fossilization (Tomescu *et al.*, 2006).

The fossil bacteria are preserved in the carbonaceous extracellular matrix of the cyanobacterial colony (Fig. 2). They are rod-shaped (bacilli) and occur as isolated cells or short filaments (Fig. 2A,D). The size of bacterial cells varies between 374 and 468 nm (mean = 417 nm) in length, and 98–149 nm (mean = 122 nm) in diameter ($n = 22$). Some of the cells have a bent appearance (Fig. 2A). The length of the bacterial filaments could not be measured, but the longest filament observed is six cells long. The bacteria are not distributed randomly in the cyanobacterial sheaths, but they occur in

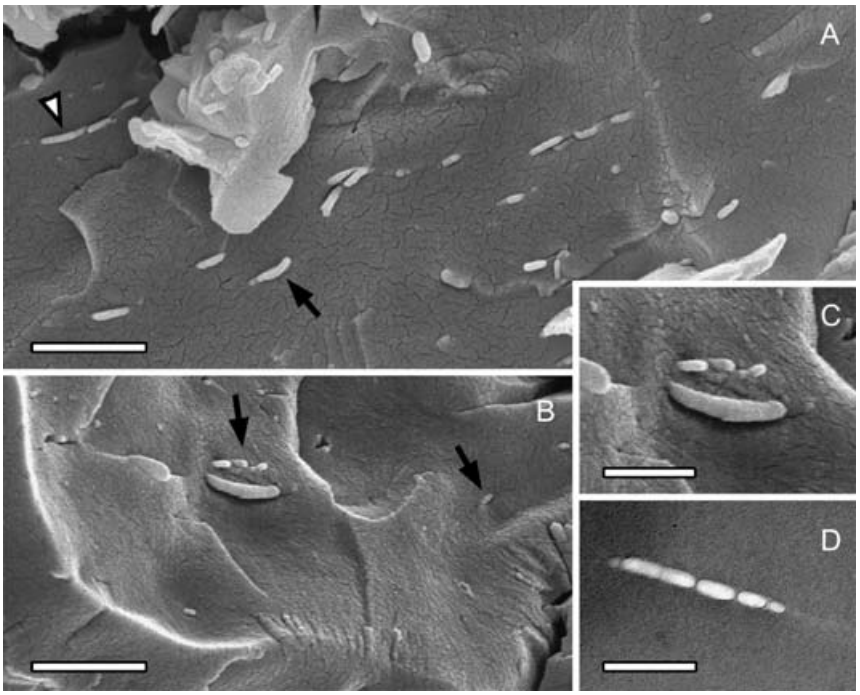

Fig. 2 Electron micrographs of carbonaceous extracellular matrix of cyanobacterial colony containing bacterial cells (Massanutten Sandstone, Llandovery, Virginia, USA). (A–C) Scanning electron microscopy showing mineral-replaced bacterial cells exposed in fresh fracture planes of the carbonaceous matrix. (A) Bacteria are present as isolated cells or filaments (arrowhead); presence of curved bacterial cells (arrow) provides additional evidence that these are not columnar crystals. Scale 2 μm. (B) Bacterial cells protruding from the carbonaceous matrix (arrows). Scale 2 μm. (C) Detail of B; the larger elongated structure below the bacterial cells is an EM artefact. Scale 1 μm. (D) Transmission electron microscopy of cellular molds left by a bacterial filament in the carbonaceous matrix of the cyanobacterial colony; the mineral filling of bacterial cells was dissolved by hydrochloric and hydrofluoric acids during preparation of the specimen; note the absence of fissures around the bacterial cells. Scale 1 μm.

layers (as seen in the fracture plane in Fig. 2A). These layers most likely follow the topology of the microfibrous gelatinous sheath of the cyanobacteria.

Like the cyanobacterial cells, the bacteria are preserved by mineral replacement, as indicated by the fact that their cell content was dissolved by hydrochloric and hydrofluoric acid treatments during preparation of specimens for TEM. The minerals replacing the bacterial cells are probably the same as those involved in the preservation of the cyanobacteria, although the very small size of the bacteria prevented precise chemical determinations.

Bacterial origin of fossils

Criteria for the recognition of microbial (including bacterial) fossils and for the differentiation of those fossils from abiogenic pseudofossils, as well as from contaminants of younger age, have been defined and refined by many (Buick, 1990; Walsh, 1992; Horodyski & Knauth, 1994; Morris *et al.*, 1999; Schopf, 1999; Southam & Donald, 1999; Westall, 1999). The essence of these criteria is to ascertain that the potential fossils are (i) indigenous to, and syngenetic with, the host rock, (ii) biogenic, and that (iii) they occur in a context consistent with the ecology of the organisms with which they are compared. As shown below, when applied to the Passage Creek specimens these criteria all support a bacterial nature.

