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Development of Quantitative PCR Assays Targeting the 16S rRNA Genes of *Enterococcus* spp. and Their Application to the Identification of *Enterococcus* Species in Environmental Samples

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The detection of environmental enterococci has been determined primarily by using culture-based techniques that might exclude some enterococcal species as well as those that are nonculturable. To address this, the relative abundances of enterococci were examined by challenging fecal and water samples against a currently available genus-specific assay (Entero1). To determine the diversity of enterococcal species, 16S rRNA gene-based group-specific quantitative PCR (qPCR) assays were developed and evaluated against eight of the most common environmental enterococcal species. Partial 16S rRNA gene sequences of 439 presumptive environmental enterococcal strains were analyzed to study further the diversity of enterococci and to confirm the specificities of group-specific assays. The group-specific qPCR assays showed relatively high amplification rates with targeted species (>98%), although some assays cross-amplified with nontargeted species (1.3 to 6.5%). The results with the group-specific assays also showed that different enterococcal species co-occurred in most fecal samples. The most abundant enterococci in water and fecal samples were *Enterococcus faecalis* and *Enterococcus faecium*, although we identified more water isolates as *Enterococcus casseliflavus* than as any of the other species. The prevalence of the Entero1 marker was in agreement with the combined number of positive signals determined by the group-specific assays in most fecal samples, except in gull feces. On the other hand, the number of group-specific assay signals was lower in all water samples tested, suggesting that other enterococcal species are present in these samples. While the results highlight the value of genus- and group-specific assays for detecting the major enterococcal groups in environmental water samples, additional studies are needed to determine further the diversity, distributions, and relative abundances of all enterococcal species found in water.

For more than a century the microbiological quality of environmental waters has been assessed using fecal indicator bacteria (FIB). While fecal coliforms and *Escherichia coli* are still widely used in environmental monitoring, enterococci are becoming a frequent target, as they can be used to estimate health risks in both recreational marine waters and bodies of freshwater. The *Enterococcus* genus includes more than 20 species, many of which are commonly associated with different mammals and birds, while some species have been isolated from nonfecal sources (1). Studies looking at the enterococci diversity in environmental waters have identified most strains as *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus casseliflavus*, *Enterococcus hirae*, *Enterococcus durans*, and *Enterococcus mundtii* (2–4). These findings have relied on the isolation of enterococcal strains on selective culturing medium (5), followed by their classification, which may involve biochemical (6, 7) and molecular (8) techniques. Culture-based techniques are also used in regulatory activities to estimate the densities of enterococci in environmental waters. Since none of the enterococcal media available can be used to discriminate between the different species, their densities are recorded as general enterococcus counts. Information on the environmental prevalence of enterococcal species is not only relevant for confirming the presence of fecal enterococci, but it has also been suggested that it can help identify primary fecal pollution sources (9). Different fecal sources can contribute to the pollution of environmental waters, and each of them carries different health risks (10). The general consensus is that human fecal sources are associated with higher risks, particularly due to host-specific pathogens, such as enteric protozoa and viruses. However, nonhuman pollution sources are increasingly receiving attention by those in the health risk community, in light of recent outbreaks in which they are implicated as the most likely sources (11) and due to their relevance in beach closures, where the economic impact can be significant.

A quantitative PCR (qPCR) assay, Entero1, was used recently to estimate the levels of enterococci in recreational waters (12). Originally developed by Ludwig and Schleifer (13), the Entero1 assay targets the 23S rRNA gene. In most bacterial species, rRNA genes are present in multiple copies per genome, and therefore, targeting such genes in environmental samples can improve assay sensitivity due to their lower detection limits. However, less sequencing information is available for the 23S rRNA gene than for the 16S rRNA gene, precluding robust *in silico* validation. As a result, validation of the Entero1 assay has relied on testing the assay against a relatively small number of environmental strains

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isolated from a limited number of different geographic locations (12, 14). Moreover, similar to selective enterococcal media, the Entero1 assay cannot be used to determine which of the major enterococcal groups are present in a given sample.

To address some of these issues, we compared the relative occurrences and abundances of environmental and fecal enterococci using the Entero1 assay and several 16S rRNA gene-based group-specific PCR assays, most of which were developed as part of this study. Due to their reported prevalences in the environment, three of the major fecal enterococcal groups (*E. faecalis*, *E. faecium*, and *E. casseliflavus*) were targeted by the group-specific assays. The study was conducted by challenging the assays against fecal samples from diverse hosts and environmental waters with a history of fecal pollution. We also identified 439 strains isolated from surface water samples using 16S rRNA gene sequence analysis.

MATERIALS AND METHODS

Bacterial strains. The following strains were used as positive and negative controls: *E. casseliflavus* (ATCC 25788), *Enterococcus dispar* (ATCC 51266), *E. durans* (ATCC 19432), *E. faecalis* (ATCC 19433), *E. faecium* (ATCC 19434), *Enterococcus gallinarum* (ATCC 49573), *E. hirae* (ATCC 8043), *Enterococcus pseudoavium* (ATCC 49372), *Aeromonas eucrenophila* (ATCC 23309), *Escherichia coli* (ATCC 25922), *Legionella sainthelensi* (ATCC 35248), *Proteus vulgaris* (ATCC 13315), *Salmonella enterica* serovar Typhimurium (ATCC 14028), *Shigella flexneri* (ATCC 29903), *Staphylococcus aureus* (ATCC 29213), *Catellicoccus marimammalium*, *Citrobacter freundii*, *E. coli* O157:H7, *Escherichia hermannii*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. Additionally, 439 presumptive *Enterococcus* sp. strains isolated on *Enterococcus* indoxyl-β-D-glucoside (mEI) agar (15) were used for evaluating the enterococcal assays. The latter strains were isolated from environmental waters collected from 15 U.S. states (AZ, CO, FL, GA, KS, MD, MN, MT, NJ, NV, NY, OK, WA, WV, and WY). The identities of the environmental enterococcal strains were confirmed using 16S rRNA gene sequencing analysis.

