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Prevalence and genetic diversity of *Bacillus licheniformis* in avian plumage

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ABSTRACT. *Bacillus licheniformis*, a soil bacterium capable of degrading the β-keratin in feathers, is present in the plumage of some wild-caught birds, but the published carriage rate is quite low. Microbial degradation could be a selective agent leading to the evolution of molt and plumage pigmentation, but we hypothesized that for *B. licheniformis* to have played an important role in avian evolution, it is likely to occur more widely in bird populations than previously reported. We sampled the plumage of 461 wild-caught birds of eight species. We designed a selective and differential culture method to isolate bacteria with the potential to degrade feathers. Putative feather-degrading bacteria were isolated from 21–59% of the individuals of each of the species tested, for an average carriage rate of 39%. 16S rRNA (*rrnA*) sequencing of 98 of these bacterial isolates indicated that 69% were *Bacillus lichenformis*, suggesting that the prevalence of this feather-degrading bacterium is 4 × higher than previously reported. We also hypothesized that interspecific variation in avian plumage, behavior, and habitat may have led to the evolution of host-specific *B. licheniformis* strains. Fingerprinting of *B. licheniformis* isolated from Northern Saw-whet Owls (*Aegolius acadicus*) and Gray Catbirds (*Dumetella carolinensis*) using REP-PCR demonstrated that for nine owls, one individual carried four different strains and eight individuals carried only one strain. Of 20 catbirds, there was a single strain found on nine birds, but four carried two different strains each.

SINOPSIS. **Prevalencia y diversidad genética de *Bacillus licheniformis* en el plumaje de aves**
Bacillus licheniformis, es una bacteria que se encuentra en los suelos que es capaz de degradar la keratina-β en la pluma de las aves. La misma está presente en algunas aves silvestres, pero son pocas las publicaciones sobre este tema. La degradación microbiana pudiera ser un agente de selección natural dando origen a la muda y la pigmentación del plumaje en las aves. Planteamos la hipótesis de que esta bacteria ha tenido un rol importante en la evolución de las aves y que debe estar presente en un gran número de especies que lo previamente informado. Examinamos a 461 aves silvestres, perteneciente a ocho especies. Diseñamos un cultivo selectivo y diferencial para aislar la bacteria que tenía el potencial de degradar las plumas. La bacteria fue aislada de 21–59% ($\bar{x}$ = 39%) de las especies de aves muestreadas. El estudio secuencial de 16srRNA (*rrnA*) de 98 de los cultivos aislados dio positivo para *B. licheniformis*, lo que sugiere que la presencia de esta bacteria en las plumas de las aves es cuatro veces mayor que lo previamente informado. Proponemos otra hipótesis en referencia a que dado que hay variaciones en el plumaje de las aves, conducta y selección de hábitat, esto puede haber dado origen a sepas especificas de estas bacterias en aves. La huella genetica de sepas aisladas del bacilo antes mencionado, de aves como *Aegolius acadicus* y *Dumetella carolinensis*, utilizando REP-PCR ha demostrado que para nueve individuos del *Aegolus*, uno de estos cargaba cuatro sepas de la bacteria y los otros ocho tan solo una. De los 20 individuos de *Dumetella*, en nueve individuos tan solo se encontró una sepa y en otros cuatro dos sepas diferentes.

Key words: *Bacillus licheniformis*, β-keratin, feather-degrading microbes, keratinolytic, REP-PCR

By digesting the β-keratin in avian plumage, feather-degrading microbial symbionts potentially decrease host fitness. In response, birds may have evolved mechanisms to reduce damage to their plumage, such as preening, anting, dusting or molt (Hart 1997). Burtt (1999; Burtt and Ichida 1999) proposed that *B. licheniformis* may be an important member of a consortium of keratin-digesting microbes that have contributed to the evolution of feather shedding through molt, plumage strengthening through increase in melanin content, and behavior that might increase fitness in the face of microbial assault on the plumage. Yet Burtt and Ichida (1999) reported that keratinolytic bacilli were detected within the plumage of only 7.4% (range across species; 0–23%) of individuals among 870 wild birds of seven species with adequate sample sizes. Such a carriage rate may be too low to be a powerful force of natural selection since plumage is not the moist, warm

