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RESEARCH ARTICLE

Preferential attachment and colonization of the keratinolytic bacterium *Bacillus licheniformis* on black- and white-striped feathers

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ABSTRACT

Feathers serve numerous functions, from flight to interspecific and intraspecific communication. Melanin has been shown to protect feathers from microbial degradation that might, for example, hinder flight or mate attraction. Most studies have focused on the physical resistance to degradation that melanin provides. However, it has yet to be addressed whether melanin alters bacterial colonization and attachment patterns before degradation. We used the common keratinolytic bacterium *Bacillus licheniformis* to test for preferential attachment and colonization on feathers with black (melanized) and white (unmelanized) stripes. Using experimental inoculation of Eurasian Three-toed Woodpecker (*Picoides tridactylus*) feathers in vitro and scanning electron microscopy, we show that *B. licheniformis* preferentially colonizes white feather stripes nearly twice as often as black feather stripes. These data suggest that melanin, in addition to strengthening feathers, may inhibit colonization of keratinolytic bacteria, with possible implications regarding the mechanisms of exceptional preservation of feathers and melanin in the fossil record.

Keywords: attachment, *Bacillus licheniformis*, colonization, feather-degrading bacteria, melanin, melanosome, striped feather, taphonomy.

Fijación preferencial y colonización de la bacteria queratinolítica *Bacillus licheniformis* en plumas a rayas blancas y negras

RESUMEN

Las plumas tienen numerosas funciones, desde el vuelo hasta la comunicación interespecífica e intraespecífica. Se ha demostrado que la melanina protege las plumas de la degradación microbiana que podría, por ejemplo, impedir el vuelo o la atracción de parejas. La mayoría de los estudios se han enfocado en la resistencia física que brinda la melanina a la degradación. Sin embargo, todavía debe evaluarse si la melanina altera la colonización bacteriana y los patrones de fijación antes de la degradación. En este estudio usamos a la bacteria queratinolítica común *Bacillus licheniformis* para evaluar la fijación preferencial y la colonización en plumas a rayas negras (con melanina) y blancas (sin melanina). Por medio de la inoculación experimental *in vitro* de plumas de *Picoides tridactylus* y microscopía de barrido electrónico, mostramos que *B. licheniformis* coloniza preferencialmente las rayas blancas de las plumas casi el doble de veces que las rayas negras de las plumas. Estos datos sugieren que la melanina, además de fortalecer las plumas, podría inhibir la colonización de bacterias queratinolíticas y tener implicancias en los mecanismos de preservación excepcional de plumas y melanina en el registro fósil.

Palabras clave: Bacillus licheniformis, bacterias degradadoras de plumas, colonización, fijación, melanina, melanosoma, pluma rayada, tafonomía

INTRODUCTION

Feathers serve numerous essential functions for birds, from flight, signaling, and camouflage to thermoregulation (for a review, see McGraw 2006). Abrasion or degradation of feathers can reduce their effectiveness in carrying out these integral functions. In particular, flight feathers weakened by physical abrasion can limit a bird's ability to escape predators or catch prey (Bonser 1995), and degradation of the keratin cortex by keratinolytic bacteria can potentially alter the observed color of a feather and, in turn, the bird's reproductive success (Shawkey et al. 2007, Gunderson et al. 2009). For these reasons, the maintenance of feathers to preserve their structural and visual integrity is an essential behavior that can directly affect fitness. Molting, preening (with uropygial oil), dusting, and sunning are all active methods that birds use to combat keratinolytic bacteria that may colonize and degrade their feathers (Hillgarth and Wingfield 1997, Shawkey et al. 2003, Ruiz-Rodríguez et al. 2009, Kent and Burtt 2016).

Some pigments deposited in feathers, such as melanin and psittacofulvins, have previously been shown to aid in resistance to degradation by keratinolytic bacteria (Goldstein et al. 2004, Gunderson et al. 2008, Burtt et al. 2010, Ruiz-de-Castañeda et al. 2012), but the mechanism for this resistance is currently unknown. Keratinolytic bacteria, such as *Bacillus licheniformis*, degrade the β-keratin matrix of a feather by secreting the hydrolytic enzyme keratinase (Santos et al. 1996, Ruiz-Rodríguez et al. 2009). In vitro, black feathers containing a high concentration of eumelanin (a ubiquitous chemical variant of melanin that produces black and brown colors) are more resistant than white feathers to bacterial degradation by *B. licheniformis* (Goldstein et al. 2004). Similarly, melanized areas within individual feathers are more resistant to bacterial degradation than unmelanized areas (Ruiz-de-Castañeda et al. 2012). Melanin may enhance resistance by thickening the feather cortex or by making the keratin matrix more difficult to break down (Goldstein et al. 2004, Gunderson et al. 2008). Additionally, melanin and melanosomes are known to inhibit bacterial and fungal growth in mammal integument (Mackintosh 2001) and may directly affect keratinolytic bacteria in birds (Gunderson et al. 2008).

