eLife

Reviewed Preprint
v2 • December 27, 2024
Revised by authors

Reviewed Preprint
v1 • July 4, 2024

Evolutionary Biology

On the nature of the earliest known lifeforms

Dheeraj Kanaparthi, Frances Westall, Marko Lampe, Baoli Zhu, Thomas Boesen, Bettina Scheu, Andreas Klingl, Petra Schwille, Tillmann Lueders

Max-Planck Institute for Biochemistry, Munich, Germany • Chair of Ecological Microbiology, BayCeer, University of Bayreuth, Bayreuth Germany • Earth and environmental sciences, Ludwig Maximilian University, Munich, Germany • CNRS-Centre de Biophysique Moléculaire, Orléans, France • Advanced Light Microscopy Facility, European Molecular Biology Laboratory, Heidelberg, Germany • Key Laboratory of Agro-Ecological Processes in Subtropical Regions, Taoyuan Agroecosystem Research Station, Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha, China • Department of Biosciences, Center for Electromicrobiology, Aarhus, Denmark • Department of Botany I, Ludwig Maximilian University, Munich, Germany

https://en.wikipedia.org/wiki/Open_access

Copyright information

eLife Assessment

This provocative manuscript presents **important** comparisons of the morphologies of Archaean bacterial microfossils to those of microbes transformed under environmental conditions that mimic those present on Earth during the same Eon. The evidence in support of the conclusions is **solid**. The authors' environmental condition selection for their experiment is justified.

https://doi.org/10.7554/eLife.98637.2.sa2

Abstract

Microfossils from the Paleoarchean Eon are the oldest known evidence of life. Despite their significance in understanding the history of life on Earth, any interpretation of the nature of these microfossils has been a point of contention among researchers. Decades of back-and-forth arguments led to the consensus that reconstructing the lifecycles of Archaean Eon organisms is the most promising way of understanding the nature of these microfossils. Here, we transformed a Gram-positive bacterium into a primitive lipid vesicle-like state and studied it under environmental conditions prevalent on early Earth. Using this approach, we successfully reconstructed morphologies and life cycles of Archaean microfossils. In addition to reproducing microfossil morphologies, we conducted experiments that spanned years to understand the process of cell degradation and how Archaean cells could have undergone encrustation minerals (in this case, salt), leading to their preservation as fossilized organic carbon in the rock record. These degradation products strongly resemble fossiliferous features from Archaean rock formations. Our observations suggest that microfossils aged between 3.8 to 2.5Ga most likely were liposome-like protocells that have evolved physiological pathways of energy conservation but not the mechanisms to regulate their morphology. Based on these observations, we propose that morphology is not a reliable indicator of taxonomy in these microfossils.

Introduction

The Pilbara Greenstone Belt (PGB), Western Australia, and Barberton Greenstone Belt (BGB), South Africa, are the oldest known sedimentary rock successions that have not undergone significant metamorphic alterations (1,2). Hence, these rock formations have been the subject of numerous scientific investigations focused on understanding the biology and biogeochemistry of Archaean Earth (1–5). Over a span of 50 years, these studies have documented various organic structures within these rock formations that resemble fossilized cells and their degradation products, with $\delta^{13}C$ composition consistent with biologically derived organic carbon (6,7). Although these observations suggest that these organic structures were fossil remnants of Archaean microorganisms, any such interpretation, together with the biological origin of these structures, has been a point of contention among researchers (8–11). Two factors currently limit wider acceptance of their biological origin – the absence of truly analogous microfossil morphologies among extant prokaryotes and an indication of an ongoing biological process, like cell division, among most microfossils (4,12). Moreover, most of the described microfossils are larger than present-day prokaryotes and often exhibit considerable cytoplasmic complexity with intracellular alveolar structures (3,5,13). These complex morphologies and relatively larger cell sizes of supposedly primitive Archaean Eon cells is not in accordance with our current understanding of how biological complexity evolved through Darwinian evolution (14,15).

Apart from the chemical and $\delta^{13}C$-biomass composition (13,16–18), one key emphasis of the studies arguing for and against the biological origin of Archaean Eon organic structures involves an extensive morphological comparison with extant prokaryotes or abiotically formed minerals (8,9,19,20). Cell morphology among extant organisms is maintained by a plethora of intracellular processes and is determined by the information encoded in their genome (21). In our opinion, drawing parallels between the present-day prokaryotes and Archaean Eon organisms inherently involves subscribing to the notion that paleo-Archaean life forms possess all the complex molecular biological mechanisms to regulate their morphology as present-day cells. Any such presumptions are not in tune with the current scientific consensus of how life could have originated on early Earth (14,22,23). It is now widely believed that life evolved in the form of protocells devoid of most molecular biological complexity (23). These primitive cells are thought to have undergone slow Darwinian evolution, resulting in present-day cells with intricate intracellular processes (24,25). Given the unlikelihood of Archaean cells possessing complex molecular biological processes, we test the possibility that complex morphologies of Archaean microfossils result from the complete absence of intracellular mechanisms regulating their morphology. (26).

To test this hypothesis, we used a top-down approach of transforming a Gram-positive bacterium (*Exiguobacterium Strain-Molly*) into a primitive lipid vesicle-like state (*EM-P*). Cells in this state can be described as a simple sack of cytoplasm devoid of all mechanisms to regulate their morphology and reproduction. Although it has not been empirically demonstrated, some studies have suggested that cells in this lipid vesicle-like state may resemble primitive protocells (27–29). Given that the reproduction of such cells is shown to be influenced by environmental conditions (28,29), we studied the life cycle of these cells under experimental conditions resembling the native environment of the Archaean microfossils.

While the precise environmental conditions of early Earth remain uncertain, a growing consensus within the scientific community suggests that surface temperatures on Archaean Earth ranged between 26° and 35°C (30–32). Moreover, most, if not all, of the known microfossils from the Archaean Eon are restricted to coastal marine environments (6,33). Coastal marine environments often exhibit higher salinity due to the constant evaporation of seawater. To replicate the high salinities of the coastal marine environments, *EM-P* was cultivated in half-

strength tryptic soy broth supplemented with 7% (w/v) Dead Sea Salt (TSB-DSS) at 30°C. We chose Dead Sea Salt over pure NaCl to better emulate complex salt compositions of natural environments.

Given that *EM-P*'s life cycle and the biophysical basis of such a reproduction process is extensively discussed in the previous paper (34), the primary focus of this manuscript will be restricted to the morphological comparison of *EM-P* cells and Archaean Eon microfossils. Below, we present the morphological comparison between *EM-P* cells and Archaean Eon microfossils. In addition to this morphological comparison, we also conducted experiments that spanned years (18 to 28 months) to understand the process of protocell degradation, how they become encrusted in salt, and how they are preserved as fossilized organic carbon in the rock record.

Results

Morphological comparison of top-down modified cells with fossilized Archaean cells

When cultured under experimental conditions likely resembling coastal marine environments of Paleoarchean Eon, *EM-P* exhibited cell sizes that were an order of magnitude larger than their original size. They also exhibited complex morphologies and reproduced by a relatively less understood process (28,34). The life cycle of these cells involves reproduction by two methods – via forming internal or a string of external daughter cells (34). *EM-P* reproducing by both these processes bears close morphological resemblance to microfossils reported from the Archaean Eon.

The first step in reproduction by intracellular daughter cells is the formation of hollow intracellular vesicles (**Fig. 1A**). These vesicles were formed by a process that resembles endocytosis (**Fig. 1A**). A similar process of vesicle formation was previously reported in protoplasts (29,37). Over time, the number of intracellular vesicles (ICVs) within *EM-P* gradually increased (**Fig. 1A-F**). No uniformity was observed in the size of ICVs within a cell or the number of ICVs among different cells (**Fig. 1E**, **1F**, S1 & S2). *EM-P* cells with such intracellular vesicles resemble spherical microfossils reported from 3.46 billion-year-old (Ga) Apex chert (35). Like the Apex-chert microfossils, ICVs of *EM-P* were hollow, and organic carbon (cytoplasm) in these cells is restricted to spaces between the vesicles (**Fig. 1F-K** & S3).

The three-dimensional STED and SEM images of *EM-P* show numerous surface depressions (**Fig. 1K**, arrow & S1E). Such depressions are formed either during vesicle formation (**Fig. 1A** & **1B**, arrow) or by the rupture of intracellular vesicles attached to the cell membrane (**Fig. 1D**, **1E**, S1G-K & S4-S6). *EM-P* cells with such surface depressions and intracellular vesicles strongly resemble morphological resemblance to microfossils reported from 3.4 Ga Strelley Pool Formation (SPF) microfossils (36) (**Fig. 1M** & Fig. S4-S6). Microfossils reported from other sites, such as the Farrel Quarzite (Fig. S7)(38), Turee Creek (Fig. S8)(39), and the Fig Tree Formations (Fig. S9)(40), likely are morphological variants of Archaean *EM-P*-like cells and the Apex Chert microfossils. For instance, *EM-P* with many but relatively smaller intracellular vesicles resemble the Fig Tree microfossils, both in cell size and shape (Fig. S9). On the other hand, *EM-P,* with a relatively larger number of intracellular vesicles squeezed into polygonal shapes, resemble microfossils reported from the SPF (**Fig. 1G-I** & S4-S6), the Farrel Quartzite microfossils with polygonal alveolar structures (Fig. S7) and the Turee creek microfossils (Fig. S8)(38).

The second step in this method of reproduction involves the formation of daughter cells into the ICVs (Fig. S10)(34). Daughter cells were formed in the intracellular vesicles by a process resembling budding (Fig S10). Over time, these bud-like daughter cells detached from the vesicle wall and were released into the ICV (Movie 1). Due to a gradual loss of cytoplasm to the daughter

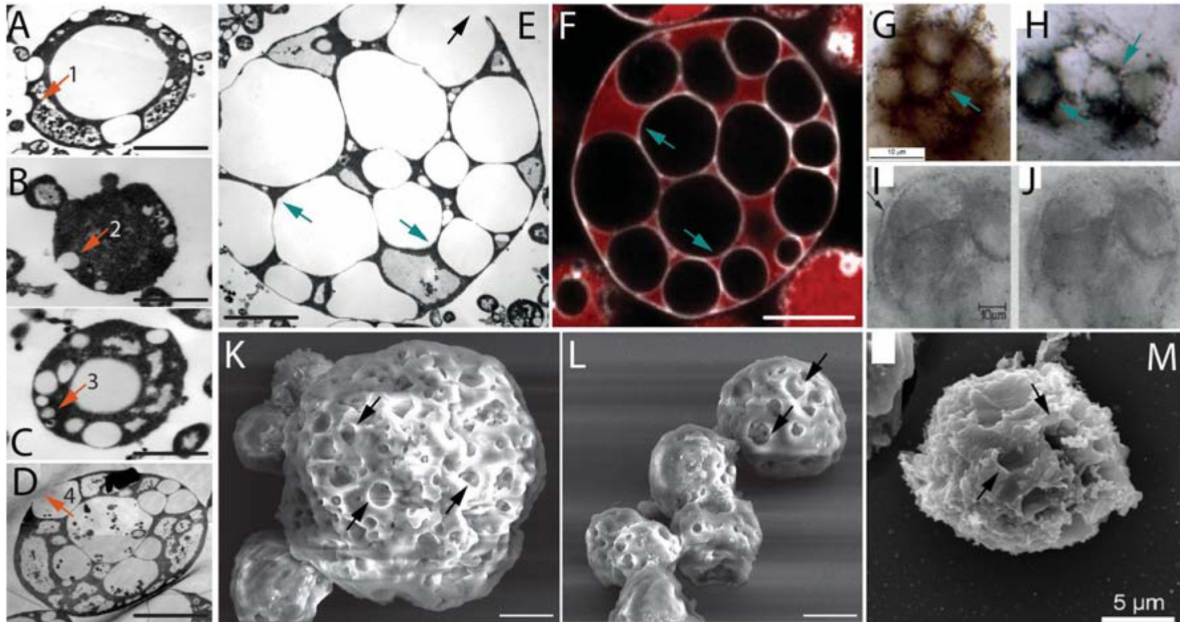

Fig 1.

Morphological comparison of the Apex Chert and the Strelley Pool Formation microfossils with *EM-P*.

Images A-D are TEM images of *EM-P* cells forming intracellular vesicles (ICVs) and intracellular daughter cells. The numbered arrows in these images point to different stages of ICV formation (see Fig. S1). Images E, F, K & L show TEM, SEM, and STED microscope images of *EM-P* cells with ICVs and surface depressions (black arrows). Cells in image F were stained with universal membrane stain, FM^{TM}5-95 (red), and DNA stain, PicoGreen (green). Images G-J & M are spherical microfossils reported from the Apex Chert and the Strelley Pool Formation, respectively (originally published by Schopf et al., 1987 & Delarue *et al.,* 2019)(35,36). Cyan arrows in images E-H point to cytoplasm sandwiched between large hollow vesicles. The arrow in the image I point to the dual membrane enclosing the microfossil. Morphologically similar images of *EM-P* cells are shown in Fig. S3. Black arrows in images K-M point to surface depressions in both *EM-P* and the Strelley Pool Formation microfossils, possibly formed by the rupture of ICV's as shown in D & E (arrows) (also see Fig. S4-S6). Scale bars: A-D (0.5µm) E, K & L (2 µm), and 5 µm (F).

cells, we observed a gradual reduction of the cytoplasmic volume of the parent *EM-P* cells and a corresponding increase in the number of daughter cells within the ICVs (Fig. S11, Movie 2-4). Over time, *EM-P* cells transformed into hollow vesicles with multiple tiny daughter cells (Fig. S11e-h, Movie 3&4). These intracellular daughter cells were released into the surroundings by a two-step process. In the first step, cells underwent lysis to release the ICVs (**Fig. 2**, S12 A & B and Movies. 5-9). In the second step, the vesicle membrane underwent lysis (Fig. S12C-E) to release the daughter cells (Fig. S12F-H). *EM-P* cells undergoing this method of reproduction closely resemble fossilized microbial cells discovered from several Archaean Eon rock formations (**Fig. 2** & S13-S23)(36,41,42). For instance, microfossils reported from Mt. Goldsworthy Formation exhibit cells with ICVs containing daughter cells, cells that underwent lysis to release these ICVs (**Fig. 2** & S13-S17), and the subsequent rupture of the vesicle membrane and release of the daughter cells (Fig. S14D-F)(34).

The microfossils reported from sites like the Farrel Quartzite (Fig. S7)(38), the Strelley Pool Formation (Fig. S18-S20), the Waterfall locality (Fig. S21-S22)(41,44,45), the Turee Creek (Fig. S23)(42), and Dresser Formation (Fig. S24-S28)(10), bear close morphological resemblance with morphologies of *EM-P* cells reproducing by this process. *EM-P* cells exhibited all the distinctive features of the Dresser formation microfossils, like the presence of hollow regions within the cell (Fig.S25) and discontinuous or thick-porous cell walls (Fig. S24 & S27). The step-by-step transformation of *EM-P* cells into these morphologies is shown in Fig. S27. Due to the lysis and release of daughter cells, most late stationary growth phase *EM-P* cells were deflated with numerous surface depressions. The morphology and surface texture of such deflated *EM-P* cells resemble the morphologies of microfossils reported from the Kromberg Formations (Fig. S29) (46).

Reproduction by external daughter cells happens by two different processes. Tiny daughter cells initially appeared as buds attached to the cell membrane (Fig. S30, S31 & Movie 10). These buds subsequently grew in size and detached from the parent cell. Depending on the size of the daughter cells (buds), *EM-P* cells appear to have been reproducing either by budding or binary fission. *EM-P* cells that appear to have been reproducing by budding resemble microfossils reported from the North-pole formations (Fig. S31). As observed in our incubations, microfossils from this site are a mix of individual spherical cells, spherical cells with pustular protuberances, cells with bud-like structures, and cells undergoing binary fission (Fig. S31). Other *EM-P* morphotypes, like individual spherical cells, hourglass-shaped cells undergoing fission, and cells in dyads, bear close morphological resemblance to microfossils reported from both the Swartkopie (Fig. S32) and the Sheba formations (Fig. S33 & S34)(12,33,48). Like the Sheba Formation microfossils, the cells undergoing binary fission were observed to be in close contact with extracellular organic carbon (clasts of organic carbon in the Sheba Formation). This extracellular organic carbon likely represents the intracellular constituents released during the lysis of cells, as described above (Movies 5-8 & Fig. S34).

In some cases, the above-described buds did not detach from the cell surface but transformed into long tentacles (Movies 11&12). These initially hollow tentacles (34) gradually received cytoplasm from the parent cells and gradually transformed into "string-of-spherical daughter cells" (**Fig. 3**, S31A-C & S33)(34). Subsequently, these filaments detached from the parent cell, and due to the constant motion of daughter cells within these filaments, the "string-of-spherical daughter cells" fragmented into smaller and smaller strings and ultimately into multiple individual daughter cells (Movies 13-15). Apart from the cells that received cytoplasm (daughter cells), we also observed hollow spherical structures within these tentacles that did not receive cytoplasm from the parent cells (**Fig. 3F** & **3G**, black arrows).

