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ULTRASTRUCTURE OF CAMBRIAN CRYPTOSPORES AND THE EARLY EVOLUTION OF THE PLANT SPORE WALL

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Abstract: Terrestrially-derived cryptospores from Cambrian deposits in eastern Tennessee were examined using transmission electron microscopy (TEM) to determine the underlying structural basis for spore wall construction in these pre-embryophytic spore types. In addition to previously-described species from the middle Cambrian Conasauga Group, we examined new specimens extracted from the Rome Formation, which are coeval to the oldest reported cryptospores from North China. Our results reveal a substantial diversity of endogenous laminated sporoderm construction, expanding on the three basic types described previously. The underlying cellular processes involved in the production of sporopollenin wall construction were in place by the middle Cambrian, but a modern form of spore wall assembly did not evolve until the canalization of plant-like sporogenesis during the Middle Ordovician.

Key words: Rome Formation, origin of land plants, sporopollenin, charophytes, terrestrialization, paleopalynology, sporoderm, Conasauga Group, synoecosporal wall

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Introduction

Palynologists use the term acritarch to refer to vesicular (unicellular) organic-walled microfossils whose systematic provenance is unknown (Downie 1963, Downie et al. 1963, Evitt 1963). Acritarchs are generally considered to be the cysts of marine phytoplankton (Vidal and Knoll 1983, Fensome et al. 1990, Martin 1993, Servais 1996, Strother 1996, Servais et al. 1997, Moczydłowska 2011, Nowak et al. 2015, Lei et al. 2019). They occur in fine-grained siliciclastic rocks of all ages, but are most common in rocks of Neoproterozoic to Devonian age (Tappan 1980, Strother 1996, 2008). Palynological assemblages from shallow marine deposits of Darriwilian age and younger often contain an admixture of plant spores (trilete spores and permanent spore tetrads) and acritarchs and chitinozoans, and usually the distinction between spores and acritarchs is evident, based on morphological features such as color, wall thickness, and ornament. In older deposits that lack cryptospore tetrads, however, the distinction between simple, smooth-walled acritarchs, known simply as leiospheres (after the genus, *Leiosphaeridia* Eisenack) or, sphaeromorphs (referring to the category, sphaeromorph acritarch) can be less than obvious. So, it behooves us to clarify, as best we can in simple morphological terms, the distinction between spore-like microfossils and leiospheres, when we are addressing palynological assemblages from lowermost Paleozoic strata. Individual spore-like palynomorphs are discoidal in shape (not spherical) with robust walls that typically impart a darker color than leiospheres, which tend to be thinner-walled and possess folds in their walls – reflecting an originally spherical shape. There are other features, as well, that we have used in the past to justify the use of the term, cryptospore (Strother and Beck 2000, Baldwin et al. 2004, Taylor and Strother 2009, Strother and Taylor 2018), to refer to spore-like palynomorphs of Cambrian age, some of which are addressed below.

Spore-like palynomorphs from Cambrian deposits have yet to be generally accepted as belonging to land plants (embryophytes) (Kenrick et al. 2012, Wellman et al. 2022), even though molecular evolutionary trees consistently posit the existence of embryophytes by middle Cambrian time (Morris et al. 2018). If the molecular timetrees that predict Precambrian and Cambrian origins to the embryophytes are correct, then one might expect to find a land plant spore record extending deeper in time. Undoubtedly, cryptospore assemblages of pre-Darriwilian age contain numerous spore-like microfossil types that are clearly morphologically distinct from embryophyte spores of younger ages. These differences are significant, including

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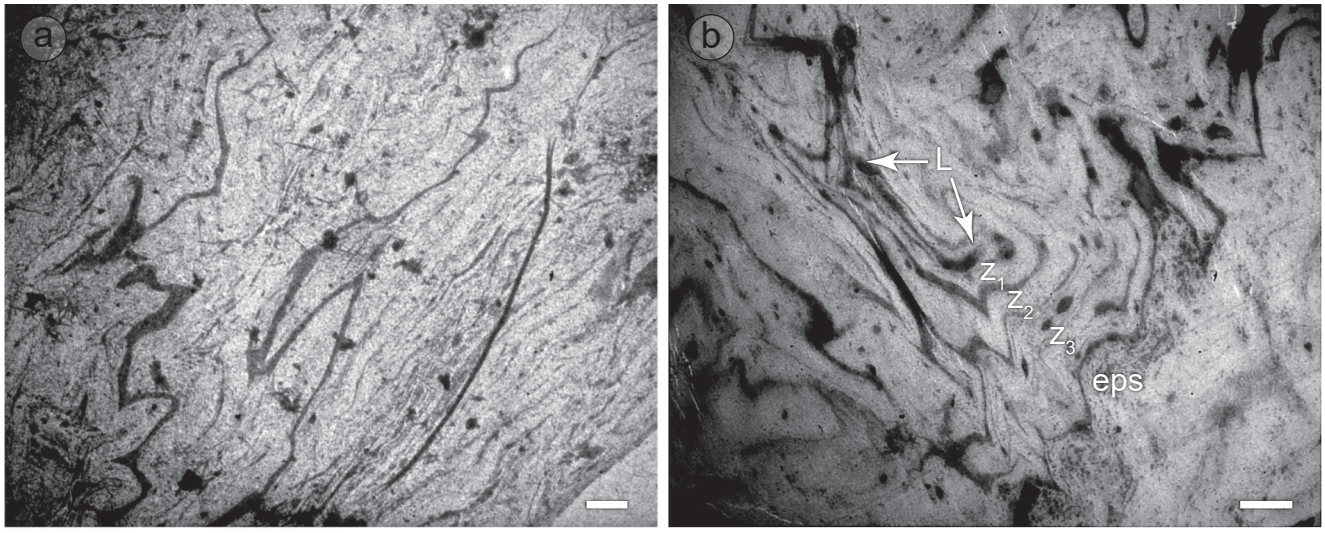
the arrangement of attached spore-bodies, the topology of enclosing sporoderm walls, the overall smaller size of spore-bodies, and the endosporic (versus tapetal) nature of spore wall development (Strother and Taylor 2018, Taylor and Strother 2024). Embracing these differences with younger spore types has led to a more nuanced understanding of Cambrian and early Ordovician cryptospores – one that now views such fossils as a record of spore evolution in terrestrial charophytic algae prior to the evolution of the embryophyte sporangium (Taylor et al. 2017, Strother and Foster 2021, Strother 2023, Taylor and Strother 2024). Recent reviews on the origin of land plants from an evo-devo perspective (Bowman 2022, McCourt et al. 2023) have incorporated this way of thinking by integrating the fossil record into Bower's (Bower 1890, 1908) antithetic hypothesis for the origin of the plant sporophyte, although (Renner and Sokoloff 2024) present an alternative explanation for the antithetic concept that considers a free-living sporophyte as primitive. The discovery of a Tremadocian cryptospore assemblage from Australia (Strother and Foster 2021), which contains a mix of Cambrian species (*Adinosporus* P.K.Strother) with more typical post-Darriwilian cryptospore types (*Dyadospora* P.K.Strother et Traverse, *Tetraplanarisporites* C.H.Wellman, Steemans et M.A.Miller, *Rimosotetras* N.D.Burgess), has reinforced this view that the fossil spores of Cambrian age belong to an evolutionary lineage that ultimately includes the embryophytes (Strother and Taylor 2018). In this study, we continue to explore a developmental approach to the evolution of the plant spore in our interpretation of early sporoderm ultrastructure based on material collected from the Conasauga Group in eastern Tennessee, USA.

Cambrian cryptospores are not simply clusters of sphaeromorph acritarchs

As mentioned above, at the outset there is an overarching issue as to why spore-like palynomorphs, or, as we prefer to call them, cryptospores, are distinct from clusters of sphaeromorph acritarchs or leiospheres. What do we know about the characteristics of Cambrian sphaeromorphs and other acritarchs in general which are recovered from normal marine assemblages? A recent thorough study of lower Cambrian assemblages from East Greenland (Wallet et al. 2023) includes well-illustrated examples of colonial taxa and cell-clusters attributed to *Synsphaeridium* Timofeev (pl 3., figs 5–11) and *Symplassosphaeridium* Timofeev (pl. 3., fig.12). All the cells in *Synsphaeridium* are distinctly circular in outline with thin, translucent walls that show folds from compression. They occur as a single layer of marginally attached cells, and without any extra-cellular enclosing membranes or partitions into sub-groups or packets. *Symplassosphaeridium* consists of a mass of distinct individual cells that form an irregularly-shaped colony, again not easily confused with cryptospores clustered together into packets like *Agamachates* W.K.Taylor et P.K.Strother or *Adinosporus* P.K.Strother. One category of colonial forms that has been clarified recently falls into the Hydrodictyeaceae within the Chlorophyta as described from the lower Cambrian Forteau Fm (Harvey 2023). However, all these colonies are composed of distinct spherical cells attached laterally into geometrically regular, planar configurations. They are quite distinct from Cambrian spore-like microfossils.

