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Use of Culture-Independent Methods to Compare Bacterial Assemblages on Feathers of Crested and Least Auklets (*Aethia cristatella* and *Aethia pusilla*) with Those of Passerines

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Abstract.—Despite recent interest in the interactions between birds and feather microbes, little is known about the identity of these microbes, and all studies of feather microbes thus far have focused on passerines living in temperate regions. Comparisons of the microbial groups living on different groups of birds may provide valuable insight into the ecological roles microbes play on feathers. We used culture-independent molecular techniques to identify the assemblages of bacteria found on the feathers of two closely related seabirds (Crested and Least Auklets (*Aethia cristatella* and *A. pusilla*)) and, for comparison, domestic Chickens (*Gallus gallus*). Some isolates were found on all three species (as well as on other species, as reported in the literature), while others were only found on Auklets. In particular, bacteria of the cold- and salt-tolerant genus *Psychrobacter* were only recovered from Crested Auklets. These results suggest that some genera of bacteria may be commonly found on birds, while others may be restricted in their distributions. *Received 25 March 2006, accepted 21 June 2006.*

Key words.—feather microbes, culture-independent, seabirds, 16S rDNA, Auklets, keratinolytic.

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The potential interactions between birds and the microbes on their feathers have received significant attention recently (Burtt and Ichida 1999; Shawkey *et al.* 2003; Goldstein *et al.* 2004; Shawkey and Hill 2004; Lucas *et al.* 2005; Shawkey *et al.* 2005). Several of these studies have focused on bacteria that are capable of degrading keratin (Burtt *et al.* 1999; Goldstein *et al.* 2004; Shawkey *et al.* 2003), while others have focused on entire bacterial assemblages (Lucas *et al.* 2005; Shawkey *et al.* 2003, 2005). These assemblages appear to be fairly diverse; Shawkey *et al.* (2005) recovered 20 unique bacterial isolates from feathers of Eastern Bluebirds (*Sialia sialis*) and 13 from House Finches (*Carpodacus mexicanus*) in a less-intensive survey (Shawkey *et al.* 2003). Until now, these studies have been entirely restricted to passerines. By examining the composition of assemblages on other groups of birds, we may improve our understanding of the diversity of bacteria associated with bird feathers. Seabirds differ from passerines in habitat, morphology and physiology; thus, we might expect their bacterial assemblages to differ as well. For example, we would expect to find salt-tolerant bacteria on these birds that spend much of their time in the ocean water.

Crested (*Aethia cristatella*) and Least (*A. pusilla*) Auklets are arctic seabirds that breed in mixed colonies on islands and coastlines around the Bering Sea and winter in flocks on nearby waters (Jones 1993a, b). The microbial assemblages found on the feathers of these or any other seabird have never been studied. Here we use culture-independent techniques to characterize bacteria found on feathers of Crested and Least Auklets and, for comparison, domestic Chickens (*Gallus gallus*). We expected the assemblages on Crested and Least Auklet feathers to be similar to one another and distinct from those on feathers of Chickens and passerines.

METHODS

Crested and Least Auklets were captured using carpet traps on St. Lawrence Island, Alaska (63.69°N, 170.48°W) in August, 2004. Birds were handled with sterile gloves,

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and approximately 25 feathers were pulled from the rump and nape of each bird. These feathers were placed in sterile 15 ml plastic tubes (Falcon, city) and stored at -20°C until being shipped on ice to Swarthmore College, where they were stored at 4°C until the time of analysis. Single wing feathers (primaries) were collected from lab-reared chicks at Swarthmore College and stored in sterile tubes at 4°C until the time of analysis.

Feathers were placed at room temperature overnight, and then homogenized in liquid nitrogen using a sterile mortar and pestle. The homogenate was then resuspended in 10 ml buffer solution (0.85% NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 8.0) and immediately processed or frozen at -20°C. To loosen bacteria, homogenates were shaken at room temperature at 200 rpm for 20 minutes, and then 1 ml aliquots were centrifuged at 17,000 g for 10 minutes. DNA was extracted from pellets using the DNeasy tissue extraction kit (Qiagen, Valencia, California) according to the manufacturers' recommendations for gram-positive bacteria.

PCR amplification of 16S rDNA signature sequences and subsequent cloning were performed following the methods of Shawkey *et al.* (2005). Following cloning, colonies containing inserts were picked and grown overnight with agitation at 37°C in LB broth with 50 µg/ml kanamycin. Aliquots (1 ml) of cultures were centrifuged at 10,000 rpm for five minutes and plasmids were purified from pellets using the QIAprep MiniPrep kit for plasmids (Qiagen, Valencia, Californai) according to the manufacturers' recommendations. Plasmids were then amplified by PCR using the vector primers M13F and M13R in 100 µl reaction volumes containing ~25 ng DNA, 1× PCR buffer, 200 µM dNTP's, 400 µM of each forward and reverse primer, and 0.025 U/µl recombinant *Taq* polymerase (Invitrogen, Carlsbad, California). The PCR consisted of an initial denaturing step (94°C, 10 min), followed by 36 cycles of denaturation (94°C, 1 min), annealing (50°C, 1 min) and extension (72°C, 2 min), and a final extension step (72°C, 10 min).

