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Chemical warfare? Effects of uropygial oil on feather-degrading bacteria

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Anti-microbial activity is a commonly suggested but rarely tested property of avian uropygial oil. Birds may defend themselves against feather-degrading and other potentially harmful bacteria using this oil. We preliminarily identified 13 bacterial isolates taken from the plumage of wild house finches *Carpodacus mexicanus*, measured bacterial production of the enzyme keratinase as an index of feather-degrading activity, and used the disc-diffusion method to test bacterial response to uropygial oil of house finches. For comparison, we performed the same tests on a type strain of the known feather-degrading bacterium *Bacillus licheniformis*. Uropygial oil inhibited the growth of three strongly feather-degrading isolates (including *Bacillus licheniformis*), as well as one weakly feather-degrading isolate and one non-feather-degrading isolate. Uropygial oil appeared to enhance the growth of one weakly feather-degrading isolate. Growth of the remaining isolates was unaffected by uropygial oil. These results suggest that birds may defend themselves against some feather-degrading bacteria using uropygial oil.

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The oily secretions of the uropygial gland of birds preserve feather flexibility, and provide an insulating and waterproofing layer when spread over the feathers (Jacob and Zisweiler 1982). The alkyl-substituted fatty acids and alcohols found in uropygial oil can retard the growth of bacteria and fungi (Jacob and Zisweiler 1982). For example, Jacob et al. (1997) showed that the fatty acid 3,7-dimethyloctan-1-ol found in Pelicaniform preen oil actively inhibited the growth of dermatophyte fungi. It has been postulated (Jacob and Zisweiler 1982, Gill 1995) that these secretions regulate the microbial flora on feathers, but this hypothesis remains virtually untested. Such regulation could be important in maintaining the integrity of feathers, as certain bacteria (e.g. Böckle et al. 1995, Burtt and Ichida 1999, Sangali and Brandelli 2000) and fungi (Santos et al. 1996) have been shown to degrade feathers *in vitro*. Microbial degradation of feathers on wild birds could decrease the insulation, lift (Burtt and Ichida 1999; Clayton 1999) and optical signaling that feathers provide. Feathers may also harbor opportunistic pathogens that could cause disease and infection (Scullion 1989). For these and other reasons, it may be important for birds to control their microbial flora.

The antimicrobial activity of uropygial oil has been tested only a few times. Baxter and Trotter (1969), Pugh and Evans (1970), and Pugh (1972) demonstrated that feather oils inhibited the growth of some keratinophilic fungi, but enhanced the growth of others. Bandyopadhyay and Bhattacharyya (1996) found that domestic fowl uropygial secretions enhanced the growth of the bacteria *Staphylococcus epidermidis*, *Streptomyces* spp. and *Proteus* sp., but inhibited the bacterium *Bacillus anthracis*. All of these bacterial isolates had been taken from the skin of these fowl. Thus, the effects of uropygial oil on microbial communities of birds appear to be complex. Uropygial oil may promote the growth of mutualists that out-compete or otherwise exclude parasitic or pathogenic microbes (Pugh and Evans 1970).

As a first step towards understanding the effects of uropygial secretions on the bacteria of feathers, we preliminarily identified 13 isolates of bacteria from house finches *Carpodacus mexicanus*, measured the feather-degrading activity of each, and used the disc-

diffusion assay (NCCLS 1997) to test the effect of house finch uropygial oil on bacterial growth. For comparison, we also used the type strain *Bacillus licheniformis* O.W.U. 138B (ATCC # 55768). This bacterium is known to degrade feathers *in vitro*, and similar strains have been isolated from house finches (Burtt and Ichida 1999).

Methods

Isolation and identification of bacteria

We captured 29 adult male house finches in mist nets and feeder traps on the campus of Auburn University in Lee County, Alabama (32°35′N, 82°28′W), USA between April 1–July 1, 2002. Wearing latex gloves, we gently rubbed a BBL™ CultureSwab™ (Becton–Dickinson, Sparks, MD, USA) dipped in sterile phosphate-buffered saline (PBS) on the breast, rump, and crown of the birds. The swabs were immediately refrigerated and then washed in 1 ml sterile PBS within 24 hours of collection. We pipetted 100 µl of inoculated PBS onto plates containing feather meal agar (FMA; Sangali and Brandelli 2000;15 g l^{-1} feather meal, 0.5 g l^{-1} NaCl, 0.30 g l^{-1} K_2HPO_4, 0.40 g l^{-1} KH_2PO_4, and 15 g l^{-1} agar), a medium selective for feather-degrading bacteria (Sangali and Brandelli 2000). We then incubated plates at 37°C for two weeks. We isolated several bacteria with distinctive colony morphology from FMA, re-streaked them on Tryptic Soy Agar (TSA, Difco, Sparks, MD, USA), a generalized medium, and then incubated them at 37°C for 48–72 hours until we could determine the purity of our cultures as evidenced by unique colony morphology characteristics.