All *EM-P* morphotypes observed undergoing this reproduction process bear close morphological resemblance to microfossils reported from the Cleaverville Formation (**Fig. 3**). All distinctive features of the Cleaverville microfossils, like the arrangement of cells as pairs within a string (**Fig.**

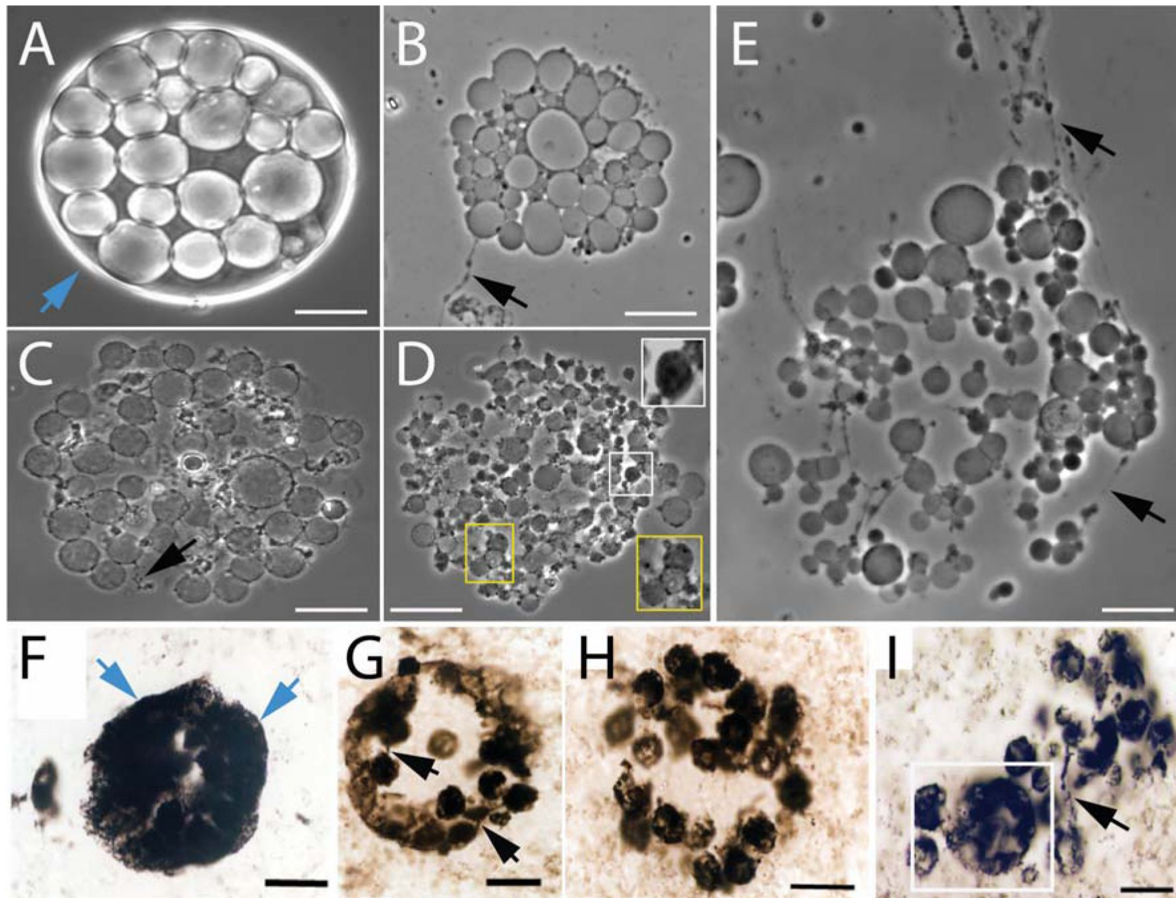

Fig 2.

Morphological comparison between the Mt. Goldsworthy microfossils and *EM-P*.

Images A-E show the process of cell lysis and release of intracellular vesicles in *EM-P*. Image A shows an intact cell with intracellular vesicles. Images B-E show lysis and gradual dispersion of these vesicles. Insert in image D shows enlarged images of individual ICVs. Images F-I show spherical microfossils reported from the Mt. Goldsworthy formation (originally published by Sugitani et al., 2009)(41). The arrow in this image, A & F, points to a cell surrounded by an intact membrane. The black arrow in these images points to filamentous extensions connecting individual vesicles. The boxed region in images D & I highlights a similar discontinuous distribution of organic carbon in ICVs and microfossils. Also see Fig. S13-S17. Scale bars: 20μm (A-E) & 20μm (F-I).

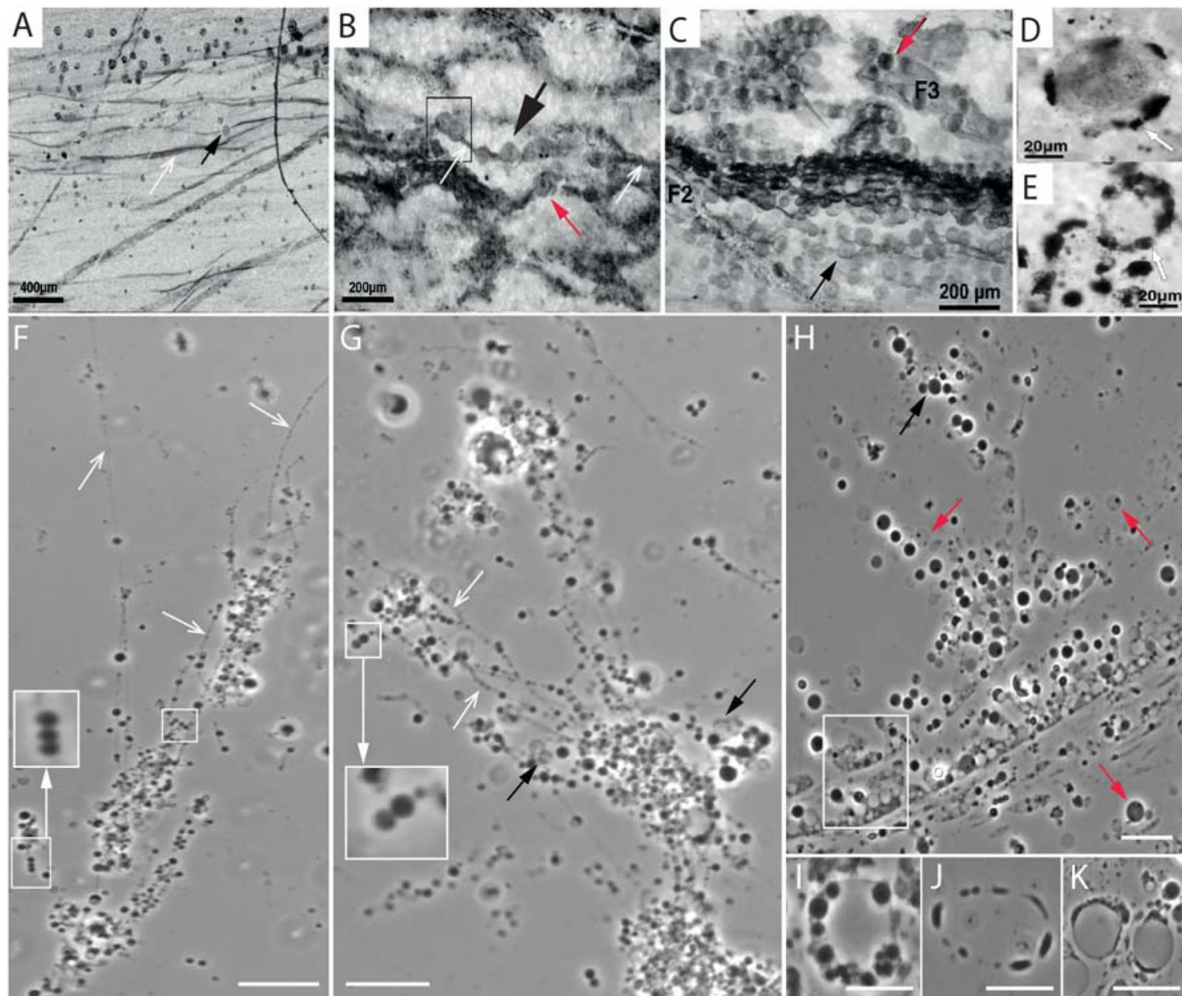

Fig 3.

Morphological comparison of the Cleaverville microfossils with *EM-P*:

Images A-E are the microfossils reported from Cleverville formation (originally reported by Ueno *et al.,* 2006). Images F-K are the *EM-P* cells morphologically analogous to the Cleverville Formation microfossils. Open arrows in images A, B, F & G point to the membrane tethers connecting the spherical cells within the filamentous extensions. Red arrows in the images point to the cells that have a similar distribution of organic carbon within the cells. Boxed and magnified regions in images B, F & G highlight the arrangement of cells in the filaments in pairs. The boxed region in image H highlights the cluster of hollow vesicles in *EM-P* incubations similar to the hollow organic structures in the Cleverville Formation, as shown in image C. Images D, E, and I-J show spherical cells that were largely hollow with organic carbon (cytoplasm) restricted to discontinuous patches at the periphery of the cell. Scale bars: 20µm (F-K).

3H, 3F & 3G), microfossils with a discontinuous layer of organic carbon at the cell periphery (Fig. 3E, 3D & 3I-K), were also observed in *EM-P*. Several hollow spherical structures devoid of organic carbon were reported from the Cleaverville formation (Fig. 3). As in *EM-P*, these structures could have been the hollow membranous structures that didn't receive the cytoplasm from the parent cells. Similar structures were also reported from other microfossil sites, such as the organic structures reported from the Onverwacht Group (Fig. S35)(6).

In addition to the Cleaverville microfossils, *EM-P* cells in our incubations also resemble filamentous structures with spherical inclusions reported from the Sulphur Spring Formations (Fig. S36-S38)(17). Based on the morphological similarities, we propose that these structures could have been the filamentous extensions with spherical daughter cells observed in *EM-P*. Similar but smaller filamentous structures were reported from the Mt. Grant Formation (Fig. S39) (39) and could have been the shorter fragments of similar "strings-of-daughter cells" (Movie 14). The spherical structures with sparsely distributed organic carbon reported from the Sheba Formations could also have been such cells undergoing fragmentation into smaller and smaller filaments (Fig. S33). In tune with this proposition, the Sheba Formation microfossils, like the *EM-P* cells, exhibited an un-uniform distribution of organic carbon within the cells and filamentous overhangs (Fig. S33, Movie 16).

Five to seven days after the start of the experiment, most cells in the incubations were the daughter cells. We observed three distinct types of daughter cells – a string of daughter cells (**Fig. 3**, S39 & Movie 14), daughter cells that were still attached to the membrane debris of the parent cell (**Fig. 4**, **5**, S40-S48 & Movie 17) and individual daughter cells (**Fig. 3h** & Movie 15). All these daughter cell morphotypes resemble – a cluster of tiny spherical globules reported from the SPF (Fig. S18), a string of daughter cells reported from Mt. Grant formation (Fig. S39)(39,41), and spherical daughter cells still attached to membrane debris of parent cell like the ones reported from the Sulphur Spring Formations, Mt.Goldsworthy, the Farrel Quartzite, the Moodies Group, the Dresser Formation, and the SPF (**Fig. 4** & S40-S48)(10,41,43,45,51).

The gradual transformation of *EM-P* cells into lamination-like structures and their comparison with Archaean organic structures

Fossilization and preservation of individual cells is considered unlikely due to the absence of rigid structures. However, recent studies indicate such a process could happen under favorable environmental conditions (52,53). However, the prevailing observations suggest that a significant portion of cell biomass undergoes taphonomic alteration and is preserved as degraded organic matter. To understand the possibility of *EM-P*-like cells forming structures similar to those observed in Archaean rocks, we studied the morphological transformation of individual *EM-P* cells and biofilms over 12 to 30 months. Below, we present the step-by-step transformation of individual cells into organic structures and their morphological resemblance to Archaean organic structures.

EM-P grew in our incubations as a biofilm at the bottom of the culture flask (**Fig. 6** & Fig. S49). The rapid biofilm formation by *EM-P* can be attributed to the presence of extracellular DNA released during the lysis of *EM-P* cells (Fig. S50)(34). DNA released by such processes is known to promote biofilm formation (54). Over time, increased cell numbers resulted in biofilms comprising multiple layers of closely packed individual spherical cells (**Fig. 6** & S50). A subsequent increase in the number of cells led to lateral compression and transformation of spherical cells into a polygonal shape (**Fig. 6**). By the late stationary growth phase, most cells underwent such a transformation, resulting in a honeycomb-like biofilm. The step-by-step transformation of individual spherical cells into these structures is shown in **Fig. 6**. Morphologically similar organic structures were reported from several paleo-Archaean sites, like

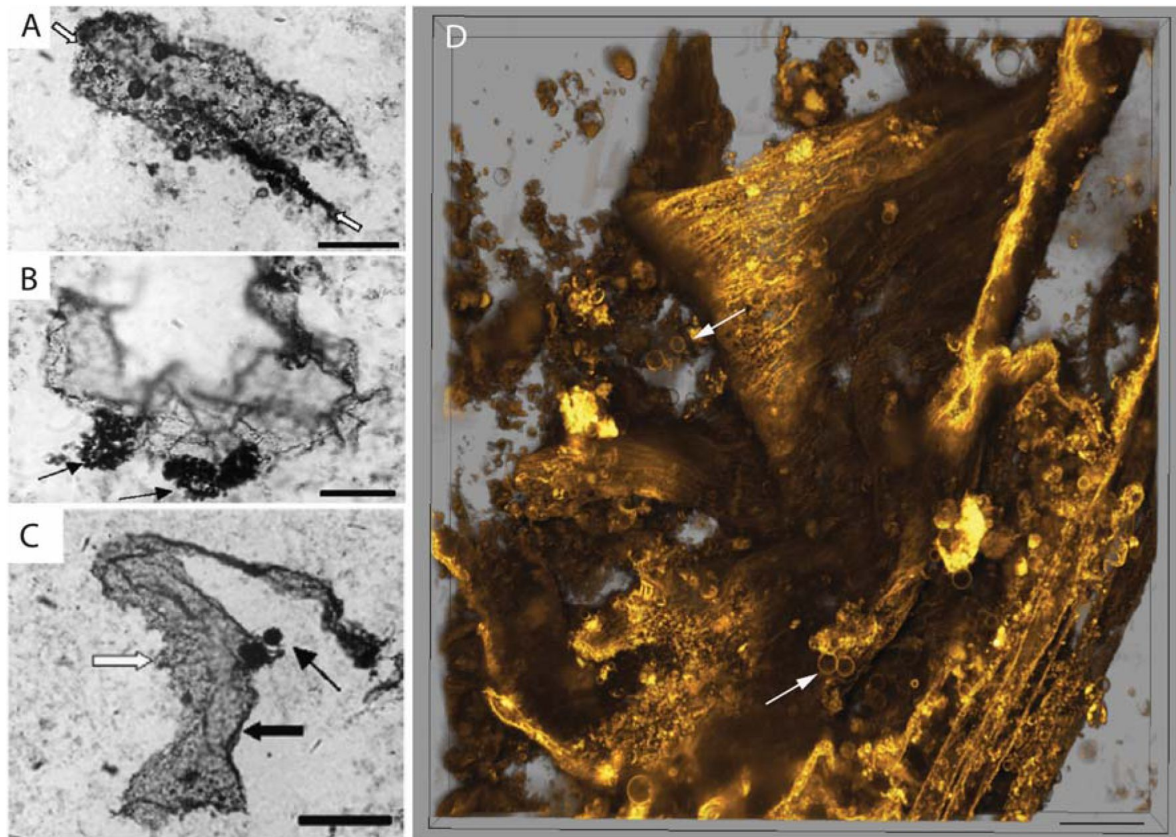

Fig. 4.

Morphological comparison between *EM-P* and the Mt. Goldsworthy microfossils.

Images A, B & C are organic structures reported from the Mt. Goldsworthy Formation (Sugitani *et al.,* 2009)(43). Image D shows morphologically analogous film-like membrane debris observed in *EM-P* incubations. Arrows in images A-D point to either clusters or individual spherical structures attached to these film-like structures. Scale bar: 50µm (A-C) & 10µm (D).

