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Bifidobacteria in Feces and Environmental Waters▽†

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Bifidobacteria have been recommended as potential indicators of human fecal pollution in surface waters even though very little is known about their presence in nonhuman fecal sources. The objective of this research was to shed light on the occurrence and molecular diversity of this fecal indicator group in different animals and environmental waters. Genus- and species-specific 16S rRNA gene PCR assays were used to study the presence of bifidobacteria among 269 fecal DNA extracts from 32 different animals. Twelve samples from three wastewater treatment plants and 34 water samples from two fecally impacted watersheds were also tested. The species-specific assays showed that *Bifidobacterium adolescentis*, *B. bifidum*, *B. dentium*, and *B. catenulatum* had the broadest host distribution (11.9 to 17.4%), whereas *B. breve*, *B. infantis*, and *B. longum* were detected in fewer than 3% of all fecal samples. Phylogenetic analysis of 356 bifidobacterial clones obtained from different animal feces showed that ca. 67% of all of the sequences clustered with cultured bifidobacteria, while the rest formed a supercluster with low sequence identity (i.e., <94%) to previously described *Bifidobacterium* spp. The *B. pseudolongum* subcluster (>97% similarity) contained 53 fecal sequences from seven different animal hosts, suggesting the cosmopolitan distribution of members of this clade. In contrast, two clades containing *B. thermophilum* and *B. boum* clustered exclusively with 37 and 18 pig fecal clones, respectively, suggesting host specificity. Using species-specific assays, bifidobacteria were detected in only two of the surface water DNA extracts, although other fecal anaerobic bacteria were detected in these waters. Overall, the results suggest that the use of bifidobacterial species as potential markers to monitor human fecal pollution in natural waters may be questionable.

Members of the *Bifidobacterium* genus have been described as some of the most common and beneficial bacteria in the intestinal tract of humans (40), constituting up to 91% of the total fecal microflora in infants (11, 21). Some important roles of bifidobacteria have recently been elucidated through the completion of the *B. longum* genome (18). For example, homologs of genes encoding numerous enzymes for processing complex carbohydrates such as xylo-oligosaccharides, pectin, and fructo-oligosaccharides have been discovered, demonstrating the adaptability of bifidobacteria to utilize a wide variety of complex carbohydrates, that are otherwise recalcitrant to humans (18, 41). Other characteristics that might contribute to bifidobacteria persistence within their hosts include exopolysaccharide production (33), secreted membrane proteins involved in cell adhesion (18), and bacteriocin production (45).

Bifidobacteria have stringent nutrient requirements and grow poorly outside of the animal gut, making this bacterial group a potentially useful indicator of recent fecal pollution (36). Some bifidobacterial species are thought to be strictly of human origin, while others have been suggested as exclusively associated with animal feces (7). Specifically, *B. dentium* and *B. adolescentis* have been suggested as useful for tracking human fecal sources in surface waters (17, 34). Group- and species-specific 16S rRNA gene assays have been developed for bifidobacterial populations frequently isolated from human feces (16, 19, 25, 27). While the presence of these bifidobacterial species have been determined in human and infant subjects (26), their presence and diversity in nonhuman hosts has yet to be tested. This is critical if these species are to be considered as useful markers for the specific detection of human fecal pollution sources in environmental waters.

The primary objective of the present study was to determine the occurrence of presumed human bifidobacteria in nonhuman hosts by using 16S rRNA gene-based PCR assays. Phylogenetic analyses of 16S rRNA gene fecal clone libraries were performed to elucidate the bifidobacterial population diversity in different animal guts. In addition, the presence of bifidobacteria in fecally impacted water samples was studied to determine the potential of this bacterial group as an indicator of human fecal pollution in environmental waters.

MATERIALS AND METHODS

Sample collection. A total of 269 fecal samples were collected from locations in West Virginia, Texas, Ohio, and Nebraska from 32 different species of animals and birds (see Table S1 in the supplemental material). Site selection of individual farms was made to represent a large variety of animal operations. The selection was also based on the goal of including as many different animal types as possible to check for host specificity, with emphasis on hosts considered to be important sources of fecal pollution in the United States. There were three main categories of fecal sources represented in this collection: human, domesticated animals, and wildlife samples. Some of the wildlife host types that are not considered impor-

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tant fecal sources were included in the present study to expand our library of potential nontarget hosts. Where individual droppings were available, sterile toothpicks were used to expose the interior of the fecal mass (i.e., 1 mm in diameter from the mass). Approximately 0.5 to 1.0 g of the exposed fecal mass was placed into individual sterile vials containing 3.5 ml of phosphate-buffered saline (pH 7.2) (2). For deer feces, a single pellet was placed into the sample vial. To collect human samples, anonymous adult volunteers were requested to place approximately 1 g of fresh feces into sterile vials containing 3.5 ml of phosphate-buffered saline, using a sterile spatula. Septic samples were collected from nine septic tanks (Plum Creek, NE) by using sterile cotton swabs, which were then placed in sterile vials containing 3.5 ml of phosphate-buffered saline. Samples were transported on ice to the laboratory and stored at −80°C for 6 to 8 weeks prior to analyses.

Water samples were collected from different sites within two different watersheds (Ohio River basin and Lower Rio Grande) known to be impacted with different fecal pollution sources (see Fig. S1 and S2 in the supplemental material for maps of sampling points). Specifically, samples associated with the Ohio River basin were collected from Twelve-Mile Creek (Alexandria, KY), which is a multiuse watershed, with cattle, human (septic and combined sewer overflow), and wildlife fecal inputs. In addition, Brush Creek, which is impaired for its designated use due to high fecal bacterial counts (from a suspected faulty wastewater treatment plant [WWTP]), feeds into Twelve-Mile Creek. Water samples were also collected on the Ohio River, approximately 200 m upstream and downstream of where Twelve-Mile Creek meets the Ohio River (confluence), in addition to the confluence point. Samples were collected from sites on the Ohio River presumed to be impacted with human pollution, since several combined sewer overflows run directly into sites near these collection points. This portion of the Ohio River is currently on the Impaired Waters 303(d) list for exceeding fecal bacteria concentrations. Duplicate water samples were collected in September of 2005 from six sites along Twelve-mile Creek (Alexandria, KY) and three sites (upstream, downstream, and confluence) along the Ohio River. Water samples from Twelve-Mile Creek (site 6) and the Ohio River (confluence point) were also collected in duplicate in September 2007. Water samples were also collected at four points along the Lower Rio Grande (Las Cruces watershed, El Paso, TX), which has also been placed on the Impaired Waters 303(d) list. Samples were collected approximately 0.75 miles upstream and downstream of Bustamante and Northwest WWTP, in addition to sampling points at Sunland Park and Courchesne Bridge. Water samples (i.e., 75 to 300 ml) were filtered onto 0.45-μm-pore-size polycarbonate filters (GE Osmonics, Minnetonka, MN), which were stored at −80°C until genomic DNA extractions were performed.

Samples (75 to 100 ml) were also collected from the effluents of three WWTPs, including the Dry Creek WWTP (Alexandria, KY), Northwest WWTP (El Paso, TX), and Bustamante WWTP (El Paso, TX). In addition, samples within different stages of wastewater treatment were collected in sterile 50-ml conical tubes, including influent wastewater, secondary aeration, and return activated sludge from the Dry Creek WWTP. To ease filtration, samples from the three latter locations were first centrifuged at 8,000 × *g* for 10 min at 4°C, and the supernatant was then filtered onto 0.45-μm-pore-size polycarbonate filters. DNA extractions from pellets and filters were performed immediately after the centrifugation-filtration process. (See Table S2 in the supplemental material for WWTP design, capacity, and location.)

DNA extraction. Total DNA was extracted with an UltraClean fecal DNA isolation kit according to the manufacturer's instructions (MO BIO Laboratories, Inc., Carlsbad, CA) using 250 μl of each fecal slurry. For water samples, DNA was extracted directly from whole filters by using an UltraClean soil DNA isolation kit (MO BIO Laboratories, Inc.). Total DNA was eluted in 50 μl of 10 mM Tris and quantified by using a NanoDrop ND-1000 UV spectrophotometer (NanoDrop Technologies, Wilmington, DE). To test for the presence of extraneous DNA contamination introduced during laboratory procedures, no-template and extraction blanks were included in the PCR assays. DNA extracts were stored at −20°C until further processing.

