APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2007, p. 32–39 Vol. 73, No. 1
0099-2240/07/$08.00+0 doi:10.1128/AEM.01224-06

Sensitive Quantitative Detection of Commensal Bacteria by rRNA-Targeted Reverse Transcription-PCR▽

Kazunori Matsuda, Hirokazu Tsuji, Takashi Asahara, Yukiko Kado, and Koji Nomoto*

Yakult Central Institute for Microbiological Research, 1796 Yaho, Kunitachi, Tokyo 186-8650, Japan

Received 28 May 2006/Accepted 13 October 2006

A sensitive rRNA-targeted reverse transcription-quantitative PCR (RT-qPCR) method was developed for exact and sensitive enumeration of subdominant bacterial populations. Using group- or species-specific primers for 16S or 23S rRNA, analytical curves were constructed for *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Clostridium perfringens*, and *Pseudomonas aeruginosa*, and the threshold cycle value was found to be linear up to an RNA amount of 10^{-3} cell per RT-PCR. The number of bacteria in culture was determined by RT-qPCR, and the results correlated well with the CFU count over the range from 10^0 to 10^5 CFU. The bacterial counts obtained by RT-qPCR were the same as the CFU counts irrespective of the growth phase in vitro, except for *C. perfringens* during starvation periods; the viable cell counts obtained by using a combination of 4′,6-diamidino-2-phenylindole (DAPI) staining and SYTO9-propidium iodide double staining were in good agreement with the RT-qPCR counts rather than with the CFU counts. The RT-qPCR method could detect endogenous *Enterobacteriaceae* and *P. aeruginosa* in feces of hospitalized patients ($n = 38$) at a level of 10^3 cells per g of feces, and for enumeration of *S. aureus* or *P. aeruginosa* spiked into human peripheral blood, the lower detection limit for RT-qPCR quantification of the bacteria was 2 cells per ml of blood, suggesting that this method was equivalent to the conventional culture method. As only 5 h was needed for RT-qPCR quantification, we suggest that rRNA-targeted RT-qPCR assays provide a sensitive and convenient system for quantification of commensal bacteria and for examining their possible invasion of a host.

For almost a century, culture techniques have been recognized as the "gold standards" for determining viable bacterial counts. As the human fecal flora has been reported to consist of approximately 400 bacterial species (12, 35) and these species are present at a concentration of 10^{11} viable microorganisms per g of contents (42), conventional culture techniques for enumeration of different populations involve the use of selective microbiological media, followed by isolation of pure cultures and the use of confirmatory biochemical tests. Recently, a number of molecular methods based on immunological and genotypic techniques have been developed (41, 48). In analyses of the gut microflora, a number of molecular methods have been used in place of cultivation-based techniques. Techniques such as the clone library method (42, 46), denaturing gradient gel electrophoresis (13), and terminal restriction fragment length polymorphism (31, 36) allow analysis of predominant bacteria that are difficult to culture. The fluorescent in situ hybridization method (18, 43) and the quantitative PCR (qPCR) method with rRNA-targeted oligonucleotide probes or primers have also been used as culture-independent methods. Among these, PCR methods targeting mainly well-conserved 16S rRNA genes have prevailed for rapid quantification of bacteria and are recognized as having two advantages, specificity and convenience. To determine the bacterial population in the human gastrointestinal tract, the applications of qPCR have been expanded (5, 16, 29, 30). The new techniques enable accurate and convenient quantification of targeted predominant anaerobic species in the microflora, such as members of *Bifidobacterium* and the *Bacteroideaceae*, that are present at levels of more than 10^9 cells per g of feces. However, it has been demonstrated that the sensitivity of PCR is around 10^5 to 10^6 cells per g of feces, which does not seem to be sufficient for accurate quantification of minor but important commensal species, such as members of the *Enterobacteriaceae*, *Enterococcus*, *Staphylococcus*, and *Clostridium perfringens* that have been implicated as potential pathogens in immunocompromised hosts. Because of the lower levels of these subdominant bacterial species in healthy intestines, it is difficult to detect them accurately in the huge total bacterial population by existing techniques. In clinical examinations, it has been demonstrated that qPCR can detect bacterial contaminants with a sensitivity of 10^1 to 10^2 CFU per ml of blood (23, 38) but usually is not able to detect contamination with only a small number of bacteria (less than 10 cells per ml of blood).