Whether melanized feathers are more difficult to degrade because melanin enhances their toughness or their antimicrobial activity is unclear, because all experimental studies thus far have examined feathers only after degradation is complete. Attachment is the first step in the degradation process, and thus some natural materials have evolved defenses, such as nanospheres, on the surface of eggshells that prevent bacterial adhesion (D'Alba et al. 2014, 2016). Melanin's known antimicrobial properties (Mackintosh 2001) suggest that it may serve a similar function in feathers, but this has not previously been tested. Here, we test this hypothesis by experimentally inoculating *B. licheniformis* onto striped feathers in vitro, predicting that the bacteria will preferentially attach to and colonize unmelanized white stripes over melanized black stripes. We also discuss potential mechanisms that may affect bacterial attachment to feathers, and we assess the implications for pre-burial taphonomic processes that may have occurred prior to the exceptional preservation of fossil feathers.

METHODS

Feather Preparation

Forty-four black- and white-striped feathers were plucked from the back of study skins of male and female Eurasian Three-toed Woodpeckers (*Picoides tridactylus*) from the University of Akron collections. The feathers were cut to approximately equal sizes (0.5 cm) so that 22 of the samples had a white region at the apical end and black at the base, while the other 22 had a black region at the apical end and white at the base. This was done to avoid any differences in keratin thickness, such as the wider circumference of keratin at the base as opposed to the tip of the feather, and to make the size of white and black regions similar. We took pictures of each feather using a binocular microscope (Leica S8AP0) as references for the location of melanized regions on each feather.

Although previous authors have autoclaved feathers used in bacterial degradation experiments (Grande et al. 2004), autoclaving may degrade feather keratin and influence the activity of feather-degrading bacteria (Gunderson et al. 2008). We instead removed excess dust and other particles by washing the feathers in deionized water and then cleaned them with 100% ethanol. Our subsequent analyses of control feathers (sham inoculated; see below) did not contain any bacteria, confirming that this method of sterilization was successful in removing any potential microbes from the surface of the feather as well as from between the barbs and barbules.

Bacteria Preparation

We used the keratinolytic bacterium *B. licheniformis* as a model organism for the study of preferential colonization of striped feathers. It is the most common feather-degrading bacterium, at times representing over 80–90% of a feather's bacterial load (Burtt and Ichida 1999). We grew *B. licheniformis* strain 138B (Burtt and Ichida 1999) on sterile tryptic soy agar (TSA) source plates for 48 hr. Twenty sealable test tubes containing 2 mL each of tryptic soy broth (TSB) were inoculated with 20 μL of bacteria from the source plates and incubated overnight in a shaking incubator at 37°C and 180 rpm. Then 2 mL of fresh TSB was mixed into each of the 20 inoculated tubes, using a vortex mixer to create a 1:1 solution of *B. licheniformis* and TSB; the tubes were then placed back in the shaking incubator for 4 hr using the same settings. This 4 hr period for bacterial growth falls within the projected optimal growth period for *B. licheniformis* (Frankena et al. 1985), and the bacteria should feed more readily on feathers during this period. The 4 hr tubes were emptied into a sterile graduated cylinder and combined with equal parts 0.9% saline solution to create a homogeneous 1:1 mixture. The saline was added to increase the likelihood that the bacteria would attach to and degrade the feathers rather than remain in solution in the nutrient-rich TSB. The absorbance of this mixture was tested using a 96-well spectrophotometer at a 530 nm wavelength of light with a SpectraMax Plus 384 Microplate Reader. We obtained an absorbance value of 0.805, which correlated to ~6,400 colonies of *B. licheniformis* (based on our previously established standard; see Appendix Table 1).

Experiment

Forty of the sampled feathers (20 with white at the apical end and 20 with black at the apical end) were sterilized and

placed into separate sterile glass test tubes and inoculated with 2 mL of the 1:1 *B. licheniformis* and saline solution. The samples were placed in the shaking incubator for 18 hr at 37°C and 180 rpm. Previous studies have reported that incubation times >18 hr led to the total degradation of feathers (e.g., Goldstein et al. 2004, Grande et al. 2004, Ruiz-de-Castañeda et al. 2012), whereas times <18 hr led to minimal attachment (Ramnani et al. 2005). We then washed the feathers using a 5-stage ethanol rinse to remove any biofilm and unattached bacteria from the feathers, leaving only bacteria that were securely adhered (Dunne 2002). The feathers were washed at 25%, 50%, 75%, and 100% ethanol for 5 min each, then at 100% ethanol for 10 min. One sample with a white base was inadvertently contaminated during the serial washing process and was therefore excluded from analysis. Samples were dried in an incubator at 37°C, then mounted with carbon tape on aluminum stubs and sputter-coated for 3 min with gold-palladium using a Polaron E5000 Sputter-Coater (Quorum Technologies, Laughton, UK) for environmental scanning electron microscopy.

