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Assessment of equine fecal contamination: the search for alternative bacterial source-tracking targets

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Abstract

16S rDNA clone libraries were evaluated for detection of fecal source-identifying bacteria from a collapsed equine manure pile. Libraries were constructed using universal eubacterial primers and *Bacteroides-Prevotella* group-specific primers. Eubacterial sequences indicated that upstream and downstream water samples were predominantly β- and γ-Proteobacteria (35 and 19%, respectively), while the manure library consisted predominantly of Firmicutes (31%) and previously unidentified sequences (60%). Manure-specific eubacterial sequences were not detectable beyond 5 m downstream of the pile, suggesting either poor survival or high dilution rates. In contrast, *Bacteroides* and *Prevotella* sp. sequences were detected both in manure and downstream using group-specific primers. Novel sequences from *Bacteroides* and *Prevotella* analysis produced an equine-specific phylogenetic cluster as compared to previous data sets obtained for human and bovine samples. While these results suggest that some anaerobic fecal bacteria might be potential identifiers for use in source-tracking applications, a comprehensive examination of environmental sequences within these species should be performed before methods targeting these bacterial groups are applied to watersheds for development of microbial source-tracking protocols.
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Keywords: 16S rDNA; Horse manure; *Bacteroides*; Watershed; Source tracking

1. Introduction

Regardless of efforts to reduce or eliminate fecal pollution in US waters, a good percentage of watersheds continue to be impaired in great part due to failure to conclusively identify non-point sources of contamination. Non-point sources can include, but are not limited to agricultural runoff, wildlife, aquaculture, ineffective sewage treatment plants, or privately owned leaking septic systems. Since fecal bacteria are among the most common biological pollutants affecting rivers and streams, fecal indicator bacteria (e.g. *Escherichia coli* and fecal enterococci) are frequently used in bacterial source tracking (BST) as primary targets. While indicator bacteria are relatively good predictors of fecal contamination, their use in BST studies has been based on ease of detection and cultivation, not their ability to differentiate among sources [1–3].

The development of culture-independent molecular methods has made possible the rapid detection of fastidious anaerobic gastrointestinal bacteria. As a consequence, the possibility of using alternative fecal source identifiers like fecal anaerobes to assess water quality for BST is not limited by the isolation and cultivation of these bacterial species. Recent research has utilized *Bacteroides* and *Bifidobacteria* in several studies to differentiate between human and animal sources [2,4–8]. However, a comprehensive study examining the potential for other organisms to be used as source indicators, based upon 16S rDNA profiles of fecal material, has not been done to date. This leaves a gap in our knowledge base regarding the potential usefulness of other bacterial groups, which may be suitable for fecal source tracking.

There has been only one study thus far that examined the composition of the equine intestine using partial sequencing analysis of eubacterial 16S rDNA [9]. Previous studies have characterized components of the microbiota using physiochemical identifications [10] or 16S rRNA-targeted oligonucleotide probes [11,12]. The occurrence of a collapsed equine manure pile in a local stream provided us

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with the opportunity to study not only the bacteria present in the manure pile itself, but also to potentially track contributed fecal bacteria in the neighboring stream. Samples of fecal material and water were obtained from which characterizations of the microbial populations were determined using 16S rDNA techniques. DNA sequences from *Bacteroides-Prevotella* genera and eubacteria were selectively polymerase chain reaction (PCR)-amplified to examine the diversity of these fecal bacterial groups in the manure pile and in the adjacent watershed. Our goal was to evaluate the 16S rDNA clone library approach for detecting novel fecal microorganisms present in fecally contaminated water that could be directly associated with a manure source.

2. Materials and methods

2.1. Collection and processing of samples

The sampling site was located within the Shepherds Creek sub-watershed, Cincinnati, OH. To the authors' knowledge, neither wastewater treatment plant nor leaking septic systems were present near the sampling sites. Water discharge was 2.24×10^{-3} m^3 s^{-1} and the average temperature was 24.57°C over the sampling period. Grab samples of free-flowing water were collected at locations 20 m upstream and 5, 10, and 20 m downstream of a distinct and discrete equine fecal contamination source (a collapsed manure pile). Initially, distances further than 20 m downstream were considered for investigation, however due to physical limitations regarding accessibility of the stream, this was not possible. The manure pile was present in and adjacent to the creek during the months of August and September 2001 and samples were collected bi-weekly. Grab samples of water were collected in sterile 1-l polypropylene bottles and were transported to the laboratory on ice [13]. Manure samples (100 g) were collected in sterile 50-ml conical tubes and transported to laboratory on ice. At the end of September 2001, the manure pile was removed from the site by the land owner and water sampling continued during October and November. Water samples (100, 10 and 1 ml) were filtered according to EPA guidelines [14] and fecal enterococci were enumerated as described by Messer and Dufour [15]. Results were compared by Student's *t*-test to determine their statistical significance. Water samples (100 ml) were also filtered through sterile polycarbonate membranes (0.2 μm) (Osmonics, Minnetonka, MN, USA) to collect microbial biomass for genomic DNA extractions. Membranes were stored at −20°C until analyzed.

2.2. DNA extraction

DNA extractions for manure samples (0.5g) and water biomass retained on filters were performed using an Ultra Clean Soil DNA Kit (MoBio Labs, Solana Beach, CA, USA) [16]. Filters from water samples containing the highest amount of contamination, as determined by enterococci enumeration, were selected for DNA extraction. Filters containing biomass were aseptically folded such that biomass faced outward and placed into 2-ml extraction tubes. DNA extractions utilized the vortex adapter (MoBio Labs) for Vortex Genie 2 (Fisher Scientific, Pittsburgh, PA, USA) to bead-beat the samples for 10 min at high speed. DNA was suspended in sterile 10 mM Tris buffer (pH 8.0) and stored at −20°C. DNA quality was checked by agarose gel electrophoresis (0.7% agarose, 0.5×TBE, 100 V for 1 h) and visualized using GelStar® nucleic acid stain (BMA, Rockland, ME, USA).

2.3. PCR

Oligonucleotide primers 27f and 1525r were used to PCR-amplify environmental population 16S rDNA genes [17], while group-specific primers Bac32f and Bac708r were used to selectively amplify *Bacteroides*-like 16S rDNA genes [18]. Each PCR reaction contained 100 ng of genomic DNA, 25 μmol of each primer, 4 μl of dNTP mixture, 5 μl of 10×ExTaq buffer, 5 μl of a 25% acetamide solution and 0.25 μl of TaKaRa ExTaq polymerase (TaKaRa, Shuzo, Otsu, Japan). The final volume was adjusted to 50 μl with sterile deionized water. PCR amplification for 16S rDNA gene: initial denaturation at 94°C for 4 min, followed by 25 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 45 s, and final extension at 72°C for 7 min using a PTC-200 DNA Engine™ thermal cycler (MJ Research, Waltham, MA, USA). *Bacteroides*-specific PCR was performed as described above except that 53°C was used as the annealing temperature.