Cell lysis experiments were conducted to assess the overall performance of the fecal extraction kit. *B. breve* cells (10^6 to 10 cells) were spiked into 100 ml of Ohio River water samples for which *B. breve* was previously undetected. The samples were filtered and processed as mentioned above. The same number of cells used in the filtration experiments was added directly into the bead beating solution of the extraction kit, and the samples were processed by using the same extraction protocol.

Group- and species-targeted 16S rRNA gene PCR assays. PCR assays were performed on all fecal and water DNA extracts using three genus- and nine species-specific PCR assays targeting the 16S rRNA gene of *Bifidobacterium* spp. Group-specific *Bacteroides-Prevotella* (4) and *Clostridium coccoides* (27) 16S rRNA gene-based PCR assays were used to determine the presence of fecal anaerobic bacteria in water samples and to determine PCR inhibition potential. The *Bifidobacterium* genus-specific primer sets Bif164-f and Bif662-r (19), Lm26-f and Lm3-r (16), and g-Bifid-f and g-Bifid-r (27) were used to detect bifidobacteria in fecal samples. Reactions for the genus-specific assays were conducted by using previously described protocols (19, 27), with the exception of the Lm26-f and Lm3-r assay, for which the following cycling conditions were used: 94°C for 5 min, followed by 35 cycles of 94°C for 20 s, 55°C for 20 s, and 72°C for 30 s, and a final extension step of 72°C for 5 min. Nine bifidobacteria species-specific primers sets were used to target *B. adolescentisis*, *B. angulatum*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. dentium*, *B. gallicum*, *B. infantis*, and *B. longum* according to the PCR conditions described elsewhere (26). These species have been previously isolated from human feces (6, 7). Fecal and water DNA template concentrations ranged between two to 21 ng of DNA per μl for each reaction. Final PCR solutions (25 μl, total volume) contained 2.5 μl of Takara ExTaq 10× buffer (20 mM Mg^{2+}), 2 μl of deoxynucleoside triphosphate mixture (2.5 mM each), 0.4 μl of 4% bovine serum albumin, 17 μl of UltraPure water, 0.5 μl of primer at 25 pmol per μl, and 0.625 U of ExTaq DNA polymerase (Takara Mirus Bio, Madison, WI). Reactions were conducted on a DNA Engine 2 Tetrad thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA). Amplification products were visualized by using 1% agarose gels and GelSTAR nucleic acid stain (Cambrex BioScience, East Rutherford, NJ).

The performance of each assay was determined in PCR experiments adding known concentrations of fecal and water DNA extracts. Using this approach it is possible to determine the detection limits of an assay for environmental DNA extracts (20). PCR assays were performed using serial fecal DNA dilutions (10^{-8} to 10^{-16} g DNA) of composite samples from animals that tested positive for each assay and that represented the different types of general sources of pollution, that is, human, domesticated animals (chicken, pig, cattle, and horse), and wildlife (deer). We performed similar assays using serial dilutions of influent, return activated sludge, secondary aeration, effluent, and environmental water DNA extracts that yielded positive signals with a given bifidobacterial assay. To determine the inhibition potential at a specific environmental DNA concentration, DNA extracts were used as a template in general and human-specific *Bacteroides* assays (4), as well as in *C. coccoides* PCR assays (27).

Detection limits were also established for seven of the nine bifidobacterial species markers using DNA extracts from pure cultures of *B. adolescentis* (DSM 20086), *B. angulatum* (DSM 20225), *B. bifidum* (DSM 20082), *B. breve* (DSM 20213), *B. catenulatum* (DSM 20103), *B. gallicum* (DSM 20093), and *B. infantis* (DSM 20088). Serial dilutions (10^{-8} to 10^{-16} g of DNA) from each pure culture were used as a template in the respective PCR assays. The potential cross-reactivity of the bifidobacteria species-specific assays was determined by using 1 ng of the aforementioned pure culture DNA extracts and nontarget species DNA extracts of additional species such as *B. pseudolongum* subsp. *globosum* (DSM 20092), *B. ruminatum* (DSM 6489), and *B. suis* (DSM 20211). Coverage of genus-specific assays was tested by using all 10 pure culture DNA extracts (1 ng) as a template in each of the three genus-specific bifidobacterial assays.

Cloning and sequencing analyses. Bif164-f/Bif662-r PCR products were used to determine the phylogenetic diversity of bifidobacteria in different hosts. Sequencing and data analysis was performed as previously described (20). Briefly, PCR products were purified by using the QIAquick PCR purification kit according to the manufacturer's instructions (Qiagen, Valencia, CA). Representative PCR products derived from 14 different human and animal feces (including alpaca, cat, cattle, chicken, deer, dog, goat, goose, human, peacock, pig, pigeon, seagull, and sheep) were cloned into pCR4.1 TOPO vector as described by the manufacturer (Invitrogen, Carlsbad, CA). Individual *Escherichia coli* clones were subcultured into 300 μl of Luria broth containing 50 μg of ampicillin/ml and screened for inserts by using M13 PCR. Clones were submitted to the Children's Hospital DNA Core Facility (Cincinnati, OH) for sequencing using BigDye sequencing chemistry (Applied Biosystems, Foster City, CA), M13 forward and reverse primers, and an Applied Biosystems Prism 3730XL DNA analyzer. Sequences were manually verified and aligned using Sequencher 4.7 software. Potential chimeric sequences detected by using Bellerophon (14) and the Mallard (3) softwares were not included in further analyses. Sequences were also subjected to BLAST homology search algorithms in order to assess sequence similarity to publicly available sequences (1). Phylogenetic analysis used ARB software and trees were inferred from 456 sequence positions (*E. coli* bases 179 to 655) by using neighbor-joining (Kimura correction) and maximum parsimony (Phylip DNAPARS tool) (24). In order to statistically evaluate branching confidence, bootstrap values were obtained from a consensus of 100 parsimony trees. *Arcanobacterium haemolyticum* rRNA 16S gene sequence (accession no. AJ234059) was used as the outgroup (35), while cultured *Bifidobacterium* species were included in the analyses as points of reference.

Community richness and diversity of the clone libraries were studied by cal-

TABLE 1. Detection limits of genus- and species-specific bifidobacterial PCR assays

