Analysis of the Gull Fecal Microbial Community Reveals the Dominance of *Catellicoccus marimammalium* in Relation to Culturable Enterococci

Amber M. Koskey, Jenny C. Fisher, Mary F. Traudt, Ryan J. Newton, Sandra L. McLellan

University of Wisconsin—Milwaukee, School of Freshwater Sciences, Milwaukee, Wisconsin, USA

Gulls are prevalent in beach environments and can be a major source of fecal contamination. Gulls have been shown to harbor a high abundance of fecal indicator bacteria (FIB), such as *Escherichia coli* and enterococci, which can be readily detected as part of routine beach monitoring. Despite the ubiquitous presence of gull fecal material in beach environments, the associated microbial community is relatively poorly characterized. We generated comprehensive microbial community profiles of gull fecal samples using Roche 454 and Illumina MiSeq platforms to investigate the composition and variability of the gull fecal microbial community and to measure the proportion of FIB. *Enterococcaceae* and *Enterobacteriaceae* were the two most abundant families in our gull samples. Sequence comparisons between short-read data and nearly full-length 16S rRNA gene clones generated from the same samples revealed *Catellicoccus marimammalium* as the most numerous taxon among all samples. The identification of bacteria from gull fecal pellets cultured on membrane-*Enterococcus* indoxyl-β-D-glucoside (mEI) plates showed that the dominant sequences recovered in our sequence libraries did not represent organisms culturable on mEI. Based on 16S rRNA gene sequencing of gull fecal isolates cultured on mEI plates, 98.8% were identified as *Enterococcus* spp., 1.2% were identified as *Streptococcus* spp., and none were identified as *C. marimammalium*. Illumina deep sequencing indicated that gull fecal samples harbor significantly higher proportions of *C. marimammalium* 16S rRNA gene sequences (>50-fold) relative to typical mEI culturable *Enterococcus* spp. *C. marimammalium* therefore can be confidently utilized as a genetic marker to identify gull fecal pollution in the beach environment.

Gulls and other shorebirds and waterfowl are prevalent in beach environments, and their feces are considered a major source of fecal indicator bacteria (FIB) in coastal and lake waters worldwide (1–5). *Escherichia coli* and enterococci are commonly used to monitor the presence of pathogenic organisms in beach environments and assess the relative human health risks associated with recreational water use (6); however, these FIB are ubiquitous, as they are found in the feces of humans and animals alike, limiting their utility to accurately predict human health risks (7). Although there is evidence to support the shedding of human pathogens (8–11), the human health risks associated with shorebird and waterfowl feces are inherently lower than those from human fecal inputs (12, 13). Furthermore, bird droppings are a more concentrated point source of contamination on beaches, while sewage is more readily dispersed, presenting a larger radius of contamination risk. Beach advisories are implemented to protect public health, but many unnecessary beach closings result from FIB seeding by gulls (2, 3). These closings can have huge economic implications, especially in coastal areas (14); therefore, the ability to discriminate the source of fecal contamination is critical for both human health risk assessments and mitigation of economic losses.

Currently, most health-related surface water monitoring programs use selective media to culture FIB, usually *E. coli* and enterococci. Gulls often shed high densities (10^5 to 10^9 CFU of *E. coli* and 10^4 to 10^8 CFU of *Enterococcus* spp. g^{-1} feces) of these organisms via their droppings to surface waters and beach environments (3, 15). Thus, gull contamination can confound the monitoring efforts of recreational water sources proposed by the U.S. Environmental Protection Agency (16). Gull populations have steadily increased in urban areas over the past 30 years because of

more readily abundant food supplies, long life spans, and few natural predators (17–19). Recreational water quality monitoring efforts are, in turn, impacted by these growing gull populations.

Traditional culture-based monitoring methods have been successful at establishing human health risk thresholds; however, these methods generally lack the ability to identify contamination sources (7). The inability to distinguish sources of fecal contamination—human sewage, avian species, domestic pets, or other urban animals—hinders management efforts and conveys an inexact measure of human health risks associated with recreational water use. Discriminating between contamination sources in beach and coastal environments can improve risk assessment and mitigation strategies and help limit unnecessary beach advisories and closings caused by sources that do not carry pathogenic organisms.

Despite the ubiquitous presence of gulls and their impact on water quality monitoring efforts, analysis of the organisms harbored in gull feces has been limited to traditional cloning methods (20–22) and a comprehensive microbial community profile has yet to be investigated. Next generation sequencing platforms have gained popularity in recent years as a cost-effective way to deeply explore the microbial community compositions of many samples, while providing taxonomic resolution nearly equivalent to full-

length 16S rRNA gene sequences (23, 24). Deeper resolution of microbial communities from gull feces can aid in discerning the gulls' contribution to the total fecal load in beach environments. Furthermore, an increased understanding of the gull fecal bacterial composition could assist in the development and validation of source-specific assays to enhance beach monitoring efforts.

Previous attempts to monitor gull pollution have resulted in the development of assays targeting specific bacteria as markers of fecal pollution. The Gull-2 PCR assay and the gull2, gull3, gull4, and LeeSeaGull real-time PCR assays target the 16S rRNA gene of *Catellicoccus marimammalium* (Gull-2, gull2, and gull4) and the genus *Streptococcus* (gull3) (21, 22, 25). The assays targeting *C. marimammalium* seem the most promising, as this organism is commonly found in gull fecal samples, is host specific compared to nonavian animals, and is detectable in water sources with suspected gull contamination (21). Geographic variability can factor into the efficiency and breadth of microbial source tracking (MST) marker use, so the regional utility of a marker must be well tested and validated. A recent nationwide, multilaboratory assessment of PCR methods targeting *C. marimammalium* discovered that MST methods designated for gull detection were cross-reactive with pigeon feces from California (26). The prevalence, geographic scope, and ecology of *C. marimammalium* in host birds warrants further investigation, and future assessments should encompass environmental samples from diverse geographic regions (26). Given these findings, it will be important to note the abundance of *C. marimammalium* and general composition of gull fecal samples from Lake Michigan.