RFLP Screening of rDNA Clones

To minimize sequencing of redundant clones were screened using a restriction fragment length polymorphism (RFLP) analysis. Aliquots (10 µl) of crude PCR product were digested to completion with 1 U each of restriction enzymes *Msp*I and *HinP*1 I in 1× NEB buffer 2 (New England Biolabs, Beverly, Massechusetts) in a final volume of 20 µl for at least one hour at 37°C. Digested products were separated on agarose gels (4% MetaPhor®, Cambrex, Baltimore, Maryland). PCR products with unique RFLP patterns were selected for sequencing. Unique products were purified using the QIAquick PCR Purification kit (Qiagen, Valencia, California) and sequenced at the Nucleic Acid/Protein Research Core Facility at Children's Hospital of Philadelphia.

Sequences were inspected manually for the presence of ambiguous base assignments. The Bellerophon program (Huber *et al.* 2004) was used to detect potentially chimeric sequences, and the Chimera Check program in the Ribosomal Database Project (Maidak *et al.* 1997) was used for verification. The BLAST algorithm was (Altschul *et al.* 1997) used to determine their approximate phylogenetic affiliation. All chimeric sequences and sequences with >99% similarity to known PCR contaminants (Tanner *et al.* 1998) were discarded. Sequences that were ≥99% similar to one another were considered as a single relatedness group.

RESULTS

Nape feathers of two Least Auklets and two Crested Auklets, rump feathers of two Least Auklets and one Crested Auklet and wing feathers of eight domestic chicks were analyzed. Approximately 10 clones from each library were screened using RFLP for a total of 150 clones. A total of 47 unique patterns were detected.

The BLAST program identified 28 different 16S rDNA gene sequences as the closest relatives of the feather bacteria sequences (Table 1). Approximately 63% of the unique sequences obtained were between 98 and 100% identical to their closest matches in GenBank, and approximately 37% of the sequences obtained were between 95 and 97% identical to their nearest match. One sequence was identified as a chimera and was discarded.

All recovered sequences were most closely related to members of the *Firmicutes* and *Proteobacteria* (Table 1). The largest number of unique sequences was recovered from nape feathers of Crested Auklets (8), followed by wing feathers of domestic Chickens (7), Least Auklet nape feathers (6), Crested Auklet rump feathers (4) and Least Auklet rump feathers (3). Isolates closely related to *Janthinobacterium* spp. were recovered from nape feathers of Crested and Least Auklets, while isolates closely related to *Burkholderia* spp. were recovered from Chicken wing feathers, Crested Auklet rump feathers and Least Auklet nape feathers. Isolates closely related to *Staphylococcus* spp. were recovered from both Chicken wing feathers and Least Auklet rump feathers.

DISCUSSION

Fairly diverse groups of bacteria were isolated from feathers of Crested and Least Auklets. Comparison of these isolates with those from domestic Chickens, Eastern Bluebirds (Shawkey *et al.* 2005) and House Finches (Shawkey *et al.* 2003) allowed identification of potential Auklet or arctic seabird specialists. Many genera of isolates from Auklets were also found either on Chickens, bluebirds or House Finches (see Table 1), suggesting that they are commonly distributed

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Table 1. Identification of clones from culture-independent survey of feathers from domestic Chickens and Crested and Least Auklets. The bacterial species in GenBank with the closest DNA sequence to each isolate (as determined by the BLAST algorithm) is presented as a preliminary identification. Whether members of each genus have been found on bluebirds (as reported in Shawkey *et al.* 2005) and/or House Finches (as reported in Shawkey *et al.* 2003) is noted in the column labeled "Also found on."