Fecal or environmental sample (n)[a]	Detection limit (g)[b]											
	Lm26-f/ Lm3-r	Bif164-f/ Bif662-r	g-Bif-f/ g-Bif-r	BiADO	BiANG	BiBIF	BiBRE	BiCAT	BiDEN	BiGAL	BiINF	BiLON
Deer (6)	10^{-8}	10^{-12}	10^{-12}	–	–	–	–	10^{-8}	10^{-10}	–	–	–
Horse (8)	–	10^{-9}	–	–	–	–	–	10^{-9}	10^{-8}	–	–	–
Cattle (8)	10^{-9}	10^{-12}	10^{-12}	10^{-9}	–	–	–	10^{-8}	–	–	–	–
Chicken (5)	–	10^{-9}	10^{-11}	–	–	–	–	–	–	–	–	–
Pig (10)	10^{-10}	10^{-14}	10^{-13}	–	10^{-8}	10^{-8}	–	10^{-10}	10^{-10}	–	–	–
Human (9)	10^{-9}	10^{-13}	10^{-14}	10^{-13}	–	10^{-11}	–	10^{-11}	–	–	–	10^{-8}
Dry Creek WWTP influent (2)	10^{-8}	10^{-11}	10^{-9}	10^{-11}	10^{-8}	10^{-10}	NA	10^{-10}	NA	NA	NA	NA
Dry Creek WWTP MLSS/RAS (2)	10^{-8}	10^{-9}	10^{-9}	10^{-10}	10^{-8}	10^{-9}	NA	10^{-9}	NA	NA	NA	NA
Dry Creek WWTP secondary aeration (2)	NA	10^{-9}	10^{-9}	10^{-10}	10^{-8}	10^{-8}	NA	10^{-8}	NA	NA	NA	NA
Dry Creek WWTP effluent (1)	NA	10^{-9}	10^{-9}	10^{-9}	10^{-8}	10^{-8}	NA	10^{-8}	NA	NA	NA	NA
NWWTP effluent (3)	10^{-8}	10^{-8}	10^{-8}	10^{-8}	NA	10^{-8}	NA	10^{-8}	NA	NA	NA	NA
Rio Grande Downstream of NWWTP (2)	NA	10^{-8}	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Upstream of Bustamante WWTP (2)	NA	10^{-8}	NA	NA	NA	NA	NA	10^{-8}	NA	NA	NA	NA
Bustamante WWTP effluent (2)	NA	10^{-9}	10^{-9}	NA	NA	NA	NA	NA	NA	NA	NA	NA
Downstream of Bustamante WWTP (2)	NA	NA	10^{-8}	NA	NA	NA	NA	NA	NA	NA	NA	NA
Sunland Park upstream of NWWTP (2)	10^{-8}	NA	10^{-8}	NA	NA	NA	NA	NA	NA	NA	NA	NA
B. adoloscentis (DSM 20086)	10^{-10}	10^{-14}	10^{-14}	10^{-14}	–	–	–	–	–	–	–	–
B. angulatum (DSM 20225)	10^{-10}	10^{-15}	10^{-15}	–	10^{-14}	–	–	–	–	–	–	–
B. bifidum (DSM 20082)	10^{-9}	10^{-14}	10^{-14}	–	–	10^{-14}	–	–	–	–	–	–
B. breve (DSM 20213)	10^{-10}	10^{-13}	10^{-14}	–	–	–	10^{-14}	–	–	–	–	–
B. catenulatum (DSM 20103)	10^{-11}	10^{-12}	10^{-14}	–	–	–	–	10^{-13}	–	–	–	–
B gallicum (DSM 20093)	10^{-12}	10^{-14}	10^{-14}	–	–	–	–	–	–	10^{-13}	–	–
B. infantis (DSM 20088)	10^{-9}	10^{-12}	10^{-14}	–	–	–	–	–	–	–	10^{-13}	–

[a] Numbers in parentheses (n) indicate the number of samples in each fecal or environmental composite (i.e., for deer there were six individual fecal samples).
[b] All detection limits are indicated as grams of DNA extract. A dash (–) indicates that the limit of detection was greater than 10^{-8} g of DNA extract. NA, no detection limit assay was performed due to the absence or very low intensity of PCR signal in previous assays.

culating rarefaction analysis (12, 15, 42) using aRarefactWin 1.3 (S. Holland [www.uga.edu/strata/software/Software.html]), abundance-based coverage estimator (ACE), Chao 1 estimator of species richness, and Shannon's and Simpson's index for diversity using EstimateS software. Rarefaction curves were produced by using individual-based Coleman methods and the sample-based Mao Tau method available through EstimateS (R. K. Colwell [http://viceroy.eeb.uconn.edu/EstimateS.

GenBank accession numbers. Representative sequences generated in the present study have been deposited in the GenBank database under accession numbers EU359826 to EU359907.

RESULTS AND DISCUSSION

Cell lysis efficiency, assay specificity, and assay detection limits. Detection limits using *B. breve* cells spiked into the extraction kit was 10 cells, while filtration of *B. breve* cells yielded a detection limit of 100 cells, suggesting that up to 90% of the cells could be lost during the filtration/bead beating process. To compensate for the impact the filtration step could have on the assay detection limits, we increased the number of cycles from 35 to 45 for every genus- and host-specific assays using as a template DNA extracts from water and wastewater samples collected 2007 (n = 14) (Table 1). Of all samples tested (i.e., n = 552), only two water samples tested positive for any given assays (i.e., *B. breve* and *B. bifidum* using Dry Creek WWTP return activated sludge DNA as a template), even after extending the protocol to 45 cycles. Adding 10 cycles is the equivalent of increasing cell detection, potentially up to 3 orders of magnitude, which should compensate for the reduced extraction and/or cell lysis performance of most nucleic extrac-

tion protocols. Consequently, failure to detect bifidobacteria using 16S rRNA gene PCR-based methods strongly suggests low survival rates for this bacterial group in environmental waters, particularly when other fecal anaerobic bacteria were detected in the same samples.

Specificity was confirmed for the species-specific assays, with the exception of the BiLON assay, which also amplified *B. pseudolongum*. All genus-specific assays amplified DNA from all bifidobacterial strains used in this study. The detection limits of the bifidobacterial assays ranged from 10^{-8} to 10^{-14} g of DNA and from 10^{-8} to 10^{-11} g of DNA when fecal and water DNA extracts, respectively, were used (Table 1). Assay detection limits with pure cultures indicated that the Bif164-f/Bif662-r (10^{-12} to 10^{-15} g of DNA) and g-Bif-f/g-Bif-r (10^{-14} to 10^{-15} g of DNA) markers had lower detection levels than the Lm26-f/Lm3-r markers (10^{-9} to 10^{-12} g of DNA) (Table 1), a finding in agreement with the lower sensitivity of the latter marker in fecal and environmental DNA extracts. In general, the bifidobacterial genus-specific markers (g-Bif-f/g-Bif-r and Bif164-f/Bif662-r) had lower detection limits than the species-specific assays in fecal and environmental matrices. The latter results are not surprising due to the fact that the densities of host-specific bacteria tend to be one to two orders of magnitude lower than general fecal bacterial groups (20).

***Bifidobacterium* genus- and species-specific PCR results.** The presence of bifidobacteria was confirmed in 25 of the 32 different animals studied. However, only 10 animal types had positive signals to all three genus-specific assays. Moreover, of the 269 total fecal samples, only 56, 98, and 87 of the DNA extracts were positive for Lm26-f/Lm3-r, Bif164-f/Bif662-r, and g-Bif-f/g-Bif-r assays, respectively (Table 2). Surprisingly, no more than five of the nineteen human fecal samples and three of the nine septic samples were positive when any of the given genus-specific assays were used. Moreover, none of the genus-specific primers produced PCR signals when fecal DNA extracts from armadillo, dove, fox, guinea pig, hedgehog, raccoon, squirrel, and vulture were used as a template. Altogether, these data suggest that these genus-specific assays may target different populations of bifidobacteria and that several assays may be needed to understand the occurrence of these populations in animal gut systems. While these results may also suggest that some species might not be found in detectable numbers in some gut types, additional samples must be analyzed to further confirm this trend. It should be noted that Resnick and Levin (36) could not isolate bifidobacteria from the feces of chickens, cows, dogs, horses, cats, sheep, beavers, goats, and turkeys, while Rhodes and Kator (38) did not find any bifidobacteria in deer, muskrat, and raccoon scat. However, our results demonstrate that culture-based techniques may fail to detect bifidobacteria in nonhuman hosts, since at least 21% of all of the chicken, dairy cattle, cat, goat, pig, and sheep fecal samples had positive signals with all genus-specific markers. *Bifidobacterium* signals were also detected in at least one coyote, deer, and dog fecal sample using genus-targeted assays.

