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Relative Abundance of *Bacteroides* spp. in Stools and Wastewaters as Determined by Hierarchical Oligonucleotide Primer Extension▽

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A molecular method, termed hierarchical oligonucleotide primer extension (HOPE), was used to determine the relative abundances of predominant *Bacteroides* spp. present in fecal microbiota and wastewaters. For this analysis, genomic DNA in feces of healthy human adults, bovines, and swine and in wastewaters was extracted and total bacterial 16S rRNA genes were PCR amplified and used as the DNA templates for HOPE. Nineteen oligonucleotide primers were designed to detect 14 *Bacteroides* spp. at different hierarchical levels (domain, order, cluster, and species) and were arranged into and used in six multiplex HOPE reaction mixtures. Results showed that species like *B. vulgatus*, *B. thetaiotaomicron*, *B. caccae*, *B. uniformis*, *B. fragilis*, *B. eggerthii*, and *B. massiliensis* could be individually detected in human feces at abundances corresponding to as little as 0.1% of PCR-amplified 16S rRNA genes. Minor species like *B. pyogenes*, *B. salyersiae*, and *B. nordii* were detected only collectively using a primer that targeted the *B. fragilis* subgroup (corresponding to ~0.2% of PCR-amplified 16S rRNA genes). Furthermore, Bac303-related targets (i.e., most *Bacteroidales*) were observed to account for 28 to 44% of PCR-amplified 16S rRNA genes from human fecal microbiota, and their abundances were higher than those detected in the bovine and swine fecal microbiota and in wastewaters by factors of five and two, respectively. These results were comparable to those obtained by quantitative PCR and to those reported previously from studies using whole-cell fluorescence hybridization and 16S rRNA clone library methods, supporting the conclusion that HOPE can be a sensitive, specific, and rapid method to determine the relative abundances of *Bacteroides* spp. predominant in fecal samples.

Approximately 10^{14} microbial cells reside in the human intestine and form close symbiotic associations with the host (18). Members of the genera *Bacteroides* and *Parabacteroides* in the order *Bacteroidales* are known to make up 14 to 40% of cultivable microorganisms in human feces (21). Species including *Bacteroides caccae*, *B. fragilis*, *B. eggerthii*, *B. uniformis*, *B. thetaiotaomicron*, *B. vulgatus*, *B. nordii*, *B. salyersiae*, *B. coprocola*, *B. massiliensis*, *B. intestinalis*, *Parabacteroides distasonis*, *P. merdae*, and *P. goldsteinii* have been successfully isolated from the human gut or human feces (3, 12, 23, 26, 38) and constitute up to 80% of the fecal bacterial isolates from the genus *Bacteroides* documented in Ribosomal Database Project II (http://rdp.cme.msu.edu).

Due to their high prevalence in the gut and fecal microbiota, *Bacteroides* spp. and *Parabacteroides* spp. have been considered to be clinically important anaerobes that are related to gastrointestinal well-being (13). For example, species like *B. vulgatus* and *B. caccae* can colonize the surface of the intestinal mucosa and inhibit the adherence of enteroinvasive pathogens (8, 18). Others like *B. thetaiotaomicron* and *B. ovatus* can break down a wide variety of indigestible polysaccharides to supply the host with 10 to 15% of the daily metabolic needs (35, 42). Besides relating to gastrointestinal functioning, certain *Bacteroides* spp. and *Parabacteroides* spp. are also suspected to be human gut specific and can act as biomarkers for identifying fecal pollutants from human and nonhuman sources (9, 27, 29). For instance, *P. distasonis*, *B. fragilis*, *B. thetaiotaomicron*, and *B. vulgatus* are reported to be 100- to 1,000-fold more abundant in human feces than in animal feces (27), while species in uncultivated animal-specific *Bacteroidales* clusters (Fig. 1) are seldom detected in human feces. Given the importance of *Bacteroides* spp. and *Parabacteroides* spp., determining the abundances of all or specific strains in the microbiota has become important for establishing a correlation to the health status of the host and for performing microbial source tracking.

At present, qualitative and quantitative analyses of *Bacteroidales* in feces-related samples are achieved mainly through cell cultivation and the use of conventional molecular tools (2, 4, 10, 19, 21, 33). Both approaches, however, have their strengths and weaknesses. The former can be time-consuming and favor the growth of selective species (40). The latter methods (e.g., the use of whole-cell fluorescence in situ hybridization [FISH], quantitative PCR [Q-PCR], and 16S rRNA gene clone libraries) can circumvent the issues related to cultivability but may also present other technical challenges. For example, FISH can be affected by the in situ accessibility of labeled probes through the cell membranes and the copies of rRNA inside inactive cells (7, 17). Clone library construction, with its resolution dependent on the total number of clones selected for analysis, can be time-consuming (15). Q-PCR can provide excellent quantitative detection sensitivity (down to a few copies of target fragments) but requires standard curves to be established for individual targets (24). Therefore, there is still a need to develop a direct and simple-to-use technique which can complement the existing molecular methods to rapidly evaluate the

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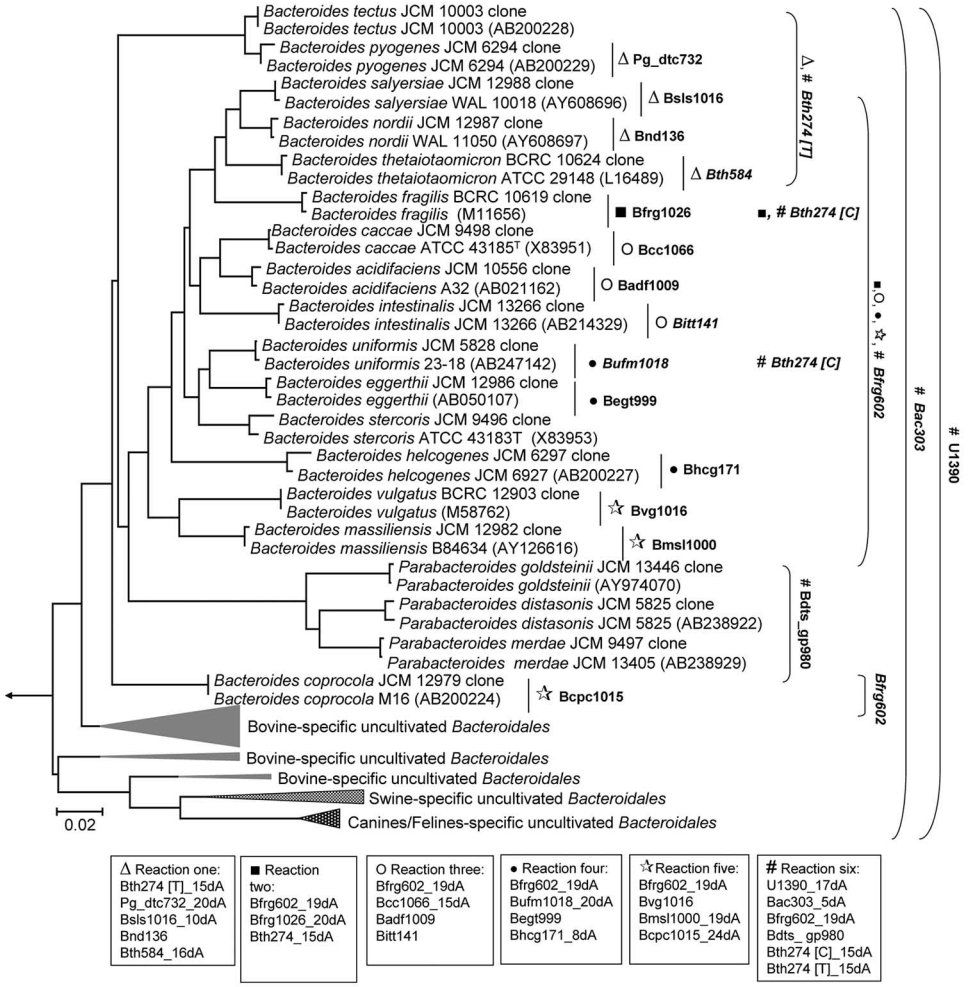

FIG. 1. Phylogenetic representation of different cultivated and uncultivated *Bacteroides* spp. In this study, a total of 19 primers were designed to target 14 functionally important *Bacteroides* spp. and *Parabacteroides* spp. at various taxonomical levels. The associated coverage of each primer is indicated on the right. Primers were arranged into six reaction tubes, with those targeting at the species level distributed across reaction tubes 1 to 5. Within reaction tubes 1 to 5, an associated higher-level primer, usually targeting not beyond the family level, was placed alongside. In the last reaction tube, all higher-level primers that were used in reaction tubes 1 to 5 (i.e., Bth274 and Bfrg602) were placed along with primers targeting at even higher taxonomical levels, like Bac303 and U1390.

relative abundances of *Bacteroides* spp. and *Parabacteroides* spp. present in complex microbial communities.