We prepared 2 additional sets of uninoculated control feathers, each consisting of 2 feathers with opposite color patterns, that were sterilized using the same methods as the experimental feathers. "Wet" control feathers were submerged in 2 mL of a 1:1 TSB and then 0.9% sterile saline solution mix. The samples were then incubated for 18 hr at 37°C and 180 rpm and washed using the same 5-stage ethanol wash procedure as experimental samples. The "dry" control samples were cut and washed in 100% ethanol, then directly placed on aluminum stubs and sputter-coated.

Electron Microscopy

We used an FEI Quanta 200 environmental scanning electron microscope (ESEM) at the University of Akron. Each image was taken under high vacuum at a standard working distance of 10 mm, with magnification 12,000×, spot size 4.5, and voltage 30 kV. The feather samples were chosen at random and imaged blindly so that the melanized and unmelanized regions could not be distinguished from one another during sampling. Four images were taken of the rachis of each sample. One image each was taken from the base, the lower central section, the upper central section, and the apical end of the rachis to ensure inclusion of both melanized and unmelanized sections on each feather. Three images were taken at the basal, middle, and apical sections of 2 barbs, 1 each on the right and left halves of the feather (Figure 1). We chose barbs that ran the full length of the feather with the assumption that these barbs would contain both melanized and unmelanized sections. Sampling locations within each region were selected at random. Additionally, we took ESEM images at lower magnification at each sampling site to later cross-reference with optical microscope images for color identification (see below).

Analysis

We counted the number of bacteria in each high-magnification ESEM image and then determined the color of each sampling location by comparing the low-magnification ESEM images to optical images of each feather. Individual barbules were counted for each ESEM and microscope image to determine the precise location and color of each sampled area. We also selected the total surface area of the feather available for attachment in each image using Image J software (National Institutes of Health; http://rsb.info.nih.gov/nih-image/index.html) and calculated density as the number of bacteria per square micron. We measured bacterial density to account for the increased surface area in the lower and middle sections of the feathers in relation to the surface area of single barbs near the apical end of the feather.

To test whether bacteria preferentially attached to certain colors or locations on the feathers, we used a zero-inflated Poisson model in R for bacterial cell count (R Development Core Team 2007). The Poisson model was zero-inflated to account for the large number of sampling sites that did not have attached bacterial cells. Sampling location was separated into 3 general regions on each feather: (1) top, which included the apical one-third of the feather; (2) middle, which included the central one-third; and (3) bottom, which included the basal one-third. We also recorded whether bacteria were present in the rachis or the barbs of each feather. Our models included bacterial density as response variable, and sampling location, region color, and feather structure (rachis or barb) as explaining factors.

RESULTS

Color and location significantly affected the number of bacterial cells attached to the feather. Of the 390 feather regions imaged, 206 were white and 184 were black. Overall, bacterial cells were attached to approximately twice as many white regions (67) as black regions (35), and there were approximately twice as many individual *B. licheniformis* attached to white stripes (1,569 cells) as to black stripes (879 cells; Figure 2). *Bacillus licheniformis* preferentially colonized white over black stripes ($z = 3.2$, $P = 0.001$). Bacterial count was also significantly lower toward the base ($n = 156$ images) than at the middle ($n = 78$ images, $z = -2.9$, $P = 0.004$) and top of the feather ($n = 156$ images, $z = -2.7$, $P = 0.006$). Density calculations of each section indicated that the base of the feather supported fewer bacteria (average density: top, 0.0172 ± 0.0041 μm^{-2}; middle, 0.0168 ± 0.0047 μm^{-2}; base, 0.0112 ± 0.0044 μm^{-2}). Additionally, there was no difference