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environment in which *B. licheniformis* thrives, and there is no evidence of their ability to damage feathers on living birds (Cristol et al. 2005). *B. licheniformis* does not require a host for survival, as it thrives in the soil. Feather-degrading bacilli may only degrade feathers when they are in contact with the soil (after molt or death of the bird) and may have no impact on avian evolution. Before the importance of feather-degrading microbes can be evaluated, we need to determine if they occur on a high enough proportion of individuals to be ecologically important. Our main objective in this study was to estimate the frequency of occurrence of *B. licheniformis* on a large sample of free-living birds to determine if carriage rates were as low as previously reported.

If our hypothesis is correct and *B. licheniformis* is found commonly on birds, it is worthwhile to ask whether *B. licheniformis* might have played an important role in avian evolution. A secondary objective was to learn more about possible routes of transmission for feather-degrading microbes. To achieve this we examined whether the *B. licheniformis* present on an individual bird were of one or many genetic strains. If one strain or a consistent consortium of strains is present on a host species, that would suggest clonal spread after infection. Understanding if birds randomly acquire feather-degrading bacterial strains from the environment (i.e., contamination) or if the bacteria are passed on by infection from conspecifics, is critical to determining the potential for coevolution between these microbes and their hosts. In the case of transmission by contamination, a variety of strains are acquired by a variety of avian species. Those *B. licheniformis* strains would likely remain generalized to degrade any feather under appropriate conditions (moist, warm environment) and may therefore remain genetically indistinct. However, if a particular strain or group of strains become associated with one species of bird because it is passed from parent to offspring each generation, those strains may become specialized to degrade feathers of a particular type, such as high melanin content. The behavior of the bird (e.g., higher affinity for sunning), may also select for one strain or a group of strains. As *B. licheniformis* strains become more adapted to living on a specific host, they may become genetically distinct from other strains living on different avian hosts. Understanding the diversity of *B. licheniformis* strains on wild birds is an initial step to understanding if *B. licheniformis* has evolved to degrade feathers while on a living bird. We carried out a preliminary investigation of the diversity of bacterial strains within individuals of a host species and across host species.

METHODS

Study species. Until 1990, *B. licheniformis* was considered a typical soil bacterium, but Williams et al. (1990) isolated this bacterium from a poultry waste digester and characterized its feather-degrading capabilities. *B. licheniformis* is a gram positive, facultatively aerobic bacterium most closely related to the well-studied bacterium, *Bacillus subtilis*. At least three species of *Bacillus* are known to degrade β-keratin including *B. licheniformis*, *B. subtilis* (Evans et al. 2000), and *B. pumilis* (Burtt and Ichida 1999). Optimal growth of *B. licheniformis* in nutrient broth occurs at 50°C and pH 7.5 (Williams et al. 1990; Cheng et al. 1995).

To determine the carriage rate of *Bacillus* species within the plumage of wild-caught birds, 40 or more individuals of eight bird species were mist netted during routine banding operations of the Coastal Virginia Wildlife Observatory in Kiptopeke State Park, Virginia (all species), and on the campus of the College of William and Mary in Williamsburg, Virginia (sparrows only), between 5 September 2001 and 18 March 2002 (Table 1).

Sampling. Bacteria were sampled by rubbing a sterile cotton-tipped applicator through each bird's feathers starting with the right side of the head and continuing over the top and left side. The applicator was then rubbed down the ventral side of the body, through the tail and across the rump. Applicators had been saturated with isotonic saline and sterilized by autoclaving. Two replicate samples were taken from the same individual bird with separate applicators. The applicator was continually rotated during sampling to ensure optimal contact with feathers. After sampling applicators were frozen at −80°C until culturing. Ethanol (70%) was used to decrease the risk of contamination during sampling. The senior author carried out all of the plumage sampling and carefully washed her hands between samples; how-

Table 1. Birds sampled and found positive for putative feather-degrading bacteria.

