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Dinosaur Tissue or Bacterial Biofilms?

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Abstract

Pliable soft tissue containing detailed cellular structures has been detected in numerous dinosaur fossils. Studies have also reported extracting and identifying several animal proteins (e.g., collagen and actin) from this tissue. Since predicted decay rates are not consistent with tissue and biomolecules being preserved for millions of years, these findings challenge the assigned ages of the dinosaur fossils. Different explanations have been offered for how tissue could survive for extended periods of time. One explanation is that this tissue is actually a bacterial biofilm with a replica imprint of dinosaur cells and the biomolecules are of bacterial origin. Bacterial biofilms have even been shown to have a significant role in the fossilization processes. However, biofilms have not been shown to replicate the cellular detail found in dinosaur tissue. Also, amino-acid sequence, antibody affinity, and microspectroscopic analysis reveals significant difference between bacterial proteins and those proteins extracted from the soft tissue. Thus there is no substantial evidence that the pliable material extracted from dinosaur fossils is contaminating biofilm.

Introduction

Numerous studies have reported the detection of pliable tissue and even biomolecule remnants (such as protein fragments) in dinosaur fossils (Thomas, 2015). These discoveries have presented evolutionists with a conflicting situation. Based upon the standard (evolution-biased) dating paradigm, dinosaurs lived and died at least 65 million years ago. Yet, the persistence of this pliable tissue and some partially intact biomolecules contradicts decay rates and decomposition studies for biological material (Allentoft et al., 2012; Bada et al., 1999; Buckley et al., 2008). The presence of this tissue indicates these dinosaur fossils are far less than 65 million years of age. In fact, an age of merely a few thousand years would be a much more consistent conclusion.

Thus, evolutionists have sought various alternative explanations for the presence of this tissue (see the discussion of DeMassa and Boudreaux, 2015; and Thomas, 2015). One of these alternatives is that the detected material is not actual preserved original tissue. Rather, it is a bacterial biofilm with general characteristics similar to animal tissue.

Kaye et al. (2008) argue that bacterial "biofilms share a closer molecular make-up" to the detected pliable "soft tissue" material than does actual bone tissue.

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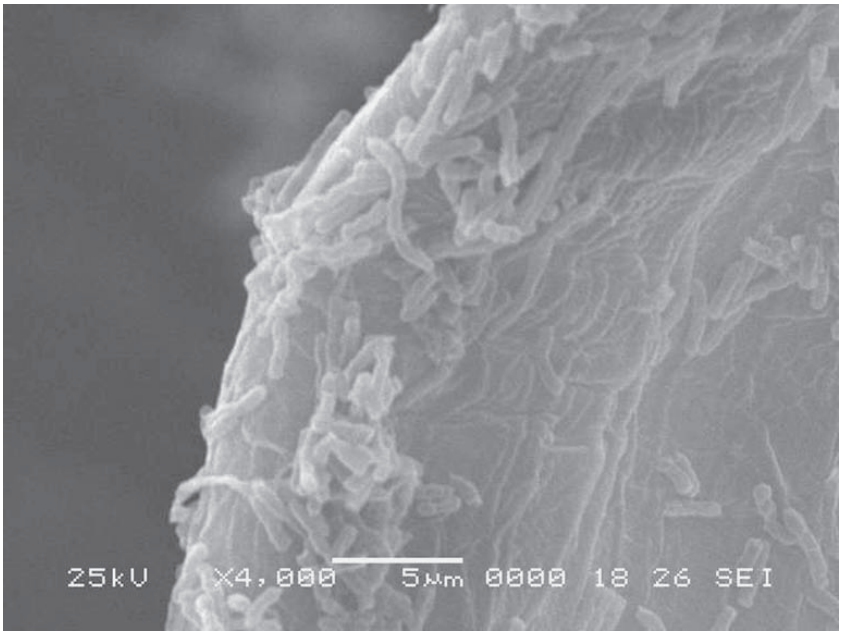

Figure 1. Electron micrograph (4,000x) of bacterial cells attaching to a surface as the first step of biofilm formation.