We investigated the presence of nine bifidobacterial species within 269 fecal samples representing 32 different animal types (Table 3). As expected, some of the species were not detected or were infrequently detected in the hosts tested. For example, the BiIFN assay was positive for only two piglet fecal samples; *B. breve* was only detected in two pig, two chicken, one dairy cow, and one rabbit fecal sample, whereas *B. longum* was detected in one human, two pig, and two sheep fecal samples. The absence of *B. infantis* and *B. breve* in the human samples and most fecal samples tested can be explained by the fact that this species is normally present only in infants. The low frequency of detection of *B. longum* in human fecal samples and in septic samples was not expected, since previous culture-dependent and culture-independent studies have indicated the incidence of *B. longum* in human feces (37). However, it should be noted that the assay used to detect *B. longum* here has been reported to depend on a higher template concentration than other bifidobacterial assays (27). In contrast, some of the species were detected frequently and in multiple hosts, as in the case of *B. bifidum*, *B. adolescentis*, *B. catenulatum*, and *B. dentium*, which were found in 7, 8, 13, and 16 different hosts, respectively. These species were also detected in the highest number of fecal samples. In addition, *B. gallicum* was detected in high frequencies in chicken and horse fecal samples. Cattle, chicken, deer, human, pig, rabbit, and sheep were among the animals showing the highest diversity of bifidobacterial species. The significance of these findings in terms of host-microbial interactions is unknown, although it suggests that some bifidobacterial species prefer a cosmopolitan lifestyle. *B. bifidum*,

TABLE 2. Results from *Bifidobacterium* species-specific PCR assays using different animal fecal DNA extracts

Animal type (n)[a]	No. of samples detected with *Bifidobacterium* sp.-targeted primers[b]		
	Lm26-f/Lm3-r	Bif164-f/Bif662-r	g-Bif-f/g-Bif-r
Alpaca (2)	–	–	1
Beef cattle (14)	1	–	1
Bobcat (1)	–	1	1
Canadian goose (20)	–	4	2
Chicken (29)	6	9	14
Coyote (11)	7	3	2
Dairy Cattle (14)	5	11	7
Deer (17)	2	4	7
Domestic cat (10)	3	5	6
Domestic dog (15)	2	3	1
Ferret (1)	–	–	1
Goat (4)	1	3	2
Hog (1)	1	–	–
Horse (16)	3	4	–
Human (19)	2	5	3
Llama (1)	–	1	–
Peacock (1)	–	1	1
Pig (43)	17	33	27
Pigeon (4)	–	1	1
Possum (2)	1	–	1
Prairie dog (2)	1	–	1
Rabbit (4)	1	–	–
Septic (9)	–	1	3
Sheep (8)	3	6	3
Turkey (10)	–	3	2

[a] n, number of total fecal samples tested for that animal type. All squirrel (n = 4), armadillo (n = 1), dove (n = 1), fox (n = 1), guinea pig (n = 1), hedgehog (n = 1), raccoon (1), and vulture (n = 1) fecal sample DNA extracts produced no PCR signal using any of the three genus-specific assays.

[b] –, no amplification product was visualized for any of the samples from a given animal type using that primer. The total numbers of amplification products for all 269 fecal samples using that genus-specific marker were as follows (percent positive): Lm26-f/Lm3-r, 56 (20.8%); Bif164-f/Bif662-r, 98 (36.4%); and g-Bif-f/g-Bif-r, 87 (32.3%).

TABLE 3. Results from *Bifidobacterium* species-specific PCR assays using different animal fecal DNA extracts

Animal type (n)[a]	No. of samples detected with *Bifidobacterium* sp.- and group-specific primer[b]								
	BiADO	BiANG	BiBIF	BiBRE	BiCAT	BiDEN	BiGAL	BiINF	BiLON
Alpaca (2)	1	–	–	–	–	–	–	–	–
Beef cattle (14)	–	–	–	–	1	1	–	–	–
Canadian goose (20)	–	–	–	–	2	2	–	–	–
Chicken (29)	1	–	2	2	–	–	3	–	–
Coyote (11)	1	–	–	–	1	1	–	–	–
Dairy cattle (14)	4	–	–	1	2	8	1	–	–
Deer (17)	1	–	–	–	2	2	2	–	–
Domestic cat (10)	–	–	2	–	5	–	–	–	–
Domestic dog (15)	–	–	1	–	2	–	–	–	–
Goat (4)	–	–	–	–	2	2	–	–	–
Guinea pig (1)	–	–	–	–	–	1	–	–	–
Hog, feral (1)	–	–	–	–	–	1	–	–	–
Horse (16)	–	–	–	–	2	3	4	–	–
Human (19)	7	–	4	–	4	1	–	–	1
Pig (43)	13	11	22	2	20	15	7	2	2
Pigeon (4)	–	–	1	–	–	–	–	–	–
Possum (2)	–	–	–	–	–	1	–	–	–
Prairie dog (2)	–	–	–	–	–	1	–	–	–
Rabbit (4)	–	1	2	1	1	1	1	–	–
Septic (9)	1	1	3	–	1	1	1	–	–
Sheep (8)	3	1	–	–	2	5	2	–	2
Squirrel (4)	–	–	–	–	–	1	–	–	–
Turkey (10)	–	1	–	–	–	–	–	–	–

[a] n, number of fecal samples tested for that animal type. The results do not include assays against armadillo ($n = 1$), bobcat ($n = 1$), dove ($n = 1$), ferret ($n = 1$), fox ($n = 1$), hedgehog ($n = 1$), llama ($n = 1$), peacock ($n = 1$), raccoon ($n = 1$), and vulture ($n = 1$) fecal DNA extracts produced since these did not produce PCR signals with any of the species-specific assays.

[b] BiADO, BiANG, BiBIF, BiBRE, BiCAT, BiDEN, BiGAL, BiINF, and BiLON refer to PCR assays targeting *B. adolescentis*, *B. angulatum*, *B. bifidum*, *B. breve*, *B. catenulatum* group, *B. dentium*, *B. gallicum*, *B. infantis*, and *B. longum*, respectively. –, no amplification product was visualized for any of the samples from a given animal type using that primer. The total numbers of amplification products for all 269 fecal samples using the indicated group- or genus-specific marker were as follows (percent positive): BiADO, 32 (11.9%); BiANG, 15 (5.6%); BiBIF, 37 (13.8%); BiBRE, 6 (2.2%); BiCAT, 47 (17.4%); BiDEN, 47 (17.4%); BiGAL, 21 (7.8%); BiINF, 2 (0.74%); and BiLON, 5 (1.9%).

B. adolescentis, and *B. catenulatum* were found in particularly high frequencies in humans and pigs. The high incidence of these species in human samples is consistent with previous studies that have shown that these are among the most frequently detected bifidobacterial species in the human adult intestinal microflora (6, 10, 26, 32). However, the *B. catenulatum* amplification frequency in human feces was lower in the present study than in a previous report, in which nearly all (92%) of Japanese adult feces indicated the presence of *B. catenulatum* (26). Differences in diet might explain these results. The low detection of *B. dentium* marker in human fecal samples is consistent with previous non-culture-based studies, in which *B. dentium* was detected in only 3 of 48 adult human fecal samples (26).

Some bifidobacterial species, for example, *B. adolescentis* and *B. dentium*, have been suggested to be good targets for tracking human fecal pollution in environmental waters (8, 34). However, previous host specificity studies of *B. adolescentis* and *B. dentium* have been performed with a limited number of fecal samples and host types. Using a larger data set, our results showed that these species were not exclusive to human feces since they were also detected in several animals (Table 3). Moreover, their detection in cattle and swine feces is significant to environmental monitoring programs in the United States, since feces from these animals are important sources of water fecal pollution. In cases in which it is necessary to discriminate between human, cattle, and/or swine pollution, assays targeting *B. adolescentis* and *B. dentium* might not be adequate. Another important finding was the relatively lower frequency of detection of bifidobacteria in environmental waters and wastewater treatment effluents compared to other anaerobic fecal bacteria tested in the present study (Table 4). For example, none of the genus- or species-specific assays were detected in more than 50% of the tested environmental samples (g-Bif-f/g-Bif-r, 0.50; Bif164-f/Bif662-r, 0.37; BiADO, 0.36; and BiCAT, 0.26). In contrast, other fecal bacteria groups were detected in nearly all samples (i.e., *Bacteroidales*, 0.89; *C. coccoides*, 0.98). Interestingly, BiDEN, a proposed human-specific fecal indicator, was not detected in any of the 46 environmental samples, even though 61% of the environmental samples tested were positive for the human *Bacteroides* spp. marker (HF183F/Bac708R). While the BiADO assay appears to be the most sensitive of all of the bifidobacterial species-specific markers, it should be noted that this marker was only detected in wastewater and absent in all surface water samples tested ($n = 34$). Nested PCR approaches have been reported to increase detection of bifidobacteria in water samples (8, 17). However, the low sensitivity of bifidobacterial species-specific assays with environmental samples containing human-specific *Bacteroidales* and *C. coccoides* (even after increasing the number of PCR cycles) suggests that bifidobacteria might not be a reliable indicator for tracking human pollution sources in natural waters.