Intriguingly, a recent study focused entirely on the issue of acritarch clustering, based on a study of assemblages from the middle Cambrian (Miaolingian) Jince Fm of the Prague Basin did not document any clusters of spore-like microfossils (Kovář et al. 2023). These authors very thoroughly considered the range of possible biological and ecological reasons for acritarch clustering, including both smooth-walled and acanthomorphic forms, and did not consider any clustering to be derived from sporangia – instead they concluded that, in this case of marine phytoplankton, clustering was due to "aggregation within algal blooms" or, in some cases colonial behavior. None of



Text-fig. 1. Some examples of TEM images of acetolyzed *Coleochaete*. a: Note the disorganized nature of the sample, without obvious structural relations to vegetative cells. b: Another example that we interpret to possess a cell lumen (L, arrows), surrounded by three layers – z_1 and z_2 are homogeneous zones enclosed by z_3 which is composed of discontinuous laminae. None of the three zones is particularly persistent over the entire specimen. The outside of the cell mass is characterized by a granular extracellular matrix, labelled "eps". Scale bar = 2 µm.

their illustrations of smooth-walled (leiosphere) clusters are similar to any of the Cambrian cryptospores documented from Laurentia (Strother and Taylor 2024) or China (Wang et al. 2022). Kovář et al. (2023) provide a comprehensive review of acritarch clustering from the Cambrian through to the Devonian, and we recommend this work to those who wish to further review the comparison of lowermost Paleozoic spore-like cryptospores to sphaeromorph clusters.

Cambrian cryptospore wall ultrastructure differs from desiccated vegetative algal walls

In a singular study of structural changes in vegetative cell walls during desiccation, (Graham et al. 2012) looked for structural similarities between extra-cellular lamination in *Coleochaete* Brébisson species subjected to desiccation and laminar walls in cryptospores. Their specimens were subjected to acetolysis, which should have removed the cellulosic component of the cell wall. Persistent laminae remaining after acetolysis would be evidence of sporopollenin or some other complex polymers not affected by the acetolysis reaction. Text-fig. 1a, is a TEM image of acetolyzed vegetative cells. Here, electron dense laminae are folded, distorted, and largely discontinuous, but the relation to the (now missing) primary cell walls is not obvious. In Text-fig. 1b we were able to interpret a section of the acetolyzed cell mass that retains lamination around what we interpret to be the original cell lumen, indicated by L in the figure. This electron dense mass is surrounded concentrically by two homogeneous layers, z_1 and z_2, and a third layer (z_3), characterized by discontinuous laminae. This layered sequence is, in turn, surrounded by a granular exterior space that we interpret to be extra-cellular polymeric substances (eps). The nature of marginal attachment and discrete cellular arrangement corresponding to traceable cell division is not evident in these images. Although desiccation in *Coleochaete* clearly has resulted in extracellular lamination, this is strikingly different from any of the laminar walls from any of the Cambrian spore-like remains we have examined to date.

Cambrian cryptospore wall ultrastructure differs from Leiosphaeridia

Several authors have reported on wall ultrastructure in leiospherid acritarchs (Arouri et al. 1999, Talyzina and Moczydłowska 2000, Javaux et al. 2004). There are a few types present; twin thin laminae (e.g., *Multifronsphaeridium pelorium* W.Zang (Arouri et al. 1999); *Leiosphaeridia crassa* (S.N.Naumova) Jankauskas (Javaux et al. 2004)), and those that possess a thicker homogenous wall that may or may not be bound by surface laminae (e.g., *Globosphaeridium cerinum* (Volkova) Moczydłowska (Talyzina and Moczydłowska 2000); *Leiosphaeridia tenuissima* Eisenack (Javaux et al. 2004)). In general, while there is some comparable complexity to Cambrian cryptospores, where the overall walls are as thick, they are homogeneous, and where they have comparable complexity (number of layers), the laminae are much thinner and less robust overall. Cambrian cryptospores show both complexity and thickness, consistent with their original function in a subaerial environment. Additionally, the vast majority of the examined leiospheres come from strictly marine deposits.

What have we learned from prior studies of Cambrian cryptospore ultrastructure?

Middle Cambrian spore-like remains, which are distinct from *Leiosphaeridia* and other sphaeromorph acritarchs, were first reported from the Bright Angel Shale (Grand Canyon, Arizona) in abstracts by Strother (1998) and Strother et al. (1998). Gordon Wood quickly recognized that the Rogersville Shale in the Conasauga Group of eastern Tennessee contained similar spore-like remains of similar age, and these were first reported in a pair of abstracts (Strother and Wood 1999, 2000), claiming that such remains were evidence of a land flora by Cambrian time. Strother and Beck (2000) published both SEM and LM images of cryptospores from the Grand Canyon and the subsequent, more general treatment (Strother et al. 2004), included the first transmission electron micrographs of a cryptospore dyad from the Bright Angel Shale. This well-preserved specimen possessed a homogeneous inner wall that was surrounded by three similar walls that enclosed both spores of the dyad pair. Each of the five wall layers was smooth on the inside with distinctly scabrate outer surfaces. In total, the multilayered sporoderm was about 1,200 nm in thickness. The innermost layer, the actual spore wall, was the thickest, at about 400 nm. The scabrate nature of the exosporal sporoderm prompted the authors to conclude that in this particular specimen, the sporoderm was applied centripetally, from the outside of the developing sporocyte.

Taylor and Strother (2008) revised the description of this early dyad, clarifying some of the wall terminology and proposing a new term, **synoecosporal**, for the wall layers that surround multiple spore bodies. The synoescoporal wall, therefore, corresponds, in part to the term **envelope** used to describe enclosed cryptospore tetrads and dyads from Ordovician and younger strata. This first dedicated TEM study of Cambrian cryptospore ultrastructure described three distinct wall types, all from a single palynological sample from the Bright Angel Shale at Tapeats Creek drainage in the Grand Canyon. In addition to the scabrate dyad, which was now shown to possess four synoescoporal walls surrounding a single inner spore wall, the other wall types included those with multiple distinct, but homogeneous, laminae, and a third sporoderm type of heterogeneous walls composed of uniform to fusiform lamellae. They further clarified proposed wall terminology to make the distinction between laminae and lamellae: **laminae** refer to discrete wall layers, which themselves can be homogeneous to somewhat granular in electron density and are generally thicker than 20 nm, whereas, **lamellae** refer to inferred white-line tripartite lamellae (TPL), structures that fuse to form laminated texture within individual walls.

Initial examination of these Cambrian cryptospores and spore-clusters revealed that many of the spore-bodies were enclosed within one or more distinct layers of sporopollenin-like material. TEM analysis also demonstrated the collapsed and folded character of many of these enclosing, synoescoporal walls (Taylor 2009). A third aspect to Cambrian cryptospore wall structure and topology as revealed in TEM is seen in the late Cambrian species, *Agamachates casearinus* (Taylor and Strother 2009). Here, sectioning showed that spore end-members could be packaged into either pairs or

quartets of enclosed dyads. This arrangement appears to indicate a decoupling between karyokinesis and cytokinesis during sporogenesis, a condition that is consistent with endoreduplication as documented in some living charophyte algae, including *Coleochaete* (Hopkins and McBride 1976, Haig 2010, 2015). Thus, basic topology of cryptospore arrangements into enclosed packets has led to a separate line of evidence supporting a developmental connection between sporogenesis in charophytic algae and dispersed Cambrian cryptospores (Strother and Taylor 2018).

In this report we will attempt to further explore the evolutionary connection between the dispersed cryptospore record and the origin of land plants, especially with respect to the evolution of the embryophytic spore wall. The basic laminated sporoderm as seen in the Cambrian species from the Grand Canyon (Strother et al. 2004, Taylor and Strother 2008) can be seen as a possible homolog to laminated sporoderm in the liverworts, which was considered to be plesiomorphic by Blackmore and Barnes (1987). However, as we discuss below, results from *Adinosporus* and slightly older cryptospores from the Rome Fm demonstrate a further level of complexity that requires interpretation in the context of the evolution of spore development over simple character placement into a morphology-based phylogenetic analysis.

Materials and methods

Geological setting and sampling regime

The geological section at Thorn Hill, eastern Tennessee is a classic Paleozoic stratigraphic section from the Southern Appalachian Mountains (Byerly et al. 1986). The middle Cambrian Conasauga Group exposed in the road cut at Thorn Hill conformably overlies the lower Cambrian Rome Formation, and is, in turn, conformably overlain by the upper Cambrian to Lower Ordovician Knox Group. Palynological samples were collected from the Thorn Hill section in 2001 and 2008. Importantly, samples were also collected from several drill cores made available to us from environmental boreholes located on the grounds of the Oak Ridge National Laboratories. In this report these include samples from the JOY No. 2 borehole, which penetrated the entirety of the Conasauga Group and the underlying Rome Fm (Hasse et al. 1985). A full description of the regional geological setting, including the section at Thorn Hill, can be found in Hasson and Haase (1988).