They further argue that studies, such as Asara et al. (2007), mistakenly conclude that the collagen detected in the fossil was of dinosaur origin. Instead, Kaye et al. (2008) counter that these studies have merely detected a collagen-like protein in the fossils, with little justification to assume this is actual dinosaur collagen. In fact, bacteria have been previously reported to make a collagen-like protein (Rasmussen et al., 2003). Thus, Kaye et al. (2008) suggest that both the pliable tissue and the proteins found in dinosaur specimens are likely not original but of bacterial origin. Many bloggers and other science commentators were quick to accept, or at least promote, this biofilm explanation (e.g., Cambell, 2008; Hecht, 2008; Smith, 2008).

More recently, Raff et al. (2008, 2013, 2014) and Hu et al. (2011) have proposed a mechanism for bacterial involvement in soft-tissue preservation. While not specifically challenging the validity of dinosaur soft-tissue reports, their work reintroduces a role for biofilms. The extent of this role warrants closer examination of the nature of biofilms and the dinosaur soft-tissue data.

Bacterial Biofilms

Most people are familiar with the aromatically fruity slime on forgotten leftovers in the refrigerator.

Bacteria frequently exist in such a microbial community, which can be found attached to a wide variety of surfaces. This community, known as a biofilm, provides a microenvironment that facilitates a synergistic interaction of bacteria within the film. As such, biofilms frequently offer a significant survival advantage for bacteria, which helps explain their common occurrence in virtually all microbial niches.

The initial stage of biofilm development involves a few bacterial cells binding to some type of surface (e.g., teeth, bone, food, tissue, rock) (Figure 1). After binding, the cells begin to replicate and can form an immature biofilm, which is usually small and contains a low number of bacteria (comprised of perhaps only one or a few species). As the biofilm matures, its population size increases, as well as the size and shape of the film (Figure 2). During maturation, the composition of the bacterial population may become very diverse (Rendueles and Ghigo, 2012). Biofilm structures also vary, depending on the nature of the surface on which the biofilm is adhering (Hochbaum and Aizenberg, 2010). Mature biofilms may ultimately be composed of a single species or multiple species, and bacterial interactions also become more complex (Anderson, 2003; Elias and Banin, 2012; Rendueles and Ghigo, 2012).

The growth of the biofilm population is affected by the availability of nutrients, environmental temperature, and the population composition. Cells near the biofilm surface have ready access to nutrients from the environment, while cells in the interior are more dependent upon metabolites from surrounding bacteria for their energy substrates. Thus, different locations in the biofilm will likely be populated by different species of bacteria. This also means that cells within a biofilm are often in a dynamic of movement and metabolism.

As the biofilm matures, the metabolism of the bacteria begins to either increase or decrease the internal pH. The pH change stimulates bacterial production of mineral precipitates (e.g., calcium carbonate and hydroxyapatite) (Cooke et al., 1999; VanGlulck et al., 2003). This situation is frequently encountered in urinary catheters (Morris et al., 1999; Stickler et al., 1993) and leachate collection systems of waste landfills (Cooke et al., 2001), causing encrustation and fouling.

Maturation of biofilms also involves bacterial secretion of a polysaccharide matrix known as an extracellular polymeric substance (EPS). In most