We have focused on rRNAs as the target for precise and sensitive quantification of commensal subdominant bacterial populations, since rRNA is a universal constituent of bacterial ribosomes and high copy numbers (10^3 to 10^4 molecules per actively growing cell) are present as housekeeping genes (1, 17). Targeting these molecules has the potential to increase the detection sensitivity compared to the sensitivity of assays based on detection of a single copy or even multiple copies of genomic sequences. Here we describe sensitive quantification of bacterial populations with lower detection limits of 10^3 cells per g of feces and 10^0 cells per ml of peripheral blood using reverse transcription-quantitative PCR (RT-qPCR) targeting rRNA, which has almost the same sensitivity as the conventional culture method but improved performance time.

* Corresponding author. Mailing address: Yakult Central Institute for Microbiological Research, 1796 Yaho, Kunitachi, Tokyo 186-8650, Japan. Phone: 81(42)577 8962. Fax: 81(42)577 3020. E-mail: koji-nomoto@yakult.co.jp.

▽ Published ahead of print on 27 October 2006.

TABLE 1. Specificity tests with newly developed primers

Taxon	Strain	Reactions with the following primers[a]:				
		En-lsu3F/ En-lsu3′R	Ec-ssu1′F/ Ec-ssu1R	STPYF/ STPYR2	PSD7F/ PSD7R	ClPER-F/ ClPER-R
Escherichia coli	ATCC 11775^T	+	−	−	−	−
Citrobacter freundii	ATCC 13316^T	+	−	−	−	−
Citrobacter koseri	ATCC 27028^T	+	−	−	−	−
Enterobacter cloacae	ATCC 13047^T	+	−	−	−	−
Enterobacter aerogenes	ATCC 13048^T	+	−	−	−	−
Enterobacter sakazakii	JCM 1233^T	+	−	−	−	−
Klebsiella pneumoniae	ATCC 13883^T	+	−	−	−	−
Klebsiella oxytoca	ATCC 13182^T	+	−	−	−	−
Serratia marcescens	ATCC 13880^T	+	−	−	−	−
Proteus mirabilis	ATCC 29906^T	+	−	−	−	−
Proteus vulgaris	JCM 1668	+	−	−	−	−
Proteus penneri	JCM 3948^T	+	−	−	−	−
Hafnia alvei	JCM 1666^T	+	−	−	−	−
Edwardsiella tarda	ATCC 15947^T	+	−	−	−	−
Providencia alcalifaciens	ATCC 9886^T	+	−	−	−	−
Providencia rettgerii	DSM 4542^T	+	−	−	−	−
Morganella morganii	JCM 1672^T	+	−	−	−	−
Salmonella choleraesuis subsp. *choleraesuis* serotype enteritidis	DSM 9898	+	−	−	−	−
Yersinia enterocolitica	DSM 4780^T	+	−	−	−	−
Enterococcus faecalis	ATCC 19433^T	−	+	−	−	−
Enterococcus faecium	ATCC 19434^T	−	+	−	−	−
Enterococcus hirae	ATCC 8043^T	−	+	−	−	−
Enterococcus avium	JCM 8722^T	−	+	−	−	−
Enterococcus gallinarum	JCM 8728^T	−	+	−	−	−
Enterococcus casseliflavus	JCM 8723^T	−	+	−	−	−
Enterococcus flavescens	DSM 7370^T	−	+	−	−	−
Staphylococcus aureus	ATCC 12600^T	−	−	+	−	−
Staphylococcus epidermidis	ATCC 14990^T	−	−	+	−	−
Staphylococcus schleiferi subsp. *coagulans*	JCM 7470^T	−	−	+	−	−
Pseudomonas aeruginosa	ATCC 10145^T	−	−	−	+	−
Pseudomonas putida	DSM 291^T	−	−	−	+	−
Clostridium perfringens	JCM 1290^T	−	−	−	−	+
Ruminococcus productus	ATCC 27340^T	−	−	−	−	−
Ruminococcus obeum	ATCC 29174^T	−	−	−	−	−
Clostridium orbiscindens	DSM 6740^T	−	−	−	−	−
Bacteriodes fragilis	DSM 2151^T	−	−	−	−	−
Bacteroides vulgatus	JCM 5826^T	−	−	−	−	−
Bifidobacterium adolescentis	ATCC 15703^T	−	−	−	−	−
Bifidobacterium longum	ATCC 15707^T	−	−	−	−	−
Collinsella aerofaciens	ATCC 25986^T	−	−	−	−	−
Eggerthella lenta	ATCC 25559^T	−	−	−	−	−
Prevotella melaninogenica	ATCC 25845^T	−	−	−	−	−
Veillonella parvula	ATCC 10790^T	−	−	−	−	−
Lactobacillus acidophilus	ATCC 4356^T	−	−	−	−	−
Lactobacillus casei	ATCC 334^T	−	−	−	−	−
Streptococcus intermedius	ATCC 27335	−	−	−	−	−
Campylobacter jejuni	ATCC 33560^T	−	−	−	−	−
Candida albicans	ATCC 18804^T	−	−	−	−	−
Bacillus cereus	JCM 2152^T	−	−	−	−	−
Bacillus subtilis	ATCC 14579^T	−	−	−	−	−