Recently, a molecular method termed hierarchical oligonucleotide primer extension (HOPE) has been developed to measure the relative abundances of multiple bacterial 16S rRNA genes among a total set of PCR-amplified 16S rRNA genes (41). In this method, multiple oligonucleotide primers that are designed with different lengths by the addition of poly(A) tails are used to target specific 16S rRNA gene sequences at different taxonomical levels (i.e., domain, phylum, order, and so on down to species). During the primer extension reaction, these primers anneal to their complementary target sequences and are subsequently extended with a fluorescently labeled dideoxynucleoside triphosphate (ddNTP) by DNA polymerase. The extended primers are identified by a DNA sequencer based on their fragment sizes and the type of the extended fluorescent ddNTP. Based on the fluorescence intensity detected, peak areas for extended specific primers are then determined, and the ratios of the areas to that for a common reference primer are calculated and used to determine the relative abundances of the bacterial targets of interest (Fig. 2).

In a previous study, a 10-plex HOPE reaction protocol targeting *Bacteroides* spp. at domain, group, and species levels was developed and applied to detect the relative abundances of *Bacteroides* spp. in the influent and effluent of a domestic wastewater treatment plant (41). It was successfully shown that HOPE could achieve single-mismatch discrimination and had the sensitivity to detect targets accounting for as little as 0.01 to 0.05% of the total set of PCR-amplified 16S rRNA genes (41). These promising results have further encouraged us to develop HOPE as a robust method to rapidly detect and measure the relative abundances of a total of 14 functionally important *Bacteroides* and *Parabacteroides* spp. at species, group, and domain levels in feces and wastewaters. To do so, we have added 12 primers specific for species in the genera *Bacteroides*

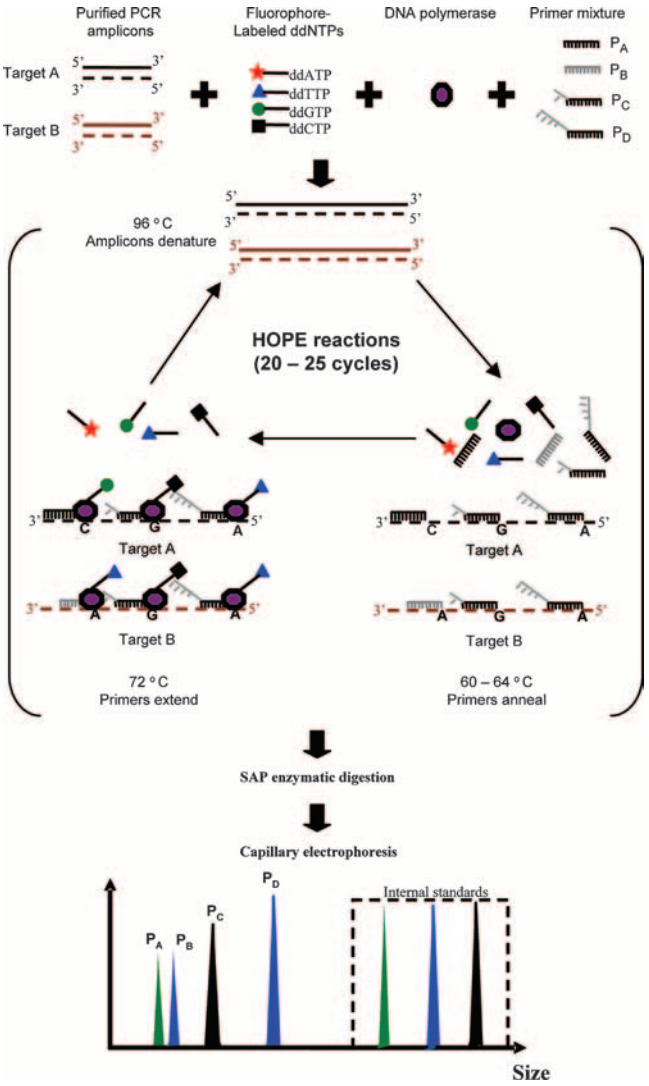

FIG. 2. Schematic illustration of HOPE. PCR amplicons, fluorophore-labeled ddNTPs, DNA polymerase, and oligonucleotide primers were mixed together to form a HOPE reaction mixture. The mixture underwent a thermal program involving 20 to 25 cycles of denaturation (96°C), annealing (60 to 64°C), and single-base extension (72°C). The products were purified and the excess ddNTP residue was removed by shrimp alkaline phosphatase (SAP) digestion. An appropriate amount of the product mixture was mixed with synthetic oligonucleotide standards and then subjected to capillary electrophoresis. From the electropherograms, the extended primers were recognized by means of consistent fragment sizes and the colors of the extended ddNTPs. P_A to P_D, primers A to D.

and *Parabacteroides* (Fig. 1 and Table 1). To further validate the ability of HOPE to determine the relative abundances of bacterial targets, we have compared the HOPE results with results from conventional molecular methods like Q-PCR.

MATERIALS AND METHODS

Bacterial strains and environmental samples. Nineteen bacterial reference strains obtained from the Japan Collection of Microorganisms (Wako, Japan) or the Bioresources Collection and Research Centre (Hsinchu, Taiwan) were used (Table 1). These bacterial strains are classified into the *B. fragilis* group (i.e., *B. uniformis*, *B. helcogenes*, *B. stercoris*, *B. caccae*, *B. acidifaciens*, *B. fragilis*, *B. thetaiotaomicron*, *B. vulgatus*, *B. coprocola*, *B. massiliensis*, *B. eggerthii*, *B. nordii*, *B. salyersiae*, *B. intestinalis*, and *B. ovatus*) and the *P. distasonis* group (i.e., *P. distasonis*, *P. merdae*, and *P. goldsteinii*). Within the *B. fragilis* subgroup, bacterial strains can be further classified into the subcluster for which primer Bth274 extended with nucleotide T (the Bth274 [T]-related subcluster, i.e., *B. pyogenes*, *B. tectus*, *B. nordii*, *B. salyersiae*, and *B. thetaiotaomicron*) and the subcluster for which primer Bth274 extended with nucleotide C (the Bth274 [C]-related subcluster, i.e., *B. uniformis* and *B. fragilis*). Fecal samples were collected from (i) three healthy donors aged 26 to 32 years old, (ii) three healthy pigs, and (iii) three healthy cows. Environmental samples were collected from the influents of a municipal treatment plant located in Singapore and a swine wastewater treatment plant in Tainan, Taiwan. Samples were collected and kept at −20°C prior to DNA extraction.

DNA extraction. Total DNA from pure cultures and influent samples was extracted according to a previously described protocol with minor modifications (36). Total DNA from fecal samples was extracted using a QIAamp DNA stool mini kit (Qiagen) (32).