2.4. 16S rDNA clone library construction

16S rDNA PCR products were cloned into the TOPO® TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA, USA) and transformants randomly picked. The recombinant plasmids were extracted using QIAquick® Miniprep Extraction Kit (Qiagen, Valencia, CA, USA). DNA inserts were amplified using M13 primers. Amplified inserts were screened by restriction enzyme digests (*Alu*I, TaKaRa) according to the manufacturer's instructions. Representative plasmids of different digestion patterns were selected and sent for sequencing at MJ Genomics Services (South San Francisco, CA, USA). Numbers associated with sample designations refer to randomly assigned sequencing order, which was used to remove any potential bias by batch processing.

2.5. Phylogenetic analysis

Editing of sequence data was performed using Sequencher 3.0 (Gene Codes, Ann Arbor, MI, USA), and

sequences were compared to the GenBank database (National Center for Biotechnology Information, Bethesda, MD, USA) using BLAST [19]. All reference sequences were obtained from the GenBank database. Sequences were analyzed by CHIMERA_CHECK v.2.7 to remove any chimeric clones [20]. Sequences were aligned using BioEdit v. 5.0.9.1 [21] and ClustalX v. 1.81 [22]. Maximum parsimony trees were constructed using Paup* v. 4.10 [23]. Statistical significance of tree branches was evaluated by bootstrap analysis using 1000 trees (values < 50 omitted from figures) [24]. The sequence data reported in this paper have been submitted to the GenBank database with accession numbers AY212516 to AY212779.

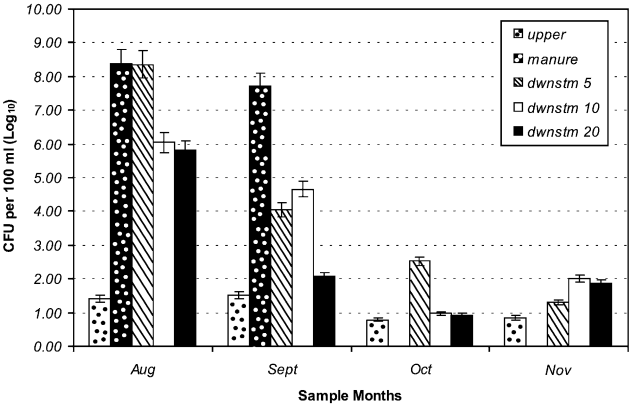

Fig. 1. Enterococci CFU 100 ml^{-1} (log_{10}) obtained from water samples and equine manure. Manure sample was not analyzed for the months of October and November as it was removed from site.

3. Results

3.1. Fecal enterococci enumeration

Fecal enterococci were enumerated as an indication of water contamination levels. Initial sample counts were seven log_{10} orders of magnitude higher 5 m downstream of the manure pile as compared with upstream values (Fig. 1). Fecal enterococci numbers decreased with distance downstream of the manure pile relative to the 5-m sampling site. By September, while the manure pile maintained relatively high numbers, levels of enterococci found in the water downstream had decreased by one to four log_{10} orders of magnitude. After the manure pile was removed, enterococci counts continued to decrease and by November, no differences ($P \leq 0.05$) in enterococci densities were found when comparing upstream and downstream samples.

3.2. Clone library of 16S rDNA recovered using universal primers

Seventy-two clones per sample were arbitrarily selected for analysis. Of the 360 clones screened, 223 clones yielded unambiguous sequence data. A large number of sequences recovered from the manure and water samples did not correspond well to entries in the GenBank database: 64% of sequences had similarity values in the range of 90–97%, while 4% showed less than 90% homology to their nearest database entry. Although 32% of the sequences recovered showed greater than 97% homology with database entries, a large portion (87%) of these were matched to other environmental clones (i.e. no specific species). The distribution of sequences recovered from both manure and water samples at the phylum level is presented in Table 1. Representative clone sequences were used to construct phylogenetic trees and reference organisms were selected as the closest relatives to sample sequences within the database (Figs. 2–4).

In the upstream water sample library (Fig. 2), the majority of sequences placed within the Proteobacteria (PROT) phylum and fell predominantly into either β or γ subgroups. Two sequences fell into the Verrucomicrobiales (VER) and another two to the *Cytophaga-Flavobacter-Bacteroides* (CFB) phyla. Low G+C Gram-positive (LGCGP) bacteria were included for comparison to the manure sample, but corresponding sequences were not recovered for upstream samples. Although most clones clustered with bacteria common to freshwater sources, some clones (representing 11% of total upstream clone popula-

Table 1
Taxonomy of 16S rDNA clone library sequences

Sample	Bacterial sequence types detected (%)									
	Proteobacteria					Other				
	α	β	δ	ε	γ	CFB[a]	Firmicute	Spirochaete	Verrucomicrobia	Unknown[b]
Upstream	7	44	2	2	20	2	4	nd	6	13
Wet manure	nd	nd	nd	nd	2	nd	31	5	2	60
Downstream 5 m	2	56	nd	nd	18	10	8	nd	nd	6
Downstream 10 m	5	35	2	2	19	8	5	nd	4	20
Downstream 20 m	6	34	nd	nd	32	14	2	nd	1	11
Total libraries	5	35	1	1	19	7	9	1	2	20

nd = none detected.

[a] *Cytophaga-Flexibacter-Bacteroides* group.

[b] Uncultured and/or unidentified environmental clones were only match in GenBank.