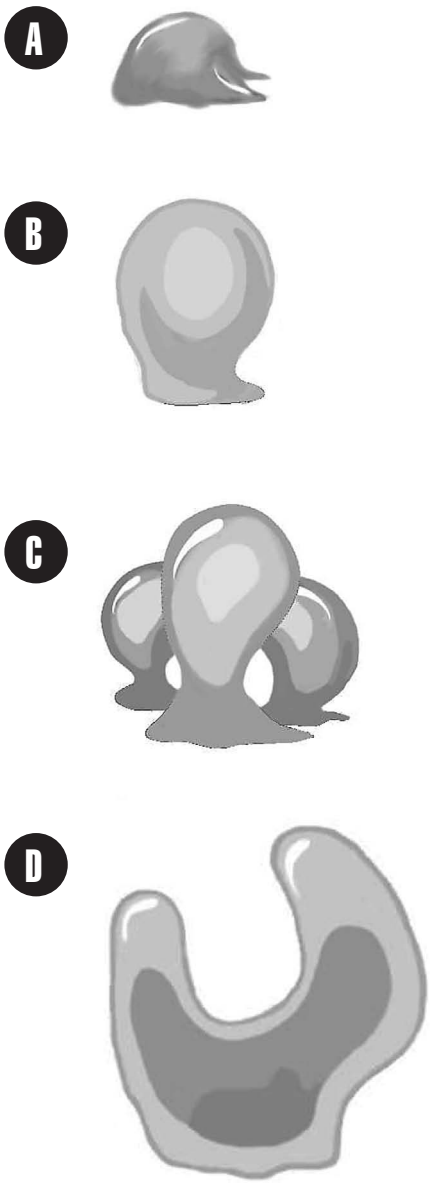

Figure 2 *(left)*. Development of bacterial biofilm structures. After initial attachment of cells to a surface, the population size steadily increases and begins production of EPS: (A) early biofilm structure following initial EPS production, (B) continued growth of the biofilm from increased accumulation of EPS, (C) biofilm begins to expand and potentially form multiple structures, and (D) maturation of the biofilm.

biofilms, EPS accounts for 90% of the matrix (Flemming and Windgender, 2010). This EPS serves to provide both a protective layer and a scaffold to hold the bacteria within the community (Flemming and Wingender, 2010). As a hydrogel, EPS consists mostly of polysaccharide (with some proteins, nucleic acids, and sometimes humic substances). The composition of the polysaccharides in the EPS varies depending upon the types of bacteria in the biofilm. EPS typically has properties similar to gums (polysaccharides that greatly increase a solutions viscosity).

Within this EPS matrix, bacterial cells are maintained in close proximity, enabling a variety of microbial interactions (e.g., nutrient sharing, water transport, and chemical protection). In addition, this structure serves as a platform for cell-to-cell communication (including a phenomenon called *quorum sensing*) (Schuster and Markx, 2014). Water columns may also form, enabling water transport to cells in the biofilm interior.

The resulting 3-dimensional EPS matrix can have a variety of shapes and sizes (Figure 3). Comparisons of different biofilms formed by different organisms and strains typically show that many, if not most, biofilms are very simple structures. Biofilm structure and shape is generally a function of population composition and environmental conditions. Typically, biofilms are flat and featureless, or they consist of simple aggregates, especially when nutrient availability is limited (Bridier et al., 2010). Some bacteria can form biofilms with a patchy characteristic, or a more complex pillar or mushroom-shaped structure (Schuster and Markx, 2014). Many biofilms are microscopic in size, but some very mature biofilms may become fairly large and readily visible.

Biofilms appear to form by random growth or simple chaotic aggregation processes. However, even in such simple models, different microenvironments can be found within the biofilm. The presence of EPS limits convective mass transfer of nutrients from the medium to the cells. It also limits movement of metabolic substrates, products, and intermediates within the matrix. Cells usually need a close juxtaposition for cell-to-cell transfer of metabolites or other chemicals. Thus, diffusion is the primary mechanism for transferring nutrients and chemicals in a biofilm, and the viscosity of the EPS causes chemical gradients to form. This tends to favor specific bacterial populations within each gradient, giving a segregation of species within a biofilm.

Biofilm or Tissue?

The relationship of bacterial biofilms and tissue preservation has been studied for several years. Raff et al. (2008) suggest that embryo preservation occurs with a biofilm consuming the embryo and simultaneously forming a replica of the embryo. In a follow-up study, they observe in greater detail the process of how microbial invasion stabilizes the embryo tissue and subsequently replaces the embryo with a three-dimensional biofilm, which mimics the embryo's morphology (Raff et al., 2013). McNamara et al. (2009) also report that detail of a Miocene frog's soft tissue is preserved by a bacterial biofilm replica.

