Nannobacteria as a by-product of enzyme-driven tissue decay

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ABSTRACT

Spheroidal features, 50 to 200 nm in size and found in sedimentary rocks, have been described as nannobacteria. The idea that they are minute fossilized life forms—and especially the discovery of such features in Martian meteorite ALH84001—sparked a lively debate with regard to identification of ancient microbial life. Because biologists consider 200–300 nm to be the lower viable size limit for microorganisms, an alternative explanation is needed for features that have been described as nannobacteria by geologists. We report here on tissue-decay experiments that produced abundant proteinaceous spheroids in the size range of nannobacteria (described as nannoballs in the remainder of this paper). Experimental conditions were comparable to those found in Earth's surface sediments, and diagenetic mineralization of these spheroids may be a common process for preservation of nannoballs that are observed in the rock record.

Keywords: nannobacteria, enzyme, soft tissue, preservation, bacteria, decay.

INTRODUCTION

Folk (1993) defined nannobacteria as objects in sedimentary rocks that have bacterial morphology but at 50–200 nm are an order of magnitude smaller than typical bacteria. Their recognition in sedimentary rocks (Folk and Lynch, 1997; Pedone and Folk, 1996), as well as purportedly extant counterparts (Kajander and Ciftcioglu, 1998), led to their interpretation as hitherto overlooked life forms. The debate that grew out of the interpretation of nannobacteria in Martian meteorite ALH84001 as evidence of extraterrestrial life (McKay et al., 1996) led a panel of experts to conclude that viable microorganisms need to be at least 200–300 nm in size (Nealson, 1999). This implied that nannobacteria observed by Folk and colleagues were probably artifacts of modes of mineral precipitation and/or sample preparation.

The debate did not end, however (Rasmussen et al., 2002). Culture experiments (Kajander and Ciftcioglu, 1998) were cited as proof for the existence of nanobacteria (the preferred spelling in a biological context). Other research, however, suggests that the supposedly biogenic hydroxyapatite mineralization associated with nanobacteria (Kajander and Ciftcioglu, 1998) is due to nucleating activities of self-propagating microcrystalline centers (Cisar et al., 2000), whose nucleation, growth, and morphology are most likely mediated by proteins (Vali et al., 2001).

In experiments designed to study iron sulfide fossilization of microbes in sediments (Schieber, 2002), we made observations relevant to the nannobacteria debate. Pieces of organic matter were allowed to decompose (Schieber et al., 2002) and examined by scanning electron microscope. We observed abundant spheroidal features in the nannobacteria size range, the likely precursors of so-called nannobacteria in sedimentary rocks. A new set of experiments was designed to examine their origin.

METHODS, MATERIALS, AND INITIAL OBSERVATIONS

Experiments (Table 1) were aimed at three questions. (1) In what way do soft tissues change as they are degraded by bacteria? (2) In what way do soft tissues change as they are degraded by enzymes? (3) Are artifacts introduced through sample preparation procedures used in these experiments?

Experiments 1 and 2

Pieces of bean, squid, and beef (5–10 mm in size) were buried in a clay layer in a tank filled with sulfate-bearing water (40 L). Samples were wrapped into pieces of nylon stocking, tied to a color-coded string, dipped for 10 min into a pond-muck suspension (for inoculation with a full spectrum of naturally occurring decay bacteria), and placed on an earlier-deposited clay bed of 20 mm thickness. The strings were taped to the outside of the tank, and then the samples were covered with a second layer of clay (20 mm thick). Samples were withdrawn (pulled out of clay bed with string) at intervals spaced 1 day, 2 days, and finally 3 days apart, over a 14 day time period.

After removal from the tank, samples were processed for electron microscopy by fixation in 2% glutaraldehyde in a 0.1*M* phosphate buffer for 2.5 h, followed by two buffer washes and postfixation overnight in a 1% solution of osmium tetroxide in 0.1*M* phosphate buffer. Specimens were then washed, dehydrated to 100% ETOH, critical point dried, affixed to stubs, and sputter coated with AuPd. The buffer rinse effectively removed clay minerals that clung to the samples during withdrawal. Samples were examined with a JEOL T-300 scanning electron microscope (SEM). The buffer is intended to prevent artifacts due to chemical changes by minimizing osmotic pressure differences between tissue samples and surrounding fluids.