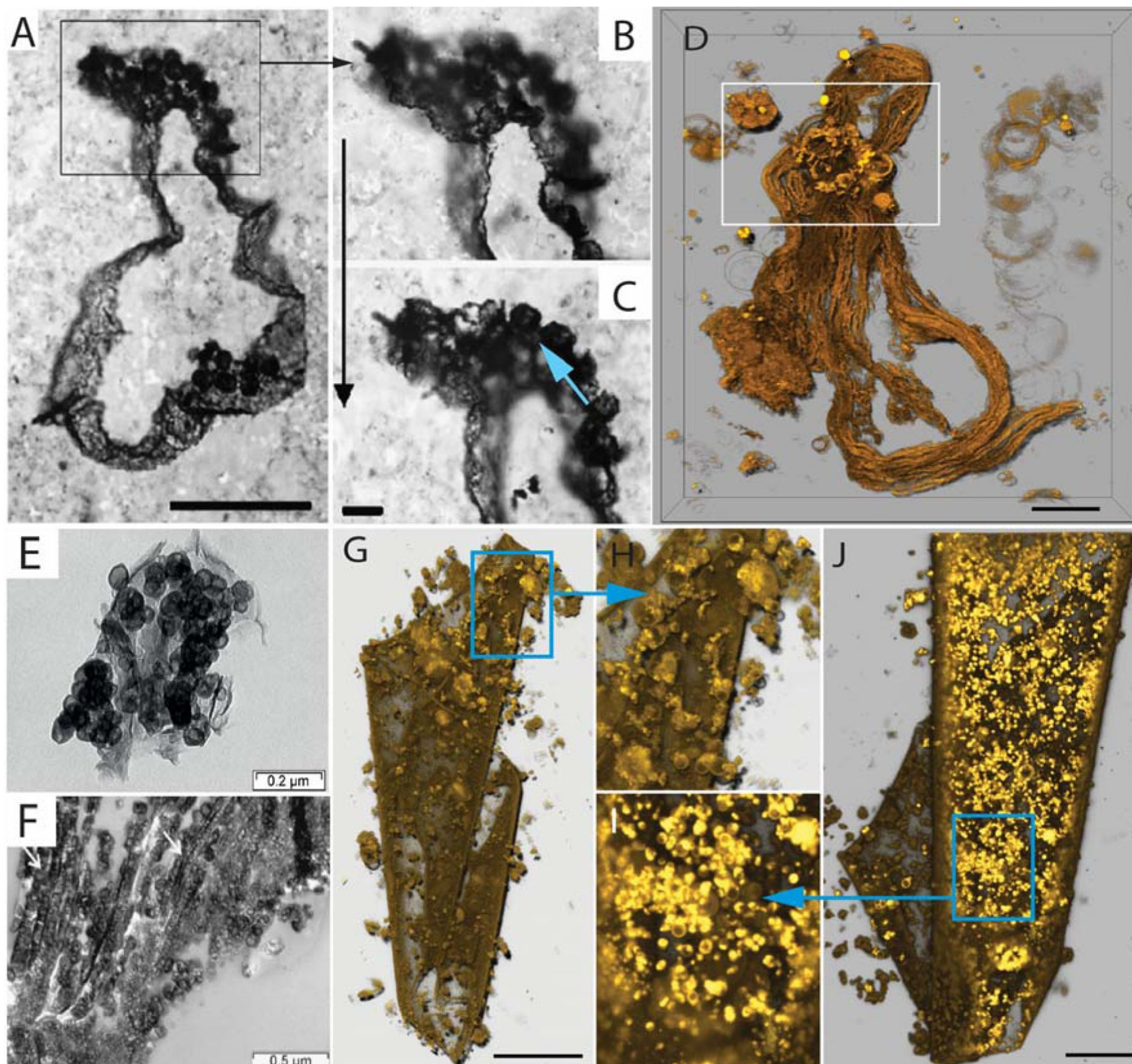

Fig. 5.

Morphological comparison of the Mt. Goldsworthy and the Sulphur Spring microfossils with *EM-P.*

Image A-C are microfossils reported from the Mt. Goldsworthy Formation (39)(Sugitani *et al.,* 2007). Image D is the 3D-rendered STED microscope images of morphologically analogous membrane debris of *EM-P* cell with attached daughter cells (highlighted region) (also see Fig. S40). Images E & F are microfossils reported from the Sulphur Spring site (59)(Duck *et al.,* 2007), showing spherical structures attached to membrane debris. Images G & J are the morphologically analogous structures observed in *EM-P* incubations. Images H & I show the magnified regions of G & J showing spherical *EM-P* daughter cells attached to membrane debris (also see Fig. S40-S47, Movie 17). Cells and membrane debris in these images were stained with the membrane stain FM^{TM}5-95 (yellow). Scale bars: A (50µm), G & J (20µm).

the North Pole Formation (Fig. S51-S62)(47). A similar taphonomic degradation of organic matter associated with a biofilm was demonstrated by previous studies (Westall et al., 2006, Figure 10B)(55).

Large aggregations of spherical cells devoid of internal organic carbon were reported from the North-Pole Formation (47) (Fig S52). These structures closely resemble the aggregations of hollow ICVs released after the lysis of *EM-P* cells (**Fig. 2A**-**2E** & S22). As observed in *EM-P*, the distribution of organic carbon in the North-Pole Formation microfossils is restricted to the periphery of the spherical cells (Fig. S52). Along with the morphological and organizational similarities, *EM-P* also exhibited all the accessory structures associated with the North Pole formation microfossils, such as the large clots of organic carbon (Fig. S51 arrows), filamentous structures originating from the spherical cells, and spherical clots of organic carbon within these filamentous (Fig. S52-S55)(47). Based on the similarities, we propose that the large clots of organic carbon would have been the membrane debris formed during the lysis and the release of intracellular vesicles (Fig. S51, Movie 5-9). As observed in *EM-P*, the filamentous structures associated with the microfossils could also have formed during the release of intracellular vesicles (Movie 5-9). The organic carbon clots within the filamentous structures could have been fossilized daughter cells (Fig. S53 & S54, arrows).

Apart from the aggregations of hollow spherical cells, honeycomb-like structures were also reported from several microfossil sites, like the SPF, the Nuga Formation, the Buck Reef Chert, the Moodies Group, and the Turee Creek formations (**Fig. 6**, S57-S62)(42,56–58). As observed in *EM-P*, these structures could have been formed by the lateral compression of cells or hollow vesicles within the biofilm (**Fig. 6**). In tune with our proposition, Archaean honeycomb-like structures are often closely associated with spherical *EM-P*-like cells (**Fig. 6**).

Spherical microfossils from the Pilbara and Barberton Greenstone Belts were often discovered within layers of organic carbon (48,51,60,61). Over a period of 2-6 months, we observed cells in our incubations gradually being enclosed by membrane debris. These structures were formed by a multi-step process (Fig S63). First, *EM-P* grew as multiple layers of cells within a biofilm (Fig. S63A). Second, the lysis of these cells led to the formation of a considerable amount of membrane debris (Fig. S63B, S64 & Movie 17&18). Subsequently, this membrane debris coalesced to form large fabric-like structures (Fig. S65). These membrane fabrics were then expelled from the biofilm (Fig. S63D, S63E & Movie 18). Over time, these expelled membrane fabrics grew in surface area to form a continuous layer of membrane enclosing a large population of cells (Fig. S65-S69 & Movie 19). This fabric-like membrane debris enclosing biofilms observed in *EM-P* incubations bear close morphological resemblance to microfossils reported from Chinaman Creek in the Pilbara (Fig. S69), and Mt. Goldsworthy Formation (**Fig. 4** & **5**)(39,43,62).

Parallel layers of organic carbon termed laminations were reported from several Archaean microfossil sites (33,51,60,63). Structures similar to these laminations were observed in our incubations. As described above, the reproduction in *EM-P* involves the lysis of cells to facilitate the release of the intracellular daughter cells, resulting in a considerable amount of cell debris (Movie 9 & 17). The parallel layers of organic carbon in our incubations (**Fig. 7** & **8**) are formed by lysis and collapse within individual biofilm layers (Fig. S49). Another way the organic carbon layers could have formed is by the lateral compression of honeycomb-like biofilms (Fig. S71-S74). Sequential steps resulting in the formation of such structures are shown in Fig. S72. Such layers of cell debris closely resemble different types of laminated structures reported from the Barberton Greenstone Belt and Pilbara Iron Formations, like the α, and β laminations (**Fig. 7**, **8** & S69-S75)(51,60,61).

Also similar to the Archaean laminations, we observed layers of cell debris in our incubations have lenticular gaps (**Fig. 8**, S69, S70-S71 & S76-S79). Within these lenticular gaps, we observed intact *EM-P* cells or honeycomb patterns, suggesting that lenticular gaps within otherwise

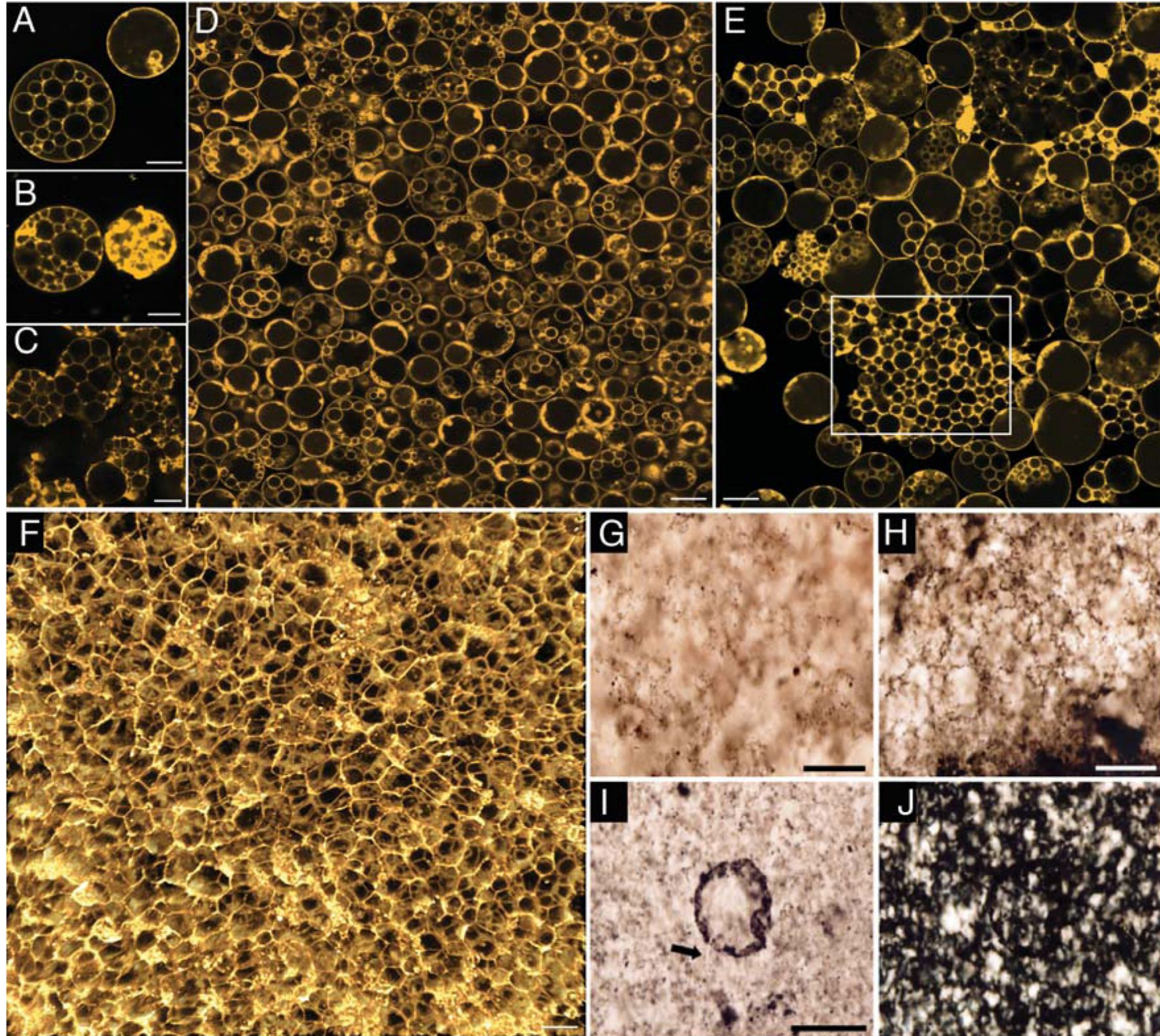

Fig 6

Sequential steps involved in the formation of honeycomb-shaped mats:

Images A-C show single *EM-P* cells that gradually transformed from spherical cells with intracellular vesicles into honeycomb-like structures. Images D-E show a similar transformation of biofilms composed of individual spherical cells into honeycomb-like structures. Cells in these images are stained with membrane stain, FM^{TM}5-95 (red), and imaged using a STED microscope. Images G-J are the microfossils reported from the SPF (originally published by Sugitani et al., 2007)(39). Scale bars: A-F (10μm), G & H (20μm), and I (50μm).

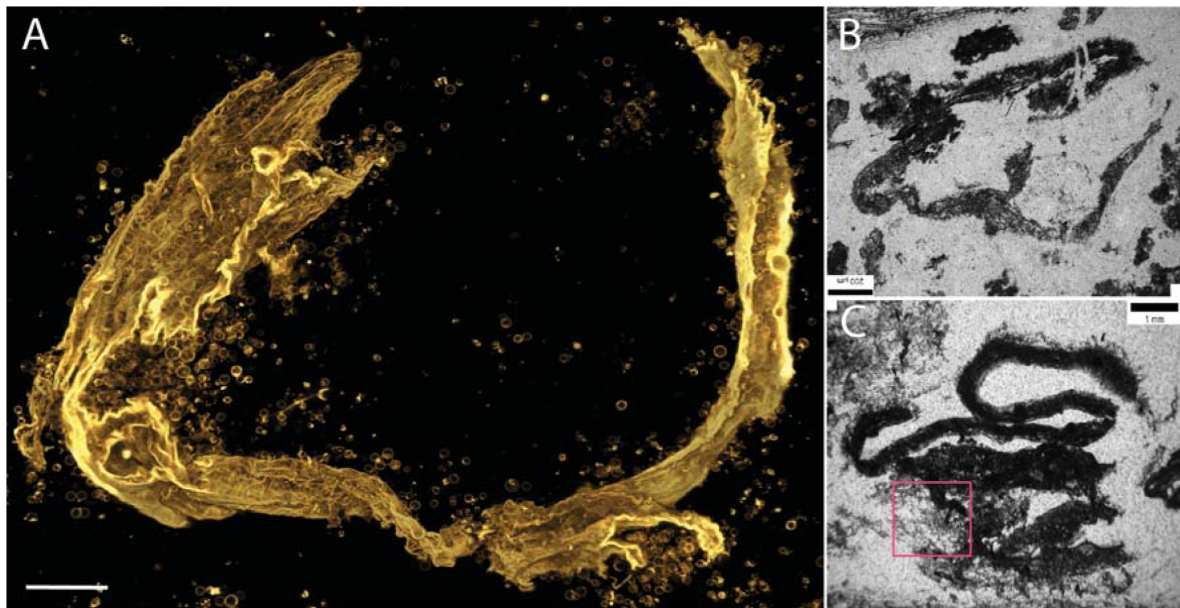

Fig. 7

Morphological comparison of the Buck Reef Chert β-laminations with *EM-P's* membrane debris.

Image A shows a 3D-rendered image of *EM-P's* membrane debris. Cells in the image are stained with membrane stain Nile red and imaged using a STED microscope. Images B & C show β-type laminations reported from Buck Reef Chert (originally published by Tice *et al.,* 2009)(61). The boxed region in image-a highlights the membrane-forming rolled-up structures containing spherical daughter cells, as described in the case of BRC organic structures. Scale bars: 50µm.

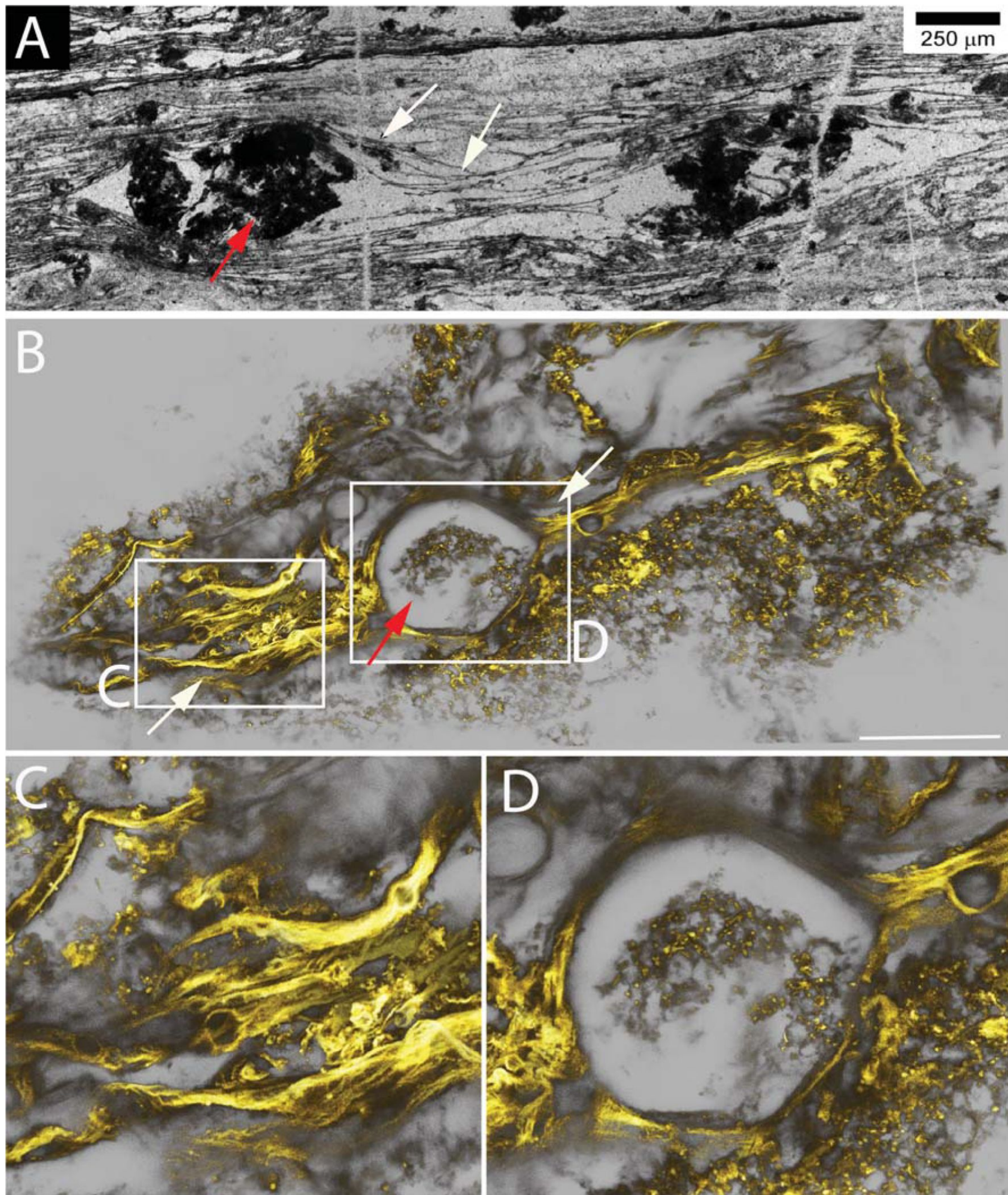

Fig. 8

Morphological comparison between laminated structures reported from the Moodies Group and structures formed by *EM-P.*

Image A shows laminated structures reported from the Moodies Group (originally published by Homann *et al.,* 2015)(63). They show parallel layers of organic carbon with lenticular gaps. Together with the quartz, these lenticular gaps consist of clumps of organic carbon. Image B is a 3D-rendered confocal image of analogous membrane debris formed by *EM-P*. Images C & D are the magnified regions of C. Like Moodies formation, filamentous membrane debris bifurcating to form spherical\lenticular gaps can be seen in several regions (S75, S76, & S77). Some spherical/lenticular gaps were hollow, and some had an organic structure within them, even exhibiting a honeycomb pattern (arrow), suggesting the presence of large spherical *EM-P* cells with intracellular vesicles (D, & S77). Membranes were stained with Nile red, and imaging was done using a STED microscope. The scales: 50µm.

uniformly parallel laminations were formed due to non-uniform lysis or incomplete deflation of cells within individual layers of *EM-P* cells (**Fig. 7**, S75-S79 & Movies 20 & 21). Although in the case of Archaean laminations, these lenticular gaps were thought to have been formed by the entrapment of air bubbles (51), based on our results, we argue that there could have been more than one way such structures could have formed. Other distinctive features of the lamination, like raised mounds or swirls (Fig. S81 - S83)(51,61), were also observed in batch cultures of *EM-P*. Given these morphological similarities, we propose that some of the laminated and other diaphanous filamentous structures could have been formed by the cell debris of the *EM-P*-like cells that inhabited these sites during the Archaean Eon. We will discuss these possibilities in more detail below.

