The Journal of Microbiology, December 2008, p. 599-607 Vol. 46, No. 6
DOI 10.1007/s12275-008-0174-3

Molecular Identification of Fecal Pollution Sources in Water Supplies by Host-Specific Fecal DNA Markers and Terminal Restriction Fragment Length Polymorphism Profiles of 16S rRNA Gene

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(Received July 11, 2008 / Accepted August 25, 2008)

Specific fecal DNA markers were investigated for major pollution sources, cow, human, and pig, and occurrence of the identified markers was analyzed in river waters using Terminal Restriction Fragment Length Polymorphism (T-RFLP) techniques and sequencing of 16S rDNA of *Bacteroides-Prevotella*. The unique and specific DNA markers for cow and human were identified as a 222 bp and 60 bp peak in *Hae*III T-RFLP profiles, respectively, and the pig-specific marker was not identified but the unique T-RFLP profile of pig could be used as a substitution. Human-specific marker was detected in most of the river waters tested (92.1%) and T-RFLP profiles of river waters were shown to be similar to those of human feces. Cluster analysis of T-RFLP data showed that the fecal sources were multiple (human plus cow and human plus dairy cow) in most of the river waters. The phylogenetic analysis for the clones recovered from the fecal and water samples showed that the clones from cow formed a discreet cluster from those of other sources. The other clones from human, pig, and river water formed two groups all together. The results of this study could be used to identify and control the fecal pollution source in the bodies of water in Korea.

***Keywords*:** 16S rDNA, *Bacteroides-Prevotella*, fecal DNA marker, T-RFLP

Since general fecal pollution indicators (coliforms, fecal coliforms, *E. coli*) exist in human and animals simultaneously, standard methods of fecal pollution in water could not tell their sources. In addition, there is still dispute about how fecal coliform group will survive and multiply after they are discharged into receiving waters (Davies *et al.*, 1995). *Bacteroides* are enteric bacteria and exist in the intestine tracts of human and animal with 1,000-fold higher density than coliform group, and therefore, from a numerical point, *Bacteroides* could serve as more sensitive fecal indicators than fecal coliform bacteria including *E. coli* (Fiksdal *et al.*, 1985). Moreover, they are Gram-negative obligate anaerobes demanding very strict nutrient condition and will not survive long outside the host, which makes them a good indicator of recent fecal pollution (Fiksdal *et al.*, 1985). Several studies have proposed that some species of *Bacteroides* should be source-specific. In other word, they are human-specific as they are the most abundant species in human feces and they present only at very low density in other animals (Allsop and Stickler, 1985; Fiksdal *et al*., 1985; Kreader, 1995). Because *Bacteroides* has very short survival time and is hard to cultivate after being discharged into natural watershed, it has been very difficult to use this species for the fecal source tracking. But with advent of molecular biological techniques such as Terminal Restriction Fragment Length Polymorphism (T-RFLP), it is more feasible to use a particular DNA of *Bacteroides* species as a source-specific molecular marker in water (Field *et al.*, 2003).

Fecal source tracking has been studied for ages in advanced countries and various methods have been used for this purpose, including molecular biological techniques, such as length heterogeneity-PCR (Bernhard and Field, 2000a), terminal restriction fragment length polymorphism (Bernhard and Field, 2000a; Sakamoto *et al.*, 2003), PCR with species specific primers (Bernhard and Field, 2000b; Matsuki *et al.*, 2002), multiplex PCR (Bonjoch *et al.*, 2004), real time PCR (Layton *et al.*, 2006), metabolic fingerprint (Ahmed *et al.*, 2005), denaturing gradient gel electrophoresis (Buchan *et al.*, 2001), subtractive hybridization (Dick *et al.*, 2005), fluorescence in situ hybridization (Dore *et al.*, 1998), pulsed field gel electrophoresis (Parveen *et al.*, 2001), and ribotyping (Carson *et al.*, 2001). In addition to the molecular techniques, some researchers utilized statistical methods to discriminate source-specific fingerprints using existence and nonexistence of some peaks in the molecular fingerprints like T-RFLP profiles (Wang *et al.*, 2004; Bower *et al.*, 2005; Fogarty and Voytek, 2005). Because of monitoring pressure to identify the origin of fecal pollution source, as exemplified by U.S. EPA's TMDL (Total Maximum Daily Load) program, there has been a steady increase in research on this subject (Stoeckel and Harwood, 2007). In Korea, coliforms and fecal coliforms have been used as fecal pollution

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indicators using various cultivation methods. The genetic fingerprinting technique such as T-RFLP was occasionally used to demonstrate microbial community (Kim *et al.*, 2004; Lee *et al.*, 2008). However, little attention has been given to the use of fecal molecular markers to track pollution sources in a watershed environment.

