Applied and Environmental Microbiology, May 2011, p. 3544–3546 Vol. 77, No. 10
0099-2240/11/$12.00 doi:10.1128/AEM.02851-10

Overestimation of the Abundance of Sulfate-Reducing Bacteria in Human Feces by Quantitative PCR Targeting the *Desulfovibrio* 16S rRNA Gene▽

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Received 6 December 2010/Accepted 20 March 2011

The dominant genus of sulfate-reducing bacteria (SRB) in humans is *Desulfovibrio*, and quantitative PCR (QPCR) targeting the 16S rRNA gene is often used in assays. We show that the 16S rRNA gene assay overestimated SRB abundance in feces from 24 adults compared to QPCR assays using primers targeting two genes involved in SRB energy metabolism.

There is growing interest in the quantification of sulfate-reducing bacteria (SRB) in humans because of reports suggesting SRB to be involved in the etiology of gastrointestinal diseases (10, 18). The principal byproduct of SRB metabolism is hydrogen sulfide (H_2S), which can be toxic to epithelial cells in the colon, where it is mainly produced. At higher levels, H_2S can inhibit butyrate oxidation (20) and phagocytosis and bacterial killing (11) and induces hyperproliferation and metabolic abnormalities in epithelial cells (5). H_2S is also produced endogenously by colonocytes (21) and is physiologically active in the brain, heart, vasculature, urogenital system, and gastrointestinal tract at nontoxic levels (8, 25, 26). Ulcerative colitis patients were reported to have higher levels of H_2S (19) and a higher abundance of SRB (12) in their feces. Use of the *Desulfovibrio* 16S rRNA gene for detection of SRB in human feces has revealed a higher abundance in elderly people compared to healthy adults (9), a positive correlation between *Desulfovibrio* abundance and smoking (14), and no difference (2) or a decrease (22) in *Desulfovibrio* levels in colorectal cancer patients compared to healthy individuals. Therefore, to determine the role of SRB in gastrointestinal health, an accurate estimate of the abundance of SRB is vital.

For quantification of SRB in environmental samples, three genes, the 16S rRNA, adenosine-5′-phosphosulfate reductase (*aps*), and dissimilatory (bi)sulfite (*dsrA*) genes, are generally targeted (3, 24). The *aps* and *dsrA* genes are involved in the energy metabolism of SRB and have been identified as reliable gene markers for SRB (24). The 16S rRNA gene is, however, considered inadequate for determinations involving environmental samples, because SRB are found in different phyla in the phylogenetic tree (4, 24). Therefore, the 16S rRNA gene assay cannot cover all the different phyla and underestimates the SRB abundance in environmental samples (3, 24). This may not be the case, however, for human fecal samples, as SRB of the genus *Desulfovibrio* can occur in large numbers in the gut and because *Desulfovibrio* has been identified as the main genus of SRB in humans (9, 12).

In this study, we used quantitative real-time PCR (QPCR) to examine the accuracy of use of the 16S rRNA gene to quantify SRB in human feces by comparing an assay employing the widely used 16S rRNA primer pair that targets *Desulfovibrio* (9) with two QPCR assays targeting the functional *aps* and *dsrA* SRB genes that have been found to give reliable quantifications of SRB in environmental samples.

Human fecal samples were collected from a group of 24 healthy individuals (14 males and 10 females) with an average age of 53.7 years (range, 33 to 67) who had not had any antibiotic treatment during the past 6 months. DNA was extracted from these samples using the repeated beat-beading and column cleanup method described by Yu and Morrison (27). *Desulfovibrio* numbers, *aps* and *dsrA*, and the total number of bacteria in human fecal samples were quantified using QPCR. Quantifications were performed using 1× Ssofast Evagreen Supermix fluorescent nucleic acid dye (Bio-Rad Laboratories, Hercules, CA) and 0.4 μl bovine serum albumin (BSA) (Promega, Madison, WI). The primers were as follows: for total bacteria (concentration, 175 nM), primers 1114f (5′-CGGCAACGAGCGCAACCC-3′) and 1275r (5′-CCATTGT AGCACGTGTGTAGCC-3′) (6); for *aps* (400 nM), primers *aps*3f (5′-TGGCAGATCATGWTYAAYGG-3′) and *aps*2r (5′-GGGCCGTAACCRTCYTTRAA-3′) (modified from reference 7); for *dsrA* (400 nM), primers Dsr1F (5′-ACSCACTG GAAGCACGGCGG-3′) and Dsr1R (5′-GTGGMRCCTGCA KRTTGG-3′) (16); and for the *Desulfovibrio* 16S rRNA assay (300 nM), primers DSV691F (5′-CCGTAGATATCTGGAG GAACATCAG-3′) and DSV826R (5′-ACATCTAGCATCCA TCGTTTACAGC-3′) (9). For quantification, a total of 10 to 30 ng of template DNA was used and cycling was performed using a Chromo-4 thermocycler (MJ Research Inc., Waltham, MA). Reaction mixtures for *aps* assays contained 1 μl dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) instead of BSA. The PCR cycling conditions were as follows: 98°C for 4 min followed by 35 cycles of 98°C for 30 s, 58 to 65°C for 15 to 30 s (for total bacteria, 60°C for 20 s; for *aps*, 58°C for 30 s; for *dsrA*, 65°C for 15 s; and for *Desulfovibrio* spp., 62°C for 30 s), and 72°C for 30 s. Elongation was followed by fluorescence

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▽ Published ahead of print on 1 April 2011.