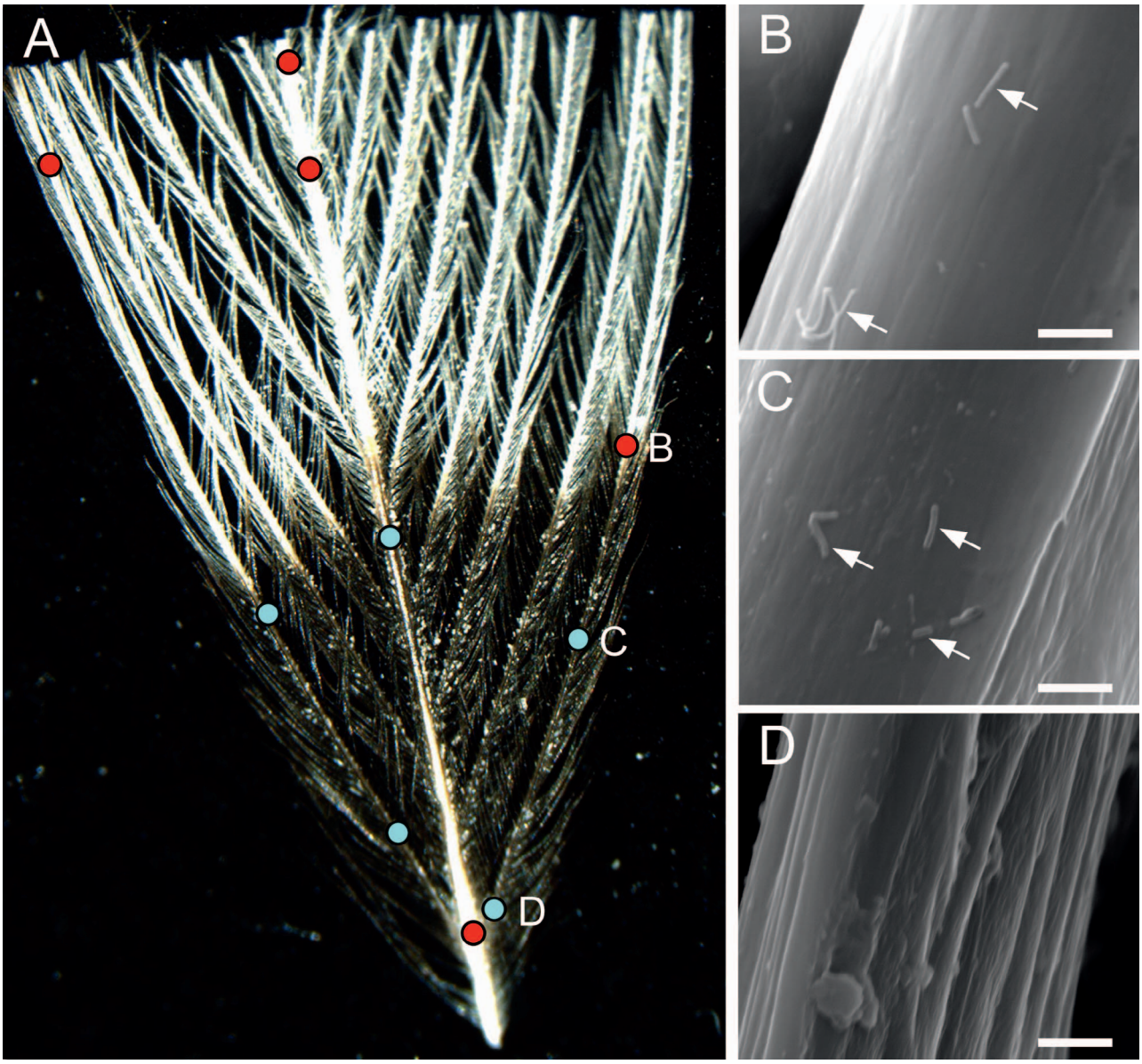

FIGURE 1. Colonization of *Bacillus licheniformis* on a white- and black-striped Eurasian Three-toed Woodpecker feather. (**A**) Light microscope image detailing the environmental scanning electron microscope (ESEM) imaging locations on 1 of the 39 feathers analyzed. Red circles indicate sampling locations in unmelanized regions, and blue circles indicate sampling locations in melanized regions. (**B**–**D**) ESEM images corresponding to similarly labeled sampling locations on the feather; B and C show *B. licheniformis* cells (arrow), whereas D shows no bacterial cells. Scale bars = 5 μm.

between the numbers of bacteria attached to the barbs of each feather vs. the rachis ($z = 1.1$; $P = 0.27$).

DISCUSSION

Previous studies have shown that keratinolytic bacteria degrade unmelanized white feathers more quickly than melanized black feathers (Goldstein et al. 2004, Gunderson et al. 2008, Ruiz-de-Castañeda et al. 2012). However, the mechanisms by which this occurs and the roles that melanin plays are unclear. Our results show that the common keratinolytic bacterium *B. licheniformis* preferentially attaches to and colonizes unmelanized white stripes of a single feather nearly twice as often as melanized black stripes in vitro. Indeed, *B. licheniformis* attached to nearly twice as many unmelanized regions, with twice the number of individual cells as unmelanized regions. It is possible that the bacteria initially colonized both feather regions equally, then reproduced more quickly in the white regions because of the lack of melanin