In this study, specific fecal DNA markers were analyzed for potential use as specific indicators of fecal pollution sources in water using molecular biological techniques. The main objectives of this study were (1) to identify source-specific DNA markers of cow, human, and pig by comparing their T-RFLP profiles for the 16S rDNA of obligate anaerobic fecal *Bacteroides-Prevotella*, (2) to analyze how these host-specific markers could be recovered in mixed sources and river waters, and (3) to investigate the occurrence of the *Bacteroides-Prevotella* group in river environment using cloning, sequencing of 16S rDNA, and phylogenetic analysis.

Materials and Methods

Sampling

Human fecal samples were taken from nine humans including adults and children for identification of host-specific 16S rDNA markers. Five each of cow, dairy cow, and pig fecal samples were collected from livestock farms in Icheon city, Gyeonggi-do. To recover the fecal *Bacteroides-Prevotella* 16S rDNA markers in natural waters, river water samples were taken from 14 sites of five rivers three times, February, April, and June 2005. The five rivers were Anseong, Jinwi, Hwangguji, Bokha, and Deokpung that are national water quality monitoring sites located in southern part of Gyeonggi-do. Two sites were selected from the Deokpung and three sites, upstream, midstream, and downstream, were chosen for others. The sources of fecal pollution of these rivers were expected to be mainly human and partly cow or pig.

DNA extraction

DNAs were extracted from the fecal samples using QIAamp stool DNA Mini kit (QIAGEN, USA) according to the manufacturer's recommendation. To collect microorganisms in the water samples, 300~1,000 ml of water was filtered using 0.22 μm pore size cylinder filter membrane (Sterivex-GP, Milipore) and a peristaltic pump (Somerville *et al.*, 1989). Approximately 1,000 ml of tap and distilled waters were filtered as above and used as negative controls. After filtration of the samples, 1 ml of CLS-TC buffer (Qbiogene, USA) was added to each filter and filters were stored at -20°C until processed.

PCR amplification

Equal portion of fecal DNAs from fecal sources was pooled to make samples representing human, cow, dairy cow, and pig. The pooled DNAs were mixed to reproduce multiple (human+cow, human+dairy cow, human+pig, cow+dairy cow, cow+pig, dairy cow+pig, human+cow+dairy cow+pig) fecal pollutions in the environmental water. PCR reactions for individual and pooled samples were performed to amplify 16S rRNA genes of *Bacteroides-Prevotella* groups from fecal and water samples with fluorescently labeled Bac32F; AACGCTAGCTACAGGCTT and Bac708R; CAATCGGAGTTCTTCGTG primers (Bernhard and Field, 2000a), yielding amplicons of approximately 690 bp.

Terminal restriction fragment length polymorphism (T-RFLP)

T-RFLP analysis was conducted on PCR product by digesting a 10 μl aliquot over 4 h at 37°C with 2.5 units of single restriction endonuclease *Hae*III and *Rsa*I (Bioneer) in the manufacturer's recommended buffer solutions. After digestion, the digested solution was purified to remove inhibitory salts using nucleotide removal kit (QIAGEN) according to the manufacturer's recommendation and approximately 50 ng of samples was dried down using a MicroVac concentrator to make pellets. An ABI 3100 DNA sequencer at Korea Ocean Research and Development Institute (KORDI) determined the precise lengths of peaks from the amplified 16S rDNA products. Results were analyzed using ABI prism GeneScan Analysis program version 3.7 (Applied Biosystem, USA).

T-RFLP data analysis

T-RFLP profiles were compared with each other to find the unique peak representing each source. To acquire the percentages of peaks in the T-RFLP profiles, the method developed by Dunbar *et al.* (2000) was used. The T-RFL of 50 to 690 bp in length and 50 fluorescence units in height were included in the analysis. The sum of all terminal restriction fragment heights in each replicate or triplicate T-RFLP profiles was calculated as a total DNA amount of each profile. The percentage of specific peak was calculated as height of specific peak divided by the total DNA height of profiles calculated as above.

Cluster analysis of T-RFLP data

T-RFLP data of *Hae*III digestion were analyzed for the presence/absence of peaks only by percent similarities as previously described (Wawrik *et al.*, 2005). A search window of ±0.5 bp was applied to match peaks between individual T-RFLP profiles. Similarity values were calculated as follows: $P_s = S_a/N_a$, where S_a is the number of peaks in sample A that are also found in sample B and N_a is the total number of peaks in sample A. Distance matrix was analyzed to draw a neighbor-joining tree using MEGA program (Version 4.0) to assess similarities among samples by means of cluster analysis.