This model for biofilm replica of tissue prompted Kaye et al. (2008) to offer an alternative interpretation of dinosaur soft tissue. They report that microscopic analysis of pliable sheets from dinosaur fossils reveals the presence of bacterial biofilms in the pores of the bone. They interpret this biofilm layer as forming an endocast of the original cell and bone

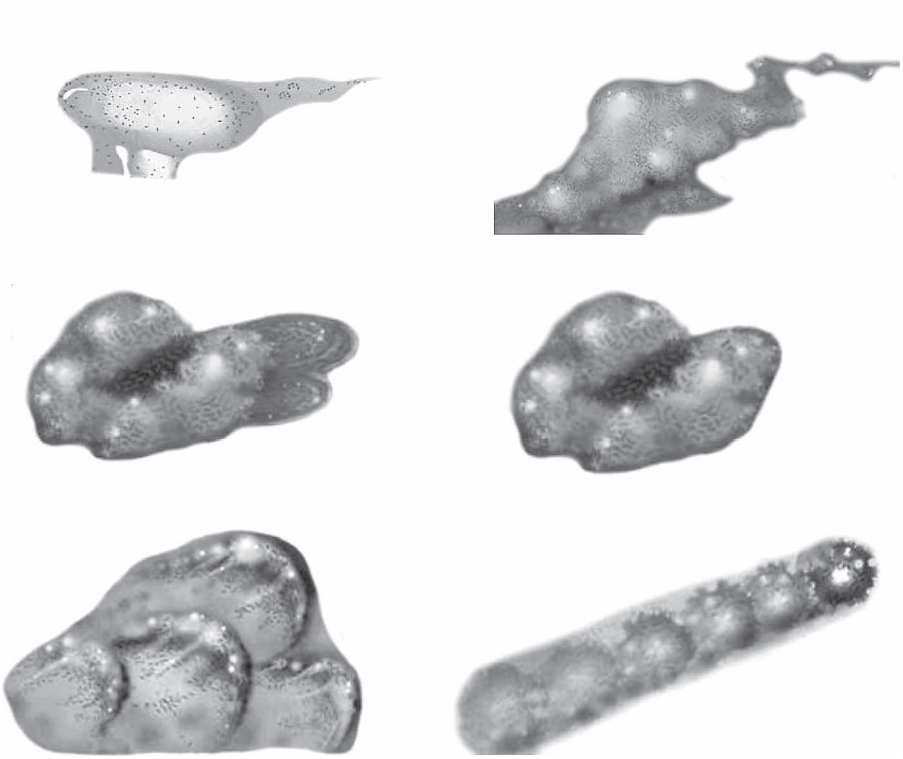

Figure 3. Examples of different mature biofilm structures/morphologies.

matrix. Thus, they suggest that these endocasts have been misinterpreted as blood vessels and pliable tissue containing osteocyte cells. The researchers further offer that the natural pliability of a biofilm enables it to retain the shape of the surface it formed upon, in this case the dinosaur tissue.

They are somewhat vague regarding the mechanism of this biofilm endocast. Presumably, they are suggesting the endocast forms upon the cells' cast, as suggested by Raff et al. (2008, 2013). The biofilm retains the structural features of the original dinosaur cells and tissue. This, combined with a biofilm's natural elasticity, gives the appearance of being soft, stretchable tissue containing cellular structures. Kaye et al. (2008) suggest that "the lack of observed cell structure in the transparent tubes is inconsistent with preserved tissues." Therefore, they conclude that such biofilms have been mistakenly identified as original dinosaur tissue that has survived millions of years without fossilizing. They further suggest that the red blood cells reported in previous studies were actually oxidized forms of pyritic frameworks.

Peterson et al. (2010) specifically address the claims of Kaye et al. (2008). They followed the development of biofilm on experimentally prepared bones, which were placed into holes in areas of the Yucatan Peninsula (Mexico). After a period of four months, the bones were removed and analyzed. The results of this study support previous suggestions that microbial activity is an important stage of fossil preservation (Briggs et al., 2003; Hollcher et al., 2001; Raff et al., 2008). Biofilms apparently metabolize organics, reducing the pH of the matrix. As a result of the lower pH, the bacteria produce minerals that form a mineral barrier (microbial masonry), which helps seal pores in the bone. This sealing may not only help accelerate the fossilization process, but also provide some protection of the inner bone tissue. In fact, microbial biofilms may actually help stabilize and preserve original tissue (Iniesto et al., 2013; Raff et al., 2014). While there is little evidence that this preservation would be sufficient for 65 million years, it may serve as at least a partial explanation for the preservation of the tissue for 4,000–5,000 years.