[a] The specificity of the RT-qPCR assay for target bacteria with each primer was investigated using RNA extracts corresponding to 10^5 cells of each strain. Specificity was judged using the criteria described in Materials and Methods.

MATERIALS AND METHODS

Reference strains and culture conditions. The strains listed in Table 1 were used. *Escherichia coli* ATCC 11775^T, *Enterococcus faecalis* ATCC 19433^T, *Staphylococcus aureus* ATCC 12600^T, and *Pseudomonas aeruginosa* ATCC 10145^T were grown aerobically in brain heart infusion (BHI) broth (Becton Dickinson, Sparks, Md.) at 37°C, and the bacterial counts were expressed in CFU after culturing on BHI agar. *C. perfringens* JCM 1290^T was grown anaerobically in MRS broth (Becton Dickinson) at 37°C, and the CFU counts were determined by culturing the organism on GAM agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) in an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI).

Development of rRNA-targeted primers. By using 16S and 23S rRNA gene sequences obtained from the DDBJ/GenBank/EMBL databases for bacteria detected in the human intestinal tract, we constructed a multiple alignment of the target groups and reference organisms with the Clustal X program (44). After comparing the sequences, we identified potential primer target sites for group-specific detection for *Enterobacteriaceae*, *Enterococcus*, *Staphylococcus*, and *Pseudomonas*. We then designed the primers for *Enterobacteriaceae*, *Enterococcus*, *Staphylococcus*, and *Pseudomonas* listed in Table 2 and checked their specificities with the database by submitting the sequences to the Probe Match program of the Ribosomal Database Project (RDP-II) (http://rdp.cme.msu.edu/) (28).

TABLE 2. Primers based on 16S or 23S rRNA sequences

Target	Primer	Sequence	Product size (bp)	Reference
Enterobacteriaceae 23S rRNA	En-lsu3F En-lsu3′R	TGCCGTAACTTCGGGAGAAGGCA TCAAGGCTCAATGTTCAGTGTC	428	This study
Enterococcus 16S rRNA	Ec-ssu1′F Ec-ssu1R	GGATAACACTTGGAAACAGG TCCTTGTTCTTCTCTAACAA	115	This study
Staphylococcus 16S rRNA	STPYF STPYR2	ACGGTCTTGCTGTCACTTATA TACACATATGTTCTTCCCTAATAA	257	This study
Pseudomonas 16S rRNA	PSD7F PSD7R	CAAAACTACTGAGCTAGAGTACG TAAGATCTCAAGGATCCCAACGGCT	215	This study
Clostridium perfringens 16S rRNA	ClPER-F ClPER-R	AGATGGCATCATCATTCAAC GCAAGGGATGTCAAGTGT	793	21

Fecal sampling. Fecal samples provided by 19 hospitalized patients were weighed and then suspended in 9 volumes of sterilized anaerobic transfer medium, which contained KH_2PO_4 (0.0225%, wt/vol), K_2HPO_4 (0.0225%, wt/vol), NaCl (0.045%, wt/vol), $(NH_4)_2SO_4$ (0.0225%, wt/vol), $CaCl_2$ (0.00225%, wt/vol), $MgSO_4$ (0.00225%, wt/vol), Na_2CO_3 (0.3%, wt/vol), L-cysteine hydrochloride (0.05%, wt/vol), resazurin (0.0001%, wt/vol), Lab lemco powder (1.0%, wt/vol; Oxoid Co., Ltd., Basingstoke, United Kingdom), and glycerol (10%, wt/vol; Wako Pure Chemical Industries, Ltd., Osaka, Japan). After serial dilution of the fecal suspensions with a buffer solution containing KH_2PO_4 (0.0225%, wt/vol), K_2HPO_4 (0.0225%, wt/vol), NaCl (0.045%, wt/vol), $(NH_4)_2SO_4$ (0.0225%, wt/vol), $CaCl_2$ (0.00225%, wt/vol), $MgSO_4$ (0.00225%, wt/vol), Na_2CO_3 (0.3%, wt/vol), L-cysteine hydrochloride (0.05%, wt/vol), and resazurin (0.0001%, wt/vol), 50-μl portions of the appropriate dilutions were spread onto the following culture media: DHL agar (Nikken Bio Medical Laboratory Inc., Kyoto, Japan) for *Enterobacteriaceae* and NAC agar (Eikenkizai Co., Ltd., Tokyo, Japan) for *P. aeruginosa*. DHL agar and NAC agar were incubated aerobically at 37°C for 24 h. The colonies on the agar plates were then counted, and the numbers of CFU of target bacteria per g (wet weight) of feces were calculated. The lower limit of bacterial detection with this procedure was 200 CFU per g of feces.

