Microbial Diversity of Wild Bird Feathers Revealed through Culture-Based and Culture-Independent Techniques

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Abstract

Despite recent interest in the interactions between birds and environmental microbes, the identities of the bacteria that inhabit the feathers of wild birds remain largely unknown. We used culture-based and culture-independent surveys of the feathers of eastern bluebirds (*Sialis sialis*) to examine bacterial flora. When used to analyze feathers taken from the same birds, the two survey techniques produced different results. Species of the poorly defined genus *Pseudomonas* were most common in the molecular survey, whereas species of the genus *Bacillus* were predominant in the culture-based survey. This difference may have been caused by biases in both the culture and polymerase chain reaction techniques that we used. The pooled results from both techniques indicate that the overall community is diverse and composed largely of members of the *Firmicutes* and β- and γ- subdivisions of the *Proteobacteria*. For the most part, bacterial sequences isolated from birds were closely related to sequences of soil-borne and water-borne bacteria in the GenBank database, suggesting that birds may have acquired many of these bacteria from the environment. However, the metabolic properties and optimal growth requirements of several isolates suggest that some of the bacteria may have a specialized association with feathers.

Introduction

The identities and ecological roles of microbes found on the feathers of wild birds are largely unknown. For a variety of reasons, we might expect to find a limited diversity and abundance of bacteria on the surface of feathers. First, birds waterproof their feathers by applying preen oil [12], thereby limiting water availability. This oil inhibits the growth of some bacteria, although it appears to enhance the growth of others [3, 27]. Second, common proteolytic enzymes produced by bacteria cannot degrade the ß-keratin sheets that constitute 90% of feather mass [37]. Thus, to utilize feathers as a nutrient source, bacteria must produce keratinolytic enzymes that convert feather keratin to peptides [37]. Such enzymes appear to be produced by fairly diverse groups of bacteria in the environment [15], but whether these bacteria are also present and active on wild bird feather is unknown. Bacteria may also use detritus or other microbes on feathers as nutrient sources, but such use has yet to be documented. There have been no inventories of the microbiota of the feathers of a wild bird, and the ecology of microbes on feathers cannot be understood until such basic inventories are obtained.

Burtt and Ichida [6] isolated keratinolytic *Bacillus* spp. from a broad spectrum of birds, Shawkey et al. [27] cultured 13 distinct isolates (determined by BLAST searches of 16S rDNA sequences) from the feathers of house finches (*Carpodacus mexicanus*). To our knowledge, however, no one has yet comprehensively characterized the microbial communities living on feathers of any species. Such a survey is needed for several reasons. First, these basic data are needed to determine how microbes interact both with one another and with birds. For example, Burtt and Ichida [6] suggested that degradation of feathers by *Bacillus* sp. may have partially driven the evolution of feather molt. They used highly selective media, and therefore did not isolate any non-*Bacillus* strains. However, different species of bacteria may act syntrophically to degrade feathers, or, conversely, the metabolic activity or antibiotic production of some bacteria may inhibit the growth of others. Some bacteria

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on feathers could be parasites, as suggested by Burtt and Ichida [6], whereas others could be commensals or even mutualists. Such communities are seen on the human skin, where the activity of bacteria utilizing the sebum of the skin lowers the pH of the skin's surface, providing an effective barrier preventing colonization of other (possibly pathogenic) bacteria [35]. Second, all studies of feather bacteria to date have relied on culture-based methods. A very small proportion (<1%) of microbes can be cultured by traditional methods [2], so culture-based studies may not provide an adequate sampling of diversity. The use of culture-independent molecular phylogenetic techniques allows us to sample a broader spectrum of bacteria on feathers, although these methods have several limitations [8, 22, 31, 32, 38] including the possibility that DNA isolation, amplification, and cloning might be biased in favor of certain phylogenetic groups. By simultaneously performing molecular and culture-based sampling, we can compare results obtained using the two methodologies.

In the current study we characterized the bacteria on the feathers of a common North American passerine, the eastern bluebird (*Sialia sialis*) using both culture-based and culture-free methods. The purpose of this study was to describe the diversity of bacteria found on feathers and to compare data collected using culture-based and culture-free methods.