Bifidobacteria population diversity. Rarefaction analysis (Mao Tau method) of 356 bifidobacterial clones generated in the present study suggested that the sequence diversity is ap-

TABLE 4. Presence of bifidobacteria in surface waters and wastewater samples

Environmental sample (yr)	Presence of bifidobacteria[a]														
	Lm26-f/ Lm3-r	Bif164-f/ Bif662-r	g-Bif-f/ g-Bif-r	BiADO	BiANG	BiBIF	BiBRE	BiCAT	BiDEN	BiGAL	BiINF	BiLON	*C. coccoides*	*Bacteroidales*	Human *Bacteroides*
Twelve-Mile Creek site 1 (2005)	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	+/–	ND
Twelve-Mile Creek site 2 (2005)	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	+/–	ND
Twelve-Mile Creek site 3 (2005)	+/–	+/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	+/–	ND
Twelve-Mile Creek site 4 (2005)	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	+/+	ND
Twelve-Mile Creek site 5 (2005)	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	+/–	ND
Twelve-Mile Creek Site 6 (2005)	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	+/+	ND
Twelve-Mile Creek Site 6 (2007)	+/–	+/+	+/+	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	+/+	–/–
Ohio River upstream (2005)	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	+/+	ND
Ohio River/Twelve-Mile Creek (2005)	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	+/+	ND
Ohio River/Twelve-Mile Creek (2007)	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/–	+/–	–/–
Ohio River downstream (2005)	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	+/+	ND
Dry Creek WWTP influent	+/–	+/+	+/+	+/+	+/–	+/+	–/–	+/–	–/–	–/–	+/–	+/–	+/+	+/+	+/+
Dry Creek WWTP RAS	–/–	–/–	+/+	+/+	–/–*	–/–	–/–*	+/+	–/–	–/–	–/–	–/–	+/+	+/+	+/+
Dry Creek WWTP secondary aeration	–/–	–/–	+/+	+/+	+/–	+/+	–/–	+/+	–/–	–/–	–/–	–/–	+/+	+/+	+/+
Dry Creek WWTP effluent	–	–	+	+	–	–	–	+	–	–	–	–	+	+	+
Rio Grande upstream of NWWTP	+/–	+/–	+/+	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	+/+	–/–
NWWTP effluent	+/+/+	+/+/+	+/+/+	+/+/+	–/–/–	+/+/+	–/–/–	+/+/+	–/–/–	–/–/–	–/–/–	–/–/–	+/+/+	+/+/+	+/+/+
Rio Grande downstream of NWWTP	–/–	+/+	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	+/+	–/–
Bustamante upstream	–/–	+/–	+/+	–/–	–/–	–/–	–/–	+/+	–/–	–/–	–/–	–/–	+/+	+/+	+/+
Bustamante WWTP effluent	–/–	+/+	+/+	–/–	–/–	–/–	–/–	+/–	–/–	–/–	–/–	–/–	+/+	+/+	+/+
Bustamante downstream	–/–	+/+	+/+	–/–	–/–	–/–	–/–	–/–	–/–	+/+	–/–	–/–	+/+	+/+	+/+
Sunland Park upstream of WWTP	+/+	+/–	+/+	–/–	–/–	–/–	–/–	–/–	–/–	+/–	–/–	–/–	+/+	+/+	+/–
Courchesne downstream of WWTP	–/–	–/–	+/–	–/–	–/–	–/–	–/–	–/–	–/–	+/–	–/–	–/–	+/+	+/+	–/–

[a] BiADO, BiANG, BiBIF, BiBRE, BiCAT, BiDEN, BiGAL, BiINF, and BiLON refer to PCR assays targeting *B. adolescentis*, *B. angulatum*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. dentium*, *B. gallicum*, *B. infantis*, and *B. longum*, respectively. "–/–" or "+/+" indicates that both duplicate samples produced either negative or positive PCR results; "+/–" indicates that only one of the duplicate samples produced a positive signal. Environmental samples were processed in duplicate with the exception of Dry Creek WWTP effluent ($n = 1$) and NWWTP effluent ($n = 3$). ND, not determined. *, samples that produced positive signals after increasing the number of PCR cycles from 35 to 45. The sensitivities, calculated by dividing the number of positive PCR results by the number of positive and negative PCR results, were as follows: Lm26-f/Lm3-r, 0.20; Bif164-f/Bif662-r, 0.37; g-Bif-f/g-Bif-r, 0.50; BiADO, 0.36; BiANG, 0.04; BiBIF, 0.15; BiBRE, 0.00; BiCAT, 0.26; BiDEN, 0.00; BiGAL, 0.09; BiINF, 0.02; BiLON, 0.02; *C. coccoides*, 0.98; *Bacteroidales*, 0.89; and human *Bacteroides*, 0.61.

proaching operational taxonomic unit (OTU) saturation (Fig. 1). Estimations of species richness and diversity were calculated for *Bifidobacterium*-related sequences obtained in the present study and *Bacteroidales* fecal bacterial populations from a previous study (20) for means of relative comparison of community species richness. Running statistical analyses using EstimateS (v5.0.1) with 100 randomizations on 356 fecal bacterial sequences from *Bacteroidales* and *Bifidobacterium* fecal bacteria communities indicated that OTU richness and diversity indices were significantly higher for *Bacteroidales* than for *Bifidobacterium* in the fecal communities tested (Table 5). Clones sharing at least 98% sequence identity to one another were placed in the same taxonomic unit. The observed number of OTUs for 356 clones from *Bifidobacterium* fecal libraries was 22, while 356 *Bacteroidales* fecal clones formed 116 OTUs. Two nonparametric estimators of OTU richness, mean Chao 1 and ACE values, were calculated to be 28.25 and 28.32 and to be 455.31 and 1,089.49 for fecal *Bifidobaterium* and *Bacteroidales* richness, respectively. The confidence intervals for the fecal community estimators did not overlap ($P < 0.05$), suggesting that there is a significant difference between *Bifidobacterium* and *Bacteroidales* fecal OTU richness. Thus, for these fecal clone libraries, *Bacteroidales* appear to have higher species richness than *Bifidobacterium* spp. However, the observed species richness of *Bacteroidales* appears to be driven by singletons (species captured once), or low-abundance classes, since nearly 75% of the observed OTUs are singletons. In contrast, fewer than 25% of the *Bifidobacterium* OTUs come

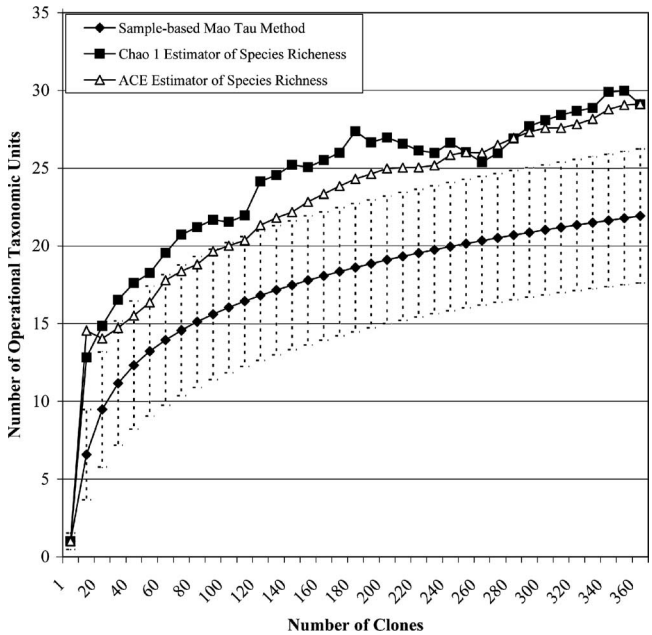

FIG. 1. Observed (◆) and estimated (■, △) OTU richness of *Bifidobacterium* spp. in animal feces versus sample size. The rarefaction curve (i.e., the number of observed phylotypes as a function of number of clones) was calculated by using the sample-based Mao-Tau, Chao 1, and ACE estimators averaged over 100 simulations. The dotted lines indicated the 95% confidence interval for the rarefaction calculations.

from singletons, and the remaining species are derived from species captured more than once. These findings have important implications in the development of assays targeting specific sources of fecal pollution and in further understanding how fecal bacterial populations adapt to a particular set of gut conditions.