Environmental sample collection and DNA extraction. The environmental monitoring values of the group-specific assays were tested against water samples ($n = 311$) collected from different locations in California, North Carolina, and Puerto Rico. The water samples from California and North Carolina were collected from estuarine sites that are primarily impacted by gulls, whereas the water samples from Puerto Rico were collected from sites within the Rio Grande de Arecibo watershed which are presumably impacted by cattle, humans, and wildlife. Additionally, the assays were challenged against fecal samples ($n = 497$) from 4 domesticated animals (goat, horse, monkey, and pig), 13 wildlife species (chipmunk, coyote, fox, marmot, yellow-bellied marmot, mule, mule deer, rabbit, jackrabbit, raccoon, snowshoe hare, squirrel, and ground squirrel), and 7 avian species (chicken, duck, guinea fowl, gull, pelican, swan, and turkey). The water samples (100 ml each) were collected and filtered onto polycarbonate membranes (0.4-μm pore size, 47-mm diameter) (GE Water and Process Technologies, Trevose, PA). The fecal samples were collected aseptically, transferred to sterile tubes, and transported to the laboratory in ice coolers. The frozen filters and fecal samples were shipped overnight on dry ice to the U.S. Environmental Protection Agency, Cincinnati, OH, and stored at −80°C until further processing. DNA extraction from the filters and fecal samples was performed using the PowerSoil DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA) according to the manufacturer's protocols. Avian fecal samples (i.e., gull and turkey) from France were extracted using the FastDNA spin kit for soil (MP Biomedical, Illkirsh, France) according to the supplier's instructions, except that an additional wash using the salt/ethanol wash solution (SEWS-M) reagent was performed. The DNA concentrations were measured using a NanoDrop ND-1000 UV spectrophotometer (NanoDrop Technologies, Wilmington, DE). The DNA extracts were stored at −20°C until further processing.

Sequencing analyses. The sequences from 16S rRNA gene PCR products that were generated using universal bacterial primers (8F, 5′-AGAG TTTGATCCTGGCTCAG-3′, and 787R, 5′-CGACTACCAGGGTATCT AAT-3′) were used to determine the identities of the 439 environmental isolates from mEI cultures and reference bacteria. Briefly, PCR assays were performed in 25 μl using the polymerase TaKaRa Ex Taq (TaKaRa Bio, Inc.) in a Tetrad2 thermal cycler (Bio-Rad, Hercules, CA) under the following cycling conditions: an initial denaturation step at 95°C for 5 min and 25 cycles of 1 min at 95°C, 1 min at 56°C, and 1 min at 72°C. The PCR products were sequenced in both directions in the Children's Hospital DNA Core Facility (Cincinnati, OH) using an Applied Biosystems Prism 3730XL DNA analyzer. The raw gene sequences were processed using Sequencher software (Gene Codes, Ann Arbor, MI). For the 16S rRNA gene sequences, homology searches of DNA sequences in the GenBank (nonredundant [NR]) database were undertaken with the National Center for Biotechnology Information (NCBI) BLASTn program (http://www.ncbi.nlm.nih.gov/BLAST/) (16).

Assay development and performance evaluation. Fifteen different assays were tested in this study: five genus-specific assays, three *E. faecalis*-specific assays, three *E. casseliflavus*-specific assays, and four *E. faecium*-specific assays (Table 1). Eleven assays were tested as conventional PCR assays, and four were tested as qPCR (TaqMan-based) assays (one genus-specific, Entero1, and one for each of the enterococcal groups, Faecalis1, Casseli1, and Faecium1). The Entero1 and Faecalis1 qPCR assays were developed and evaluated in previous studies (12, 17). To develop new enterococcal assays, a phylogenetic tree that included the 16S rRNA gene sequences from reference enterococcal strains (8) and environmental strains was generated using a neighbor-joining algorithm in ARB (18). Unique phylogenetic clades were identified (Fig. 1), and candidate primers were then chosen to target three major environmental clades (*E. faecalis*, *E. faecium*, and *E. casseliflavus*) using the primer design algorithm in ARB (Table 1). Additionally, 16S rRNA gene enterococcal sequences were used to design two group-specific qPCR assays using the Primer Express software (Applied Biosystems, Foster City, CA) (Table 1). The assays were optimized through the use of temperature gradients and were tested for their specificities and sensitivities against the reference bacterial strains and environmental enterococcus isolates described above. The applicabilities of the PCR and qPCR assays in environmental monitoring were also evaluated against the aforementioned set of water and fecal samples.

For the conventional PCR assays, all water and fecal samples were tested as described previously (19), with the following modifications: 0.5 to 1 ng/μl of DNA extracts was used as a template, and 10-fold dilutions of each DNA extract were used to test for PCR inhibition. The PCR assays were performed in 25 μl using TaKaRa Ex Taq (TaKaRa Bio, Inc.) in a Bio-Rad Tetrad2 Peltier thermal cycler (Bio-Rad, Hercules, CA) under the following cycling conditions: an initial denaturation step at 95°C for 5 min and 25 cycles of 1 min at 95°C, 1 min at optimum annealing temperature (Table 1), and 1 min at 72°C. The PCR products were visualized in 1.5% agarose gels using GelStar nucleic acid gel stain (Lonza, Rockland, ME).