Blood sampling. Human peripheral blood was collected from three healthy adult volunteers, and then sodium citrate (0.38%, wt/vol) was added and the preparations were mixed immediately. Tenfold serial dilutions of *S. aureus* or *P. aeruginosa* were added to the peripheral blood. After serial dilution of the samples with physiological saline, 500-μl samples of the appropriate dilutions were spread onto BHI agar and then incubated aerobically at 37°C for 24 h. The colonies on the agar plates were then counted, and the numbers of CFU of target bacteria per ml of blood were calculated. The lower limit of bacterial detection with this procedure was 2 CFU per ml of blood.

Isolation of total RNA. For RNA stabilization, fresh cultures of each bacterial strain (50 μl), fecal homogenate samples (200 μl), or blood samples (500 μl) were added to 2 volumes of RNAprotect bacterial reagent (QIAGEN GmbH, Hilden, Germany), and then the preparations were incubated for 5 min at room temperature. After centrifugation of each mixture at 5,000 × *g* for 10 min, the supernatant was discarded, and the pellet was stored at −80°C until it was used for extraction of RNA. RNA was isolated using a modified acid guanidinium thiocyanate-phenol-chloroform extraction method (8). Briefly, a thawed sample was resuspended in a solution containing 346.5 μl RLT lysis buffer (catalog no. 79216; QIAGEN Sciences, Germantown, MD), 3.5 μl β-mercaptoethanol (Sigma-Aldrich Co., St. Louis, MO), and 100 μl Tris-EDTA buffer (pH 8.0). Then 300 mg of glass beads (diameter, 0.1 mm; BioSpec Products, Inc., Bartlesville, OK) was added to the suspension, and the mixture was vortexed vigorously for 60 s using a FastPrep FP 120 (BIO 101, Vista, CA) at a power level of 5.0. Then 500 μl acid phenol (Wako Pure Chemical Industries, Ltd.) was added, and the mixture was incubated for 10 min at 60°C. After incubation, the mixture was cooled on ice for 5 min and added to 100 μl chloroform-isoamyl alcohol. After centrifugation at 12,000 × *g* for 10 min at 4°C, 450 μl of the supernatant was collected and added to an equal volume of chloroform-isoamyl alcohol. After centrifugation at 12,000 × *g* for 5 min, 400 μl of the supernatant was collected and subjected to isopropanol precipitation. Finally, the nucleic acid fraction was suspended in 50 μl nuclease-free water. To remove contaminating genomic DNA from the RNA fraction, 0.5 U RNase-free DNase I (TaKaRa Bio Inc., Shiga, Japan) per μg RNA was added to each sample in a solution containing 1× DNase I buffer (TaKaRa Bio Inc.), which was then incubated at 37°C for 20 min. After incubation, the DNase was inactivated and removed twice by acid-phenol and chloroform-isoamyl alcohol extraction as described above, and the RNA in the resultant supernatant was collected by isopropanol precipitation. Finally, the RNA was suspended in 50 μl nuclease-free water. The quantity of RNA was confirmed spectrophotometrically.