Phylogenetic analyses of *Bifidobacterium* clones. A total of 366 partial 16S rRNA gene sequences derived from 14 different animal feces were analyzed. The final phylogenetic analysis included 55 pig, 53 chicken, 51 cattle, 51 human, 43 cat, 22 deer, 19 pigeon, 17 seagull, 12 sheep, 11 peacock, 10 dog, 6 goose, 3 alpaca, and 3 goat sequences, as well as sequences from cultured *Bifidobacterium* strains. A total of 32 chimeric sequences were excluded from the analysis. More than half (i.e., 56%) of all unique clone sequences exhibited low sequence similarity (≤96%) to *Bifidobacterium*-related 16S rRNA gene sequences, indicating that the phylogenetic diversity of *Bifidobacterium*-like sequences may be currently underrepresented in the publicly available databases.

The overall phylogenetic tree topology was in agreement with previous studies with cultured strains (23, 39). For instance, sequences having >97% sequence similarity with cultured strains indeed formed specific clusters with species such as the *B. catenulatum*, *B. pseudocatenulatum*, *B. longum*, *B. coryneforme*, *B. asteroides*, and *B. pseudolongum* groups (23, 31, 39). The overall topology of the neighbor-joining tree was supported by parsimony trees with 100 resamplings (data not shown). Most major branching orders of the phylogenetic tree were supported by bootstrap values of at least 63% of the parsimony bootstrap resamplings, while two branches of the unidentified clades containing fecal sequences distantly related to cultured *Bifidobacterium* species were not supported by high bootstrap values (43 and 21%). These low bootstrap values may be a result of the use of partial 16S rRNA sequences limiting phylogenetic resolution in a comparative analysis (13). All other subclusters in the parsimony analysis were supported by bootstrap values of 90% and higher.

Phylogenetic analysis of the fecally derived bifidobacterial clones revealed previously unidentified host-microbial distributions (Fig. 2). For example, ca. 67% of all of the sequences (groups I and III) were associated with cultured bifidobacteria. *Bifidobacterium group* I contains several reference strains, as well as mammalian and avian-derived clones. A total of 24 of 51 human fecal clones clustered with *B. ruminatum*, *B. adolescentis*, *B. pseudocatenulatum*, and *B. longum* (>97% sequence similarity), while 16 other human fecal clones formed subclusters related to identifiable strains. In addition, all 43 domestic cat sequences were found within group I, sharing high sequence similarity with *B. pseudocatenulatum*, *B. longum*, and unidentified human fecal clones. Close sequence identity among human and cat bifidobacterium-related clones suggests that the close interaction shared among domesticated animals and their owners may serve as a pathway for sharing gut microflora. Interestingly, dog sequences were missing from the predominantly "human" clade, perhaps due to the low number of clones examined. Another interesting host distribution pattern was noted in group I, in which 21 chicken, 3 geese, and 2 peacock clones formed a subcluster with *B. gallinarum*, a strain commonly isolated from the chicken cecum (22). This bird-derived clade suggests that *B. gallinarum* and closely related populations may exhibit host specificity.

Group II contained cultured strains of bifidobacteria, including *B. coryneforme*, *B. indicum*, *B. asteroides*, *B. minimum*, and *B. subtile* (Fig. 2). None of the 356 clones clustered with these bacteria, suggesting these species may not be common intestinal members in the animal types used in the study. Group III comprised cultured strains including *B. pseudolongum*, *B. animalis*, *B. choerinum*, *B. bifidum*, *B. thermophilum*, and *B. boum*, in addition to fecal sequences derived from eight different animal fecal samples. Most notably, *B. pseudolongum* formed a subcluster (>97% similarity) with 53 fecal sequences from seven different animal types. Previous studies have characterized *B. pseudolongum* strains isolated from pig, chicken, bull, calf, guinea pig, rabbit, lamb, and cattle feces, further supporting the seemingly cosmopolitan lifestyle of species within this clade (5, 30, 44). In contrast, two subclusters within group III

TABLE 5. *Bifidobacterium* and *Bacteroidales* sequence diversity and richness estimators

Parameter	Fecal population	
	Bifidobacterium	*Bacteroidales*
No. of sequences	356	366
No. of OTUs (98%)	22	116
No. of singletons	5	85
Chao 1 estimator of OTU richness	28	455
Chao 1 95% confidence interval	23–62	280–821
ACE estimator of OTU richness	28	1,089
ACE SD	0.4	121
Shannon's index of diversity	2.5	3.7
Simpson's index of diversity	9.5	16.1