Paleoenvironmental interpretations of the Rome Formation and Conasauga Group

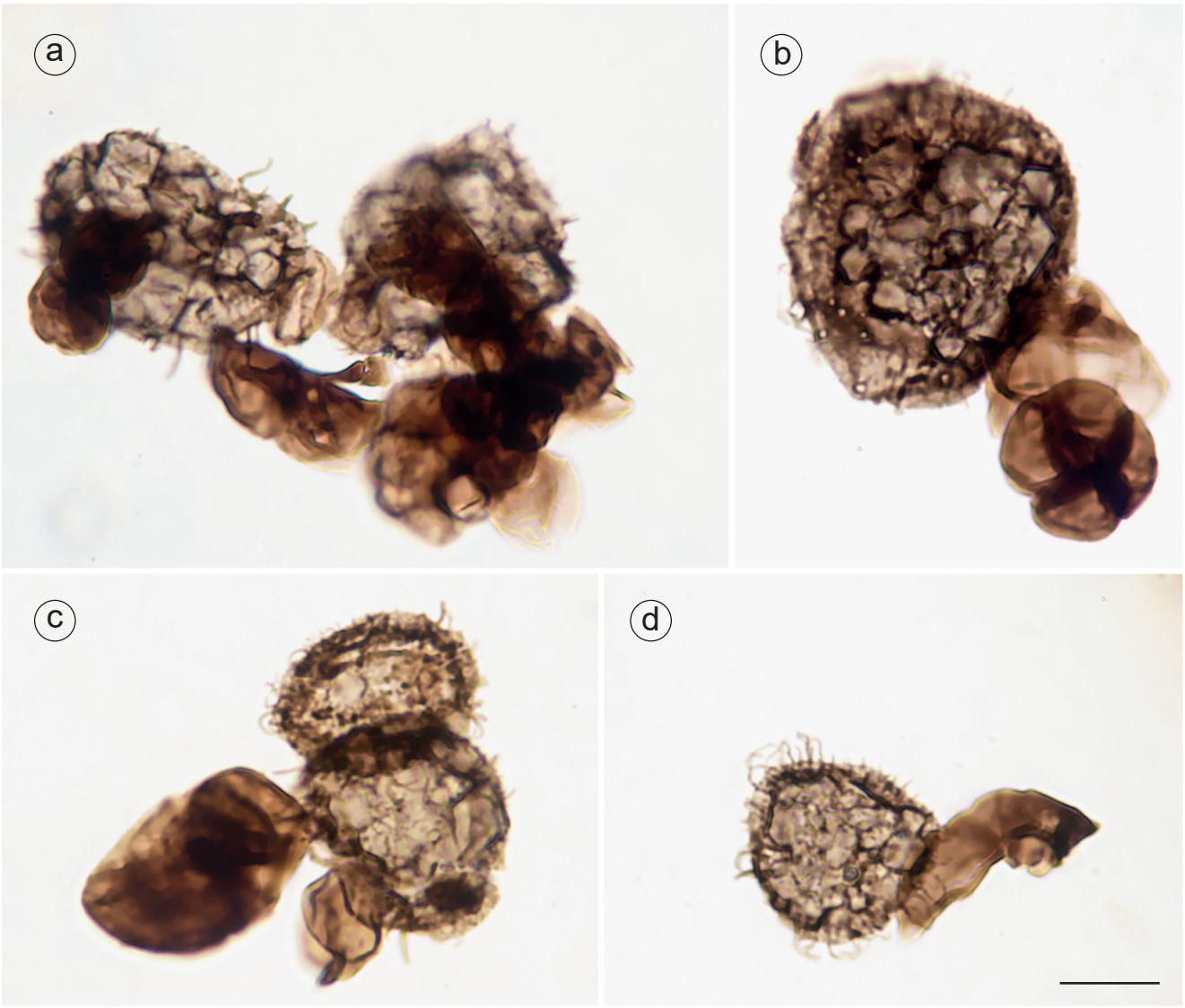
Sediments marginal to Laurentia during the middle Cambrian occur as narrow belts of continentally-derived siliciclastics and shallow marine to paralic carbonates. The geological section at Thorn Hill belongs to an "inner clastic belt" as designated by Lochman-Balk (1971). The sequence begins with the Rome Fm, which is siliciclastic in nature and contains evidence of very shallow deposition and subaerial exposure such as red beds, desiccation cracks (Text-fig. 2), and rare halite casts (Brooks 1955). The subsequent Conasauga Group has long been considered to be a marine

Text-fig. 2. An example of mudcracks, indicative of subaerial exposure, preserved in red mudstones of the Rome Formation, Thorn Hill section, east Tennessee.

unit, but it is regionally heterogeneous with intertonguing siliciclastistics derived from the craton to the west and carbonates to the east and south (Rodgers 1953, Markello 1981, Hasse et al. 1985, Hasson and Haase 1988).

Brooks (1955) interpreted halite casts as the result of temporal aridity in tidal pools that were subsequently gently flushed with normal seawater, not with freshwater. He considered the possibility that the dissolution of halite could have occurred as a result of freshwater influx from terrestrial sources onto a tidal flat, but he favored a marine model, citing, in part, the presence of trilobite trails in the same beds. Trilobites have long been considered as stenohaline indicators, but this has changed in recent years with extensive paleoenvironmental research in ichnofacies analysis by Gabriella Mángano and Luis Buatois (Mángano et al. 2021, Buatois et al. 2022), who have clearly demonstrated that some trilobites inhabited low salinity and even freshwater paralic environments. In any case, we should not be too surprised that the Rome "mudflats", which contain clear evidence of subaerial exposure, might have received some degree of freshwater influx, bringing with it terrestrially derived sediments and organic matter.

Prior palynologic study on the shale interbeds within the lower Conasauga Group does not support an open marine provenance (Strother and Beck 2000, Baldwin et al. 2004, Strother et al. 2004, Strother 2016). Here, the assemblages are overwhelmingly dominated by clusters of spore-like cells, cryptospores sensu Strother and Beck (2000), with only minor acritarchs, including *Auritusphaera bifurcata* Strother (Strother 2008), *Revinotesta* Vanguest., *Leiosphaeridia* and *Michrystridium* spp. that are quite small (8 to 12 μm in diameter). A few examples of re-worked early Cambrian acritarchs (*Retisphaeridium* Staplin, Janson. et S.Pocock and *Skiagia* S.Downie) have been recovered and are illustrated here in Text-fig. 3. These are easily recognizable because of often fragmentary condition, ashen-gray color, and walls that are impressed with damage from pyrite inclusions. Reworking, in this case, is an indication that the source area for the fine-grained siliciclastic component of these Conasauga sediments included an actively eroding continental source with earlier Cambrian deposits. The very existence of re-working indicates a terrestrial source to the recovered palynological assemblage.



Text-fig. 3. Some examples of re-worked lower Cambrian acritarchs admixed with middle Cambrian cryptospores from JOY-2 core, depth 1578 feet (Rogersville Shale). In all cases note the greyish granular nature of the acritarch walls in contrast to the smooth brownish wall of the cryptospores. a: *Skiagia* sp. with unnamed cryptospores. b: *Skiagia* ? with isometric cryptospore tetrad, *Spissuspora laevigata*. c: *Skiagia* sp. with unnamed cryptospores. d: *Skiagia* sp. with cryptospore fragment. Scale bar is 10 µm and corresponds to all images.

Sample preparation and palynomorph recovery

The rock samples used in this report were processed at Boston College and at the University of Sheffield, with TEM preparation performed by WAT at the University of Wisconsin – Eau Claire. All samples were processed using normal palynological processing techniques as described in Barss and Williams (1973) and Traverse (2008). This included, crushing approximately 20 g of rock in an iron mortar and pestle, treatment in dilute HCL, followed by treatment in concentrated HF for up to one week. After a second HCl treatment and water washing, samples were sieved through a 20 µm screen and mounted on glass microscope slides either in glycerine jelly or Evacite mounting medium.

For TEM, organic residues from the Rogersville Formation were washed from glycerine jelly with hot water, or taken directly from residues stored in ethanol, and completely dehydrated to pure ethanol. Strew specimens from the Rome Formation were first viewed in the SEM. Identified specimens of interest were picked from the SEM stubs individually and embedded in 4% agar blocks. Using acetone as an intermediate solvent, specimens were run through a graded sequence of acetone:fresh epoxy monomer (3:1, 1:1, 1:3, pure epoxy, 8–12 hours between changes; Spurr or Spurr replacement epoxy (Ellis 2006)) then flat embedded in aluminum weighing dishes. Polymerization was carried out at 70C overnight. Specimens were then selected from the resulting epoxy discs, cut out with the proper orientation, and sectioned using a Leica UCT-B ultramicrotome into sections approximately 100 nm in thickness. Imaging of Rogersville spores was carried out using a JEOL 2010 TEM operating at 80 kv that was fitted with an Advantage digital camera manufactured by Advanced Microscopy Techniques Corp., Danvers, MA, at the University of Wisconsin – Eau Claire. Some Rome specimens were imaged with an Hitachi H-7500 TEM fitted with a Gatan ES1000W Erlangshen CCD 11 MP camera at the University of Wisconsin – Stevens Point.