PCR amplification of 16S rRNA genes. Each PCR mixture (50 μl in volume) contained 50 to 100 ng of genomic DNA in 1× TaKaRa *Ex Taq* buffer, 200 nM (each) forward primer (11F; 5′-GTT TGA TCC TGG CTC AG-3′ [25]) and reverse primer (1512R; 5′-GGC TAC CTT GTT ACG ACT T-3′ [28]), 200 mM deoxynucleoside triphosphate, and 0.5 U of *Ex Taq* DNA polymerase (TaKaRa). The optimal number of thermal cycles required to obtain a proportional amplification of the DNA of the microbial community was examined by varying the cycle number of the thermal program (denaturation, 95°C for 30 s; annealing, 55°C for 45 s; and extension, 72°C for 60 s) from 10 to 35 cycles at an increment of 5 cycles. Amplicons obtained at each cycle were concentrated and purified using a QIAquick PCR purification kit (Qiagen), and the concentrations were determined by UV absorbance measurement using a DU730 spectrophotometer (Beckman Coulter).

Cloning and sequencing. To ensure culture purity, 16S rRNA genes of reference bacterial strains were separately cloned into the pCRII vector by using the TA cloning kit (Invitrogen Corporation, Carlsbad, CA). For each clone library, 20 colonies were selected, and the presence of DNA insertion was confirmed by direct PCR amplification with M13R and M13F primers. The inserted DNA fragment was then sequenced using an ABI PRISM 3130 genetic analyzer (Applied Biosystems) and a BigDye Terminator sequencing kit (Applied Biosystems). The sequences were compared against the entries in the 16S rRNA gene database by using BLAST software (1).

Species-specific primer design. A total of 12 new primers specifically targeting different human-specific *Bacteroides* spp. were designed using the probe design function of ARB software (30). An updated ssu_jan04.arb database (http://www.arb-home.de), which contained 28,289 nearly complete 16S rRNA sequences (with lengths of >1,450 nucleotides [nt]), was used together with 189 aligned sequences of cultivated *Bacteroides* spp. obtained from Ribosomal Database Project II and nearly complete 16S rRNA sequences of the 19 reference bacterial strains. To enhance the specificity of HOPE primers, each primer was designed with mismatch bases corresponding to nontargets located at the 3′ terminus. The specificity of the designed primers was verified first in silico against entries in the Ribosomal Database Project II database and subsequently in HOPE reactions against DNA from all reference strains to ensure that primers did not extend when DNA from nontarget bacterial strains was used as the DNA template.

Hierarchical primer arrangement. Table 1 lists the names, sequences, and specificities of the 19 different HOPE primers used in this study. These primers were assigned to six different multiplex HOPE reaction mixtures based on the phylogenetic affiliations of the bacterial targets (Table 1 and Fig. 1). Reaction mixtures 1 to 5 contained one of two higher-level primers that targeted both the *B. fragilis* group and its subgroups (i.e., primers Bfrg602 and Bth274), together with 14 species-specific primers. Thus, the abundance of the individual bacterial target targeted by each species-specific primer relative to those of targets targeted by higher-level primers could be determined. With reaction mixture 6, the relative abundances of *Bacteroidales*, the *B. fragilis* group and its subgroups, and the *Parabacteroides* group as represented by Bac303, Bfrg602, Bth274, and Bdts_gp980 relative to the total community as represented by all the 16S rRNA gene amplicons could be determined. To clearly differentiate extended primers in the same HOPE reaction mixture, primers that extended with the same ddNTP were further modified with poly(A) tails of different lengths (5 to 24 nt) at the 5′ ends.

Internal standard oligonucleotide mixture. The internal standard oligonucleotide mixture contained four oligonucleotides of different lengths [5′-$(GT)_x$-3′, where *x* equals 18, 20, 22, and 24], which were synthesized and labeled at the 5′ ends with four different fluorophores (i.e., dR110, dR6G, deoxy-6-carboxytetramethylrhodamine [dTAMRA], and deoxy-6-carboxy-X-rhodamine [dROX]; Ap-

TABLE 1. HOPE primers designed to target human-specific *Bacteroides* spp. at various hierarchical levels (species, group, order, and domain levels)

Primer	Target	Sequence (5′–3′)	Length of poly(A) tail (nt)	Type of ddNTP added	HOPE reaction tube no.	Reference
Bnd136	*B. nordii* (JCM12987)	AGC CTA TCC CCG AGT AAA A	0	G	1	This study
Bsls1016	*B. salyersiae* (JCM12988)	GCC TTG CGG CTA TGC CG	10	T	1	This study
Pg_dtc732	*B. pyogenes* (JCM6294)	GTT CCG GCC CGG TGA GCT	20	G	1	This study
Bth584	*B. thetaiotaomicron* (BCRC10624)	CAA CTG ACT TAA CTG TCC AC	16	C	1	41
Bfrg1026	*B. fragilis* (BCRC10619)	TCA CAG CGG TGA TTG CTC	20	A	2	This study
Bcc1066	*B. caccae* (JCM9498)	CGT ATG GGT TTC CCC ATA A	15	T	3	This study
Badf1009	*B. acidifaciens* (JCM10556)	CGG CTA ACA TGT TTC CAC	0	A	3	This study
Bitt141	*B. intestinalis* (JCM13266)	CGA AAG GCT ATC CCG GAA	0	T	3	41
Begt999	*B. eggerthii* (JCM12986)	GTT TCC ACT ACA TTC CGC	0	T	4	This study
Bhcg171	*B. helcogenes* (JCM6297)	TTT CAG TGC CAT CGG GCA T	8	T	4	This study
Bufm1018	*B. uniformis* (JCM5828)	CTG CCT TGC GGC TGA CA	20	T	4	41
Bvg1016	*B. vulgatus* (BCRC12903)	ATG CCT TGC GGC TTA CGG C	0	T	5	This study
Bcpc1015	*B. coprocola* (JCM12979)	CGC CTT GCG GCT TAC AAG T	24	T	5	This study
Bmsl1000	*B. massiliensis* (JCM12982)	GCG TTT CCG CCA TAT TCG G	19	T	5	This study
Bdts_gp980	*P. distasonis* (JCM5825) *P. merdae* (JCM9497) *P. goldsteinii* (JCM13446)	CGT TCA AAC CCG GGT AA	12	G	6	This study
Bth274	*B. fragilis* subgroups	CCC CTA TCC ATC GAA GG	15	C or T	6, 2, 1	41
Bfrg602	*B. fragilis* cluster	GAG CCG CAA ACT TTC ACA A	19	C	6, 5, 4, 3, 2	19
Bac303	Most *Bacteroidales*	CCA ATG TGG GGG ACC TT	5	C	6	31
U1390	Most *Bacteria*	YGA CGG GCG GTG TGT	17	A	6	43

plied Biosystems). The concentrations of individual oligonucleotides at 260 nm were measured and subsequently diluted to 3 nM for 5′-dR110-$(GT)_{20}$.3′, 15 nM for 5′-dTAMRA-$(GT)_{22}$.3′, 18 nM for 5′-dROX-$(GT)_{24}$.3′, and 5 nM for 5′-dR6G-$(GT)_{18}$.3′.

HOPE reactions. Unless stated otherwise, each HOPE reaction mixture (in total, 5 μl) contained 2.5 μl of SNaPshot premix, 5 to 30 fmol of the DNA template, 10 to 60 pmol of oligonucleotide primers (Sigma-Proligo, Singapore, and Mission Biotech, Taiwan), and various amounts of deionized water. The SNaPshot premix consisted of DNA polymerase, ionic buffer, and fluorescently labeled dideoxynucleotides (dROX-ddTTP, dTAMRA-ddCTP, dR110-ddGTP, and dR6G-ddATP). The HOPE thermal program consisted of 20 cycles of denaturation (96°C for 10 s), annealing (64°C for 30 s), and extension (72°C for 15 s). After the primer extension reaction, 1 U of shrimp alkaline phosphatase (Roche Applied Science, Penzberg, Germany) was added and the mixture was incubated at 37°C for 60 min in order to remove unincorporated dye terminators. The reaction was terminated by heating at 75°C for 10 min.

