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Phylogenetic Diversity and Molecular Detection of Bacteria in Gull Feces▽

Jingrang Lu,[1] Jorge W. Santo Domingo,[1]* Regina Lamendella,[2] Thomas Edge,[3] and Stephen Hill[3]

U.S. Environmental Protection Agency, Office of Research and Development, National Risk Management Research Laboratory, 26 Martin Luther King Drive, MS-387, Cincinnati, Ohio 45268[1]; Department of Environmental Engineering, University of Cincinnati, Cincinnati, Ohio 45220[2]; and National Water Research Institute, Environment Canada, 867 Lakeshore Road, P.O. Box 5050, Burlington, Ontario L7R 4A6, Canada[3]

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In spite of increasing public health concerns about the potential risks associated with swimming in waters contaminated with waterfowl feces, little is known about the composition of the gut microbial community of aquatic birds. To address this, a gull 16S rRNA gene clone library was developed and analyzed to determine the identities of fecal bacteria. Analysis of 282 16S rRNA gene clones demonstrated that the gull gut bacterial community is mostly composed of populations closely related to *Bacilli* (37%), *Clostridia* (17%), *Gammaproteobacteria* (11%), and *Bacteriodetes* (1%). Interestingly, a considerable number of sequences (i.e., 26%) were closely related to *Catellicoccus marimammalium*, a gram-positive, catalase-negative bacterium. To determine the occurrence of *C. marimammalium* in waterfowl, species-specific 16S rRNA gene PCR and real-time assays were developed and used to test fecal DNA extracts from different bird ($n = 13$) and mammal ($n = 26$) species. The results showed that both assays were specific to gull fecal DNA and that *C. marimammalium* was present in gull fecal samples collected from the five locations in North America (California, Georgia, Ohio, Wisconsin, and Toronto, Canada) tested. Additionally, 48 DNA extracts from waters collected from six sites in southern California, Great Lakes in Michigan, Lake Erie in Ohio, and Lake Ontario in Canada presumed to be impacted with gull feces were positive by the *C. marimammalium* assay. Due to the widespread presence of this species in gulls and environmental waters contaminated with gull feces, targeting this bacterial species might be useful for detecting gull fecal contamination in waterfowl-impacted waters.

Gulls are common shore waterfowl species, and consequently, their feces could be considered a major source of contamination in coastal and lake waters worldwide. The health risks associated with waterfowl fecal pollution are largely unknown, although they are presumed to be lower than those associated with human fecal pollution. However, because of the migratory character and feeding behavior of feral birds (3), there are increasing public health concerns regarding waterfowl fecal contamination in environmental waters due to the potential spread of microbial pathogens to humans, domesticated animals in close contact with humans, and human food sources. Indeed, several studies have shown that waterfowl feces may carry human pathogens like *Campylobacter* spp. (35), *Salmonella* spp. (2), pathogenic *Escherichia coli* (19, 24), microsporidia (33), and *Cryptosporidium* spp. (37). The role of wild birds in spreading drug-resistant genes has also been recently documented (9), further suggesting the importance of avian pollution in zoonosis. Aquatic birds are also natural reservoirs of influenza viruses and therefore are an important link in the evolution of these viruses and their spreading in the environment (18).

Most studies describing the gut microbiota of waterfowl have used culture-based methods and have focused on targeted pathogens (6, 15). As culture-based studies can provide only a limited view of natural microbial communities, recently developed molecular methods can be used to better describe the composition of waterfowl gut systems. This information is critical in order to recognize potential hazards associated with waterfowl fecal pollution and to help distinguish waterfowl fecal sources from other animal sources. Waterfowl feces has been suggested as an important source of fecal contamination in several studies (27, 34) and therefore is potentially responsible for many beach closures every year (10). Although fecal pollution in recreational waters is traced with fecal indicator bacteria, whether contamination in water is primarily associated with human, waterfowl, or other fecal sources is often undetermined. While microbial source-tracking (MST) methods that use fingerprint databases of water and fecal bacterial isolates may be able to differentiate bird feces from other fecal sources (10, 14), these methods can be laborious and time-consuming and have yet to clearly discriminate among different avian fecal sources. Alternatively, PCR-based methods that detect host-specific 16S rRNA genes and functional genes directly in water DNA extracts are becoming more popular (4, 31, 32). The latter methods have been used mainly to differentiate human and ruminant from other animal fecal sources, primarily targeting *Bacteroidetes*. Studies tracking sources of gull feces based on the detection of *Bacteroidetes* genes have had little success (12), perhaps because of the low prevalence of *Bacteroidetes* in bird feces

* Corresponding author. Mailing address: U.S. Environmental Protection Agency, NRMRL/WSWRD/MCCB, 26 W. Martin Luther King Dr., MS 387, Cincinnati, OH 45268. Phone: (513) 569-7085. Fax: (513) 569-7328. E-mail: santodomingo.jorge@epa.gov.
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and the limited knowledge of the composition of the normal microbiota of gull feces.

The aims of this study were to study microbial community composition and structure in the fecal DNA of gulls by 16S rRNA gene sequencing analysis and to develop host-specific assays for detecting gull fecal community DNA in waters.