Table 1. Compilation of wall layer types found in Cambrian cryptospores. Measurements in nm.

taxon/specimen	number wwof laminae	min lam thickness	avg lam thickness	max lam thickness	number of measurements	overall wall thickness	reference
A. voluminosus	4	63.9	124.8	208.1	50	600–800	this study
A. bullatus							
Rog 2ox2-4	1+?	61	81	113	21		this study
Rog 2-2-1	1+?	14	31,5	43	32		this study
A. geminus Rog 2ox3-3	>4	62	78	112	15	400–700	this study
lamellated monad/dyad	12–15	22	39	85	40		this study
Rome							
lamina type 1		68	129	188	30		this study
lamina type 2		98	170	250	25		this study
lamina type 3 - thick		96	121	146	10		this study
lamina type 3 - thin		15	32	47	20		this study
lamina type 4 - beads		89	165	307	10		this study
lamina type 4 - thin		12,5	17	22	20		this study
Other Cambrian laminae							
A. casearius	1	44		125			Taylor 2009
BAS polyads	4	50		110			Taylor 2009
BAS scabrate dyads		50		300			Taylor 2009
Rog monad	multiple	25		90			Taylor 2009

All white light microscopy was performed at the Weston Observatory Paleobotanical laboratory using a Zeiss Universal microscope, modified to remove the IR filter in the substage light path and mounted onto a pneumatic vibration isolation table. The objective used in this report was a Leitz PLEZY apochromatic 40/0.95 lens with a correction collar. Illumination was from a 100W/12V Halogen-type bulb with a color temperature of 3450 K. Photomicrography was accomplished using a Nikon D700 digital SLR camera back attached to the microscope phototube, with a separate electronic shutter placed after the field stop in the substage light path. Photomicrographs were taken by first opening the camera shutter for a four-second-long exposure, and then operating the substage shutter to control actual exposure time. Image processing workflow included initial cropping and setting color balance in Camera Raw, followed by white level adjustments in Photoshop in which the maximum range was set to 248.

Results

On the characterization of laminated sporoderm

We will use the term **laminated** for units of spore wall construction (lamina[e]) that have an average minimum thickness of 20 nm. This contrasts with the term **lamellated** wherein the units (lamella[e]) are thinner and usually possess a characteristic three-layered construction consisting of a central lightly-staining layer, bounded above and below by more darkly staining layers (= tripartite lamellae = TPL). The term **continuous** will be used to refer to a lamina in a spore wall that forms a complete covering over the contained spore body. In the TEM, one to several of these may combine to form a spore wall, but at any point around the periphery, the same number of walls will be seen in cross-section. This presumably is the result of a single pulse of secretion off of the plasma membrane of the entire cell per lamina. Successive pulses result in successive continuous laminae where the number of pulses equals the number of laminae. The terms heterogenous and homogenous refer to the internal appearance of constituent wall layers in the TEM, mostly where no laminae are visible.

Ultrastructural examination of cryptospores from the Conasauga Group

The systematic basis for these fossils was established on characteristics seen under white light microscopy (LM), although prior work on Cambrian cryptospore wall ultrastructure was used in the interpretation of how spore-bodies were assembled into irregular spore packets.

Adinosporus voluminosus Strother, 2016

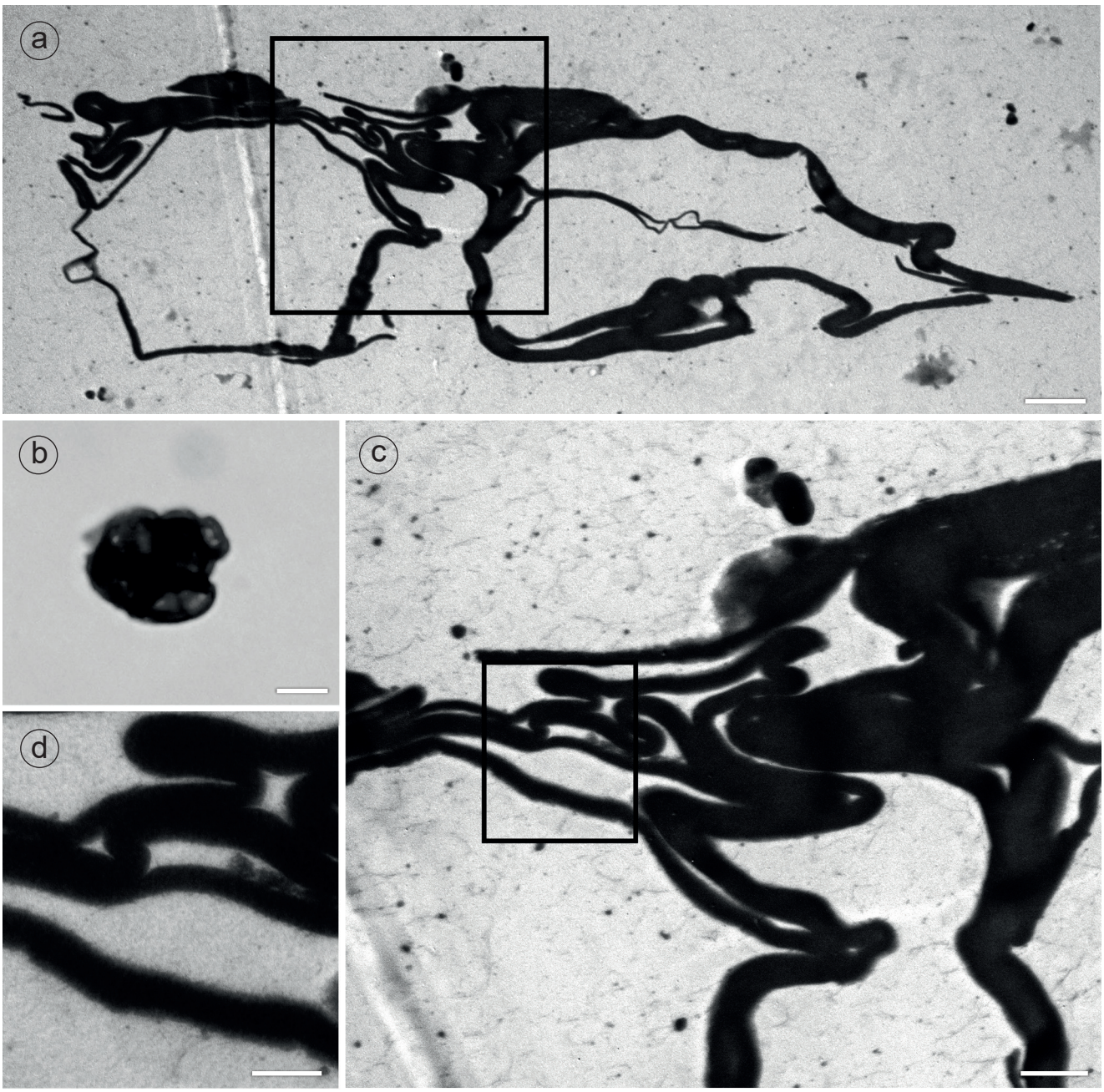
Text-fig. 4a–d

The spore walls of this species consist solely of laminae. The specimen illustrated in Text-fig. 4a–d (Rog 2ox3-1) has no more than four laminae that range from 60–320 nm thick, and line the entire spore lumen – i.e., they are continuous laminae. The fragile laminae often break and fold back on themselves. This causes the spores to appear mottled when viewed in the light microscope (Text-fig. 4b).

Adinosporus bullatus Strother, 2016

Text-fig. 5a–f

In this taxon the spore walls have an outer layer that appears to be homogeneous, although we recognize that, in



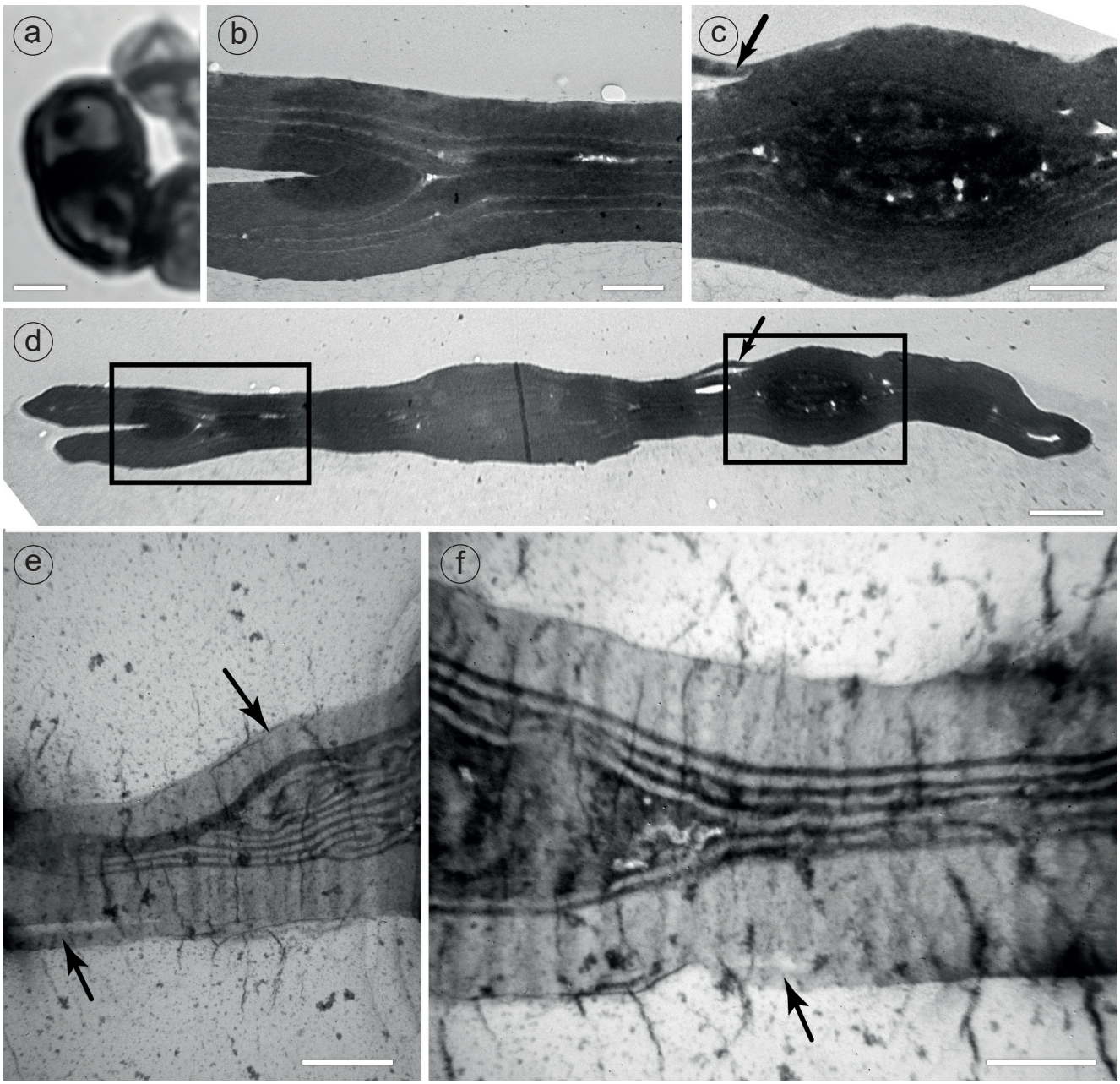
Text-fig. 4. ***Adinosporus voluminosus*** **Strother, specimen Rog 2ox3-1. a: Low magnification TEM cross section of entire dyad. Note numerous broken and folded laminae. Box inset is shown in (b). b: Light micrograph of the specimen viewed through thick epoxy disc prior to sectioning. c: Central region of contact face shown in inset box in (a). Note interdigitation of laminae of adjacent spore-bodies. d: Detail of central region seen in (c) (inset box). Scale bar = 10 µm (b), 2 µm (a), 1 µm (c), 500 nm (d).**