We grew thirteen bacterial isolates with unique colony morphology overnight at 37°C in tryptose soy broth (Becton–Dickinson, Sparks, Maryland, USA). We extracted DNA from each bacterium with the Instagene DNA purification matrix (Bio-Rad laboratories, Hercules, CA, USA) as previously described (Pillai et al. 2001). We used primers 63F (5′ CAGGCCTAACACATGCAAGTC 3′) and 1389R (5′ACGGGCGGTGTGTACAAG 3′), corresponding to *Escherichia coli* 16SrDNA gene sequence in 50 µl reaction volumes according to the protocol of Osborn et al. (1999). The 16s rDNA PCR products were detected by electrophoresis in 2% agarose gels containing 0.6 ng/ml of ethidium bromide. Agarose gel electrophoresis was run for 3 h at 100 V, and the gels were photographed under ultraviolet illumination using a digital image analysis system (ChemiImager, Alpha Innotech Corporation, San Leandro, CA, USA).

Amplified PCR products from isolates were purified from agarose gels with a QIAquick gel extraction kit (Qiagen Inc., Valencia, CA, USA). These purified gel products were sequenced at the Auburn University Genomics and Sequencing Laboratory using the same primers as above. Automated sequencing was done using the Big Dye Terminator cycle sequencing kit with an ABI Prism 3100 genetic analyzer and associated software (Applied Biosystems, Foster City, CA, USA). Bacterial 16srDNA sequences were deposited in GenBank (accession numbers AY269864 – AY269876), and the BLAST algorithm was used to search for homologous sequences in the GenBank nr database (NCBI, Bethesda, MD, USA). In addition to the isolates taken from house finches, we used *Bacillus licheniformis* strain O.W.U.138B (ATCC # 55768) as a positive control in our studies.

Measurement of keratinase

We tested for feather-degrading activity by measuring the quantity of keratinase produced by each bacterial isolate. This enzyme catalyzes the hydrolysis of keratin (Böckle et al. 1995, Santos et al. 1996) and is thus probably produced in proportion to a bacterium's feather-degrading activity. First, we washed house finch rump feathers three times in distilled water, dried them overnight at 65°C and placed 5 mg of feathers in each of 45 separate tubes. To control for the possibility that differently-colored feathers may be differentially resistant to degradation, we only used feathers taken from birds whose rump hue scored between 9 and 11 (corresponding to an orange-red color) when measured on a Colortron™ portable spectrophotometer (Hill 1998). We autoclaved these feathers for 5 min at 120°C. This sterilization period was kept short to prevent breakdown of feather keratin before addition of the bacteria. We then added 5 ml of sterile PBS (pH 7.3) to each tube.

Colonies of each unique bacterial isolate were grown for 24 h on TSA and added to sterile PBS until a turbidity comparable to McFarland standard # 1.0 (equivalent to about 3×10^8 bacterial cells/ml) was reached. For each isolate, we inoculated three replicate tubes with 200 µl of suspension. These tubes were incubated at 37°C with agitation at 200 rpm (C24 Incubator Shaker, New Brunswick Scientific, Edison Park, NJ, USA) for three weeks. To ensure that our samples were not contaminated, we streaked 10 µl of solution from each tube on TSA at the beginning of the experiment and at the end of each week (four times total) and compared colony morphology to our original plates. No obvious contamination was detected. Following this incubation period, intact feathers were removed using sterile forceps and the remaining solution was filtered through 0.2 µm Durapore membranes (Millipore, Billerica, MA, USA).

Next, we measured keratinase concentration in these samples following the method of Santos et al. (1996). Briefly, we added 2 ml of filtrate from each sample to 4

mg of keratin azure (Sigma, St. Louis, USA) and incubated them at 42°C with constant agitation for 21 h. The release of the blue dye, indicating keratin hydrolysis, was measured using a spectrophotometer (Tecan Rainbow Therma, Tecan U.S., Research Triangle Park, NJ, USA) at 595 nm. Results were expressed as mean units of keratinase per ml, with 1 unit (U) of keratinase defined as the activity required for 1.0 A_{595} increase in 3 h incubation.