Capillary electrophoresis. HOPE products (0.5 to 1 μl) were mixed with 0.125 μl of GeneScan Liz 120 standard (Applied Biosystems), 0.25 μl of the standard oligonucleotide mixture, and 12 μl of Hi-Di formamide (Applied Biosystems). The electrophoresis program included a denaturation step (60°C), an injection step with an applied voltage of 1.0 to 2.1 kV for 12 to 40 s, and a separation step. Fluorescence data were subsequently analyzed by the fragment analysis software GeneMapper, in which the fragment sizes and peak areas for the extended primers and internal standard oligonucleotides were recorded.

Calculation of relative abundances of HOPE products. The concentrations of extended primers were quantified as follows:

$$\frac{\text{Peak area for extended primer}}{(C_p)(\text{volume of injected sample})} = \frac{\text{Peak area for associated internal oligonucleotide}}{(C_{std})(\text{volume of internal oligonucleotide})} \quad (1)$$

where C_p represents the molar concentration of a specific primer extended in the HOPE reactions for the samples and C_{std} represents the molar concentration of the associated standard oligonucleotide.

Calibration factors (CFs) for each specific primer with respect to higher-level primers could be obtained using the M13 amplicons from associated reference strains as templates and calculating as follows:

$$CF_{A-B} = \frac{C_A}{C_B} \quad (2)$$

where primer B targets a higher hierarchical level than primer A, CF_{A-B} is the calibration factor for primer A with respect to primer B, C_A is the concentration of extended primer A, and C_B is the concentration of extended primer B. The relative abundances of 16S rRNA gene amplicons targeted by primer A with respect to those of amplicons targeted by primer B can then be calculated as follows:

$$\text{Relative abundance of target (\%)} = \frac{C_A}{C_B \times CF_{A-B}} \times 100 \quad (3)$$

Quantification of *Bacteroides* spp. with Q-PCR. Standard curves used for the detection of *Bacteroides* spp. at different taxonomical levels were obtained using replicates with 400 nM of forward and reverse primers (Table 2), 1× iQ SYBR green mastermix (Bio-Rad), and various concentrations (1 to 10,000 pg per 25 μl of reaction mixture) of DNA from two independent reference strains (*B. fragilis* BCRC10619 and *B. uniformis* JCM5825). Forty cycles of the thermal program (denaturation at 96°C for 10 s, annealing at 64°C for 30 s, and extension at 72°C for 30 s) were performed with iCycler (Bio-Rad). Standard curves were generated by plotting the concentration of the genomic DNA template against mean numbers of threshold cycles. Similarly, real-time quantitative measurement of *Bacteroides* spp. present in human fecal samples was subsequently performed using replicates with 1,000 pg of genomic DNA.

Nucleotide sequence accession numbers. The sequences determined in this study have been deposited in the GenBank database under the accession numbers EU136678 to EU136697.

RESULTS

PCR amplification. PCR amplification was first examined at various cycle intervals to determine the appropriate number of thermal cycles needed to generate amplicons that were representative of the actual microbial community. With 2 ng of genomic DNA/μl as the starting template in PCR, it was observed that after 15 to 20 thermal cycles, the amplicons obtained were proportionally amplified and the use of these amplicons for downstream analyses could minimize the effect of PCR bias. After 20 to 35 cycles of PCR, the amplification curve deviated from the exponential shape and underwent a quasi-linear phase. Amplification eventually reached a plateau beyond 35 cycles, as the amount of accumulated amplicons could not be increased further (Fig. 3). Thus, amplicons that under-

TABLE 2. Primers targeting *Bacteroides* spp. at various taxonomical levels that were used in Q-PCR to validate HOPE results[a]

Target	Primer	Primer sequence (5′–3′)	Standard curve	Amplification efficiency (%)
Total *Bacteria*	1390F 1512R	ACA CAC CGC CCG TCR GGC TAC CTT GTT ACG ACT T	$y = -3.309x + 26.069$	100.5
Bacteroides-Prevotella	Bac303F 530R	AAG GTC CCC CAC ATT GG CCG CGG CKG CTG GCA C	$y = -3.306x + 24.345$	100.7
B. fragilis cluster	Bfrg602F 797R	TTG TGA AAG TTT GCG GCT C GGA CTA CCA GGG TAT CTA ATC CTG TT	$y = -3.4043x + 25.999$	96.7
P. distasonis cluster	Bdts_gp980F 1055R	TTA CCC GGG TTT GAA CG CAR CCA TGC AGC ACC	$y = -3.375x + 29.742$	97.8
Bth274[C]- and Bth274[T]-related subclusters	Bth274F 530R	CCT TCG ATG GAT AGG GG CCG CGG CKG CTG GCA C	$y = -3.565x + 28.925$	90.8

[a] Appropriate reverse primers were chosen to result in an average amplicon size of less than 250 bp.

went 20 thermal amplification cycles were subsequently used as the DNA templates for all HOPE reactions in this study.

Specificity of primer extension. The specificities of primer extension were validated against reference strains. Results showed that all species-specific primers correctly extended with the same nucleotide as the in silico analysis predicted when DNA from their targeted species was used as the DNA template. Likewise, no species-specific primers were extended when nontargets were used. Group-specific primer Bth274 extended with nucleotide C in the presence of *B. uniformis* and *B. fragilis* and with nucleotide T in the presence of *B. tectus*, *B. pyogenes*, *B. nordii*, *B. salyersiae*, and *B. thetaiotaomicron*. Bfrg602, which was designed to target *Bacteroides* spp. in the *B. fragilis* cluster, extended with nucleotide C, and Bdts_gp980, which was designed to target the *P. distasonis* group, correctly extended with nucleotide G. Bac303 and U1390 were designed to target all reference bacterial strains at the order and domain levels, respectively, and extended with C and A, respectively (Table 1). However, minimal extension of the Bac303 primer in the presence of *P. goldsteinii* JCM13446 was observed. Resequencing of the 16S rRNA gene of *P. goldsteinii* revealed that the target sequence region had a single mismatch relative to Bac303 at the fifth position from the 3′ end of the primer and that this mismatch could significantly reduce the primer extension efficiency in comparison to that obtained with the perfect-match sequence (37). Since *P. goldsteinii* is not a predominant member in the fecal microbiota, its mismatch to Bac303 should not affect the measurement of the relative abundances of *Bacteroidales* in the fecal microbiota as represented by the total set of PCR-amplified 16S rRNA genes.

Calculation of CFs. CFs were established in the presence of 16S RNA gene amplicons derived from the bacterial reference clone. For instance, when the *B. thetaiotaomicron* 16S rRNA gene amplicon was used as the HOPE template, only primers in reactions mixtures 1 and 6 were extended, and they were correctly identified by size and the type of the extended fluorescent ddNTP (Fig. 4A). Peak areas for individual primers Bth584 and Bth274 [T] in reaction 1 and Bth274, Bfrg602, Bac303, and U1390 in reaction 6 were noted, and the individual extended-primer concentrations were calculated. The concentration of the extended *B. thetaiotaomicron*-specific primer (i.e., primer Bth584) was normalized against that of the higher-level primer in the same reaction mixture (i.e., Bth274 [T]) to obtain the CF (i.e., $CF_{Bth584-Bth274\ [T]}$). By repeating this procedure for all tested reference bacterial strains, CFs for all primers were obtained (Table 3). These values were subsequently used for calculating the relative abundances of bacterial targets present in the fecal and wastewater samples.

Electropherograms for tested samples. Figure 4B shows the electropherograms obtained after HOPE was carried out in the presence of the total set of 16S rRNA gene amplicons derived from human stool sample H3. As higher-level primers had more targets for priming, the peak heights and areas were considerably larger than those corresponding to species-specific primers. Furthermore, as primers should be extended at a specific fragment size, all other peaks that did not coincide with the anticipated size could be treated as background noise and removed from subsequent analyses.