Methods

Collection of Materials. In July 2003, using mist nets and box traps, we trapped two adult male (M1 and M2) and two adult female (F1 and F2) Eastern Bluebirds (*Sialia sialis*) in Lee County, Alabama (32°35′N, 82°28′W). Wearing sterile rubber gloves, we pulled contour feathers from the breast, belly, and rump of the birds and placed them in separate sterile tubes. All tubes were transported to the laboratory within 3 h and processed immediately. For each bird, we created two sets of 15 feathers using 5 feathers from each of the three body areas sampled. One set was analyzed using culture-independent methods and the other was analyzed by the culture-based method.

Cultures-Independent Method

DNA Extraction, Amplification, and Cloning. The feathers from each bird were homogenized in liquid nitrogen using a sterile mortar and pestle [39], and resuspended in 10 mL sterile 0.85% NaCl solution. After settling of particulate matter, 1 mL of the suspension was transferred to a sterile microcentrifuge tube and pelleted for 1 min at $13,000 \times g$. The supernatant was removed and the pellet was re-suspended in 100 μL of sterile 0.85% NaCl. DNA was extracted from the pellet with the DNeasy®

Tissue Kit (Qiagen, Valencia, CA), and used as a template for polymerase chain reaction (PCR) amplification of the bacterial 16S rDNA gene.

To generate libraries of PCR-amplified 16S rDNA sequences from DNA isolated from feathers, we used "universal" primers 515F (5′-GTGCCAGCMGCCGC GGTAA-3′) and 1492R (5′-GGTTACCTTGTTACG ACTT-3′) (5). All PCRs were performed in 50-μL reaction volumes containing between 1 ng and 50 ng purified DNA template and (as final concentrations) 1 × PCR buffer, 2.5 mM MgCl₂, 200 μM dNTP's, 100 μM of each forward and reverse primer, and 0.025 U/μL *AmpliTaq* DNA polymerase (Applied Biosystems, Foster City, CA). All reactions were incubated on a model PT-100 thermal cycler (MJ Research, Inc., Waterstown, MA) for an initial denaturation step at 94°C for 5 min, followed by 36 cycles of denaturation (94°C, 1 min), annealing (55°C, 1 min) and extension (72°C, 2 min). An extension step of 10 min at 72°C was added after the last cycle to promote A-tailing of PCR products prior to cloning. Three reactions were performed for each sample. The PCR products were checked on 1.5% agarose gels and the 1-kbp amplicons were excised from gels and purified with the Gel Extraction Kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. Then, 20–50 ng of this purified PCR product was cloned into the pCR-II® TOPO® vector (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations.

Plasmid DNAs containing inserts were amplified by colony PCR using either the vector primers M13F and M13R, or primers M13F and 1492R in 50 μL reaction volumes containing 1× PCR buffer, 2.5 mM MgCl2, 400 μM dNTP's, 400 μM of each forward and reverse primer, and 0.025 U/μL AmpliTaq polymerase (Applied Biosystems, Foster City, CA). Recombinant colonies were inoculated directly in to the PCR mixture using sterile pipette tips. The PCR consisted of a denaturing step (94°C, 5 min), followed by 36 cycles of denaturation (94°C, 1 min), annealing (52°C, 1 min), and extension (72°C, 2 min), and a final extension step (72°C, 5 min). To verify the success of PCR, 7 μL of each PCR product was checked by electrophoresis.