Species	*N*	% Positive
Northern Saw-whet Owl (*Aegolius acadicus*)	51	59
Hermit Thrush (*Catharus guttatus*)	44	21
Gray Catbird (*Dumetella carolinensis*)	65	35
Black-throated Blue Warbler (*Dendroica caerulescens*)	58	43
Yellow-rumped Warbler (*Dendroica coronata*)	103	33
American Redstart (*Setophaga ruticilla*)	40	35
Dark-eyed Junco (*Junco hyemalis*)	40	58
White-throated Sparrow (*Zonotrichia albicollis*)	60	27

ever, it should be noted that birds were removed from mist nets by numerous people who generally did not wash their hands between birds.

Culturing. A multi-step process was used to eliminate most bacteria other than *B. licheniformis*. Bacteria were tested for their ability to secrete proteases, such as the keratinase used in degrading feathers, by using a swab from each bird to inoculate a petri dish containing a nutrient agar medium with 1% skim milk, incubated at 37°C for 24 h. Only those colonies that were capable of degrading the casein in milk were transferred to nutrient agar media containing 7.5% NaCl, which was used to select for bacteria that could tolerate high salt conditions (i.e., halotolerant), a characteristic of the bacterial genus, *Bacillus*. The inoculated high salt medium was incubated at 50°C in a humidified chamber for up to 7 d to select for mildly thermophillic bacteria. These differential and selective steps in the culturing process eliminated the growth of a wide variety of bacteria, especially any gram-negative bacteria that may have resulted from fecal contamination. Bacterial colonies that were capable of hydrolyzing the skim milk protein (i.e., proteolytic) and could tolerate the high salt environment at 50°C (i.e., halotolerant and mildly thermophilic) were then gram stained to determine morphology and gram reaction status. Those determined to be gram-positive, proteolytic, halotolerant, mildly thermophillic bacilli were considered putative feather degraders and were stored on agar slants at room temperature.

To obtain genomic DNA (gDNA) for further species identification, isolates of mildly thermophilic, halotolerant, proteolytic bacilli were grown in 10 ml Luria-Bertani medium, incubated overnight at 37°C with aerobic aeration, and then lysed as follows. Pelleted cells were resuspended in 300 μl of 10 mM Tris-HCl (pH 8.0) and incubated for 48 h with 10% lysozyme at 55°C. The lysis solution was then incubated at 100°C for 10 min. The lysed isolates were then centrifuged at 14,000 × gravity for 5 min and the supernatant transferred to a sterile tube. Polymerase chain reaction (PCR) was performed using 5 μl of the supernatant as template as described below.

Isolates were identified by sequencing a PCR amplified section of the bacterial 16S ribosomal RNA gene (*rrnA*). This procedure ensured that the selective and differential culture scheme used was efficient at isolating potentially keratinolytic bacilli. A highly conserved section (~900 bp) of the 16S *rRNA* was amplified from bacterial lysates by PCR. The 50 μl reaction consisted of 1× Reaction Buffer (Promega), 2 mM Mg^{2+}, 0.2 mM each dATP, dCTP, dGTP, dTTP, 400 ng of reverse primer (5′CCCGGGATCCAAGCTTACGGCTACCTTGTTACGACTT-3′) , 400 ng of forward primer (5′-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3′), and 2.5 U Taq DNA polymerase (Promega). The thermal cycling conditions used were 25 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 1.5 min.