They further report that "fractured bones have a less-likely chance of preserving primary soft-tissues" (Peterson et al., 2010, p. 11). The researchers propose that fractured bones allow greater penetration of bacteria that can degrade any biological material. In contrast, intact bone provides only limited access of bacteria through the pores in the bone and actually enhances biofilm production of EPS and hydroxyapaptite. This polysaccharide and mineral fabric seals the pores, helping protect the tissue from degradative processes.

Other studies also offer similar biofilm activity as part of the fossilization process (Briggs, 2003; Dunn et al., 1997; Daniel and Chin, 2010; Hu et al., 2011; Iniesto et al., 2013). In addition, Raff et al. (2014) propose that the type of bacteria comprising the biofilm has a significant effect on preservation quality. Hence, some tissue may be preserved and other tissue not preserved, strictly as a consequence of the bacterial population present at the time of fossilization. Hu et al. (2011) even suggest this microbial masonry event enabled body-part preservation of fossils with assigned ages of over 250 million years.

Interestingly, the study by Peterson et al. (2010) failed to provide any support for the proposal of Kaye et al. (2008). Rather, they observed a general lack of morphological similarity between

samples from soft-tissue controls compared to biofilm structures (Peterson et al., 2010). Following the four months of burial, the biofilms they observed did not present any detailed vessel or cellular structures (Peterson et al., 2010), both of which have been repeatedly detected in soft tissue from dinosaur fossils (Armitage, 2015; Armitage and Anderson, 2013; Schweitzer et al., 2007, 2013). In fact, close scrutiny of microscopic biofilm images obtained by Kaye et al. (2008) reveals no intricate cellular structures with filopodia or other features that closely mimic previously reported dinosaur cells. Kaye et al. (2008) offer little explanation for the cellular detail reported by other studies, a glaring omission in their conclusions.

In addition, Peterson et al. (2010) were able to microscopically observe specific bacterial cells and EPS structures for the biofilms on their test fossils. The presence of bacterial cells in an EPS layer has never been observed in any reported examinations of pliable tissue extracted from dinosaur fossils. Rather, osteocytes, which are much larger cells than bacteria, are readily observed even without electron microscopy (Armitage and Anderson, 2013; Schweitzer et al., 2013). Thus, results from Kaye et al. (2008) are not consistent with those from other soft-tissue studies.

It should be noted that bacteria are ubiquitous on any geologic specimen. A wide variety of bacteria can participate in biofilm formation, but the simple presence of bacteria does not automatically indicate the presence of significant levels of biofilms. Under specific conditions, many bacteria appear to prefer colonizing within a biofilm community, but large and mature biofilms will not always occur. While bacteria are certainly present on the extracted pliable tissue, no distinct mature biofilms were evident in microscopic examination of various dinosaur soft/pliable tissue samples (Armitage and Anderson, 2013; Schweitzer et al. 2009, 2013). Also, as mentioned above, few biofilms become large enough to mimic the size and textural features of the extracted soft tissue.