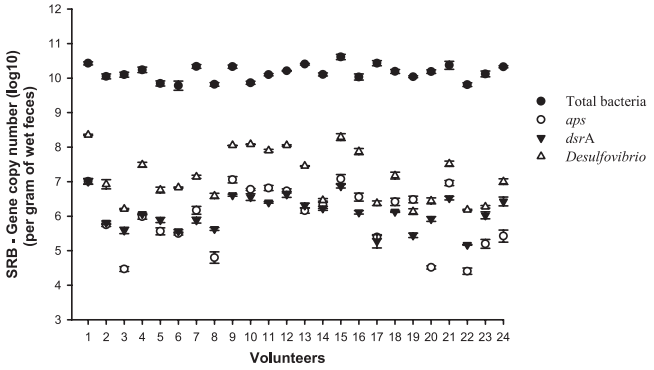

FIG. 1. Abundance of SRB (mean ± SD) in feces from 24 healthy volunteers measured with QPCR using primers that target the *Desulfovibrio* 16S rRNA gene (Δ), the adenosine-5′-phosphosulfate reductase (*aps*) gene (○), and the dissimilatory (bi)sulfite (*dsrA*) gene (▼). QPCR estimates of total number of bacteria are also represented (●).

acquisition; however, a further elongation step at 83°C for 15 s was performed before fluorescence acquisition for *dsrA*, and a final melt-curve analysis was performed after completion of all cycles, with fluorescence acquired at 0.5°C intervals between 55 and 95°C to verify that only the expected fragment was amplified. PCR products were also visualized on a 1.5% agarose gel. Nontemplate controls were included, and assays were performed in technical triplicate experiments by analyzing the same DNA sample in 3 independent reactions. A series of eight 10-fold dilutions of a sample derived-plasmid construct (Topo chemical competent cells; Invitrogen) containing the target amplicon were analyzed in parallel with DNA samples for estimations of absolute abundance and PCR efficiency for all assays. Results were analyzed using Opticon Monitor software (version 3.1; Bio-Rad Laboratories) for absolute abundance estimates. All calculations were done using an assay specific for PCR efficiency. Using a clone library, a specificity test was performed on the 16S rRNA gene primers. Twenty-four clones were sequenced using a 96-capillary 3730xl DNA analyzer and putatively identified using the Basic Local Alignment Search Tool (1). Statistical analysis was performed using the Primer 6 with Permanova$^+$ package (PRIMER-E Ltd., Plymouth, United Kingdom). Natural-logarithm-normalized data were used for statistical analysis of absolute quantities.

The absolute quantification of SRB in human feces by use of the 3 primer sets showed the following average abundance values (no. of copies × gram wet feces^{-1} [range]): for *aps*, 3.26×10^6 (2.54×10^4 to 1.19×10^7); for *dsrA*, 2.21×10^6 (1.48×10^5 to 1.01×10^7); and for *Desulfovibrio* spp., 4.53×10^7 (1.36×10^6 to 2.26×10^8). These abundances revealed a significantly higher absolute abundance (Fig. 1) for *Desulfovibrio* compared to *aps* ($P = 0.00005$) and *dsrA* ($P = 0.00001$), but no significant differences were observed between *aps* and *dsrA* results. Similar differences relative to the total numbers of bacteria were found when data were analyzed using Qbase$^+$ (13, 23) (data not shown). The PCR efficiency values for the assays were as follows: for total bacteria, 103%; for *Desulfovibrio*, 103%; for *dsrA*, 105%; and for *aps*, 99%. The efficiency results were similar for plasmid-derived DNA and stool-derived DNA. The clone library revealed that 18 clones (75%) were 98 to 100% similar and 4 clones (17%) were between 92% and 97% similar to known *Desulfovibrio* sequences, whereas 2 clones (8%) were putatively identified as *Papillibacter cinnamivorans*. It was also noted that all 24 healthy volunteers had detectable numbers of SRB.

This report supports previous evidence indicating that *Desulfovibrio* is the dominant genus of SRB found in stool samples from humans on the basis of the clone library and suggests that using the *Desulfovibrio* 16S rRNA gene overestimates the abundance of SRB in human feces compared to SRB abundances estimated using the *aps* or the *dsrA* gene. Hence, caution has to be exercised in analyzing and reporting SRB abundances when quantifications are performed using the 16S rRNA gene. However, the abundances of *aps* and *dsrA* also differ between individuals (Fig. 1) because some SRB may carry both genes and some only one (3). According to other studies, both *aps* and *dsrA* are genes suitable for reliable quantification of SRB populations and are specific for the SRB energy metabolism. The absolute quantities of *Desulfovibrio* spp., as determined in this study by use of the 16S rRNA primers, are similar to the findings of Fite et al. (9), and, given the results showing PCR efficiencies of around 100% (99% to 105%), we are confident that the 16S rRNA gene primers used in this study overestimated the abundance of SRB in human feces. Those results differ from what is found in experiments using environmental samples but are in line with the general idea that 16S rRNA primers do often overestimate abundances due to a higher copy number of the 16S rRNA gene compared to *aps* and *dsrA* copy numbers. According to the rRNA Operon Copy Number Database (15, 17), members of the genus *Desulfovibrio* have, on average, 4.5 copies of the 16S rRNA gene. Another reason for the overestimation of the abundance of *Desulfovibrio* spp. by 16S rRNA gene primers is the specificity of the primers. The clone library showed that the primers did amplify two fragments that did not match *Desulfovibrio* sequences, even though *in silico* tests showed the 16S rRNA gene primers to have several mismatches. In conclusion, the overestimation of abundance observed when using 16S rRNA gene primers compared to *aps* and *dsrA* primers in this study was almost certainly due to unspecific priming of the 16S rRNA gene primers and a higher copy number of the 16S rRNA gene compared to the *aps* and *dsrA* gene.

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