RT-qPCR. The RT-qPCR analysis was conducted with one-step reactions using a QIAGEN OneStep RT-PCR kit (QIAGEN GmbH). Each reaction mixture (20 μl) was composed of 1× QIAGEN OneStep RT-PCR buffer, each deoxynucleoside triphosphate at a concentration of 400 μM, a 1:100,000 dilution of SYBR green I (catalog no. 50513; BioWhittaker Molecular Applications, Rockland, ME), 1 μl QIAGEN OneStep RT-PCR enzyme mixture, each of the specific primers at a concentration of 0.6 μM, and 2 μl template RNA. The reaction mixture was incubated at 50°C for 30 min for reverse transcription. The continuous amplification program consisted of one cycle at 95°C for 15 min, followed by 40 cycles at 94°C for 20 s, 60°C for 20 s, and 72°C for 50 s and finally one cycle at 94°C for 15 s. The fluorescent products were detected in the last step of each cycle. A melting curve analysis was performed after amplification to distinguish the target from the nontargeted PCR products. The melting curve was obtained by slow heating at temperatures from 60 to 95°C at a rate of 0.2°C/s with continuous fluorescence collection. Amplification and detection were performed in 96-well optical plates with an ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA).

DNA extraction and qPCR. DNA extraction was performed by using the method described by Matsuki et al., with minor modifications (29). Briefly, DNA was extracted from a fresh culture of each bacterium (50 μl) and suspended in 50 μl Tris-EDTA buffer. qPCR were conducted using a QIAGEN OneStep RT-PCR kit (QIAGEN GmbH). Each qPCR was performed in a 20-μl reaction mixture containing DNA and SYBR green I (Molecular Probes) by using the same conditions that were used for RT-qPCR except for the reverse transcription step. qPCR amplification and detection were performed in 96-well optical plates with an ABI PRISM 7900HT sequence detection system (Applied Biosystems).

Determination of bacterial number by RT-qPCR. A standard curve was generated with the RT-qPCR data (using the threshold cycle [C_T] value, the cycle number when the threshold fluorescence was reached) and the corresponding cell count, which was determined microscopically with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Inc., Burlingame, CA) staining using the method of Jansen et al. (20), for dilution series of the following standard strains: *E. coli* ATCC 11775^T (for *Enterobacteriaceae*), *E. faecalis* ATCC 19433^T (for *Enterococcus*), *S. aureus* ATCC 12600^T (for *Staphylococcus*), *C. perfringens* JCM 1290^T (for *C. perfringens*), and *P. aeruginosa* ATCC 10145^T (for *Pseudomonas*). For determination of the bacteria present in samples, three serial dilutions of an extracted RNA sample were used for RT-qPCR, and the C_T values in the linear range of the assay were applied to the standard curve generated in the same experiment to obtain the corresponding number of bacteria in each nucleic acid sample and then converted to the number of bacteria per sample.

The specificity of the RT-qPCR assay was determined as follows. Total RNA fractions extracted from the cells of 50 bacterial strains corresponding to 10^5 cells were assessed for the RT-qPCR using the group- or species-specific primers shown in Table 2. Using the standard curve for the representative strain of each

group obtained as described above, the amplified signal was considered positive when it was greater than the signal for 10^4 standard cells and negative when it was less than the signal for 10^{-1} standard cell.

In situ viability staining. The viability of the bacteria was assessed using a LIVE/DEAD *Bac*Light bacterial viability kit (catalog no. L7012; Molecular Probes, Eugene, OR). Fresh bacterial cultures were incubated with 5 μM SYTO9 (Molecular Probes) and 30 μM propidium iodide (PI) (Molecular Probes) at 30°C for 10 min in the dark. SYTO9 and PI bind to DNA, and the complexes have an excitation maximum of 480 nm and an emission maximum of 500 nm for SYTO9 and an excitation maximum of 490 nm and an emission maximum of 635 nm for PI. SYTO9 is a green fluorescent dye that penetrates both viable and nonviable cells, while PI penetrates only bacteria with damaged plasma membranes (such as heat-treated or chemically treated, nonviable cells), quenching the green SYTO9 fluorescence (40). Thus, bacterial cells with compromised membranes fluoresce red, and bacterial cells with intact membranes fluoresce green. After incubation, cell suspensions were mixed with VECTASHIELD mounting medium (Vector Laboratories, Inc.) and then trapped between a glass slide and a square coverslip. The cells were imaged with a fluorescence microscope (Olympus BX-50; Olympus, Napa, CA) with a BX-FLA reflected-light fluorescence attachment using a combined fluorescein isothiocyanate-tetrarhodamine isothiocyanate filter set (catalog no. 51004v2; Chroma Technologies Corp, Brattleboro, VT). Images were then produced by using the image analysis software Image-Pro Plus, version 4 (Media-Cybernetics, Silver Spring, MD), and the ratio of the number of cells with green fluorescence (viable cells) to the total number of cells detected in each field (with both green and red fluorescence) was calculated. At the same time, the DAPI staining method was used to determine the total cell count in the bacterial culture. By multiplying the ratio for the viable cells by the total bacterial count obtained by DAPI staining, the number of viable cells in the culture was calculated with the following equation: number of viable bacteria = (number of cells labeled with SYTO9/number of cells labeled with both SYTO9 and PI) × (number of cells stained with DAPI).