The TaqMan qPCR assays were performed in 25-μl reaction mixtures containing 1× TaqMan universal PCR master mix with AmpErase uracil-*N*-glycosylase (Applied Biosystems, Foster City, CA), 0.2 μg/μl bovine serum albumin, 0.2 μM (final concentration) of each primer, and a 6-FAM (6-carboxyfluorescein)-labeled hydrolysis probe. The amplification protocol involved an initial incubation step at 50°C for 2 min to activate uracil-*N*-glycosylase, followed by 10 min of incubation at 95°C to activate AmpliTaq Gold enzyme, and then 40 cycles of 95°C for 15 s and 60°C for 1 min. The qPCR assays were performed using a 7900 HT Fast real-time sequence detector (Applied Biosystems, Foster City, CA). All assays were performed in triplicate in MicroAmp Optical 96-well reaction plates with MicroAmp Optical Caps strips (Applied Biosystems, Foster City, CA). The PCR data were analyzed using ABI's Sequence Detector software (version 2.2.2). Four independent standard curves for each qPCR assay were generated by plotting the threshold cycle (C_T) values against the numbers of target copies corresponding to serially diluted

TABLE 1 Summary of oligonucleotide primers and probes for PCR and TaqMan qPCR

Target organism(s)	Assay[a]	Primer	P sequence (5′ to 3′)	T_a (°C)[b]	Size (bp)[c]	Reference or source
Enterococcus spp.	Entero1	ECST748F	AGAAATTCCAAACGAACTTG	60	92	18
		ENC854R	CAGTGCTCTACCTCCATCATT			
		GPL813TQ	6FAM-TGGTTCTCTCCGAAATAGCTTTAGGGCTA-TAMRA			
	Ent1	Ent151F	ACACTTGGAAACAGGTGC	65	243	This study
		Ent376R	TCGGTCAGACTTKCGTCC			
	Ent2	Ent151F	ACACTTGGAAACAGGTGC	65	445	This study
		Ent578R	TTAAGAAACCGCCTGCGC			
	Ent3	Ent240F	TGCATTAGCTAGTTGGTG	63	356	This study
		Ent578R	TTAAGAAACCGCCTGCGC			
	Ent4	Ent376F	GGACGMAAGTCTGACCGA	65	220	This study
		Ent578R	TTAAGAAACCGCCTGCGC			
Enterococcus faecalis	Faecalis1	FaecalF	CGCTTCTTTCCTCCCGAGT	60	143	32
		FaecalR	GCCATGCGGCATAAACTG			
		FaecalP	6FAM-CAATTGGAAA GAGGAGTGGCGGACG-TAMRA			
	Faecalis2	Ent151F	ACACTTGGAAACAGGTGC	64	318	This study
		Faecal449R	AGTTACTAACGTCCTTGTTC			
	Faecalis3	Ent240F	TGCATTAGCTAGTTGGTG	63	229	This study
		Faecal449R	AGTTACTAACGTCCTTGTTC			
Enterococcus casseliflavus	Casseli1	CasselF	GGAGCTTGCTCCACCGAA	60	132	This study
		CasselR	TTTCTTCCATGCGGAAAATAGT			
		CasselP	6FAM-CGAACGGGTGAGTAACACGTGGGTAA-TAMRA			
	Casseli2	Cassel190F	GGAAGAAAGTTGAAAGGC	60	204	This study
		Ent376R	TCGGTCAGACTTKCGTCC			
	Casseli3	Cassel190F	GGAAGAAAGTTGAAAGGC	60	406	This study
		Ent578R	TTAAGAAACCGCCTGCGC			
Enterococcus faecium	Faecium1	CiumF	TTCTTTTTCCACCGGAGCTT	60	141	This study
		CiumR	AACCATGCGGTTTYGATTG			
		CiumP	6FAM-AGTAACACGTGGGTAACCTGCCCATCAGA-TAMRA			
	Faecium2	Cium84F	TGCTCCACCGGAAAAAGA	63	174	This study
		Ent240R	CACCAACTAGCTAATGCA			
	Faecium3	Cium84F	TGCTCCACCGGAAAAAGA	64	310	This study
		Ent376R	TCGGTCAGACTTKCGTCC			
	Faecium4	Cium84F	TGCTCCACCGGAAAAAGA	65	512	This study
		Ent578R	TTAAGAAACCGCCTGCGC			

[a] Entero1 targets the 23S rRNA gene, whereas the other assays target the 16S rRNA gene.
[b] Optimum annealing temperatures determined using temperature-gradient PCR.
[c] Approximate product size determined from *in silico* data.

plasmid standards purchased from Integrated DNA Technologies (IDT; Coralville, Iowa). The target copy numbers (T) were estimated by the equation $T = [D/(PL \times 660)] \times 6.022 \times 10^{23}$, where D (g/μl) is plasmid DNA concentration and PL (in base pairs) is plasmid length. Each standard curve was generated from at least five 10-fold plasmid dilutions in triplicate. The percent amplification efficiencies were calculated by the instrument manufacturer's instructions (Applied Biosystems). Two no-template controls per PCR plate were used to check for cross-contamination.

Venn diagrams. The relationships among the genus- and species-specific qPCR assays against fecal and water samples were determined using Venn diagrams as described previously (20). Briefly, two Venn diagrams were constructed sequentially: the first diagram was used for calculating the prevalences of three species-specific markers, and the second diagram was used to establish the relationship between the genus-specific assay and the three species-specific assays combined.

Nucleotide sequence accession numbers. The representative sequences were deposited in GenBank under the following accession numbers: JQ804941 to JQ804949.