To see the potential impact of decay bacteria that had already resided in the specimens (e.g., from the butcher shop and fish market) prior to the experiment, autoclaved (sterilized) samples were used for experiment 2. The only noticeable difference was that initial decay did not proceed as vigorously in the autoclaved sample set.

In experiments 1 and 2, microbial decay of organic tissues produced anaerobic conditions in the clay layer within 2 days, as indicated by H_2S gas bubbles and precipitation of black iron sulfides around tissue samples. SEM examination of successively withdrawn samples showed explosive bacterial growth and rapid tissue degradation (Schieber et al., 2002), as well as widespread occurrence of spheroids in the 40–120 nm size range that compare well with published examples of nannobacterial textures (Figs. 1 and 2). We call these spheroids ''nannoballs.''

Experiment 3

In experiment 3 (Table 1) autoclaved pieces of bean, squid, and beef (same source materials as experiments 1 and 2) were immersed

TABLE 1. EXPERIMENTAL MATRIX

	No. of samples	Autoclaved	Fixation (glutaraldehyde and osmium tetroxide)	Nannoball formation
Experiment 1: Tissues buried in mud 1	21	No	After removal from tank	Yes
Experiment 2: Tissues buried in mud 2	21	Yes	After removal from tank	Yes
Experiment 3: Tissues in enzyme solutions	12	Yes	After 1 week	Yes
Experiment 4A: Blind 1	3	Yes	Immediately	No
Experiment 4B: Blind 2	3	No	Immediately	No

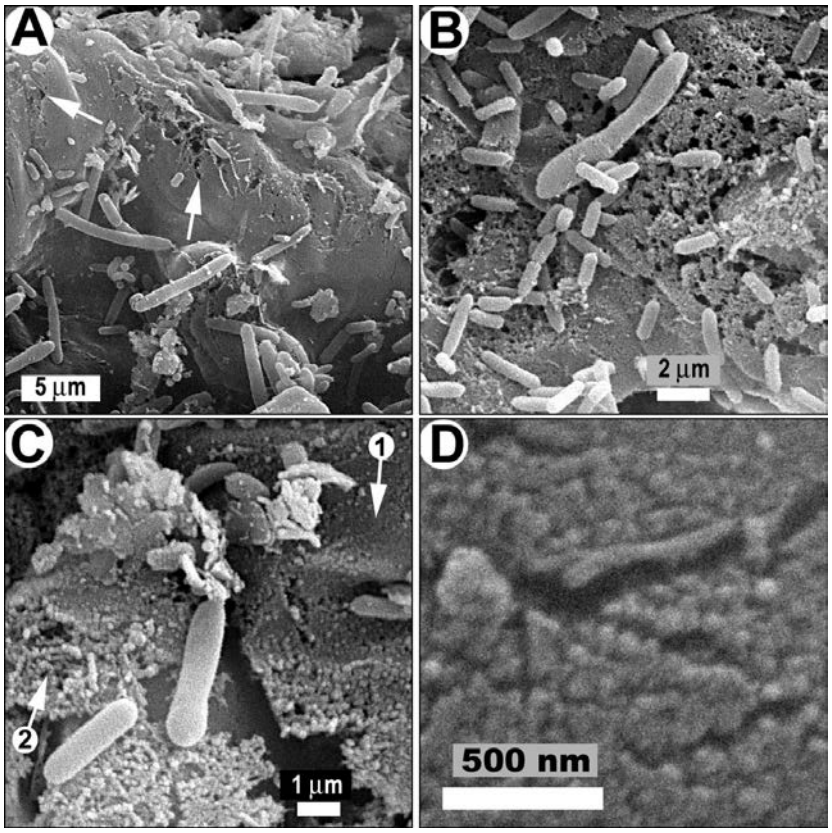

Figure 1. Progressive tissue decay in bean. A: Cell wall covered with rod-shaped bacteria. In places, holes (arrows) have formed in originally smooth surface. B: Closer view of area with holes. Cell wall resembles corroded metal foil. C: Closer view. Corroded areas change from smooth (arrow 1) to granular (arrow 2) texture. D: Close-up of granular-textured area shows it to consist of spheroidal bodies ranging in size from 40 to 80 nm. These are nannoballs.

in purified protein-degrading enzymes (proteinase, protease, pepsin, trypsin; Sigma-Aldrich Corp.) for 1 week at room temperature (23 °C). We did not go through the same withdrawal schedule as in experiments 1 and 2 because we were primarily interested in whether enzyme digestion does or does not produce nannoballs. We assumed that as long as some sample remained in the vial, we would be able to examine degradation products. After removal, samples were rinsed in phosphate buffer and processed for electron microscopy exactly like the samples in experiments 1 and 2.