this case, diagenetic fusion may be masking an underlying laminar construction. This interpretation is supported by areas where the surface appears to be delaminating (Text-fig. 5c, d, e, f, arrows). The outer layer ranges in thickness from 150 to 700 nm (Text-fig. 5b–f). Lining each of the lumens of the dyads is a single (or possibly two) lamina(e) 30–60 nm in thickness. The dark spots (bullae) that characterize this taxon are formed by darkly staining, likely organic deposits, that form in spaces where the inner laminae have folded up and/or collapsed toward the contact area (Text-fig. 5b, c, e, f). Two specimens (Rog 2ox2-4, Rog 2-2-1) are figured here.

Adinosporus geminus Strother, 2016

Text-fig. 6a–d

The spore walls of this species consist solely of laminae. The specimen illustrated in Text-fig. 6a–d (Rog 2ox3-3) has more than four laminae that range from 60–120 nm thick (Text-fig. 6b, d), and line the entire spore lumen – i.e., they are continuous laminae. Paired dyads with aligned contact face planes characterize this taxon (Text-fig. 6c). The outermost laminae of the paired dyads interdigitate (Text-fig. 6a, b). There is no fusion of the laminae of adjacent spore bodies.



Text-fig. 5. ***Adinosporus bullatus*** **Strother; specimen Rog 2ox2-4 (a–d), specimen Rog 2-2-1, (e, f). a: Light micrograph of specimen viewed through thick epoxy disc prior to sectioning. b: Detail of inset rectangle in (d). Arrow indicates area of surface delamination. c: Detail of inset rectangle in (d). This is a bulla, which appears to consists of dark carbonaceous deposits. Arrow indicates area of surface delamination. d: Low mag TEM cross-section showing whole dyad and locations of high-mag insets. Arrow indicates area of surface delamination. e: High-contrast image of specimen Rog 2-2-1 showing folded central laminae. Arrows indicate areas of surface delamination. f: High-contrast image of specimen Rog 2-2-1 showing folded central laminae. There is a bulla is to right side of image. Arrow indicates area of surface delamination. Scale bar is approximately 10 µm (a), is 2 µm (d), 500 nm (b, c, e, f).**

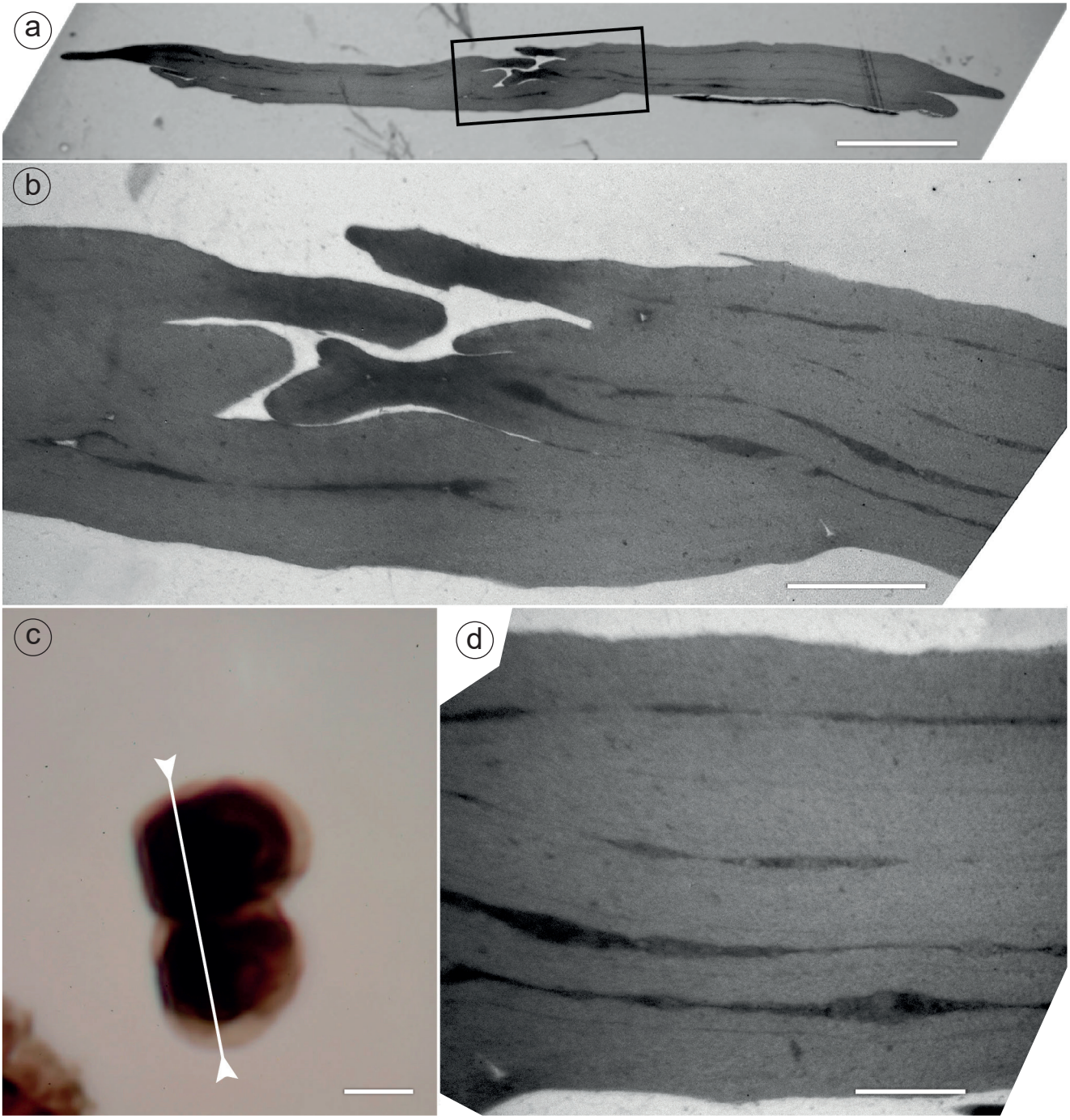
Unnamed lamellated dyads

Text-fig. 7a, b

Specimen Rog 2ox-1-1 is a pair of two dyads with highly convoluted walls composed of multiple thin laminae. (Or, it is possible this specimen is simply a single dyad pair as the convolution of the constituent laminae complicates interpretation). The multiple convoluted laminae (22 [38.3] 68 µm thick, N = 40) appear to be continuous, and number 12–15 in each wall. The laminae are, for the most part, distinct, forming walls of parallel laminae; however, some regions, particularly toward the spore margins, appear more uniform. Here the laminae have fused, perhaps a reflection of changes during burial and preservation. In any case, the spore-bodies here are highly laminar in spite of the difficulty in determining the spore lumen.