Indigenousness and syngenicity

The cyanobacterial colony that hosts the bacteria is a compression fossil indigenous to, and syngenetic with, the Massanutten Sandstone (Tomescu & Rothwell, 2006; Tomescu *et al.*, 2006). The bacteria always occur embedded in the carbonaceous matrix of the colony and not along fissures as contaminants, as shown by electron microscopy. This indicates that the bacteria are indigenous to the cyanobacterial colony and that they were already embedded in the exopolysaccharide matrix of the colony at the time of fossilization (syngenetic). In SEM bacterial cells can be seen protruding from fresh breaks in the carbonaceous matrix (Fig. 2B,C). Sections examined in TEM are thought to provide some of the best information for the syngenicity, as well as the biogenicity, of bacterial fossils (Westall, 1999). In our case, TEM shows bacterial fossils completely embedded deep within the carbonaceous matrix (Fig. 2D), and absence of fissures in the carbonaceous matrix around bacterial cells in all of the specimens observed.

Biogenicity

The carbon fingerprint characteristic of biogenic structures is absent in fossil bacteria preserved by mineral replacement. As a result, in these fossils biogenicity is established based on size, shape and, if present, evidence of cell division and patterns of cell organization or colonial distribution (Westall, 1999). The Passage Creek specimens fall within the size range of bacteria (the smallest known free-living bacteria are no larger than 100 nm; Gorbushina & Krumbein, 2000). Although rod-shaped bacteria (bacilli) are less easily confused with abiogenic structures, which tend to be spherical, some rod-shaped minerals can be confused with bacilli. However, the rounded ends that characterize our bacteria (Fig. 2C,D) exclude

rod-shaped crystals that typically do not exhibit rounded terminations even upon acid etching (Westall, 1999). Additionally, the presence of specimens with a bent appearance (Fig. 2A) is evidence for a soft nature characteristic of living organisms and not of crystals.

Ecology and preservation

Also supporting the biogenicity of the Passage Creek specimens are the living environment and the type of preservation of these fossil bacteria, both of which are consistent with those documented for living and fossil bacteria by many previous studies. The Passage Creek bacteria populated the extracellular slime of cyanobacteria, a niche in which bacteria are known to thrive in modern environments (Paerl, 1982, 1992; Rai, 1990; Paerl *et al.*, 2000; Lopez-Garcia *et al.*, 2005). Replacement of cells by pyrite and iron oxides is a common type of fossil preservation for bacteria (Westall, 1999 and references therein).

Taphonomy makes it difficult to discern with certainty whether the bacteria at Passage Creek were metabolically interactive with their cyanobacterial hosts, as is the case in most modern bacterial–cyanobacterial consortia, or whether they might represent parasites or saprotrophs. The relatively low frequency of the bacteria, along with their position that likely follows structural features of the cyanobacterial sheaths, nevertheless suggests a symbiotic association (be it mutualistic or commensal, or possibly parasitic) much rather than saprotrophy. If the bacteria were saprotrophic, one would expect to find locally much richer bacterial populations, since in instances where bacteria are saprotrophic they form concentrations and they proliferate in such high numbers that they replace significant amounts of the biomass of the organic remains that they colonize (e.g. Wuttke, 1983; Liebig *et al.*, 1996). Or, this is not the case in the cyanobacterial colony documented here, where bacteria are relatively evenly distributed in the cyanobacterial extracellular polysaccharide matrix and they do not form concentrations. Furthermore, none of the numerous Passage Creek compression fossils investigated in electron microscopy shows such cases of saprotrophic colonization. If the bacteria were indeed saprotrophic, then we are looking at such an early stage of saprotrophic colonization that one would be hard pressed to distinguish it from parasitism. Based on these considerations the interpretation of the bacteria having been symbiotic with the cyanobacteria at the time of fossilization is the most parsimonious one.

CONCLUSIONS

The early Silurian Passage Creek biota is one of the earliest extensive biotas known from a continental environment, outside the marine realm, and hosts the oldest cyanobacteria recorded in such a setting (Pratt *et al.*, 1978; Tomescu & Rothwell, 2006; Tomescu *et al.*, 2006). As documented here, this biota also produced the earliest fossil evidence for bacteria associated with cyanobacteria and living in the exopolysaccharide matrix of the latter. Documentation of this association was possible at Passage Creek because of the particular type of fossil preservation combining carbonaceous compression and mineral replacement of the cells. The fact that heterotrophic bacteria had colonized the niche provided by cyanobacterial exopolysaccharides as early as 440 Ma might not come as a surprise given the much older age of both types of organisms (Golubic & Knoll, 1993; Schopf, 2000). However, direct association of bacteria and cyanobacteria had not been documented previously in the fossil record and the evidence that such associations can be fossilized suggests that their origin can be tracked deeper in the fossil record.