16S rDNA sequencing and phylogenetic analysis

Equal portion of DNAs extracted from each fecal source was pooled to make three samples representing human, cow (cow+dairy cow), and pig. DNAs from river water samples were also pooled to represent five rivers tested. The pooled DNAs from fecal and water samples were amplified with non-labeled Bac32F and Bac708R primers. The PCR product was cloned into plasmid pCR2.1-TOPO using a TOPO TA PCR Cloning Kit (Invitrogen, USA) according to the manufacturer's recommendation. Positive colonies were selected and the inserted region was amplified with M13F and M13R primers. The PCR products were digested with *Hae*III and *Rsa*I restriction enzymes as in the T-RFLP analysis to select unique RFLP patterns. The RFLP patterns for each clone

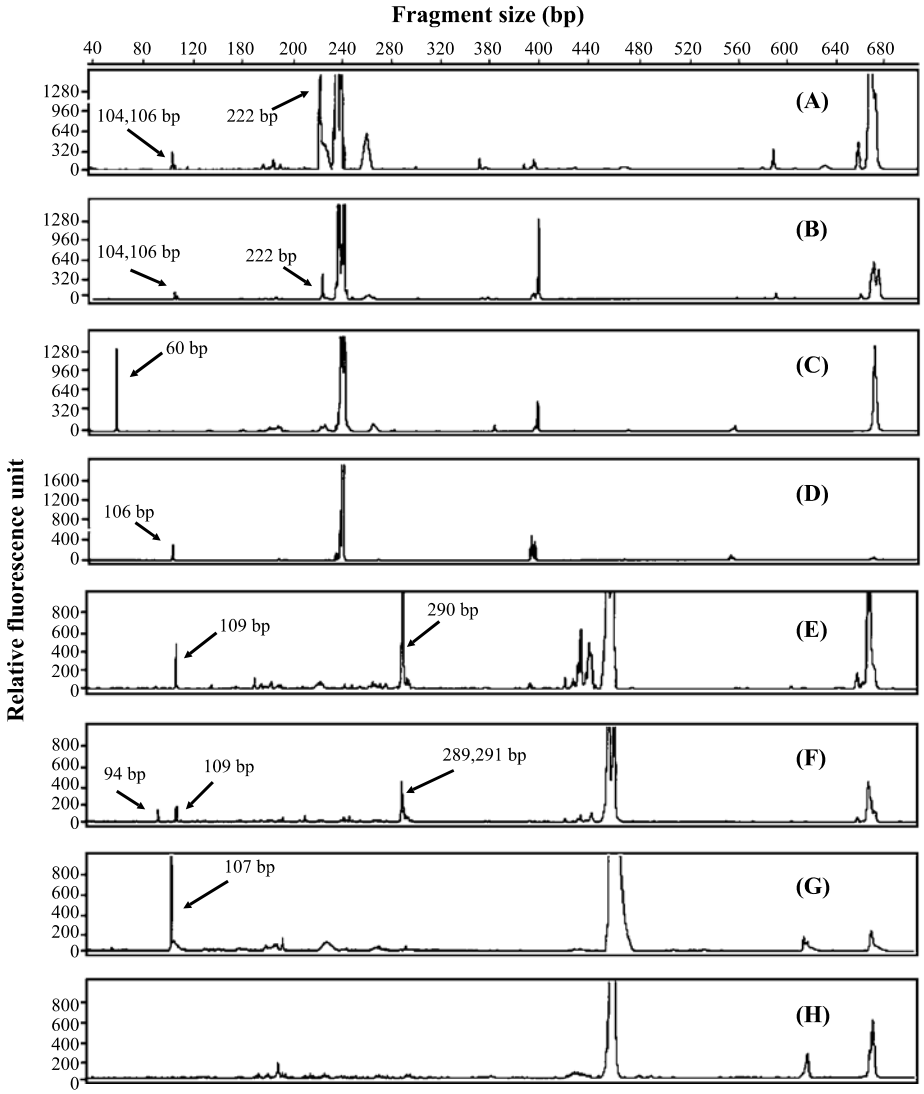

Fig. 1. Profiles of potential source-specific molecular markers in T-RFLP analyses of 16S rDNA fragments amplified with Bac32F-FAM and Bac708R followed by digestion with *Hae*III (A, B, C, D) and *Rsa*I (E, F, G, H). Cow (A, E); Dairy cow (B, F); Human (C, G); Pig (D, H).

were grouped visually, and representative clones were selected for sequencing. The PCR products of each representative clone were analyzed with ABI 3100 DNA sequencer using ABI prism BigDye Terminator Cycle sequencing V2.0 Ready Reaction with AmpliTaq DNA polymerase, FS at KORDI. Sequences of approximately 690 bases were compared with sequences available in the GenBank by using the NCBI-BLAST to determine their phylogenetic affiliation. A phylogenetic tree was built including type strains and sequences from previous studies using neighbor-joining method in the MEGA program (Version 4.0).