MATERIALS AND METHODS

Bacterial strains. DNA extracts from the following bacteria were used to test the specificity of the *Catellicoccus marimammalium* PCR assay: *C. marimammalium* DSMZ M35/04/3T (obtained from the Culture Collection of the University of Göteborg, Göteborg, Sweden), *Aeromonas hydrophila* ATCC 7966, *Bacillus cereus* ATCC 10876, *B. subtilis* ATCC 21332, *Enterobacter aerogenes* ATCC 13048, *Enterococcus faecalis* ATCC 29212, *E. faecium* ATCC 19433, *Lactobacillus acidophilus* ATCC 43121, and *Streptococcus pyogenes* ATCC 19615. With the exception of *C. marimammalium*, biomass from the aforementioned strains was directly harvested from agar plates and used for DNA extractions. Cells of *C. marimammalium* were harvested directly from lyophilized cultures.

Sample collection and DNA extraction. Gull fecal samples used to develop the 16S rRNA gene clone library were collected in West Virginia. Fecal DNA extracts from deposited fecal samples from the following animals were used in host specificity studies: *Sus scrofa* (pig), *Bos taurus* (bovine), *Homo sapiens* (human), *Capra aegagrus* (domestic goat), *Ovis aries* (sheep), *Equus caballus* (horse), *Felis catus* (cat), *Canis familiaris* (dog), *Canis latrans* (coyote), *Sciurus carolinensis* (gray squirrel), *Odocoileus virginianus* (whitetail deer), *Didelphis virginiana* (possum), *Loragyps atratus* (black vulture), *Lynx rufus* (bobcat), *Procyon lotor* (raccoon), *Erinaceus* sp. (hedgehog), *Pongo pygmaeus* (red ape), *Elephas maximus* (Asian elephant), *Zalophus californianus* (California sea lion), *Callorhinus ursinus* (northern fur seal), *Phoca vitulina* (Pacific harbor seal), *Physeter macrocephalus* (sperm whale), *Megaptera novaeangliae* (humpback whale), *Phocoenoides dalli* (Dall's porpoise), *Phocoena phocoena* (harbor porpoise), *Lagenorhynchus obliquidens* (Pacific white-sided dolphin), *Branta canadensis* (Canadian goose), *Anser* sp. (goose), *Meleagris gallopavo* (turkey), *Treron* sp. (pigeon), *Aix sponsa* (duck), *Gallus gallus* (chicken), *Pygoscelis* sp. (penguin), *Psittacus* sp. (parrot), *Collumba livia* (dove), *Pelicanus* sp. (pelican), *Eudocimus ruber* (ibis), *Larus atricilla* (laughing gull), and *L. delawarensis* (ring-billed gull). In addition, feces collected from gulls located in Georgia, Ohio, West Virginia, Florida, and Ontario (Canada) were used in host distribution studies. Feces from marine mammals were obtained directly from the animals, while samples from other animals were collected from previously deposited fecal samples. All samples were collected aseptically, placed into sterile 50-ml conical tubes with screw caps, and stored at −80°C until required. Total DNA was extracted from individual fecal samples. The Mo Bio Fecal kit (Mo Bio Laboratories, Inc., Carlsbad, CA) and the FastDNA kit (Q-Biogene, Carlsbad, CA) were used by following the protocols provided by the manufacturers. Total DNA was eluted in 50 (Mo Bio Fecal kit) or 100 μl (Fast DNA kit) of water, and DNA concentrations were measured with a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Inc., Berlin, Germany).

Water samples presumed to be impacted by gull feces were used to evaluate the potential of gull-specific assays as a source-tracking tool (see below). Freshwater samples were collected in sterile bottles from beaches in or near the Great Lakes (Grant Park Beach, Wisconsin; Lake Erie, Ohio; Lake Ontario, Canada) and from a pond adjacent to the San Juan Creek next to Doheny State Beach (Dana Point, California). Additionally, samples were collected from sites in northern Georgia and northern Ohio with no previous history of gull contamination, as well as from sites in northeastern Ohio impacted by non-waterfowl sources (i.e., chicken, cattle, swine). Water samples were transported to the laboratory in ice coolers and filtered (i.e., 100 to 300 ml) onto 47-mm polycarbonate membranes (0.2-μm pore size) as previously described (23). Membranes were then transferred into sterile conical tubes and kept at −80°C until further processing. Total community DNA was extracted from water samples with a Mo Bio Fecal kit and a FastDNA kit.

16S rRNA gene clone library analysis. Sequence analysis of 16S rRNA gene clones was used to describe the phylogenetic affiliations of bacterial populations in gull fecal extracts. The 16S rRNA gene was amplified with general bacterial primers 27F (*E. coli* numbering positions 8 to 27: 5′-AGAGTTTGATCMTGG CTCAG-3′) and 785R (*E. coli* numbering positions 785 to 804: 5′-ACTACCR GGGTATCTAATCC-3′). DNA extracted from eight gull fecal samples from West Virginia was pooled and used as the PCR template. PCR amplifications were performed in a PTC-240 DNA Engine Tetrad 2 Cycler (MJ Research, Inc., Alameda, CA). Reaction mixtures were prepared in a 25-μl volume and subjected to the following cycling conditions: 3 min at 95°C, followed by 22 cycles of 30 s at 95°C, 30 s at 58°C, and 60 s at 72°C and a final 10-min primer extension step at 72°C. PCR products from five reactions were pooled and cloned into pCR4.1 TOPO (Invitrogen). Individual clones were sequenced by using BigDye Terminator chemistry and an Applied Biosystems PRISM 3730XL as described by Lu et al. (23).

