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Evaluation of Broiler Litter with Reference to the Microbial Composition as Assessed by Using 16S rRNA and Functional Gene Markers

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Very little is known about the microbial composition of animal bedding wastes, including poultry litter, and what is known has been deduced from standard culture methods, by which some fastidious organisms that exist in the environment may not be detected. We evaluated the bacterial composition of poultry litter by using a combination of culture and molecular detection. Total aerobic bacteria in poultry litter were detected by culture at 10^9 CFU/g of material. Enteric bacteria such as *Enterococcus* spp. and coliforms composed 0.1 and 0.01%, respectively, of the total aerobic cultivatable bacteria in poultry litter; no *Salmonella* strains were detected by culture. In order to characterize the most abundant bacterial groups, we sequenced 16S ribosomal DNA (rDNA) genes amplified by PCR with microbial community DNA isolated from poultry litter as the template. From the 16S rDNA library, 31 genera were identified. Twelve families or groups were identified with lactobacilli and *Salinococcus* spp. forming the most abundant groups. In fact, 82% of the total sequences were identified as gram-positive bacteria with 62% of total belonging to low G+C gram-positive groups. In addition to detection of 16S rDNA sequences associated with the expected fecal bacteria present in manure, we detected many bacterial sequences for organisms, such as *Globicatella sulfidofaciens*, *Corynebacterium ammoniagenes*, *Corynebacterium urealyticum*, *Clostridium aminovalericum*, *Arthrobacter* sp., and *Denitrobacter permanens*, that may be involved in the degradation of wood and cycling of nitrogen and sulfur. Several sequences were identified in the library for bacteria associated with disease in humans and poultry such as clostridia, staphylococci, and *Bordetella* spp. However, specific PCR targeting other human and veterinary pathogens did not detect the presence of *Salmonella*, pathogenic *Escherichia coli*, *Campylobacter* spp., *Yersinia* spp., *Listeria* spp., or toxigenic staphylococci. PCR and DNA hybridization revealed the presence of class 1 integrons with gene cassettes that specify resistance to aminoglycosides and chloramphenicol. Only from understanding the microbial community of animal wastes such as poultry litter can we manage animal disease and limit the impact of animal waste on the environment and human and animal health.

Poultry litter is a mixture of excreted manure mixed with bedding material. According to the U.S. Food and Drug Administration, 5.6 million tons of litter dry matter is produced per year in the United States (16). One type of poultry waste is broiler litter resulting from the production of commercial broiler chickens on wood chips, sawdust, wheat straw, peanut, and rice hulls (61). Analysis has shown that broiler litter contains potentially valuable plant nutrients, including nearly 30% crude protein and high levels of minerals and some heavy metals (36). For this reason, litter is commonly used as a fertilizer but environmental concerns, such as nutrient, nitrate, and phosphate runoff to streams, ponds, and groundwater have limited its acceptability (53, 54). Another disadvantage is the tendency for ammonia and other volatile gases to escape into the atmosphere, resulting in unpleasant odors.

In addition to its use as fertilizer, poultry litter has nutritional value in cattle. Growth performance for cattle on feed supplemented with poultry litter is similar to or higher than a diet with good-quality legume hays (53). The U.S. Food and Drug Administration estimates that 20 to 25% of the broiler litter is fed, but there are concerns regarding the safety of feeding large quantities of poultry litter to cattle, which are susceptible to infection by pathogenic microorganisms such as *Listeria monocytogenes* and *Salmonella* and *Campylobacter* spp. that may be present (36). Botulism outbreaks have also been described in cattle herds fed contaminated litter (44).

These concerns necessitate knowledge about the composition of the bacterial microflora present in broiler litter. Martin et al. performed a survey of pathogenic microorganisms in poultry litter with selective medium and found *Staphylococcus xylosus* to be a predominant species (36). However, we have not yet cultured the majority of the bacteria in the environment because many microorganisms are difficult to culture (60, 63) and selective media are often not truly specific or are too selective for certain bacteria (23, 42, 65). In the present study, we characterized the bacterial composition of broiler litter in order to assess its safety as fertilizer or feed amendment for cattle, employing 16S ribosomal DNA (rDNA) sequencing and PCR screens for pathogens and antibiotic resistance genes.