Relative abundances of *Bacteroides* spp. in feces as determined by HOPE. Table 3 and Fig. 5 describe the relative abundances of the 14 tested *Bacteroides* spp. present in the three tested human feces samples at various taxonomical levels. At the higher taxonomical levels, the relative abundances of the *Bacteroidales* group targeted by the Bac303 primer corresponded to a range from 28.1 to 44.4% of PCR-amplified 16S rRNA genes. The *B. fragilis* Bth274 [C]-related subcluster (including *B. uniformis* and *B. fragilis*) accounted for 0.8 to 1.3% of amplified genes, while the *B. fragilis* Bth274 [T]-related subcluster (including *B. thetaiotaomicron*, *B. tectus*, *B. caccae*, *B. pyogenes*, *B. nordii*, and *B. salyersiae*) accounted for 0.2 to 1.1% of amplified genes. The abundance of the *B. fragilis* cluster, which varied across human samples, corresponded to a range from 5.0 to 12.1% of PCR-amplified 16S rRNA genes.

Within the *B. fragilis* group, *B. vulgatus* was observed to be the predominant species (accounting for 14.8 to 49.8% of amplified 16S rRNA genes from the *B. fragilis* group), and *B. caccae* and *B. uniformis* were present at various relative abundances (corresponding to 1.3 to 8.9% of amplified genes) in microbiota from different human fecal samples. Other species, like *B. fragilis*, *B. eggerthii*, *B. intestinalis*, and *B. massiliensis*, were detected to account for between 0.6 and 3.1% of amplified genes from samples H2 and H3, but their levels were too

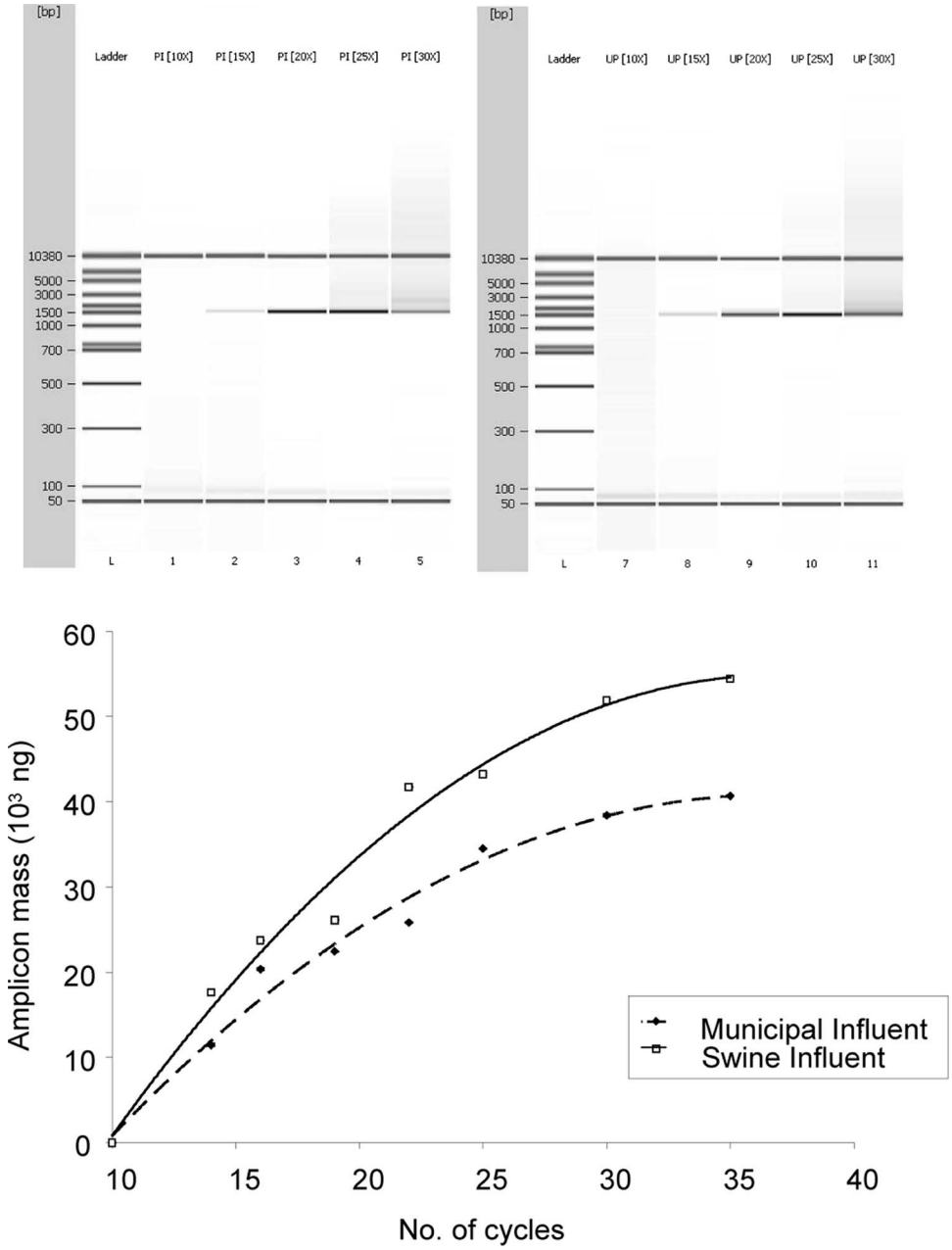

FIG. 3. Graphical representation of PCR amplicon masses obtained with different numbers of thermal cycles. Exponential amplification was achieved from cycles 10 to 20, and a plateau was reached after 20 cycles. To minimize PCR bias that can affect the determination of the relative abundances of bacterial targets, the total set of PCR-amplified 16S rRNA genes was obtained after 20 cycles of PCR amplification, purified, and then used for HOPE. Lanes 1 and 7 represent the DNA size ladder. Lanes 2 to 6 show the PCR amplicons obtained from the piggery wastewater influent after 10, 15, 20, 25, and 30 cycles, respectively. Lanes 8 to 12 show the PCR amplicons obtained from the municipal wastewater after 10, 15, 20, 25, and 30 cycles, respectively.

low to be detected in sample H1. In total, these targeted *Bacteroides* spp. accounted for 26.6 to 62.6% of amplified 16S rRNA genes from the *B. fragilis* group. These observations further suggest that other important and unknown *Bacteroides* spp. from this group were present in the human fecal microbiota (Fig. 5B). Within the *B. fragilis* Bth274 [T]-related subcluster, *B. thetaiotaomicron* was detected as the predominant and major species (58.9 to 97.5%) (Table 3).

In contrast, with the swine and bovine fecal microbiota, none of the 14 species-specific primers extended, suggesting that the *Bacteroides* spp. examined in this study were present at abundances below the detection sensitivity of HOPE. These results further suggest that the *Bacteroides* spp. examined in this study are probably host-specific to human fecal microbiota. In the animal fecal microbiota, most *Bacteroidales* could account for only 4.6 to 5.2% and 1.8 to 2.9% of PCR-amplified 16S rRNA