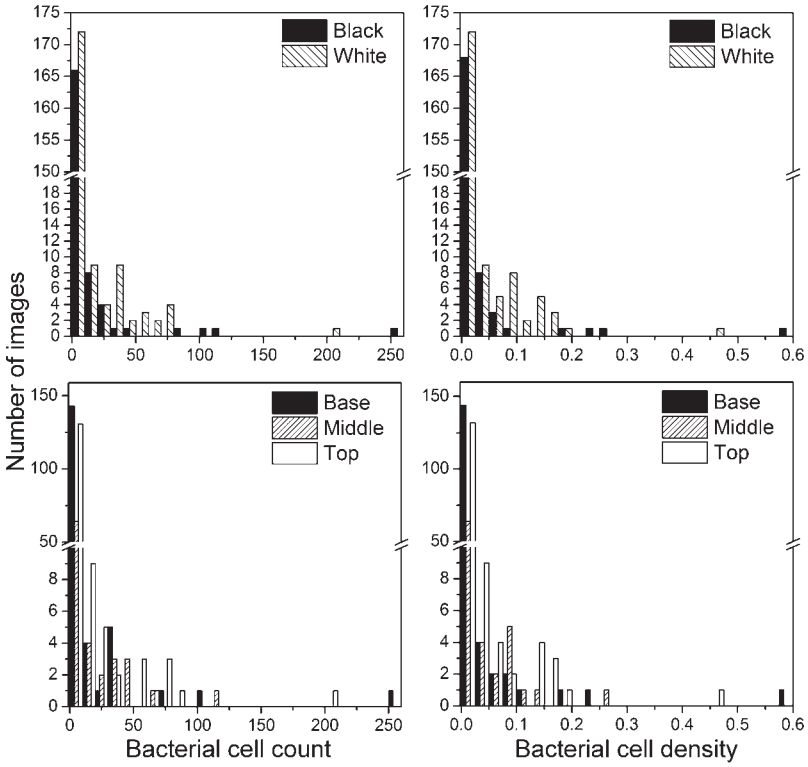

FIGURE 2. Histograms comparing bacterial cell count and cell density between black and white feather regions and location on the feather.

and therefore greater availability of keratin. However, given our short incubation time and scant evidence of keratin degradation, it is more likely that the bacteria initially preferentially colonized the unmelanized regions. We also used density of bacteria to ensure that this effect was not due to changing surface area over the feather (i.e. thinner feather tip vs. wider feather base). Our results show that *B. licheniformis* is more likely to colonize the tip of a feather than the base. However, in vivo colonization may differ from our experimental results, because feathers often contain a greater diversity of bacteria than a single species (Burtt and Ichida 2004, Shawkey et al. 2005, Kent and Burtt 2016). Additionally, our feathers were experimentally inoculated in solution rather than through contact with soil, as would occur in vivo (Burtt and Ichida 1999). Therefore, we cannot rule out the possibility that the range of other bacteria found on feathers of birds in their natural environment and differing inoculation methods may affect the colonization and degradation of the feathers (Czirják et al. 2013).

Preferential colonization on unmelanized areas may partially explain the greater degree of bacterial degradation of unmelanized vs. melanized feathers (Santos et al. 1996, Ruiz-Rodríguez et al. 2009). If bacteria attach more quickly and easily to unmelanized feathers, they should degrade them more quickly. The presence of melanin may thus reduce the number of keratinolytic bacteria that adhere to feathers, whether attached to live birds or molted, through several possible mechanisms. Melanin may indirectly affect the ability of a bacterium to attach to a feather, such as by increasing the roughness of the keratin surface. Nanoscale differences in roughness can have a significant negative impact on bacterial adherence and ability to form biofilms (Mitik-Dineva et al. 2009, Singh et al. 2011). However, we saw no obvious difference in roughness between the different feather locations or between black and white sections of the feather, although these regions exhibit some texture. The presence of melanosomes may also reduce colonization by making feather keratin thicker and harder, or by influencing the surface chemistry. This is accomplished by increasing the number of disulfide bonds present, which also makes these bonds more difficult to break down by bacterial keratinases during degradation (Goldstein et al. 2004, Ramnani et al. 2005).

Melanin may also directly interact with bacteria. For example, because melanosomes are lysosomal organelles (Raposo and Marks 2002), melanized feather regions may contain lysosomal enzymes that could interfere with bacterial processes and make feathers less suitable for bacterial proliferation (Burkhart and Burkhart 2005). Melanin may also inhibit degradation by actively binding keratinases secreted by bacteria and limiting their ability to

break down feather keratin (Gunderson et al. 2008). Indeed, there is evidence that melanin decreases the effect of bacterial proteases on fungi, including those of *B. subtilis* (Kuo and Alexander 1967). Melanin may thus help slow degradation through prevention of both bacterial colonization and growth. Additionally, melanin may directly affect a bacterial cell's ability to attach to and degrade a feather via electrostatic repulsion. Melanin is highly negatively charged, and melanization of fungal cells has been shown to increase their negative charge, making them more resistant to phagocytosis by, for example, macrophages (Nosanchuk and Casadevall 1997). Melanization of feathers may similarly increase the negative charge of the keratin matrix, particularly if the melanosomes are located close to the surface of the feather (i.e. just below the outer keratin cortex), as in glossy or iridescent feathers (Maia et al. 2010). It is unlikely, however, that these direct methods could affect the attachment or colonization of bacteria on the surface of the feather prior to degradation, because the bacteria are not in direct contact with the melanin or melanosomes. These methods may, however, affect growth of bacteria after attachment. Whether direct or indirect, all these potential mechanisms could limit bacterial colonization and degradation of feathers, regardless of the latter's environment (i.e. attached to a live bird or molted). Further experiments should test these mechanistic hypotheses.