Sequence editing and alignment were completed with Sequencher (Gene Codes Corporation, Ann Arbor, MI). The 16S rRNA gene sequences were screened for chimeras with the Check Chimera program of the Ribosome Database Project and by manual alignments of secondary structure. The Bellerophon program (http://foo.maths.uq.edu.au/~huber/bellerophon.pl) (16) was also used to check for chimeras by comparing each sequence against the sequences from the same library. As a final check for chimeras, each sequence was split into 5′ and 3′ fragments, which were analyzed separately by BLAST searching of the GenBank database. Sequences for which either the 5′ or the 3′ fragment had significantly different closest relatives were considered probable chimeras and were removed from the data set. For 16S rRNA gene sequences, homology searches of DNA sequences in the GenBank (NR) database were done with National Center for Biotechnology Information (NCBI) BLASTn (http://www.ncbi.nlm.nih.gov/BLAST/) (1).

Conventional PCR and real-time assays. *C. marimammalium* PCR primers were designed by aligning 16S rRNA gene sequences from closely related species by using Primer Designer software (version 2.01; Scientific & Educational Software, Durham, NC) and the following conditions: no hairpin, no primer dimer formation, and an annealing temperature of 64 to 65°C. Assays were optimized through temperature gradients and with various concentrations of fecal DNA templates. Primers were tested for host specificity against fecal DNA composites for each of the animal types listed above. DNA composites were generated by combining equal amounts of DNA from the individual fecal samples. Assays that showed host specificity to gull composites were further tested against DNA extracts of individual avian, human, pig, and cow fecal samples. Gull samples were also used to determine the host distribution of potential markers. Detection limits of PCR assays were determined by three different approaches: (i) 10-fold dilutions of plasmid DNA (6 to 6×10^{-7} ng) containing a targeted insert in reaction mixtures with no fecal DNA background; (ii) 10-fold dilutions of plasmid DNA (6 to 6×10^{-7} ng) containing a targeted insert in reaction mixtures spiked with bird fecal DNA (10 ng/μl) made of equal amounts of chicken, turkey, and Canadian goose fecal DNA extracts; and (iii) 10-fold dilutions of gull fecal DNA (6 to 6×10^{-7} ng). PCR assays specific to *Bacteroides* spp. and *Clostridium coccoides* were used to determine the presence of potential PCR inhibitors in DNA extracts used as templates in PCR assays (4, 25). All of the assays were performed with 1 and 10 ng μl^{-1} fecal DNA extracts. The presence of PCR products was visualized by 2% agarose gel electrophoresis with GelStar as the nucleic acid stain (FMC BioProducts; Rockland, ME). The cycling conditions for the PCR assays were 3 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 64°C, and 60 s at 72°C and a final 10-min primer extension step at 72°C. The *C. marimammalium* species-specific primer sequences used in this study were TG CATCGACCTAAAGTTTTGAG and GTCAAAGAGCGAGCAGTTACTA for the forward and reverse primers, respectively. We refer to this assay as Gull-2.

The *C. marimammalium* PCR assay was also used in real-time assays, with SYBR green as the detection dye. The assays were performed with a 7900 HT Fast Real-Time Sequence Detector (Applied Biosystems). Reaction mixtures (20 μl) contained 10 μl 2x SYBR Premix Ex *Taq*, 0.4 μl ROX reference dye (Takara Bio Inc., Shiga, Japan), 0.2 μM (final concentration) primers, and either 100 ng genomic DNA (fecal and water samples), a series of dilutions of plasmid DNA with a target insert with a log copy number of 8.8 to 1.8 (i.e., a copy number of 6.3×10^8 to about 63) per reaction mixture, or a series of dilutions of gull fecal DNA ranging from 60 to 6×10^{-7} ng per reaction mixture. All reaction mixtures were prepared in triplicate in MicroAmp Optical 96-well reaction plates with MicroAmp Optical Caps (Applied Biosystems). The amplification protocol consisted of 50°C for 2 min, followed by 95°C for 2 min and then 40 cycles of 95°C for 5 s, 64°C for 15 s, and a 72°C extension for 10 s and an additional disassociation at 60°C for 15 s. Data were initially analyzed with Sequence Detector software (version 2.2.2) with a 0.2 threshold. Gene copies in fecal samples were calculated from standard curves based on the log transformation of a known concentration versus the threshold cycle (C_T). Comparison tests between gull species (laughing gull and ring-billed gull) were performed with Statistical Analysis Software (SAS Institute Inc., Cary, NC) by using PROC GLM with the contrast statement feature.

Nucleotide sequence accession numbers. Representative 16S rRNA gene sequences from cloning experiments were deposited in GenBank with accession numbers EU181006 to EU181122.