RFLP Screening of rDNA Clones. To avoid sequencing redundant clones, we screened clones by means of a restriction fragment length polymorphism (RFLP) analysis. Aliquots (10 μL) of crude PCR product were digested to completion with the restriction enzymes *Msp*I and *Hin*P1 I in 1× NEB buffer 2 (New England Biolabs, Beverly, MA) in a final volume of 20 μL for 3 h at 37°C. Digested products were separated on agarose gels (4% MetaPhor®, Cambrex, Baltimore, MD). Using digital images of these gels in Adobe Photoshop® 4.0 LE, we aligned the RFLP patterns obtained for each bird visually and selected representatives from each group for sequencing.
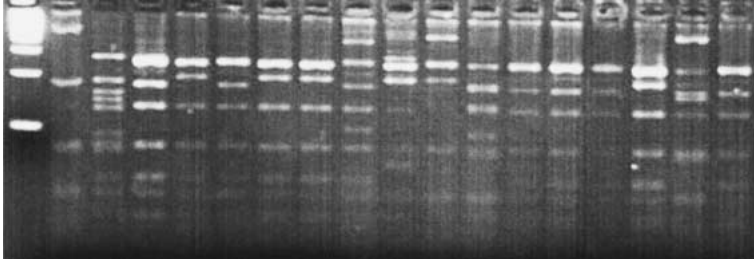
Figure 1. Typical restriction fragment length polymorphism analysis of cloned bacterial sequences from eastern bluebird feathers. The well on the far left is a 100 bp ladder, while the remaining lanes are individual sequences digested with the 4-base cutting restriction enzymes *Msp*I and *Hin*P1 I.

The PCR products from representative clones were sequenced at the Auburn University Genomics and Sequencing Laboratory using the M13F and 1492R primers.

Phylogenetic Analyses. Sequences were inspected manually for the presence of ambiguous base assignments, and chimeric sequences were identified with the Chimera Check program in the Ribosomal Database Project (RDP [16]). The BLAST algorithm (1) was used to determine the sequences' approximate phylogenetic affiliation. Sequences were then aligned with known rDNA sequences in the RDP with the Sequence Match function. All chimeras and sequences with >99% similarity to known PCR contaminants [34] were discarded. Sequences that were ≥99% similar to one another were considered as a single relatedness group, and we chose the most complete and unambiguous representative for further analysis. Unique bacterial 16S rDNA sequences were deposited in GenBank (accession numbers AY581128–AY581144).

Unique sequences were then compiled with known sequences of ATCC type cultures from GenBank and the RDP in MacClade v. 4.0 [17], and aligned in ClustalX v.1.83 [36]. Only homologous nucelotide positions with unambiguous bases in every sequence were used in phylogenetic analyses. Distance-based methods were used to construct bootstrap Neighbor-Joining trees in PAUP* 4. 0b10 [33]. Separate trees were constructed for each major phylogenetic subdivision (Firmicutes, α-Proteobacteria, γ-Proteobacteria).

Culture-Based Method. The second set of feathers from each bird was homogenized in sterile phosphate-buffered saline with a sterile all-glass tissue grinder (Kontes, Vineland, NJ). Then, 100 μL of raw homogenate, as well as serial dilutions, was transferred onto two distinct media. Tryptic soy agar (TSA, Difco, New Jersey) is a generalized medium, whereas feather meal agar (FMA; 15 gL^{-1} feather meal, 0.5 gL^{-1} NaCl, 0.30 gL^{-1} K$_2$HPO$_4$, 0.40 gL^{-1} KH$_2$PO$_4$, and 15 gL^{-1} agar), is selective for keratinolytic bacteria [25]. Both media types contained 100 μL/mL of cycloheximide to inhibit fungal growth [29], and all plates were incubated at room temperature (∼28°C) for 1 week. The rationale for selecting this growth temperature was that mesophilic bacteria should all grow at a temperature of 28°C.

Eighty colonies were chosen at random. Colonies with unusual or infrequently detected morphologies were always selected, to increase the probability of obtaining a diverse sampling. Colonies were re-streaked on TSA at least three times, and incubated at room temperature for 48–72 h each time until the purity of culture was confirmed by examination of colony morphology.

Pure cultures were re-streaked on TSA and incubated at 28°C for 48 h in preparation for identification. A loopful of cell material of late-log phase cells was harvested, and fatty acids were extracted and methylated according to the procedure described by the manufacturer (Microbial ID, Inc., Newark, DE). Samples were analyzed in a Hewlett-Packard (Palo Alto, CA) 5890 series II gas chromatograph with a 7673 autosampler, a 3396 series II integrator, and a 7673 controller. With the Sherlock (Microbial ID, Inc.) program on a Hewlett-Packard Vectra QS/20 computer, the chromatograms were compared to a database of reference cultures previously grown on TSA.