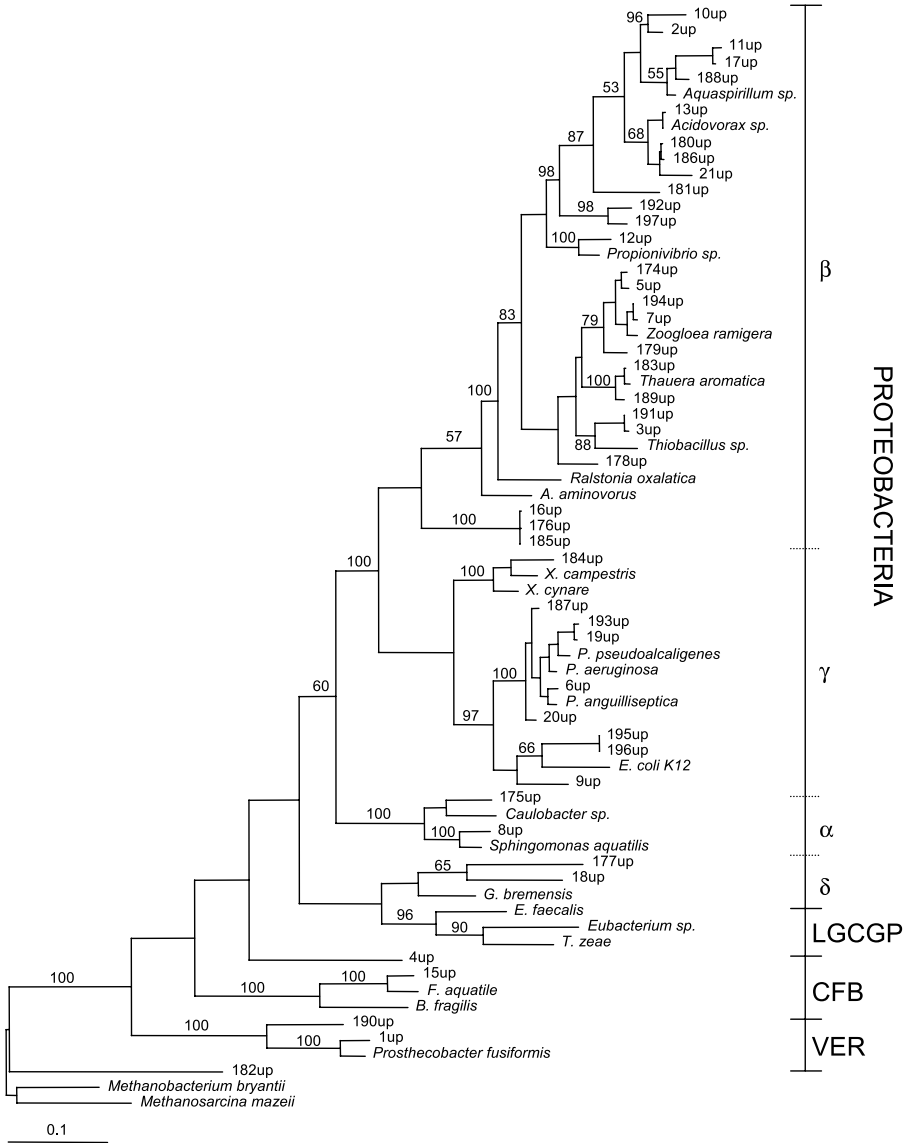

Fig. 2. Phylogenetic tree derived from 16S rDNA sequence data from upstream water samples (clone number followed by 'up'). Bootstrap values are shown as percent of 1000 trees and values below 50% are omitted. Sequences from *Methanobacterium bryantii* and *Methanosarcina mazeii* are used as outgroup for rooting the tree. Known species sequences obtained from GenBank are italicized. Scale bar represents substitutions per nucleotide position. Key: Proteobacteria (α, β, γ, and δ subgroups); *Cytophaga-Flexibacter-Bacteroides* (CFB); low G+C Gram-positive bacteria (LGCGP); and Verrucomicrobiales (VER).

tion), clustered with *Zoogloea ramigera*, a species commonly found in organically contaminated freshwater and aerobically treated sewage.

For the manure sample library (Fig. 3), the majority of the sequences were placed within the LGCGP phylum. Within the LGCGP phylum, sequences occurred predominantly (84%) within a cluster of the Clostridiaceae, which contained cellulolytic *Clostridium* spp. along with *Ruminococcus* spp., *Butyrivibrio* spp. and *Eubacterium* spp. Four sequences grouped with the Spirochaetaceae (SPIR) (15%) clustering closely with *Treponema bryantii* and one sequence grouped to the PROT associated with *Acinetobacter lwolfii*. Three sequences clustered with *Holdemania filiformis*, and two clustered with *Carnobacterium inhibens*. CFB bacteria were included in the phylogeny for comparison purposes, but corresponding sequences were not recovered from manure samples.

The downstream water sample libraries were similar to those of the upstream samples (example library shown in Fig. 4). Again, the majority of sequences placed within the Proteobacteria phylum and fell predominantly into either β or γ subgroups. However, increasing numbers of α and δ sequences were detected with increasing distance from the manure pile. VER sequences were detected only at the 10-m downstream site and were most closely associated with *Prosthecobacter* spp. LGCGP bacteria associated with Clostridia and enterococci were identified at the 5-m site, but were not detected thereafter. Additionally, sequences