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REFERENCES

Bazzichelli G, Abdelahad N, Ventola F (1985) Structural modifications in the extracellular investment of *Nostoc commune* Vauch. during the life cycle. I. Motile and nonmotile hormogonium, biseriate stage. *Journal of Ultrastructure Research* **91**, 174–181.

Buick R (1990) Microfossil recognition in Archean rocks: an appraisal of spheroids and filaments from a 3500 My old chert-barite unit at North Pole, Western Australia. *Palaios* **5**, 441–459.

Golubic S, Knoll AH (1993) Prokaryotes. In *Fossil Prokaryotes and Protists* (ed. Lipps JH). Blackwell, Boston, MA, pp. 51–76.

Gorbushina AA, Krumbein WE (2000) Subaerial microbial mats and their effects on soil and rock. In *Microbial Sediments* (eds Riding RE, Awramik SM). Springer, Berlin, Germany, pp. 161–170.

Horodyski RJ, Knauth LP (1994) Life on land in the Precambrian. *Science* **263**, 494–498.

Liebig K, Westall F, Schmitz M (1996) A study of fossil microstructures from the Eocene Messel Formation using transmission electron microscopy. *Neue Jahrbucher fur Geologie und Palaontologie Monatshefte* **4**, 218–231.

Lopez-Garcia P, Kazmierczak P, Benzerara K, Kempe S, Guyot F, Moreira D (2005) Bacterial diversity and carbonate precipitation in the giant microbialites from the highly alkaline Lake Van, Turkey. *Extremophiles* **9**, 263–274.

Morris PA, Wentworth SJ, Allen CC, Thomas-Keprta K, Westall F, Bell MS, Gibson EK, McKay DS (1999) Methods for determining biogenicity in Archean and other ancient rocks. *Lunar and Planetary Science* **30**.

Paerl HW (1982) Interactions with bacteria. In: *The Biology of Cyanobacteria* (eds Carr NG, Whitton BA). University of California Press, Berkeley, CA, pp. 441–461.

Paerl HW (1992) Epi- and endobiotic interactions of cyanobacteria. In *Algae and Symbioses* (ed. Reisser W). Biopress, Bristol, UK, pp. 537–565.

Paerl HW, Pinckney JL, Steppe TF (2000) Cyanobacterial–bacterial mat consortia: examining the functional unit of microbial survival and growth in extreme environments. *Environmental Microbiology* **2**, 11–26.

Pratt LM, Phillips TL, Dennison JM (1978) Evidence of non-vascular land plants from the Early Silurian (Llandoverian) of Virginia, U.S.A. *Review of Palaeobotany and Palynology* **25**, 121–149.

Rai AN (1990) *Handbook of Symbiotic Cyanobacteria*. CRC Press, Boca Raton, FL.

Schopf JW (1999) Fossils and pseudofossils: lessons from the hunt for early life on Earth. In *Size Limits of Very Small Microorganisms*. National Academy Press, Washington, pp. 88–93.

Schopf JW (2000) The fossil record: tracing the roots of the cyanobacterial lineage. In *The Ecology of Cyanobacteria* (eds Whitton BA, Potts M). Kluwer, Dordrecht, the Netherlands, pp. 13–35.

Southam G, Donald R (1999) A structural comparison of bacterial microfossils vs. 'nanobacteria' and nanofossils. *Earth-Science Reviews* **48**, 251–264.

Takeda M, Nishiyama T, Nomoto S, Shinmaru S, Suzuki I, Koizumi J-I (2002) Commensal relationship between a sheath-forming bacterium, *Sphaerotilus natans*, and a sheath-degrading bacterium, *Paenibacillus* sp. *Bioscience, Biotechnology, and Biochemistry* **66**, 458–463.

Tomescu AMF, Rothwell GW (2006) Wetlands before tracheophytes: thalloid terrestrial communities of the Early Silurian Passage Creek biota (Virginia). In *Wetlands Through Time* (eds Greb SF, DiMichele WA). *Geological Society of America Special Paper* **399**, 41–56.

Tomescu AMF, Rothwell GW, Honegger R (2006) Cyanobacterial macrophytes in an Early Silurian (Llandovery) continental biota: Passage Creek, lower Massanutten Sandstone, Virginia, USA. *Lethaia* **39**, 329–338.

Walsh MM (1992) Microfossils and possible microfossils from the Early Archaean Onverwacht Group, Barberton Mountain Land, South Africa. *Precambrian Research* **54**, 271–293.

Westall F (1999) The nature of fossil bacteria: a guide to the search for extraterrestrial life. *Journal of Geophysical Research* **104** (E7), 16437–16451.

Wuttke M (1983) 'Weichteil-Erhaltung' durch lithifizierte Mikroorganismen bei mittel-eozänen Vertebraten aus den Ölschiefern der 'Grube Messel' bei Darmstadt. *Senckenbergiana Lethaea* **64**, 509–527.