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MATERIALS AND METHODS

Origin and collection of litter samples. Litter was collected from four geographically separate farms in Northeast Georgia and poultry houses already stocked with broiler chickens. Birds present on these four farms ranged in age from a few days old to 6-week-old, market-weight size animals, and the litter had not been removed from the house between flocks. All farms were contracted to raise birds for a single poultry company. The company provided the farm with the birds and feed. Wood shavings from softwoods were used as bedding; material commonly used in poultry houses in the southeastern United States. Litter samples were randomly removed from underneath the nipple-drinker, water lines, and along the length of the flock house and then pooled. Five such samples were collected from each house. Five grams of chicken litter was resuspended in 30 ml of phosphate-buffered saline and then mixed for 5 min with a "wrist-action" shaker set on the maximum setting. Debris was removed by low-speed centrifugation (50 × *g* for 15 min at 4°C). The bacteria were pelleted by high-speed centrifugation (3,650 × *g* for 15 min at 4°C) and resuspended in 1 ml of phosphate-buffered saline. A 0.5-ml aliquot was transferred to two 1.5-ml microfuge tubes, and the bacteria were pelleted once again by high-speed centrifugation (3,650 × *g* for 15 min at 4°C). The bacterial pellet for one tube was resuspended in Superbroth (48) with 15% glycerol and stored at −80°C, whereas nucleic acid was extracted from the second bacterial pellet as described for DNA extraction.

Viable counts of bacteria present in poultry litter. Frozen bacterial suspensions were serially diluted in buffered saline gelatin (12). The dilutions were plated on MacConkey, mEnterococcus, and mannitol-salt agar for enumeration of gram-negative enterics, enterococci, and staphylococci, respectively. Dilutions were also plated on brain heart infusion (BHI) agar to obtain the total aerobic plate count for each litter sample. The litter samples, diluted 10^{-4} to 10^{-7}, were plated on the MacConkey or Enterococcus agar; those diluted 10^{-8} to 10^{-11} were plated on BHI or mannitol-salt agar. Plates were incubated overnight at 37°C. Selective agar medium was acquired as dehydrated powder from Difco (Detroit, Mich.).

The environment of four, commercial broiler farms was sampled for *Salmonella* by using drag swabs: gauze pads soaked with double-strength skim milk that were then dragged across the birds' bedding material (9). Each swab was then placed in 100 ml of tetrathionate brilliant green broth (Difco) and incubated at 41.5°C for 18 h (6). A loop full of the tetrathionate brilliant green enrichment broth was streaked onto a XLT4/BGN biplate (Difco), followed by incubation at 37°C overnight (24).

DNA extraction. The bacteria collected from chicken litter were resuspended in cell lysis solution (40% sucrose, 10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, lysozyme [4 mg/ml], lysostaphin [30 μg/ml], RNase [250 μg/ml]) and incubated at 37°C for 1 h. After incubation, bacteria were pelleted by high-speed centrifugation (13,000 × *g* for 15 min at 4°C) and then resuspended in 10 mM Tris–1 mM EDTA. The subsequent steps were done according to the CTAB procedure for isolating bacteria DNA (2). DNA concentration (1.3 μg μl^{-1}) was measured by using a Beckman DU640 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). Unless otherwise indicated, chemical reagents were purchased from Sigma Chemical Co. (St. Louis, Mo.).

PCR detection of pathogens and antibiotic resistance genes. DNA extracted from litter was used in PCR to detect the presence of *Salmonella* spp. (34), *Escherichia coli* O157 (37), *Campylobacter* spp. (14, 21), *Yersinia* spp. (58), *Listeria* spp. (8), pathogenic *E. coli* (17, 46), and toxigenic staphylococci (29) and *Clostridium perfringens* (64). The specific genes, primer sequences, conditions, expected size of each amplicon, and PCR references are given in Table 1. Litter DNA was used at 2.5 and at 25 ng/10 μl of PCR volume. Clinical isolates from the veterinary diagnostic laboratory served as positive controls for the PCRs.