Our experiment has additional implications regarding the pre-burial taphonomy of fossilized feathers. Although preservation of feathers in terrestrial *Konservat-Lagerstätten*—deposits with soft-tissue preservation—is not uncommon (Davis and Briggs 1995, Schweitzer 2011), our understanding of feather taphonomy is limited. Previous studies have shown that melanin (Colleary et al. 2015, Lindgren et al. 2015) and melanosomes (Vinther et al. 2008, Clarke et al. 2010, Li et al. 2010, 2012, 2014, Carney et al. 2012, Field et al. 2013, Huang et al. 2016, Peteya et al. 2016) are preserved in fossil feathers, but few have examined the potential effects of bacteria on pre-burial degradation of feathers (see Moyer et al. 2014). Indeed, feather-degrading bacteria were likely involved in the degradation of theropod dinosaur integument. Our results and those of others (Goldstein et al. 2004, Gunderson et al. 2008, Ruiz-de Castañeda et al. 2012) suggest that eumelanin inhibits the colonization and degradation of feather keratin by bacteria. White feather stripes or whole white feathers therefore would have been more susceptible to bacterial colonization and degradation than melanized feathers prior to burial.

We have shown that keratinolytic bacteria preferentially colonize unmelanized white regions of striped feathers over melanized regions. The processes that occur prior to the degradation of a feather by keratinolytic bacteria are largely uncharacterized. Examining these processes is essential to understanding feather degradation by keratinolytic bacteria; the mechanism whereby melanin inhibits bacterial degradation; the pre-burial processes that may have affected the preservation of feathers in the fossil record; and avian ecology.

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LITERATURE CITED

Bonser, R. H. C. (1995). Melanin and the abrasion resistance of feathers. The Condor 97:590–591.

Burkhart, C. G., and C. N. Burkhart (2005). The mole theory: Primary function of melanocytes and melanin may be antimicrobial defense and immunomodulation (not solar protection). International Journal of Dermatology 44:340–342.

Burtt, E. H., Jr., and J. M. Ichida (1999). Occurrence of feather-degrading bacilli in the plumage of birds. The Auk 116:364–372.

Burtt, E. H., Jr., and J. M. Ichida (2004). Gloger's rule, feather-degrading bacteria, and color variation among Song Sparrows. The Condor 106:681–686.

Burtt, E. H., Jr., M. R. Schroeder, L. A. Smith, J. E. Sroka, and K. J. McGraw (2010). Colourful parrot feathers resist bacterial degradation. Biology Letters 7:214–216.

Carney, R. M., J. Vinther, M. D. Shawkey, L. D'Alba, and J. Ackermann (2012). New evidence on the colour and nature of the isolated *Archaeopteryx* feather. Nature Communications 3:637.

Clarke, J. A., D. T. Ksepka, R. Salas-Gismondi, A. J. Altamirano, M. D. Shawkey, L. D'Alba, J. Vinther, T. J. DeVries, and P. Baby (2010). Fossil evidence for evolution of the shape and color of penguin feathers. Science 330:954–957.

Colleary, C., A. Dolocan, J. Gardner, S. Singh, M. Wuttke, R. Rabenstein, J. Habersetzer, S. Schaal, M. Feseha, M. Clemens, B. F. Jacobs, et al. (2015). Chemical, experimental, and morphological evidence for diagenetically altered melanin in exceptionally preserved fossils. Proceedings of the National Academy of Sciences USA 112:12592–12597.

Czirják, G. Á., P. L. Pap, C. I. Vágási, M. Giraudeau, C. Mureşan, P. Mirleau, and P. Heeb (2013). Preen gland removal increases plumage bacterial load but not that of feather-degrading bacteria. Naturwissenschaften 100:145–151.

D'Alba, L., D. N. Jones, H. T. Badawy, C. M. Eliason, and M. D. Shawkey (2014). Antimicrobial properties of a nanostructured eggshell from a compost-nesting bird. Journal of Experimental Biology 217:1116–1121.

D'Alba, L., R. Maia, M. E. Hauber, and M. D. Shawkey (2016). The evolution of eggshell cuticle in relation to nesting ecology. Proceedings of the Royal Society B 283:20160687.

Davis, P. G., and D. E. G. Briggs (1995). Fossilization of feathers. Geology 23:783–786.

Dunne, W. M., Jr. (2002). Bacterial adhesion: Seen any good biofilms lately? Clinical Microbiology Reviews 15:155–166.

Field, D. J., L. D'Alba, J. Vinther, S. M. Webb, W. Gearty, and M. D. Shawkey (2013). Melanin concentration gradients in modern and fossil feathers. PLoS ONE 8:e59451.

Frankena, J., H. W. van Verseveld, and A. H. Stouthamer (1985). A continuous culture study of the bioenergetic aspects of growth and production of exocellular protease in *Bacillus licheniformis*. Applied Microbiology and Biotechnology 22: 169–176.

Goldstein, G., K. R. Flory, B. A. Browne, S. Majid, J. M. Ichida, and E. H. Burtt, Jr. (2004). Bacterial degradation of black and white feathers. The Auk 121:656–659.

Grande, J. M., J. J. Negro, and M. J. Torres (2004). The evolution of bird plumage colouration: A role for feather-degrading bacteria? Ardeola 51:375–383.