Results

Molecular Phylogenetic Method. By RFLP, approximately 220 clones were screened from each library for a total of 909 clones. Typically, 5 to 15 bands resulted from each rDNA digest in the discernible fragment size range of 50–300 bp (Fig. 1). Twenty unique banding patterns in library F1, 25 in F2, 34 in M1, and 18 in F2 were detected. When a banding pattern was faint or unclear, the corresponding PCR product was sequenced.

The chimera-detection program of the RDP was used to detect chimeras. The most serious limitation of this program is that it depends on the presence of sequences of the parent molecules in the database. Because the sequences in this study were for the most part closely related to known sequences (see below), this limitation should not affect the detection of chimeras in our samples. Two chimeric sequences were detected and discarded, and one sequence was identified as a known contaminant [34] and discarded. The BLAST program identified 14 different 16S rDNA gene sequences as the closest relatives of the feather bacteria sequences (Table 1). Approximately 53% of the unique sequences obtained were between 98% and 100% identical to their closest matches in GenBank. Approximately 35% of the

Table 1. Identification of unique cloned bacterial sequences from the feathers of eastern bluebirds in Lee County, Alabama

Clone	Highest BLAST identity (Accession number)	Base pairs matched	Division	RFLP pattern detection frequency
9	*Pseudomonas poae* (AJ492829)	752/765 (98%)	γ-proteobacteria	36
432	*Stenotrophomonas maltophila* (AF137357)	927/937 (99%)	γ-proteobacteria	45
753	*Acinetobacter venetianus* (AVE295007)	763/790 (96%)	γ-proteobacteria	34
73	*Pseudomonas brennerii* (AF268968)	771/784 (98%)	γ-proteobacteria	9
791	*Ewingella americana* (U29438)	950/967 (98%)	γ-proteobacteria	33
85	*Pseudomonas lundensis* (AB021395)	941/961 (98%)	γ-proteobacteria	47
78	*Aeromonas veronii* (X60414)	788/802 (98%)	γ-proteobacteria	10
15	*Pseudomonas fluorescens* (AF094725)	951/959 (99%)	γ-proteobacteria	305
531	*Pseudomonas fragi* (AF094733)	806/840 (95%)	γ-proteobacteria	85
2	*Pseudomonas lundensis* (AB021395)	871/897 (97%)	γ-proteobacteria	67
60	*Janthinobacterium lividum* (AF174648)	836/837 (96%)	β-proteobacteria	53
252	*Rhodoferax ferrireducens* (AF435948)	706/752 (93%)	β-proteobacteria	20
47	*Janthinobacterium lividum* (AF174648)	766/802 (95%)	β-proteobacteria	35
96	*Janthinobacterium lividum* (AF174648)	699/710 (98%)	β-proteobacteria	20
88	*Streptococcus vestibularis* (AY188353)	765/772 (99%)	Firmicutes	5
4	*Streptococcus salivarius* (AF459433)	785/807 (97%)	Firmicutes	7
191	*Lactobacillus gasseri* (AF519171)	806/863 (93%)	Firmicutes	10

The species in GenBank with the closest DNA sequence to each isolate (as determined by the BLAST algorithm) is presented as a preliminary identification. RFLP: restriction fragment length polymorphism.

sequences obtained were between 95% and 97% identical to their nearest match, and 12% were ≤93% identical. Seventeen unique sequences were used in subsequent phylogenetic analyses.