Ultrastructural examination of cryptospores from the Rome Formation

Three specimens from the Rome Fm consisting of clusters of various numbers of spore packets were examined with TEM. These forms are not readily distinguished from *A. voluminosus* when examined using LM, but none of these



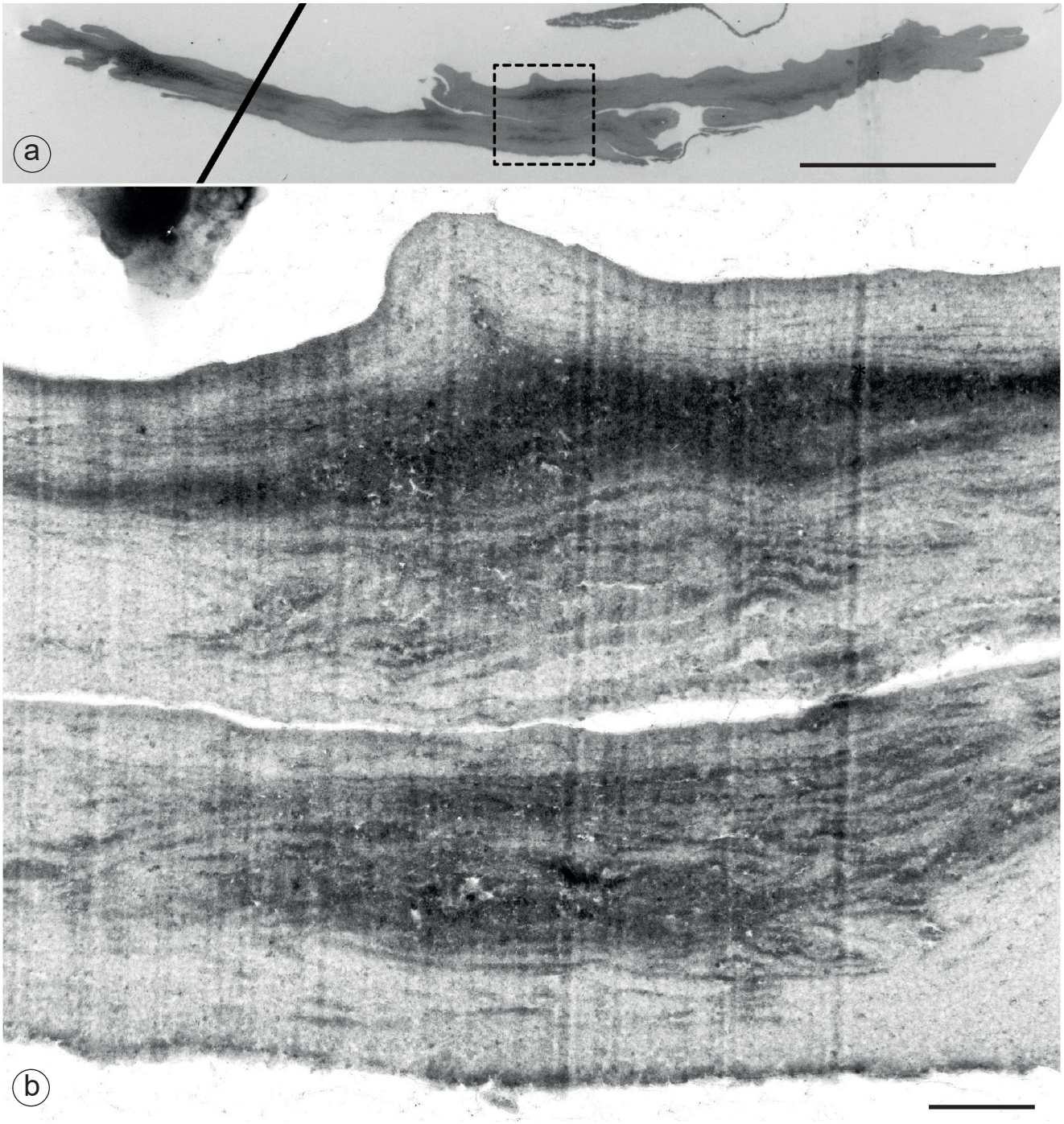
Text-fig. 6. ***Adinosporus geminus*** **Strother, specimen Rog 2ox3-3. a: Low magnification TEM cross section of entire specimen of paired dyads. Inset is magnified in (b). Note the numerous folded laminae. b: Central region of contact face shown in inset in (a). Note interdigitation of laminae of adjacent spore-bodies. c: Low resolution light micrograph of the specimen viewed through thick epoxy disc prior to sectioning. Line shows the approximate location of cross-section depicted in (a). d: Detail of the central region of a single spore-body. Note numerous continuous laminae. Scale bar = 10 µm (c), 5 µm (a), 1 µm (b), 500 nm (d).**

has a wall ultrastructure that matches that seen in the various species of *Adinosporus* described above. These forms are therefore kept in open nomenclature and are indicated here as *Adinosporus*?.

***Adinosporus*? sp.**

Text-figs 8a–d, 9a, b

Specimen R10 is a clump of 40 or more packets, each approximately 30 µm in diameter (Text-fig. 8a). When viewed in TEM cross-section (Text-fig. 8b, c, d), the packets consist of multiple spore-bodies with highly compressed walls that are approximately 1.5–2.0 µm thick. The former lumen can be difficult to identify with certainty, but based on the symmetry of the surrounding laminar zones, an approximation can be made (marked with an arrow on Text-fig. 8 and Text-fig. 9). The outer half of the wall is densely constructed with hints of lamination throughout and some more lightly staining voids (Text-fig. 8c, d). The inner half consists of multiple continuous laminae of variable construction. Few if any of the individual laminae are of consistent thickness along their length.



Text-fig. 7. Dyad pair from the Rogersville Formation, specimen Rog 2ox1-1. a: Low mag cross-section of pair of dyads (?). b: Detail of central region box in (a). Scale bar = 10 µm (a), 1 µm (b).

There are several types of laminar construction present:

Type 1 laminae are approximately 100 nm thick, smooth on the inside, but with an undulating surface on the outside (labeled "1" on Text-fig. 9a, b).

Type 2 laminae occur as thicker units (up to 200 nm) that undulate on both the inside and the outside surface (labeled "2" on Text-fig. 9a, b).

Type 3 laminae are found in zones of alternating thick and very thin laminae (labeled "3" on Text-fig. 9a, b).

Type 4 laminae consist mostly of beads connected by zones of very thin laminae (labeled "4" on Text-fig. 8c, d).

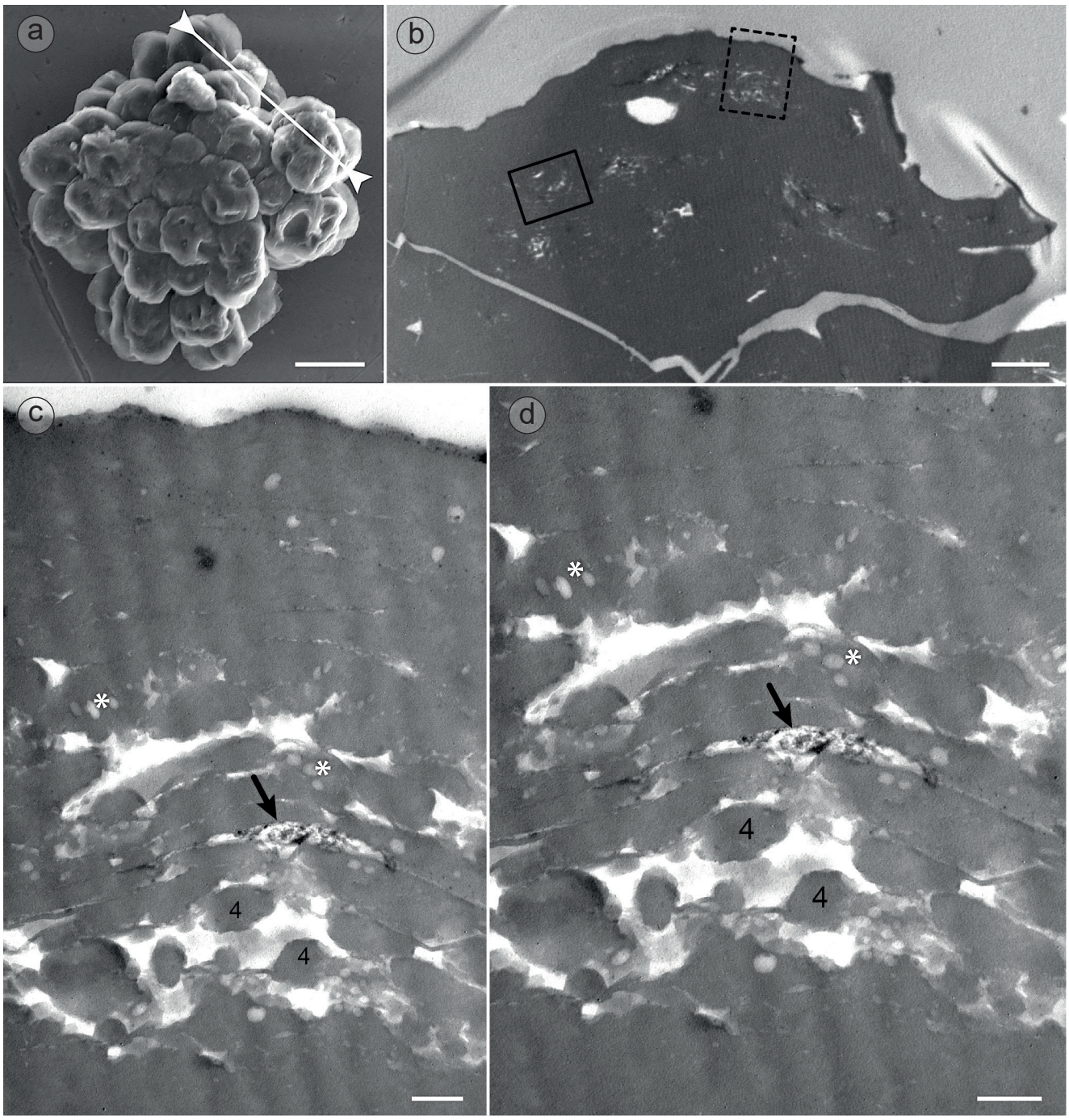
Other extraneous fragments of wall material (beads, laminae, etc.) also occur throughout the wall but are difficult to track to individual laminar zones.