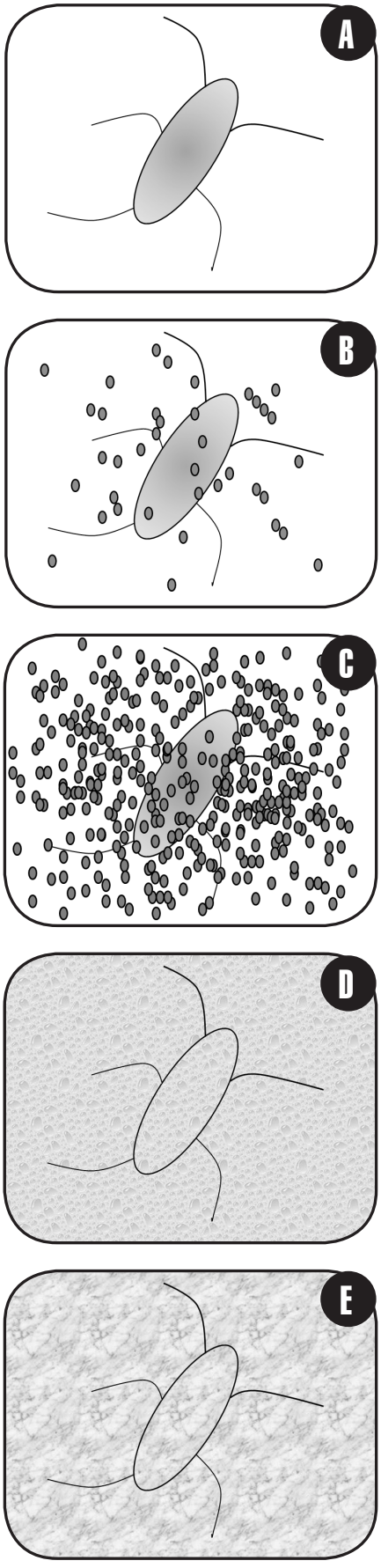

Figure 4 *(right)*. Pseudomorphing model proposed by Raff et al. (2008, 2013). (A) An intact osteocyte within bone tissue. (B) During fossilization, bacteria invade bone tissue and begin to replicate their cells. (C) As the bacterial population grows, it begins releasing EPS. (D) Sufficient EPS production leads to formation of a biofilm layer over the bone tissue, which captures the impression of the osteocyte before the cell decomposes. (E) The mature biofilm is subsequently fossilized, retaining the osteocyte impression.

Kaye's model (Kaye, et al., 2008) requires that the biofilm EPS matrix can mimic characteristics of dinosaur tissue (including intact osteocytes). However, the model is vague regarding whether this structural impression occurred prior to the fossilization of the osteocytes or if the impression is that of an already fossilized cell. Raff et al. (2008, 2013) suggest that when bacteria digest tissue, they can also form a biofilm imprint of that tissue. If Kaye's model is suggesting the tissue/cell impression is made prior to fossilization, this requires the biofilm to retain extraordinary morphological detail for millions of years. It should be noted that the results of recent studies reported by Raff et al. (2008, 2013, 2014) deal with fresh specimens, and any replica or imprint they detected in the biofilm was of recent formation.

It also should be noted that the model offered by Raff and coworkers proposes what they call "pseudomorphing" (Raff et al., 2013, 2014). This involves a bacterial biofilm stabilizing the soft body parts of a dead organism and then forming an endocast of the tissue.

Subsequent mineralization preserves the biofilm as a fossilized impression of the tissue (Figure 4). They even suggest that the three-dimensional biofilm could retain some of the detailed features of the cells within the tissue (e.g., organelles, cell boundaries, etc), which allows these features to eventually be preserved in a fossilized form. However, they admit that any internal cellular detail would likely be "voids within the cells" and thus pseudomorphing may only offer a "potential" for preserving detail (Raff et al., 2013, p. 255). Regardless, this "pseudomorphing" model addresses potential mineralization leading to fossilization of soft-body parts. It does not adequately address a mechanism for preserving them as soft/pliable tissue. As such, it does not provide an adequate mechanism for Kaye's model.

Thus, biofilms may be able to retain some temporary general impressions of a surface, but there is no evidence that this impression could possess intricate cellular detail. What is more, there is certainly no evidence that a biofilm can retain an impression of detailed structures for millions of years. In fact, such a concept fully contradicts known biofilm behavior, where the biofilm structure constantly changes as a consequence of maturity, temperature, species composition, and other environmental factors. None of these factors could ever be suggested to have remained constant over a multimillion-year period. Also, Peterson et al. (2010) found no microscopic indication that biofilms could be mistakenly identified as tissue or complex cellular structures, such as osteocytes.

Close scrutiny of micrographs reported by Raff et al. (2008, 2013) reveal that bacteria and bacterial aggregates can be clearly identified within the preserving biofilm. Reports by Schweitzer et al. (2007, 2013) and Armitage and Anderson (2013) do not reveal any clearly seen bacteria or bacterial aggregates in the extracted tissue. In fact, *Triceratops* horn tissue at 800x magnification (Armitage and Anderson, 2013; figure 8–10) compared to micrographs of similar magnification reported by Raff et al. (2008, 2013) or Kaye et al. (2008) reveals very distinct differences. The pliable horn tissue possesses several cellular structures of distinct osteocyte-type cells. This is not observed in any of the biofilm images reported by Raff et al. (2008, 2013) or Kaye et al. (2008).