RESULTS AND DISCUSSION

Phylogenetic analysis of 16 rRNA gene sequences. Of the 282 gull fecal 16S rRNA gene sequences analyzed, there were 85 different operational taxonomic units (i.e., 97% identical to previously deposited sequences). These sequences represent 76% coverage of the gull fecal community diversity as calculated by Lu et al. (22), indicating that the majority of the predominant populations of the fecal microbial community in gulls was represented in the clones analyzed. Excluding the sequences classified as unknowns ($n = 9$), 38 bacterial genera were represented in the clone library (Table 1; Fig. 1). *Bacilli* sequences were the most common (37%), particularly sequences closely related to the low-G+C gram-positive bacterium *C. marimammalium* (i.e., 99% identity), which constituted 26% of the clone library. The *C. marimammalium*-like sequences were nearly identical to each other, with <1% showing sequence heterogeneity among the clones recovered. Other *Bacilli*-like sequences were closely related to *Lactobacillus aviarius* and *Vagococcus camiphilus*. *Clostridia* sequences were relatively numerous (17%), specifically, sequences closely related to *Clostridium* sp. and *Eubacterium tortuosum*. Mollicute sequences (9%) pertaining to an unknown genus were also numerous. Sequences homologous to *Gammaproteobacteria* (identity, ≥98%) were also numerous (11%), and many were closely related to *Klebsiella pneumoniae*, *Enterobacter* sp., *Pseudomonas* sp., and *E. coli*. In contrast, sequences homologous to *Bacteroides* represented only 1% of the total number of clones.

Although there are very limited data on the composition of bird fecal microbial communities, some trends are emerging from 16S rRNA gene sequence analyses of chicken (22) and turkey (30) intestines and from this study. For example, the predominant bacteria in avian gut microbial communities are low-G+C gram-positive bacteria, particularly *Clostridia* and *Bacilli* in chicken and turkey systems. In contrast to other gut systems, *Bacteroidetes* bacteria represent a small fraction of the bacteria in avian feces (e.g., as little as ~1% of the total community). This is an important finding to those developing markers to track avian fecal pollution in environmental waters, as it suggests that *Bacteroidetes* bacteria might not be practical targets for the development of avian-specific assays due to their low densities in the avian gut and considering that *Bacteroidetes* host-specific populations represent less that 1 to 10% of the total *Bacteroidetes* populations (4, 20).

Our data show that the dominant low-G+C gram-positive bacteria in gull feces are closely related to *C. marimammalium*. This finding is interesting, as *C. marimammalium* was recently described as a new group of low-G+C gram-positive bacteria isolated from a porpoise and a gray seal (21). This group is part of the *Bacilli* class and specifically belongs in the *Enterococcaceae* family, which houses the genera *Enterococcus*, *Melissococcus*, *Tetragenococcus*, and *Vagococcus*. *Enterococcus* and *Vagococcus* spp. are commonly found in animal feces, and in fact, *Enterococcus* spp. are often used as indicators of fecal pollution in freshwater and marine waters and as targets in source-tracking studies (8, 28). Several studies have shown that some *Enterococcus* populations might be host specific (5), although the ecology of this genus is still poorly understood.

TABLE 1. Distribution of 16S rRNA genes in the gull clone library

Class or group (% clones of total)	Genus	No. of clones
Actinobacteria (6.4)	*Arthrobacter*	4
	Corynebacterium	8
	Microbacterium	1
	Propionibacterium	2
	Unknown	3
Bacilli (37.2)	*Bacillus*	3
	Catellicoccus	74
	Enterococcus	1
	Lactobacillus	9
	Paenibacillus	1
	Staphylococcus	1
	Vagococcus	9
	Unknown	7
Bacteroidetes (1.1)	*Bacteroidetes*	1
	Unknown	2
Clostridia (17.31)	*Clostridium*	44
	Eubacterium	1
	Ruminococcus	2
	Unknown	2
Fusobacteria (0.7)	*Cetobacterium*	2
Mollicutes (8.8)	Unknown	25
Alphaproteobacteria (6.7)	*Agrobacterium*	1
	Bosea	1
	Devosia	1
	Fulvimarina	1
	Mesorhizobium	1
	Paracoccus	8
	Rhizobium	1
	Rhodobacter	1
	Unknown	4
Betaproteobacteria (4.3)	*Acidovorax*	6
	"Panaciterramonas"	2
	Polynucleobacter	3
	Zoogloea	1
Gammaproteobacteria (11.3)	*Acinetobacter*	13
	Enterobacter	6
	Escherichia	6
	Klebsiella	5
	Pantoea	1
	Pseudomonas	1
Deltaproteobacteria (0.4)	Unknown	1
Epsilonproteobacteria (0.4)	*Campylobacter*	1
Planctomycetes (0.4)	*Planctomyces*	1
Spirochaetes (1.1)	*Leptospira*	3
Cyanobacteria (0.4)	*Synechococcus*	1
Archaea (0.4)	Unknown	1
Unknown class (3.2)	Unknown	9