Gunderson, A. R., M. H. Forsyth, and J. P. Swaddle (2009). Evidence that plumage bacteria influence feather coloration and body condition of Eastern Bluebirds *Sialia sialis*. Journal of Avian Biology 40:440–447.

Gunderson, A. R., A. M. Frame, J. P. Swaddle, and M. H. Forsyth (2008). Resistance of melanized feathers to bacterial degradation: Is it really so black and white? Journal of Avian Biology 39:539–545.

Hillgarth, N., and J. C. Wingfield (1997). Testosterone and immunosuppression in vertebrates: Implications for parasite-mediated sexual selection. In Parasites and Pathogens (N. E. Beckage, Editor). Chapman & Hall, New York, NY, USA.

Huang, J., X. Wang, Y. Hu, J. Liu, J. A. Peteya, and J. A. Clarke (2016). A new ornithurine from the Early Cretaceous of China sheds light on the evolution of early ecological and cranial diversity in birds. PeerJ 4:1765.

Kent, C. M., and E. H. Burtt, Jr. (2016). Feather-degrading bacilli in the plumage of wild birds: Prevalence and relation to feather wear. The Auk: Ornithological Advances 133:583–592.

Kuo, M.-J., and M. Alexander (1967). Inhibition of lysis of fungi by melanins. Journal of Bacteriology 94:624–629.

Li, Q., J. A. Clarke, K. Gao, C. Zhou, Q. Meng, D. Li, L. D'Alba, and M. D. Shawkey (2014). Melanosome evolution indicates a key physiological shift within feathered dinosaurs. Nature 507: 350–353.

Li, Q., K. Gao, Q. Meng, J. A. Clarke, M. D. Shawkey, L. D'Alba, R. Pei, M. Ellison, M. A. Norell, and J. Vinther (2012). Reconstruction of *Microraptor* and the evolution of iridescent plumage. Science 335:1215–1219.

Li, Q., K. Gao, J. Vinther, M. D. Shawkey, J. A. Clarke, L. D'Alba, Q. Meng, D. E. G. Briggs, and R. O. Prum (2010). Plumage color patters on an extinct dinosaur. Science 327:1369–1372.

Lindgren, J., P. Sjövall, R. M. Carney, A. Cincotta, P. Uvdal, S. W. Hutcheson, O. Gustafsson, U. Lefèvre, F. Escuillié, J. Heimdal, A. Engdahl, et al. (2015). Molecular composition and ultrastructure of Jurassic paravian feathers. Scientific Reports 5:13520.

Mackintosh, J. A. (2001). The antimicrobial properties of melanocytes, melanosomes and melanin and the evolution of black skin. Journal of Theoretical Biology 211:101–113.

Maia, R., L. D'Alba, and M. D. Shawkey (2010). What makes a feather shine? A nanostructural basis for glossy black colours in feathers. Proceedings of the Royal Society B 278:1973–1980.

McGraw, K. J. (2006). Mechanics of melanin-based coloration. In Bird Coloration, vol. 1: Mechanisms and Measurements (G. E. Hill and K. J. McGraw, Editors). Harvard University Press, Cambridge, MA, USA.

Mitik-Dineva, N., J. Wang, V. K. Truong, P. Stoddart, F. Malherbe, R. J. Crawford, and E. P. Ivanova (2009). *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* attachment patterns on glass surfaces with nanoscale roughness. Current Microbiology 58:268–273.

Moyer, A. E., W. Zheng, E. A. Johnson, M. C. Lamanna, D. Li, K. J. Lacovara, and M. H. Schweitzer (2014). Melanosomes or microbes: Testing an alternative hypothesis for the origin of microbodies in fossil feathers. Scientific Reports 4:4233.

Nosanchuk, J. D., and A. Casadevall (1997). Cellular charge of *Cryptococcus neoformans*: Contributions from the capsular polysaccharide, melanin, and monoclonal antibody binding. Infection and Immunity 65:1836–1841.

Peteya, J. A., J. A. Clarke, Q. Li, K. Gao, and M. D. Shawkey (2016). The plumage and colouration of an enantiornithine bird from the Early Cretaceous of China. Palaeontology 60:55–71.

Ramnani, P., R. Singh, and R. Gupta (2005). Keratinolytic potential of *Bacillus licheniformis* RG1: Structural and biochemical mechanism of feather degradation. Canadian Journal of Microbiology 51:191–196.

Raposo, G., and M. S. Marks (2002). The dark side of lysosome-related organelles: Specialization of the endocytic pathway for melanosome biogenesis. Traffic 3:237–248.

R Development Core Team (2007). R: A Language for Data Analysis and Graphics. Vienna, Austria. http://cran.r-project.org/

Ruiz-de-Castañeda, R., E. H. Burtt, Jr., S. González-Braojos, and J. Moreno (2012). Bacterial degradability of an intrafeather unmelanized ornament: A role for feather-degrading bacteria in sexual selection? Biological Journal of the Linnean Society 105:409–419.