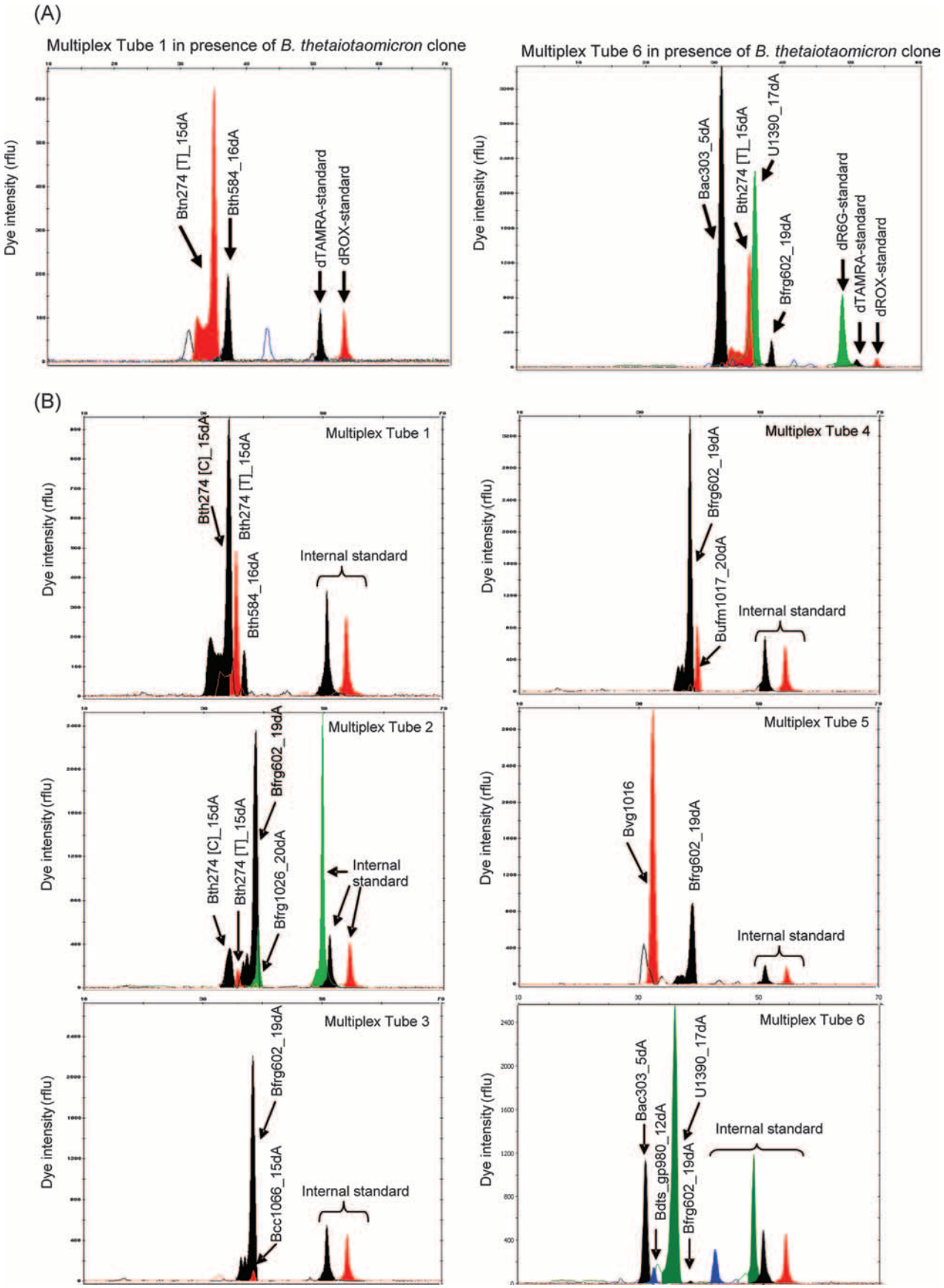

FIG. 4. Actual electropherograms for HOPE products obtained in the presence of the total set of PCR-amplified 16S rRNA genes from the *B. thetaiotaomicron* BCRC10624 clone (A) and human feces sample H3 (B). Black, red, and green peaks represent primers that were extended with dTAMRA-ddCTP, dROX-ddTTP, and dR6G-ddATP, respectively. rflu, relative fluorescence units.

TABLE 3. Relative abundances of bacterial targets[a]

Group	Species	Relative abundance (%) of bacteria of indicated species in sample[e]:										
		H1	H2	H3	P1	P2	P3	C1	C2	C3	Municipal WW	Pig WW
Bfrg602-related group	*B. fragilis*	ND[d]	3.1 ± 0.1	2.9 ± 0.2	ND	ND	ND	ND	ND	ND	3.6 ± 0.3	ND
	B. caccae	8.9 ± 1.5	1.3 ± 0.8	1.6 ± 0.2	ND	ND	ND	ND	ND	ND	2.7 ± 0.4	ND
	B. acidifaciens	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	B. intestinalis	ND	ND	1.2 ± 0.3	ND	ND	ND	ND	ND	ND	ND	ND
	B. uniformis	7.5 ± 0.5	3.9 ± 0.4	7.1 ± 0.3	ND	ND	ND	ND	ND	ND	18.3 ± 0.7	ND
	B. eggerthii	ND	0.6 ± 0.1	ND	ND	ND	ND	ND	ND	ND	ND	ND
	B. helcogenes	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	B. vulgatus	28.4 ± 0.9	14.8 ± 0.3	49.8 ± 2.2	ND	ND	ND	ND	ND	ND	31.5 ± 1.2	ND
	B. massiliensis	ND	2.9 ± 1.0	ND	ND	ND	ND	ND	ND	ND	5.2 ± 0.2	ND
	B. coprocola	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Bth274 [T]-related group	*B. nordii*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	B. salyersiae	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	B. pyogenes	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	B. thetaiotaomicron	72.2 ± 0.3	58.9 ± 0.7	97.5 ± 0.4	ND	ND	ND	ND	ND	ND	95.2 ± 2.0	ND
U1390-related domain	Bac303-related species	28.1 ± 0.3	44.4 ± 2.0	29.9 ± 3.0	5.2 ± 0.4	4.6 ± 0.9	5.1 ± 0.7	2.3 ± 0.3	2.9 ± 0.2	1.8 ± 0.2	10.4 ± 1.6	4.6 ± 0.6
	Bfrg602-related species	5.0 ± 0.9	11.8 ± 1.0	12.1 ± 1.2	ND	ND	ND	ND	ND	ND	6.2 ± 0.2	ND
	Bdts_gp980-related species	2.4 ± 0.1	2.5 ± 0.2	4.1 ± 0.2	1.3 ± 0.1	1.4 ± 0.1	1.7 ± 0.1	0.7 ± 0.2	0.7 ± 0.2	0.5 ± 0.1	5.1 ± 0.2	1.8 ± 0.1
	Bth274 [C]-related species[b]	0.8 ± 0.1	1.2 ± 0.2	1.3 ± 0.2	ND	ND	ND	ND	ND	ND	1.5 ± 0.8	ND
	Bth274 [T]-related species[c]	1.1 ± 0.3	0.5 ± 0.1	0.2 ± 0.1	ND	ND	ND	ND	ND	ND	0.3 ± 0.2	ND

[a] Relative abundances of bacterial targets in the human (H1 to H3), pig (P1 to P3), and cow (C1 to C3) feces, together with those present in the influents of municipal and swine wastewater treatment plants (municipal WW and pig WW, respectively), were determined individually by using a multiplex HOPE method. CFs obtained for primers Bth274 [T]_15dA, Bnd136, Bsls1016_10dA, Pg_dtc732_20dA, and Bth584_16dA in multiplex tube 1 were as follows: 1:2.05:0.39:0.36:0.19. CFs were obtained using clones from bacterial strains that included *B. nordii*, *B. salyersiae*, *B. pyogenes*, and *B. thetaiotaomicron* strains. CFs obtained for primers Bfrg602_19dA, Bfrg1026_20dA, Bcc1066_15dA, Badf1009, Bitt141, Bufm1018_20dA, Begt999, Bhcg171_8dA, Bvg1016, Bmsl1000_19dA, and Bcpc1015_24dA in multiplex tubes 2 to 5 were as follows: 1:0.17:2.96:0.36:16.51:4.61:8.17:13.86:8.37:3.13:3.06. CFs were obtained using clones from bacterial strains that included *B. fragilis*, *B. caccae*, *B. acidifaciens*, *B. intestinalis*, *B. uniformis*, *B. eggerthii*, *B. helcogenes*, *B. vulgatus*, *B. massiliensis*, and *B. coprocola* strains. CFs obtained for primers U1390_17dA, Bac303_5dA, Bfrg602_19dA, Bdts_gp980_12dA, Bth274 [C]_15dA, and Bth274 [T]_15dA in multiplex tube 6 were as follows: 1:1.206:0.091:1.998:0.158:0.432. CFs were obtained using clones from bacterial strains that included *B. fragilis*, *B. thetaiotaomicron*, *B. tectus*, *B. uniformis*, *B. nordii*, *P. distasonis*, and *P. merdae* strains.