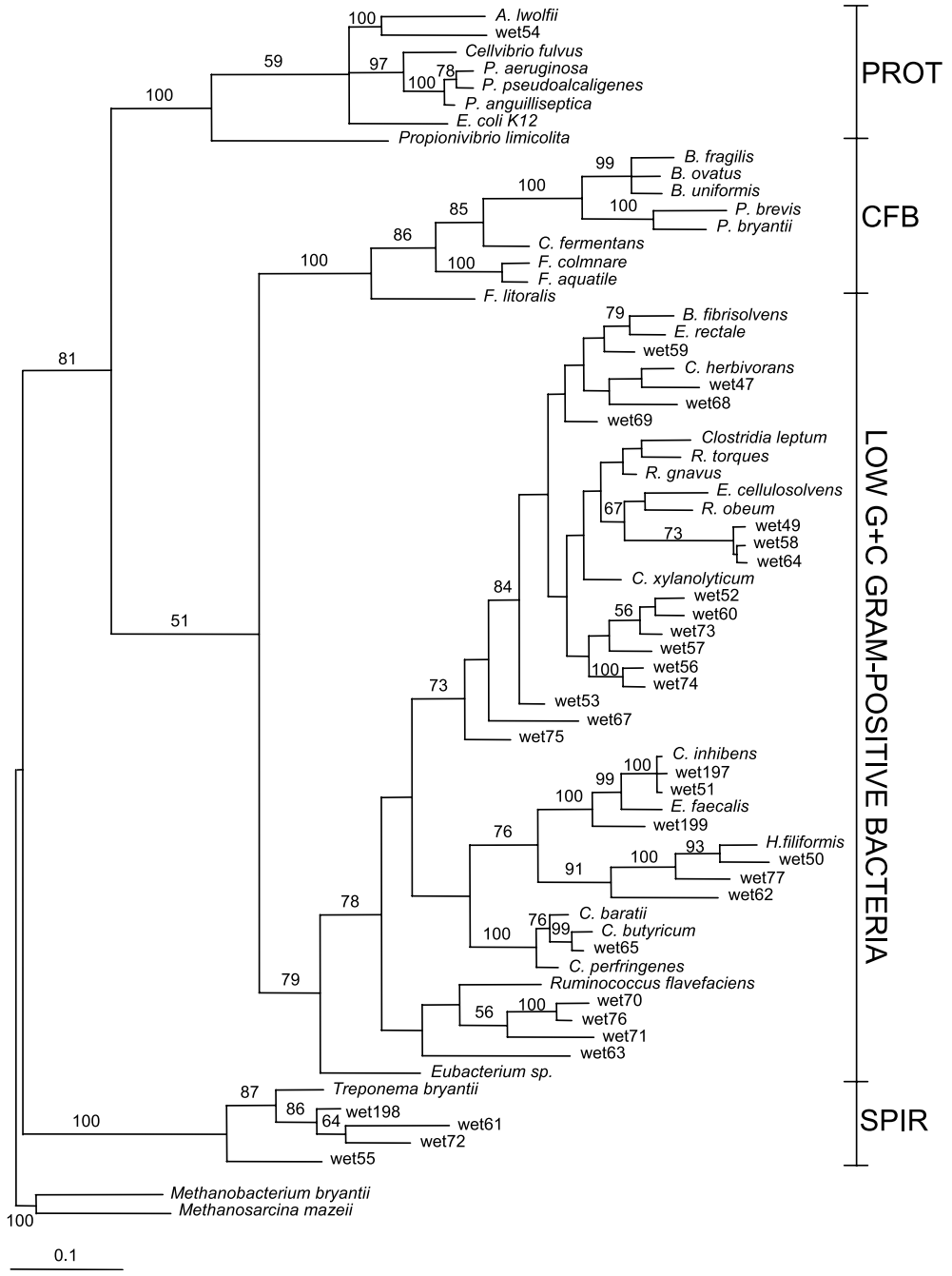

Fig. 3. Phylogenetic tree derived from 16S rDNA sequence data from manure samples ('wet' followed by clone number). Bootstrap values are shown as percent of 1000 trees and values below 50% are omitted. Sequences from *Methanobacterium bryantii* and *Methanosarcina mazeii* are used as outgroup for rooting the tree. Known species sequences obtained from GenBank are italicized. Scale bar represents the number of substitutions per nucleotide position. Key: Proteobacteria (PROT); *Cytophaga-Flexibacter-Bacteroides* (CFB); and Spirochaetaceae (SPIR).

corresponding to Eikelboom type 021N (filamentous bacteria common in activated sludge) were isolated and comprised 23% of sequences obtained.

3.3. Bacteroides-Prevotella-*specific 16S rDNA clone libraries*

Bacteroides-Prevotella-specific primers produced the expected PCR product (approximately 700 bp) in all samples, including the upstream site. In total, 180 clones were picked and screened (36 per sample), resulting in 76 clones yielding unambiguous sequence data. Results showed that 41% of sequences had similarity values of 90–97%, while 9% showed less than 90% homology with database sequences. As compared to the 16S rDNA clone library, a greater proportion of sequences recovered corresponded to entries in the GenBank database: 50% showed greater than 97% homology to their nearest database entry. Although the

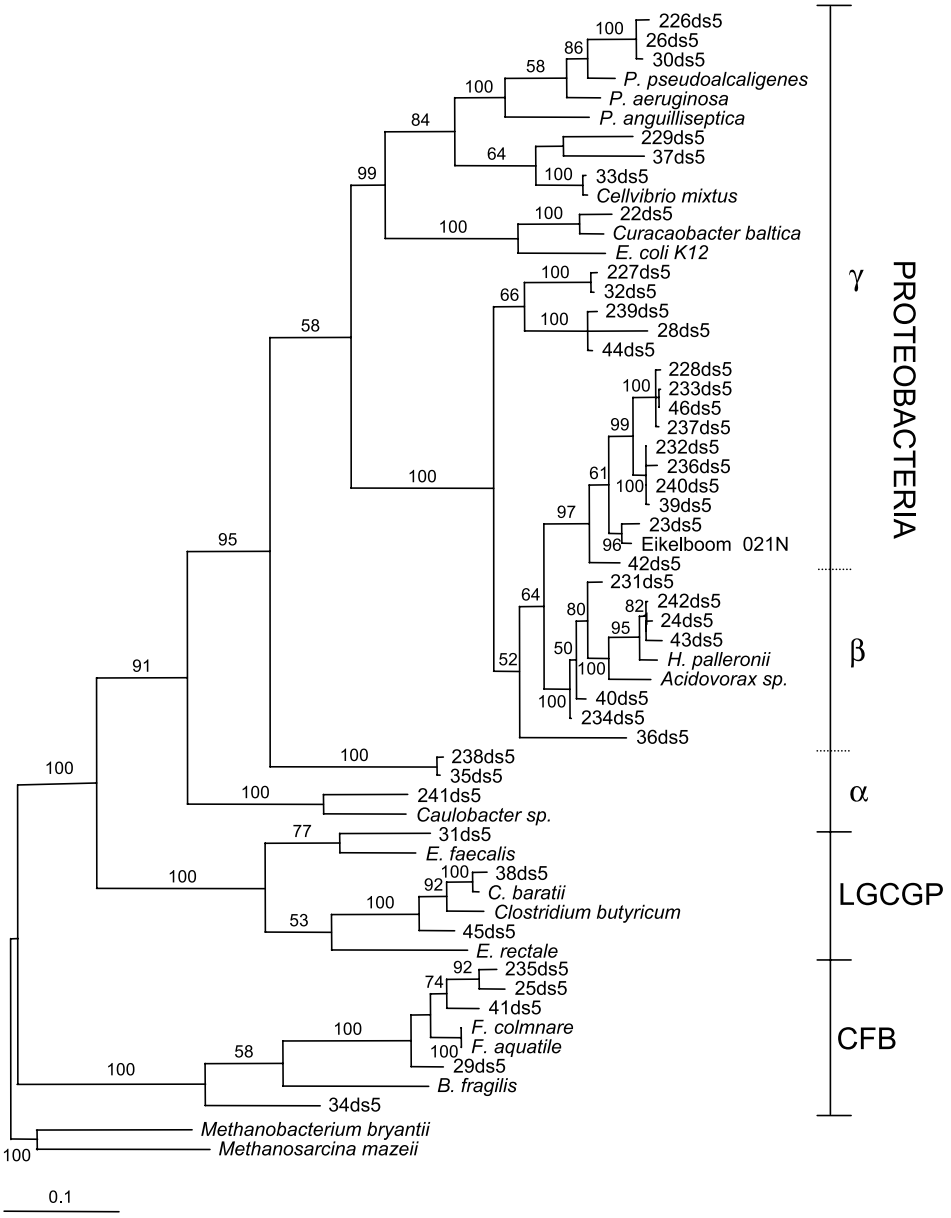

Fig. 4. Phylogenetic tree derived from 16S rDNA sequence data from downstream water samples (clone number followed by 'ds5'). Bootstrap values are shown as percent of 1000 trees and values below 50% are omitted. Sequences from *Methanobacterium bryantii* and *Methanosarcina mazeii* are used as outgroup for rooting the tree. Known species sequences obtained from GenBank are italicized. Scale bar represents the number of substitutions per nucleotide position. Key: Proteobacteria (α, β, and γ subgroups); *Cytophaga-Flexibacter-Bacteroides* (CFB); and low G+C Gram-positive bacteria (LGCGP).

sequences recovered showed greater homology with database entries, 57% of these were matched to other uncultured environmental clones. Only four sequences were identified as either *Bacteroides* or *Prevotella* spp. from the upstream clones.

