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Improvement of the Representation of Bifidobacteria in Fecal Microbiota Metagenomic Libraries by Application of the *cpn60* Universal Primer Cocktail▽

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Actinobacteria, particularly bifidobacteria, are widely observed to be underrepresented in metagenomic studies of microbial communities. We have compared human fecal microbiota clone libraries based on 16S rRNA and *cpn60* PCR products. Taxonomic profiles were similar except that the *cpn60* libraries contained large numbers of bifidobacterial sequences.

Bifidobacteria are Gram-positive, high G+C members of the phylum *Actinobacteria* that are a focus of study for their role in intestinal metabolism as well as their potential as probiotics. Culture-based studies of mammalian intestinal and fecal microbiota consistently indicate the presence of large numbers of bifidobacteria, with viable counts of 10^8 to 10^{10} CFU/g in feces (3, 15, 16, 34).

The advent of sequence-based methods for studying microbial communities has led to characterizations of intestinal microbiota based on sequencing of cloned PCR amplicons derived from the 16S rRNA gene or, more recently, the direct sequencing of 16S rRNA PCR products using pyrosequencing methods. The results of these studies almost invariably provide a description of the distal intestinal or fecal microbiota as a population in which *Firmicutes* and *Bacteroidetes* dominate, accounting for as much as 98% of the observed sequences (7, 23, 24). These descriptions conflict with the results of investigations in which bifidobacteria are cultured from samples or detected using taxon-specific methods, such as fluorescent *in situ* hybridization (FISH) (22, 36) or taxon-specific PCR (34, 35). There are a few exceptions to the problem of "missing bifidobacteria" in metagenomic studies, and these have employed modified PCR protocols involving higher annealing temperatures (30) or "universal" 16S rRNA primers known to be more sensitive for *Actinobacteria* (2). PCR primer bias also has been observed as a problem in amplification of *Actinobacteria* sequences from environmental samples (13).

An alternative gene target for metagenomic studies of microbial communities is the *cpn60* gene that encodes the universal 60-kDa chaperonin protein (also known as GroEL or Hsp60) (19). This target has been exploited in microbial ecology studies involving clone libraries of PCR products (6, 10, 11, 17, 18, 20, 28), pyrosequencing of PCR amplicons (31), and quantitative real-time PCR (4, 9). Recently, we published a modification of the *cpn60* universal primer PCR protocol which includes the use of a cocktail of degenerate primers optimized to give proportional amplifications of sequences across a broad range of G+C contents (21). This approach has subsequently been used in the characterization of human vaginal microbiota (31) and feline fecal microbiota (6). Although in the feline study a large proportion of sequences observed corresponded to bifidobacteria, there is no corresponding 16S rRNA-based study of the samples for comparison. Here, we have applied a *cpn60*-based approach to the characterization of human fecal microbiota and conducted a direct comparison to 16S rRNA clone libraries generated from the same samples.

Fecal samples were collected from healthy adult volunteers (aged 18 to 65 years) enrolled in a study of the effects of whole chickpea or raffinose on intestinal health (14). Total genomic DNA was extracted from fecal samples as described previously (18) and pooled according to diet protocol (control [diet A], raffinose [diet B], and chickpea [diet C]) such that 12 individuals were represented in each diet library. *cpn60* PCR was conducted as described previously (21). For 16S rRNA amplification, universal bacterial primers F1 (5′-GAGTTTGATCC TGGCTCAG-3′) and R2 (5′-GWATTACCGCGGCKGCTG-3′) (8) were used to amplify the region corresponding to nucleotides 11 to 536 of the *Escherichia coli* 16S rRNA gene. PCR amplifications were performed with 50-μl reaction mixtures containing 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris HCl (pH 8.75), 0.1% Triton X-100, 0.1 mg/ml bovine serum albumin (BSA), 2 mM $MgSO_4$, 0.2 mM deoxynucleoside triphosphates (dNTPs), 0.4 μM concentrations of each primer, and 1 U of *Taq* DNA polymerase (UBI Life Sciences, Calgary, AB, Canada) using an Eppendorf Mastercycler EP. The amplification program was 3 min at 95°C followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s and a final extension of 10 min at 72°C. The resulting PCR products from each template pool were purified and ligated into cloning vector pGEM-T Easy (Promega). Ligation reactions were used to transform *E. coli* JM109-competent cells, and recombinants

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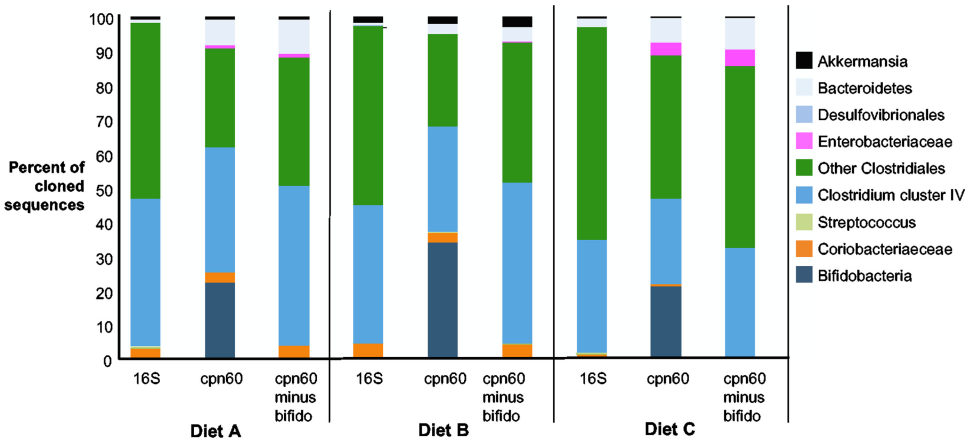

FIG. 1. Proportional representation of major taxonomic groups in the PCR product clone libraries generated from three diets, based on either 16S rRNA or *cpn60*. Each pair of libraries was generated from the same sample pool and DNA extract, as described in the text. *cpn60* libraries are shown with and without inclusion of bifidobacterial sequences.