Results

Potential host-specific DNA markers for each source

To be a host-specific marker, a peak had to be present in all of the samples from the host and absent in all of the samples from the other sources. When source-specific markers were identified by digesting labeled PCR products with *Hae*III, a potential cow and dairy cow specific marker was found at 222 bp in the replicate of pooled and all individual cow and dairy cow feces, which accounted for approximately 5% of total DNA (Fig. 1A and B). A human specific peak was identified as a peak of 60 bp in the replicate of pooled and all individual samples that occupied 7∼8% of the total DNA (Fig. 1C). While a pig-specific DNA marker was not found in the *Hae*III T-RFLP profile, a peak of 106 bp observed from pig was in common with cow and dairy cow (Fig. 1D). When labeled PCR amplicons were digested with *Rsa*I endonuclease, promising cow-specific peaks were found at 109 bp and 290 bp in the replicate of pooled and 4 out of 5 individual samples that occupied 1.4% and 4.2% of total DNA, respectively, while dairy cow-specific markers were identified at 94 bp and 289∼291 bp in the replicate of pooled and all individual samples that accounted for 1.7% and 7.6% of total DNA, respectively (Fig. 1E and F). A

Table 1. Total coliforms and fecal coliforms[a] estimated by standard MPN method in the study sites

River	Site	February		April		June	
		Total Coliform	Fecal Coliform	Total Coliform	Fecal Coliform	Total Coliform	Fecal Coliform
Bokha	1	900	4	1600	22	17	7
	2	3000	110	5000	1100	7000	700
	3	230	20	9000	90	9000	90
Deokpung	1	9000	80	9000	20	40	20
	2	1700	110	500	110	110	40
Anseong	1	2200	40	40	20	1100	200
	2	16000	20	400	200	5000	200
	3	1600	6	1400	20	220	20
Jinwi	1	NA	NA	27	2	80	20
	2	3000	20	110	20	110	40
	3	16000	20	11000	200	8000	400
Hwangguji	1	16000	40	110000	14000	50000	7000
	2	13000	200	80000	2000	13000	2000
	3	300	20	16000	20	27000	2000

[a] All data are the means for two replicate measurements (MPN/100 ml).
NA, not available

human-specific marker was identified as a 107 bp peak in the replicate of pooled and 6 out of 9 T-RFLP profiles when digested with *Rsa*I (Fig. 1G). However, in case of pig, there were no significant peaks at all below fragment size of 400 bp in the *Rsa*I T-RFLP profile, which may be a unique pattern different from the others (Fig. 1H). The results suggested that fecal DNAs from 4 different sources, human, cow, dairy cow, and pig, could be discriminated by source-specific peaks and T-RFLP profiles obtained after restriction enzyme digestions.

Detection of source-specific fecal markers in the river waters

The PCR reactions for river water samples with primers targeting *Bacteroides-Prevotella* produced 11, 14, and 13 positive results out of each 14 water samples of February, April, and June, respectively. From this result, most of river water samples appeared to be polluted with feces, which was in agreement with relatively high MPN values for total coliform and fecal coliforms measured by cultivation methods (Table 1). The positive PCR products were analyzed to investigate fecal DNA markers identified in the above

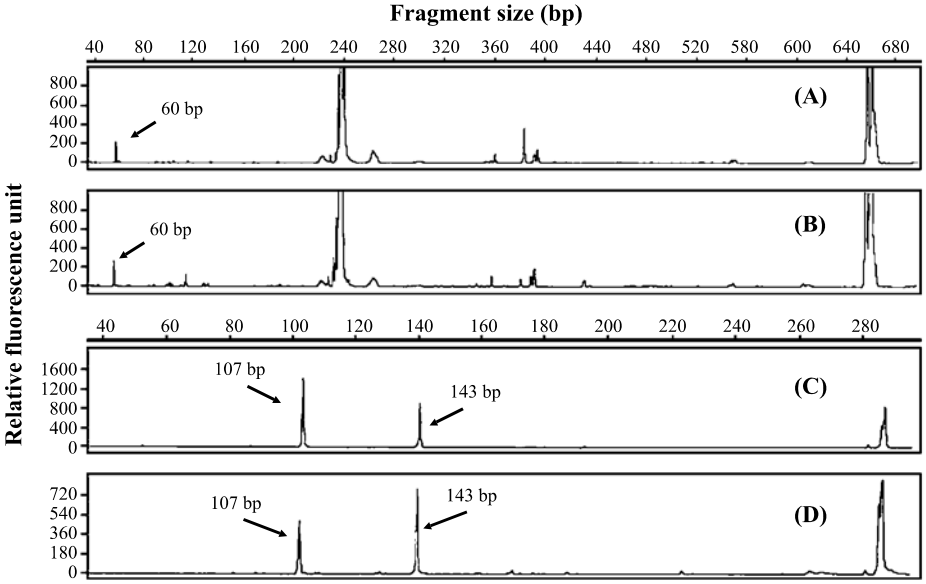

Fig. 2. Representative T-RFLP profiles of 16S rDNA fragments from Anseong 1 (upstream) and 2 (midstream) river waters (April) amplified with Bac32F-FAM and Bac708R followed by digestion with *Hae*III (A, B) and *Rsa*I (C, D).