The PCR amplicon was purified using the Qiagen PCR purification kit by the manufacturer's suggested protocol. A 20 μl Fluorescent Dye Terminator sequencing reaction (Big-Dye Terminator, Applied Biosystems Inc.) was performed on the purified 16S-PCR product using 500 ng of the PCR amplicon, 1× Ready Reaction mix, and 200 ng of the reverse primer. The thermal cycling conditions used were 25 cycles of 96°C for 10 s, 50°C for 5 s, 60°C for 4 min. The reaction was then purified using a

Table 2. Species of *Bacillus* isolated using selective and differential culture methods.[a]

Bird species	*N*	*B. l.*[b]	*B. m.*[c]	*B. p.*[d]	*B. s.*[e]	*B. sp.*[f]	*B. f.*[g]	*V. sp.*[h]
Northern Saw-whet Owl	27	17	5	1	0	4	0	0
Gray Catbird	31	27	0	1	0	3	0	0
Yellow-rumped Warbler	25	20	1	2	2	0	0	0
Dark-eyed Junco	15	4	0	7	2	0	1	1
Total	98	68	6	11	4	7	1	1

[a] Isolates were identified to species level through sequencing of a 900 bp portion of the 16s rRNA.
[b] *Bacillus licheniformis*.
[c] *B. megaterium*.
[d] *B. pumilis*.
[e] *B. subtilis*.
[f] Bacilli that could not be identified to the species level.
[g] *B. flavothermus*.
[h] Member of genus *Virgibacillus* that could not be identified at the species level.

DTR Gel Filtration Cartridge kit (Edge Biosystems). Complete sequencing reactions were processed in an ABI 3100 Avant Gene Sequencer.

BOX-PCR, a method of Repetitive Extragenic Polymorphism (REP) PCR, was used to differentiate strains of *B. licheniformis*. BOX-PCR utilizes a single oligonucleotide primer to produce a strain specific array of bands by amplifying the sequences between repetitive extragenic repeats conserved throughout most bacterial genomes. The 50 μl reaction consisted of 1× Reaction Buffer (Promega), 2 mM Mg^{2+}, 0.2 mM each dATP, dCTP, dGTP, dTTP, 400 ng of primer (5′CTACGGCAAGGCGACGCTGA3′) (van Belkum et al. 1996), and 2.5 U Taq polymerase (Promega). The thermal cycling conditions used were 30 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 1.5 min. The strain specific banding patterns were visualized on 5% neutral polyacrylamide gels stained with ethidium bromide.

RESULTS

Carriage rate. We cultured bacteria from the plumage of 461 individuals of eight bird species using a selective method that favored growth of *B. licheniformis* and other protein-digesting, gram-positive bacteria that tolerate salty, warm conditions. We detected putative *B. licheniformis* colonies in an average of 39% of the samples for each species (range, 21–59%, Table 1).

To determine whether our selective and differential culture scheme was effective in selecting for *B. licheniformis*, we identified 98 of the 461 isolates to the species level by amplifying and sequencing a ~900 bp portion of the 16s rRNA gene that is diagnostic for bacterial species (Wilson et al. 1990). We found that 69% of the 98 isolates were *B. lichenformis*, suggesting that overall carriage rate of this bacterial species was 26%. Overall, 92% of the bacteria we isolated were known keratinolytic *Bacillus* species, as identified by 16s *r*RNA sequencing (Table 2). We also isolated, albeit at a much lower frequency, two congeneric bacterial species known to degrade keratin, *B. subtilis* (Suh and Lee 2001) and *B. pumilis* (Burtt and Ichida 1999), and three additional *Bacillus* that have not been characterized as keratinolytic: *B. megaterium*, *B. flavothermus*, *Virgibacillus* sp. (Table 2). Finally, we isolated one gram positive coccus, *Kocuria roseus*, which has been characterized as keratinolytic (Vidal et al. 2000).