[b] Primer Bth274 [C]_15dA extends with ddCTP when annealed to bacterial targets in this group.

[c] Primer Bth274 [T]_15dA extends with ddTTP when annealed to bacterial targets in this group.

[d] ND, not detected.

[e] The relative abundances of all bacterial targets were averaged from triplicate measurements and are expressed as means ± standard deviations ($n = 3$).

genes from the swine and bovine feces, respectively. These abundances were lower than those in human fecal microbiota by factors of 5 to 25 and were clustered significantly apart from the latter. Furthermore, the interindividual differences among the animal fecal microbiota were less apparent than those among the human fecal microbiota (Fig. 5C).

Abundances of *Bacteroides* spp. as quantified by Q-PCR. To validate the relative abundances of *Bacteroides* spp. as determined by HOPE, Q-PCR was also employed to determine the abundances of *Bacteroidales*, the *B. fragilis* group and its subgroups, and the *Parabacteroides* group that were present in the human fecal samples H1 to H3. As the 16S rRNA operon copy numbers of different *Bacteroides* spp. are not well defined yet, the quantitative expression of Q-PCR calculations was based on the genomic mass (in picograms). Standard curves generated showed a linear relationship between mean numbers of threshold cycles and the logarithmic mass over the range of 1 to 10,000 picograms, and the amplification efficiency at an annealing temperature of 64°C was 90.8 to 100.7%. The relative abundances of most *Bacteroidales* in the feces of all three human subjects as targeted by primer Bac303 were 30.1 to 50.9%. Likewise, the *B. fragilis* group, the *Parabacteroides* group, and the *B. fragilis* subgroups accounted for 4.7 to 14.8%, 3.1 to 5.4%, and 1.7 to 2.0% of amplified genes, respectively (Table 4). These results were in close proximity to those obtained using HOPE.

Relative abundances of *Bacteroides* spp. in wastewaters. Table 3 and Fig. 5 indicate the relative abundances of *Bacteroides* spp. detected in the influents of swine and municipal wastewater treatment plants. *Bacteroides* spp. that were previously detected in the human feces were also present in municipal wastewater, with *B. fragilis*, *B. caccae*, *B. uniformis*, *B. vulgatus*, and *B. massiliensis* accounting for 3.6, 2.7, 18.3, 31.5, and 5.2% of the *B. fragilis* group, respectively. *B. thetaiotaomicron* remained the predominant species in the *B. fragilis* Bth274 [T]-related subcluster (95.2%). The relative abundances of these *Bacteroides* spp. in the municipal wastewater therefore approximate those found in human fecal microbiota. However, the relative abundances of most *Bacteroidales* decreased by more than twofold compared to those in human fecal samples, possibly due to a die-off effect for these *Bacteroides* spp. and the proliferation of other microbial populations during the transportation in the sewage system.

With the influent of the swine wastewater treatment plant, none of the primers targeting *Bacteroides* spp. at the species level could be detected. The relative abundances of targets in the order *Bacteroidales* were also about twofold lower than those in municipal wastewater.

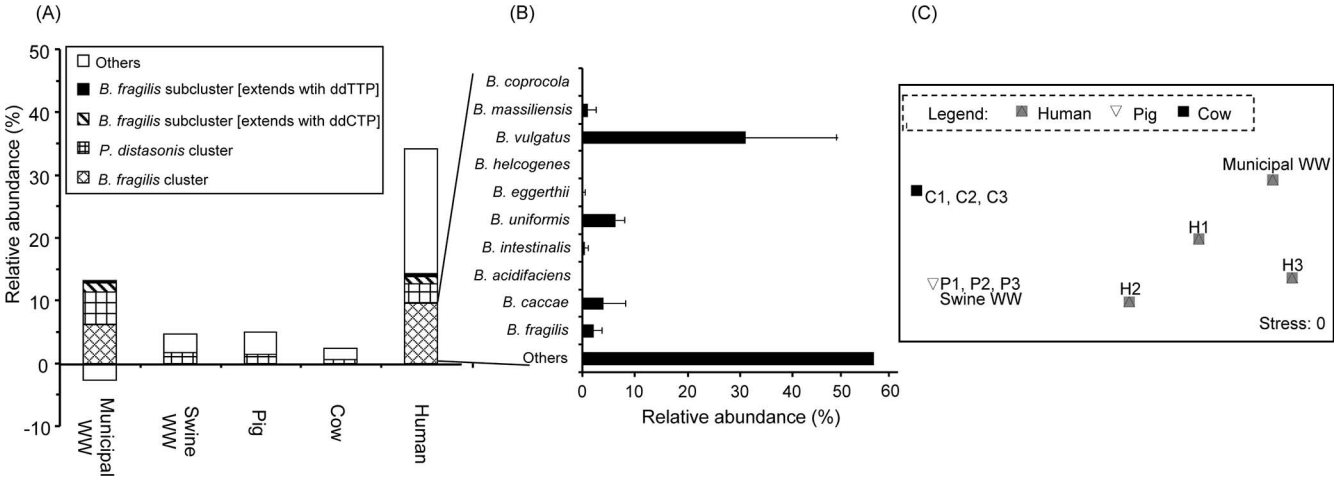

FIG. 5. Statistical interpretation of the HOPE results. (A) Abundances of the *B. fragilis* cluster, the *B. fragilis* subclusters, and the *P. distasonis* cluster relative to the total set of amplified 16S rRNA genes were averaged for the individual sample types. WW, wastewater. (B) *B. massiliensis*, *B. vulgatus*, *B. eggerthii*, *B. uniformis*, *B. intestinalis*, *B. caccae*, and *B. fragilis* were detected, and their average relative abundances within the *B. fragilis* cluster are illustrated. A predominant portion within the *B. fragilis* cluster remains unidentified. (C) Multidimensional scaling plot obtained by first performing a Euclidean distance square-root transformation of the raw data to generate a similarity matrix. The matrix then underwent a 100-iteration multidimensional scaling analysis. C1 to C3, cow fecal samples; P1 to P3, pig fecal samples.

DISCUSSION

In this study, HOPE, with its advantages in detection sensitivity, specificity, rapidity, and throughput, has been successfully demonstrated to be a method that can improve understanding of the relative abundances of *Bacteroides* and *Parabacteroides* spp. present in fecal microbiota. Findings from the analysis of human feces suggested that the fecal microbiota contain a relatively stable profile of *Bacteroides* spp., with major species like *B. vulgatus*, *B. fragilis*, *B. thetaiotaomicron*, *B. uniformis*, and *B. caccae* consistently detected in all tested samples. Clear interindividual differences in the diversity of *Bacteroides* spp. were observed, presumably due to differences in living habits, age, and diet (20, 22). The total abundances of the *Bacteroides* spp. within the *B. fragilis* cluster targeted by the species-specific primers were 27 to 63%, further suggesting the existence of other *Bacteroides* spp. in human fecal microbiota. It is possible that a small fraction of these unidentified *Bacteroides* spp. may be *B. ovatus* and *B. stercoris*, which were not detected in this study. However, these two species are unlikely to be predominant, as previous FISH studies have reported a low frequency of detection of these species (34). The remaining, largely unidentified fraction may represent the yet-to-be-cultured species in the *B. fragilis* cluster as described by Eckburg and coworkers (11). Based on a 16S rRNA gene clone library, they detected 65 *Bacteroidetes*-related phylotypes, and as many as 65% are classified as novel.