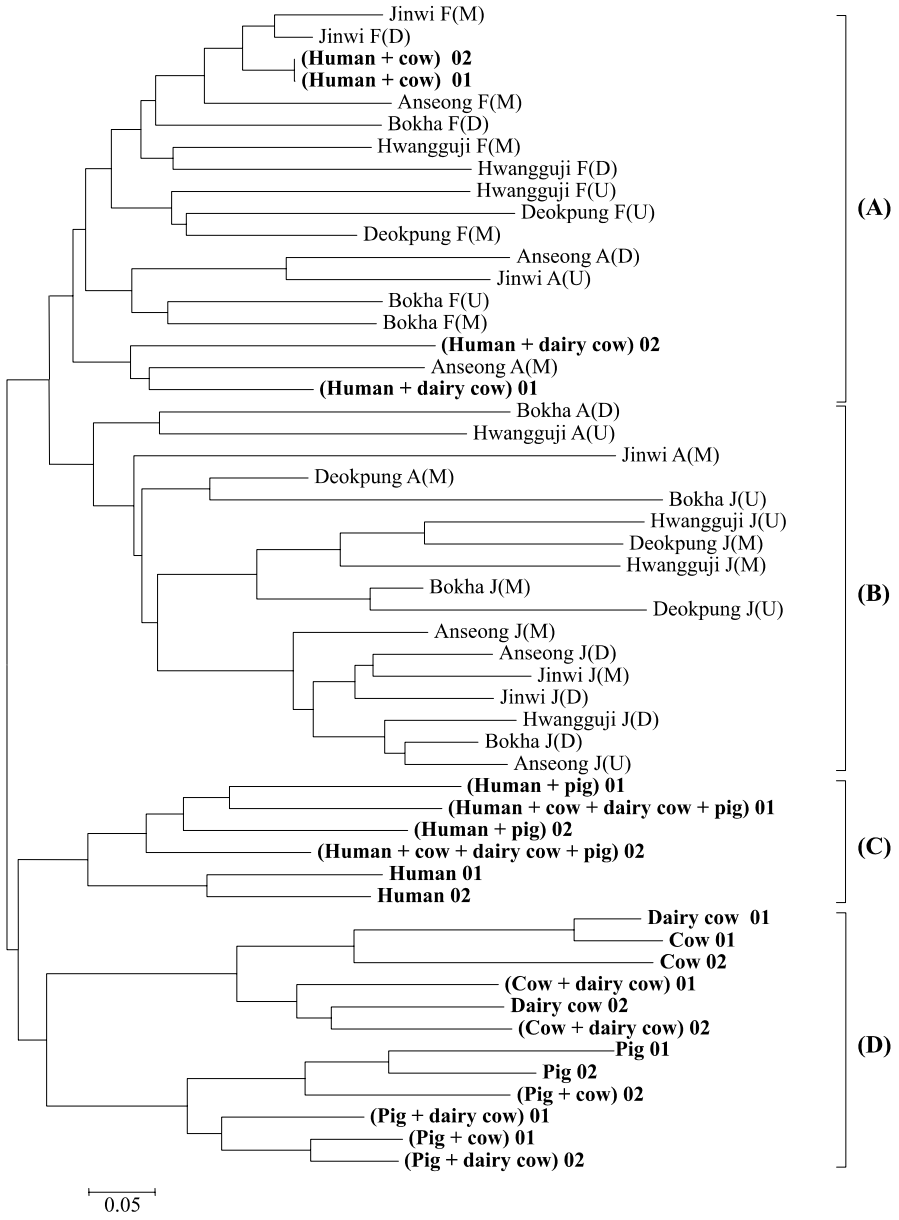

Fig. 3. T-RFLP dendrogram. Labels indicate the identity of single and multiple fecal sources and water sample where F (February), A (April), and J (June) are sampling time. U (Upstream), M (Midstream), and D (Downstream) are sampling sites for each river.