PCR was also used to detect the presence of class 1 integrons and associated cassettes. The class 1 integrase (*int1I*) was detected by PCR as described by Goldstein et al. (20) by using litter DNA at 2.5and at 25 ng/10 μl of PCR volume. Associated antibiotic resistance cassettes were amplified by using 5′-3′ conserved sequence (CS) PCR with primers targeted to the 5′ and 3′ conserved regions of the class 1 integron sequences (33). The resistance cassettes were detected in a PCR-enzyme-linked immunosorbent assay (ELISA) as previously described (43). This consisted of the 5′-3′ CS PCR by using digoxigenin-labeled nucleotides (Roche Molecular Biochemicals, Indianapolis, Ind.) and biotinylated cassette-specific probes. The probe sequences and specificities are shown in Table 2; all of the probes possessed a T_m of 46 to 51°C and were used at high stringency (50°C annealing temperature). The 5′-3′ CS PCR mixture (50 mM Tris [pH 8.3], 3 mM $MgCl_2$, 0.25 mg of bovine serum albumin/ml, 0.5% Ficoll) contained a 0.1 fM concentration of each 3′-biotinylated probe. After 30 cycles the amplicons were denatured by incubation for 1 min at 96°C with probe annealing for 15 min at 50°C. Probe-amplicon hybrids were captured in streptavidin-coated wells and detected by using anti-digoxigen antibody-conjugate as described by the manufacturer (Roche Molecular Biochemicals). Oligonucleotide probes were pooled in the hybridization step of the PCR-ELISA in order to detect resistance genes of certain antibiotic classes. *Salmonella enterica* serovar Typhimurium DT104, which possesses two class 1 integrons containing *blaP1* or *aadA1*, was used as the positive control for the aminoglycoside and β-lactam resistance pool (7). Plasmid R388, which possesses a class 1 integron containing *dfrB2*, was used as the positive control for the trimethoprim resistance pool (56). Tn*1696*, which possesses a class 1 integron containing *cmlA*, was used as the positive control for the chloramphenicol resistance pool (4).

PCR for construction of 16S rDNA clone libraries. For construction of the 16S rRNA gene clone libraries, three sets of primers, which target the domain *Bacteria*, were used (26): primer set A, 8F [(5′-AGA GTT TGA TCC TGG CTC AG-3′)/1492R] [5′-TAC GG(C/T) TAC CTT GTT ACG ACT T-3′]; primer set B, 8F/1522R (AAG GAG GTG ATC CAN CCR CA); and primer set C, 8F/926R (ACC GCT TGT GCG GGC CC). Primer 1492R was synthesized as a mixture of oligonucleotides with either T or C at position 1497 (*E. coli* numbering). Primer sets A and B are frequently used in molecular diversity studies because they result in a nearly full-length 16S rDNA product and are considered universal for the domain *Bacteria* and for the prokaryotes, respectively (32). Primer set C was used to minimize the effect of template concentration on PCR bias (11). The final reaction mixture included 25 or 100 ng of template DNA with primer set C and 25 ng with other primer sets, 1′ PCR buffer, 2.0 mM $MgCl_2$, a 0.2 mM concentration of deoxynucleoside triphosphate, a 1 μM concentration of each primer, and 0.05 U of *Taq* DNA polymerase (Roche Molecular Biochemicals) in a final reaction volume of 25 μl. Initial DNA denaturation was performed at 94°C for 2 min in a PTC200 thermocycler (M. J. Research, Inc., Watertown, Mass.), followed by 15 cycles of denaturation at 94°C for 1 min, annealing at 54°C (primer set A), 48°C (primer set B), and 58°C (primer set C), respectively, for 30 s, and then elongation at 72°C for 1 min, which was followed in turn by a final elongation at 72°C for 10 min. Fifteen cycles of PCR were performed rather than 30 cycles to minimize the risk of certain 16S rDNA types being preferentially amplified (63). Three separate PCR amplifications were performed to minimize potential bias due to PCR drift (11) and to increase the DNA yield for subsequent cloning. The nine PCRs generated with the three PCR primer sets were pooled together for cloning. The amplified PCR products were purified with the Wizard PCR product purification kit (Promega, Madison, Wis.). The purified products were ligated into pGEM-T-Easy (Promega). Ligation was done at 4°C overnight, followed by transformation into competent *E. coli* JM109 cells by heat shock (45 s at 42°C). The clones were screened for α-complementation of β-galactosidase by using X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside) (2).