Likewise, when HOPE was applied to animal feces and wastewaters, all of the species-specific primers were undetected and a reduction in the abundances of *Bacteroidales* in the wastewater treatment influent compared to those in human fecal samples was observed. These results reconfirmed previous reports that those *Bacteroides* spp. predominant in human fecal microbiota are present in much lesser abundance in animal stools (27). Therefore, certain *Bacteroides* 16S rRNA genes have been suggested and used as effective biomarkers to differentiate the origin and duration of fecal contamination, thereby achieving microbial source tracking (5, 9, 14).

We further compared the HOPE results on the diversity and abundances of those fecal bacterial populations with results obtained previously using 16S rRNA clone libraries and FISH (11, 16, 19, 34, 39) (Table 4). Though the abundances of bacterial targets as determined by 16S rRNA clone library and FISH analyses were expressed based on different normalization units (i.e., the percentage of the total number of clones selected and the percentage of the total number of cells hybridized with a domain *Bacteria*-specific probe) and interindividual variation in the composition of the *Bacteroides* flora exists, both molecular methods showed ranges of abundances of *Bacteroides-Prevotella* bacteria (14.5 to 48%) in the human fecal microbiota similar to those shown by HOPE (28.1 to 44.4%). FISH results showed that the *B. fragilis* cluster and the *P. distasonis* group together accounted for 4.3 to 21%, respectively, of the total *Bacteria* and that *B. vulgatus* accounted for 4.2% of the total *Bacteria* (16, 19, 34). Similarly, after the selection and sequencing of 300 to 700 clones to construct a clone library, the *B. fragilis* group and the *P. distasonis* group were found to account for 13.3 and 2.3% of total *Bacteria*, respectively, while predominant species like *B. vulgatus*, *B. thetaiotaomicron*, *B. uniformis*, and *B. caccae* accounted for 2.1 to 15%, 1.4 to 6.2%, 4.9%, and 1.1% of total *Bacteria* (11, 39).

To further validate the reliability of HOPE results obtained in this study, we used Q-PCR analysis of the same set of human fecal samples analyzed by HOPE to determine the abundances of selected *Bacteroides* spp. The relative abundances of *Bacteroidales*, the *B. fragilis* cluster and its subclusters, and the *P. distasonis* cluster as determined by Q-PCR and HOPE were in close approximation (Table 4). This outcome suggests that when intersample differences in the composition of the *Bacteroides* spp. are minimal, the two PCR-based molecular meth-

TABLE 4. Comparison of the abundances of *Bacteroides* spp. at different hierarchical levels as reported in the published literature

Reference	Method	Sampling size	Relative abundance (%) of bacteria classified as:							
			Bacteroides-Prevotella	*B. fragilis* subgroup	*P. distasonis* subgroup	Bth274-related subgroup	*B. vulgatus*	*B. thetaiotaomicron*	*B. uniformis*	*B. caccae*
Franks et al. (16)	FISH[a]	Fecal samples from 9 subjects	NA[d]	21 (Bfra602 and Bdis656 related)	21 (Bfra602 and Bdis656 related)	ND[e]	ND	ND	ND	ND
Harmsen et al. (19)	FISH	Fecal samples from 11 subjects	27.7 (Bac303 related)	ND	ND	ND	ND	ND	ND	ND
Rigottier-Gois et al. (34)	FISH	Fecal samples from 20 subjects	14.5 (Bac303 related)	3.9 (Bfra998 related)	0.4 (Bdist1025 related)	ND	4.2 (Bvg1017 related)	ND	ND	ND
Suau et al. (39)	16S rRNA clone library analysis[b]	Fecal samples from 1 subject; 284 clones	31	13.3	2.3	NA	2.1	1.4	4.9	1.1
Eckburg et al. (11)	16S rRNA clone library analysis	Mucosa and fecal samples from 3 subjects; total of 395 bacterial phylotypes	48	NA	NA	NA	15	6.2	NA	NA
This study	HOPE[c]	Fecal samples from 3 subjects	28.1–44.4 (Bac303 related)	5.0–12.1 (Bfrg602 related)	2.4–4.1 (Bdts_gp980 related)	1.5–1.9	1.4–6.0 (Bvg1016 related)	0.2–0.8 (Bth584 related)	0.4–0.9 (Bufm1018 related)	0.2–0.4 (Bcc1066 related)
This study	Q-PCR[c]	Fecal samples from 3 subjects	30.1–50.9 (Bac303 related)	4.7–14.8 (Bfrg602 related)	3.1–5.4 (Bdts_gp980 related)	1.7–2.0	ND	ND	ND	ND

[a] Results from FISH analyses are expressed in terms of hybridized cell counts normalized against Eub338.
[b] Results from clone library analyses are expressed with respect to the total numbers of clones selected.
[c] Results from HOPE and Q-PCR are expressed with respect to the total set of PCR-amplified 16S rRNA genes.
[d] NA, information not available in the literature.
[e] ND, not determined.

ods complement each other in determining the abundances of specific bacterial targets.

It is clear through this comparison that HOPE, with its advantages in detection sensitivity, specificity, and throughput, can complement other available molecular methods and aid microbial studies. It has been demonstrated previously that HOPE is able to detect bacterial targets at different phylogenetic levels (i.e., species, genus, and so on up to domain) and at a sensitivity corresponding to 0.1% of the total set of PCR-amplified bacterial targets (41). This sensitivity approximates that obtained by constructing a 300-clone library (39), and thus, HOPE can easily detect bacterial targets like *Bacteroides* spp. in the human feces at the species level. Unlike clone library analysis, in which the abundance of a particular bacterial target is dependent on the ultimate number of clones picked, HOPE analysis examines the entire set of PCR-amplified products from the microbial community and can better reveal the relative abundances of bacterial targets. The comparability of the results obtained by HOPE to results obtained by other molecular tools (Table 4) suggests that the specificity and reliability of HOPE were also not compromised. The entire HOPE procedure for the identification and quantification of those 14 *Bacteroides* and *Parabacteroides* spp. after primary DNA extraction and PCR amplification took less than 120 min and was significantly shorter than that required for FISH or clone library construction. Furthermore, the total number of bacterial targets can be easily increased simply by adding HOPE reactions or by adding primer(s) to individual reactions (Fig. 1).

At present, HOPE has been demonstrated to determine the relative abundances of only known bacterial targets and, therefore, does not aid in further understanding the diversity of the microbial community. The complexity of making multiplex tube arrangements and analyzing the electropherograms can also increase with an increase in the number of applied primers. Thus, there is a strong need to automate both processes. It should be noted that the determination of the relative abundances of bacterial targets by HOPE can be subject to PCR bias. To minimize this bias, one can apply amplicons obtained at the exponential phase of PCR amplification as DNA templates in HOPE reactions. The sensitivity of the method may also be compromised by an excessively long poly(A) tail (41) and by possible interference from the nontarget DNA in complex environmental samples. To circumvent these effects, it is therefore essential to perform a systematic study that looks into an optimal poly(A) tail length and to perform thorough purification of the HOPE products prior to capillary electrophoresis.

In summary, this study has demonstrated the potential of HOPE as a rapid and high-throughput detection method that is able to examine the relative abundances of bacterial targets at various taxonomical levels. It can be used to identify disease-related bacterial populations that are present in the gut or feces over temporal and spatial intervals. Likewise, the present set of human-specific *Bacteroides* HOPE primers can be improved to include primers targeting animal-specific uncultivated *Bacteroidales* (5, 6, 9, 10, 14, 29) and be used to detect the presence of host-specific *Bacteroidales* in water bodies that may be contaminated with feces from multiple sources. The simplicity and versatility of this method can further facilitate the attempts to understand microbial diversity in different ecological systems when HOPE is used to complement other existing molecular tools. For example, one can use fingerprinting methods to quickly evaluate the microbial diversity of an environmental sample. Once the key microbial populations are identified using the fingerprinting methods, HOPE can be subsequently applied to effectively monitor the dynamics of those key populations at a much-refined resolution.

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