When compared to microbial decay, degradation of tissue samples in enzyme solutions was slower, but degradation effects (discoloration of solution, fuzzy films on samples) were nonetheless visible within 24–48 h. After 1 week in the enzyme solutions, the samples tended to break into smaller pieces when handled. Although degradation rates for a given tissue type varied depending on the enzyme used, degradation was again accompanied by production of nannoballs (Fig. 3).

Experiments 4A and 4B

Experiments 4A and 4B (Table 1) were conducted to ensure that sample processing (autoclaving, fixation, critical-point drying, sputter coating) had not caused the features that we interpreted as related to bacterial and enzyme degradation. Samples were processed for electron microscopy exactly like those in experiments 1, 2, and 3, but were not exposed to bacterial decay or enzyme degradation prior to fixation. We surmised that if the purported decay and/or degradation features from experiments 1, 2, and 3 were indeed artifacts, then these samples should show them as well because they went through identical sample-processing steps.

RESULTS AND DISCUSSION

In the debate about Martian meteorite ALH84001 (McKay et al., 1996), one suggestion was that the observed nanofossils had resulted from deposition of a conductive coating on the specimen (Bradley et al., 1997; Bradley, 1999). Testing of various coating approaches, however, showed that AuPd coatings of 10–20 nm thickness do not produce artifacts that could be mistaken for microbial remains (Clanton and Morrison, 1979; McKay et al., 1997).

Beam heating of small areas in high-magnification mode can cause thermal expansion and formation of nanoscale platelets (Fig. DR1[1]). We adjusted beam current, spot size, and acceleration voltage to avoid this effect and minimize specimen heating. High magnification inspection of thermally cracked and curled-up coatings on smooth surfaces (e.g., glue coverings of SEM stubs) showed the coatings to be ~20 nm thick and established that coating did not produce nanoscale spheroidal bodies (Fig. DR1, see footnote one).

Because gradual decay of tissues always led to formation of nannoballs (Figs. 1 and 2), we surmised that the latter resulted when microbial enzymes interacted with the buried samples. Enzymes attack specific bonds within organic molecules. For example, trypsin cleaves

[1]GSA Data Repository item 2003102, Figure DR1, effects of beam heating on AuPd coating, is available from Documents Secretary, GSA, P.O. Box 9140, Boulder, CO 80301-9140, editing@geosociety.org, or at www.geosociety.org/pubs/ft2003.htm.

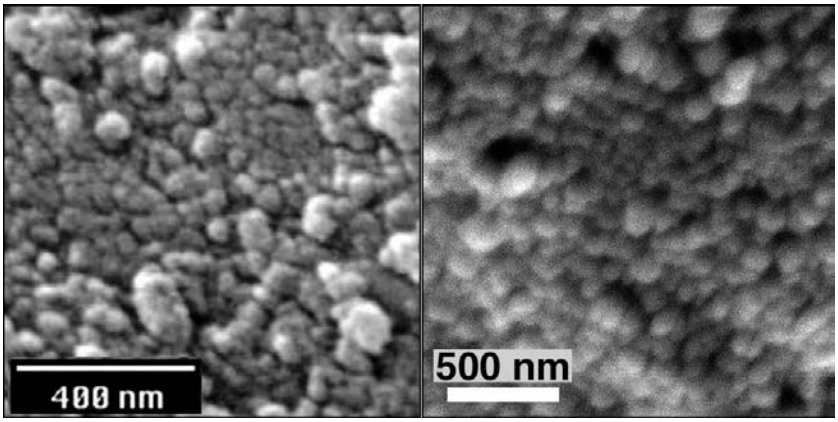

Figure 2. Comparison of nannobacterial textures. At left, picture is from nannobacteria Web site (http://www.msstate.edu/dept/geosciences/4site/nannobacteria.htm) maintained by Leo Lynch; image is described by Folk and Lynch as Jamaican microbialite with abundant nannobacterial textures. At right, granular nannoball texture in decaying squid muscle (nannoballs range in size from 50 to 120 nm). These nannoballs were produced in experiment 1 and are clearly quite similar in appearance to Folk's nannobacteria.