RESULTS AND DISCUSSION

Rationale for assay development. Phylogenetic trees that included sequences from reference and environmental enterococcal strains were generated to identify the 16S rRNA gene sequences that could be used to develop multiple enterococcal species-specific assays (Fig. 1). This approach indicated that it was difficult to develop assays that discriminated *E. faecium* from *E. mundtii*, *E. durans*, *E. hirae*, and *E. dispar* and *E. casseliflavus* from *E. gallinarum*. However, nonribosomal genes can be used to discriminate between different enterococcal species (21, 22). However, only a handful of nonribosomal genes have been used in environmental studies to detect or identify enterococci (14, 23, 24). More importantly, the sequence database for the function-specific genes of environmental enterococci and other phylogenetically related genera is much more limiting than that for the 16S and 23S rRNA genes. Moreover, sequence conservancy in functional genes is considerably lower than that in rRNA genes, which explains why it

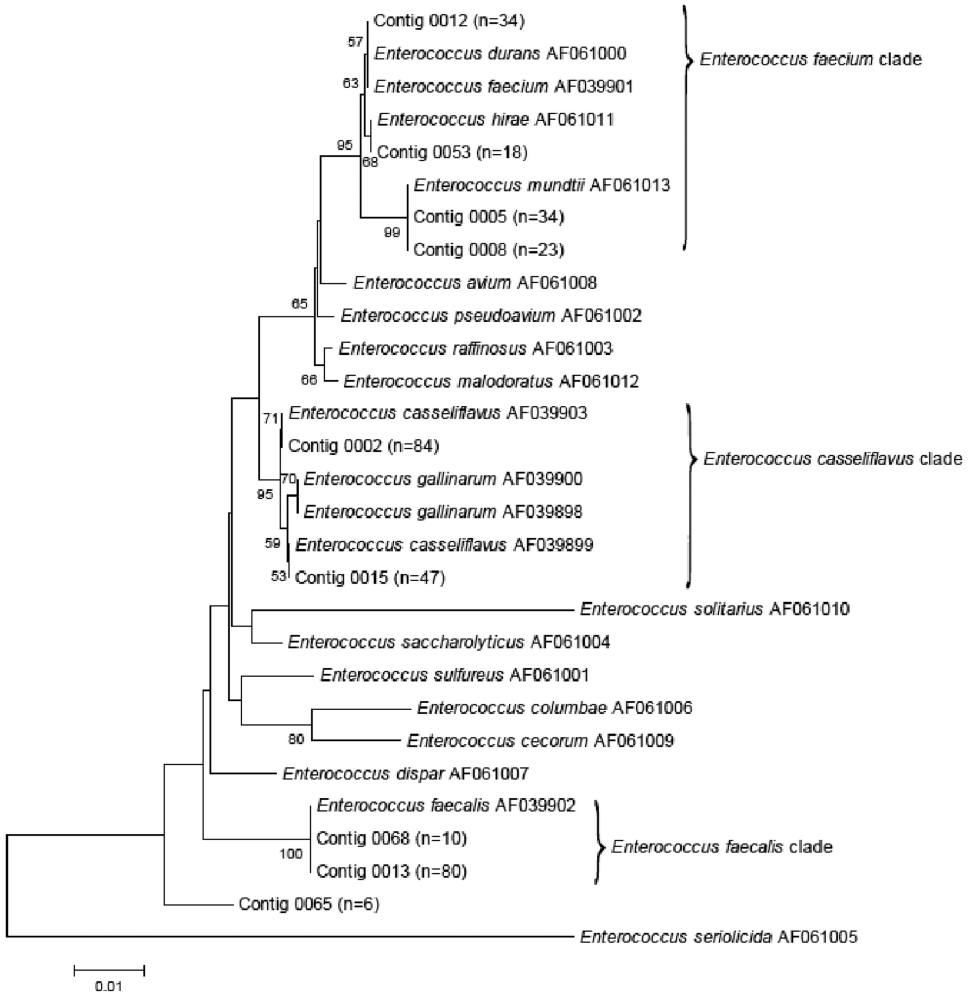

FIG 1 Unrooted neighbor-joining tree of 16S rRNA gene sequences obtained from *Enterococcus* environmental isolates. The number of sequences for each contig is included in parentheses, and the contigs of fewer than 5 sequences are not presented in the phylogenetic tree. The reference bacteria with their GenBank accession numbers and 1,000-replicate bootstrap values are shown in the tree. The bootstrap values reported are the percentages greater than 50%. The scale bar corresponds to 0.01 changes per nucleotide.

is difficult to develop genus- and group-specific assays unless comprehensive sequence databases are developed.

Identification of environmental strains. Based on the 16S rRNA gene sequence analyses of the 439 environmental isolates used in this study, approximately 91% were identified as *Enterococcus* spp., whereas others were classified as nonenterococci (7%) or unclassified bacteria (2%). These results are in agreement with other studies using mEI agar as the isolation medium for environmental enterococci (15, 25), although Nayak et al. (26) reported relatively lower false-positive rates (1.6%) in subtropical waters. The study by Nayak et al. was based on 61 strains isolated from two lakes on two different dates, which may explain the lower false-positive rate.

Based on the sequence identities of the environmental isolates tested in our study, the most dominant enterococcal species were *E. casseliflavus* (34%), *E. faecalis* (25%), and *E. mundtii* (15%), while *E. faecium* and *E. hirae* were identified to a lesser extent (5%) (Table 2). Several enterococcal species have been detected in environmental waters, but their overall prevalences have varied considerably. For example, Mote et al. (25) found that the most dom-

TABLE 2 Classification of environmental isolates from mEI cultures using 16S rRNA gene sequencing

Bacteria	No. (%) of isolates	GenBank accession no.[a]
Enterococcus casseliflavus	152 (34)	DQ333294.1
Enterococcus faecalis	111 (25)	AB534553.1
Enterococcus mundtii	68 (15)	NR_024906.1
Enterococcus faecium	23 (5)	EU003447.1
Enterococcus hirae	20 (5)	Y17302.1
Enterococcus spp.	21 (5)	NR_036922.1, NR_037082.1, and NR_042054.1
Aerococcus spp.	24 (5)	HM582941.1
Lactococcus garvieae	3 (0.7)	AY699289.1
Pediococcus pentosaceus	4 (0.9)	CP000422.1
Streptococcus gallolyticus subsp. *pasteurianus*	4 (0.9)	AB457024.1
Unclassified	9 (2)	Not available
Total	439 (100)	

[a] All sequences for enterococcus isolates are >99% identical to GenBank reference sequences.