Over a period of 3-12 months, we observed the biofilms solidifying into a solid crust (Fig. S84 & S85). The SEM-EDX characterization of these solidified biofilms showed the presence of potassium and magnesium minerals on the surface, suggesting that these structures were formed by the gradual adsorption of positively charged cations on the negatively charged biofilms (Fig. S84). Most Archaean microfossils are restricted to coastal marine environments. Compared to open oceans, these coastal marine environments harbor higher concentrations of salt due to higher evaporation rates. Hence, these microfossils could have undergone a similar encrustation process as observed in our incubations. Moreover, solidified *EM-P* biofilms resemble the mineral-encrusted structures reported from the Kromberg Formation (Fig. S85)(64). Like the Kromberg Formations structures, solidified *EM-P* biofilms are composed of desiccation cracks and spherical cells beneath the surface (Fig. S85).

When these salt-encrusted cells were transferred into fresh media, we observed a gradual increase in cell numbers (results not shown). However, given that these cells are encrusted in a layer of salts, we observed early growth-phase cells breaking out of the thick salt crust, resulting in stellar morphologies (Fig. S86 & S87). The observed morphologies of these cells closely resemble the morphologies of microfossils reported from the Strelley Pool and other North Pole cherts (Fig. S86 & S87)(41,47). All distinctive features of these microfossils, like the stellar-shaped cells undergoing binary fission and a string of daughter cells extending out of such stellar cells (Movie 22), were also observed in our incubations.

Discussion

Advances in microscopic (FIB-SEM) and analytical (NanoSIMS) techniques over the past few decades have facilitated better imaging and precise determination of chemical and isotopic compositions of microfossils (13,65). Nevertheless, there is considerable disagreement among researchers regarding the interpretation of this information (9). Given the importance of morphology in determining the biogenicity and taxonomic affiliation of the microfossils, reconstructing the lifecycles of Archaean Eon organisms is considered crucial to understanding the nature of these microfossils (43). Our study is the first to reconstruct all known spherical microfossil morphologies and their lifecycles from extant bacteria. Furthermore, we have shown that many of the taphonomic structures observed in our study closely resemble the controversial structures observed in rocks of the Palaeo-Mesoarchaean age (3.6-3.0 Ga) and even in the Neoarchaean (3.0-2.4 Ga). These similarities help us answer long-standing questions regarding the origin and the nature of Archaean microfossils.

The nature of Archaean organic structures is currently being debated among researchers (7,9,10). While some studies suggest that these structures could be remnants of Archaean microorganisms, others suggest that they may have been abiotic minerals that formed due to volcanic activity (10). The argument for this proposition is based on the fact that these organic structures share more similarities with inorganic mineral structures than with extant prokaryotes. To establish the biogenicity of a microfossil, it is essential to either find a convincing

morphological analog among extant bacteria or establish a biogenic process through which they are formed (3). The biogenicity of microfossils reported from several sites like the Swartkoppoie Formation, Kitty's Gap Chert, and the Josephdal Formation is widely accepted among the scientific community due to the discovery of spherical microfossils in different stages of their lifecycle (Fig. S32)(12,55,64). However, such a step-by-step biological process through which Archean Eon organic structures could have formed has never been demonstrated empirically.

The biological origin of microfossils reported from several sites, like the Dresser Formation, to date, remains a matter of debate (10). Several morphological features of these organic structures, like the presence of organic carbon only at the periphery, the absence of internal cell constituents, the presence of pyrite and silicate minerals inside the cells, and the presence of a thick porous or discontinuous cell wall, were all argued as claims for their abiotic origin (10). Justifiably, these morphological features have never been observed in any living organism. Nevertheless, all spherical microfossils reported from the Dresser Formation resemble *EM-P* cells, especially those with a single large ICV (Fig. S24-S27). What was thought to have been a thick, porous cell wall in the microfossils could have been the cytoplasm with tiny ICVs sandwiched between the cell and vesicle membrane (Fig. S24 & S27). Similarly, the hollow cells with a discontinuous cell wall could either have been the ICVs released by cell lysis or the late-growth stage cells with little cytoplasm (Fig. S26). In such cells, the presence of cytoplasm is restricted to discontinuous patches around the periphery of the cells (Fig. S26). The sequence of steps leading to these cell morphologies that resemble the Dresser Formation microfossils is shown in Fig. S27. A closer inspection of the Dresser Formation microfossils shows the ICVs membrane rupture and the daughter cell release (Fig. S28). Morphologies indicating this method of reproduction among microfossils is not unique to the Dresser Formation. Microfossils with similar morphological features were reported from sites like the Strelley Pool, the Waterfall region, and Mt. Goldsworthy Formation (Fig. S3-S14)(3,43). These similarities suggest that microfossil morphologies observed in the Dresser Formation are in tune with other microfossils of similar geological time periods, suggesting their biological origin.

Spherical structures half-coated with pyrite are reported from the Dresser Formation (Fig.S25) (10). These structures could have been the iron-reducing *EM-P*-like cells with hollow ICV constituting half their volume. The selective co-localization of pyrite and carbon could be explained by the Fe(III) reduction happening at the cell surface. The Fe(II) produced from this metabolic reaction could have reacted with environmental sulfide, converted to insoluble pyrite, and precipitated onto the cell surface. Given the absence of this metabolic process within the hollow ICV, these structures remained pyrite-free (Fig.S25). In addition to the Dresser Formation, organic structures coated with pyrite have also been reported from other microfossil sites like the Sulphur Spring Formations (17). The selective presence of pyrite on these microfossils could also be explained by a similar mechanism (Fig. S36-S38). Apart from pyrite, minerals like anatase were reported to have been present within the cells (10). The presence of anatase within these cells could be explained by the transport of these minerals into the cells during the ICV formation (**Fig. 1B&D**). ICVs are formed by a process similar to endocytosis, which involves the intake of salt-rich media and minerals into the cells (Fig. S88). Like the Dresser Formation microfossils, we often observed the presence of salts and minerals within *EM-P*s vesicles (Fig. S88). Moreover, the presence of minerals within cells is not unique to the Dresser Formation microfossils (10) and was reported previously from several bonafide microfossils from Gunflint Iron Formations (66). Additionally, we observed remarkable similarities between the *EM-P* cell debris and all the organic structures closely associated with the microfossil, such as the wavy lamellar and pumice-like structures (Fig. S59, S61 & S89). The step-by-step transformation of cell debris into pumice-like structures is shown in **Fig. 6**. Based on these morphological similarities between the Dresser Formation organic structures and *EM-P*, we hypothesize that these organic structures are the fossil remnants of *EM-P*-like bacteria rather than mineral aggregates.

Morphological similarity between microfossils from far-flung sites like Western Australia and Southern Africa could be explained by the similarity in the environmental conditions in both sites (5). This relationship between cell morphology, reproductive processes, and environmental conditions was discussed extensively in our previous work (34,67). The experimental conditions that we employed in our study are likely similar to the environmental conditions faced by Archaean organisms from both these sites at the time of their fossilization. All sites from which microfossils were reported are shallow intertidal regions. Evidence for periodic evaporation and flooding with sea water was presented from the Barberton and Pilbara Greenstone Belts (6,68), suggesting that the original microorganisms experienced high salinities. The salinities of our experiments are broadly similar to those of Archaean oceans (5-10% w/v)(31). To our knowledge, the exact salt composition of the Archaean Ocean has not been elucidated. Hence, we used a complex mixture of salts (DSS) as a proxy to reproduce these salinities in our experiments. Salts like Mg^{+2}, Ca^{+2}, Na^{+}, and K^{+} or their oxides were also reported to be present and constitute 1-5% by weight in both Pilbara and Barberton greenstone belt microfossil sites (6,68). Moreover, these salts were shown to be closely associated with microfossils (68). The spatial distribution of these salts resembles the spatial distribution pattern of organic carbon, possibly indicating the chelation of these salts to the cell membrane, which is also in agreement with our observations (9). The presence of potassium phyllosilicates and NaCl crystals within the microfossils (68) is also in agreement with our hypothesis that internal structures of the microfossils should have formed by invagination of cell-membrane taking in salt-rich water (Fig. S86). As observed in the microfossils (68), salt crystals on the cell surface, within the membrane invaginations, or cell debris were often observed in *EM-P* (Fig. S89).

The above-presented results suggest that Archaean Eon cells are likely primitive lipid-vesicle-like protocells that lack a cell wall. From a physiological perspective, it would have been unlikely for primitive cells to possess a cell wall given the substantial number of genes required to synthesize individual building blocks, to mediate its assembly, and its constant modification to facilitate cell growth and reproduction (21,69,70). Furthermore, a cell wall could impede the transport of physiologically relevant compounds in and out of the cells. To overcome this limitation, present-day microorganisms (with a cell wall) had to develop extensive molecular biological processes for transporting nutrients and metabolic end products across the cell wall (71,72). This could not have been the case for primitive Archaean life forms. Hence, rather than drawing parallels between the microfossils and life as we know it today, we propose that these microfossils could have been liposome-like protocells, as proposed by the theory of chemical evolution (26). Indeed, it has been recently shown that liposome-like molecules could be produced in some of the hydrothermal settings proposed for the emergence of life (73). To the best of our knowledge, this is the first study to provide a link between theoretical propositions and geological evidence for the existence of protocells on early Earth.

According to the theory of chemical evolution, biological organic compounds are formed by abiotic processes (74). These compounds then self-assembled to form lipid vesicles, which grew in complexity and eventually evolved into self-replicating protocells (75,76). These protocells are believed to have undergone Darwinian evolution, resulting in the emergence of bacteria, archaea, and eukaryotes (77). It was previously thought that the fragility of protocells made it unlikely for them to be preserved in rock formations. However, later studies showed the preservation of cellular features by a rapid encrustation of cells with cationic minerals (52,53). The rapid encrustation and preservation of cells observed in our study (Fig. S84-S85) is in accordance with the proposition that environmental conditions influence the extent of cellular preservation. Our study aligns with the interpretations from these studies that environmental conditions play a pivotal role in determining the extent of cellular preservation.

Conclusion

For the first time, our investigations have been able to reproduce morphologies of most Archaean microfossils from wall-less, extant cells. Apart from reproducing the morphologies, we also presented a step-by-step biological process by which Archaean organic structures could have formed. Based on these results, we propose that Archaean microfossils were likely liposome-like cells, which had evolved mechanisms for energy conservation but not for regulating cell morphology and replication. In an earlier study, we have shown that the morphologies of such primitive cells are determined by environmental conditions (34,67) rather than the information encoded in their genome. Given this lack of intrinsic ability to regulate their morphology, we argue that morphological features such as cell size, shape, or cytological complexity are reliable factors in interpreting either the phylogeny or the physiology of microfossils (at least from Archaean Eon). Rather than attempting to assign present-day taxonomies to these microfossils, we suggest that these microfossils represent primitive protocells proposed by the theory of chemical evolution. To the best of our knowledge, ours is the first study to provide paleontological evidence of the possible existence of protocells on the Palaeoarchaean Earth.

Methods

Isolation of cells and their transformation to protoplasts

Exiguobacterium strain-Molly (*EM*) was isolated from submerged freshwater springs within the Dead Sea (78). The taxonomic identification of the isolate to the genus *Exiguobacterium* was determined by 16S rRNA gene sequencing (79,80). *EM* cells were transformed into protoplasts following a previously documented protocol(28). The resulting *EM-P* cells were cultured in half-strength TSB with 7% Dead Sea Salt (7%DSS-TSB).

Microscopic observation of *EM-P* cells

Morphology *EM-P* was routinely assessed using an Axioskop 2plus microscope (Carl Zeiss, Germany) with a Plan-NEOFLUAR 100X/1.3 objective. Images were captured using a Leica DSF9000 camera (Leica Microsystems, Mannheim, Germany). STED microscopy was performed with an inverted TCS SP8 STED 3X microscope (Leica Microsystems, Mannheim Germany) using an 86x/1.2 NA water immersion objective (Leica HC PL APO CS2 -STED White). Fluorophores were excited with 488, 561nm, 594nm, or 633m laser light derived from an 80 MHz pulsed White Light Laser (Leica Microsystems, Mannheim Germany). For stimulated emission, either a pulsed 775 nm laser or a 592nm CW laser (Leica Microsystems, Mannheim, Germany) was used depending on the fluorophore. Photon counting mode and line accumulation were used for image recording, and Huygens Professional (SVI, Hilversum, The Netherlands) performed image deconvolution on selected images and movies.

Spinning Disk Microscopy was performed using an Olympus SpinSR10 spinning disk confocal microscope (Olympus, Tokyo, Japan) equipped with a 100x/NA1.35 silicone oil immersion objective (Olympus UPLSAPO100XS, Tokyo, Japan), a CSU-W1-Spinning Disk-Unit (Yokogawa, Tokyo, Japan) and ORCALFlash 4.0 V3 Digital CMOS Camera (Hamamatsu, Hamamatsu City, Japan).

Transmission electron microscopy was conducted utilizing a Zeiss EM 912 (Zeiss, Oberkochen, Germany) equipped with an integrated OMEGA filter, operating at 80 kilovolts (kV). Image acquisition was carried out using a 2k x 2k pixel slow-scan CCD camera (TRS, TrÖndle Restlichtverstrkersysteme, Moorenweis, Germany) with ImageSP software (SysProg, Minsk, Belarus).

Acknowledgements

We want to thank Gabriella Berthal for her excellent technical support and Christian Sibert for providing the Dead Sea samples from which *EM* was isolated. We thank the Advanced Light Microscopy Facility at EMBL, Heidelberg, Ulf Schwartz from Leica Microsystems, and colleagues at the departments of Ecological Microbiology (Bayreuth University) and of Cellular and Molecular Biophysics (Max Planck Institute for Biochemistry) for their support throughout the work.

Additional information

Competing interests

The authors declare that there are no conflicts of interest regarding the publication of this article.

Data availability statement

Dr. Dheeraj Kanaparthi will share all data, materials, and methods upon reasonable request.

Funding

This research was funded by the European Research Council (ERC) grant agreement 616644 (POLLOX) and by the Deutsche Forschungsgemeinschaft (DFG) grant agreements DFG-TRR174 and Seed funding from Excellence Cluster ORIGINS EXC2094 – 390783311.