Although all sequences identified were representatives of the eubacteria, the overall community was diverse. Most (10/17 or 62%) of the unique sequences used in phylogenetic analyses were representatives of the γ subdivision of the Proteobacteria (Table 1; Fig. 2). Many of these sequences were most closely related to bacteria of the genus *Pseudomonas*, but this genus is poorly defined, with representatives in the α, β, and γ subdivisions of the Proteobacteria [14]. Most of our *Pseudomonas*-like sequences were closest to the fluorescent Pseudomonads in the γ-Proteobacteria (Fig. 2a). Approximately 23% (4/17) of unique sequences were closely related to bacteria in the β subdivision of the Proteobacteria (Fig. 2b). The remaining unique sequences (3/17 or 16%) were most closely related to bacteria in the Firmicute division (Fig. 2c).

Culture-Based Method. Eighty randomly picked colonies were analyzed by gas chromatography of cellular fatty acids. Similarity indices ranged from 0.140 to 0.904, with an average of 0.491, which is considered robust for this type of analysis [30]. Identifications with similarity values <0.300 (11 samples) were not used in any analysis. Firmicutes represented the largest portion (50 of 69 or 72%, Table 2) of the identified organisms, followed by γ-Proteobacteria (17 of 69 or 25%; Table 2), α-Proteobacteria and Actinobacteridae (both 1/69 or 1.4%).

These results stand in sharp contrast to those obtained using the culture-independent method. The difference between the results obtained by these methods

was unexpected, so the possibility that either (1) the GC results were inaccurate or (2) we were unable to amplify the sequences corresponding to the GC results was tested. Ten colonies that had been analyzed by gas chromatography were grown overnight on TSA, and 16S rDNA sequences were obtained from them by PCR with primers 515F and 1492R. These sequences were compared to known sequences in GenBank with BLAST, and the results are displayed in Table 2. Most of the 16S rDNA sequence identifications corresponded to the GC identifications, indicating that we were able to amplify sequences from these organisms and that our GC results were accurate.

Discussion

The primary purpose of this study was to inventory the microbial diversity of wild bird feathers. In other studies researchers have focused on particular groups of microbes (e.g. *Bacillus* [6]) or on keratinolytic bacteria [27], but here we surveyed total bacterial diversity with both culture-based and culture-independent methods. Such a survey is a necessary first step toward a fuller understanding of the microbial ecology of bird feathers and the potential interactions between birds and feather microbes. Members of the genus *Pseudomonas* were most heavily represented in the molecular analysis, and members of the genus *Bacillus* were most heavily represented in the culture-based analysis. Given the simple nutritional requirements and growth conditions of the *Pseudomonas* spp. from our molecular survey, it is surprising that we did not detect them in our culture-based survey. Perhaps the number of isolates we identified was too small for
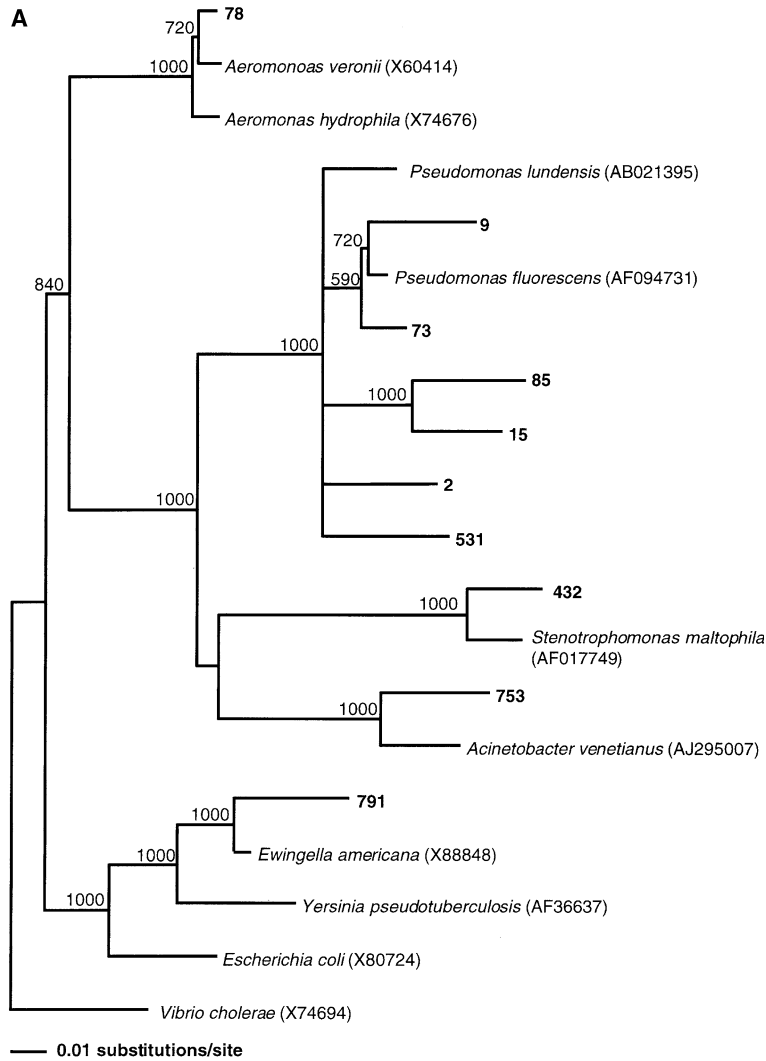
A