TABLE 3 Number (percentage) of positives by the *Enterococcus* assays against environmental isolates and ATCC strains

Target organism(s)	Assay	No. (%) of positives						
		Enterococcus casseliflavus ($n = 50$)[a]	*Enterococcus faecalis* ($n = 39$)	*Enterococcus faecium* ($n = 11$)	*Enterococcus hirae* ($n = 5$)	*Enterococcus mundtii* ($n = 40$)	Other enterococcal species ($n = 8$)[b]	Nonenterococcal species ($n = 17$)[c]
Enterococcus spp.	Entero1[c]	50 (100)	39 (100)	11 (100)	5 (100)	40 (100)	8 (100)	1 (5.9)
	Ent1	50 (100)	39 (100)	11 (100)	5 (100)	39 (98)	7 (88)	4 (24)
	Ent2	33 (66)	19 (49)	8 (73)	4 (80)	18 (45)	8 (100)	0 (0)
	Ent3	50 (100)	37 (95)	11 (100)	4 (80)	39 (98)	8 (100)	0 (0)
	Ent4	50 (100)	39 (100)	11 (100)	5 (100)	40 (100)	8 (100)	2 (12)
Enterococcus faecalis	Faecalis1[d]	5 (10)	39 (100)	2 (18)	0 (0)	6 (15)	1 (13)	0 (0)
	Faecalis2	0 (0)	16 (41)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Faecalis3	0 (0)	25 (64)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Enterococcus casseliflavus	Casseli1[d]	49 (98)	0 (0)	1 (9.1)	1 (20)	0 (0)	0 (0)	0 (0)
	Casseli2	29 (58)	4 (10)	1 (9.1)	0 (0)	4 (10)	0 (0)	0 (0)
	Casseli3	48 (96)	0 (0)	1 (9.1)	0 (0)	0 (0)	0 (0)	0 (0)
Enterococcus faecium	Faecium1[d]	2 (4.0)	0 (0)	11 (100)	5 (100)	40 (100)	8 (100)	0 (0)
	Faecium2	27 (54)	2 (5.1)	10 (91)	3 (60)	35 (88)	8 (100)	0 (0)
	Faecium3	30 (60)	1 (2.6)	11 (100)	5 (100)	28 (70)	8 (100)	0 (0)
	Faecium4	0 (0)	0 (0)	11 (100)	4 (80)	19 (48)	7 (88)	0 (0)

[a] All enterococcal species were identified by NCBI BLAST, with the exception of *E. casseliflavus*, the sequences of which are nearly identical to those of *E. gallinarum*.
[b] Sequences of the isolates are affiliated with the *E. faecium* clade (see Fig. 1).
[c] Seven ATCC strains (*Aeromonas eucrenophila*, *Escherichia coli*, *Legionella sainthelensi*, *Proteus vulgaris*, *Salmonella enterica* serovar Typhimurium, *Shigella flexneri*, and *Staphylococcus aureus*), six laboratory strains (*Catellicoccus marimammalium*, *Citrobacter freundii*, *Escherichia coli* O157:H7, *Escherichia hermannii*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*), and four environmental strains (*Aerococcus* species, *Lactococcus garvieae*, *Pediococcus pentosaceus*, and *Streptococcus pasteurianus*).
[d] TaqMan qPCR assays.

inant enterococcal species were *E. faecalis* (31%), *E. mundtii* (31%), and *E. casseliflavus* (16%), while *E. faecium* and *E. gallinarum* were identified less frequently (10% and 4%, respectively). Moore et al. (4) and Grammenou et al. (27) also found different environmental enterococcal species, but *E. faecalis* and *E. faecium* were the most dominant species in many water samples. In spite of these differences, these results clearly indicate that multiple enterococcal species can be present in the same water sample. The differences in the occurrences of enterococcal species may be associated with different *in situ* growth and environmental survival rates (28) and with preferential host distributions of different enterococcal species in different animals. Other studies have suggested that that the environmentally relevant enterococcal species detected in this study are found in a wide variety of hosts (29). Altogether, these data suggest that the identification of enterococcal species might not be an adequate approach to fecal source identification.

In this study, four nonenterococcal species were identified (i.e., >99% identical to reference sequences) among the environmental isolates, namely, *Aerococcus* sp., *Lactococcus garvieae*, *Pediococcus pentosaceus*, and *Streptococcus gallolyticus* subsp. *pasteurianus*. Other studies have reported on the presence of some of these genera in mEI medium. For example, Maraccini et al. (30) showed that most nonenterococcal mEI isolates were identified as *Aerococcus viridans* (17%), with fewer isolates identified as *Streptococcus mutans*, *S. gallolyticus*, *Leuconostoc* spp., and *Pediococcus acidilactici*. The samples from the latter study were collected within a 3-day period from one marine site. While it is not known how predominant these nonenterococcal species are during an entire beach season, these data suggest that some nonenterococcal species may be highly abundant in recreational marine waters, potentially resulting in the overestimation of enterococcus densities when using culture-based methods. In another study, Viau and Peccia (31) showed that mEI medium also supported the growth of bacteria from biosolids that were identified as *Bacillus* spp., *Vagococcus* spp., and *Desemzia incerta*. As biosolids and animal fecal waste (i.e., treated manure) are used in agricultural activities, the results from these studies suggest that nonenterococcal species might interfere with the culture-based methods that are used to estimate fecal pollution levels. In our study, the bacterial strains found in the water tested were isolated from waters presumed to be impacted by wastewater treatment plants and, to a lesser extent, by agricultural activities, although wildlife fecal pollution sources cannot be ruled out. Our results suggest further that culture-based methods can support the growth of nonenterococcal species present in freshwater samples and that additional studies are needed to determine better the identities and prevalences of these nontargeted species in fecal and water samples.