In this study, we used the Roche 454 and Illumina MiSeq deep sequencing platforms to examine the composition and relative abundance of bacteria in gull fecal samples. We obtained 16S rRNA gene sequences for bacterial species cultured on mEI plates using Sanger sequencing and determined their proportion in the overall community. We explored the potential for gull fecal contamination to confound current water quality monitoring efforts through their contribution of commonly cultured FIB. Last, we evaluated the regional utility of the Gull-2 assay for beaches along Lake Michigan using gull fecal samples and environmental samples with elevated mEI plate counts.

MATERIALS AND METHODS

Study area, sample collection, and DNA extraction of gull fecal samples. Fresh gull fecal samples were collected aseptically with sterile spatulas from parking lot surfaces at Bradford Beach (Milwaukee, WI) and Grant Park (South Milwaukee, WI). Samples were collected within 2 to 3 min after deposition to prevent overgrowth with nonfecal bacteria. In total, 57 gull fecal samples were collected at Bradford Beach (n = 42) and Grant Park (n = 13), and Point Beach (n = 2) in Two Rivers, WI, between December 2011 and October 2012. The collected samples were stored in sterile 2-ml tubes, transported to the laboratory within 2 h, and stored at −80°C. DNA extractions were performed on 200 mg of the fecal pellets using the Qiagen stool kit (Qiagen, Valencia, CA) according to the manufacturer's protocol with minor modifications (doubling the amount of proteinase K, transferring 2× supernatant volumes, and eluting with both DES [ultrapure water; MP Biomedicals, Solon, OH] and AE buffer). Extracted DNA was stored at −20°C until further analysis. Four samples from Grant Park (2012) and four from Bradford Beach (two each from 2011 and 2012) were further purified using the Mo Bio PowerClean DNA cleanup kit for sequencing (Mo Bio Laboratories, Carlsbad, CA). DNA concentration and purity were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Pittsburg, PA).

Next-generation sequencing and 16S rRNA gene data set analysis. 454 pyrosequencing targeting the V4-V6 hypervariable regions of the 16S rRNA gene generated ~7,000 quality reads per gull fecal sample. Forward primer 518F (5′-CCAGCAGCYGCGGTAAN-3′) and reverse primer 1064R (5′-CGACRRCCATGCANCACCT-3′) amplified DNA, and primer 565F (5′-TGGGCGTAAAG-3′) allowed for bioinformatic trimming (27) of raw sequence reads from the Roche genome sequencer GS-FLX. Illumina sequencing was also performed for six of eight gull samples (gull 1 and gulls 4 to 8). The V4-V5 hypervariable regions were amplified in these samples according to protocols developed at the Josephine Bay Paul Center at the Marine Biological Laboratory, Woods Hole, MA (28). Sequences from both data sets were trimmed, quality controlled, and aligned; data were stored in the Visualization and Analysis of Microbial Population Structures (VAMPS) database (http://vamps.mbl.edu). Taxonomic assignments were made for all sequences using Global Alignment for Sequence Taxonomy (GAST) (23). VAMPS taxonomic count tables were normalized to maximum for microbial community comparisons.

***Enterococcus columbae* and *Catellicoccus marimammalium* reference sequence comparisons and Gull-2 alignment.** Similarity entropy plots comparing full-length sequences of *E. columbae* (GAST annotation of the most abundant species of bacteria in gull samples) and *C. marimammalium* (identified by Lu et al. [21] as the dominant species in gulls and target for the Gull-2 assay) were made with Vector NTI software (Life Technologies, Grand Island, NY). NCBI reference sequences (*E. columbae* [AF061006] and *C. marimammalium* [AJ854484]) were used for comparison.

16S rRNA clone library generation from gull fecal samples. Three of the eight gull fecal samples (gulls 1, 2, and 5) were used to generate 16S rRNA gene clone libraries to enable a more definitive classification of the most abundant taxa in the gull microbial communities. PCR was performed with universal primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). Products were purified and cloned as described previously (20); sequencing was carried out with both the M13F and M13R primers using the ABI BigDye terminator kit (Applied Biosystems, Foster City, CA) on an ABI Prism 3700xi genetic analyzer (Applied Biosystems, Foster City, CA). Nearly full-length (~1,400-bp) contigs were constructed from forward and reverse sequence reads with sufficient overlap (CLC Genomics Workbench, Cambridge, MA). A total of 223 clones were analyzed; incomplete and partial sequences were removed from the data set. Quality sequences were aligned with mother software program (29) using the SILVA alignment as a reference. Chimeras were identified using DECIPHER (30) and removed. Quality sequences were uploaded into Ribosomal Database Project (RDP) for classification (31). BLAST (32) was used to obtain species-level identification of sequences identified by RDP as *Catellicoccus*. Sequence alignment comparisons were done using MEGA5 software program (33) to evaluate the number of times the Gull-2 assay would match our nearly full-length *C. marimammalium* contigs (n = 176).