T-RFLP and species distribution of *Bacteroides-Prevotella* in the river water samples. Detection rates of the human-specific marker were 100% (11/11), 71% (11/14), and 100% (13/13) for February, April, and June, respectively. T-RFLP profiles of positive water samples were very similar to those of human feces, exhibiting the human-specific marker and major peaks except 143 bp peak (*Rsa*I-digest) from an unidentified source (Fig. 2). Interestingly two clones that have TRF of 60 bp and 143 bp in Fig. 4 were recently recovered from the human intestines (Eckburg *et al.*, 2005).

The proportions of the human specific marker, 60 bp peak (*Hae*III), and major peaks, 240 bp and 242 bp were calculated in water samples. In February, the *Hae*III T-RFLP profiles were generally similar among different water samples and the human fecal marker accounted 2.9% of total DNA on the average. The two major peaks, 240 bp (20%) and 242 bp (56%), which were presumed to originate from human and/or pig, occupied 76% of total DNA overall. The T-RFLP profiles of April samples were slightly different from those of February. Percentages of two major peaks decreased to 60%, 240 bp (33%) and 242 bp (27%), and the relative proportion of the 240 bp peak increased in April. In June, the human-specific marker was observed 1.6%, with 240 bp and 242 bp occupied 34% and 41%, respectively. Overall, the human fecal maker was detected in most of river water samples (92.1%) and 240 bp and 242 bp peaks were found to be the two major peaks as in the human fecal samples. From these results, it could be inferred that major

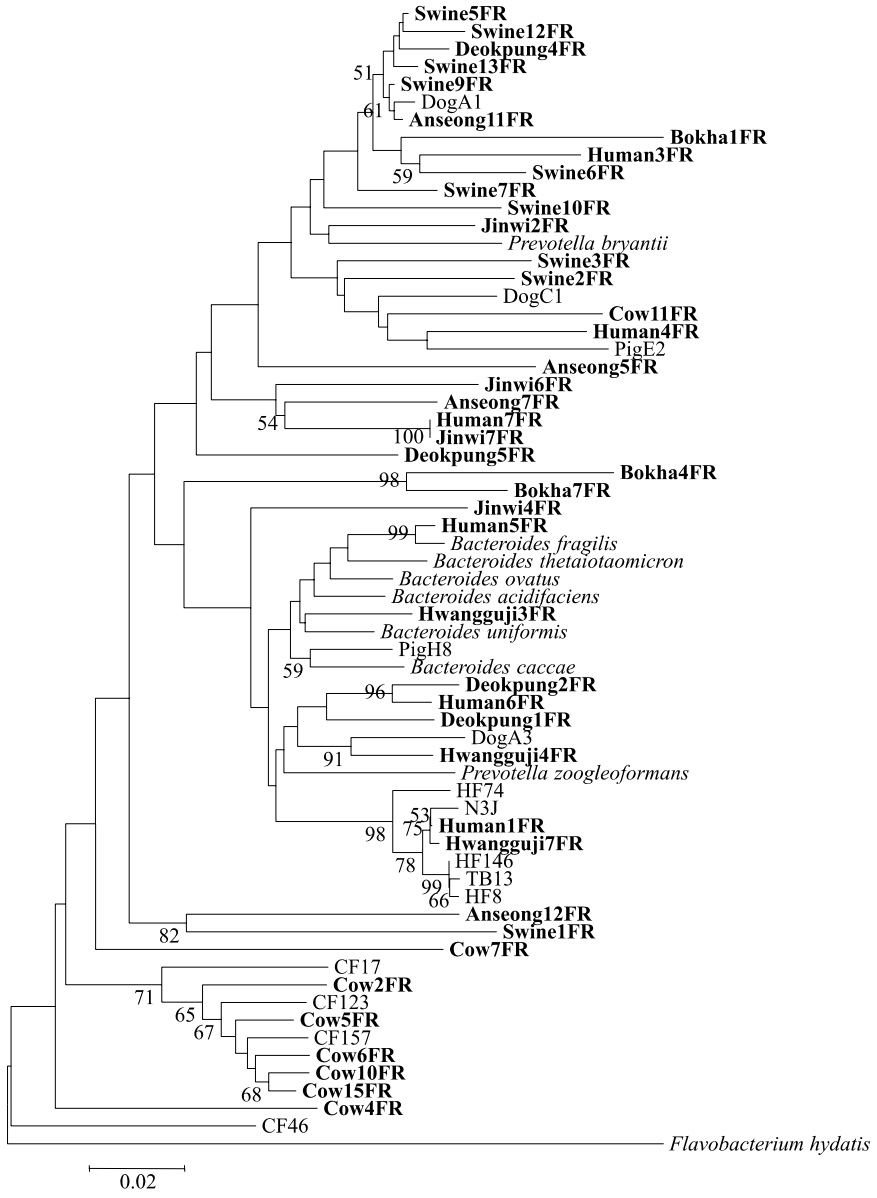

Fig. 4. Phylogenetic relationship among 16S rDNA sequences from this study (bold letters), previous study, and type strains (in italic). Bootstrap values less than 50% are not included. The tree was built by using the neighbor-joining method in MEGA program. *Flavbacterium hydatis* was used to root the tree. Each clone sequence from fecal sources was labeled as cow, human and swine. Clones from river water samples were named after rivers they were detected. HF (Human), CF (Cow), Pig, and Dog, etc. were clones identified in the previous studies.

fecal pollution source in the river waters examined was human. It was not certain whether the changes of relative ratios of 240 bp and 242 bp peaks were due to the different pollution sources or environmental factors, such as water temperature and predation.