All *Bacteroides*-like sequences obtained from each sample site were incorporated into a single phylogenetic analysis. These results showed that sequences were divided into two *Bacteroides* spp. and *Prevotella* spp. 'superclusters' and one minor cluster related to *B. distasonis* (Fig. 5). Other CFB bacteria included for comparison separated from the superclusters and rooted deeply with *Cytophaga fermentans*. Within the *Bacteroides* supercluster, there were two groups that did not associate with any of the known sequences. Unknown group 1 contained eight sequences, which were mainly isolated from water samples with nearest known sequence being a cluster associated with *B. vulgatus*. Unknown group 2 contained eight sequences all from the manure pile. This group was associated within the *Bacteroides*, but was not closely related to any other phylotype within the supercluster, supported by a bootstrap value of 100%. The remaining clone sequences clustered with *B. fragilis*, *B. eggerthii* or *B. vulgatus*. Within the *Prevotella* supercluster, four *Prevotella* clones from

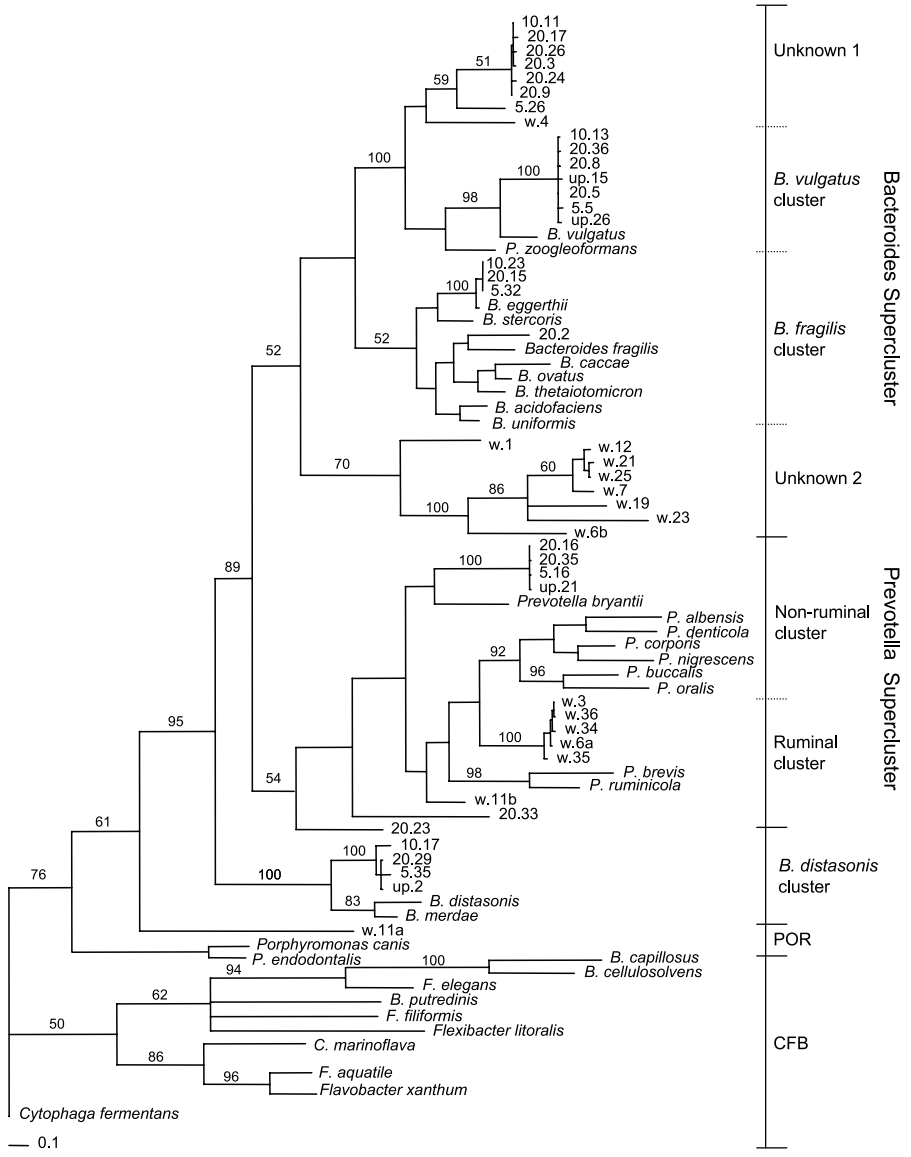

Fig. 5. Phylogenetic tree derived from 16S rDNA sequence data obtained using *Bacteroides-Prevotella*-specific primers from water samples (first number = distance [5, 10, 20 m] and second number = clone ID) and manure samples (W plus clone #). Bootstrap values are shown as percent of 1000 trees and values below 50% are omitted. Sequence from *Cytophaga fermentans* was used as outgroup for rooting the tree. Known species sequences obtained from GenBank are italicized. Scale bar represents the number of substitutions per nucleotide position. Key: *Cytophaga-Flexibacter-Bacteroides* (CFB); and *Porphyromonas* (POR).

water samples associated with non-ruminal *P. bryantii* and five clones from the manure sample formed a novel cluster distantly related to ruminant *P. brevis* and *P. ruminicola*, supported by bootstrap values of 100%. The remaining clones clustered within the *Prevotella* supercluster, but were not closely associated with any of the subgroups. A minor cluster containing four water-associated clones was related to *B. distasonis* and *B. merdae* by a bootstrap value of 100%.

4. Discussion

A total of 540 16S rDNA clones were screened from five different environmental libraries (four water samples and one manure sample). The water libraries contained sequences from bacteria commonly associated with freshwater and the library from the manure pile was mainly composed of bacterial sequences commonly associated with fecal matter. Although the number of sequences retrieved cannot be regarded as a complete inventory, each water library contained sequences corresponding to environmental clones predominantly from freshwater studies [25,26]. Unexpectedly, the libraries from downstream water samples did not contain sequences from the manure pile based on the universal eubacterial primers used. Considering that the water was still visibly contaminated at 20 m (water was turbid and brown as contrasted to clear

upstream), it was unexpected that after 5 m the majority of the detected downstream eubacterial clone populations resembled the upstream populations. These data suggest that the dilution factors were such that fecal organisms were rapidly becoming a relatively minor portion of the microbial populations. These results argue against the use of total community-based tools to identify sources of fecal pollution, as it is unlikely that fecal bacteria will be well represented in universal clone libraries derived from environmental waters.