References

1. Shields G. A (2007) **Chapter 7.6 The Marine Carbonate and Chert Isotope Records and Their Implications for Tectonics, Life and Climate on the Early Earth** *Dev. Precambrian Geol.*

2. van Zuilen M. A, Van Kranendonk M. J., Bennett V. C., Hoffmann J. E (2019) **Chapter 38 - The Significance of Carbonaceous Matter to Understanding Life Processes on Early Earth** *Earth's Oldest Rocks* Elsevier :945–963 https://doi.org/10.1016/B978-0-444-63901-1.00038-1

3. Sugitani K., et al. (2015) **Early evolution of large microLorganisms with cytological complexity revealed by microanalyses of 3.4 Ga organicLwalled microfossils** *Geobiology* **13**:507–521

4. Hickman-Lewis K., Westall F. A (2021) **Southern African perspective on the co-evolution of early life and environments** *South Afr. J. Geol* **124**:225–252

5. Oehler D. Z., Walsh M. M., Sugitani K., Liu M.-C., House C. H (2017) **Large and robust lenticular microorganisms on the young Earth** *Precambrian Res* **296**:112–119

6. Walsh M. W (1992) **Microfossils and possible microfossils from the early archean onverwacht group, barberton mountain land, South Africa** *Precambrian Res* **54**:271–293

7. Schopf J. W., Kitajima K., Spicuzza M. J., Kudryavtsev A. B., Valley J. W (2018) **SIMS analyses of the oldest known assemblage of microfossils document their taxon-correlated carbon isotope compositions** *Proc. Natl. Acad. Sci* **115**:53–58

8. Schopf J. W., Kudryavtsev A. B (2012) **Biogenicity of Earth’s earliest fossils: a resolution of the controversy** *Gondwana Res* **22**:761–771

9. Wacey D., Saunders M., Kong C., Brasier A., Brasier M (2016) **3.46 Ga Apex chert ‘microfossils’ reinterpreted as mineral artefacts produced during phyllosilicate exfoliation** *Gondwana Res* **36**:296–313

10. Wacey D., Noffke N., Saunders M., Guagliardo P., Pyle D. M (2018) **Volcanogenic Pseudo-Fossils from the ∼3.48 Ga Dresser Formation, Pilbara, Western Australia** *Astrobiology* https://doi.org/10.1089/ast.2017.1734

11. Gee H (2002) **That’s life?** *Nature* **416**

12. Knoll A. H., Barghoorn E. S (1977) **Archean microfossils showing cell division from the Swaziland System of South Africa** *Science* **198**:396–398

13. Lepot K., et al. (2013) **Texture-specific isotopic compositions in 3.4 Gyr old organic matter support selective preservation in cell-like structures** *Geochim. Cosmochim. Acta* **112**:66–86

14. Adami C., Ofria C., Collier T. C (2000) **Evolution of biological complexity** *Proc. Natl. Acad. Sci* **97**:4463–4468

15. Wolf Y. I., Katsnelson M. I., Koonin E. V (2018) **Physical foundations of biological complexity** *Proc. Natl. Acad. Sci. U. S. A* **115**:E8678–E8687

16. Malaterre C., et al. (2023) **Is There Such a Thing as a Biosignature?** *Astrobiology* **23**:1213–1227

17. Wacey D., et al. (2014) **Geochemistry and nano-structure of a putative~ 3240 million-year-old black smoker biota, Sulphur Springs Group, Western Australia** *Precambrian Res* **249**:1–12

18. Hickman-Lewis K., et al. (2016) **Carbonaceous microstructures from sedimentary laminated chert within the 3.46 Ga Apex Basalt, Chinaman Creek locality, Pilbara, Western Australia** *Precambrian Res* **278**:161–178

19. Schopf J. W (1993) **Microfossils of the Early Archean Apex chert: new evidence of the antiquity of life** *Science* **260**:640–646

20. Wacey D., Saunders M., Kong C (2018) **Remarkably preserved tephra from the 3430 Ma Strelley Pool Formation, Western Australia: Implications for the interpretation of Precambrian microfossils** *Earth Planet. Sci. Lett* **487**:33–43

21. Adams D. W., Errington J (2009) **Bacterial cell division: assembly, maintenance and disassembly of the Z ring** *Nat. Rev. Microbiol* **7**:642–653

22. Westall F., et al. (2018) **A Hydrothermal-Sedimentary Context for the Origin of Life** *Astrobiology* **18**:259–243

23. Szostak J. W. (2017) **The narrow road to the deep past: in search of the chemistry of the origin of life** *Angew. Chem. Int. Ed.* **56**:11037–11043

24. Lane N., Martin W (2010) **The energetics of genome complexity** *Nature* **467**:929–934

25. Lane N (2014) **Bioenergetic constraints on the evolution of complex life** *Cold Spring Harb. Perspect. Biol*

26. Oparin A. I (1969) **Chemistry and the origin of life** *R. Inst. Chem. Rev* **2**:1–12

27. Errington J (2013) **L-form bacteria, cell walls and the origins of life** *Open Biol* **3**

28. Kanaparthi D, Lampe M, Krohn JH, Zhu B, Klingl A, Lueders T (2023) **The reproduction of gram-negative protoplasts and the influence of environmental conditions on this process** *iScience* **26**

29. Kanaparthi D, Lampe M, Krohn JH, Zhu B, Hildebrand F, Boesen T, et al. (2024) **The reproduction process of Gram-positive protocells** *Sci Rep* **14**

30. Knauth L. P (2005) **Temperature and salinity history of the Precambrian ocean: implications for the course of microbial evolution** *Palaeogeogr. Palaeoclimatol. Palaeoecol* **219**:53–69

31. Knauth L. P (1998) **Salinity history of the Earth's early ocean** *Nature* **395**

32. Catling D. C., Zahnle K. J. (2020) **The Archean atmosphere** *Sci. Adv* **6**

33. Hickman-Lewis K., Westall F., Cavalazzi B (2019) **Traces of Early Life From the Barberton Greenstone Belt, South Africa** *Earth's Oldest Rocks* :1029–1058

34. Kanaparthi D, Lampe M, Krohn JH, Zhu B, Hildebrand F, Boesen T, et al. (2024) **The reproduction process of Gram-positive protocells** *Sci Rep* **14**

35. Schopf J. W., Packer B. M (1987) **Early Archean (3.3-billion to 3.5-billion-year-old) microfossils from Warrawoona Group, Australia** *Science* **237**:70–73

36. Delarue F., et al. (2019) **Out of rock: a new look at the morphological and geochemical preservation of microfossils from the 3.46 Gyr-old Strelley Pool Formation** *Precambrian Res* **336**

37. Kapteijn R, Shitut S, Aschmann D, Zhang L, de Beer M, Daviran D, et al. (2022) **Endocytosis-like DNA uptake by cell wall-deficient bacteria** *Nat Commun* **13**

38. Retallack GJ, Krinsley DH, Fischer R, Razink JJ, Langworthy KA (2016) **Archean coastal-plain paleosols and life on land** *Gondwana Res* **40**:1–20

39. Sugitani K., Grey K., Nagaoka T., Mimura K., Walter M. R (2009) **Taxonomy and biogenicity of Archaean spheroidal microfossils (ca. 3.0 Ga) from the Mount Goldsworthy–Mount Grant area in the northeastern Pilbara Craton, Western Australia** *Precambrian Res.* **173**:50–59

40. Schopf JW, Barghoorn ES (1967) **Alga-like fossils from the early Precambrian of South Africa** *Science* **156**:508–12

41. Sugitani K, Mimura K, Nagaoka T, Lepot K, Takeuchi M (2013) **Microfossil assemblage from the 3400 Ma Strelley Pool Formation in the Pilbara Craton, Western Australia: results form a new locality** *Precambrian Res* **226**:59–74

42. Barlow E. V., Van Kranendonk M. J (2018) **Snapshot of an early Paleoproterozoic ecosystem: Two diverse microfossil communities from the Turee Creek Group, Western Australia** *Geobiology* **16**:449–475

43. Sugitani K, Grey K, Nagaoka T, Mimura K, Walter MR (2009) **Taxonomy and biogenicity of Archaean spheroidal microfossils (ca. 3.0 Ga) from the Mount Goldsworthy–Mount Grant area in the northeastern Pilbara Craton, Western Australia** *Precambrian Res* **173**:50–9

44. Sugitani K, Mimura K, Takeuchi M, Yamaguchi T, Suzuki K, Senda R, et al. (2015) **A Paleoarchean coastal hydrothermal field inhabited by diverse microbial communities: the Strelley Pool Formation, Pilbara Craton, Western Australia** *Geobiology* **13**:522–45

45. Wacey D, Kilburn MR, Saunders M, Cliff J, Brasier MD (2011) **Microfossils of sulphur-metabolizing cells in 3.4-billion-year-old rocks of Western Australia** *Nat Geosci* **4**:698–702

46. Kaźmierczak J, Kremer B (2019) **Pattern of cell division in~ 3.4 Ga-old microbes from South Africa** *Precambrian Res* **331**

47. Buick R (1990) **Microfossil Recognition in Archean Rocks: An Appraisal of Spheroids and Filaments from a 3500 M.Y. Old Chert-Barite Unit at North Pole, Western Australia** *Palaios* **5**:441–459

48. Homann M (2019) **Earliest life on Earth: Evidence from the Barberton Greenstone Belt, South Africa** *Earth-Sci Rev* **196**

49. Ueno Y, Isozaki Y, McNamara KJ (2006) **Coccoid-like microstructures in a 3.0 Ga chert from Western Australia** *Int Geol Rev* **48**:78–88

50. Kazmierczak J, Altermann W, Kremer B, Kempe S, Eriksson PG (2009) **Mass occurrence of benthic coccoid cyanobacteria and their role in the production of Neoarchean carbonates of South Africa** *Precambrian Res* **173**:79–92

51. Homann M, Sansjofre P, Van Zuilen M, Heubeck C, Gong J, Killingsworth B, et al. (2018) **Microbial life and biogeochemical cycling on land 3,220 million years ago** *Nat Geosci* **11**:665–671

52. Orange F., et al. (2009) **Experimental silicification of the extremophilic archaea pyrococcus abyssi and methanocaldococcus jannaschii: Applications in the search for evidence of life in early earth and extraterrestrial rocks** *Geobiology* **7**:403–418

53. Orange F., Disnar J. R., Westall F., Prieur D., Baillif P (2011) **Metal cation binding by the hyperthermophilic microorganism, Archaea Methanocaldococcus Jannaschii, and its effects on silicification** *Palaeontology* **54**:593–564

54. Gödeke J, Paul K, Lassak J, Thormann KM (2011) **Phage-induced lysis enhances biofilm formation in Shewanella oneidensis MR-1** *ISME J* **5**:613–636

55. Westall F, De Vries ST, Nijman W, Rouchon V, Orberger B, Pearson V, et al. (2006) **The 3.466 Ga "Kitty's Gap Chert," an early Archean microbial ecosystem** *Spec Pap Geol Soc Am.* **405**

56. Gamper A, Heubeck C, Demske D, Hoehse M. (2012) **Composition and Microfacies of Archean Microbial Mats (Moodies Group, ca. 3.22 Ga, South Africa)** *Microbial Mats in Silicilastic Depositional Systems Through Time* :65–74

57. Schopf JW, Kudryavtsev AB, Osterhout JT, Williford KH, Kitajima K, Valley JW, et al. (2017) **An anaerobic ~3400 Ma shallow-water microbial consortium: Presumptive evidence of Earth's Paleoarchean anoxic atmosphere** *Precambrian Res* **299**:309–318

58. Tice MM, Lowe DR (2006) **The origin of carbonaceous matter in pre-3.0 Ga greenstone terrains: A review and new evidence from the 3.42 Ga Buck Reef Chert** *Earth-Sci Rev* **76**:259–300

59. Duck LJ, Glikson M, Golding SD, Webb RE (2007) **Microbial remains and other carbonaceous forms from the 3.24 Ga Sulphur Springs black smoker deposit, Western Australia** *Precambrian Res* **154**:205–20

60. Tice M. M., Lowe D. R (2004) **Photosynthetic microbial mats in the 3,416-Myr-old ocean** *Nature* **431**:549–552

61. Tice MM (2009) **Environmental controls on photosynthetic microbial mat distribution and morphogenesis on a 3.42 Ga clastic-starved platform** *Astrobiology* **9**:989–1000

62. Brasier MD, Green OR, Lindsay JF, McLoughlin N, Steele A, Stoakes C (2005) **Critical testing of Earth's oldest putative fossil assemblage from the ~3.5 Ga Apex chert, Chinaman Creek, Western Australia** *Precambrian Res* **141**:55–102

63. Homann M., Heubeck C., Airo A., Tice M. M (2015) **Morphological adaptations of 3.22 Ga-old tufted microbial mats to Archean coastal habitats (Moodies Group, Barberton Greenstone Belt, South Africa)** *Precambrian Res* **266**:47–64

64. Westall F, de Wit MJ, Dann J, van der Gaast S, de Ronde CEJ, Gerneke D (2001) **Early Archean fossil bacteria and biofilms in hydrothermally-influenced sediments from the Barberton greenstone belt, South Africa** *Precambrian Res* **106**:93–116

65. Brasier M. D., Antcliffe J., Saunders M., Wacey D (2015) **Changing the picture of Earth's earliest fossils (3.5–1.9 Ga) with new approaches and new discoveries** *Proc. Natl. Acad. Sci* **112**:4859–4864

66. Lepot K., et al. (2017) **Iron minerals within specific microfossil morphospecies of the 1.88 Ga Gunflint Formation** *Nat. Commun* **8**

67. Kanaparthi D, Lampe M, Zhu B, Klingl A, Lueders T, Schwille P (2021) **On the reproductive mechanism of Gram-negative protocells** *bioRxiv*

68. Alleon J, Bernard S, Le Guillou C, Beyssac O, Sugitani K, Robert F (2018) **Chemical nature of the 3.4 Ga Strelley Pool microfossils** *Geochem Perspect Lett* **7**:37–42

69. Vollmer W., Joris B., Charlier P., Foster S (2008) **Bacterial peptidoglycan (murein) hydrolases** *FEMS Microbiol. Rev* **32**:259–286

70. Egan A. J. F., Cleverley R. M., Peters K., Lewis R. J., Vollmer W (2017) **Regulation of bacterial cell wall growth** *Febs J* **284**:851–867

71. Dijkstra A. J., Keck W (1996) **Peptidoglycan as a barrier to transenvelope transport** *J. Bacteriol* **178**:5555–5562

72. Prajapati J. D., Kleinekathöfer U., Winterhalter M (2021) **How to Enter a Bacterium: Bacterial Porins and the Permeation of Antibiotics** *Chem. Rev* **121**:5158–5192

73. Purvis G., et al. (2024) **Generation of long-chain fatty acids by hydrogen-driven bicarbonate reduction in ancient alkaline hydrothermal vents** *Commun. Earth Environ* **5**

74. Powner M. W., Gerland B., Sutherland J. D (2009) **Synthesis of activated pyrimidine ribonucleotides in prebiotically plausible conditions** *Nature* **459**:239–242

75. Zhu T. F., Szostak J. W (2009) **Coupled growth and division of model protocell membranes** *J. Am. Chem. Soc* **131**:5705–5713

76. Schwille P (2019) **Division in synthetic cells** *Emerg. Top. Life Sci* **52**:3307–3325

77. Woese C. R., Kandler O., Wheelis M. L (1990) **Towards a natural system of organisms: Proposal for the domains Archaea, Bacteria, and Eucarya** *Proc. Natl. Acad. Sci. U. S. A* **87**:4576–4579

78. Häusler S., et al. (2014) **Microenvironments of reduced salinity harbour biofilms in Dead Sea underwater springs** *Environ. Microbiol. Rep* **6**:152–158

79. Kanaparthi D., Reim A., Martinson G. O., Pommerenke B., Conrad R (2017) **Methane emission from feather moss stands** *Glob. Change Biol* **23**:4884–4895

80. Kanaparthi D., Conrad R (2015) **Role of humic substances in promoting autotrophic growth in nitrate-dependent iron-oxidizing bacteria** *Syst. Appl. Microbiol* **38**:184–188

Author information

Dheeraj Kanaparthi
Max-Planck Institute for Biochemistry, Munich, Germany, Chair of Ecological Microbiology, BayCeer, University of Bayreuth, Bayreuth Germany, Earth and environmental sciences, Ludwig Maximilian University, Munich, Germany
ORCID iD: 0000-0003-1009-4103

For correspondence: kanaparthi@biochem.mpg.de

Frances Westall
CNRS-Centre de Biophysique Moléculaire, Orléans, France

Marko Lampe
Advanced Light Microscopy Facility, European Molecular Biology Laboratory, Heidelberg, Germany

Baoli Zhu
Chair of Ecological Microbiology, BayCeer, University of Bayreuth, Bayreuth Germany, Key Laboratory of Agro-Ecological Processes in Subtropical Regions, Taoyuan Agroecosystem Research Station, Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha, China

Thomas Boesen
Department of Biosciences, Center for Electromicrobiology, Aarhus, Denmark

Bettina Scheu
Earth and environmental sciences, Ludwig Maximilian University, Munich, Germany

Andreas Klingl
Department of Botany I, Ludwig Maximilian University, Munich, Germany

Petra Schwille
Max-Planck Institute for Biochemistry, Munich, Germany

Tillmann Lueders
Chair of Ecological Microbiology, BayCeer, University of Bayreuth, Bayreuth Germany

For correspondence: Tillmann.Lueders@uni-bayreuth.de

Editors

Reviewing Editor
George Perry
Pennsylvania State University, University Park, United States of America

Senior Editor
George Perry
Pennsylvania State University, University Park, United States of America

Joint Public Review:

Summary:

Microfossils from the Paleoarchean Eon represent the oldest evidence of life, but their nature has been strongly debated among scientists. To resolve this, the authors reconstructed the lifecycles of Archaean organisms by transforming a Gram-positive bacterium into a primitive lipid vesicle-like state and simulating early Earth conditions. They successfully replicated all morphologies and life cycles of Archaean microfossils and studied cell degradation processes over several years, finding that encrustation with minerals like salt preserved these cells as fossilized organic carbon. Their findings suggest that microfossils from 3.8 to 2.5 billion years ago were likely liposome-like protocells with energy conservation pathways but without regulated morphology.