Statistical analysis. We employed the SPSS14.0 software (SPSS Japan Inc., Tokyo, Japan). A regression analysis was performed to determine the statistical correlation of the results, and Pearson's product-moment correlation coefficient was calculated. A P value of <0.05 was considered significant.

RESULTS

Quantitative detection of bacteria by RT-qPCR compared with detection by qPCR. As shown in Fig. 1, the bacterial count obtained by direct staining (x axis) and the RT-qPCR value (C_T value, y axis) were found to correlate well over the range of RNA dilutions corresponding to bacterial counts ranging from 10^5 to 10^{-3} cell per reaction for *E. faecalis*, *S. aureus*, *C. perfringens*, and *P. aeruginosa* (Fig. 1B to E) and ranging from 10^5 to 10^{-1} cell per reaction for *E. coli* (Fig. 1A) (R^2, >0.99). Although there was nonspecific amplification of *E. coli* DNA or RNA that may have resulted from RT-qPCR reagents such as *Taq* DNA polymerase (data not shown), *E. coli* at a concentration of 10^{-1} cell per reaction was distinguishable (Fig. 1A). A comparison of the analytical curves for RT-qPCR with those for qPCR revealed no significant differences in slopes for the same target bacteria, indicating that the amplification efficiencies of RT-qPCR and qPCR were nearly equal, while the y-axis intercepts (C_T values) of the RT-qPCR analytical curve were 6 to 10 cycles less than those of the qPCR curve, indicating that the RT-qPCR assay was 64- to 1,024-fold more sensitive than the qPCR assay.

Next, total RNA extracts corresponding to 10^5 cells of 50 strains belonging to 50 species (Table 1) were assessed for specific detection of the target bacteria by RT-qPCR with the group-specific primers En-lsu3F and En-lsu3′R (for *Enterobacteriaceae*), Ec-ssu1′F and Ec-ssu1R (for *Enterococcus*), STPYF and STPYR2 (for *Staphylococcus*), and PSD7F and PSD7R (for *Pseudomonas*). As shown in Table 1, the primers gave

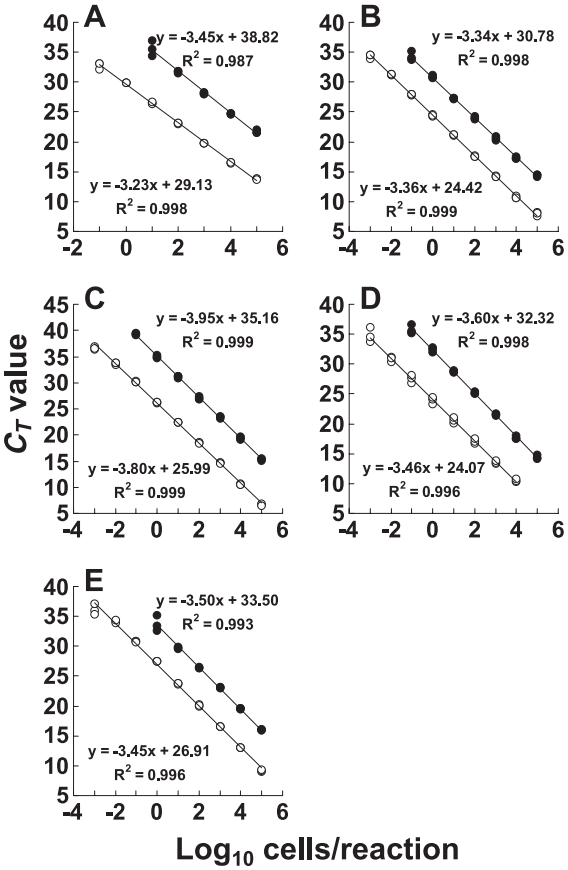

FIG. 1. Quantitative detection of bacteria by RT-qPCR and by qPCR. *E. coli* ATCC 11775^T (A), *E. faecalis* ATCC 19433^T (B), *S. aureus* ATCC 12600^T (C), *C. perfringens* JCM 1290^T (D), and *P. aeruginosa* ATCC 10145^T (E) were cultivated separately in BHI or MRS broth. RNA and DNA fractions were extracted from culture samples in the early stationary phase (18 h), and the bacterial counts were determined microscopically with DAPI staining. Based on the bacterial counts, 10-fold serial dilutions of RNA or DNA from 10^5 to 10^{-3} bacteria were assessed by RT-qPCR (○) and qPCR (●) assays. The C_T values for triplicate samples obtained were plotted against the $\log_{10}$ number of bacterial cells subjected to each reaction.

positive RT-qPCR results only for the corresponding target bacterial species and did not cross-react with any of the nontarget microorganisms tested. The specificity of primers ClPER-F and ClPER-R for *C. perfringens* reported previously (21) was also confirmed.