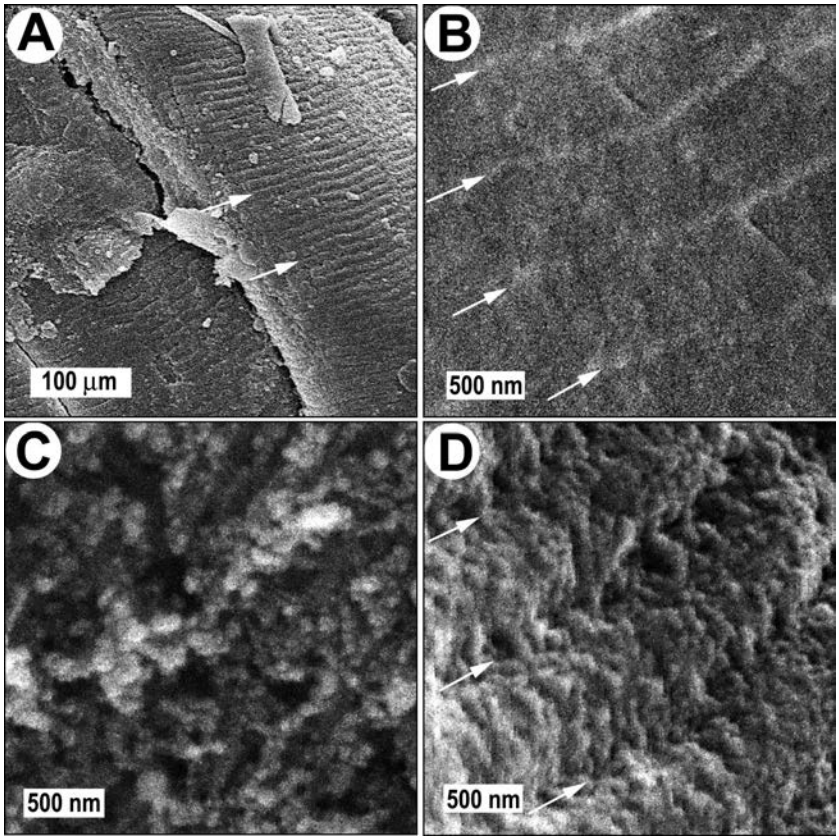

Figure 3. A: Low-magnification view of beef muscle fibers (extending from upper left to lower right) with characteristic striations (arrows). B: Close-up of muscle fiber in control sample. Striations (arrows) oriented as in A. Note absence of nannoballs. C: Surface of muscle fiber exposed to bacterial-decay processes. Note formation of granular masses of nannoballs. Striations oriented as in A. D: Surface of muscle fiber exposed to enzyme solution (proteinase). Striations (arrows) oriented as in A. Note granular nature of muscle fiber when compared to B, as well as formation of nannoballs of comparable size as in C.

peptide bonds associated with lysine and arganine residues, whereas chymotrypsin breaks the peptide bonds associated with phenylalanine, tryptophan, and tyrosine (Lodish et al., 1995). When enzymes cleave polymer structures, such as a muscle fiber or cell wall, it seems reasonable that fragments of a smaller and then smaller size range should be produced.

In experiment 3 (Table 1) we tested whether enzyme-driven tissue degradation was at the root of the observed nannoballs. Finding nannoballs (Fig. 3) in samples that were exposed to different types of purified protein-degrading enzymes confirmed this hypothesis. Because control samples (experiment 4) did not show nannoball formation (Fig. 3B), we conclude that nannoballs were not by-products of the sample preparation procedure.