Strengths:

The authors have crafted a compelling narrative about the morphological similarities between microfossils from various sites and proliferating wall-deficient bacterial cells, providing detailed comparisons that have never been demonstrated in this detail before. The extensive number of supporting figures is impressive, highlighting numerous similarities. While conclusively proving that these microfossils are proliferating protocells morphologically akin to those studied here is challenging, we applaud this effort as the first detailed comparison between microfossils and morphologically primitive cells.

Summary of reviewer comments on this revision:

Each of the original reviewers evaluated the revised manuscript and were complimentary about how the authors addressed their original concerns. One reviewer added: "It is a thought-provoking manuscript that will be well received." We encourage readers of this version of the paper to consider the original reviewer comments and the authors' responses: https://elifesciences.org/reviewed-preprints/98637/reviews

https://doi.org/10.7554/eLife.98637.2.sa1

Author response:

The following is the authors’ response to the original reviews.

> ***eLife Assessment***
>
> *This provocative manuscript from presents valuable comparisons of the morphologies of Archaean bacterial microfossils to those of microbes transformed under environmental conditions that mimic those present on Earth during the same Eon, although the evidence in support of the conclusions is currently incomplete. The reasons include that taphonomy is not presently considered, and a greater diversity of experimental environmental conditions is not evaluated -- which is important because we ultimately do not know much about Earth's early environments. The authors may want to reframe their conclusions to reflect this work as a first step towards an interpretation of some microfossils as 'proto-cells,' and less so as providing strong support for this hypothesis.*

Regarding the taphonomic alterations: The editor and reviewers are correct in pointing out this issue. Taphonomic alteration of the microfossils attains special significance in the case of microorganisms, as they lack rigid structures and are prone to morphological alterations during or after their fossilization. We are acutely aware of this issue and have conducted long-term experiments (lasting two years) to observe how cells die, decay, and get preserved.

A large section of the manuscript (pages 11 to 20) and a substantial portion of the supplementary information is dedicated to understanding the taphonomic alterations. To the best of our knowledge, these are among the longest experiments done to understand the taphonomic alterations of the cells within laboratory conditions.

Recent reports by Orange et al. (1,2) showed that under favorable environmental conditions, cells could be fossilized rather rapidly with little morphological modifications. We observed a similar phenomenon in this work. Cells in our study underwent rapid encrustation with cations from the growth media. We have analyzed the morphological changes over a period of 18 months. After 18 months, the softer biofilms got encrusted entirely in salt and turned solid (Fig.). Despite this transformation, morphologically intact cells could still be observed within these structures. This suggests that the cells inhabiting Archaean coastal marine environments could undergo rather rapid encrustation, and their morphological features could be preserved in the geological record with little taphonomic alteration.

Regarding the environmental conditions: We are in total agreement with the reviewers that much is unknown about Archaean geology and its environmental conditions. Like the present-day Earth, Archaean Earth certainly had regions that greatly differed in their environmental conditions—volcanic freshwater ponds, brines, mildly halophilic coastal marine environments, and geothermal and hydrothermal vents, to name a few. Our experimental design focuses on one environment we have a relatively good understanding of rather than the rest of the planet, of which we know little. Below, we list our reasons for restricting to coastal marine environments and studying cells under mildly halophilic experimental conditions.

(1) Very little continental crust from Haden and early Archaean Eon exists on the presentday Earth. Much of our geochemical understanding of this time period was a result of studying the Pilbara Iron Formations and the Barberton Greenstone Belt. Geological investigations suggest that these sites were coastal marine environments. The salinity of coastal marine environments is higher than that of open oceans due to the greater water evaporation within these environments. Moreover, brines were discovered within pillow basalts within the Barberton greenstone belt, suggesting that the salinity within these sites is higher or similar to marine environments.

(2) We are not certain about the environmental conditions that could have supported the origin of life. However, all currently known Archaean microfossils were reported from coastal marine environments (3.8-2.4Ga). This suggests that proto-life likely flourished in mildly halophilic environments, similar to the experimental conditions employed in our study.

(3) The chemical analysis of Archaean microfossils also suggests that they lived in saltrich environments, as most, if not all, microfossils are closely associated, often encrusted in a thin layer of salt.

However, we concur with the reviewers that our interpretations should be reassessed if Archaean microfossils that greatly differ from the currently known microfossils are to be discovered or if new microfossils are to be reported from environments other than coastal marine sites.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *Microfossils from the Paleoarchean Eon represent the oldest evidence of life, but their nature has been strongly debated among scientists. To resolve this, the authors*

> *reconstructed the lifecycles of Archaean organisms by transforming a Gram-positive bacterium into a primitive lipid vesicle-like state and simulating early Earth conditions. They successfully replicated all morphologies and life cycles of Archaean microfossils and studied cell degradation processes over several years, finding that encrustation with minerals like salt preserved these cells as fossilized organic carbon. Their findings suggest that microfossils from 3.8 to 2.5 billion years ago were likely liposome-like protocells with energy conservation pathways but without regulated morphology.*
>
> *Strengths:*
>
> *The authors have crafted a compelling narrative about the morphological similarities between microfossils from various sites and proliferating wall-deficient bacterial cells, providing detailed comparisons that have never been demonstrated in this detail before. The extensive number of supporting figures is impressive, highlighting numerous similarities. While conclusively proving that these microfossils are proliferating protocells morphologically akin to those studied here is challenging, we applaud this effort as the first detailed comparison between microfossils and morphologically primitive cells.*
>
> *Weaknesses:*
>
> *Although the species used in this study closely resembles the fossils morphologically, it would be beneficial to provide a clearer explanation for its selection. The literature indicates that many bacteria, if not all, can be rendered cell wall-deficient, making the rationale for choosing this specific species somewhat unclear. While this manuscript includes clear morphological comparisons, we believe the authors do not adequately address the limitations of using modern bacterial species in their study. All contemporary bacteria have undergone extensive evolutionary changes, developing complex and intertwined genetic pathways unlike those of early life forms. Consequently, comparing existing bacteria with fossilized life forms is largely hypothetical, a point that should be more thoroughly emphasized in the discussion.*
>
> *Another weak aspect of the study is the absence of any quantitative data. While we understand that obtaining such data for microfossils may be challenging, it would be helpful to present the frequencies of different proliferative events observed in the bacterium used. Additionally, reflecting on the chemical factors in early life that might cause these distinct proliferation modes would provide valuable context.*

Regarding our choice of using modern organisms or this particular bacterial species:

Based on current scientific knowledge, it is logical to infer that cellular life originated as protocells; nevertheless, there has been no direct geological evidence for the existence of such cells on early Earth. Hence, protocells remain an entirely theoretical concept. Moreover, protocells are considered to have been far more primitive than present-day cells. Surprisingly, this lack of sophistication was the biggest challenge in understanding protocells. Designing experiments in which cells are primitive (but not as primitive as non-living lipid vesicles) and still retain a functional resemblance to a living cell does pose some practical challenges. Laboratory experiments with substitute (proxy) protocells almost always come with some limitations. Although not a perfect proxy, we believe protocells and protoplasts share certain characteristics. Having said that, we would like to reemphasize that protoplasts are not protocells. Our reasons for using protoplasts as model organisms and working with this bacterial species (Exiguobacterium Strain-Molly) are based on several scientific and practical criteria listed below.

(1) Irrespective of cell physiology and intracellular complexity, we believe that protoplasts and protocells share certain similarities in the biophysical properties of their cytoplasm. We explained our reasoning in the manuscript introduction and in our previous manuscripts

(Kanaparthi et al., 2024 & Kanaparthi et al., 2023). In short, to be classified as a cell, even a protocell should possess minimal biosynthetic pathways, a physiological mechanism of harvesting free energy from the surrounding (energy-yielding pathways), and a means of replicating its genetic material and transferring it to the daughter cells. These minimal physiological processes could incorporate considerable cytoplasmic complexity. Hence, the biophysical properties of the protocell cytoplasm could have resembled those of the cytoplasm of protoplasts, irrespective of the genomic complexity.

(2) Irrespective of their physiology, protoplasts exhibit several key similarities to protocells, such as their inherent inability to regulate their morphology or reproduction. This similarity was pointed out in previous studies (3). Despite possessing all the necessary genetic information, protoplasts undergo reproduction through simple physiochemical processes independent of canonical molecular biological processes. This method of reproduction is considered to have been erratic and rather primitive, akin to the theoretical propositions on protocells. Although protoplasts are fully evolved cells with considerable physiological complexity, the above-mentioned biophysical similarities suggest that the protoplast life cycle could morphologically resemble that of protocells (in no other aspect except for their morphology and reproduction).

(3) Physiologically or genomically different species of Gram-positive protoplasts are shown to exhibit similar morphologies. This suggests that when Gram-positive bacteria lose their cell wall and turn into a protoplast, they reproduce in a similar manner independent of physiological or genome-based differences. As morphology and only morphology is key to our study, at least from the scope of this study, intracellular complexity is not a key consideration.

(4) This specific strain was isolated from submerged freshwater springs in the Dead Sea. This isolate and members of this bacterial genus are known to have been well acclimatized to growing in a wide range of salt concentrations and in different salt species. This is important for our study (this and previous manuscript), in which cells must be grown not only at high salt concentrations (1-15%) but in different salts like NaCl, $MgCl_2$, and KCl.

(5) Our initial interest in this isolate was due to its ability to reduce iron at high salt concentrations. Given that most spherical microfossils are found in Archaean-banded iron formations covered in pyrite, this suggests that these microfossils could have been reducing oxidized iron species like Fe(III). Nevertheless, over the course of our study, we realized the complexities of live cell staining and imaging under anoxic conditions. Given that the scope of the manuscript is restricted only to comparing the morphologies, not the physiology, we abandoned the idea of growing cells under anoxic conditions.

Based on these observations, cell physiology may not be a key consideration, at least within the scope of studying microfossil morphology. However, we want to emphasize again that “We do not claim present-day protoplasts are protocells.”

Regarding the absence of quantitative data:

We are unsure what the reviewer meant by the absence of quantitative data. Is it from the cell size/reproductive pathways perspective or from a microfossil/ecological perspective? At the risk of being portrayed in a bad light, we admit that we did not present quantitative data from either of these perspectives. In our defense, this was not due to our lack of effort but due to the practical limitations imposed by our model organism.

If the reviewer means the quantitative data regarding cell sizes and morphology: In our previous work, we studied the relationship between protoplast morphology, growth rate, and environmental conditions. In that study, we proposed that the growth rate is one factor that regulates protoplast morphology. Nevertheless, we did not observe uniformity in the sizes of the cells. This lack of uniformity was not just between the replicates but even among the cells grown within the same culture flask or the cells within the same microscopic field. Moreover,

cells are often observed to be reproducing either by forming internal or external or by both these processes at the same time. The size and morphological differences among cells within a growth stage could be explained by the physiological and growth rate heterogenicity among cells.

Bacterial growth curves and their partition into different stages (lag, log & stationary), in general, represent the growth dynamics of an entire bacterial population. Nevertheless, averaging the data obscures the behavior of individual cells (4,5). It is known that genetically identical cells within a single bacterial population could exhibit considerable cell-to-cell variation in gene expression (6,7) and growth rates (8). The reason for such stochastic behavior among monoclonal cells has not been well understood. In the case of normal cells, morphological manifestation of these variations is restricted by a rigid cell wall. Given the absence of a cell wall in protoplasts, we assume such cell-to-cell variations in growth rate is manifested in cell morphology. This makes it challenging to quantitatively determine variations in cell sizes or the size increase in a statically robust manner, even in monoclonal cells.

Although this lack of uniformity in cell sizes should not be perceived as a limitation, this behavior is consistently observed among microfossils. Spherical microfossils of similar morphology but different sizes were reported from different microfossil sites (9,10). In this regard, both protoplasts and microfossils are very similar.

If the reviewer means the quantitative data from an ecological perspective:

Based on the elemental composition and the isotopic signatures of the organic carbon, we can deduce if these structures are of biological origin or not. However, any further interpretation of this data to annotate these microfossils to a particular physiology group is fraught with errors. Hence, we refrain from making any inferences about the physiology and ecological function of these microfossils. This lack of clarity on the physiology of microfossils reduces the chance of quantitative studies on their ecological functions. Moreover, we would like to re-emphasize that the scope of this work is restricted to morphological comparison and is not targeted at understanding the ecological function of these microfossils. This narrow objective also limits the nature of the quantitative data we could present.

Moreover, developing a quantitative understanding of some phenomena could be technically challenging. Many theories on the origin of life, like chemical evolution, started with the qualitative observation that lightning could mediate the synthesis of biologically relevant organic carbon. Our quantitative understanding of this process is still being explored and debated even to this day.

> ***Reviewer #2 (Public Review):***
>
> *Summary:*
>
> *In summary, the manuscript describes life-cycle-related morphologies of primitive vesiclelike states (Em-P) produced in the laboratory from the Gram-positive bacterium Exiguobacterium Strain-Molly) under assumed Archean environmental conditions. Em-P morphologies (life cycles) are controlled by the "native environment". In order to mimic Archean environmental conditions, soy broth supplemented with Dead Sea salt was used to cultivate Em-Ps. The manuscript compares Archean microfossils and biofilms from selected photos with those laboratory morphologies. The photos derive from publications on various stratigraphic sections of Paleo- to Neoarchean ages. Based on the similarity of morphologies of microfossils and Em-Ps, the manuscript concludes that all Archean microfossils are in fact not prokaryotes, but merely "sacks of cytoplasm".*
>
> *Strengths:*

The approach of the authors to recognize the possibility that "real" cells were not around in the Archean time is appealing. The manuscript reflects the very hard work by the authors composing the Em-Ps used for comparison and selecting the appropriate photo material of fossils.

Weaknesses:

While the basic idea is very interesting, the manuscript includes flaws and falls short in presenting supportive data. The manuscript makes too simplistic assumptions on the "Archean paleoenvironment". First, like in our modern world, the environmental conditions during the Archean time were not globally the same. Second, we do not know much about the Archean paleoenvironment due to the immense lack of rock records. More so, the Archean stratigraphic sections from where the fossil material derived record different paleoenvironments: shelf to tidal flat and lacustrine settings, so differences must have been significant. Finally, the Archean spanned 2.500 billion years and it is unlikely that environmental conditions remained the same. Diurnal or seasonal variations are not considered. Sediment types are not considered. Due to these reasons, the laboratory model of an Archean paleoenvironment and the life therein is too simplistic. Another aspect is that eucaryote cells are described from Archean rocks, so it seems unlikely that prokaryotes were not around at the same time. Considering other fossil evidence preserved in Archean rocks except for microfossils, the many early Archean microbialites that show baffling and trapping cannot be explained without the presence of "real cells". With respect to lithology: chert is a rock predominantly composed of silica, not salt. The formation of Em-Ps in the "salty" laboratory set-up seems therefore not a good fit to evaluate chert fossils. Formation of structures in sediment is one step. The second step is their preservation. However, the second aspect of taphonomy is largely excluded in the manuscript, and the role of fossilization (lithification) of Em-Ps is not discussed. This is important because Archean rock successions are known for their tectonic and hydrothermal overprint, as well as recrystallization over time. Some of the comparisons of laboratory morphologies with fossil microfossils and biofilms are incorrect because scales differ by magnitudes. In general, one has to recognize that prokaryote cell morphologies do not offer many variations. It is possible to arrive at the morphologies described in various ways including abiotic ones.

Regarding the simplistic presumptions on the Archaean Eon environmental conditions, we provided a detailed explanation of this issue in our response to the eLife evaluation. In short, we agree with the reviewer that little is known about the Archaean Eon environmental conditions at a planetary scale. Hence, we restricted our study to one particular environment of which we had a comparatively good understanding. The Archaean Eon spanned 2.5 billion years. However, most of the microfossil sites we discussed in the manuscript are older than 3 billion years, with one exception (2.4 billion years old Turee Creek microfossils). We presume that conditions within this niche (coastal marine) environment could not have changed greatly until 2Ga, after which there have been major changes in the ocean salt composition and salinities.

In the manuscript, we discussed extensively the reasons for restricting our study to these particular environmental conditions. Further explanations of these choices are presented in our response to the eLife evaluation (also see our previous manuscript). In short, the fact that all known microfossils are restricted to coastal marine environments justifies the experimental conditions employed in our study. Nevertheless, we agree with the reviewer that all lab-based studies involve some extent of simplification. This gap/mismatch is even wider when it comes to studies involving origin or early life on Earth.

We are not arguing that prokaryotes are not around at this time. The key message of the manuscript is that they are present, but they have not developed intracellular mechanisms to regulate their morphology and remained primitive in this aspect.

The sizes of the microfossils and cells from our study were similar in most cases. However, we agree with the reviewer that they deviated considerably in some cases, for example, S70, S73, and S83. These size variations are limited to sedimentary structures like laminations rather than cells. These differences should be expected as we try to replicate the real-life morphologies of biofilms that could have extended over large swats of natural environments in a 2ml volume chamber slide. More specifically, in Fig. S70, there is a considerable size mismatch. But, in Fig. S73, the sizes were comparable between A & C (of course, the size of our reproduction did not match B). In the case of Fig. S83, we do not see a huge size mismatch.

> ***Reviewer #1 (Recommendations For The Authors):***
>
> *We would like to provide several suggestions for changes in text and additions to data analysis.*
>
> *39-41: It has been stated that reconstructing the lifecycle is the only way of understanding the nature of these microfossils. First of all, I would rephrase this to 'the most promising way', as there are always multiple approaches to comparing phenomena.*

We agree with the reviewer's suggestion. The suggested changes have been made (line 41).