Cluster analysis of T-RFLP profiles

Cluster analysis using percent similarities was performed using the T-RFLP profiles of fecal sources and river waters (Fig. 3). Four major clusters resulted from the analysis. Cluster A composed of river waters of February, April and human+cow or dairy cow feces. Cluster B contained water samples of April and June. Cluster C included all the mixed fecal samples that contained human feces. Cluster D is comprised of multiple sources containing cow, dairy cow, and pig. While source-specific markers and major peak profiles could only indicate that the river waters were mainly polluted with human feces, the cluster analysis of T-RFLP data told more about it. That is, river waters would be polluted with also other sources rather than only human feces. Because the T-RFLP profiles of replicate of same fecal source were closely related in the T-RFLP dendrogram, it was convinced that the T-RFLP analysis was pretty reproducible and reliable. The cluster analysis showed that the

river waters sampled on February and April might be polluted with multiple fecal sources (human+cow or dairy cow). It also could be inferred that the main fecal pollution source of April and June would be human+cow or dairy cow rather than human, cow, dairy cow, pig alone or cow+pig (Fig. 3). The fact that the water samples from February, April, and June formed different clusters maybe reflects seasonal variations or different combination of fecal sources.

Identification and phylogenetic analysis of clones recovered from fecal and water samples

To investigate species distribution of the *Bacteroides-Prevotella* group, 16S rDNA fragments PCR-amplified with Bac32F and Bac708R primers were cloned from the host feces and river waters and screened for T-RFLP source-specific pattern. Blast search indicated that the 16S rDNA sequences of 59 clones recovered from the fecal and water samples belonged to the *Bacteroides-Prevotella*g group (89∼100% similarities). Sequences from water samples were all similar (70∼100% similarities), but not identical, to clones from cow, human, and pig feces except Jinwi7FR that was 100% matched to Human7FR. Many 16S rDNA sequences of clones were shown to have the major peaks described in the previous section. For examples, Human4FR and Swine7FR were shown to have 240 bp peaks in *Hae*III-digests, and blast search indicated that these sequences were identical to the uncultured *bacterial* clones with 98% and 96% matches, respectively. Interestingly, three clones, Anseong3FR, Bokha5FR, and Hwangguji7FR, had 62 bp peaks (*Hae*III-digested) instead of the 60 bp peak, the human fecal marker. These three clones, Anseong3FR, Bokha5FR, and Hwangguji7FR, were 99%, 100%, and 93% identical to the partial 16S rDNA sequence of an uncultured bacterium, respectively. In addition, Anseong3FR, Bokha5FR, and Hwangguji7FR were found to have 455 and 457 bp terminal fragments that were major peaks of human and pig feces. In *Hae*III digests, some clones, such as Anseong12FR, Swine3FR, and Deokpung4FR, showed new peaks of 106, 110, and 394 bp, which were not detected in the previous section. In *Rsa*I digests, some clones, such as Jinwi2FR and Human6FR, also showed additional peaks of 96 and 110 bp, besides major peaks.

A phylogenetic tree was built using the clones of this study, previously known clones, and *Bacteroides* type strains (Fig. 4). One of the most distinct features in this phylogenetic tree was that most of clones from the cow formed a discrete group together with clones recovered from cow feces of previous studies. The other clones from human, pig, and river water formed two groups all together. The separate clustering of *Bacteroides-Prevotella* species in cow feces from those in the other sources may be related to the nutritional property of cow as a ruminant animal.