(672 per library) were picked, placed in 96-well plates, and sequenced with the T7 sequencing primer.

Sequences were processed and warehoused using Another Portal for Examining DNA (APED) (25) as described previously (6). For taxonomic identification, *cpn60* nucleotide and translated peptide sequences were compared to the reference *cpn60* sequence database within cpnDB (19) (http://cpndb.cbr.nrc.ca) using FASTA (29) and BLASTp (1). 16S rRNA sequences were identified using the Ribosomal Database Project (RDP) Classifier provided by the RDP (http://rdp.cme.msu.edu). Additionally, unique sequences identified from the *cpn60* and 16S rRNA libraries were subjected to phylogenetic analysis to support their taxonomic identification (data not shown). A total of 1,763 *cpn60* clones (618 from library A, 554 from library B, 591 from library C) and 1,314 16S rRNA clones (459 from library A, 440 from library B, 415 from library C) were included in the analysis. The number of cloned sequences available for each library was influenced by sequencing success rates, as only full-length, high-quality insertion sequences were included in the analysis. To compensate for library size variation, all comparisons were done based on the proportional representation of sequences in each library.

Figure 1 shows the proportional representation of the major taxonomic groups in the clone libraries. Differences in taxonomic profiles between the different diets are discussed in detail elsewhere (14). *Firmicutes* (including *Streptococcus*, *Clostridium*, *Faecalibacterium*, and *Eubacterium*) accounted for 93 to 96% of the sequences in the 16S rRNA clone libraries. *Bacteroidetes* comprised only 1 to 2% of the 16S rRNA libraries. This profile is similar to those previously reported for human fecal microbiota, for which the *Firmicutes* and *Bacteroidetes* combined account for up to 98% of sequences (24). *Bacteroidetes* were somewhat more abundant in the *cpn60* libraries, where they accounted for 3 to 8% of the clones. The major difference between the libraries based on the two gene targets was in the *Actinobacteria* (including *Coriobacterineae* and *Bifidobacterium*). While sequences from the *Coriobacterineae* family constituted 0.5 to 4% of each of the libraries regardless of the gene target, *Bifidobacterium* sequences were detected in 16S rRNA libraries from diets B (0.5% of clones) and C (0.2% of clones) only. In the *cpn60* libraries, *Bifidobacterium* sequences accounted for 22%, 34%, and 21% of libraries from diets A, B, and C, respectively. When bifidobacterial sequences are removed from each of the *cpn60* libraries, their taxonomic profiles are very similar to those of the corresponding 16S rRNA libraries.

Real-time PCR was used to quantify *Clostridium* cluster IV and *Bifidobacterium* spp. in the genomic DNA extracts used to create the PCR product libraries (26, 27). Similar quantities of the two groups were detected in all samples: $10^{8.8}$ to $10^{9.3}$ copies/g of feces for *Clostridium* cluster IV and $10^{8.8}$ to $10^{9.8}$ copies/g for *Bifidobacterium* spp. (14). *Clostridium* cluster IV is a group within the *Firmicutes* that includes *Faecalibacterium prausnitzii*, *C. orbiscindens*, *C. leptum*, and *C. methylpentosum* (5, 12, 32). This group accounted for 47%, 40%, and 33% of the cloned 16S rRNA sequences in libraries A, B, and C, respectively, and 47%, 47%, 32% of the corresponding nonbifidobacterial *cpn60* sequences. However, despite similar abundances indicated by taxon-specific PCRs, only 0%, 0.5%, and 0.2% of 16S rRNA clones were from bifidobacteria, illustrating a gross underrepresentation of these organisms in the 16S rRNA clone libraries.

In a recent study of human intestinal microbiota in lean and obese twins that utilized deep pyrosequencing of multiple regions of the 16S rRNA gene, sequencing of full-length 16S rRNA amplicons, and shotgun metagenome sequencing, Turnbaugh et al. (33) found that in almost all samples, <10% of sequences were from *Actinobacteria*. However, they also determined that 75% of the obesity-enriched genes were from *Actinobacteria*. Given this observation and the acknowledged importance of bifidobacteria in health and nutrition, any assessment of intestinal or fecal microbiota would ideally be conducted with a method that allows detection and monitoring of this taxon. The potential biases of PCR primers must be carefully considered. None of the other targets that are routinely exploited for bacterial speciation, such as *rpoB* and *gyrB*, have been applied in metagenomic studies of microbial communities, so it is unknown if they would offer improved representation of bifidobacteria. Our experiment, in which we compared directly the microbial population profiles generated

from three samples, demonstrates that the *cpn60* target offers a useful alternative or complement to 16S rRNA and alleviates the "missing bifidobacteria" problem.

Nucleotide sequence accession numbers. The 1,763 *cpn60* clones and 1,314 16S rRNA clones analyzed were deposited in GenBank under accession numbers GQ178291 to GQ179638.

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