Fecal-based clone libraries have been examined from several gastrointestinal systems including human [27–29], bovine rumen [30,31], porcine [32], equine [9] and domestic pets [28]. The majority of the sequences obtained from these studies did not place well within specific taxa of characterized isolates in the databases (similarities ranged from >90 to $<97\%$, irrespective of which bacterial primer set was used). Based on the phylogenetic analyses from these studies, the predominant organisms which could be expected to be found in manure and/or fecally contaminated water would be: *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Eubacterium*, *Fusobacterium*, *Peptococcus*, *Peptostreptococcus*, *Prevotella*, *Ruminococcus*, and to a lesser extent, *Butyrivibrio*, *Selenomonas*, *Treponema* and *Verrucomicrobia* species. The majority of these genera were represented among the eubacterial sequences obtained from the horse manure samples examined in this study. Proportions detected may have varied, but most species were similar to those found in other studies. Manure pile sequences typically corresponded to clones from either rumen or fecal samples [31,33]. In addition, sequences corresponding to *H. filiformis* and *C. inhibens* were also detected which are common to human and fish feces respectively and have not previously been reported in horse feces [34,35]. Results from the manure clone library closely matched results reported by Daly et al. [9] with one notable exception. Daly et al. reported a large population of *Bacteroides* and *Prevotella* detected throughout the horse digestive tract. This portion of the CFB bacteria was not detected in our horse manure clone library using the selected universal PCR primers.

The lack of *Bacteroides* spp. in the eubacterial libraries was noted as unusual, particularly since they were expected to be found based on previous reports and as *Bacteroides* spp. are among the most predominant obligate anaerobes in the colon of mammals [9,36–39]. Although bacterial sequences similar to previous reports were detected in varying proportions in fecal samples, it must be noted that our library was derived from an aged manure pile, not a fresh fecal sample. Shifts in population densities and die-off of many commensal bacteria were expected to have occurred during the time the manure was on the stream bank. Yet, the collapse of the pile exposed a presumably micro-aerophilic, if not anaerobic core, from which anaerobic colonic bacteria DNA was obtained. Whether or not these organisms may have been active is unknown, but the conditions were such that they were able to persist with DNA intact. A large proportion of the organisms detected were cellulolytic bacteria, which may have been able to survive on the intercalated undigested feed material and possibly were incubated due to internal heat generation via fermentative processes (composting).

In contrast to eubacterial data, *Bacteroides* group-specific primers produced PCR products in which the resultant sequences had higher similarity values than those obtained via universal primers and confirmed that these organisms were present in the samples. All *Bacteroides* and *Prevotella* spp. detected were of fecal origin and did not group with non-fecal representatives of the species. Specifically, two subsets were predominant, those matching other fecal and environmental clone sets (32%) [18] and those matching rumen clone sets (25%) [31,33]. *Prevotella* spp. consisted of both ruminant and non-ruminant clones as defined by Ramšak et al. [40]. Especially noted was that not a single match was made to other equine fecal clones available in the GenBank database [9]. Sequences detected in the water clustered closely with each other, as did those of manure origin, and little cross-clustering was observed. This distinct separation by source (water vs. manure) is puzzling, as they must have all been sloughed off the manure pile at some point in time, considering that only a few sequences were obtained from the upstream site. One hypothesis would be that the organisms obtained from the center of the pile had differentiated from those initially deposited, and in contrast, those sloughed off the collapsed portion were more likely to have been recent additions placed on top of the manure pile, thus providing for two different subgroups to be detected from only 'one' actual source. Alternatively, it is possible that these sequences belong to organisms that are associated with other fecal sources (i.e. wildlife).

Since *Bacteroides-Prevotella* sequences had been used previously to differentiate human and bovine samples [6,18], it was of interest to note that two 'unknown' sequence clusters formed within the *Bacteroides* supercluster. To determine if these primers could also be used to differentiate equine *Bacteroides-Prevotella*-like sequences, GenBank data obtained from previous studies were downloaded and added to the phylogenetic analysis (Fig. 6). Sequences included in the hf8 cluster (human), cf123 cluster (cow), and cf151 (cow) cluster were from Bernhard and Field [6,18] (designated here as BF-). Sequences from the current study (up.15, up.26, 5.5, 10.13, 20.5, 20.8, 20.36), which had grouped with the *B. vulgatus* cluster, were now more closely associated with BF-hf74, yet still distinctly separate, with bootstrap support of 100%. Unknown group 1 (5.26, 10.11, 20.3, 20.9, 20.17, 20.24, 20.26, and w4) clustered more closely to the *B. vulgatus* group and the rest of the BF clones, yet still remained distinctly separate, with a support bootstrap value of 99%. Unknown group 2 (w.1, w.6b, w.7, w.12, w.19, w.21, w.23, and w.25)