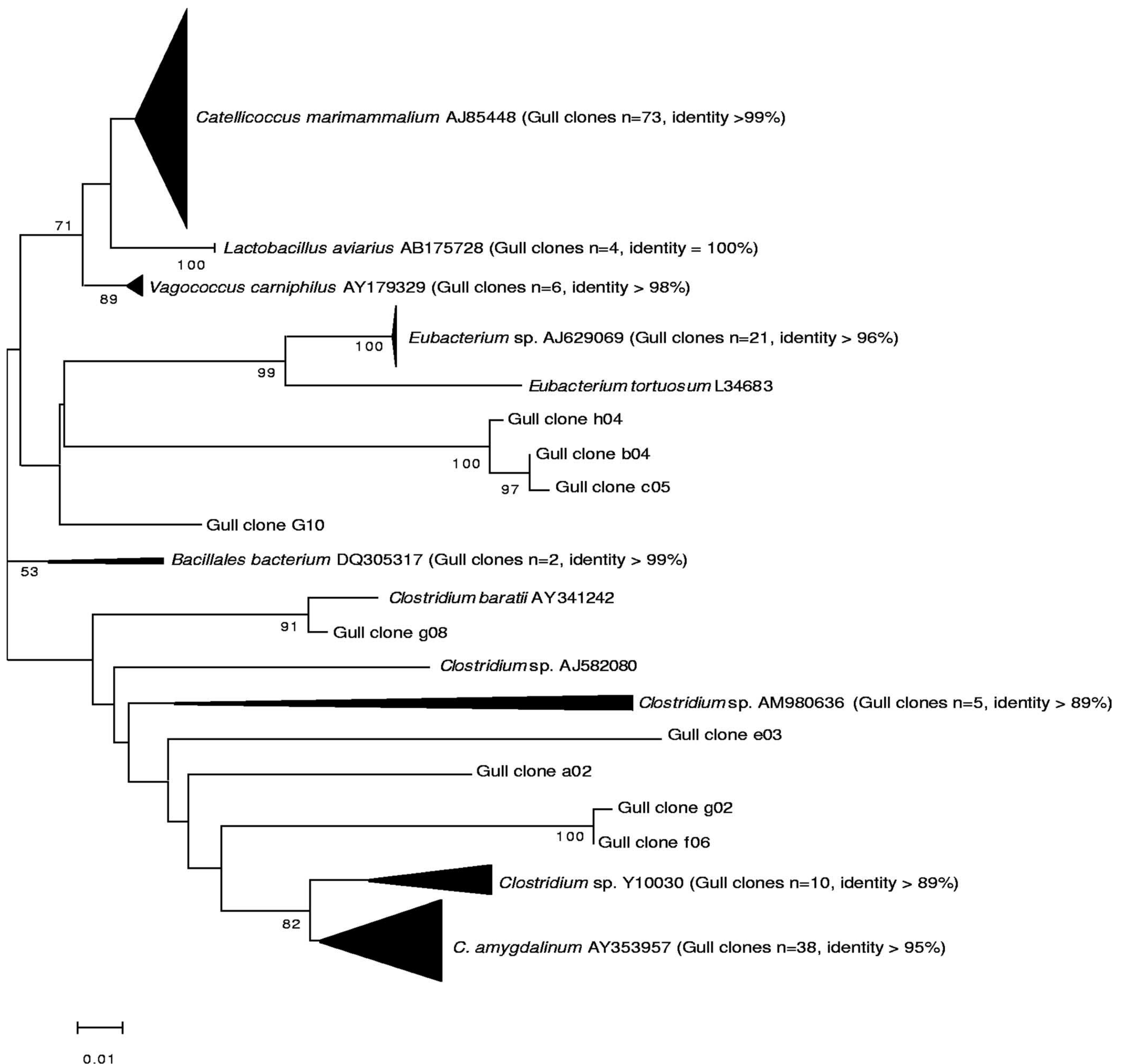

FIG. 1. Unrooted neighbor-joining tree of 16S rRNA gene sequences of low-G+C gram-positive bacteria, including *C. marimammalium*-like sequences obtained from clone libraries. Sequences were aligned, and a bootstrap consensus tree was created with MEGA 3.1 (1% divergence).

Since no strains of *C. marimammalium* have been isolated from avian systems thus far, we decided to determine if these results were unique to gulls or if this species is also present in other gut systems.

Host specificity PCR studies. The Gull-2 PCR assay was designed to target *C. marimammalium* by using publicly available sequences and closely related clone sequences obtained in this study. In silico analysis showed that the primers have no mismatches to the *C. marimammalium* 16S rRNA gene. Only a limited number of unrelated 16S rRNA gene bacterial sequences in the NCBI database (e.g., *B. vulgatus*, *Prochlorococcus marinus*, *Mycobacterium* sp. strain JLS, and *Jannaschia* sp. strain CCS1) (as of 23 August 2007) showed similarity to either one of the primers, but these sequences contain several mismatches in the 3′ end of the sequence. Moreover, none of the primers perfectly annealed sequences from the same species, suggesting a relatively low potential for cross-hybridization with bacterial species other than *C. marimammalium*.