Sequencing and phylogenetic analysis of gull fecal bacterial isolates cultured on mEI plates. Fresh fecal samples were collected aseptically, as described above, from Grant Park, South Milwaukee, WI, on 18 October 2012. Fecal pellets were resuspended in a 1:1 (wt/vol) of phosphate-buffered saline (PBS); further dilutions (~1:10 and 1:100) were made using sterile water. The diluted fecal solutions were filtered onto 47-mm-diameter, 0.45-μm-pore-size nitrocellulose filters (Millipore, Billerica, MA) and placed on membrane-*Enterococcus* indoxyl-β-D-glucoside (mEI) agar plates (34) and incubated for 24 h at 41°C. Plates that generated enough colonies, without being too numerous to count (TNTC), were selected for colony PCR with universal primers 8F and 1492R to amplify the 16S rRNA gene. PCR products were cleaned up using ExoSAP-IT (Affymetrix, Santa Clara, CA), and sequencing was carried out as described above, but with only the 8F primer.

Phylogenetic analyses of high-quality forward reads from the mEI isolates, ~874 bp long, were conducted using MEGA5 (33). Clones representing the 111 unique sequences were selected out of a total of 342. Four
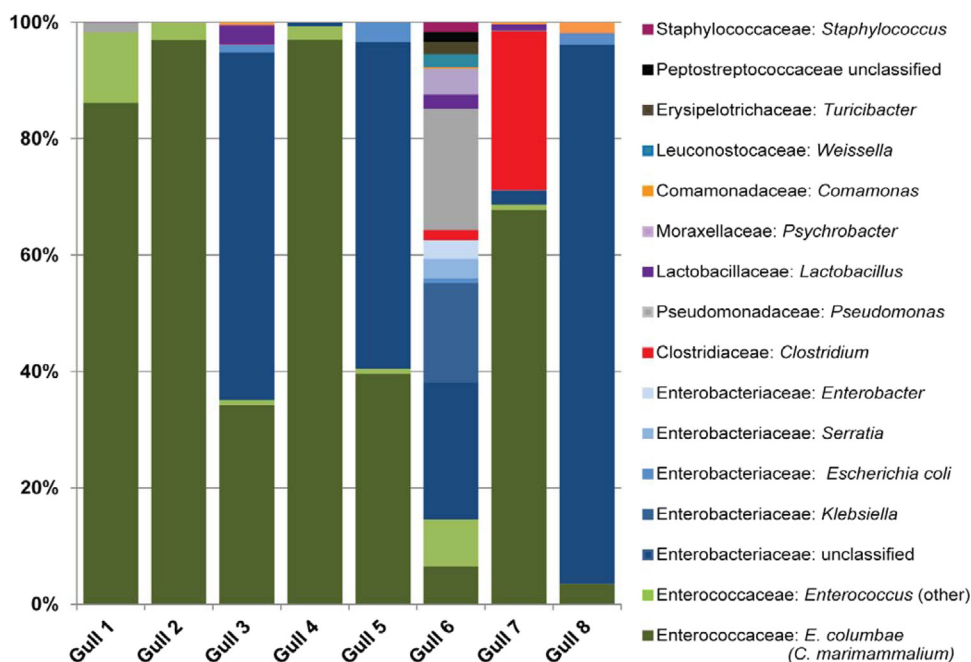
FIG 1 Microbial community populations of eight gull fecal samples. The V4-V6 hypervariable regions of the 16S rRNA gene from fecal genomic DNA were amplified and pyrosequenced. Taxonomy was assigned to sequences using GAST, and taxonomic counts were normalized to the maximum number of sequences. Only the most abundant genera (≥1% of at least one sample) are presented. Fecal samples from gulls 1 to 4 were collected from Bradford Beach, Milwaukee, WI, on 20 December 2011 (gulls 1 and 2) and 5 January 2012 (gulls 3 and 4), and samples from gulls 5 to 9 were collected from Grant Park, South Milwaukee, WI, on 1 January 2012.

isolates that were identified by BLAST (32) as *Streptococcus lutetiensis* were removed from the final phylogenetic tree. A neighbor-joining phylogenetic tree based on evolutionary distances estimated using the *p*-distance method was generated with the unique sequence representatives and reference sequences in MEGA5 (33); distances are given as the number of base differences per site. A bootstrap test was performed with 1,000 replicates. Isolates that clustered around a known reference sequence, with 99% sequence identity or greater, were grouped together and annotated as such. Isolates that clustered with two or more reference sequences were left in the phylogenetic tree without classification.

An alignment and cluster analysis was performed using mothur (29) to verify the proportion of mEI culturable organisms within our data set. Sequence reads from the Illumina data set and *Enterococcus* species sequences obtained from the mEI isolates (albeit not the same gull samples as were sequenced, but from the same study area) were compared; only exact matches were counted.

Sequences within the Illumina data set identified as *C. marimammalium* ($n = 224,082$) were compared to sequences identified as an exact match to our cultured mEI isolates to generate the proportional relationship between *C. marimammalium* and culturable *Enterococcus* spp. Proportions of culturable *Enterococcus* identified to the species level (e.g., *E. faecium*, *E. faecalis*, *E. hirae*, and *E. durans*; $n = 142$) and sequences that may represent organisms that might be cultured on mEI but were not identified to the species level (i.e., *Enterococcus* species not available [species N/A]; $n = 4,132$), were also compared to *C. marimammalium* sequences.

Detection and prevalence of *C. marimammalium* gull fecal and environmental samples from the Milwaukee region. Gull-2 assay (primers Gull-2F [5′-TGCATCGACCTAAAGTTTTGAG-3′] and Gull-2R [5′-GT CAAAGAGCGAGCAGTTACTA-3′]) PCR amplifications were carried out as described by Lu et al. (21) on fecal samples collected between December 2011 and October 2012 ($n = 57$). Preliminary tests revealed that the Gull-2 assay amplified with better efficiency when bovine serum albumin (BSA) was added to the reaction mixture. Sensitivity tests were also

conducted on the four gull fecal samples with the highest concentrations of DNA to test the detection limit of the Gull-2 assay. PCR was done on serial dilutions (10^0 to 10^6) from a 1:1 (wt/vol) mixture of fecal matter and PBS.