> *125: Please rephrase "under the environmental condition of early Earth" to "under experimental conditions possibly resembling the conditions of the Paleoarchean Eon". Now it sounds like the exact environmental conditions have been produced, which has already been debated in the discussion.*

We agree with the reviewer's suggestion. The suggested changes have been made (line 127).

> *125: Please mention the fold change in size, the original size in numbers, and whether this change is statistically significant.*

In the above sections of this document, we explained our reservations about presenting the exact number.

> *128: Have you found a difference in the relative percentages of modes of reproduction? In other words, is there a difference in percentage between forming internal daughter cells or a string of external daughter cells?*

We explained our reservations about presenting the exact number above. But this has been extensively discussed in our accompaining manuscript. We want to reemphasize that the scope of this manuscript is restricted to comparing morphologies rather than providing a mechanistic explanation of the reproduction process.

> *151: A similar model for endocytosis has already been described in proliferating wall-less cells (Kapteijn et al., 2023). In the discussion, please compare your results with the observations made in that paper.*

This is an oversight on our part. The manuscript suggested by the reviewer has now been added (line 154 & 155).

> *163: Please use another word for uncanny. We suggest using 'strong resemblance'.*

We changed this according to the reviewers' suggestion (line 168).

> *433: Please elaborate on why the results are not shown. This sounds like a statement that should be substantiated further.*

To observe growth and simultaneously image the cells, we conducted these experiments in chamber slides (2ml volume). Over time, we observed cells growing and breaking out of the salt crust (Fig. S86, S87 & Movie 22) and a gradual increase in the turbidity of the media. Although not quantitative, this is a qualitative indication of growth. We did not take precise measurements for several reasons. This sample is precious; it took us almost two years to solidify the biofilm completely, as shown in Fig. S84A. Hence, it was in limited supply, which prevented us from inoculating these salt crusts into large volumes of fresh media. Given a long period of starvation, these cells often exhibited a long lag phase (several days), and there wasn't enough volume to do OD measurements over time.

We also crushed the solidified biofilm with a sterile spatula before transferring it into the chamber slide with growth media. This resulted in debris in the form of small solid particles, which interfered with our OD measurements. These practical considerations made it challenging to determine the growth precisely. Despite these challenges, we measured an OD of 4 in some chamber slides after two weeks of incubation. Given that these measurements were done haphazardly, we chose not to present this data.

> *456: Could you please double-check whether the description is correct for the figure? 8C and 8D are part of Figure 8B, but this is stated otherwise in the description.*

We thank the reviewer for pointing it out. It has now been rectified (line 461-472).

> ***Reviewer #2 (Recommendations For The Authors):***

We thank Reviewer #2 for carefully reading the manuscript and such an elaborate list of questions. The revisions suggested have definitely improved the quality of the manuscript. Here, we would like to address some of the questions that came up repeatedly below. One frequently asked question is regarding the letters denoting the individual figures within the images. For comparison purposes, we often reproduced previously published images. To maintain a consistent figure style, we often have to block the previous denotations with an opaque square and give a new letter.

The second question that appeared repeatedly below is the missing scale bars in some of the images within a figure. We often did not include a scale bar in the images when this image is an enlarged section of another image within the same figure.

> *Title: Please consider being more precise in the title. Microfossils are only one fossil group of "oldest life". Perhaps better: "On the nature of some microfossils in Archean rocks". (see also Line 37).*

Authors’ response: The title conveys a broader message without quantitative insinuations. If our manuscript had been titled "On the nature of all known Archaean microfossils,” we should have agreed with the reviewer's suggestion and changed it to "On the nature of some microfossils in Archean rocks". As it is not, we respectfully decline to make this modification.

> *Abstract:*

> *Line 41: "one way", not "the only way"*

We agree with the reviewer's comment, and necessary changes have been made (line 41).

> *Introduction:*
>
> *Line 58f: "oldest sedimentary rock successions", not "oldest known rock formations". There are rocks of much older ages, but those are not well preserved due to metamorphic overprint, or the rocks are igneous to begin with. Minor issue: please note that "formations" are used as stratigraphic units, not so much to describe a rock succession in the field.*

We agree with the reviewer's comment and have made necessary changes (line 58).

> *Line 67: Microfossils are widely accepted as evidence of life. Please rephrase.*

We agree with the reviewer's comment, and necessary changes have been made.

> *Line 71 - 74: perhaps add a sentence of information here.*

We agree with the reviewer's comment, and necessary changes have been made (line 71).

> *Line 76: which "chemical and mineralogical considerations"?*

This has been rephrased to "Apart from the chemical and δ^{13}C-biomass composition" (line 76).

> *Line 84ff: This is a somewhat sweeping statement. Please remember that there are microbialites in such rocks that require already a high level of biofilm organization. The existence of cyanobacteria-type microbes in the Archean is also increasingly considered.*

We are aware of literature that labeled the clusters of Archaean microfossils as biofilms and layered structures as microbialites or stromatolite-like structures. However, the use of these terms is increasingly being discouraged. A more recent consensus among researchers suggests annotating these structures simply as sedimentary structures, as microbially induced sedimentary structures (MISS).

We respectfully disagree with the reviewer's comment that Archaean microfossils exhibit a high level of biofilm organization. We are not aware of any studies that have conducted such comprehensive research on the architecture of Archaean biofilms. We are not even certain if these clusters of Archaean cells could even be labeled as biofilms in the true sense of the term. We presently lack an exact definition of a biofilm. In our study, we do see sedimentation and bacteria and their encapsulation in cell debris. From a broader perspective, any such aggregation of cells enclosed in cell debris could be annotated as a biofilm. However, more in-depth studies show that biofilm is not a random but a highly organized structure. Different bacterial species have different biofilm architectures and chemical composition. The multispecies biofilms in natural environments are even more complex. We do agree with the reviewer that these structures could broadly be labeled as biofilms, but we presently lack a good, if any, understanding of the Archaean biofilm architecture.

Regarding the annotation of microfossils as cyanobacteria, we respectfully disagree with the reviewer. This is not a new concept. Many of the Archaean microfossils were annotated as cyanobacteria at the time of their discovery. This annotation is not without controversy. With

the advent of genome-based studies, researchers are increasingly moving away from this school of thought.

> *Line 101ff: The conditions on early Earth are unknown - there are many varying opinions. Perhaps simply state that this laboratory model simulates an Archean Earth environment of these conditions outlined.*

This is a good idea. We thank the reviewer for this suggestion, and we made appropriate changes.

> *Line 112: manuscript to be replaced by "paper"?*

This change has been made (line 114).

> *Line 116: "spanned years" - how many years?*

We now added the number of years in the brackets (line 118).

> *Results:*
>
> *Line 125: see comment for 101ff.*

we made appropriate changes.

> *Figure 1: Caption: Please write out ICV the first time this abbreviation is used. Images: Note that some lettering appears to not fit their white labels underneath. (G, H, I, J0, and M).*

We apologize; this is an oversight on our part. We now spell complete expansion of ICV, the first time we used this abbreviation.

We took these images from previously published work (references in the figure legend), so we must block out the previous figure captions. This is necessary to maintain a uniform style throughout the manuscript.

> *Line 152ff.: here would be a great opportunity to show in a graph the size variations of modern ICVs and to compare the variations with those in the fossil material.*

In the above sections of this document, we explained our reservations about presenting the exact number.

> *Line 159f.: Fig.1K - what is to see here? Maybe a close-up or - better - a small sketch would help?*

Fig. 1K shows the surface depressions formed during the vesicle formation. The surface characteristics of EM-P and microfossils is very similar.

> *Line 161f.: reference?*

The paragraph spanning lines 159 to 172 discusses the morphological similarities between EM-P and SPF microfossils. We rechecked the reference no 35 (Delarue 2019). This is the correct reference. We do not see a mistake if the reviewer meant the reference to the figures.

> *Line 164ff.: A question may be asked, how many fossils of the Strelley Pool population would look similar to the "modeled" ones. Questions may rise in which way the*

> *environmental conditions control such morphology variations. Perhaps more details?*

This relationship between the environmental conditions and the morphology is discussed extensively in our previous work (11).

> *Line 193: what is meant by "similar discontinuous distribution of organic carbon"?*

This statement highlights similarities between EM-P and microfossils. The distribution of cytoplasm within the cells is not uniform. There are regions with and devoid of cytoplasm, which is quite unusual for bacteria. Some previous studies argued that this could indicate that these organic structures are of abiotic origin. Here, we show that EMP-like cells could exhibit such a patchy distribution of cytoplasm within the cell.

> *Line 218 - 291: The observations are very nice, however, the figures of fossil material in Figures 3 A, B, and C appear not to conform. Perhaps use D, E and I to K. Also, S48 does not show features as described here (see below).*

We did not completely understand the reviewer's question. As mentioned in the figure legend, both the microfossils and the cells exhibit string with spherical daughter cells within them. Moreover, there are also other similarities like the presence of hollow spherical structures devoid of organic carbon. We also saw several mistakes in the Fig. S48 legend. We have rectified them, and we thank the reviewer for pointing them out.

> *Line 293f: Title with "." at end?*

This change has been made.

> *Line 298: predominantly in chert. In clastic material preservation of cells and pores is unlikely due to the common lack of in situ entombment by silica.*

We rephrased this entire paragraph to better convey our message. Either way, we are not arguing that hollow pore spaces exist. As the reviewer mentioned, they will, of course, be filled up with silica. In this entire paragraph, we did not refer to hollow spaces. So, we are not entirely sure what the question was.

> *Line 324, 328-349: Please see below comments on the supplementary figures 51-62. Some of the interpretations of morphologies may be incorrect.*

Please find our response to the reviewer's comments on individual figures below.

> *Figure 5 A to D look interesting, however E to J appear to be unconvincing. What is the grey frame in D (not the white insert).*

The grey color is just the background that was added during the 3D rendering process.

> *Figure 6 does not appear to be convincing. - Erase?*

We did not understand the reviewer's reservations regarding this figure. Images A-F within the figure show the gradual transformation of cells into honeycomb-like structures, and images G-J show such structures from the Archaean that are closely associated with microfossils. Moreover, we did not come up with this terminology (honeycomb-like). Previous manuscripts proposed it.

> *Line 379ff: S66 and 69, please see my comments below. Microfossils "were often discovered" in layers of organic carbon.*

Please see our response below.

> *Line 393-403: Laminae? There are many ways to arrive at C-rich laminae, especially, if the material was compressed during burial. Basically, any type of biofilm would appear as laminae, if compressed. The appearance of thin layers is a mere coincidence. Note that the scale difference in S70, S73, as well as S83, is way too high (cm versus μm!) to allow any such sweeping conclusions. What are α- and β- laminations, the one described by Tice et al.? The arguments are not convincing.*

We propose that cells be compressed to form laminae. We answered this question above about the differences in the scale bars. Yes, we are referring to α- and β- laminations described by Tice et al.

> *Figure 7: This is an interesting figure, but what are the arguments for B and C, the fossil material, being a membrane? Debris cannot be distinguished with certainty at this scale in the insert of C. B could also be a shriveled-up set of trichomes.*

We agree with the reviewer that debris cannot be definitely differentiated. Traditionally, annotations given to microfossil structures such as biofilm, intact cells, or laminations were all based on morphological similarities with existing structures observed in microorganisms. Given that the structures observed in our study are very similar to the microfossil structures, it is logical to make such inferences. Scales in A & B match perfectly well. The structure in C is much larger, but, as we mentioned in reply to one of the reviewer's earlier questions, some of the structures from natural environments could not be reproduced at scale in lab experiments. Working in a 2 ml chamber slides does impose some restrictions.

> *Figure 8: The figure does not show any honeycomb patterns. The "gaps" in the Moodies laminae are known as lenticular particles in biofilms. They form by desiccated and shriveledup biofilm that mineralizes in situ. Sometimes also entrapped gases induce precipitation. Note also that the modelled material shows a kind of skin around the blobs that are not present in the Moodies material.*

We agree that entrapped gas bubbles could have formed lenticular gaps. In the manuscript, we did not discount this possibility. However, if that is the case, one should explain why we often find clumps of organic carbon within these gaps. As we presented a step-by-step transformation of parallel layers of cells into laminations, which also had similar lenticular gaps, we believe this is a more plausible way such structures could have formed. In the end, there could have been more than one way such structures could have been formed.

We do see the honeycomb pattern in the hollow gaps. Often, the 3D-rendering of the STED images obscures some details. Hence, in the figure legend, we referred to the supplementary figures also show the sequence of steps involved in the formation of such a pattern.

> *Line 405-417: During deposition of clastic sediment any hollow space would be compressed during burial and settling. It is rare that additional pore space (except between the graingrain-contacts) remains visible, especially after consolidation. The exception would be if very early silicification took place filling in any pore space. What about EPS being replaced by mineralic substance? The arguments are not convincing.*

We are suggesting that EPS or cell debris is rapidly encrusted by cations from the surrounding environment and gets solidified into rigid structures. This makes it possible for the structures to be preserved in the fossil record. We believe that hollow structures like the lenticular gaps will be filled up with silica.

We do not agree with the reviewer's comment that all biological structures will be compressed. If this is true, there should be no intact microfossils in the Archaean sedimentary structures, which is definitely not the case.

> *Line 419-430: Lithification takes place within the sediment and therefore is commonly controlled by the chemistry of pore water and chemical compounds that derive from the dissolution of minerals close by. Another aspect to consider is whether "desiccation cracks" on that small scale may be artefacts related to sample preparation (?).*

We agree that desiccation cracks could have formed during the sample preparation for SEM imaging, as this involves drying the biofilms. However, we observed that the sample we used for SEM is a completely solidified biofilm (Fig. S84), so we expect little change in its morphology during drying. Moreover, visible cracks and pointy edges were also observed in wet samples, as shown in Fig. S87.

> *Line 432 - 439: Please see comments on the supplementary material below.*

Please find our response to the reviewer's comments on individual figures below.

> *Discussion:*
>
> *Line 477f: "all known microfossil morphologies" - is this a correct statement? Also, would the Archean world provide only one kind of "EM-P type"? Morphologies of prokaryote cells (spherical, rod-shaped, filamentous) in general are very simple, and any researcher of Precambrian material will appreciate the difficulties in concluding on taxonomy. There are papers that investigate putative microfossils in chert as features related to life cycles. Microfossil-papers commonly appear not to be controversial give and take some specific cases.*

We made a mistake in using the term "all known microfossil morphologies." We have now changed it to "all known spherical microfossils" from this statement (line 483). However, we do not agree with the statement that microfossil manuscripts tend not to be controversial. Assigning taxonomy to microfossils is anything but controversial. This has been intensely debated among the scientific community.

> *Line 494-496: This statement should be in the Introduction.*

We agree with the reviewer's comment. In an earlier version of the manuscript this statement was in the introduction. To put this statement in its proper context, it needs to be associated with a discussion about the importance of morphology in the identification of microfossils. The present version of the manuscript do not permit moving an entire paragraph into the introduction. Hence, we think making this statement in the discussion section is appropriate.

> *Line 484ff. The discussion on biogenicity of microfossils is long-standing (e.g., biogenicity criteria by Buick 1990 and other papers), and nothing new. In paleontology, modern prokaryotes may serve as models but everyone working on Archean microfossils will agree that these cannot correspond to modern groups. An example is fossil "cyanobacteria" that is thought to have been around already in the early Archean. While*

morphologically very similar to modern cyanobacteria, their genetic information certainly differed - how much will perhaps remain undisclosed by material of that high age.

Yes, we agree with the reviewer that there has been a longstanding conflict on the topic of biogenicity of microfossils. However, we have never come across manuscripts suggesting that modern microorganisms should only be used as models. If at all, there have been numerous manuscripts suggesting that these microfossils represent cyanobacteria, streptomycetes, and methanotrophs. Regarding the annotation of microfossils as cyanobacteria, we addressed this issue in one of the previous questions raised by the reviewer.

Line 498ff: Can the variation of morphology and sizes of the EM-Ps be demonstrated statistically? Line 505ff are very speculative statements. Relabeling of what could be vesicles as "microfossils" appears inappropriate. Contrary to what is stated in the manuscript, the morphologies of the Dresser Formation vesicles do not resemble the S3 to S14 spheroids from the Strelley Pool, the Waterfall, and Mt Goldsworthy sites listed in the manuscript. The spindle-shaped vesicles in Wacey et al are not addressed by this manuscript. What roles in mineral and element composition would have played diagenetic alteration and the extreme hydrothermal overprint and weathering typical for Dresser material? S59, S60 do not show what is stated, and the material derives from the Barberton Greenstone Belt, not the Pilbara.

Please see the comments below regarding the supplementary images.

We did not observe huge variations in the cell morphology. Morphologies, in most cases, were restricted to spherical cells with intracellular vesicles or filamentous extensions. Regarding the sizes of the cells, we see some variations. However, we are reluctant to provide exact numbers. We have presented our reasons above.

We respectfully disagree with the reviewer's comments. We see quite some similarities between Dresser formation microfossils and our cells. Not just the similarities, we have provided step-by-step transformation of cells that resulted in these morphologies. We fail to see what exactly is the speculation here. The argument that they should be classified as abiotic structures is based on the opinion that cells do form such structures. We clearly show here that they can, and these biological structures resemble Dresser formation microfossils more closely than the abiotic structures.