Some laminar zones also have a concentration of lightly staining circular to elliptical voids within (marked with an asterisk on Text-fig. 8c, d and Text-fig. 9a, b).

***Adinosporus*? sp.**

Text-fig. 10a–d

Specimen R2A is a clump of several spore packets (Text-fig. 10a) shown in cross-section in Text-fig. 10b–d. The single wall cross-section of one of these packets (Text-



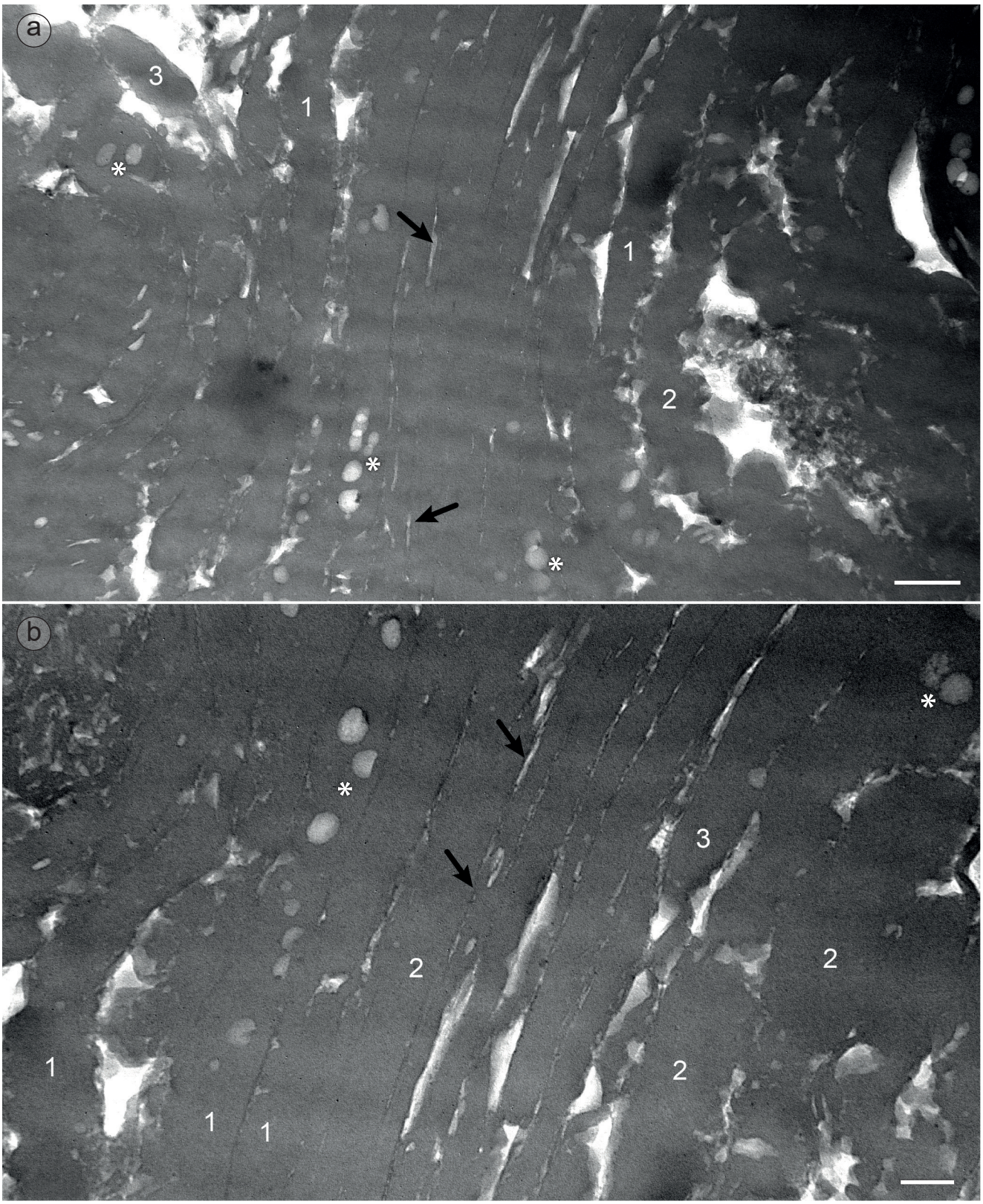
Text-fig. 8. Rome Formation packets, specimen R1O. a: SEM image of sectioned clump of packets. Line shows approximate plane of section in Text-figs 8, 9. b: TEM cross-section of single packet from clump in (a). Dotted inset box shows location of regions enlarged in (c, d). Black inset box 5 shows approximate location of images on Text-fig. 9. c: Enlarged zone corresponding to the inset box c in (b) showing the spore mass to its margin. Asterisk (⁎) denotes zones with a concentration of light staining voids. Numbers 1 through 4 correspond to laminar types described in the text. Arrow indicates the probable location of the lumen. d: Further enlargement of the spore mass central region. Asterisk (⁎) denotes zones with a concentration of light staining circular voids. Number 4 corresponds to laminar wall type described in the text. Arrow indicates the probable location of the lumen. Scale bar = 25 µm (a), 2 µm (b), 200 nm (c, d).

fig. 10d) shows a similar assortment of layer types as the specimen above.

Discussion

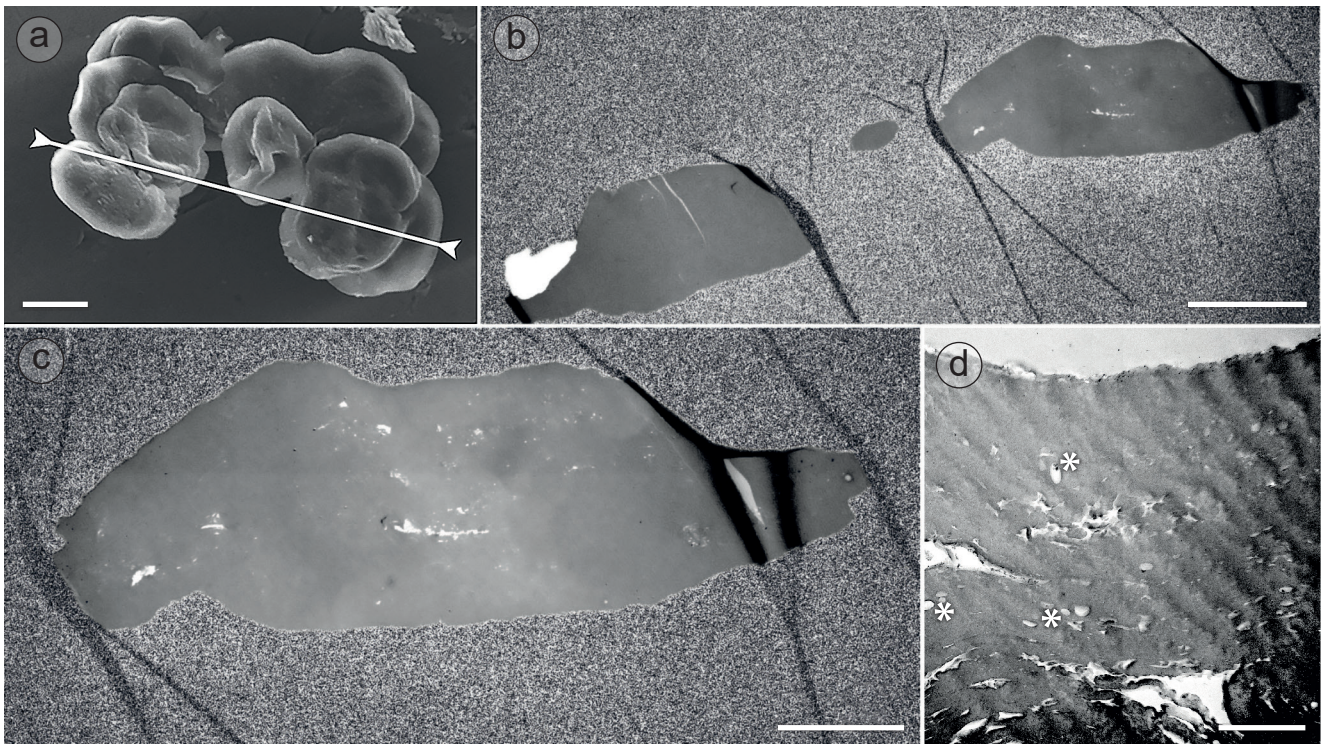
On the evolution of plant spore wall formation

It seems reasonable to infer that production of a thick sporopollenin wall around spore-like bodies evolved for protection of dispersive propagules from desiccation and/or UV radiation. That this character first appears in the Cambrian is only controversial insofar as it concerns attribution of the producer to embryophytes. We have argued, rather, that charophyte ancestors to the embryophytes adopted this character much earlier. Among bona fide embryophytes, the canalized pattern that produces mature spore walls consists of two separate and largely successive modes; 1) white-line centered TPL secreted from the surface of developing spores,



Text-fig. 9. Rome Formation packets, specimen R1O. a: Detail of laminar construction and types corresponding to the solid inset box in Text-fig. 8b. Asterisk (⁎) denotes zones with a concentration of light staining circular voids. Numbers 1 through 4 correspond to laminar types described in the text. Arrow indicates the approximate location of the lumen, now completely collapsed. b: Higher detail of laminar construction and types corresponding to solid inset box in Text-fig. 8b. Asterisk (⁎) denotes zones with a concentration of light staining circular voids. Numbers 1 through 4 correspond to laminar types described in the text. Arrow indicates the approximate location of the lumen. Scale bar = 200 nm (a),100 nm (b).

and 2) deposition of bulk sporopollenin from a surrounding tapetum or tapetum-like layer within a sporangium. The former is widely considered to be the primitive mode, and is common to nearly all embryophytes. Variable combinations of these two modes produce the entire range of wall ultrastructures present throughout the embryophytes.