Our experiments suggest that when proteinaceous organic tissues are exposed to protein-degrading enzymes, either through bacterial activity or via immersion into purified enzyme solutions, nannoballs are natural by-products of the ensuing degradation process. Nannoballs were observed within 2 days after the onset of decay experiments, and were abundant throughout as long as fixable organic material remained. Initial enzymatic degradation apparently breaks down larger tissue elements (e.g., cell walls, muscle fibers) into subunits tens to a few hundred nanometers in size. The unique shapes of enzymes determine their function and what types of bonds they can sever. The narrow size range of subunits in a given case probably relates to the repeat distance of the bonds that are affected at that initial stage.

Ubiquitous occurrence of ball-shaped nanoscale subunits may be related to elastic forces inherent in larger structures (such as muscle fibers or cell walls) composed of coiled and folded-up proteins or crystalline and noncrystalline zones of cellulosic fibers. A possible analogue might be an interconnected network of deformed springs that appears like a flat layer when intact and viewed from a distance. When the structure is cut into smaller subunits, however, now unbalanced forces cause the subunits to deform and contract into sphere-like structures.

In our experiments, tissue samples shrank while bacterial populations expanded, indicating consumption of samples by bacteria. Bacteria are osmotrophs and can only take in dissolved molecules liberated by exoenzymes utilized outside of the cell (Wyman and Stevenson, 2001). Seeing no subunits smaller than our nannoballs, we assume that in the subsequent degradation step, the nannoballs are broken down by further enzyme action into soluble molecules that can be ingested by bacteria. Bacteria seem to degrade organic tissues in a step-wise process. Enzyme action first disassembles tissues into solid subunits (tens to hundreds of nanometers in size) that give rise to the observed nannoballs, and the latter are then processed into ingestible soluble molecules.

If this process goes to completion, all soft tissue, including the nannoballs, should be consumed. However, in instances where the proper conditions for tissue mineralization exist, one might expect to find nannoballs a pervasive feature of mineralized tissues. In fact, in publications on soft-tissue preservation in fossils (e.g., Briggs and Kear, 1994; Wilby and Briggs, 1997; Hof and Briggs, 1997; Gabbott, 1998), researchers note that, for example, phosphatized soft tissues often display microspherulitic texture. The latter denotes a substrate microfabric that consists of densely packed microspheres of apatite or clay minerals, ranging in size from $<$30 nm (Wilby and Briggs, 1997) to 150 nm (Gabbott, 1998). Published SEM images of this texture in muscle fibers (e.g., Fig. 15H in Briggs and Kear, 1994; or Fig. 4C in Gabbott, 1998) resemble muscle fibers from our experiments (Fig. 3). Although magnification in the fossilized muscle-fiber images (Briggs and Kear, 1994; Gabbott, 1998) is not as high as in our images of bacterially (Fig. 3C) and enzymatically (Fig. 3D) degraded beef muscle, the textural similarities are striking.

Do nannoballs from our experiments have a potential to become mineralized and preserved? Briggs and Kear (1994) reported formation of mineralized muscle tissue within 2 weeks after onset of decay. Sagemann et al. (1999) reported that within 1 week of the onset of decay, steep chemical gradients were established that drive diffusion of ions needed for the mineralization process. Although energy-dispersive spectrometry analysis indicates that our nannoballs were not yet mineralized, our samples persisted for at least 2 weeks despite their small size (5–10 mm). Depending on sample size and the right chemical conditions (Sagemann et al., 1999), there is clearly a window of several weeks in which the nannoballs could have been mineralized. Considering the close textural similarity between the microspherulitic texture in mineralized muscle tissue (Briggs and Kear, 1994; Gabbott, 1998) and the nannoballs formed in degrading beef muscle during our experiments (Fig. 3), one might

venture that microspherules are nothing but mineralized nannoballs.

The majority of microbiologists do not consider nannobacteria as living entities (Nealson, 1999; Cisar et al., 2000), although the possibility that they could be viable life forms has not been abandoned entirely (e.g., Kajander and Ciftcioglu, 1998; Madigan et al., 2002). Yet, even under acceptance of this mainstream microbiological perspective, we still have to explain the origin of nannobacteria described from the rock record (Folk, 1993; Folk and Lynch, 1997; Pedone and Folk, 1996; McKay et al., 1996). One possible alternative explanation, namely that they are mineralized by-products of bacterial decay of organic tissues, is provided by our observations.