Validation of genus-specific enterococcal PCR assays. The specificities of the *Enterococcus* genus- and group-specific PCR assays were evaluated against a subset of the enterococcal strains sequenced in this study. This subset ($n = 153$) included several strains from the most common *Enterococcus* species identified in this study, nonenterococcal species obtained from culture collections ($n = 13$), and nonenterococcal strains isolated from this study ($n = 4$) (Table 3). All of the genus-specific assays successfully amplified the enterococcus-type strains (from the ATCC). Additionally, four of the genus-specific assays generated positive signals with more than 97% of the environmental strains tested in this study and in most cases cross-amplified relatively few nonenterococcal strains (0 to 24%) (Table 3). Two of the assays, Ent2 and Ent3, did not show cross-amplification with nonenterococcal

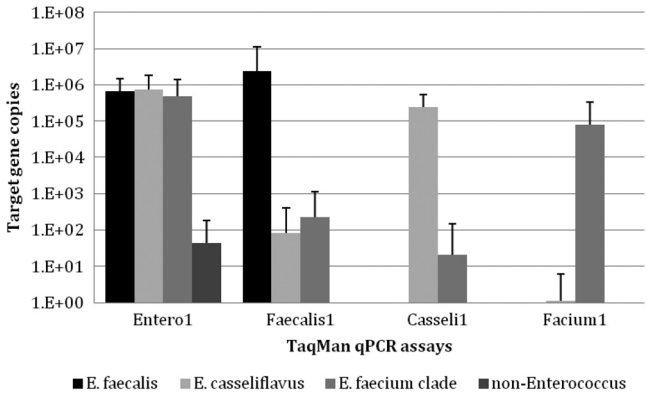

FIG 2 Mean copy numbers of target markers against environmental isolates of *Enterococcus* species and non-*Enterococcus* bacteria. To calculate the mean concentrations, the values below detection limits were treated as 0. The error bars represent 1 SD.

strains and therefore may prove useful as confirmatory tests. However, Ent2 only detected 59% of the enterococcal strains tested, suggesting that it cannot be used as a stand-alone enterococcal assay.

Interestingly, the Entero1 assay showed a low level of cross-amplification with *C. marimammalium*. Several gull-specific assays target the *C. marimammalium* 16S rRNA gene. Indeed, the signals with these gull assays have frequently been detected in environmental waters with a history of gull fecal contamination (20, 32). Signals detected against *C. marimammalium* DNA were approximately 4 orders of magnitude lower than those against the enterococcal species tested (Fig. 2), suggesting that *C. marimammalium* may not contribute significantly to false-positive signals. Entero1 cross-amplification signals have been observed with other lactobacilli species (33). These results are relevant to environmental monitoring, as the Entero1 assay has been proposed as an alternative method for the rapid detection of *Enterococcus* spp. in recreational waters (34). As the overestimation of the Entero1 assay due to nontargeted bacteria could result in unnecessary beach closures, additional studies are needed to determine more accurately the levels of false-positive signals in recreational settings. Future studies also need to determine whether these nontargeted populations are present in environmental waters frequently enough to interfere with risk assessment models.

Validation of group-specific enterococcal PCR assays. Overall, the group-specific assays indicated a relatively high amplification with targeted enterococcal species and low cross-amplification with nonenterococcal species (Table 3). Noteworthy is that the Faecalis2 and Faecalis3 assays showed 100% specificities and amplified the *E. faecalis* type strain, but they did not amplify some of the *E. faecalis* environmental strains, suggesting that they may be used in limited cases as group-specific assays. The Faecalis1 TaqMan qPCR assay successfully amplified all of the tested *E. faecalis* strains ($n = 39$) and did not cross-react with seven non-*E. faecalis* ATCC strains, as was observed previously (17). Although the Faecalis1 assay cross-reacted with a low number of non-*E. faecalis* environmental strains (Table 3), the signal intensities of these nontarget bacteria were more than 4 orders of magnitude lower than those of *E. faecalis* strains (Fig. 2). Tracking signal intensities will be important to determine the value of these assays in environmental applications. As cross-amplification signals are relatively low for some of these assays, the scenarios showing high levels of environmental signals are likely to be the result of true-positive signals rather than false-positive signals, unless cross-amplification targets are present in high abundance in a given environmental sample. This assumption needs to be tested with these newly developed assays and with most published FIB-targeting assays.

The *E. casseliflavus*-specific assays showed relatively high specificities (i.e., low cross-amplification rates against nontarget species) compared to the other group-specific assays. The Casseli1 TaqMan qPCR assay successfully amplified 98% (49/50) of *E. casseliflavus* environmental strains and showed 1.7% (2/120) cross-amplification with nontarget species. Moreover, the Casseli1 and the Casseli3 assays did not cross-amplify with any of the non-*Enterococcus* bacteria tested in this study. The Casseli2 assay showed the least specificity and sensitivity (i.e., higher cross-amplification rate with nontarget species and lower amplification rate with *E. casseliflavus*).

Most *E. faecium* assays primarily amplified *E. faecium*, *E. durans*, *E. hirae*, and *E. mundtii* strains. This is compatible with the facts that these species formed a cohesive clade and that it is difficult to differentiate these species using 16S rRNA gene sequences (Fig. 1). Specifically, the Faecium1 TaqMan qPCR assay amplified *E. faecium*, *E. hirae*, and *E. mundtii* strains. The Faecium2 and Faecium3 assays cross-reacted with *E. casseliflavus*, whereas the Faecium4 assay showed the best specificity (i.e., lower cross-amplification rate with *E. casseliflavus*) (Table 3). Thus, the *E. faecium* assays developed in this study might be used as a multispecies-specific assay. Future studies should focus on assessing the value of the conventional PCR assays developed in this study for use as qPCR assays.