When the Gull-2 PCR assay was used to test DNA extracts from eight bacterial strains, including *Firmicutes* closely related to *C. marimammalium* (i.e., enterococci), no cross-amplifica-

TABLE 2. Host specificity results of Gull2 assay against feces from various animals

Animal	Location of sample	No. of samples/no. of composites tested[a]	Gull2	
			PCR	Real-time C_T
Pig	Delaware	10/2	−	BDL[b]
Cow	West Virginia	17/3	−	BDL
Cow	Delaware	11/1	−	BDL
Human	West Virginia	16/3	−	BDL
Goat	Delaware	10/2	−	BDL
Sheep	Delaware	11/3	−	BDL
Horse	West Virginia	5/1	−	BDL
House cat	West Virginia	11/1	−	BDL
Domestic dog	West Virginia	13/1	−	BDL
Coyote	Texas	10/1	−	BDL
Gray squirrel	Texas	4/1	−	BDL
Deer	West Virginia	6/1	−	BDL
Possum	Texas	2/1	−	BDL
Black vulture	Texas	1/1	−	BDL
Raccoon	Texas	1/1	−	BDL
Hedgehog	West Virginia	1/1	−	BDL
Bobcat	Texas	1/1	−	BDL
Red ape	Ohio	1/1	−	BDL
Asian elephant	Ohio	1/1	−	BDL
California sea lion	California	10/10	−	ND[c]
Northern elephant seal	California	8/8	−	ND
Pacific harbor seal	California	6/6	−	ND
Sperm whale	California	2/2	−	ND
Humpback whale	California	1/1	−	ND
Dall's porpoise	California	1/1	−	ND
Harbor porpoise	California	2/2	−	ND
Pacific white-sided dolphin	California	1/1	−	ND
Canadian goose	West Virginia	20/20	−	BDL
Canadian goose	New Jersey	5/5	−	BDL
Canadian goose	Georgia	16/16	−	BDL
Canadian goose	Oregon	4/4	−	BDL
Canadian goose	Ohio	4/4	−	BDL
Geese	Ohio	13/13	−	BDL
Turkey	Delaware	11/1	−	BDL
Turkey	Ohio	8/8	−	BDL
Pigeon	West Virginia	2/1	−	BDL
Pigeon	Ohio	3/3	−	BDL
Duck	Georgia	21/21	−	BDL
Duck	Ohio	4/4	−	BDL
Chicken	West Virginia	14/1	−	BDL
Penguin	Ohio	3/3	−	BDL
Parrot	Ohio	4/4	−	BDL
Dove	Ohio	2/2	−	BDL
Pelican	Ohio	1/1	−	BDL
Ibis	Ohio	1/1	−	BDL
Seagull	West Virginia	8/1	+	23.41

[a] The number of samples tested and the number of composites provided for each sample type are shown.
[b] BDL, below detection limit.
[c] ND, not determined.

tion signals were obtained, further suggesting that the assay is highly species specific. DNA from the *C. marimammalium* type strain produced a PCR band of the expected size (i.e., approximately 412 bp). When the PCR assay was used to test composite fecal DNA extracts from a variety of animals, including marine mammals, only gull fecal DNA extracts generated a PCR product of the correct size (Table 2). As *C. marimammalium* was originally isolated from marine mammals, our results suggest that *C. marimammalium* might not be a normal or abundant inhabitant of a marine mammal's gut. Considering that this species was originally isolated from deceased animals, this organism might be an opportunistic pathogen, which will also explain the negative results. All of the fecal samples tested yielded PCR signals with *Bacteroides-Prevotella* 16S rRNA gene-specific primers (4), suggesting that PCR inhibition could not explain the absence of *C. marimammalium*-like signals in fecal samples that tested negative for the species-specific primers. The detection limit of both positive control tests (i.e., assays containing plasmid DNA in spiked and nonspiked bird fecal DNA) was 6×10^{-6} ng DNA per PCR. The PCR assay showed a detection limit of 0.006 ng of gull fecal DNA per reaction (Fig. 2).

The geographic and host distribution of *C. marimammalium* was determined by PCR assays against individual gull fecal samples ($n = 58$) collected from Florida (*Larus atricilla*), West Virginia (*L. delawarensis*), Ohio (*L. delawarensis*), Georgia (*L.