Comparison of the bacterial counts in culture determined by RT-qPCR and the culture method. The bacterial counts in the serial dilutions of in vitro cultures were determined by RT-qPCR and compared with the corresponding CFU counts. As shown in Fig. 2, specific amplification was detected for all the samples of five species at levels less than 10^1 CFU, and the C_T values and CFU counts were found to correlate well in the range from 10^0 to 10^5 CFU (R^2, >0.90) (Fig. 2). Based on these results, we suggest that rRNA-targeted RT-qPCR can determine the number of bacteria sensitively with a detection limit of 10^0 CFU.

Effect of growth phase on bacterial counts determined by RT-qPCR. The numbers of *E. coli*, *E. faecalis*, *S. aureus*, and *C. perfringens* cells in in vitro cultures were evaluated periodically

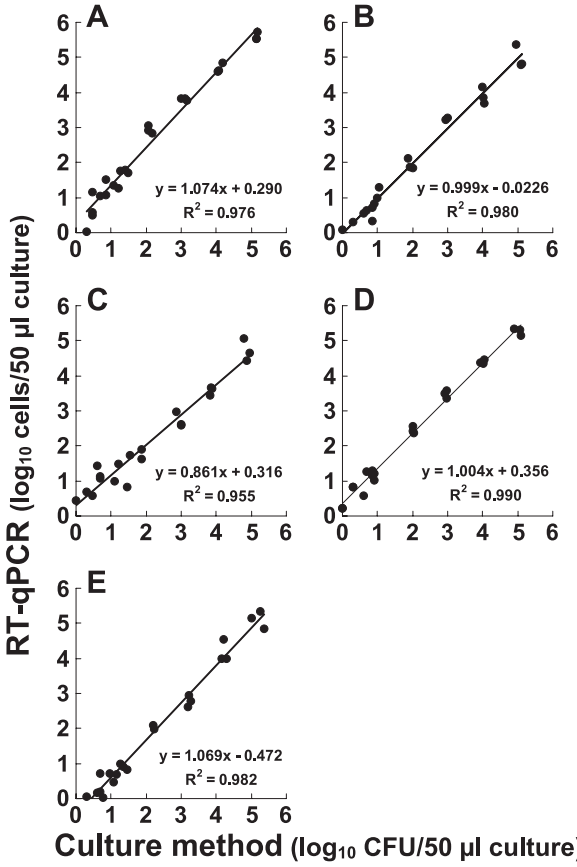

FIG. 2. Comparison of bacterial counts in cultures determined by RT-qPCR and by the culture method. *E. coli* ATCC 11775^T (A), *E. faecalis* ATCC 19433^T (B), *S. aureus* ATCC 12600^T (C), *C. perfringens* JCM 1290^T (D), and *P. aeruginosa* ATCC 10145^T (E) were cultivated in BHI or MRS broth. RNA fractions were extracted from 10-fold serial dilutions of each bacterial culture (50 μl) in the range from 10^0 to 10^5 CFU. The number of bacteria in each sample was determined by RT-qPCR and then plotted against the CFU count for the same sample determined on BHI (for *E. coli*, *E. faecalis*, *S. aureus*,and *P. aeruginosa*) or GAM (for *C. perfringens*) agar plates; data for single samples from each of the three different cultures are shown for each dilution. For RT-qPCR, an analytical curve generated with the RNA dilution series for each target strain (Fig. 1) was used.