As the oldest life forms on the planet (Schopf, 1993), bacteria were engaged in enzymatic breakdown of organic matter from the beginning. Nannoballs should have been common intermediate by-products of their enzyme-assisted metabolism, regardless of whether they degraded other microbes or tissues of more advanced organisms. Bacterial degradation is ubiquitous in dead organisms, and in the case of early diagenetic soft-tissue preservation, nannoballs should thus be preserved in abundance as well. Recognition of nanoscale microspheres in mineralized soft tissues supports this conjecture (Briggs and Kear, 1994; Wilby and Briggs, 1997; Gabbott, 1998). Basically, any sediment in which organic matter decayed in tandem with early diagenetic mineral precipitation should show mineralized nannoballs when examined at high enough magnification. In essence, this is exactly what Folk and collaborators (Folk, 1993, 1999; Folk and Lynch, 1997, 2001; Pedone and Folk, 1996) found when they conducted high-magnification studies of soils, sediments, minerals, and rocks. Although their interpretation that the nannobacteria constitute erstwhile microorganisms is highly controversial (Nealson, 1999), the actual existence of the nannoballs is not in question.

If proponents of extant nanobacteria (Kajander and Ciftcioglu, 1998) can overcome existing doubts (Cisar et al., 2000) and prove that such life forms indeed exist, nanobacteria nonetheless seem to require a very specialized and nutrient-rich environment (e.g., human and cow sera). It seems unlikely that they should be abundant in more dilute natural environments.

Medical researchers report nanobacteria from various mineralization-related ailments such as kidney stones, arterial obstructions, and cataracts (Rasmussen et al., 2002; Kajander and Cifticioglu, 1998). Detractors of nanobacterial interpretations have proposed that biomineralization attributed to nanobacteria was instead initiated by nonliving macromolecules (Cisar et al., 2000). With regard to these medical examples of nanobacteria, we suggest that mineralization of nannoballs produced by enzyme-driven bacterial-decay processes presents yet another alternative to the nanobacterial mineralization mechanism proposed by Kajander and Ciftcioglu (1998). The observation that nannoballs are usually associated with bona fide bacteria in these ''medical'' examples (Robert Folk, 2001, personal commun.) is consistent with the view that the nannoballs are by-products of bacteria-driven degradation processes.

CONCLUSION

The fossil record of diagenetic mineralization related to microbial decay should show prolific preservation of nannoballs. Most if not all alleged nannobacterial structures in sedimentary rocks are probably by-products of bacterial degradation of organic matter and not evidence for minute life forms called nannobacteria. Nonetheless, mineralized nannoballs may indicate bacterial enzyme action on organic tissues and serve as a visual proxy for microbial activity.

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REFERENCES CITED

Bradley, J., 1999, Investigation of biomineralization at the nanometer scale by using electron microscopy, *in* Steering Group, eds., Size limits of very small microorganisms: Proceedings of a workshop: Washington, D.C., National Academy Press, National Research Council, Space Studies Board, p. 103–106.

Bradley, J.P., Harvey, R.P., and McSween, H.J., 1997, No ''nanofossils'' in martian meteorite: Nature, v. 390, p. 454.

Briggs, D.E.G., and Kear, A.J., 1994, Decay and mineralization of shrimps: Palaios, v. 9, p. 431–456.

Cisar, J.O., Xu, D.Q., Thompson, J., Swaim, W., Hu, L., and Kopecko, D.J., 2000, An alternative interpretation of nanobacteria-induced biomineralization: National Academy of Sciences Proceedings, v. 97, p. 11,511–11,515.

Clanton, U.S., and Morrison, D.A., 1979, Hypervelocity impact craters <1000Å diameter: Lunar and Planetary Sciences Conference, 10th, Houston, Abstract Volume, p. 212–214.

Folk, R.L., 1993, SEM imaging of bacteria and nannobacteria in carbonate sediments and rocks: Journal of Sedimentary Petrology, v. 63, p. 990–999.

Folk, R.L., 1999, Nannobacteria and the precipitation of carbonates in unusual environments: Sedimentary Geology, v. 126, p. 47–56.

Folk, R.L., and Lynch, F.L., 1997, The possible role of nannobacterial (dwarf bacteria) in clay-mineral diagenesis and the importance of careful sample preparation in high-magnification SEM study: Journal of Sedimentary Research, v. 67, p. 583–589.