Preliminary evidence of strain diversity. Seventeen isolates from nine Northern Saw-whet Owls were identified as *B. licheniformis* (Table 2) via 16s *r*RNA sequencing. Fifteen of those 17 isolates were DNA-fingerprinted using BOXA1R-PCR, which produced complex and easily identifiable banding patterns in all cases (Fig. 1). Overall, seven distinct strains were identified from the 15 isolates. Seven isolates from the same bird produced four distinct amplicon array patterns, suggesting that one individual carried four genetically distinct strains of *B. licheniformis*. The remaining eight isolates were from eight different birds. Of these, four produced identical band patterns, indicating

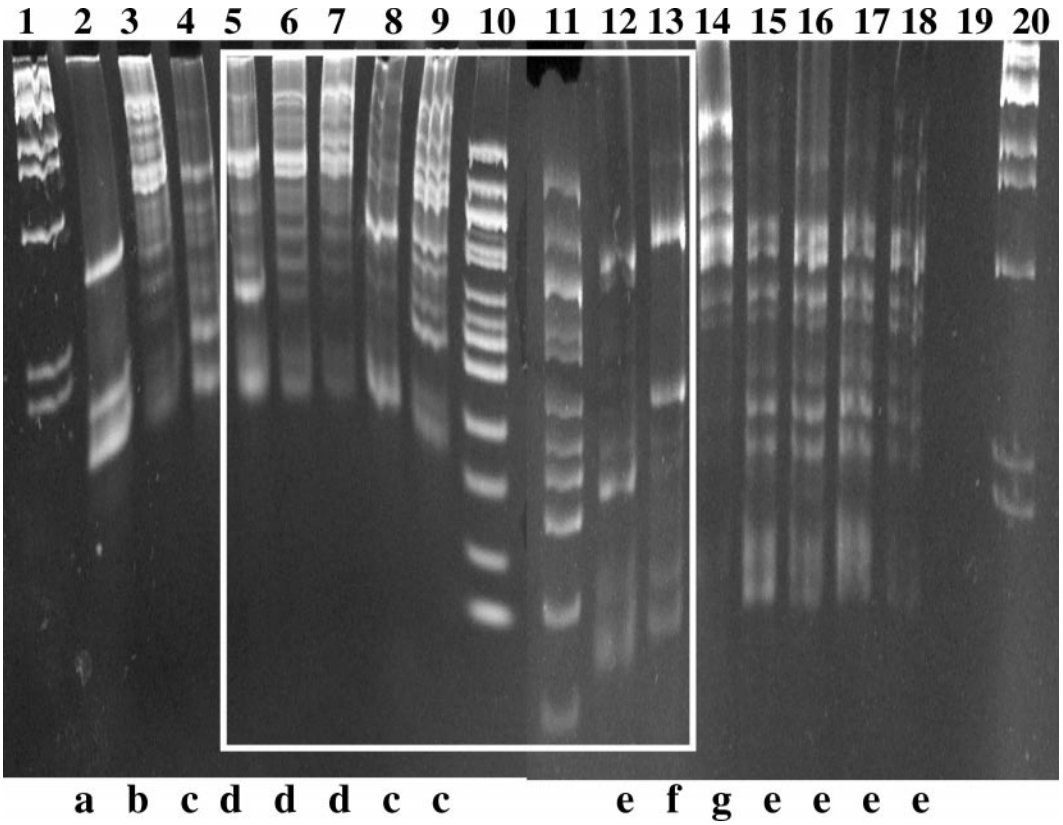

Fig. 1. BOX-A1R PCR analysis of a subset of *B. licheniformis* isolates from Northern Saw-whet Owls. Seven different *B. licheniformis* genotypes (a-g) were isolated from Northern Saw-whet Owls. The box enclosing lanes 5–13 indicates isolates from the same bird. Each of the other isolates is from a different bird. **1.** 1 kB DNA ladder; **2.** 080; **3.** 083; **4.** 084; **5.** 088- isolate #1; **6.** 088 isolate #2; **7.** 088 isolate #3; **8.** 088 isolate #4; **9.** 088 isolate #5; **10** & **11.** 100 bp DNA ladder; **12.** 088 isolate #6; **13.** 088 isolate #7; **14.** 091; **15.** 859-; **16.** 475-; **17.** 529; **18.** 519; **19.** Blank; **20.**100 bp DNA ladder.

that the same strain was found on four different owls.