Effects of uropygial oil

We sacrificed 46 captive house finches from Auburn, AL, immediately extirpated their uropygial glands and refrigerated them at 4°C for a maximum of one week. All birds were sacrificed under federal banding permit 21661 with Auburn University Institutional Animal Care and Use Committee approval. To ensure that captivity did not affect the properties of uropygial oil, we also sacrificed two wild-caught house finches. After washing the surface of each gland three times with 100% ethanol, we used sterile forceps to squeeze oil onto sterile paper discs (diameter 3 mm; Becton-Dickinson, New Jersey, USA). Because the oil was viscous and difficult to handle, it was not possible to precisely measure the amount placed on each disc. Therefore, we added oil to each side until it was entirely discolored. Most glands contained enough oil to saturate 3–4 paper discs.

These impregnated discs were sterilized under UV radiation for 30 minutes on each side. This procedure was fairly effective, as we only observed contamination (i.e. growth of bacteria around the disk different from that on the rest of the plate) on three out of 149 discs. The plates with these contaminated discs were discarded, and the tests were repeated.

Colonies of each unique bacterial isolate grown for 24 h on TSA were suspended in sterile PBS (pH 7.3) and adjusted to a turbidity comparable to McFarland standard # 0.5 (equivalent to 1.5×10^8 bacterial cells/ml). Mueller–Hinton agar (Difco, New Jersey) plates were then inoculated with these suspensions using sterile cotton swabs.

Each of the 14 bacterial isolates was then tested against oil from five different male and five different female birds (10 birds total). No bacterial isolate was tested against oil from the same bird twice. We placed paper discs saturated with oil from individual birds, as well as one blank control disc, on inoculated plates. No more than eight discs were placed on each plate. Plates were incubated at 37°C for 18 hours and were photographed using a digital image analysis system (ChemImager, Alpha Innotech Corporation, San Leandro, CA). We then measured the diameter of zones of inhibition using AlphaImager software.

Effects of UV sterilization

To ensure that the sterilization process did not affect the outcome of our tests, we repeated the disc diffusion assays described above without UV sterilization, using two individual birds for each isolate (14 isolates × two birds/isolate for a total of 28 disks). Although 54% (15/28) of the discs were clearly contaminated, the results we obtained were similar to those obtained using UV-sterilized discs (see Table 1).

Results

Identification

We identified 13 bacterial isolates from swabs of house finches, with 95–99% matches to known 16srDNA sequences in GenBank in all cases. Identification results are summarized in Table 1. These identifications are preliminary, and are considered accurate only to the genus level.

Keratinase production

Keratinase assay results are shown in Table 1. Isolate 1 produced a quantity of keratinase comparable to that of *B. licheniformis*. Nine other isolates produced smaller amounts of keratinase, while three did not produce any detectable amount of keratinase.

Effects of uropygial oil

Typical results are shown in Fig. 1. Bacteria grew normally around control discs, suggesting that they did not affect bacterial growth. Uropygial oil from every bird tested inhibited isolates 1, 2, 6 and 9, while oil from eight of ten birds inhibited *B. licheniformis* (Table 1). Based on the diameters of zones of inhibition, oil appeared to most strongly inhibit isolate 6. These results should be viewed with caution, however, as we did not control for the amount of oil placed on each disk. Uropygial oil did not inhibit any of the other isolates.

Oil appeared to enhance the growth of isolate 5, as bacterial growth was more dense in the zone surrounding the discs.

Effects of gender

Diameters of zones of inhibition were not significantly different between males and females for any isolate (all $P > 0.5$). Of the two samples that did not inhibit *B. licheniformis*, one was a male and the other was a female.

Table 1. Identification, keratinase production, and inhibition by house finch uropygial oil of 13 bacteria isolated from the feathers of house finches in Auburn, AL. *Bacillus licheniformis* O.W.U.138B (ATCC # 55768) is a type strain with known feather-degrading properties. The species in GENBANK with the closest DNA sequence to each isolate (as determined by the BLAST algorithm) is presented as a preliminary identification. Zone diameter was measured as the area of bacterial clearing around the disk impregnated with preen oil, with the exception of isolate five. Zone diameter for isolate five was measured as the area of enhanced bacterial growth around the disk. The final column contains results of inhibition tests in which the preen oil was not UV-sterilized before use.