Plasmid extraction and sequencing. DNA preparations for sequencing were made with the QIAprep spin plasmid kit (Qiagen, S.A.) as specified by the manufacturer. Plasmids were eluted with 50 μl of water, and the products were stored at −70°C. Sequencing reactions were performed with a PE-ABI BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif.) as described by the manufacturer, and electrophoresis and readout were done with an ABI Prism 3700 DNA analyzer (Applied Biosystems). Primers T7 and SP6 were used in the sequencing reactions to sequence both strands of each PCR product.

Analysis of DNA sequences. Resulting DNA sequences were edited to exclude primer-binding sites and ambiguous bases and assembled into contiguous sequences (570 to 650 bp) by using the Sequencher program, version 4.10 (Gene Code Corp., Ann Arbor, Mich.). The resulting DNA sequence information was analyzed by using the programs FASTA (47) and BLAST (1). Chimeric sequences were detected as described by Suau et al. (55). The estimate of sample size and coverage were conducted according to the formula for coverage as described by Good (22) and applied in quantitative comparisons of 16S rRNA gene sequence libraries as described by Singleton et al. (52). The same definition for the variables in the formula $Cx = 1 - (Nx/n)$ used by Singleton et al. (52) was used here, i.e., where Cx is the "homologous" coverage of sample X; Nx is the number of unique sequences, and n is the total number of sequences in the sample. Coverage was considered at two levels of minimum match percentage, 97 and 99%, respectively, for 16 S rDNA sequences (350 to 410 bp), which resulted from the three primer sets (38, 55). Based on 231 consistent sequences, Nx was analyzed by using Sequencher program, and Cx was calculated by the method of Good (22). Representative sequences were submitted to GenBank as accession numbers AY080963 to AY080994.

TABLE 1. PCR detection of potential pathogens among DNA sequences extracted from the bacterial microflora of broiler chicken litter[a]

Organism	PCR target	Primer sequence[b]	PCR conditions ($MgCl_2$ concn [mM], annealing temp [°C])	Expected size (bp)[c]	Reference
Salmonella sp.	*inv*	F: CTGTTGAACAACCCATTTGT R: CGGATCTCATTAATCAACAAT	3, 42	437	34
Escherichia coli	O157 *rfb*	F: CGTGATGATGTTGAGTTG R: AGATTGGTTGGCATTACTG	2, 55	420	37
	LT1	F: TGGATTCATCATGCACCACAAGG R: CCATTTCTCTTTTGCCTGCCATC	3, 63	360	46
	VT1 (*stx1*)	F: ACGTTACAGCGTGTTGCTGGGATC R: TGTGGCTGGGTTCGTTAATACGGC	3, 63	121	46
	VT2 (*stx2*)	F: TGTTGGCTGGGTTCGTTAATACGG R: TCCGTTGTCATGGAAACCGTTGTC	3, 63	102	46
	STI	F: TTTCCCCTCTTTTAGTCAGTCAACTG R: GGCAGGATTACAACAAAGTTCACAG	3, 63	160	46
	STII	F: CCCCCTCTCTTTTGCACTTCTTTCC R: TGCTCCAGCAGTACCATCTCTAACCC	3, 63	423	46
	CNF1	F: GGCGACAAATGCAGTATTGCTTGG R: GACGTTGGTTGCGGTAATTTTGGG	3, 63	552	46
	eaeA	F: TGAGCGGCTGGCATGAGTCATAC R: TCGATCCCCATCGTCACCAGAGG	3, 63	241	46
	K99	F: TATTATCTTAGGTGGTATGG R: GGTATCCTTTAGCAGCAGTATTTC	3, 50	314	17
	F41	F: GCATCAGCGGCAGTATCT R: GTCCCTAGCTCAGTATTATCACCT	3, 50	380	17
Staphylococcus aureus	*sea*	F: TTGGAAACGGTTAAAACGAA R: GAACCTTCCCATCAAAAACA	2, 55	120	29
	seb	F: TCGCATCAAACTGACAAACG R: GCAGGTACTCTATAAGTGCC	2, 55	478	29
	sec	F: GACATAAAAGCTAGGAATTT R: AAATCGGATTAACATTATCC	2, 55	257	29
	sed	F: CTAGTTTGGTAATATCTCCT R: TAATGCTATATCTTATAGG	2, 55	317	29
	tsst1	F: ATGGCAGCATCAGCTTGATA R: TTTCCAATAACCACCCGTT	2, 55	350	29
Campylobacter jejuni	*ceuE*	F: CCTGCTACGGTGAAAGTTTTGC R: GATCTTTTTGTTTGTGCTGC	3.5, 57	793	21
Campylobacter coli	23S rDNA	F: TATTCCAATACCAACATTAGT R: GCGAAGCATAATCCTAAAT	3, 54	390	14
Yersinia spp.	16S rDNA	F: GCGGCAGCGGGAAGTAGTTA R: TACAGCGTGGACTACCAGGGT	4, 63	749	58
Listeria spp.	*iap*	F: ATGAATATGAAAAAGCAAC R: TTATACGCCACCGAAGCCAAC	1.5, 50	1,600	8
Clostridium perfringens	α toxin	F: GTTGATAGCGCAGGACATGTTAAG R: CATGTAGTCATCTGTTCCAGCATC	2, 55	402	64
	β toxin	F: ACTATACAGACAGATCATTCAACC R: TTAGGAGCAGTTAGAACTACAGAC	2, 55	236	64
	ε toxin	F: ACTGCAACTACTACTCATACTGTG R: CTGGTGCCTTAATAGAAAGACTCC	2, 55	541	64
	ι toxin	F: GCGATGAAAAGCCTACACCACTAC R: GGTATATCCTCCACGCATATAGTC	2, 55	317	64