Nineteen Gray Catbirds yielded 27 *B. licheniformis* isolates (Table 2). Twenty of those 27 isolates were genetically fingerprinted by BOXA1R-PCR, resulting in six strain types each yielding an indistinguishable amplicon array in this assay (Fig. 2). The same strain was cultured from multiple individuals in several cases (Fig. 2, e.g., lanes 7, 8, 9 11, 15, and 16). Of the four Gray Catbirds yielding more than one *B. licheniformis* isolate, one individual yielded bacterial strains of different genotypes (Fig. 2, lanes 17 and 18). The remaining three birds each yielded two indistinguishable *B. licheniformis* isolates.

DISCUSSION

The low carriage rate of the feather-degrading bacterium *B. licheniformis* (7%) reported by Burtt and Ichida (1999) suggested to us the possibility that feather-degraders are not widespread. This, combined with our subsequent demonstration of the absence of detrimental effects of *B. licheniformis* on the plumage of live birds (Cristol et al. 2005), raises questions regarding the ecological or evolutionary importance of this intriguing guild of microbes. We developed a selective and differential culture scheme to favor mildly thermophilic, halotolerant, proteolytic, gram-positive bacilli (considered possible agents of feather damage) from samples of migratory birds. We detected such

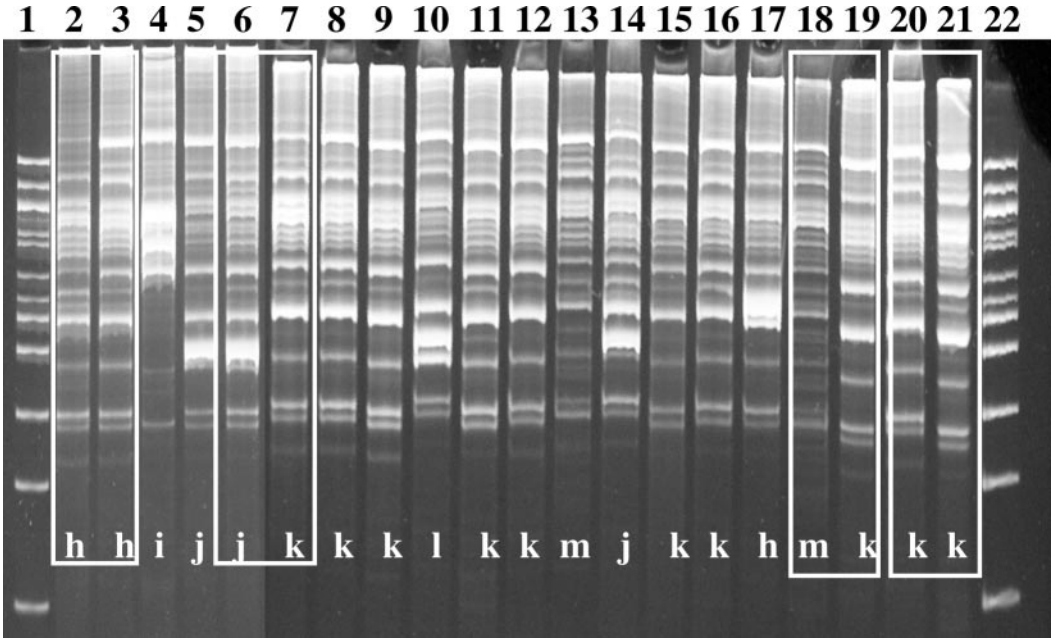

Fig. 2. BOX-A1R PCR analysis of a subset of *B. licheniformis* isolates from Gray Catbirds. Six different *B. licheniformis* genotypes (h–m) were isolated from Gray Catbirds. Lane numbers (except 1 and 22) represent individual *B. licheniformis* isolates. Each box encloses two strains isolated from the same individual bird. **1.** 100 bp ladder; **2.** 156 isolate #1; **3.** 156 isolate #2; **4.** 092; **5.** 190 isolate #1; **6.** 190 isolate #2; **7.** 153; **8.** 138; **9.** 938; **10.** 937;**11.** 929; **12.** 928; **13.** 127; **14.** 917; **15.** 898; **16.** 896; **17.** 894 isolate #1; **18.** 894 isolate #2; **19.** 893; **20.** 878 isolate #1; **21.** 878 isolate #3; **22.** 100 bp ladder.