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            78
      720 ┌─────
     1000 └── Aeromonoas veronii (X60414)
          └── Aeromonas hydrophila (X74676)

                    ┌── Pseudomonas lundensis (AB021395)
                    │        9
              720 ┌─┤
              590 │ └── Pseudomonas fluorescens (AF094731)
                  │  73
             1000 │        85
                  │ 1000 ┌──
                  │      └── 15
                  │  2
                  └── 531

              1000 ┌── 432
                   └── Stenotrophomonas maltophila (AF017749)
              1000 ┌── 753
                   └── Acinetobacter venetianus (AJ295007)

         1000 ┌── 791
         1000 ├── Ewingella americana (X88848)
         1000 └── Yersinia pseudotuberculosis (AF36637)
              └── Escherichia coli (X80724)
         └── Vibrio cholerae (X74694)
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— 0.01 substitutions/site

Figure 2. Neighbor-Joining trees of unique cloned bacterial sequences from eastern bluebird feathers and related sequences from GenBank and the Ribosomal Database Project. (A) γ-proteobacteria; (B) β-proteobacteria; (C) firmicutes. Bold letters indicate unique cloned sequences. Numbers close to the nodes represent bootstrap values obtained from 1000 bootstrap replicates.

detection. However, the apparent numerical dominance of *Pseudomonas*-like sequences in our molecular survey suggests that they should also be widespread in our culture-based survey. Similarly, we would expect the dominance of *Bacillus* in our culture-based method to be reflected in the results of our molecular survey. These conflicting results may be caused by the biases of both PCR methods and culture-based methods. The guanine-plus-cytosine (G + C) content of template DNA has been reported to influence gene amplification by PCR [7, 23]. Reysenbach et al. (23) found that low G + C rDNA was preferentially amplified from a mixture of low and high G + C rDNA. Firmicutes of the genus *Bacillus* have fairly low G + C content (∼40–48%), whereas many of the *Pseudomonas* spp. that we identified, in particular *P. fluorescens* have high G + C content (∼66% for P. *fluorescens*). Thus, our pattern is the opposite of what we expected from G + C content bias. Our successful amplification of DNA isolated from cultured *Bacillus* sp. with the Qiagen kit suggests that the bias was not introduced at the DNA-

extraction phase. Differences in secondary structure affecting either the availability of the priming sites or the polymerization reaction may cause amplification bias [11]. In any case, our results emphasize that either multiple primer pairs or both molecular and culture-based approaches should be used when characterizing microbial communities [11, 26].