[a] No target gene or organism was detected in litter by PCR.
[b] F, forward; R, reverse.
[c] A total of 10 pg of target DNA was detected by the *E. coli* O157 (37) and *C. perfringens* (64) PCRs; 10 fg could be detected in the *Yersinia* (58) and *Salmonella* PCRs. The detection range of 10 pg to 10 fg translates to 10^4 to 10 cells, respectively.

RESULTS AND DISCUSSION

Poultry litter used in the present study contained 10^9 aerobic bacteria per g of material as determined by CFU on BHI agar. Staphylococci accounted for the majority of the total aerobic plate counts (13%), whereas enteric bacteria such as enterococci (0.1%) and gram-negative enterics (0.11%) were only minor components of the total bacteria cultured from poultry litter. The levels of total bacteria in our samples were much

TABLE 2. PCR primers and oligonucleotides used to probe for class 1 integrons and integron-associated antibiotic resistance cassettes among DNA sequences extracted from the bacterial microflora of broiler chicken litter[a]

Gene cocktail and target gene(s)	Oligonucleotide probe sequence (5′-3′)	Presence (+) or absence (−) of antibiotic resistance genes in litter
Aminoglycoside resistance		+
aadA1, aadA2	GCAGCGCAATGACATTCTTG	
aacA7	TTTCGATCCGCCCGTATG	
aac6′Ia, aac6′Iq	AAACCAGATATTTGGATGTGGAAA	
aac6′2a, aac6′2b	GCSATACGCTGCTATGAGAAGGC	
aac3′Ia, aac3′Ib	YGTGCAAGCAGAYTACGGTG	
aacA4	GGCATCCAAGCAGCAAG	
aacC1	GATTACCTCGGGAACTTGCTCCAA	
aadB, aac6′Id	CCGCAGCTAGAATTTTGGCTCCAA	
Trimethoprim resistance		−
dfrA14(Ib), dfr5	CCTTCGAAGTTGTTTTTGAGCAA	
dfrB1, dfrB2	TCCTGTKGCKGCRCTTGAAC	
dfrB3	ATCTCCCAGGCCAAACGTG	
dfr7, dfr12, dfr15	AATGGYCCWRATATYCCMTGG	
dfrA1	AGCTGTTCACCTTTGGC	
Chloramphenicol resistance		+
catB2, catB3, catB4, catB5, catB6	GCCATGRTYATGCCCGGVATCAA	
cmlA	CTAGGTTTGGGCATGATCGC	
β-Lactam resistance		−
blaP1, *blaP2*, *blaP3*	TCAGGTGCTGGCGGATTTG	
bla_{imp}, bla_{esp}	ACAGCACGGGCGGAATAGAG	
bla_{veb1}	ATTTCCCGATGCAAAGCG	
oxa20	CGAAGCCAAACGCGATTG	
oxa5, oxa7, oxa10, oxa19	RTCCTGGTGTCGCWTGGTG	
oxa9	AACCCGTCTCCGAGAGATCG	
oxa1	CGGATTAACAGAAGCATGGCTCAA	
oxa2, oxa3, oxa15, oxa21	GCCGATCCTTCGACAAGTAATGAA	