Detection of enterococci in fecal and environmental water samples. The Entero1, Faecalis1, Casseli1, and Faecium1 assays were used in more studies based on the overall specificity and sensitivity results and the fact that they can provide quantification data. Specifically, the assays were used to investigate the presence and abundances of enterococci in 497 fecal samples collected from four different geographic locations and from 24 different animals and in 311 environmental water samples collected from California, North Carolina, and Puerto Rico (Table 4). To our knowledge, this represents the largest study in which different enterococcal species have been detected from fecal samples via PCR assays without the need for an enrichment step.

The range of quantification (ROQ) for the Entero1 and Faecalis1 qPCR assays was 10^1 to 10^6 DNA copies per reaction. For the Faecium1 and the Casseli1 assays, 10 copies per reaction were below the detection limit of the assay; therefore, the ROQ of these assays was determined to be from 10^2 to 10^6 DNA copies. In order to evaluate assay sensitivities, four independent standard curves were used to calculate the percent amplification efficiency average. The Entero1 assay showed the greatest amplification efficiency, followed by the Faecalis1, Faecium1, and Casseli1 assays (averages ± SD, 94.8 ± 0.8, 90.9 ± 1.1, 88.5 ± 2.1, and 85.2 ± 1.3, respectively). All of the no-template controls were negative, indicating the absence of cross-contamination in the qPCR experiments.

Approximately 74%, 41%, 25%, and 49% of the fecal samples were positive for the Entero1, Faecalis1, Casseli1, and Faecium1 markers, respectively (Table 4). However, when excluding the gull samples, the number of positive samples for enterococci increased

TABLE 4 Detection of enterococcal species in different fecal and water samples using TaqMan qPCR assays

Sample type	Sampling location(s)	No. of samples	No. (%) of positive samples with Entero1 (G)	No. (%) of positive samples with Faecalis1 (A)	No. (%) of positive samples with Casseli1 (B)	No. (%) of positive samples with Faecium1 (C)	Relationship between different assays (% positive)[a]		
							$n(S)$	$n(G \cap S)$	$n(G \cup S)$
Fecal									
Goat	Puerto Rico	32	32 (100)	18 (56)	7 (22)	30 (94)	32 (100)	31 (97)	32 (100)
Horse	Puerto Rico	28	28 (100)	7 (25)	3 (11)	22 (79)	24 (86)	24 (86)	28 (100)
Monkey	Puerto Rico	9	9 (100)	7 (78)	9 (100)	6 (67)	9 (100)	9 (100)	9 (100)
Pig	Puerto Rico	30	30 (100)	26 (87)	26 (87)	29 (97)	30 (100)	30 (100)	30 (100)
Wildlife[b]	California	77	61 (79)	13 (17)	9 (12)	39 (51)	49 (64)	49 (64)	61 (79)
Chicken	Puerto Rico	35	35 (100)	24 (69)	19 (54)	31 (89)	34 (97)	34 (97)	35 (100)
Duck	Puerto Rico	16	16 (100)	9 (56)	13 (81)	15 (94)	16 (100)	16 (100)	16 (100)
Guinea fowl	Puerto Rico	11	11 (100)	1 (9.1)	2 (18)	6 (55)	6 (55)	6 (55)	11 (100)
Gull[c]	California, Delaware, and France	220	108 (49)	82 (37)	10 (4.5)	33 (15)	87 (40)	83 (38)	112 (51)
Pelican	California	10	10 (100)	10 (100)	7 (70)	9 (90)	10 (100)	10 (100)	10 (100)
Swan	Puerto Rico	22	22 (100)	6 (27)	11 (50)	18 (82)	19 (86)	19 (86)	22 (100)
Turkey	France and Puerto Rico	7	7 (100)	2 (29)	6 (86)	7 (100)	7 (100)	7 (100)	7 (100)
Total fecal		497	369 (74)	205 (41)	122 (25)	245 (49)	NA[d]	NA	NA
Water									
Estuarine water	California	65	55 (85)	24 (37)	3 (4.6)	24 (37)	31 (48)	31 (48)	55 (85)
Estuarine water	North Carolina	109	107 (98)	68 (62)	9 (8.3)	34 (31)	76 (70)	76 (70)	107 (98)
Surface[e]	Puerto Rico	137	69 (50)	32 (23)	4 (2.9)	24 (18)	37 (27)	33 (24)	73 (53)
Total water		311	231 (74)	124 (40)	16 (5.1)	82 (26)	NA	NA	NA

[a] Results were calculated using a Venn diagram approach, where $n(\cup)$ is the total number of samples, $n(A)$ is the number of positive samples with Faecalis1, $n(B)$ is the number of positive samples with Casseli1, $n(C)$ is the number of positive samples with Faecim1, and $n(G)$ is the number of positive samples with Entero1; $n(S) = n(A \cup B \cup C)$.
[b] Thirteen different animals: chipmunk, coyote, fox, marmot, yellow-bellied marmot, mule, mule deer, rabbit, jackrabbit, raccoon, snowshoe hare, squirrel, and ground squirrel.
[c] Three species of gull from California (*Larus californicus*), Delaware (*Larus atricilla* and *Larus smithsonianus*), and France (*Larus argentatus*).
[d] NA, not available.
[e] Water samples were collected from 12 sampling locations in the Arecibo watershed, Puerto Rico, between September 2010 and January 2011 (representing 13 sampling events).