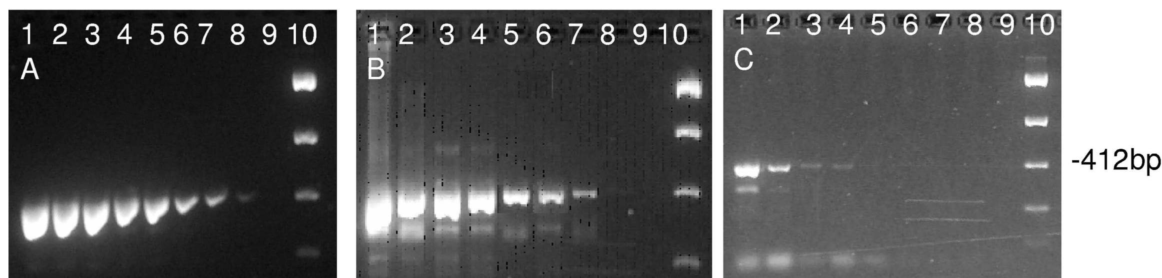

FIG. 2. Detection limits of the Gull-2 PCR assay. (A) Plasmid DNA (10-fold dilutions of plasmid DNA ranging from 6 to 6 $\times$ 10^{-7} ng) containing a targeted insert in reaction mixtures with no fecal DNA background from lane 1 to lane 8 (negative control in lane 9). (B) Tenfold dilutions of plasmid DNA (6 to 6 $\times$ 10^{-7}ng) containing a targeted insert in reaction mixtures spiked with bird fecal DNA (10 ng/μl) made of equal amounts of chicken, turkey, and Canadian goose fecal DNA extracts from lane1 to lane 8 (only bird DNA in lane 9). (C) Tenfold dilutions of seagull fecal DNA ranging from 6 to 6 $\times$ 10^{-7} ng/PCR mixture from lane1 to lane 8 (negative control in lane 9).

atricilla and *L. delawarensis*), and Ontario, Canada (*L. delawarensis*) (Table 3). Positive signals in the Gull-2 PCR assay were obtained with approximately 71% of the gull fecal specimens tested in this study. Wide distribution and high prevalence of the gull marker were obtained for the gull species tested, regardless of the locations at which the samples were collected. *Larus atricilla* and *L. delawarensis* are among the most common gulls in North America, and therefore, the *C. marimammalium*-specific PCR assay results suggested that this bacterial species is ubiquitous in the gull gastrointestinal tract as it was detected in gull feces and gull-impacted water samples from different geographic locations. The results also suggested that this species has restricted host specificity, as it was detected only in gulls and not in any other animal fecal samples, including several avian species. It should be noted that *C. marimammalium* was originally isolated from the mesentery, kidney, pericardial fluid, peritoneum, and small intestine of a dead harbor porpoise exhibiting severe enteritis and peritonitis. This is interesting from the standpoint that it suggests that some *C. marimammalium* strains are potentially pathogenic and that gull fecal contamination could be implicated in the transmission of aquatic mammal pathogens. As both *C. marimammalium* strains were isolated in mammals swimming in coastal waters of Scotland, their global distribution also merits future attention.

Real-time PCR assays. When the gull primer sets were used in real-time PCR assays, only the composite gull samples tested positive, while the C_T values for other fecal DNA extracts were below the detection limit. Standard curves for real-time PCR assays were constructed with plasmid inserts with an equivalent of 6.3 $\times$ 10^8 to 63 plasmid copies. There was a linear relationship between C_T values and copy numbers of the targeted DNA fragment according to plasmid standard curves for a wide range of targets (r^2 = 0.98; data not shown). C_T values were linear from 60 ng to 6 pg for the primer when using fecal DNA dilutions (Fig. 3), suggesting that neither PCR inhibitors nor the presence of large amounts of heterologous DNA inhibited amplifications (7). The detection limit of the real-time PCR assay was similar to that of the conventional PCR assay with the original composite gull DNA extract (i.e., 6 pg fecal DNA; Fig. 2). These results allowed us to estimate the copy numbers of fecal DNA signals from several locations (West Virginia, Georgia, Ohio, Florida, and Ontario, Canada) and in different gull species.

The copy number of the targeted sequence varied considerably among individual gull fecal samples from the same species collected in the same location or at different locations and between different gull species (Table 3). Overall, the range was 2 to 166,701 DNA copies per ng of fecal DNA, indicating that the densities of *C. marimammalium* could greatly fluctuate in the gull gut system. The copy number in the ring-billed gulls was, on average, 10 times higher than in laughing gulls from Georgia (P = 0.0094, n = 25). Factors influencing *C. marimammalium* fecal densities are yet to be determined, but it is important to know whether physiological changes in the gull due to age, diet, or the surrounding environment affect the densities of *C. marimammalium* in the gull intestine. These fluctuations are relevant to source-tracking studies for several

TABLE 3. Host distribution and estimated average copy number of gull-specific marker

Target	Sampling location	No. of fecal samples tested	Gull2 assay		
			No. of PCR-positive samples	Quantitative PCR	
				No. of samples detected	Avg copy no./ng DNA ± SD
Larus domesticus	Georgia	13	10	10	6,117 ± 12,428
Larus atricilla	Georgia	20	10	12	905 ± 1,040
Larus atricilla	Ohio	3	3	3	414 ± 496
Larus domesticus	Ohio	3	2	3	52 ± 73
Larus domesticus	West Virginia	8	7	6	896 ± 932
Larus atricilla	Florida	7	5	5	216 ± 171
Larus domesticus	Ontario, Canada	4	4	4	93,044 ± 71,792

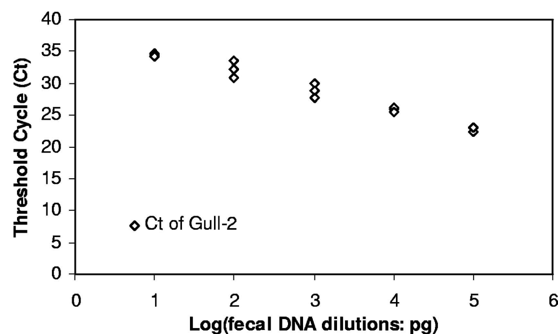

FIG. 3. Performance of *C. marimammalium* 16S rRNA gene PCR assay against 10-fold dilutions of seagull fecal DNA starting with 60 ng (indicated as 5 log units on the *x* axis) to 6 pg (indicated as 1 log unit on the *x* axis).