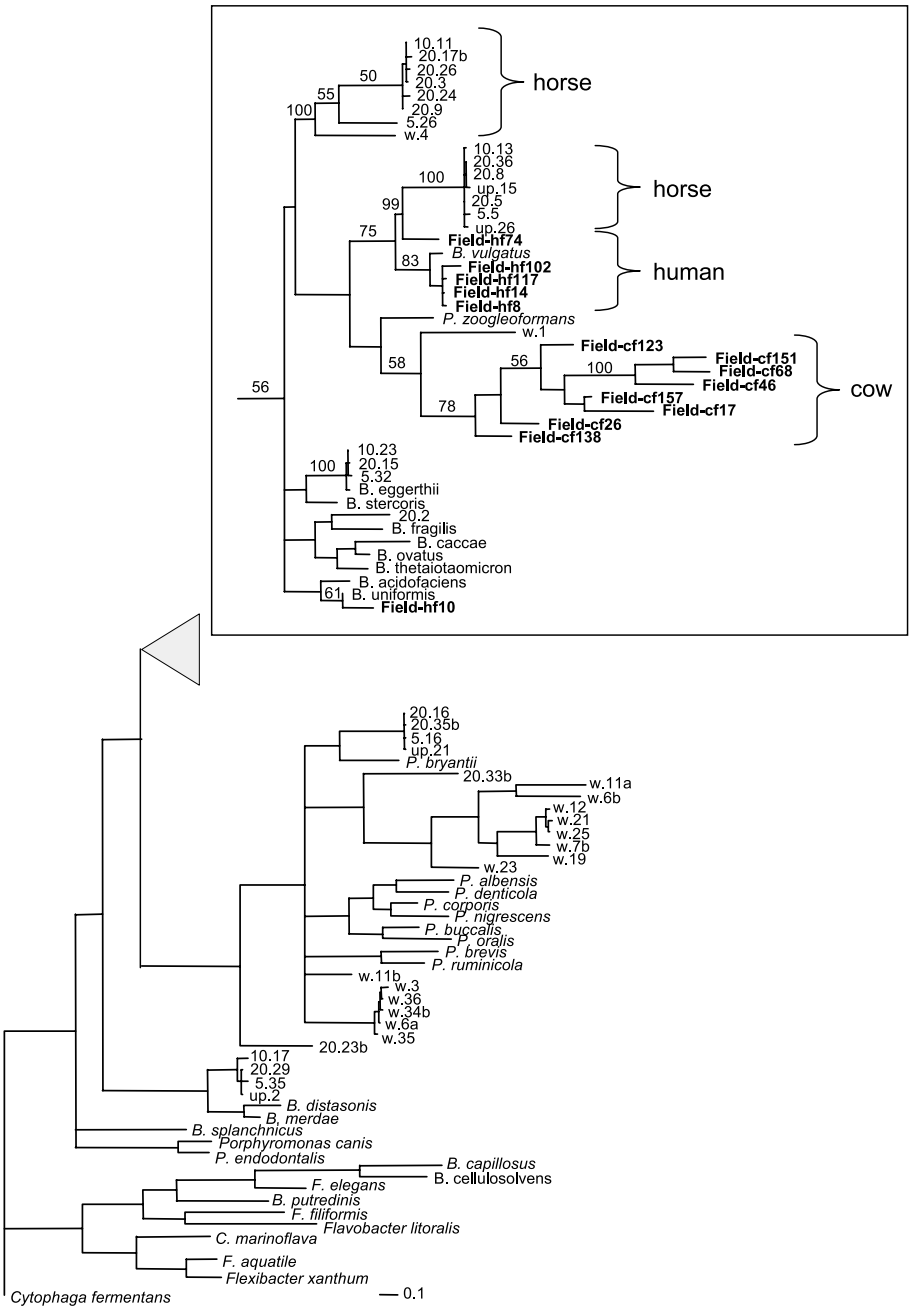

Fig. 6. Phylogenetic tree derived from 16S rDNA sequence data obtained using *Bacteroides-Prevotella*-specific primers from water samples (first number = distance [5, 10, 20 m] and second number = clone ID) and manure samples (W plus clone #). Bootstrap values are shown as percent of 1000 trees and values below 50% are omitted. Sequence from *Cytophaga fermentans* was used as outgroup for rooting the tree. Known species sequences obtained from GenBank are italicized. Scale bar represents the number of substitutions per nucleotide position. Samples designated (Field-) are sequences downloaded from GenBank from other studies using these primer sets.

clustered more closely to *Prevotella* species, although it remained distinctly separate from known sequences. Inclusion of sequences from previous studies suggests that this region of the 16S rDNA gene may contain enough sequence variability to be specific enough for determination of source hosts and leads to optimistic speculation that indeed *Bacteroides* and perhaps *Prevotella* are good candidates for source tracking. The level of distinct separation of the clusters by host animal, backed by high bootstrap values, leads one to hypothesize that similar results may be obtainable from other domestic animals.

Our goal was to evaluate the use of 16S rDNA clone libraries to detect novel fecal organisms in contaminated water that could be linked to an associated manure source. The results indicated that although fecal contamination was detected downstream by fecal enterococci enumeration, analysis of universal eubacterial clone libraries from water samples was unable to detect fecal organisms be-

yond 5 m downstream. This lack of detection may have been influenced by several factors, not the least of which was choice of universal primers selected, as fecal organisms were detected downstream upon use of genus-specific primers. Although the results of clone libraries developed with the universal primers did not propose any outstanding new target organisms, some bacteria identified may be worth a further look, such as *Clostridium*, *Eubacterium*, and *Ruminococcus*. These bacteria are among the dominant members in higher mammals examined and they represent highly diverse groups of organisms at the 16S rDNA level. It is tempting to speculate that other genera, like *Treponema*, *Holdemania*, *Carnobacterium* and *Eikelboom* might also be potential BST targets. However, their presence in other animal hosts, as well as their genetic diversity, is unknown and therefore precludes us from predicting their potential for BST. Thus, future studies should consider them for further evaluation. The *Bacteroides-Prevotella* data confirmed that uncharacterized *Bacteroides* species seem to separate amongst themselves in a manner, which suggests a host–microbe association which may be used to advantage in source-tracking applications. While this suggests that anaerobic fecal bacteria may be potential identifiers for use in source-tracking applications, a comprehensive examination of anaerobic environmental sequences within these species should be performed before methods targeting these bacterial groups are applied to watersheds on a large scale.

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References

[1] Simpson, J.M., Santo Domingo, J.W. and Reasoner, D.J. (2002) Microbial source tracking: State of science. Environ. Sci. Technol. 36, 5279–5288.

[2] Sinton, L.W., Finlay, R.K. and Hannah, D.J. (1998) Distinguishing human from animal faecal contamination in water: a review. N.Z. J. Mar. Freshwater Res. 33, 323–348.

[3] Gordon, D.M. (2001) Geographical structure and host specificity in bacteria and the implications for tracing the source of coliform contamination. Microbiology 147, 1079–1085.

[4] Rhodes, M.W. and Kator, H. (1999) Sorbitol-fermenting *Bifidobacteria* as indicators of diffuse human faecal pollution in estuarine watersheds. J. Appl. Microbiol. 87, 528–535.

[5] Satokari, R., Vaughan, E., Akkermans, A., Saarela, M. and De Vos, W. (2001) Polymerase chain reaction and denaturing gradient gel electrophoresis monitoring of fecal *Bifidobacterium* populations in a prebiotic and probiotic feeding trial. Syst. Appl. Microbiol. 24, 227–231.

[6] Bernhard, A.E. and Field, K.G. (2000) A PCR assay to discriminate human and ruminant feces on the basis of host differences in *Bacteroides-Prevotella* genes encoding 16S rRNA. Appl. Environ. Microbiol. 66, 4571–5474.

[7] Kreader, C.A. (1998) Persistence of PCR-detectable *Bacteroides distasonis* from human feces in river water. Appl. Environ. Microbiol. 64, 4103–4105.

[8] Rijal, G., Bonilla, A. and Fujioka, R.S. (2002) Detection of *Bacteroides* species by polymerase chain reaction (PCR) for identifying sewage contamination in recreational water. Presented at the Water Environment Federation 75th Annual Technical Exhibition and Conference, Chicago, IL.

[9] Daly, K., Stewart, C., Flint, H. and Shirazi-Beechey, S. (2001) Bacterial diversity within the equine large intestine as revealed by molecular analysis of cloned 16S rRNA genes. FEMS Microbiol. Ecol. 38, 141–151.

[10] Mackie, R.I. and Wilkins, C.A. (1988) Enumeration of anaerobic bacterial microflora of the equine gastrointestinal tract. Appl. Environ. Microbiol. 54, 2155–2160.