to 44% for the Casseli1 marker and 74 to 94% for the other markers, clearly suggesting that enterococci are normal inhabitants of most of the hosts tested here. The results of group-specific assays showed that different enterococcal species coinhabit most hosts, although the high prevalences of multiple enterococcal species were evident in some hosts more than others. For example, each of the three group-specific markers was detected in more than 87% of pig feces, while a specific group predominated in gulls, horses, and wildlife. The prevalence of the Entero1 marker [i.e., $n(G)$] was in agreement with the combined number of positive signals [i.e., $n(S) = n(A \cup B \cup C)$] determined by the three species-specific markers in fecal samples (Table 4). In other words, combining the results from the genus- and group-specific assays [i.e., $n(G \cup S)$] did not increase the number of enterococcus-positive samples in most feces types, with the exception of gull fecal samples, in which an increased prevalence was observed [i.e., $n(G \cup S) > n(G) > n(S)$]. There are two scenarios that might explain the lower prevalences of the species-specific markers in gull feces. First, it is possible that there are environmental enterococcal species that are detected by the Entero1 assay but not detected with the group-specific assays tested in this study. This suggests that additional group- or species-specific assays are needed to study further the abundances and dynamics of these species in fecal samples and perhaps in environmental waters impacted by gulls. This may be important if these nontargeted enterococcal species are noted to be important in recreational waters. A second scenario relates to the Entero1 assay cross-reacting with some of the indigenous non-*Enterococcus* bacteria; this may be the case for *C. marimammalium*, which resides in gull feces and for which signals have been detected in gull-impacted waters. If the latter situation is of any significance, the Entero1 assay may overestimate enterococcal levels but show a positive correlation with the presence of gull feces contamination. Thus, further validation of the specificity of Entero1 against a broad range of non-*Enterococcus* bacteria is needed; this is particularly the case for members of the *Lactobacillales* family, as overestimation due to false-positive signals is relevant in scenarios in which molecular assays are used as an alternative to culture-based assays that are used to monitor recreational water quality.

Most water samples tested in this study (74%) contained detectable enterococcal signals. In general terms, among the group-specific assays used, *E. faecalis* was detected more frequently (40%) in the water samples than *E. faecium* (26%) and *E. casseliflavus* (5.1%), regardless of the sample origin. The prevalences of the genus- and group-specific enterococcal assays in estuarine water samples from California and North Carolina were higher than those in tropical surface water samples (Table 4). The estuarine water samples tested in this study have historically been impacted by gulls, while surface waters in Puerto Rico are primarily impacted by wastewater and cattle fecal sources and, to a lesser extent, by domesticated animal sources, such as chickens, pigs, horses, and goats. Interestingly, the Casseli1 marker was seldom

detected in gull fecal samples, which could explain the relatively low prevalence of the Casseli1 marker in the temperate water samples. However, the fact that the Casseli1 marker was also seldom detected in tropical waters not impacted by gulls suggests that the low detection rate of *E. casseliflavus* may not be indicative of low levels of waterfowl in environmental waters. The data suggest that some of the major enterococcal species are cosmopolitan (i.e., present in various hosts); therefore, the use of microbial source-tracking (MST) methods targeting enterococcal species might be difficult to justify in source allocation applications.

Unlike fecal samples, the prevalences of the Entero1 marker [i.e., $n(G)$] were higher than the combined numbers of positive signals determined by three species-specific markers [i.e., $n(S) = n(A \cup B \cup C)$] in all tested water samples from three different geographical locations (Table 4). Several factors could have contributed to these results. For example, some of the numerically dominant species were not detected with the group-specific assays used in this study. This means that species such as *Enterococcus raffinosus*, *Enterococcus saccharolyticus*, *Enterococcus avium*, *E. pseudoavium*, and *Enterococcus cecorum* might be present in some of these samples and contributed significantly to the genus-specific signals. Using Slanetz-Bartley agar, one study showed that a relatively high number of *E. raffinosus*, *E. avium*, and *E. saccharolyticus* strains were isolated from environmental waters (35), while in another study, 25% of the isolates were nontypical enterococcal species and were only classified as *Enterococcus* sp. (36). Grammenou et al. (27) also isolated *E. avium* from water samples, but the strains represented approximately 2% of all enterococcus isolates. Altogether, these results suggest that mEI (the medium used in our study) favors the growth of some enterococci, which explains why *E. faecalis*, *E. casseliflavus*, and *E. faecium* are often isolated from mEI plates. On the other hand, Suzuki et al. (37) recently showed that *E. faecalis* and *E. faecium* combined did not represent more than 32% of the mEI isolates from five Japanese rivers, potentially implicating the prevalences of other enterococcal species.

An alternative explanation for the differences in prevalence between Entero1 and the collective group-specific markers is that novel enterococci might have also been responsible for a fraction of the signals in the water samples. Indeed, novel enterococcal species have been identified in recent years from water (38–40) and fecal (41, 42) samples. While the relative abundances of novel enterococcal species in water samples are unknown, these results indicate that there is a need for further investigation of enterococcal diversity in both fecal and environmental samples. The results also suggest that some enterococcal species might be more adapted to persist outside of the gut environment than others (2), which might lead to the adaptation of fecal bacteria to secondary habitats (43). The latter scenario has important implications in conventional microbial water quality monitoring and in microbial source-tracking applications using enterococci as targeted populations.

Conclusion. Overall, the results from this study are in agreement with previously published data demonstrating that animals frequently implicated in the fecal contamination of environmental waters shed different enterococcal species in their feces. This study also suggests that while three major enterococcal groups (i.e., *E. faecalis*, *E. faecium*, and *E. casseliflavus*) tend to dominate in fecally contaminated waters, additional enterococcal species may be present and yet not detected with the currently available genus- and group-specific qPCR assays. Better understandings of the molecular diversity and the occurrence of enterococcal species in fecal samples and environmental waters will be critical in the future evaluation studies of conventional and molecular detection methods used in the application of ambient microbial water quality recommendations. The approach used herein is also suitable when studying the fate and transport of targeted microbial groups in environmental waters and therefore in the improvement of current quantitative microbial risk assessment models. Future studies are needed to determine whether enterococcal species (group)-specific assays correlate better with risks than genus-specific assays and can then be of value in public health and environmental monitoring studies.

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