reasons. For example, average signals obtained in one locality might not be applicable to another study site due to inter- and intraspecies fluctuations. This is critical to presence/absence assays from the standpoint of assay sensitivity, as it suggests that detection limits in environmental waters might vary among different sites. When using quantitative PCR assays, it will also be necessary to understand the level of variability between hosts in order to better estimate fecal loads for a given source, information that is needed for regulatory activities and for microbial quantitative risk analysis (29). Fluctuations in *E. coli* and *Enterococcus* densities in gull feces have previously been documented (11). However, this is the first study showing variations in gull fecal bacterial populations other than fecal bacterial indicators.

Gull PCR-based signals in water samples. *C. marimammalium* was found to be ubiquitous and specific to gull feces, suggesting that assays targeting this bacterial species might be used as an indicator of gull fecal pollution in MST studies. Gull contamination is prevalent in many coastal areas, as well as in recreational waters in the Great Lakes. However, thus far, no assays have been published that can determine the presence of gull fecal contamination in surface waters. To address the value of the *C. marimammalium* assay in detecting gull feces in environmental waters, we tested DNA extracts from waters presumed to have a history of gull contamination against the Gull-2 assay (Table 4). All samples suspected to have been impacted with gull feces showed strong PCR signals in the Gull-2 assay, suggesting that this assay can be used to track sources in different geographic locations. Additionally, we used the assay to test waters that are impacted by other waterfowl fecal sources (i.e., Canadian geese). Water samples collected from the Toledo Botanical Garden pond (Toledo, OH), which is known to be impacted by Canadian geese (i.e., as the only waterfowl species), showed an average of 26,900 fecal coliform CFU/100 ml (W. Von Sigler, personal communication) and were negative by the Gull2 assay. Samples taken from environmental waters known to be impacted by non-waterfowl sources (i.e., swine, cattle, chickens) were also negative. In contrast, all three samples taken from a site near the Toledo Botanical Garden pond where gulls are often seen (near Lake Erie) were positive. Similar results were obtained with freshwater samples collected from Wisconsin, California, and Ontario beaches known to be frequented by gulls. More importantly, the Gull-2 assay was positive for water samples collected over beach seasons at three different Lake Ontario beaches known to be highly contaminated by gull droppings (10). A library-dependent MST study at Bayfront Park Beach on Lake Ontario demonstrated that most of the *E. coli* contamination at this beach was from birds rather than municipal wastewater or pets (10). Our results are relevant to the latter study, as the previous assays used could not discriminate between the importance of gull droppings and that of Canada goose droppings at these sites. Overall, our results showed that the Gull-2 assay can be used to detect the presence of gull fecal impacts at different geographic locations and that it can be used across multiple seasons (i.e., it is temporally stable).

Nonpoint fecal pollution sources are increasingly being recognized as important contributors to elevated levels of *E. coli* and *Enterococcus* indicator bacteria in recreational waters. As waterfowl are an important source of pollution in beach areas (17, 26) and can serve as potential reservoirs of human infections (13, 36), the assays described herein would be useful in health risk-based analyses (i.e., epidemiological studies and quantitative microbial risk studies) of nonhuman fecal pollu-

TABLE 4. Detection of gull feces in environmental samples by the Gull2 assay

Sampling location(s)	Sample type	Collection time	No. of water samples	No. of samples Gull2 assay positive	Presumed gull contamination[a]
Grant Park Beach, Milwaukee, Wisconsin (Lake Michigan)	Freshwater	September–October 2007	8	8	Yes
Maumee Bay, Oregon; Lake Erie, Ohio	Freshwater	October 2007	3	3	Yes
Toledo Botanical Garden pond, Toledo, Ohio	Freshwater	October 2007	2	0	No
Northeastern Ohio	Chicken pit	May 2007	9	0	No
Northeastern Ohio	Swine pit	February 2008	3	0	No
Northeastern Ohio	Cow manure lagoon	February 2008	1	0	No
Northern Georgia	Freshwater	May 2006	9	0	No
Bayfront Park Beach, Toronto (Lake Ontario, Canada)	Freshwater	May–August 2007	10	10	Yes
Bluffers Park Beach, Toronto (Lake Ontario, Canada)	Freshwater	May–August 2007	10	10	Yes
Sunnyside Beach (Lake Ontario, Canada)	Freshwater	May–August 2007	10	10	Yes
Doheny State Beach pond (Dana Point, California)	Freshwater	June–July 2007	7	7	Yes

[a] For all sites presumed positive, there is historical knowledge that gulls are present during a significant part of the year, particularly during warm months. At the Canadian sites, the number of gulls present on collection dates ranged from 2 to 220. No numbers were available for the other sites.

tion in recreational waters. Additionally, having gull-specific markers in the fecal source-tracking toolbox will help beach managers better assess potential causes of beach postings beyond familiar fecal pollution sources such as municipal wastewater and therefore implement remediation practices that target the most relevant sources of pollution.

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