Folk, R.L., and Lynch, F.L., 2001, Organic matter, putative nannobacteria, and the formation of ooids and hardgrounds: Sedimentology, v. 48, p. 215–229.

Gabbott, S.E., 1998, Taphonomy of the Ordovician Soom Shale lagerstätte: An example of soft tissue preservation in clay minerals: Paleontology, v. 41, p. 631–667.

Hof, C.H.J., and Briggs, D.E.G., 1997, Decay and mineralization of mantis shrimps (Stomatopoda: Crustacea)—A key to their fossil record: Palaios, v. 12, p. 420–438.

Kajander, E.O., and Ciftcioglu, N., 1998, Nanobacteria: An alternative mechanism for pathogenic intra- and extracellular calcification and stone formation: National Academy of Sciences Proceedings, v. 95, p. 8274–8279.

Lodish, H., Baltimore, D., Berk, A., Zipursky, S.L., Matsudaira, P., and Darnell, J., 1995, Molecular cell biology (third edition): New York, W.H. Freeman and Co., Scientific American Books, 1152 p.

Madigan, M.T., Martinko, J.M., and Parker, J., 2002, Brock biology of microorganisms (10th edition): Upper Saddle River, New Jersey, Prentice Hall, Pearson Education, Inc., 1104 p.

McKay, D.S., Gibson, E.K., Thomas-Keprta, K.L., Vali, L.H., Romanek, C.S., Clemett, S.J., Chillier, Z.D.F., Maechling, C.R., and Zare, R.N., 1996, Search for past life on Mars: Possible relic biogenic activity in Martian meteorite ALH84001: Science, v. 273, p. 924–926.

McKay, D.S., Gibson, E.K., Thomas-Keprta, K.L., and Vali, L.H., 1997, No ''nanofossils'' in martian meteorite: Reply: Nature, v. 390, p. 455.

Nealson, K., 1999, Discussion, *in* Steering Group, eds., Size limits of very small microorganisms: Proceedings of a workshop: Washington, D.C., National Academy Press, National Research Council, Space Studies Board, p. 39–42.

Pedone, B.A., and Folk, R.L., 1996, Formation of aragonite cement by nannobacteria in the Great Salt Lake, Utah: Geology, v. 24, p. 763–765.

Rasmussen, T., Kirkland, B., Charlesworth, J., Rodgers, G., Severson, S., Rodgers, J., Folk, R., and Miller, V., 2002, Electron microscope and immunological evidence of nanobacterial structures in calcified carotid arteries, aortic aneurysms and cardiac valves: American College of Cardiology Journal, v. 39, p. 107–109.

Sagemann, J., Bale, S.J., Briggs, D.E.G., and Parkes, R.J., 1999, Controls on the formation of authigenic minerals in association with decaying organic matter: An experimental approach: Geochimica et Cosmochimica Acta, v. 63, p. 1083–1095.

Schieber, J., 2002, Sedimentary pyrite: A window into the microbial past: Geology, v. 30, p. 531–534.

Schieber, J., Arnott, H.J., and Coviello, M., 2002, Various occurrences of potential microbial fossils in mudstones and investigations into bacterial taphonomy: Lunar and Planetary Sciences Conference, 33rd, Houston, March 11–15, 2002, Abstract Volume (CD).

Schopf, J.W., 1993, Microfossils of the Early Archean Apex chert: New evidence of the antiquity of life: Science, v. 260, p. 640–646.

Vali, L.H., McKee, M.D., Ciftcioglu, N., Plows, F.L., Sears, S.K., Chevet, E., Plavsic, M., Kajander, E.O., and Zare, R.N., 2001, Nanoforms: A new type of protein-associated mineralization: Geochimica et Cosmochimica Acta, v. 65, p. 63–74.

Wilby, P.R., and Briggs, D.E.G., 1997, Taxonomic trends in the resolution of detail preserved in fossil phosphatized soft tissues: Geobios, Mémoire spécial, v. 20, p. 493–502.

Wyman, B., and Stevenson, L.H., 2001, The facts on file dictionary of environmental science (second edition): New York, Checkmark Books, 236 p.

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