Environmental samples collected during the 2010 and 2011 field seasons from Bradford, Atwater, Point, and McKinley beaches in and near Milwaukee, WI, with elevated plate counts of both *E. coli* and enterococci were selected for Gull-2 screening. Sand and water samples were previously extracted using the Mo Bio soil isolation kit (Mo Bio Laboratories Inc., Carlsbad, CA) and stored at $-80°C$. PCR amplifications of the Gull-2 assay were carried out as described above using 5 μl of DNA in a final reaction volume of 25 μl.

Nucleotide sequence accession numbers. Isolate and clone library 16S rRNA gene sequences were deposited into GenBank under the following accession numbers: KF250762 to KF250872 (mEI isolate sequences) and KF250873 to KF251018 (clone library sequences). The 454 pyrosequences and Illumina sequences are available through the National Center for Biotechnology Information (NCBI) Sequence Read Archive (www.ncbi.nlm.nih.gov/sra) under project SRP033277.

RESULTS

Microbial community structure in gull fecal samples. A total of 56,428 pyrosequences were generated from eight gull fecal samples. *Enterococcaceae* and *Enterobacteriaceae* were the two most abundant families, comprising 58% (*Enterococcaceae*) and 29% (*Enterobacteriaceae*) of the samples (Fig. 1). Nearly all samples contained ≥50% *Enterococcaceae* or *Enterobacteriaceae*, with the exception of the fecal sample from gull 6, which was also composed of *Pseudomonadaceae*, *Erysipelotrichaceae*, *Moraxellaceae*, *Lactobacillaceae*, *Leuconostocaceae*, *Clostridiaceae*, and *Staphylococcaceae* and had the highest diversity by far of any of the fecal samples.

Catellicoccus was the most abundant genus on average, ac-

TABLE 1 Comparison of taxonomic assignments for gull fecal samples

Sequence and taxonomic assignment	Proportion (%) of bacteria from fecal sample from:							
	Gull 1	Gull 2	Gull 3	Gull 4	Gull 5	Gull 6	Gull 7	Gull 8
Pyrosequences								
Enterococcus	12.3	3.4	1.1	2.5	1.0	7.5	1.1	0.1
Staphylococcus						1.8		
Catellicoccus	85.4	96.4	33.6	96.7	39.3	5.9	67.0	3.4
Lactobacillus	0.1		3.2			2.5	1.1	0.1
Leuconostoc						0.4		
Weissella						2.1		
Clostridium						1.6	27.0	0.1
Turicibacter						1.9		
Comamonas			0.5			0.3	0.3	1.7
Enterobacter						3.0		
Escherichia			1.2		3.3	0.8	0.1	1.8
Escherichia/Shigella			58.6	0.6	55.7	21.1	2.3	90.7
Klebsiella						15.4		
Serratia						3.1		
Pasteurella						0.7	0.3	0.1
Psychrobacter						4.0		
Pseudomonas	1.6	0.1		0.1		18.8	0.2	0.2
Clone library sequences								
Catellicoccus[a]	98.6	100.0	N/A[b]	N/A	83.8	N/A	N/A	N/A
Escherichia/Shigella			N/A	N/A	9.4	N/A	N/A	N/A
Salmonella	1.4		N/A	N/A	5.4	N/A	N/A	N/A
Streptococcus			N/A	N/A	1.3	N/A	N/A	N/A

[a] *Catellicoccus* sequences ($n = 210$) uploaded to BLAST were a 99% ($n = 208$) and 96% ($n = 2$) match to *C. marimammalium* reference sequence NR042357.
[b] N/A, not analyzed by the clone library method.

counting for 55% of the total pyrosequences recovered from the eight gull fecal samples (Table 1). *Enterococcus*, *Escherichia/Shigella*, *Clostridium*, *Klebsiella*, and *Pseudomonas* were also common genera, each accounting for >10% of the sequences obtained in at least one fecal sample. *Catellicoccus* and *Enterococcus* were the only genera present in all samples. The fecal sample from gull 6 had a more heterogeneous distribution of genera than the others, with relatively high proportions of *Pseudomonas*, *Klebsiella*, and *Psychrobacter*. A few typically nonfecal genera were also relatively common in our samples, including *Serratia*, *Staphylococcus*, and *Comamonas*.

Not only was the general taxonomic diversity in V4-V6 reads from gull fecal samples low, but sequences within the *Catellicoccus* group shared high similarity. A single sequence accounted for over 16% of the *Catellicoccus* sequence reads. Furthermore, ~33% of the pyrosequences shared ≥99% sequence identity over a >400-bp read length.

Clone libraries constructed from a subset of the same DNA samples verified the pyrosequencing results. Over 94% of the 223 clone library-based sequences were classified as *Catellicoccus*. *Catellicoccus* accounted for >98% of clones in the fecal sample from gull 1 (1.4% as *Salmonella*), 100% in the fecal sample from gull 2, and 84% in the fecal sample from gull 5 (9% *Escherichia/Shigella*, 5% *Salmonella*, and 1% *Streptococcus*; Table 1). Nearly all sequence reads identified as *Catellicoccus* (208/210) were ≥99% match to the *C. marimammalium* reference sequence NR042357. The two remaining clones were a 96% match to the same reference sequence. A comparison between the clone libraries and 454 pyrosequences revealed that more than 90% of the *Catellicoccus* sequences shared ≥99% sequence identity in the V5-V6 regions.