At even greater magnifications, the results reported by Kaye et al. (2008) and Raff et al. (2008, 2013) reveal no morphologically intricate cells in the biofilm replica. None of these studies offer electron micrographs with the level of cellular detail reported for dinosaur soft tissue (e.g., Armitage, 2015; Armitage and Anderson, 2013; and Schweitzer et al., 2013). This includes fine cellular detail to a 500-nm scale, extensive filipodia (20 µg in length), and even evidence of internal contents. The absence of such cellular detail strongly indicates a significant difference between what Kaye et al. (2008) and Raff et al. (2008, 2013) observed and what has been observed in the pliable tissue extracted from dinosaur fossils.

Bacterial Proteins?

Collagen

Kaye et al. (2008) further challenge that findings of dinosaur collagen detected in fossil samples (Asara et al., 2007) are not original proteins. Instead, they document that bacteria make a collagen-like protein (Rasmussen et al., 2003; Yu et al., 2014). Kaye's model proposes that this bacterial protein is the true origin of the collagen detected in dinosaur specimens.

To further support this position, Kaye et al. (2008) obtained C-14 dates of the pliable tissue from dinosaur specimens. Their C-14 results indicate that carbon in the biomolecules of the tissue (mostly collagen) is of recent origin. They suggest this young age is more consistent with recent bacterial origin of biomolecules in the fossil, since evolutionary interpretation would require any dinosaur tissue to be "carbon dead" (i.e., greater than one million years of age; for a more detailed discussion of the significance of C-14 in dinosaur fossils, see Thomas and Nelson, 2015).

Collagen is the most abundant protein in the animal kingdom. It is a heterogeneous family of glycoproteins that contain at least one triple helical domain. All animal collagens are composed of three alpha-chains consisting of a triple helix motif. This motif consists of a repeating sequence of three amino acids, specifically glycine followed by two other amino acids. The motif is commonly designated as $(Gly\text{-}Xaa\text{-}Yaa)_n$ (Exposito et al., 2010).

Many bacteria appear to make a collagen-like protein that also possesses a triple helical motif (Rasmussen et al., 2003). This protein can have a similar structural matrix as animal collagen. Thus, it can potentially be misinterpreted by microscopic analysis as animal collagen.

However, all known vertebrate collagen possess hydroxyproline (a modification of proline), and it is frequently found in the third position (the Yaa position) of the triple helix motif (Shoulders and Raines, 2009). Bacteria often place proline in the second position (Xaa) (Yu et al., 2014), and threonine and glutamine are frequently in the Yaa position of the helix (Rasmussen et al., 2003). In addition, bacteria lack the ability to convert proline to hydroxyproline and therefore lack this modified form of proline in their collagen-like proteins (Xu et al., 2010; Yu et al., 2011). The absence of the hydroxyproline appears to make bacterial collagen-like protein more susceptible to destabilization by temperature and pH changes (Mohs et al., 2007). Asara et al. (2007) report that approximately 50% of proline residues in putative dinosaur collagen is hydroxylated. This constitutes a clear distinction between bacterial collagen-

like protein and the collagen detected in the dinosaur tissue. Thus, the proposal by Kaye et al. (2008) is contradicted by the chemical differences between bacterial protein and the collagen extracted from the fossils.

As additional confirmation of collagen, Lindgren et al. (2011) used antibody specificity to detect type I avian collagen. This antibody has an affinity for collagen isolated from dinosaur fossils but lacks affinity to bacterial "collagen" or negative controls. Thus, there is no evidence of bacterial collagen-like proteins in the pliable tissue samples from the fossils examined by these researchers.

Immunoassays of antibody affinity can be subject to false results as a consequence of (1) lack of antibody epitope specificity, and (2) inadequate microscope sensitivity. Lindgren et al. (2011) addressed the first concern by use of several positive and negative controls and use of an antibody with high specificity for animal collagen (esp. avian). The second concern was addressed by using confocal microscopy, enabling a very precise detection of antibody binding.