Text-fig. 10. Rome Formation packets, specimen R2A. a: SEM of spore cluster showing the plane of cross-section depicted in (b). b: Low mag cross-section along plane shown in (a). The dark lines running from lower left to upper right in the image are folds in the section. c: Enlargement of lower packet shown in (b). d: Detail of single wall layer of packet shown in (c). Space at bottom is close to the position of the cell lumen (L). Several types of laminae are visible below the outer dense layer (under the dark line). Scale bar = 10 µm (a), 8 µm (b), 4 µm (c), 400 nm (d).

Wall formation in primitive embryophytes

Extant liverworts possess a one-cell-thick sporangial wall that effectively functions as a tapetum (Levins et al. 2024) by secreting sporopollenin that thickens multiple lamellae originating centrifugally from the developing spore surface. These thickened lamellae dynamically form the continuous laminae that comprise the mature liverwort spore wall. There is variation within the group in the sub-layers that make up the spore wall, with laminae ranging in thickness up to 700 nm (Tab. 1). However, none of this variation observed in extant liverworts can explain the developmental origin of either thick homogeneous, or non-laminate spore walls.

Today, the polymerization of sporopollenin is widely considered to involve an interplay between physical and chemical forces, all of which occurs within an enclosed space, the sporangium. We know of no mechanism for the formation of thick walls in plant spores that does not involve some form of chamber that is isolated from the environment.

Wall formation in Adinosporus

With completely continuous separate, distinct laminae making up its wall, *Adinosporus* clearly lacks any contribution of exogenous sporopollenin by a tapetum. This does not preclude production of these spores inside a primitive sporangium, but it does hamper illuminating their development based on modern plant analogs since no such analog is known. Among extant green algae, *Mychonastes desiccatus* S.W.Brown (Margulis et al. 1988) produces a multilayered resistant wall in response to desiccation, but the layers, while continuous, are much thinner. The mode of deposition displayed by this chlorophyte alga is likely via TPL effectively extruded through the cell membrane to form an outer wall.

Wall formation in Rome packets

The variable, continuous laminae that make up the spores of the Rome packets must have originated from sporocytes via successive waves of deposition, forming a sporopolleninous wall surrounding the once-living cell. However, the means by which they acquired their different (ultrastructural) morphologies is unknown. The sequence of lamination styles (type 1 through 4) as laid down to form a spore wall, does not fall into a consistent pattern (inside to outside), although only a few walls, themselves, have been examined to date. So, at this point the differing ultrastructural laminations may simply be due to variation within the dynamic nature of deposition and polymerization during the process of wall development.

Once again, there is no extant analog for this kind of sporopolleninous wall formation among any extant algae. But among contemporaneous fossil spores, the scabrate dyads from the Bright Angel Shale (Taylor and Strother 2008) have sublayers of similar construction (Type 4); thinner laminate zones alternating with thicker beaded zones. Thus, cryptospore producers ranging across Laurentia from the Grand Canyon (AZ) to Tennessee had evolved a similar mode of wall formation as of Middle Cambrian time.

The circular to ellipsoidal holes that are found in small clusters within some of the laminae are curious. These features are marked by an asterisk in Text-figs 8, 9, but upon

examination of these plates, one can see several related fields of low numbers (say 3 to 6) of such holes. They appear to be partially occluded (electron dense), but in some cases, where two holes overlap, the overlapping portions are completely clear. The smooth nature of the holes seems to belie a chemical degradation origin, rather they are discrete structures that appear unrelated to the general structure of the laminations. One possibility is that they have a biological origin, perhaps the holes bored by chytrid fungi or actinomycetes, both of which are known to be capable of sporopollenin ingestion.

The thick, non-laminated portions of the walls of the spore-bodies that form the Rome packets were not formed by developmental processes known to occur today in extant green algae adapted to subaerial environments. Thus, we have to conclude that developmental mechanisms that no longer exist were responsible for the formation of these walls.

Wall formation in unnamed laminated dyad/monad

The continuous nature of the laminae in these spores is difficult to trace, likely due to the effects of fossilization. However, in zones where an entire thickness of one wall is visible, the laminae are continuous and mostly separate. In other areas, there may be some fusion between adjacent laminae. These effects also complicate determining the presence of one (monad) or two (dyad) lumens in each spore. The walls of this specimen are remarkably similar to those seen in several extant liverworts (e.g., *Riccia* L.; Steinkamp and Doyle 1979). We are not claiming that these spores were produced by a Cambrian liverwort, but it does demonstrate that some Cambrian organisms had evolved a developmental program capable of producing a spore wall comparable to that seen in an extant liverwort.

Hypothetical scenario of pre-embryophyte spore wall formation

Given the absence of a tapetum, we can envision two ways in which an ancient developing sporocyte produced an endogenous sporopolleninous wall: 1) extrude sporopollenin or its precursor molecules through the sporocyte membrane to create a wall surrounding and enclosing the cell membrane, 2) form TPL and subsequent laminae entirely within the cell cytoplasm and then plaster the material up against the inner face of the cell membrane of the developing sporocyte. Both scenarios result in a centripetal form of dynamic wall formation in which the outermost layer is formed first, and subsequent laminae become progressively younger toward the center of the sporocyte.

If the first deposited lamella on the spore surface were supplied with sporopollenin precursors effectively extruded through the cell membrane via fusion with golgi-derived, secretory vesicles, the surface extent of nucleation sites in the apoplast may have varied considerably. Nucleation and subsequent polymerization in the apoplast would have been somewhat variable, so that the final wall was thicker in some places and thinner in others. Subsequently, this initial layer of sporopollenin polymerization would have isolated the inner developing sporocyte from its external environment, allowing the formation of other types of laminae within this controlled space. And space between the outermost wall layer and the sporocyte cell membrane could have acted as an "incubator" of sporopollenin, in much the same manner as a true sporangium in embryophytes. If one (or more) such wall(s) were formed by the spore mother cell prior to partitioning into daughter spores, this would explain the origin of the synoecosporal wall. This scenario could explain the formation of successive, variable laminae in spore walls, but explaining the formation of thicker, homogeneous or non-laminated walls poses more of a challenge.

It is assumed that thicker layers of sporopollenin would require a substantial supply of biochemical precursor molecules, all of which were derived from the developing sporocyte. In extant embryophytes, spores undergo a phase of enlargement after release from the meiotic tetrad and prior to environmental release, with resources supplied by the parent sporophyte. Absent that source, as well as the space provided by the expansive sporangium locule, these hypothetical pre-embryophytes would be both space and resource limited. It is interesting to note that Cambrian cryptospores are notably smaller than their younger counterparts (Strother and Taylor 2018), which is consistent with the hypothetical developmental scenario presented here.

Conclusion

There is surprising variation in the diversity of cryptospore wall types in the Cambrian. They are clearly more robust than the walls that surround contemporaneous leiospheres, a paraphyletic group of acritarchs generally considered to be the cysts of marine algae (Lindgren 1981, Servais et al. 1997). The cryptospores described herein derive from terrestrial or marginal marine (terrestrially derived) sediments, so they are unlikely to have structural analogs in the marine phytoplankton. They occur in a wide range of packets and polyads which possess considerable topological variation with respect to common enclosures, and that differ from the more organized arrangements present in most post-Cambrian cryptospore taxa. These properties point to their interpretation as the equivalent of meiotic phases in the life cycles of terrestrial charophytic algae, a group that shows considerable variation in reduction division and the decoupling of karyokinesis and cytokinesis (Haig 2010, 2015, Strother 2016, Strother and Taylor 2018).

Given that these cryptospores were likely not produced in a sporangium (as in extant embryophytes) the wall layers that originate as lamellae, and subsequently thicken to laminae, do so by some developmental mechanism that is no longer expressed in most extant terrestrial algae. It's not that terrestrial green algae do not produce resistant-wall cells today, but neither modified vegetative cells (Graham et al. 2012), nor zygospores produce walls that mimic the multilaminar nature of these Cambrian forms. We conclude that an evolving complex of terrestrial charophytic algae contained species that were capable of producing robust spore walls, similar to that seen in some extant embryophytes. However, such spore walls were produced endogenously, in the absence of a surrounding sporangium that secreted sporopollenin. It is likely that the biochemical pathways and cellular mechanisms of sporopollenin wall construction seen here were an evolutionary response to environmental stress

in aeroterrestrial settings during Cambrian and possibly earlier times. The genomic basis of sporopollenin deposition was retained in those lineages that eventually evolved complex embryonic multicellularity that is the hallmark of the land plants today.

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