Isolate #	Isolated from	Highest BLAST match	Base pairs matched	Keratinase production ($U/ml \times 10^{-3} \pm 1$ SE)	Zone diameter ($mm \pm 1$ SE)	No UV: Zone diameter ($mm \pm 1$ SE)
1	Rump	*Kocuria rhizophila*	567/595 (95%)	4.17 ± 0.45	12.20 ± 0.79	12.00 ± 1.41
2	Rump	*Bacillus* sp.	999/1009 (99%)	3.15 ± 0.53	10.28 ± 0.43	10.03 ± 0.71
3	Rump	*Pseudomonas stutzeri*	1004/1014 (99%)	2.87 ± 0.67	0.00	0.00
4	Rump	*Pseudomonas fulva*	1018/1028 (99%)	2.73 ± 0.32	0.00	0.00
5	Breast	*Micrococcus nishinomyaensis*	1039/1070 (97%)	2.49 ± 0.33	11.00 ± 1.66 (E)	10.8 ± 0.70 (E)
6	Breast	*Staphylococcus epidermidis*	982/1011 (97%)	2.30 ± 0.21	15.12 ± 3.05	13.46 ± 1.50
7	Breast	*Bacillus pumilus*	1039/1060 (98%)	1.58 ± 0.45	0.00	0.00
8	Breast	*Enterococcus fecalis*	1095/1116 (98%)	0.87 ± 0.15	0.00	0.00
9	Crown	*Staphylococcus hemolyticus*	1012/1022 (99%)	0.49 ± 0.30	10.91 ± 0.67	11.23 ± 0.98
10	Breast	*Staphylococcus hominis*	1001/1021 (98%)	0.20 ± 0.10	0.00	0.00
11	Breast	*Bacillus anthracis*	1010/1030 (98%)	0.00 ± 0.11	0.00	0.00
12	Rump	*Bacillus thuringiensis*	1012/1032 (98%)	0.00 ± 0.10	0.00	0.00
13	Breast	*Rothia amarae*	944/991 (95%)	0.00 ± 0.06	0.00	0.00
		Bacillus licheniformis O.W.U.138B	–	3.25 ± 0.30	10.78 ± 0.75	10.42 ± 0.99 10.4 ± 0.84

Fig. 1. Disc diffusion assay tests of house finch uropygial oil against an isolate of the bacterial genus *Staphylococcus* isolated from wild house finches. Bacteria-free zones around the three discs impregnated with uropygial oil indicate inhibition of bacterial growth by the oil. The disc with no zone of inhibition is an unimpregnated control.

The inhibitory properties of this oil thus did not appear to vary with gender.

Effects of captivity

We tested uropygial oil from wild-caught birds against isolates 6 and 8 and *B. licheniformis* and found that, consistent with our results from captive birds, it inhibited the former two isolates but not the latter. Zones of inhibition were 12.32 ± 2.00 for isolate 6, 10.33 ± 1.33 for *B. licheniformis*, comparable to those for captive birds (see Table 1). Thus, captivity did not appear to affect the antimicrobial properties of this oil.

Discussion

Uropygial oil clearly inhibited several isolates, including three that strongly degraded feathers, but did not inhibit other isolates. The anti-microbial agent may be one of the many short-chain fatty acids and alcohols found in preen oil (Jacob and Zisweiler 1982, Jacob et al. 1997). Identifying these anti-microbial agents should be an exciting avenue for future research.

We predicted that growth of harmful bacteria would be inhibited whilst that of neutral or beneficial bacteria would either be unaffected or enhanced. At this point, however, too little data exist on the effects of bacteria on wild birds to test this prediction.

Of the type strain and isolates that were inhibited, three (*B. licheniformis*, isolates 1 and 2) produced large amounts of keratinase. The feather-degrading activity of these isolates has yet to be tested on live birds. The other two inhibited isolates were both of the genus *Staphylococcus*. *S. epidermidis* has also been associated with the

disease bumblefoot in birds (Jennings 1954, Scullion 1989), and species of this genus are known to be opportunistic infectious agents (Keymer 1958a,b). However, these data are too general to conclude that birds specifically defend themselves against *Staphylococcus*. Only the growth of isolate 5 was enhanced by uropygial oil and as far as we are aware, no data exist on the effects of bacteria of the genus *Micrococcus* on birds. No other isolates were affected by uropygial oil. Some *Bacillus* spp. are known to have detrimental effects on mammals (Parker 1984), although data are lacking for birds. *Pseudomonas* spp. have been associated with respiratory tract infections in ostriches (Momotani et al. 1995). *Rothia* spp. have been associated with infections in humans (Salamon and Prag 2002), but not in birds. Thus, at this point we can not say with certainty whether the inhibited bacteria are harmful or whether the non-inhibited bacteria are harmless. More research on the effects of bacteria on wild birds is critically needed.

The study of the interactions between birds and bacteria is barely in its infancy but already we can see the potential for such studies to help us better understand the life histories, ecology and physiology of birds and the distribution and abundance of microbes. Several fundamental lines of investigation are needed. We must determine the effects of isolated bacteria on wild birds. Once we have determined that an inhibited bacteria is harmful, for example, we can say with more certainty that the bird actively defends itself against it using uropygial oil. We can then begin testing the effectiveness of this defense through experimental manipulations. Ultimately, we may find evidence for the co-evolution of microbes or microbial community and the uropygial oil of their avian hosts. Our results here suggest that birds defend themselves against some bacteria using uropygial oil.

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