Taxonomic resolution of *Catellicoccus marimammalium*, the dominant taxonomic group in gull feces. *Enterococcus columbae* was the initial taxonomic assignment of the most numerous sequence group in the 454 pyrosequence data set. However, comparison to the RDP and NCBI databases showed that this sequence group matched most closely to *Catellicoccus marimammalium* (Table 1). The majority of nearly full-length (~1,400-bp) 16S rRNA gene sequences of these libraries exactly matched the pyrosequences in the V4-V6 region, confirming that *C. marimammalium* was the dominant species in our gull fecal samples.

E. columbae and *C. marimammalium* reference sequences shared the highest identity (>97%) at the end of the 16S rRNA gene within the 499-bp hypervariable region targeted for pyrosequencing (V4-V6). The highest variability occurred within the V1-V3 regions, which are targeted by the Gull-2 PCR assay (21) (Fig. 2). The high sequence identity in the V4-V6 regions of the two species explained the incorrect annotation of *E. columbae* within our 454 pyrosequence data set.

Identification and comparison of bacteria isolated on mEI plates. The quantity of enterococci cultured on mEI from four gull fecal samples ranged from 1.08×10^4 to 4.80×10^4 CFU g^{-1} (Table 2). The majority (338/342) of these isolates were identified as *Enterococcus* spp., and four were identified as *Streptococcus* spp. Both *E. durans* and *E. faecalis* were the most abundant species identified in each gull sample, but the distribution and proportions varied slightly between individuals. The overall *Enterococcus* species distribution was as follows: *E. durans* (52%), *E. faecalis* (36%), *E. faecium* (4%), *E. mundtii* (1%), and *E. hirae* (2%) (Fig. 3). All isolates shared ≥99% sequence identity to a reference sequence, with the exception of 11 *Enterococcus* isolates whose iden-

Gull-2 Forward Assay (5' to 3') **Gull-2 Reverse Assay (5' to 3')**
TGC**TACACC**GAAA**GAAAG**TGAG   **G**AGT**GGAAAG**T**T**CA**T**CC**T**ATGA   *E. columbae*
TGC**ATCGACC**TAAA**GTTT**TGAG   TAGT**AACTGC**T**C**GC**T**C**T**T**T**GAC   *C. marimammalium*
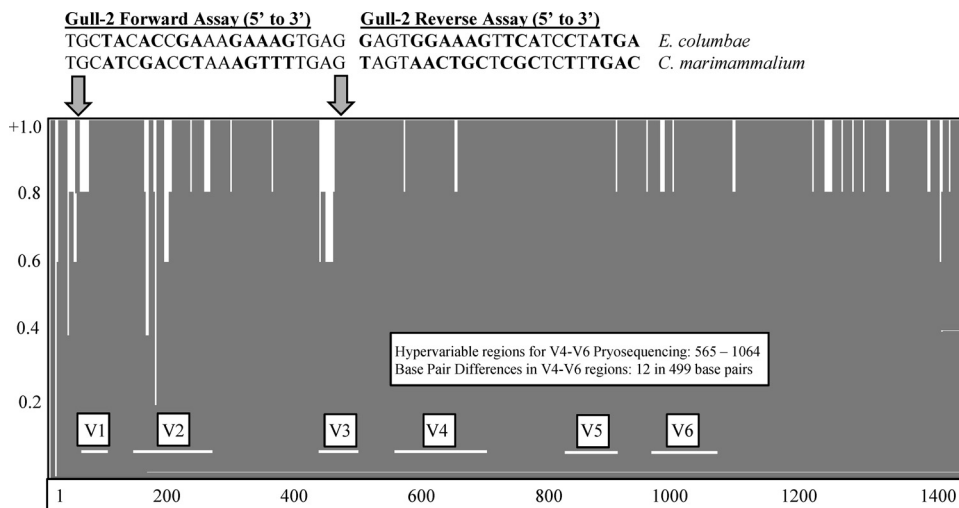

FIG 2 Entropy plot comparison of *E. columbae* and *C. marimammalium* reference sequences and Gull-2 PCR assay alignment. The entropy plot illustrates base pair agreement and dissimilarity in hypervariable regions (V1-V6). The entropy plot was generated using Vector NTI; identical base pairs have a value of +1.0, similar base pairs have a value of 0.5, and weakly similar base pairs have a value of 0.2, as indicated on the *y* axis. Variable regions, shown on the *x* axis, were identified using the *E. coli* system of nomenclature noted in Chakravorty et al. (37). Variable regions targeted by the Gull-2 PCR assay (21) include V1 and V3, and hypervariable regions selected for 454 pyrosequencing span the V4-V6 regions. Within the V1 and V3 regions targeted by the Gull-2 assay, there was 41% sequence identity within 44 bp, and 98% sequence identity with the V4-V6 regions, which spanned 499 bp. The GenBank accession numbers of the compared reference sequences are as follows: AF061006 for *E. columbae* and AJ854484 for *C. marimammalium*. Nucleotides in boldface signify mismatches between *E. columbae* and *C. marimammalium*.

tity could not be confidently resolved to the species level. No isolates were closely related to *C. marimammalium*, the most abundant species from our sequencing approaches.