Regarding the figures S3-S14. We think they are morphologically very similar. Often, it's not just comparing both images or making exact reproductions (which is not possible). We should focus on reproducing the distinctive morphological features of these microfossils.

We agree with the reviewer that we did not reproduce all the structures reported by Wacey's original manuscript, such as spherical structures. We are currently preparing another manuscript to address the filamentous microfossils. These spindle-like structures will be addressed in this subsequent work.

We agree with the reviewer, we often have difficulties differentiating between cells and vesicles. This is not a problem in the early stages of growth. During the log phase, a significant volume of the cell consists of the cytoplasm, with hollow vesicles constituting only a minor volume (Fig. 1B or S1A). During the later growth stages (Fig. 1E7F or S11), cells were almost hollow, with numerous daughter cells within them. These cells often resemble hollow vesicles rather than cells. However, given these are biologically formed structures, and one could argue that these vesicles are still alive as there is still a minimal amount of cytoplasm (Fig. S27). Hence, we should consider them as cells until they break apart to release daughter cells.

Regarding Figures S59 and S60, we did not claim either of these microfossils is from Pilbara Iron Formations. The legend of Figure S59 clearly states that these structures are from Buck Reef Chert, originally reported by Tice et al., 2006 (Figure 16 in the original manuscript). The legend of Figure S60 says these structures were originally reported by Barlow et al., 2018, from the Turee Creek Formation.

> *Line 546f and 552: The sites including microfossils in the Archean represent different paleoenvironments ranging from marine to terrestrial to lacustrine. References 6 and 66 are well-developed studies focusing on specific stratigraphic successions, but cannot include information covering other Archean worlds of the over 2.5 Ga years Archean time.*

All the Archaean microfossils reported to date are from volcanic coastal marine environments. We are aware that there are rocky terrestrial environments, but no microfossils have been reported from these sites. We are unaware of any Archaean microfossils reported from freshwater environments.

> *Line 570ff: The statements may represent a hypothesis, but the data presented are too preliminary to substantiate the assumptions.*

We believe this is a correct inference from an evolutionary, genomic, and now from a morphological perspective.

> *Figures:*
>
> *Please check all text and supplementary figures, whether scale bars are of different styles within the figure (minor quibble).*
>
> *S3 (no scale in C, D); S4, S5: Note that scale bars are of different styles.*

We believe we addressed this issue above.

> *S6 D: depressions here are well visible - perhaps exchange with a photo in the main text? Note that scale bars are of different styles.*

We agree that depressions are well visible in E. The same image of EM-P cell in E is also present in Fig. 1D in the main text.

> *S7: Scale bars should all be of the same style, if anyhow possible. Scale in D?*

We believe we addressed this issue above.

> *S9: F appears to be distorted. Is the fossil like this? The figure would need additional indicators (arrows) pointing toward what the reader needs to see - not clear in this version. More explanation in the figure caption could be offered.*

We rechecked the figure from the original publication to check if by mistake the figure was distorted during the assembly of this image. We can assure you that this is not the case. We are not sure what further could be said in the figure legend.

> *S13: What is shown in the inserts of D and E that is also visible in A and B? Here a sketch of the steps would help.*

We did not understand the question.

> *S14: Scale in A, B?*

We believe we addressed this issue above.

> *S15: Scales in A, E, C, D*

We believe we addressed this issue above.

> *S16: scales in D, E, G, H, I, J?*

We believe we addressed this issue above.

> *S17: "I" appears squeezed, is that so? If morphology is an important message, perhaps reduce the entire figure so it fits the layout. Note that labels A, B, C, and D are displaced.*

As shown in several subsequent figures, the hollow spherical vesicles are compressed first into honeycomb-like structures, and they often undergo further compression to form lamination-like structures. Such images often give the impression that the entire figure is squashed, but this is not the case. If one examines the figure closely, you could see perfectly spherical vesicles together with laterally sqeezed structures. Regarding the figure labels, we addressed this issue above.

> *S18: The filamentous feature in C could also be the grain boundaries of the crystals. Can this be excluded as an interpretation? Are there microfossils with the cell membranes? That would be an excellent contribution to this figure. Note that scale bars are of different styles.*

If this is a one-off observation, we could have arrived at the reviewer's opinion. But spherical cells in a “string of beads” configuration were frequently reported from several sites, to be discounted as mere interpretation.

> *S19: The morphologies in A - insert appear to be similar to E - insert in the lower left corner. The chain of cells in A may look similar to the morphologies in E - insert upper right of the image. B - what is to see here? D - the inclusions do not appear spherical (?). Does C look similar to the cluster with the arrow in the lower part of image E? Note that scale bars are of different styles (minor quibble). A, B, C, and D appear compressed. Perhaps reduce the size of the overall image?*

The structures highlighted (yellow box) in C are similar to the highlighted regions in E—the agglomeration of hollow vesicles. It is hard to get understand this similarity in one figure. The similarities are apparent when one sees the Movie 4 and Fig. S12, clearly showing the spherical daughter cells within the hollow vesicle. We now added the movie reference to the figure legend.

> *S20: A appears not to contribute much. The lineations in B appear to be diagenetic. However, C is suitable. Perhaps use only C, D, E?*

We believe too many unrecognizable structures are being labeled as diagenetic. Nevertheless, we do not subscribe to the notion that these are too lenient interpretations. These interpretations are justified as such structures have not been reported from live cells. This is the first study to report that cells could form such structures. As we now reproduced these structures, an alternate interpretation that these are organic structures derived from microfossils should be entertained.

> *S 21: Note that scale bars are of different styles.*

We believe we addressed this issue above.

> *S22: Perhaps add an arrow in F, where the cell opened, and add "see arrow" in the caption? Is this the same situation as shown in C (white arrow)? What is shown by the white arrow in A? Note that scale bars are of different styles.*

We did the necessary changes.

> *S23: In the caption and main text, please replace "&" with "and" (please check also the other figure captions, e.g. S24). Note that scale bars are of different styles. What is shown in F? A, D - what is shown here?*

We replaced “&” with “and.”

> *S24: Note that scale bars are of different styles. Note that Wacey et al. describe the vesicles as abiotic not as "microfossils"; please correct in figure caption [same also S26; 25; 28].*

We are aware of Prof. Dr. Wacey’s interpretations. We discuss it at length in the discussion section our manuscript. Based on the similarities between the Dresser formation structures and structures formed by EM-P, we contest that these are abiotic structures.

> *S25: Appears compressed; note different scale bars.*

We believe we addressed this issue above.

> *S28: The label in B is still in the upper right corner; scale in D? What is to see in rectangles (blue and red) in A, B? In fossil material, this could be anything.*

These figures are taken from a previous manuscript cited in the figure legend. We could not erase or modify these figures.

> *S33: "L"ewis; G appears a bit too diffuse - erase? Note that scale bars are of different styles.*

We believe we addressed this issue above.

> *S34: This figure appears unconvincing. Erase?*

There are considerable similarities between the microfossils and structures formed by EM-P. If the reviewer expands a bit on what he finds unconvincing, we can address his reservations.

> *S35: It would be more convincing to show only the morphological similarities between the cell clusters. B and C are too blurry to distinguish much. Scales in D to F and in sketches? A appears compressed (?).*

We rechecked the original manuscript to see if image A was distorted while making this figure, but this is not the case. Regarding B & C, cells in this image are faint as they are hollow vesicles and, by nature, do not generate too much contrast when imaged with a phase-contrast microscope. There are some limitations on how much we can improve the contrast.

We now added scale bars for D-I. Similarly, faint hollow vesicles can be seen in Fig. S21 C & D, and Fig. 3H.

> *S36: Very nice; in B no purple arrow is visible. Note that scale bars are of different styles. S37 and S36 are very much the same - fuse, perhaps?*

We are sorry for the confusion. There are purple arrows in Fig. S37B-D.

> *S38: this is a more unconvincing figure - erase?*

Unconvincing in wahy sense. There are considerable similarities between the microfossils and structures formed by EM-P. If the reviewer expands a bit on what he finds unconvincing, we can address his reservations.

> *S39: white rectangle in A? Arrow in A? Note that scale bars are of different styles.*

These are some of the unavoidable remnants from the image from the original publication.

> *S40: in F: CM, V = ?; Note that scale bars are of different style.*

It's an oversite on our part. We now added the definitions to the figure legaend. We thank the reviewer for pointing it out.

> *S41: Rectangles in D, E, F, G can be deleted? Scales and labels missing in photos lower right.*

Those rectangles are added by the image processing software to the 3Drendered images. Regarding the missing scale bars in H & I they are the magnified regions of F. The scale bar is already present in F.

> *S42: appears compressed. G could be trimmed. Labels too small; scale in G?*

This is a curled-up folded membrane. We needed to lower the resolution of some images to restrict the size of the supplement to journal size restrictions. It is not possible to present 85 figures in high resolution. But we assure you that the image is not laterally compressed in any manner.

> *S43: This figure appears to be unconvincing. Reducing to pairing B, C, D with L, K? Spherical inclusions in B? Scales in E to G? Similar in S44: A, B, E only? Note that scale bars are of different styles.*

Figures I to K are important. They show not just the morphological similarities but also the sequence of steps through which such structures are formed. We addressed the issue of the scale bars above.

> *S45: A, B, and C appear to show live or subrecent material. How was this isolated of a rock? Note that scale bars are of different styles.*

It is common to treat rocks with acids to dissolve them and then retrieve organic structures within them. This technique is becoming increasingly common. The procedure is quite extensively discussed in the original manuscript. We don't see much differences in the scale bars of microfossils and EM-P cells, they are quite similar.

> *S46: A: what is to see here? Note that scale bars are of different styles.*

There are considerable similarities between the folded fabric like organic structures with spherical inclusions and structures formed by EM-P. If the reviewer expands a bit on what he finds unconvincing, we can address his reservations.

> *S47: Perhaps enlarge B and erase A. Note that scale bars are of different styles.*
>
> *S48: Image B appears to show the fossil material - is the figure caption inconsistent? There are no aggregations visible in the boxes in A. H is described in the figure caption but missing in the figure. Overall, F and G do not appear to mirror anything in A to E (which may be fossil material?).*
>
> *S51; S52 B, C, E; S53: these figures appear unconvincing - erase?*

Unconvincing in what sense? The structures from our study are very similar to the microfossils.

> *S54: North "Pole; scale bars in A to C =?*

These figures were borrowed from an earlier publication referenced in the figure legend. That is the reason for the differences in the styles of scale bars.

> *S55: D and E appear not to contribute anything. Perhaps add arrow(s) and more explanation? Check the spelling in the caption, please.*

D & E show morphological similarities between cells from our study and microfossils (A).

> *S56: Hexagonal morphologies may also be a consequence of diagenesis. Overall, perhaps erase this figure?*

I certainly agree that could be one of the reasons for the hexagonal morphologies. Such geometric polygonal morphologies have not been observed in living organisms. Nevertheless, as you can see from the figure, such morphologies could also be formed by living organisms. Hence, this alternate interpretation should not be discounted.

> *S57: The figure caption needs improvement. Please add more description. What show arrows in A, what are the numbers in A? What is the relation between the image attached to the right side of A? Is this a close-up? Note that scale bars are of different styles.*

We expanded a bit on our original description of the figure. However, we request the reviewer to keep in mind that the parts of the figure are taken from previous publication. We are not at liberty to modifiy them, like removing the arrows. This imposes some constrains.

> *S58: There are no honeycomb-shaped features visible. What is to see here? Erase this figure?*

Clearly, one can see spherical and polygonal shapes within the Archaean organic structures and mat-like structures formed by EM-P.

> *S59 and S60: What is to see here? - Erase?*

Clearly, one can see spherical and polygonal shapes within the Archaean organic structures and mat-like structures formed by EM-P in Fig. S59. Further disintegration of these honeycomb shaped mats into filamentous struructures with spherical cells attached to them can be seen in both Archaean organic structures and structures formed by EM-P.

> *S61: This figure appears to be unconvincing. B and F may be a good pairing. Note that scale bars are of different styles.*

There are considerable similarities between the microfossils and structures formed by EM-P. If the reviewer expands a bit on what he finds unconvincing, we might be able to address his reservations.

> *S62: This figure appears to be unconvincing - erase?*

There are considerable similarities between the microfossils and structures formed by EM-P. If the reviewer expands a bit on what he finds unconvincing, we might be able to address his reservations.

> *S66: This figure is unconvincing - erase?*

There are considerable similarities between the microfossils and structures formed by EM-P. If the reviewer expands a bit on what he finds unconvincing, we might be able to address his reservations.

> *S68: Scale in B, D, and E?*

Image B is just a magnified image of a small portion of image A. Hence, there is no need for an additional scale bar. The same is true for images D and E.

> *S69: This figure appears to be unconvincing, at least the fossil part. Filamentous features are visible in fossil material as well, but nothing else.*

We are not sure what filamentous features the reviewer is referring to. Both the figures show morphologically similar spherical cells covered in membrane debris.

> *S70 [as well as S82]: Good thinking here, but scales differ by magnitudes (cm to µm). Erase this figure? Very similar to Figure S73: Insert in C has which scale in comparison to B? Note that scale bars are of different styles.*

We realize the scale bars are of different sizes. In our defense, our experiments are conducted in 1ml volume chamber slides. We don't have the luxury of doing these experiments on a scale similar to the natural environments. The size differences are to be expected.

> *S71: Scale in E?*

Image E is just a magnified image of a small portion of image D. Hence, we believe a scale bar is unnecessary.

> *S72: Scale in insert?*

The insert is just a magnified region of A & C

> *S75: This figure appears to be unconvincing. This is clastic sediment, not chert. Lenticular gaps would collapse during burial by subsequent sediment. - Erase?*

Regarding the similarities, we see similar lenticular gaps within the parallel layers of organic carbon in both microfossils, and structures formed by EM-P.

> *S76: A, C, D do not look similar to B - erase? Similar to S79, also with respect to the differences in scale. Erase?*

Regarding the similarities, we see similar lenticular gaps within the parallel layers of organic carbon in both microfossils, and structures formed by EM-P. We believe we addressed the issue of scale bars above.

> *S80: A appears to be diagenetic, not primary. Erase?*

These two structures share too many resemblances to ignore or discount just as diagenic structures - Raised filamentous structures originate out of parallel layers of organic carbon (laminations), with spherical cells within this filamentous organic carbon.

> *S85: What role would diagenesis play here? This figure appears unconvincing. Erase?*

We do believe that diagenesis plays a major role in microfossil preservation. However, we also do not suscribe to the notion that we should by default assign diagenesis to all microfossil features. Our study shows that there could be an alternate explanation to some of the observations.

> *S86 and S87: These appear unconvincing. What is to see here? Erase?*

The morphological similarities between these two structures. Stellarshaped organic structures with strings of spherical daughter cells growing out of them.

> *S88: Does this image suggest the preservation of "salt" in organic material once preserved in chert?*

That is one inference we conclude from this observation. Crystaline NaCl was previously reported from within the microfossil cells.

> *S89: What is to see here? Spherical phenomena in different materials?*

At present, the presence of honeycomb-like structures is often considered to have been an indication of volcanic pumice. We meant to show that biofilms of living organisms could result in honeycomb-shaped patterns similar to volcanic pumice.

> *References*
>
> *Please check the spelling in the references.*

We found a few references that required corrention. We now rectified them.

References

(1) Orange F, Westall F, Disnar JR, Prieur D, Bienvenu N, Le Romancer M, et al. Experimental silicification of the extremophilic archaea pyrococcus abyssi and methanocaldococcus jannaschii: Applications in the search for evidence of life in early earth and extraterrestrial rocks. Geobiology. 2009;7(4).

(2) Orange F, Disnar JR, Westall F, Prieur D, Baillif P. Metal cation binding by the hyperthermophilic microorganism, Archaea Methanocaldococcus Jannaschii, and its effects on silicification. Palaeontology. 2011;54(5).

(3) Errington J. L-form bacteria, cell walls and the origins of life. Open Biol. 2013;3(1):120143.

(4) Cooper S. Distinguishing between linear and exponential cell growth during the division cycle: Single-cell studies, cell-culture studies, and the object of cell-cycle research. Theor Biol Med Model. 2006;

(5) Mitchison JM. Single cell studies of the cell cycle and some models. Theor Biol Med Model. 2005;

(6) Kærn M, Elston TC, Blake WJ, Collins JJ. Stochasticity in gene expression: From theories to phenotypes. Nat Rev Genet. 2005;

(7) Elowitz MB, Levine AJ, Siggia ED, Swain PS. Stochastic gene expression in a single cell. Science. 2002;

(8) Strovas TJ, Sauter LM, Guo X, Lidstrom ME. Cell-to-cell heterogeneity in growth rate and gene expression in Methylobacterium extorquens AM1. J Bacteriol. 2007;

(9) Knoll AH, Barghoorn ES. Archean microfossils showing cell division from the Swaziland System of South Africa. Science. 1977;198(4315):396–8.

(10) Sugitani K, Grey K, Allwood A, Nagaoka T, Mimura K, Minami M, et al. Diverse microstructures from Archaean chert from the Mount Goldsworthy–Mount Grant area, Pilbara Craton, Western Australia: microfossils, dubiofossils, or pseudofossils? Precambrian Res. 2007;158(3–4):228–62.

(11) Kanaparthi D, Lampe M, Krohn JH, Zhu B, Hildebrand F, Boesen T, et al. The reproduction process of Gram-positive protocells. Sci Rep. 2024 Mar 25;14(1):7075.

https://doi.org/10.7554/eLife.98637.2.sa0