putative feather-degrading bacilli on the plumage of an average of 38% of the individuals of eight bird species sampled. Genetic characterization of these bacterial isolates indicated that *B. licheniformis* was the dominant member of this microbial consortium isolated from the plumage, comprising 69% of our isolates. Taken together, this suggests that 26% of the birds sampled carried *B. licheniformis*, about 4× the previously reported carriage rate. Although the mist nets used at the banding station to capture the birds are a possible source of contamination, sampling was performed primarily within the plumage, as opposed to sampling only on the surface of the feathers. Areas within the plumage would be less likely to be contaminated from handling. The cotton swabs used in sampling are slightly anti-microbial and may have caused our sampling to be somewhat conservative. Also, because we did not sample the entire plumage, and because at least one of the steps in the culturing process, freezing, may have killed bacteria, the true carriage rate may be somewhat higher.

The difference between our results and those of Burtt and Ichida (1999) may be attributable to the different sampling and culturing techniques we used, but because we changed many other variables this is speculation. Sampling in Burtt and Ichida's (1999) study was accomplished by rubbing three separate Dacron-tipped applicators through the feathers. Each applicator was used to sample a different area of the plumage. We used one cotton applicator to obtain bacteria from the entire bird. Burtt and Ichida (1999) cultured keratinolytic bacilli in modified nutrient broth (7.5% NaCl) at 50°C. Our culture scheme consisted of using two types of media and incubation temperatures. In addition to applying the applicator to high salt agar incubated at 50°C, we also used skim milk media, which was incubated at 37°C. The addition of the skim milk medium allowed some bacteria to grow that may otherwise not have grown when removed from the bird and cultured under harsh conditions. The inclusion of milk allowed a facile examination of the ability of bacterial isolates to secrete proteolytic en-

zymes. This was viewed as a potential surrogate for the breakdown of keratinase.

We also preliminarily investigated the genetic diversity of the *B. licheniformis* cultured from two of the bird species. If *B. licheniformis* is growing vegetatively in the plumage of birds and degrading feathers, then we might expect to see evidence of a single strain of *B. licheniformis* indicative of an infection followed by clonal spread. Infection could result from vertical transfer of *B. licheniformis* from parent to offspring or horizontally from adult to adult. An alternative mechanism would be contamination, for example, random acquisition of *B. licheniformis* strains through contact with the environment. In the case of contamination, we might expect a heterogeneous population of *B. licheniformis* isolates acquired through repeated contact with a variety of sources such as soil and plants. Our results were equivocal. Of nine Saw-whet Owls sampled, one carried four different *B. licheniformis* strains, while the other eight each apparently carried only a single strain, and four of these shared the same strain. Among 16 Gray Catbirds, there was a dominant strain found on many birds, but four birds were each carrying two different strains. Further studies of this type are needed, but our preliminary data suggest that individual birds are not necessarily infected with a single strain specific to that host, which then spreads clonally throughout the plumage. While early infection may occur, the presence of four strains on one individual owl, and inconsistent strain consortia across individuals of the same species, suggest that contamination from the environment also occurs. An important next step will be to compare the *B. licheniformis* strains from different avian species to determine whether there are host-specific consortia of strains that might be involved in host-parasite coevolution. If strains are specific to particular hosts, a useful follow-up would be a comparison of the feather-degrading abilities of different strains when grown on host feathers versus those of other bird species.

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