Use of Illumina deep sequencing to explore the rare occurrence of mEI-culturable bacteria in the gull fecal microbial community. MiSeq Illumina sequencing provided greater sequencing depth (~65,000 reads per sample) and further confirmed *C. marimammalium* as the dominant taxonomic group within our gull fecal samples. The identity and abundance of microbial taxa were generally in good agreement with the distributions seen in the pyrosequence data set. This deeper sequencing method highlighted the disparity between *C. marimammalium* and the low-abundance mEI-culturable *Enterococcus* spp.: *E. faecium*, *E. faecalis*, *E. durans*, and *E. hirae*. Of the 389,838 bacterial sequences generated by MiSeq Illumina sequencing, 57% were identified as *C. marimammalium*, <2.0% of sequences represented organisms commonly cultured on mEI, and 0.01% were an exact match to mEI-cultured isolates. Comparison of the mEI isolate sequences to the Illumina data set revealed only four unique matches, representing 46 total sequences (45 *Enterococcus* species N/A sequences and 1 *E. durans* sequence). *Escherichia coli* made up 0.05% of the

Illumina data set, and sequences that could not be distinguished between *Escherichia* and *Shigella* made up 24% (Table 3).

Validation of the Gull-2 PCR assay with clone library contigs, gull fecal samples, and environmental samples. The primers for the Gull-2 PCR assay were an exact match to 170 of the 176 nearly full-length *C. marimammalium* sequences generated from gull fecal samples. Four of the contigs had a single base pair mismatch in either the forward or reverse primer regions, and two had base pair mismatches in both the forward and reverse primer regions. Fifty-seven gull fecal samples were tested with the Gull-2 PCR assay; 54 (95%) tested positive. Sensitivity tests on the four samples with the highest DNA concentrations revealed that the Gull-2 assay was detected down to the 1:1 × 10⁵ dilution. However, only 6 (27%) of 22 environmental samples (sand and water) tested with the Gull-2 assay were positive (Table 4).

DISCUSSION

Enterococcaceae and Enterobacteriaceae families dominant within gull fecal samples. *Enterococcaceae* and/or *Enterobacteriaceae* dominated the 454 pyrosequencing libraries, with most *Enterococcaceae* sequences matching the *C. marimammalium* reference strain. With the exception of a few sequences, *Enterobacteriaceae* sequences could not be classified beyond the family level. There appeared to be a dichotomy between gull populations at the two sampling locations, Bradford Beach (Milwaukee, WI) (gulls 1 to 4) and Grant Park (South Milwaukee, WI) (gulls 5 to 8). Samples collected at Bradford Beach consisted almost entirely of *Enterococcaceae*, while those collected at Grant Park were generally more diverse and were dominated by *Enterobacteriaceae* species. Variations in gull gut microflora can occur even within small geographic regions of a city (20) and highlight the need for an assay with wide geographic applicability. While both Bradford Beach and Grant Park are beaches on Lake Michigan, differences be-

TABLE 2 Enterococcal counts from gull fecal samples cultured on mEI

Gull	Fecal mass (g)	Enterococcal count (CFU g⁻¹ feces)[a]
Gull 9	0.45	2.06 × 10⁴
Gull 10	0.72	1.08 × 10⁴
Gull 11	0.76	4.80 × 10⁴
Gull 12	0.64	1.25 × 10⁴

[a] CFU counts were obtained from 1:10 or 1:100 dilutions plated on mEI (see Materials and Methods). Isolates for 16S rRNA sequencing were harvested from plates with well-dispersed colonies.
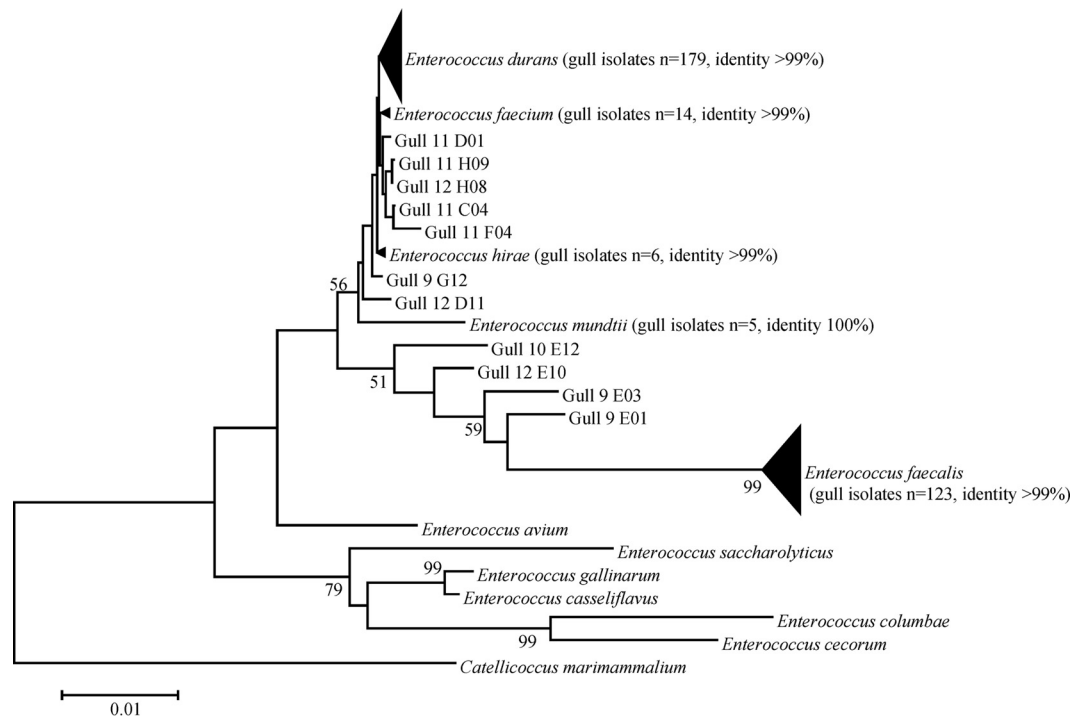
FIG 3 Phylogenetic tree of gull fecal samples cultured on mEI. The unrooted consensus phylogram from neighbor-joining phylogenetic analysis shows evolutionary relationships among bacterial isolates cultured on mEI from fecal pellets collected on 18 October 2012 from Grant Park, South Milwaukee, WI. *Enterococcus* species reference sequences (GenBank accession numbers AF061006, AB012212, AJ301830, AF039900, AF133535, AF061009, Y17302, AF061013, AF039903, AJ276354, and AF061004) were used for isolate clustering and classification. *Catellicoccus* reference sequences (NR042357 and AJ854484), which clustered together, were used as an outgroup. *Enterococcus* species reference sequences are listed next to the isolate name or cluster, along with the isolate's percent identity to the reference sequence. Gull fecal isolates were classified to the species level if they were >99% identical to GenBank reference sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. Only nodes with ≥50% confidence during bootstrapping are labeled. The scale bar indicates 1% sequence divergence.