[a] Integron gene cassette(s) were amplified by PCR by using consensus sequences flanking the integration site *attC*. Oligonucleotide probes were used in a cocktail of capture probes in a PCR-ELISA to detect gene(s) that confer resistances to aminoglycosides, β-lactams, chloramphenicol, and trimethoprim. The reference for all gene cocktails was the present study.

higher than those detected by Martin and McCann (36) in which the total bacterial counts ranged between 1,200 and 8.4 $\times 10^7$. Martin and McCann also detected low plate counts of gram-negative bacteria and high plate counts of *Staphylococcus* spp. (36). The plate counts for *Enterococcus* spp. and facultative anaerobic, gram-negative bacteria appeared to reflect their relative abundance among the chicken's normal flora of the gastrointestinal tract (66). However, accurately discerning the microbial composition is limited by the chosen culture methods and the physiological state and nutritional or atmospheric requirements of the microorganism(s) targeted for growth. In order to obtain an alternative assessment of the major microorganisms that inhabit chicken manure, we generated 16S rDNA libraries from the microbial community DNA of broiler litter.

A total of 340 contiguous sequences originating from the 16S gene were retrieved from the 16S clone library. The library consisted of 265 sequences based on primer set A (78% of total clones) and 31 sequences from primer set B (9%) and 44 sequences from primer set C (13%). The sequences from the three primer sets were very similar in composition and abundance (data not shown); therefore, we used the contiguous sequences from primer set A as the main source for sequence analysis. For calculation of the coverage, 231 consistent sequences (350 to 410 bp) were analyzed. There were 84 and 103 distinct sequences at similarity levels of 97 and 99%, respectively. The coverage calculated for the 231 sequences was 71% ± 2% at 97% identity and 62% ± 3% at 99% identity, suggesting that the majority of unique sequences of the microbial community in chicken litter were reflected.

Based on analysis of the 340 clones isolated from the 16S rDNA libraries of bacteria collected from broiler litter, we identified four major phyla. These phyla included low and high G+C gram-positive bacteria, proteobacteria, and the CFB (*Cytophaga-Flexibacter-Bacteroides*) group (Table 3). A total of 12 families or groups and 31 genera were identified among the 16S rDNA sequences analyzed. The broiler litter bacterial microbiota consisted predominantly of gram-positive bacteria (87%), with low G+C gram-positive bacteria accounting for 62% of the total 16S rDNA sequences in the libraries. The low G+C gram-positive bacteria consisted of four families or groups represented by 15 genera. Identification of members of *Lactobacillaceae* and *Clostridiaceae* was not unanticipated for the environment expected to contain these fecal bacteria (66). However, we did not anticipate finding that 2% of the 16S rDNA sequences from the library would be *Enterococcus* specific, since this group comprised only a small percentage of the total cultivatable aerobic bacteria in poultry litter. There was good agreement between 16S rDNA frequencies and culture results for staphylococci. However, unlike Martin and McCann

TABLE 3. Composition and 16S rDNA sequence abundance in chicken litter

Group	Organism	No. of clones (n = 340)	% Total clones	% Group sequences
Low GC gram positive				
Lactobacillaceae and/or *Aerococcaceae* and/or *Carnobacteriaceae*	*Pediococcus* spp., *Pediococcus urinaeequi, Lactobacillus* spp., *Trichoccoccus* spp., and *Aerococcus viridans*	59	17.35	62.05
	Facklamia spp.	34	10.00	
Bacillus and/or *Staphylococcus*	*Bacillus* spp.	9	2.65	
	Staphylococcus cohnii, Staphylococcus succinus, Staphylococcus lentus, and *Staphylococcus arlettae*	30	8.82	
	Salinicoccus spp.	60	17.65	
	Globicatella sulfidofaciens	1	0.29	
Clostridiaceae	*Clostridium, Desulfotomaculum, Georgenia,* and *Ruminococcus* spp.	10	2.94	
Enterococcaceae	*Enterococcus faecalis, Enterococcus cecorum,* and *Vagococcus* spp.	8	2.35	
High GC gram positive				
Corynebacteriaceae	*Corynebacterium ammoniagenes* and *Corynebacterium urealyticum*	33	9.71	24.7
Micrococcaceae	*Arthrobacter* spp.	8	2.35	
Microcinaceae	*Brevibacterium avium, Brevibacterium iodinum,* and *Brevibacterium epidermidis*	24	7.06	
	Brachybacterium spp. and *Brachybacterium faecium*	18	5.29	
	Cellulomonas spp.	1	0.29	
Proteobacteria				
α	*Paracoccus* and *Aquamicrobium* spp.	3	0.88	12.94
β	*Alcaligenes, Bordetella,* and *Denitrobacter* spp.	34	10.00	
γ	*Lysobacter, Xanthomonas,* and *Stenotrophomonas* spp.	6	1.76	
δ	*Geobacter grbiciae*	1	0.29	
CFB group	*Sphingobacterium*	1	0.29	0.29