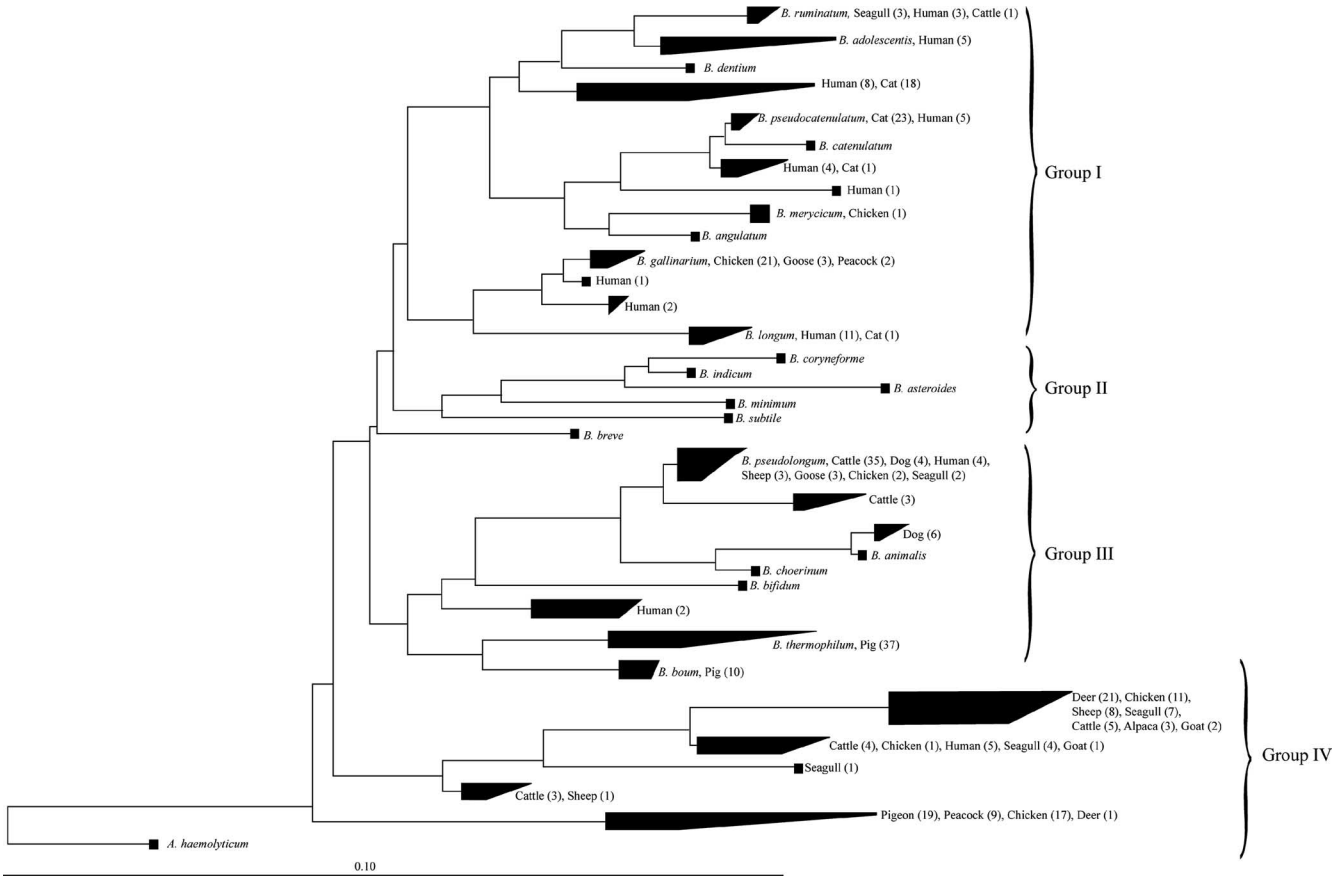

FIG. 2. Phylogenetic tree of *Bifidobacterium* 16S rRNA gene sequences ($n = 356$) derived from 14 different mammalian and avian hosts, based on a neighbor-joining algorithm. Numbers in parentheses indicate the number of sequences associated in each clade for a given host. Clone libraries were generated using the genus-specific primer set Bif164-f and Bif662-r. Sequences for cultured bifidobacteria genera were added to the analyses as reference points, while the 16S rRNA sequence of *Arcanobacterium haemolyticum* (accession no. AJ234059) was used as the outgroup.

were composed of 37 and 18 pig fecal clones. These are closely related to *B. thermophilum* and *B. boum*, species which have been previously isolated from swine feces (7, 36). Culture-based studies previously indicated that *B. suis* (closely related to *B. longum*) was the predominant bifidobacterial species in the gastrointestinal tracts of pigs (28). However, Mikkelsen et al. (29) found that most bifidobacterial isolates had restriction patterns nearly identical (>99.5%) to *B. boum*, supporting our finding that these populations may be endemic to the swine gut (29).

Group IV contained nearly one-third of the total fecal clones (Fig. 2). Nine of the 14 different animal feces were represented within this group. Approximately 22% of the sequences formed a supercluster within group IV that did not associate with any cultured *Bifidobacterium* species and exhibited some of the lowest similarities to publicly available sequences. Interestingly, most of the pseudoruminant (multigastric) fecal clones were members of these unidentified clades. For example 22 of 22 deer, 9 of 12 sheep, 3 of 3 goat, and 3 of 3 alpaca sequences were associated with these distant subclusters. These results suggest that this cluster comprises a novel bifidobacterium-like group common to the pseudoruminant gut (three-chamber stomach typical of horses, llamas, camels, and alpacas). The rest of the sequences in group IV were primarily bird-derived clones with high sequence similarity to *Aeriscardovia aeriphila*, previously known as *B. aerophilum* (43).

The results obtained in the present study suggested that certain bifidobacterial species might prefer a cosmopolitan lifestyle, while others appear to exhibit preferential host distribution. These findings are relevant to monitoring microbial water quality from the standpoint that certain bifidobacterial species might not be good targets for the development of methods to determine human fecal pollution in watersheds impacted with different fecal sources as suggested in previous studies. This is the case for *B. adolescentis* and *B. dentium*, since these species were detected by molecular means in several nonhuman hosts. On the other hand, some bifidobacterial groups might represent good target populations for assessing the specific contribution of fecal sources such as swine and avian hosts. At the 16S rRNA gene level, fecal bifidobacteria do not appear to be as diverse as other fecal anaerobic bacteria such as *Bacteroides*, according to our diversity calculations. From a detection standpoint, low sequence diversity suggests that molecular methods could be used to comprehensively study the dynamics of bifidobacteria in different environmental scenarios. Such low phylogenetic diversity might also indicate that as a group bifidobacteria has a relatively narrow environ-

mental niche, which is compatible with their poor survival skills outside of the animal gut. The relatively low incidence of bifidobacteria detected with the species-specific assays in the present study suggests that their overall densities in sources of fecal pollution is also low, and consequently the levels of bifidobacteria reaching environmental waters might not always correlate with the densities of traditional indicators of fecal pollution or with health risks. In fact, bifidobacteria have not been detected in waters showing evidence of pollution as determined by the presence of fecal indicators that exhibit higher environmental survival rates (9). Similarly, Rhodes and Kator (38) failed to detect bifidobacteria in the summer months, when water temperatures were between 23 and 30°C, an important fact considering the potential for higher exposure to waterborne pathogens due to the increase in recreational activities during this period. Moreover, in the latter study bifidobacteria were isolated in only 11 of the 250 water samples tested, even though the overall fecal coliform average for the samples was approximately 374 per 100 ml. In our study the majority of the water samples tested positive to fecal anaerobic bacteria (*Bacteroidales* and *C. coccoides*) but negative for bifidobacteria. Hence, the use of bifidobacteria as indicators of fecal pollution in environmental waters might only be applicable in a limited number of circumstances (9), such as fecal contamination associated with rainstorm events or nearby specific sources of pollution when there are high loads of recent fecal contamination.

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SUPPLEMENTAL MATERIALS

TABLE S1. Description of fecal samples used in this study

Animal Type	Location	Sample Size	Type of operation	Size range of operation(s)	Range of Diet	Number of Locations Sampled
Alpaca	Berkeley County, WV	2	-	-	-	-
Beef Cattle	Berkeley County, WV	14	small beef farm (1)	25 beef cattle	hay, grass	1
Canadian Goose	Berkeley County, WV	20	-	-	-	-
Chicken	Berkeley County, WV	29	Small dairy farm (2), small family farm (2)	10-75 chickens	commercial feed, cracked corn, scratch	4
Dairy Cattle	Berkeley County, WV	14	Small commercial dairy farm (3)	75-200 dairy cattle	hay, grain	3
Domestic Cat	Berkeley County, WV	10	Houses (3)	4-6 cats	commercial feed	3
Domestic Dog	Berkeley County, WV	15	Houses (4)	1-4 dogs	commercial feed	-
Ferret	Berkeley County, WV	1	House (1)	-	-	-
Goat	Berkeley County, WV	4	-	-	-	-
Guinea Pig	Berkeley County, WV	1	House (1)	-	-	-
Hedgehog	Berkeley County, WV	1	House (1)	-	-	-
Horse	Berkeley County, WV	11	small dairy farm (2), family hobby (1)	2-5 horses	hay, grass, sweat feed	3
Human	Berkeley County, WV	19	households (2), workplace (1)	-	-	2
Llama	Berkeley County, WV	1	small dairy farm	1 llama	Corn, alfalfa/hay mix	1
Peacock	Berkeley County, WV	1	family hobby	4 peacocks	commercial feed	1
Pig	Berkeley County, WV	24	small pig farm (1), large pig farm (1)	55-300 swine	Wheat, soy, barley mix, hay	2
Pigeon	Berkeley County, WV	4	-	-	-	-
Prairie Dog	Berkeley County, WV	2	-	-	-	-
Rabbit	Berkeley County, WV	3	-	-	-	-
Whitetail Deer	Berkeley County, WV	15	-	-	-	-
Pig	Lodounville, OH	19	small pig farm (2), large pig farm (1)	-	corn, commercial feed, scraps	3
Whitetail Deer	Waco, TX	2	-	-	-	2
Coyote	Waco, TX	11	-	-	-	3
Raccoon	Waco, TX	1	-	-	-	1
Squirrel, Grey	Waco, TX	4	-	-	-	2
Armadillo	Waco, TX	1	-	-	-	1
Turkey, Wild	Waco, TX	2	-	-	-	1
Turkey, Wild	West Virginia	8	-	-	-	1
Horse	Waco, TX	1	Ranch (1)	-	-	1
Horse	Plum Creek, NE	4	Ranch (1)	-	-	1
Vulture	Waco, TX	1	-	-	-	1