Discussion

DNA molecular fecal markers were identified for the three major fecal pollution sources, cow, human, and pig, and the specific DNA markers in the river waters were analyzed using T-RFLP of 16S rDNA of the *Bacteroides-Prevotella* group, obligate anaerobic fecal bacteria. When the PCR amplicons obtained with *Bacteroides-Prevotella* specific primers were digested with *Hae*III, host-specific DNA markers were identified as 60 and 222 bp peaks for human and cow, respectively, and no specific DNA marker was found for pig. In the previous study, an 119 bp peak, instead of the 60 bp peak, was reported to be a human specific fecal DNA marker, but the 222 bp peak as a cow specific maker was found to be the same (Bernhard and Field, 2000a). The 119 bp peak was not detected at any T-RFLP profiles in this study and some other previous researches, which may reflect a racial or dietary difference among people (Fogarty and Voytek, 2005). In *Rsa*I digests, T-RFLP analysis of the labeled PCR products showed a 107 bp human-specific fecal marker, 109 and 290 bp cow-specific peaks, and 94, 109, and 289∼291 bp dairy cow-specific markers, respectively. By contrast, pig had not any specific marker and even no significant peaks were observed below 400 bp in the *Rsa*I T- RFLP profile. In the previous study, Bernhard and Field reported that difference between fecal sources was not in existence and nonexistence of specific species but in composition of species (Bernhard and Field, 2000a). Fogarty and Voytek could not find the source specific fecal markers in their study and proposed a statistical method to identify the fecal pollution sources

Application of identified source-specific makers to river waters showed that the human fecal marker was widespread in almost all sites examined in this study. In addition, the proportions of major peaks and T-RFLP profiles in river waters were similar to those of human feces while there were seasonal variations. On the other hand, the cow specific marker was found only in Bokha2 and Hwangguji3 at April as a minor peak (0.2% of total amplified DNA). Our observation is in contrast with the previous report that the cow-specific marker detection frequency was much higher than the human-specific marker using source-specific primers (Shanks *et al.*, 2006). There have been several studies demonstrating the survival and detection by PCR of *Bacteroides* spp. in water environment. Fogarty and Voytek demonstrated that there was little change in the T-RFLP profiles after inoculation of fecal sample into pond water during 12 days at 18°C (Fogarty and Voytek, 2005). Kreader suggested that the detection of *Bacteroides* species by PCR is dependent on water temperature and predation (Kreader, 1998). For examples, *B. distaonis* was detected for 14 days at 4°C and only 1 to 2 days at 24°C. The water temperatures of February, April, and June were 0∼9°C, 11∼20°C, and 10∼29°C, respectively. The observation that the human-specific marker, but not cow-specific marker, was detected in almost all of the river waters suggested that human fecal pollution was predominant over fecal pollution by cow in river waters examined in this study.

The source identification by analysis of T-RFLP data obtained from an environmental water sample contaminated with single source was relatively straightforward (Fogarty and Voytek, 2005). In this study, we could tell that the most of river waters were contaminated with human feces using source specific DNA marker. But in many water environments, there would be multiple source rather than single. Cluster analysis using T-RFLP profiles of multiple fecal

sources and river water samples indicated that the river waters mainly were contaminated with human source but partly with other sources such as cow or pig. There have been debates on the usefulness and reproducibility of genetic fingerprinting techniques including T-RFLP to elucidate on microbial community analysis (Bent *et al.*, 2007; Pandey *et al.*, 2007). Despite several limitations of T-RFLP technique, many studies have confirmed that T-RFLP is a stout and reproducible methodology for the simple and rapid analysis of microbial community structure (Bernhard and Field, 2000a; Osborn *et al.*, 2000; Saikaly *et al.*, 2005). The size of source-specific fecal makers and profiles resulted from the T-RFLP were very reproducible in this study.

The 16S rDNA cloning, sequencing, and phylogenetic analysis of *Bacteroides-Prevotella* was performed to investigate distribution of the *Bacteroides* species in fecal and water samples. Only one clone, Bokha5FR, was 100% matched to a known sequence. Moreover, almost all of the clone sequences from fecal samples and river waters were different to each other except Jinwui7FR that were shown to have 100% sequence similarity to Human7FR that was from human source. These two identical clones were 93% matched to a known sequence and could be used as a target sequence for human source. Most of the clones were shown to have terminal restriction fragments of predominant peaks of T-RFLP profiles for fecal samples. Interestingly, most of clones from cow feces formed a discreet cluster with other cow fecal clones from the previous study (Bernhard and Field, 2000a), while the other clones from human, pig, dog, and river waters formed together a separate cluster. Primers targeting cow clones that formed a discreet cluster could be designed and utilized as a cow-specific marker to enhance detection sensitivity of cow fecal pollution.

In conclusion, the fecal pollution sources could be tracked with the source-specific molecular markers, proportions of major peaks, and T-RFLP profiles. Human and cow were shown to have unique DNA markers that could be used to determine the fecal pollution source. Pig had a distinctive T-RFLP profile that was different from the others. The information of this work on various DNA markers identified using proper restriction enzymes and primers for specific sources could be useful for rapid and accurate tracking of the fecal pollution sources in water supplies.

Acknowledgements

We thank Mr. Seung-Seob Bae for T-RFLP experiments. We thank Dr. Boris Wawrik for advice with the T-RFLP clustering analysis.

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