As further support of the detection of animal collagen, they employed synchrotron, radiation-based infrared microspectroscopy (Lindgren et al., 2011), which can analyze specific microstructures of biomolecules. This microspectroscopy found that the amino acids were located in the bone matrix fibrils. This is consistent with typical animal collagen characteristics but differs significantly from bacterial collagen-like proteins (Lindgren et al., 2011).

In addition, Schweitzer et al. (2009) also used antibodies with a specific reactivity to type I avian collagen. They found these antibodies attach to segments of the isolated dinosaur collagen samples but not to control samples. They also did a BLAST analysis of the amino acid sequences of the short collagen fragments they obtained from the fossil. While several of the sequences had some alignment with chicken, alligator, or *T. rex* proteins, none aligned with any bacterial protein sequences. Plus, Bern et al. (2009) used mass spectroscopy to reanalyze samples used by Asara et al. (2007), confirming the presence of "bird-like" hemoglobin and collagen with no evidence of contamination found in the samples. Thus, in each of these studies, there is no evidence that the detected collagen was of bacterial origin.

Actin and Tubulin

Other proteins have also been detected in dinosaur specimens. Schweitzer at al. (2013) detected proteins such as actin and tubulin. In eukaryotic cells, actin and tubulin serve as cytoskeletal proteins. Bacteria are also known to possess a cytoskeleton partly comprised of MreB and ParM—proteins that have a three-dimensional structure similar to that of actin but only a weak amino-acid-sequence similarity (Carballido-López, 2006). Bacteria also produce a protein (FtsZ) that has some tubulin-like functions and properties, but again, the amino-acid similarity with eukaryotic tubulin is very low (van den Ent et al., 2001).

There is no indication that any of these bacterial proteins (MreB, ParM, and FtsZ) would react with the immunoassay used by Schweitzer et al. (2013). In fact, these researchers analyzed both bacterial and surrounding soil samples and detected no proteins with the same antibody affinity as the actin and tubulin extracted from the pliable tissue. Thus, there is no evidence that the researchers misidentified bacterial proteins as animal actin or tubulin. Bacterial biofilms fail to offer an appropriate explanation for the presence of these proteins. This further weakens challenges that the biomolecules are bacterial contamination rather than original dinosaur proteins.

Original Tissue

As increased care has been taken to eliminate possible contamination and to account for non-dinosaur biomolecules, the results of recent studies have consistently shown (1) the presence of pliable tissue containing intact bone cells with detailed structures, (2) the presence of biomolecules, such as collagen, that appear to be original to the fossil, (3) a complete absence of evidence for bacterial biofilms mimicking tissue or dinosaur collagen, and (4) the lack of significant evidence that either the pliable tissue or the biomolecules it contains are from a contaminating source.

As a body of work, the evidence for unfossilized tissue in dinosaur fossils is significant. Attempts to suggest the tissue is contamination, biofilm, or an error of analysis can no longer be viewed as viable. That this pliable material is original dinosaur tissue sets in motion the need for explanations of its remarkable preservation. Several models have been proposed, all with the clear objective of explaining how otherwise fairly labile biological material could withstand degradation processes for multimillions of years. So far, all explanations offered fail to provide an adequate explanation (see DeMassa and Boudreaux, 2015, and Thomas, 2015 for more extensive discussion). This leaves the alternative—that dinosaur fossils are far younger than millions of years of age—as the most viable and consistent interpretation.

Acknowledgments

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Addendum

Butler et al. (2015) have recently added to the pseudomorphoring model presented above. They note that the best preservation of soft-body parts is frequently abdominal tissue. Hence, they speculate this preservation may be a consequence of intestinal bacterial activity. Studying the decay of shrimp, the authors conclude that under specific conditions the intestinal bacteria can produce biofilms that form molds of abdominal tissue. These biofilm molds are then gradually fossilized, enabling detailed features of the shrimp's soft interior body parts (which are normally degraded quickly) to be preserved in a fossilized state. However, as mentioned above, this pseudomorphoring mechanism does not account for the prolonged preservation of tissue in a pliable, unfossilized form. Therefore, this model does not address the pliable tissue described by the iDINO project.

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