tween them may account for the observed differences in gull fecal communities. Grant Park is relatively isolated and minimally impacted by human activities. Bradford Beach, within the Milwaukee city limits, is a well-utilized and highly impacted tourist beach. Human activities affect the abundance and diversity of food sources available to gulls. Furthermore, when gulls inhabit areas where contact with human and domestic pet excrement may occur, host-enteric bacterial interactions and cosmopolitan population structures can develop (35).

Catellicoccus marimammalium is numerically dominant, but not culturable on mEI. *C. marimammalium* has been identified in multiple studies (21, 22) as the dominant species in gull fecal microbial communities. Our study confirms those findings with a comprehensive analysis using three separate sequencing platforms, each targeting a different region and length of sequence. Results were generally in good agreement but did show that taxonomic classification was influenced by amplification region and assignment method. Proper taxonomic classification is limited by the length of the sequence read, the region targeted, and reference sequences available in the taxonomic databases. Targeting different hypervariable regions within the same sequence can result in different taxonomic annotations (the V4-V6 regions compared to the V1-V3 regions of the same sequence) (36, 37). We were able to demonstrate the benefits of pairing smaller, full-length sequence libraries with high-abundance, shorter sequence read libraries to elucidate the abundance and proper classification

of *C. marimammalium*. Sequence comparisons illustrated that the 5′ end of the 16S rRNA gene sequences, notably the V1-V3 regions, appear to be more discriminatory for taxonomic classification than the central portion of the gene (V4-V6 region).

We identified near clonal populations of *C. marimammalium* species in our gull fecal samples as the most highly abundant sequences. Lu et al. (21) also found predominance (26%) of high-identity (≥99%) *C. marimammalium* sequences in gulls from West Virginia. Collectively, these findings suggest that *C. marimammalium* has relatively low sequence diversity in gull fecal samples across large temporal and spatial gradients. High abundance of *C. marimammalium* in our gull fecal samples collected from Milwaukee beaches, combined with near clonal populations within the species, further supports the selection of *C. marimammalium* as the target for the microbial source tracking of gulls in this region.

The abundance of *C. marimammalium* in DNA samples suggests that the gull gut is good habitat for this organism, yet we failed to detect it on any of the mEI plates that we screened, despite the fact that *C. marimammalium* is within the *Enterococcaceae* family. Identification of gull fecal bacteria cultured on mEI plates revealed that the intestinal tracts of gulls harbor several other, lower abundance, *Enterococcaceae* spp. that are readily culturable. The mEI medium selects for the growth of *Enterococcus* spp., notably *E. faecalis*, *E. casseliflavus*, and *E. faecium*, but other enterococci (*E. mundtii*, *E. hirae*, *E. gallinarum*, *E. avium*,

TABLE 3 Relative abundance comparisons of *C. marimammalium* and commonly cultured FIB[a]

FIB type	No. of sequences from gull fecal specimen						Total no. of sequences[b]
	Gull 1	Gull 4	Gull 5	Gull 6	Gull 7	Gull 8	
Enterobacteriaceae							
Escherichia/Shigella[c]	28	80	23,921	20,996	333	48,545	**93,903**
Escherichia coli	0	0	41	45	1	121	**208**
mEI-culturable organisms[d]							
E. faecium	1	0	0	105	0	0	**105**
E. faecalis	0	0	0	28	0	0	**28**
E. hirae	0	0	0	0	0	0	**0**
E. durans	0	0	0	9	0	0	**9**
Enterococcus species N/A[e]	74	90	43	3854	59	12	**4,132**
Identical to mEI isolates[f]							
E. durans	0	0	0	1	0	0	**1**
Enterococcus species N/A	0	0	0	45	0	0	**45**
C. marimammalium	64,376	64,731	40,366	5,561	41,965	7,083	**224,082**
Total Illumina sequences from gulls	**64,974**	**64,983**	**64,980**	**64,938**	**64,971**	**64,992**	**389,838**

[a] Totals are shown in boldface type.
[b] Gull samples 2 and 3 were not analyzed by Illumina sequencing.
[c] Sequences not distinguishable between *Escherichia* and *Shigella*.
[d] Species previously observed to grow on mEI.
[e] Sequences that may represent organisms that are typically cultured on mEI but could not be assigned.
[f] Subset of *Enterococcaceae* whose sequences exactly matched our mEI isolate sequences.

and *E. durans*) have also been isolated on mEI ([38], [39]). The gull fecal isolates cultured on our mEI plates were similar to those identified in a previous study by Layton et al. ([40]) in which *C. marimammalium* was notably absent as well, suggesting that it is not mEI culturable.