Hog, Feral	Waco, TX	1	Ranch (1)	-	-	1
Rabbit, Jack	Waco, TX	1	-	-	-	1
Bobcat	Waco, TX	1	-	-	-	1
Fox, Grey	Waco, TX	1	-	-	-	1
Raccoon	Waco, TX	2	-	-	-	2
Septic Tanks	Plum Creek, NE	9	Houses (9)	2-10 people per house	variable	9
Sheep	Deleware	6	grazing farm	-	-	1
Dove	Plum Creek, NE	1	-	-	-	1
Possum	Waco, TX	2	-	-	-	-

TABLE S2. Description of wastewater treatment plants sampled in this study

WWTP Sample Type	Area Served	Sample Size	Volume (ml)	Average Daily Flow Million Gallons per Day (MGD)	Wastewater type	Treatment Technology	Effluent Discharge
Northwest WWTP Effluent	North and West El Paso, TX	Triplicate	75	17.5 MGD	Residential, Industrial	Extended aeration activated sludge, air scrubbers for odor control, UV disinfection of effluent	Rio Grande
Bustamante WWTP Effluent	South, East, and Lower Valley El Paso	Duplicate	75	39 MGD	Residential, Industrial	Extended aeration activated sludge, air scrubbers for odor control	Rio Grande
Dry Creek WWTP Effluent	Northern Kentucky (Boon, Campbell, Kenton County	Single	100	33 MGD	Residential, Industrial, commercial	Conventional activated sludge, chlorination for odor control	Ohio River
Dry Creek WWTP Influent		Duplicate	40				
Dry Creek WWTP Return Activated Sludge		Duplicate	40				
Dry Creek WWTP Secondary Aeration		Duplicate	40				

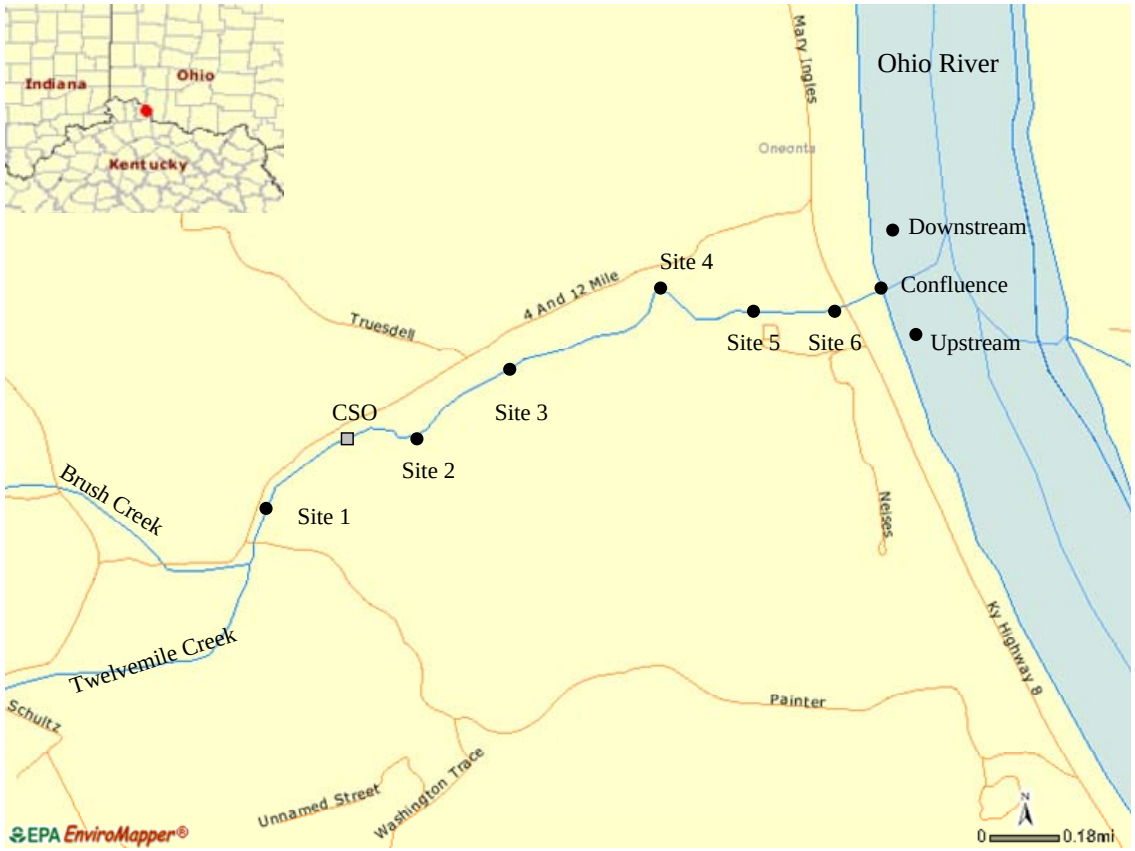

FIG. S1. Map of Twelve-mile Creek and Ohio River (Area mapped using Enviromapper; http://www.epa.gov/enviro/emef/). Water samples were collected from six sites along the Twelve-mile Creek downstream of Brush Creek and three sites located on the Ohio River. The location of a combined sewer overflow is designated by "CSO".

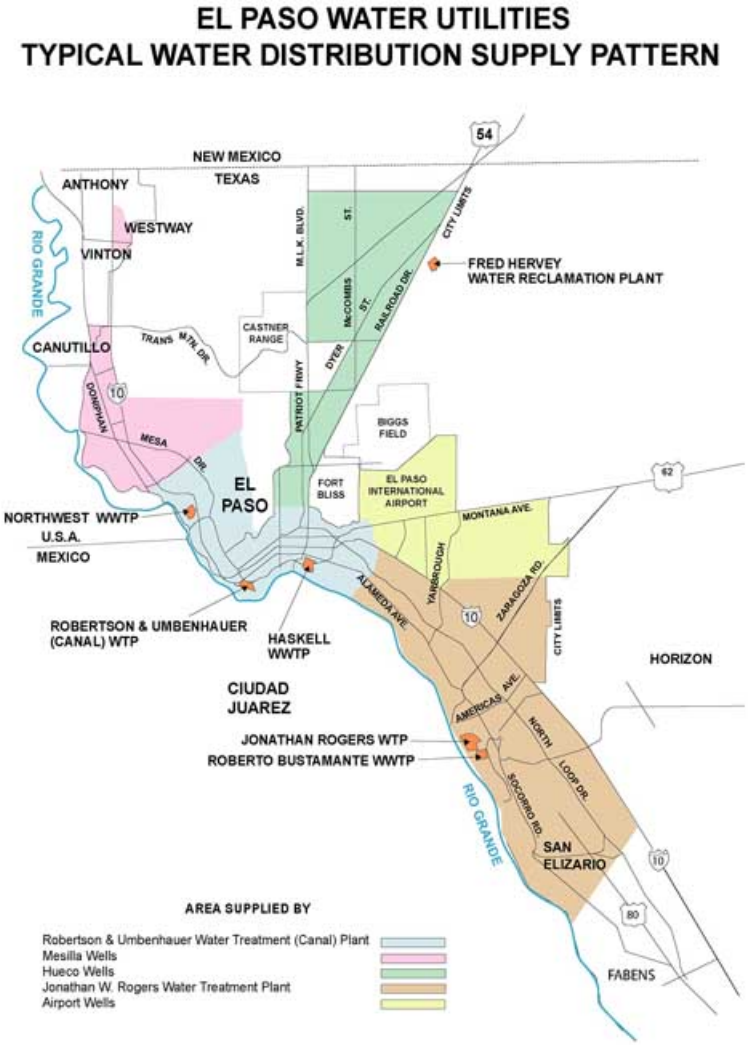

FIG. S2. Map of the Rio Grande/El Paso area (courtesy of El Paso Water Utilities). Samples were collected at upstream, effluent, and

downstream from the Northwest and Bustamante wastewater treatment plants. The Sunland Park and Courchesne samples were taken from the river just upstream from the Northwest plant in New Mexico.