[11] Lin, C. and Stahl, D. (1995) Taxon-specific probes for the cellulolytic genus *Fibrobacter* reveal abundant and novel equine-associated populations. Appl. Environ. Microbiol. 61, 1348–1351.

[12] Julliand, V., de Vaux, A., Millet, L. and Fonty, G. (1999) Identification of *Ruminococcus flavefaciens* as the predominant cellulolytic bacterial species of the equine cecum. Appl. Environ. Microbiol. 65, 3738–3741.

[13] USEPA (1978) Microbiological methods for monitoring the environment: Water and wastes. Office of Research and Development EPA-600-8-78-017, U.S. Environmental Protection Agency.

[14] USEPA (1986) Test methods for *Escherichia coli* and Enterococci in water by the membrane filter procedure. Office of Research and Development EPA-500-4-85-076, U.S. Environmental Protection Agency.

[15] Messer, J.W. and Dufour, A.P. (1998) A rapid, specific membrane filtration procedure for enumeration of enterococci in recreational water. Appl. Environ. Microbiol. 64, 678–680.

[16] Clement, B. and Kitts, C. (2000) Isolating PCR-quality DNA from human feces with a soil DNA kit. BioTechniques 28, 640–645.

[17] Lane, D.J. (1991) in: Nucleic Acid Techniques in Bacterial Systematics (Stackebrandt, E. and Goodfellow, M., Eds.), pp. 115–147. John Wiley and Sons, New York.

[18] Bernhard, A.E. and Field, K.G. (2000) Identification of nonpoint source of fecal pollution in coastal waters by using host-specific 16S ribosomal DNA genetic markers from fecal anaerobes. Appl. Environ. Microbiol. 66, 1587–1594.

[19] Altschul, S., Madden, T., Schäffer, A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–11825.

[20] Maidak, B. et al. (2001) The RDP-II (Ribosomal Database Project). Nucleic Acids Res. 29, 173–174.

[21] Hall, T. (2001) BioEdit, v. 5.0.9. North Carolina State University, Raleigh, NC.

[22] Thompson, J., Gibson, T., Plewniak, F., Jeanmougin, F. and Higgins, D. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.

[23] Swofford, D. (2002) PAUP* – Phylogenetic Analysis Using Parsimony (*and Other Methods), v. 4.10. Sinauer Associates, Sunderland, MA.

[24] Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.

[25] Zwart, G., Crump, B., Kamst-van Agterveld, M., Hagen, F. and Han, S.-K. (2002) Typical freshwater bacteria: an analysis of available 16S rRNA gene sequence from plankton of lakes and rivers. Aquat. Microb. Ecol. 28, 141–155.

[26] Zwart, G., Hiorns, W., Methe, B., van Agterveld, M., Huismans, R., Nold, S., Zehr, J. and Laanbroek, H. (1998) Nearly identical 16S

rRNA sequences recovered from lakes in North America and Europe indicate the existence of clades of globally distributed freshwater bacteria. Syst. Appl. Microbiol. 21, 546–556.

[27] Suau, A., Bonnet, R., Sutren, M., Godon, J.-J., Gibson, G., Collins, M. and Doré, J. (1999) Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Appl. Environ. Microbiol. 65, 4799–4807.

[28] Wang, R.-F., Cao, W.-W. and Cerniglia, C. (1996) PCR detection and quantitation of predominant anaerobic bacteria in human and animal fecal samples. Appl. Environ. Microbiol. 62, 1242–1247.

[29] Wilson, K. and Blitchington, R. (1996) Human colonic biota studied by ribosomal DNA sequence analysis. Appl. Environ. Microbiol. 62, 2273–2278.

[30] Kocherginskaya, S.A., Aminov, R.I. and White, B.A. (2001) Analysis of the rumen bacterial diversity under two different diet conditions using denaturing gradient gel electrophoresis, random sequencing and statistical ecology approaches. Anaerobe 7, 119–134.

[31] Tajima, K., Aminov, R., Nagamine, T., Ogata, K., Nakamura, M., Matsui, H. and Benno, Y. (1999) Rumen bacterial diversity as determined by sequence analysis of 16S rDNA libraries. FEMS Microbiol. Ecol. 29, 159–169.

[32] Pryde, S., Richardson, A., Stewart, C. and Flint, H. (1999) Molecular analysis of the microbial diversity present in the colonic wall, colonic lumen, and cecal lumen of a pig. Appl. Environ. Microbiol. 65, 5372–5377.

[33] Tajima, K., Arai, S., Ogata, K., Nagamine, T., Matsui, H., Nakamura, M., Aminov, R.I. and Benno, Y. (2000) Rumen bacterial community transition during adaptation to high-grain diet. Anaerobe 6, 273–284.

[34] Willems, A., Moore, W., Weiss, N. and Collins, M. (1997) Phenotypic and phylogenetic characterization of some Eubacterium-like isolates containing a novel type B wall murein from human feces: description of *Holdemania filiformis* gen. nov., sp. nov. Int. J. Syst. Bacteriol. 47, 1201–1204.

[35] Jöborn, A., Dorsch, M., Christer, O., Westerdahl, A. and Kjelleberg, S. (1999) *Carnobacterium inhibens* sp. nov., isolated from the intestine of Atlantic salmon (*Salmo salar*). Int. J. Syst. Bacteriol. 49, 1891–1898.

[36] Macy, J.M. (1981) in: The Prokaryotes: A Handbook on Habitats, Isolation, and Identification of Bacteria, Vol. 2 (Starr, M., Stolp, H., Trüper, H., Balows, A. and Schlegel, H., Eds.), pp. 1450–1463. Springer-Verlag, New York.

[37] Salyers, A.A. (1984) *Bacteroides* of the human lower intestinal tract. Annu. Rev. Microbiol. 38, 293–313.

[38] Wood, J., Scott, K.P., Newbold, C.J. and Flint, H.J. (1998) Estimation of the relative abundance of different *Bacteroides* and *Prevotella* ribotypes in gut samples by restriction enzyme profiling of PCR-amplified 16S rRNA gene sequences. Appl. Environ. Microbiol. 64, 3683–3689.

[39] Salyers, A.A. and Shoemaker, N.B. (1997) in: Gastrointestinal Microbiology, Vol. 2 (Mackie, R.I., White, B.A. and Isaacson, R.E., Eds.), pp. 299–320. Chapman and Hall, New York.

[40] Ramšak, A., Peterka, M., Tajima, K., Martin, J.C., Wood, J., Johnston, M.E.A., Aminov, R.I., Flint, H.J. and Avguštin, G. (2000) Unravelling the genetic diversity of ruminal bacteria belonging to the CFB phylum. FEMS Microbiol. Ecol. 33, 69–79.