Taken together, our methods identified a diverse group of bacteria from the feathers of eastern bluebirds. Many of the organisms were closely related to common soil bacteria. *P. fluorescens* is a highly heterogeneous "species" that can be subdivided by various taxonomic criteria into subspecies, biotypes, or biovars [20]. Indeed, *P. lundensis* was independently discovered as both a separate species [18] and as a well-defined subgroup of *P. fluorescens* [4]. The various forms of P. *fluorescens*, along with the other related *Pseudomonas* species in this study appear to be ubiquitous in soil [20]. *Janthinobacterium* spp. are also commonly found in soil and water [13, 21], although they typically comprise only a small
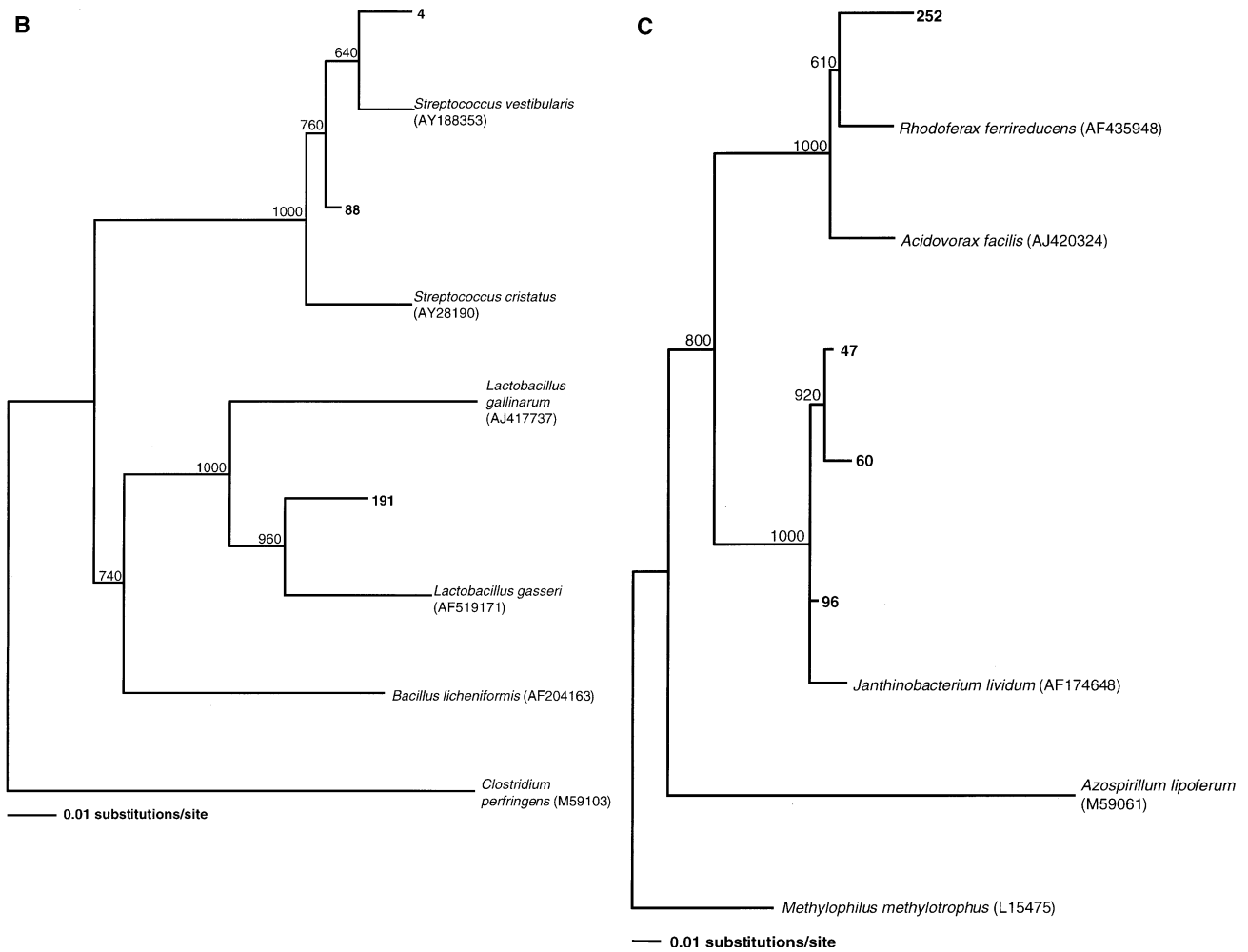
Figure 2. Continued

portion of the total microflora. This distribution suggests that birds may acquire them while landing or foraging on the ground.