Use of deep sequencing to explore the rare occurrence of culturable *Enterococcus* spp. in relation to high-abundance *C. marimammalium*. A noteworthy finding from our study is the rare occurrence of mEI-culturable *Enterococcus* spp. in relation to the high abundance of *C. marimammalium* within the Illumina data set. Only a small proportion (46/180,888) of Illumina sequences assigned to the *Enterococcus* genus were an exact match to our mEI isolates. Using the relative abundances of taxonomic counts from our six Illumina samples, we can extrapolate the proportional relationship between *C. marimammalium* and culturable *Enterococcus* spp. to infer that for each colony of enterococci observed on mEI plates, there are ~4,900 *C. marimammalium* present in gull fecal samples. If we take into account all *Enterococcus* spp., and not just our exact sequenced isolate matches, the relative fold abundance is ~50 *C. marimammalium* per *Enterococcus* CFU. *Enterococcus* spp. typically have four to six *rrnA* operons, whereas *C. marimammalium* have only one, so these are minimum estimates of the relative fold enrichment ([41], [42]). Comparing the quantity of enterococci cultured from our four gull fecal samples, which averaged 2.3×10^4 CFU g^{-1}, we can estimate that there would be over 3 orders of magnitude greater density ($\sim 1.1 \times 10^8$ cells g^{-1}) of *C. marimammalium* also present. Our results may help to better understand the relationship between organisms identified by traditional culture methods versus culture-independent molecular techniques.

Implications for screening assays, source tracking, and human health. The prevalence of *C. marimammalium* in fecal samples (55/57; 95%) was higher than previously reported (71%) ([21]), based on screening with the Gull-2 PCR assay. Detection of *C. marimammalium* in 27% of environmental samples was considerably lower than observed ([21]). Lu et al. ([21]) reported that 96% of 48 freshwater samples with presumed gull contamination, including eight samples from Grant Park Beach in South Milwaukee, WI, were positive by the Gull-2 PCR assay ([21]); however, our environmental samples were not necessarily presumed to have gull contamination. Factors affecting detection can include different fecal loads at the time of sampling, seasonal effects on bacterial survival, lower proportions of gull fecal pollution, or dilution of *C. marimammalium* DNA sequences to below the limit of detection of the assay. Our serial dilutions showed an average detection limit of 0.3 pg of gull fecal DNA per reaction mixture, which is 20 times lower than the value reported by Lu et al. ([21]), indicating that the gulls we sampled contained a higher concentration of *C. marimammalium*, a possible explanation for the increased Gull-2 assay efficiency on our gull fecal samples. Although the Gull-2 assay is sensitive to gull fecal contamination ([21], [22]), the high volume of water in beach environments, partic-

TABLE 4 Detection of Gull-2 PCR assay in gull fecal and environmental samples

Source	No. of samples	
	Tested	Positive
Gull feces		
Grant Park	13	12
Bradford Beach	42	40
Point Beach	2	2
Lake Michigan[a]		
Beach water	9	4
Sand	13	2

[a] Bradford Beach, Atwater Beach, Point Beach, and McKinley Beach.

ularly Lake Michigan beaches, may dilute *C. marimammalium* DNA to below detectable limits. Sand and water sources may additionally impact the persistence and survival of *C. marimammalium*, which would also affect assay detection.

Geographic variability and host specificity can factor into the applicability of microbial source tracking markers. The gull fecal samples used in the Lu et al. (21) study were from West Virginia, Georgia, Ohio, Florida, and Ontario, Canada; our gull fecal samples were collected on Lake Michigan in Wisconsin. A more recent study assessed the prevalence of gull markers in animal feces collected from California, Ohio, Arkansas, Georgia, Delaware, and South Africa and detected *C. marimammalium* in >85% of the gull fecal samples tested (22). *C. marimammalium* is specific to gulls, compared to other poultry, waterfowl, and nonavian species, including pigs, dogs, cows, and humans (21, 22, 25, 43). Aside from *C. marimammalium* in the feces of pigeons, which was detected with the same sensitivity and specificity as that of gulls, most nontarget hosts have been near or below the lower limit of quantification or nondetectable (26). The detection of *C. marimammalium* in our gull fecal samples, the amplification of *C. marimammalium* with the Gull-2 PCR assay in our environmental samples, and the alignment of the Gull-2 primers (21) with our nearly full-length clone library contigs demonstrates the regional utility of this microbial source tracking marker for gull contamination in the Great Lakes region.

Our research provides information on the diversity and variability of the gull fecal microbial community, including the dominance of *Enterococcaceae* or *Enterobacteriaceae* spp. in most samples analyzed. Using three separate sequencing platforms, we were able to demonstrate that although taxonomic assignments can be biased by primer choice, amplification region, and sequence length, all methods clearly show *C. marimammalium* to be the dominant taxon within our gull fecal samples.

Plate count assays such as mEI agar are the industry standard for detection of FIB in sand and water samples. However, as is often the case with environmental samples, the most abundant organisms are not readily culturable (44). Although the gull gut appears to be a good habitat for organisms in the *Enterococcaceae* family, *Enterococcus* spp. that are readily cultured on mEI accounted for 0.04% of our sequence reads. Establishing the relationship between *C. marimammalium* and culturable enterococci allows better quantification of the total gull fecal load to beaches and coastal waters, which may help in understanding the true impact gulls have on water quality monitoring efforts. These findings also highlight the advantages of using culture-independent detection techniques for environmental monitoring, as they are more encompassing, time efficient, and increasingly more cost-effective. Although gulls harbor traditional FIB that can be observed in plate assays, non-mEI-culturable *C. marimammalium* dominates the gull fecal microbial community and is a more suitable genetic marker for tracking gull fecal pollution.

ACKNOWLEDGMENTS

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