(36), we did not detect *S. xylosus* as the prevalent staphylococcal species (Table 3). Several 16S rDNA sequences were identified with homology to other gram-positive bacteria, including *Arthrobacter*, *Brevibacterium*, and *Cellulomonas* spp. (Table 3), organisms that may be involved in the decomposition of organic material including wood (18, 50, 57). 16S rDNA sequences were also identified with homology to sequences for *Corynebacterium urealyticum*, *Desulfotomaculum*, and *Globicatella sulfidofaciens*, which are anaerobic, reductive bacteria (31, 51, 59).

We identified many 16S sequences demonstrating homology to bacteria potentially pathogenic for humans and animals (Table 4). Several sequences with homology to *Bordetella* spp. were detected, however we could not identify the species since

TABLE 4. Potential pathogenic bacterial species and rDNA sequence abundance in chicken litter

Genus	Closest relative species in GenBank (accession no.)	% Identity	No. of sequences (n = 90)	% Total
Clostridium	*Clostridium* spp. (M23929, AJ308598, X71853, and Y10028)	93–96	7	7.78
Staphylococcus	*Staphylococcus cohnii* (AB009936)	98–99	12	33.33
	Staphylococcus succinus (AF004219)	98–99	12	
	Staphylococcus lentus (D83370)	99	1	
	Staphylococcus arlettae (AB009933)	98–99	3	
	Staphylococcus aureus (X363367)	99	2	
Facklamia	*Facklamia* sp. (Y17820)	98–99	34	37.78
Bordetella	*Bordetella bronchiseptica* (AJ278452)	98	3	3.33
Enterococcus	*Enterococcus faecalis* (AF440333) *Enterococcus cecorum* (AF061009), and *Enterococcus faecium* (AJ276355)	99	6	6.67
Brevibacterium	*Brevibacterium avium*	98–99	8	8.89
Vagococcus	*Vagococcus fluvialis* (X54258)	97–98	2	2.22

there are no discernible differences in 16S rDNA among *Bordetella pertussis*, *B. parapertussis*, *B. bronchisepticum*, and *B. avium* (39). None of the poultry farms from which the litter was collected had reported any present or past history of avian bordetellosis (*B. avium*), and there are no published reports of the human pathogenic bordetellae (*B. pertussis* and *B. parapertussis*) associated with birds. These sequences may represent strains causing subclinical avian disease or uncharacterized species. Other sequences were homologous to the organisms potentially virulent for animals, including *Brevibacterium avium* (45) and *Clostridium* sp. (35, 62). *Clostridium perfringens* is an important cause of necrotic enteritis in broilers, and it is generally managed or controlled with growth-promoting antibiotics (19, 35). Clostridia can also cause gangrenous dermatitis in poultry (62). However, sequences related to *Clostridium* sp. exhibited less than 96% homology to known *Clostridium* sp., suggesting that they may be uncharacterized strains. Staphylococcal 16S rDNA sequences were also frequently identified from the poultry litter library. The *Staphylococcus* 16S sequences were homologous to *S. cohnii*, *S. succinus*, *S. lentus*, and *S. arlettae*, and we did detect 16S rDNA sequences exhibiting high homology to *S. aureus*, the etiological agent of arthritis and gangrenous dermatitis in chickens (10, 41) and foodborne gastroenteritis in humans. However, PCR for specific enterotoxin or toxic shock syndrome toxin genes did not indicate that pathogenic staphylococci were prevalent in the litter. Although not pathogenic themselves, there are concerns that normal flora staphylococci and enterococci of animals may serve as a reservoir for transmitting antibiotic resistances to human commensal flora (30).