Ruiz-Rodríguez, M., E. Valdivia, J. J. Soler, M. Martín-Vivaldi, A. M. Martín-Platero, and M. Martínez-Bueno (2009). Symbiotic bacteria living in the hoopoe's uropygial gland prevent feather degradation. The Journal of Experimental Biology 212:3621–3626.

Santos, R. M., A. A. Firmino, C. M. de Sá, and C. R. Felix (1996). Keratinolytic activity of *Aspergillus fumigatus fresenius*. Current Microbiology 33:364–370.

Schweitzer, M. H. (2011). Soft tissue preservation in terrestrial Mesozoic vertebrates. Annual Review of Earth and Planetary Sciences 39:187–216.

Shawkey, M. D., K. L. Mills, C. Dale, and G. E. Hill (2005). Microbial diversity of wild bird feathers revealed through culture-based

and culture-independent techniques. Microbial Ecology 50: 40–47.

Shawkey, M. D., S. R. Pillai, and G. E. Hill (2003). Chemical warfare? Effects of uropygial oil on feather-degrading bacteria. Journal of Avian Biology 34:345–349.

Shawkey, M. D., S. R. Pillai, G. E. Hill, L. M. Siefferman, and S. R. Roberts (2007). Bacteria as an agent for change in structural plumage color: Correlational and experimental evidence. The American Naturalist 169 (Supplement 1):S112–S121.

Singh, A. V., V. Vyas, R. Patil, V. Sharma, P. E. Scopelliti, G. Bongiorno, A. Podestà, C. Lenardi, W. N. Gade, and P. Milani (2011). Quantitative characterization of the influence of the nanoscale morphology of nanostructured surfaces on bacterial adhesion and biofilm formation. PLoS ONE 6:e25029.

Vinther, J., D. E. G. Briggs, R. O. Prum, and V. Saranathan (2008). The colour of fossil feathers. Biology Letters 4:522–525.

APPENDIX

Additional Methods

Prior to experimentation, we calculated absorbance values based on serial dilutions of *B. licheniformis* to determine the number of colony-forming units (CFU) that inoculated our feather samples. We incubated 0.01 mL of bacteria in 2 mL of sterile TSB overnight at 37°C. From this source tube, we took 2 mL bacteria and mixed it with 2 mL TSB (1:1 solution). The 1:1 bacteria:saline solution was incubated for 4 hr at 37°C, which falls within the optimal growth period, and therefore the optimal feeding period, for *B. licheniformis* (Frankena et al. 1985). After 4 hr, the 1:1 tube was removed from the incubator and mixed thoroughly with a Vortex-Genie mixer. We created 10 dilutions with 2 replicates each, using a homemade 0.9% saline solution. We made the saline solution using deionized water and a fine sea salt lacking iodide and anticaking agent, which were mixed thoroughly with heat until dissolved, then autoclaved for 15 min at 121°C. The first dilution included 2 mL of the bacterial solution and 2 mL of sterile saline (1:1). Then 2 mL of this solution was mixed with 2 mL of saline in a second tube, which generated a 1:2 bacteria:saline solution. The process was repeated for the remaining tubes, generating dilutions of 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, and 1:512.

To calculate the absorbance values, 600 μL of each dilution was placed in sterile, untreated 96-well microplates for 2 replicates (300 μL per well). Each tube was mixed prior to placement within the wells using the Vortex-Genie mixer. Absorbance values were calculated using a 96-well SpectraMax Plus 384 Microplate Reader with a basic endpoint procedure and a wavelength of 530 nm. Absorbance values between the 2 replicates were averaged.

We used a spread-plating technique to count the bacteria. Twenty sterile plates with TSA were prepared prior to the experiment. To remain consistent between the absorbance calculations and the plate counting, we took 300 μL from each dilution and placed it in the center of each respective plate. Each dilution had 2 replicate plates. The solution was spread evenly across the surface of each plate, using a metal cell spreader and a rotating disk. We heat-sterilized the spreader and let it cool, then placed it on the agar plate and rotated the plate counterclockwise for one full rotation, then sterilized the spreader again and rotated the plate clockwise for one full rotation. This process was repeated for each plate, and then the plates were placed in an incubator for 2.5 hr at 37°C. The number of CFU was counted for each plate separately by N.M.J. and J.A.P. under a dissecting microscope. These results were then averaged for each dilution (Appendix Table 1). The number of CFU for the 1:2 dilution was adjusted by dividing the 8,224 CFU present in the 1:1 solution by 2 to account for lack of bacterial growth on 1 of the 2 replicate plates.

APPENDIX TABLE 1. Results of the serial dilution of *Bacillus licheniformis* strain 138B, including the number of colony-forming units (CFU) and the associated absorbance value by dilution.

Dilution	CFU	Absorbance
1:1	~8,224	0.908
1:2	4,112 [a]	0.520
1:4	2,036	0.349
1:8	1,298	0.163
1:16	1,086	0.109
1:32	830	0.073
1:64	331	0.057
1:128	332	0.057
1:256	2	0.050
1:512	0	0.048

[a] Adjusted value (original value = 1,348 CFU).