Sample	Species	Feather	Highest BLAST identity (Accession #)	Also found on	Division	Base pairs matched
Chick 1B	Chicken	wing	*Shigella boydii* (AY696681)		γ-proteobacteria	477/479 (99%)
Chick 2D	Chicken	wing	*Escherichia coli* (AE005174)		γ-proteobacteria	823/831 (99%)
Chick 3A-1	Chicken	wing	*Staphylococcus* sp. (AM084016)	House finch	Firmicutes	851/864 (98%)
Chick 4A-1	Chicken	wing	*Enterococcus canintestini* (AJ888906)	House finch, bluebird	Firmicutes	863/870 (99%)
Chick 4C-1	Chicken	wing	*Enterococcus gallinarum* (AF277567)	House finch, bluebird	Firmicutes	699/713 (98%)
Chick 5A-2	Chicken	wing	*Enterococcus faecium* (AY735408)	House finch, bluebird	Firmicutes	842/845 (99%)
Chick 5E	Chicken	wing	*Burkholderia* sp. (AY040359)		β-proteobacteria	545/548 (99%)
CR62N-1	Crested Auklet	nape	*Bacillus subtilis* (AY775778)	House finch, bluebird	Firmicutes	987/994 (99%)
CR67B	Crested Auklet	nape	*Psychrobacter luti* (AJ430828)		γ-proteobacteria	852/871 (97%)
CR7C-2	Crested Auklet	nape	*Psychrobacter fozii* (AY771717)		γ-proteobacteria	814/821 (99%)
AUBURN-3	Crested Auklet	nape	*Pseudomonas* sp. (AY69069)	House finch, bluebird	Proteobacteria	732/750 (97%)
AUBURN-5	Crested Auklet	nape	*Aeromonas* sp. (ASU88662)	bluebird	γ-proteobacteria	676/696 (97%)
AUBURN-1	Crested Auklet	nape	*Janthinobacterium* sp. (AJ864846)	bluebird	β-proteobacteria	694/728 (95%)
AUBURN-7	Crested Auklet	nape	*Janthinobacterium lividum* (JL16SRRN)	bluebird	β-proteobacteria	760/776 (97%)
AUBURN-9	Crested Auklet	nape	*Stenotrophomonas maltophilia* (AY169434)	bluebird	γ-proteobacteria	775/801 (96%)
CR50R-3	Crested Auklet	rump	*Burkholderia* sp. (AF452132)		β-proteobacteria	950/987 (96%)
CR50R-4	Crested Auklet	rump	*Acinetobacter* sp. (AY663435)	bluebird	γ-proteobacteria	647/649 (99%)
CR50R-5	Crested Auklet	rump	*Acinetobacter* sp. (AS16SRRNB)	bluebird	γ-proteobacteria	978/979 (99%)
CR50R-6	Crested Auklet	rump	*Acinetobacter* sp. (AB167212)	bluebird	γ-proteobacteria	956/957 (99%)
LE53R-6	Least Auklet	rump	*Paenibacillus dendritiformis* (AY359885)		Firmicutes	802/810 (99%)
LE53R-7	Least Auklet	rump	*Paenibacillus thiaminolyticus* (AB073197)		Firmicutes	828/834 (99%)
LE55R-1	Least Auklet	rump	*Staphylococcus equorum* (DQ232735)	House finch	γ-proteobacteria	844/852 (99%)
LE60-1	Least Auklet	nape	*Burkholderia* sp. (AY178061)		β-proteobacteria	850/857 (99%)
LE60-4	Least Auklet	nape	*Burkholderia* sp. (BSU37344)		β-proteobacteria	830/835 (99%)
LE60-8	Least Auklet	nape	*Burkholderia* sp. (AY178061)		β-proteobacteria	837/840 (99%)
LE63-1	Least Auklet	nape	*Acinetobacter* sp. (AB167206)		γ-proteobacteria	830/832 (99%)
LE63-2	Least Auklet	nape	*Tiedjeia arctica* (DQ107523)		γ-proteobacteria	834/840 (99%)
LE63-6	Least Auklet	nape	*Janthinobacterium* sp. (AJ846272)		β-proteobacteria	847/851 (99%)

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among birds. However, two genera, *Psychrobacter* and *Paenibacillus*, were only isolated from Auklet feathers. Members of *Psychrobacter* are psychrotolerant or psychrophilic and halotolerant and are widely distributed in the Arctic (Cavanagh *et al.* 1996), while members of *Paenibacillus* are found in a large number of environments (e.g., the gut of earthworms (Horn *et al.* 2005) and in industrial wastewater (Meehan *et al.* 2001)). Thus, the genus *Psychrobacter* is more likely to be found exclusively on arctic birds. Whether these bacteria play any unique role in the microecology of feathers or have merely been picked up from the environment is an interesting question that will be addressed in future research. In particular, it would be interesting to know if bacteria play a role in production of the tangerine odor of Crested Auklets. Crested Auklets exhibit a characteristic tangerine-like odor that appears to serve social (Hagelin *et al.* 2003; Jones *et al.* 2004) and/or anti-parasitic functions (Douglas *et al.* 2001) while the congeneric Least Auklets do not. The chemical compounds in this odor, simple aldehydes (octanal, 2-decenel) and alcohols (Hagelin *et al.* 2003), could be derived from bacterial cleavage of more complex fatty acids such as those found in preen gland secretions (Jacob and Ziswiler 1982). Future research will address this possibility.

These data, while limited, add to the rapidly growing field of bird-microbe interactions. By using modern molecular methods to survey arctic seabirds and comparing these results with those from similar studies of passerines, we have shown that certain groups of bacteria (e.g., *Pseudomonas*, *Janthinobacterium*) are found on diverse groups of birds and may represent a "normal microflora" for feathers. Others, such as halotolerant *Psychrobacter* species may be unique to seabirds. The ecology and evolution of these microbes remains a fertile ground for future discovery.

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