The molecular characterization of microbial ecosystems often involves the random PCR amplification, cloning, and sequencing of the 16S rDNA library. The chance that a unique clone was detected depends on its abundance in the library and on the total number of clones sequenced. Therefore, the limitation of this technique is that this method may miss unique clones representing very minor populations. We therefore chose PCR to target specific pathogens, which may exist as minor components of the litter microbiota (Table 1). PCR screens did not detect zoonotic or bovine specific pathogens including *Salmonella*, *Campylobacter*, *Yersinia*, and *Listeria* spp. or virulence genes associated with pathogenic *E. coli* or *S. aureus. Salmonella* was neither detected by PCR nor culture in the present study, and our results agree with those of a previous study (36). These findings were not entirely unexpected since the prevalence of *Salmonella* spp. on poultry farms is sporadic (34). Litter DNA did not interfere or reduce the sensitivity of PCR since detection limits were similar for litter DNA spiked with purified *Salmonella* DNA versus purified *Salmonella* DNA alone (data not shown). The absence of enterotoxigenic staphylococci, enterotoxigenic *E. coli*, enteropathogenic *E. coli*, and *E. coli* O157 in poultry litter was not unexpected due to their reported low frequency or absence in poultry flocks (5, 25, 28). We also did not detect genes associated with *E. coli* K99, F41, or EaeA adhesins that are required for *E. coli*'s colonization of a bovine or porcine host (13). However, it should be noted that, depending on the primers, the detection limits of PCR range from 10^4 cells to as few as 10 cells. Therefore, these organisms may be present in litter but well below the detection limit of PCR.

From our analysis of poultry litter microbiota, many bacterial species were identified that may be actively involved in composting of organic matter, and this may explain absence of several important veterinary and human pathogens from this environment. Poultry farms may or may not remove the old litter before placement of every new flock. Several poultry farms visited in the present study remove poultry litter only twice a year, placing fresh litter shavings on top of the old litter, which may allow the microbial activity to sufficiently compost the litter (27, 28). Comparisons of litter microflora from farms with different litter management practices may identify practices or conditions that reduce or eliminate harmful vegetative bacteria.

However, we did detect antibiotic resistance cassettes associated with class 1 integrons. The integron is potentially a major agent in the dissemination of multidrug resistance among gram-negative bacteria. The integron contains an integrase gene and a site-specific integration site where the integrase can link antibiotic resistance gene cassettes in tandem in the integration site if the circular cassette molecules possess a 59-base element (33, 49). More than 60 distinct antibiotic resistance gene cassettes have been characterized within the class 1 integron, and as many as 7 of them have been found in a single integron at one time (15, 40). In our experiments, PCR targeting of the class 1 integrase gene produced a product of the appropriate size, indicating that integrons were present in the community DNA. Past studies have shown a high prevalence of integrons in *E. coli* isolated from broiler chickens (3, 20), and culture of the litter in our study showed that coliforms were present at 250,000 CFU/g of litter. Bass et al. (3) demonstrated that the streptomycin resistance gene cassette, *aadA1*, was commonly found in the class 1 integrons possessed by avian *E. coli*. The class 1 integron cassettes can be amplified by PCR with primers anchored in the 5′ and 3′ conserved regions of the class 1 integron (33). We designed oligonucleotide probes to detect the presence of cassettes associated with aminoglycoside, β-lactam, chloramphenicol, and trimethoprim resistance (Table 2). These were used to determine whether the litter DNA contained integron associated-antibiotic resistance genes, which are potentially transferable to other ecological environments. We did not detect cassettes encoding β-lactam resistance or trimethoprim resistance but did detect the presence of genes encoding aminoglycoside resistance and chloramphenicol resistance. Antibiotics of these classes were not administered to the flocks raised on the litter used in the present study. In fact, chloramphenicol has not been used in food animals for more than 15 years, and no formulation exists on the market for its administration to flocks of birds. The detection of these resistance cassettes further illustrates the potential for antibiotic resistance genes to be present at detectable levels in environments in which the antibiotic's administration may not be recent. Subsequent application of poultry litter, as either a soil amendment or cattle feed, may not immediately negatively impact cattle or humans. However, its use may disseminate increasing loads of resistance genes that may be amplified in new environments.

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