The metabolic properties and optimal growth requirements of some of the identified bacteria suggest that they may play roles in the ecology of feathers. Members of

Table 2. Cultured isolates from the feathers of four eastern bluebirds (*Sialia sialis*) captured in Lee County, Alabama

Identification	Division	Number of isolates	Highest BLAST identity (Accession number)
Acinetobacter calcoaceticus	Firmicutes	1	*Acinetobacter calcoaceticus* (M34139)
Arthrobacter mysorens	Firmicutes	1	
Bacillus cereus	Firmicutes	14	*Bacillus anthracis* (AY043083)
Bacillus licheniformis	Firmicutes	4	*Bacillus cereus* (Z84581)
Bacillus mycoides	Firmicutes	6	
Bacillus pumilus	Firmicutes	15	*Bacillus* sp. (X81132)
Bacillus sphaericus	Firmicutes	1	
Cellulomonas flavigena	Firmicutes	1	
Exiguobacterium acetylicum	Firmicutes	6	*Exiguobacterium oxidotolerum* (AB105164)
Enterococcus durans	Firmicutes	1	*Bacillus cereus* (Z84581)
Microbacterium liquefaciens	Actinobacteridae	1	*Microbacterium esteraromaticum* (AB099658)
Erwinia chrysanthemi	γ-proteobacteria	1	
Pantoea ananas	γ-proteobacteria	1	
Serratia marcescens	γ-proteobacteria	15	*Serratia marcescens* (AY395011)
Sphingomonas capsulata	α-proteobacteria	1	

Note: Isolates were identified by gas chromatography of cellular fatty acids and, in some cases, by BLAST searches of 16S rDNA sequences.

the genus *Lactobacillus* are extremely fastidious organisms [10], so it is not surprising that we did not obtain them in culture. The optimal growth of many *Lactobacillus* spp. under microaerobic conditions suggests that they may reside on the portion of the rachis lying beneath the skin. Similarly, aerotolerant anaerobes of the genus *Streptococcus* may reside near the skin, as they do in humans [24]. Further analyses of different sections of feathers are needed to investigate the location of these bacteria.

One of our sequences was closely related to *Rhodoferax ferrireducens*, a recently isolated bacterium capable of breaking down acetate [9], most likely via the glyoxylate bypass pathway. This pathway may enable the bacteria to break down the components of preen oil on feathers. If so, this raises the possibility that preen oil may inhibit the growth of some bacteria while providing a carbon source for others. Other studies have provided evidence of this type of effect *in vitro* [3, 27].

Most studies of feather bacteria have focused on the genus *Bacillus* [6, 19], because strains of *B. licheniformis*, *B. pumilus*, and *B. megaterium* have been shown to have significant keratinolytic activity *in vitro*. *Bacillus spp.* were fairly abundant in our culture-based survey (see Table 2), suggesting that they may be common inhabitants of bird feathers. Strains of *Kocuria kristinae* have similar keratinolytic properties [27]. Further tests are needed to determine whether these bacteria can colonize feathers and express keratinolytic enzymes on feathers of live birds. Because they can form spores, *Bacillus* spp. may reside on feathers in a resting state, and they may not become active until feathers are molted and drop to the ground. Like *Pseudomonas*, these species are common in soil [28], and this may be the source of acquisition by birds.

Our results show that the microbial composition of bird feathers is diverse. The interactions of these bacteria with one another and, potentially, with birds themselves should prove a fascinating avenue for continued research. First, we need to determine which bacteria are active on feathers and how they acquire nutrition, whether from the feathers themselves, from detritus, from other microbes, or from other sources yet to be identified. Second, we should investigate interactions between bacteria on the feathers of birds and determine how bacterial communities may be controlled through, for example, the application of preen oil. Finally, we should examine whether these communities can affect birds through the degradation of feathers or possibly by acting as opportunistic pathogens. By doing so, we may gain some insight into the ecological roles of these bacteria and their potential co-evolution with birds.

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