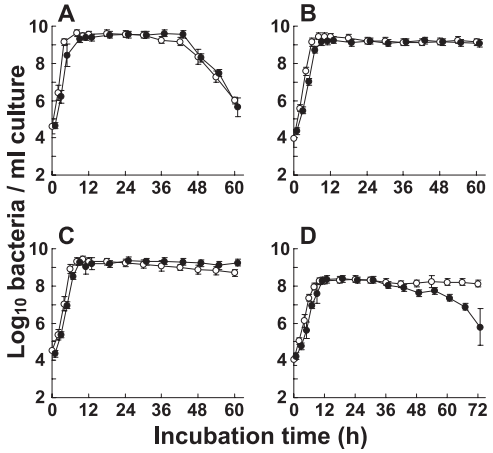

FIG. 3. Effect of growth phase on bacterial counts determined by RT-qPCR. Throughout the growth phase in broth culture, the numbers of *E. coli* ATCC 11775^T (A), *E. faecalis* ATCC 19433^T (B), *S. aureus* ATCC 12600^T (C), and *C. perfringens* JCM 1290^T (D) cells were determined by RT-qPCR (○) and the culture method (●). The analytical curves generated with the RNA dilution series for each target strain in the stationary phase (18 h) (Fig. 1) were used to quantify the bacteria. The CFU counts were determined on BHI (for *E. coli*, *E. faecalis*, and *S. aureus*) or GAM (for *C. perfringens*) agar plates. The results are the means and standard deviations of triplicate samples.

throughout the growth phases until 60 h (72 h for *C. perfringens*) both by RT-qPCR and by the culture method using a starting concentration of around 10^4 CFU per ml (Fig. 3). The RT-qPCR counts were calculated using the analytical curve for each standard strain at the early stationary phase (18 h) obtained in the experiment described above (Fig. 1). Throughout the growth phase until the stationary phase, the bacterial counts obtained by RT-qPCR were in good agreement with the counts obtained by the culture method for all the bacterial species tested (Fig. 3). For *E. coli*, the RT-qPCR counts decreased rapidly from 42 to 60 h during incubation, showing much the same pattern as the CFU counts (Fig. 3A). For *E. faecalis* and *S. aureus*, the population levels remained unchanged for 60 h during the stationary phase without any dissociation between the RT-qPCR counts and the CFU counts (Fig. 3B and C). On the other hand, for *C. perfringens*, although no significant difference between the two methods was detected until 42 h, dissociation was observed during the starvation period from 42 to 72 h (Fig. 3D); the CFU counts were found to be clearly lower than the RT-qPCR counts.

Comparison of RT-qPCR counts and viable bacterial cell counts by using a combination of DAPI staining and SYTO9-PI double staining of cultured bacteria. To further investigate the dissociation of the RT-qPCR counts and CFU counts for *C. perfringens* at the later stages of culture as described above, we determined the viable cell counts under conditions in which starved *C. perfringens* cultures were unable to form colonies. We used the SYTO9-PI double staining method, which has been reported to be able to differentiate live and dead bacteria based on differences in plasma membrane permeability (3, 15). The number of live cells stained only with SYTO9 remained 10^8 throughout the test period, while the CFU counts decreased markedly, demonstrating that most of the bacteria that lost the ability to form colonies on an agar plate were still alive and maintained their cell membrane integrity and that the numbers of cells in the population that could be detected were nearly equal to those detected by RT-qPCR (Fig. 4).

Comparison of the bacterial counts in human feces and blood determined by RT-qPCR and the culture method. In the next series of experiments, the applicability of the RT-qPCR method for enumeration of limited bacterial populations in the fecal flora or peripheral blood was examined. As shown in Fig. 5, members of the *Enterobacteriaceae* were detected in 38 samples from 19 hospitalized patients and *P. aeruginosa* was detected in seven samples, and linear regression was performed for the number of bacteria obtained by RT-qPCR and the number of bacteria obtained by the culture method. The experimental curve obtained for the *Enterobacteriaceae* had a slope of 0.887 and a correlation coefficient of 0.901 (Fig. 5A), and the curve for *P. aeruginosa* had a slope of 0.979 and a

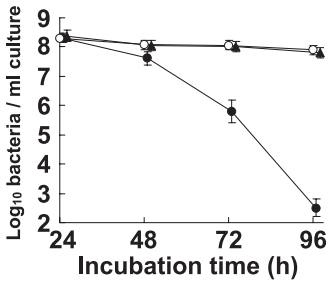

FIG. 4. Comparison of RT-qPCR counts and viable bacterial cell counts determined by a combination of DAPI staining and SYTO9-PI double staining of cultured bacteria. *C. perfringens* JCM 1290^T was incubated in MRS broth for 4 days and examined by RT-qPCR (○), SYTO9-PI double staining (▲), and the culture method (●) at 24, 48, 72, and 96 h. The viable bacterial count was calculated with the following equation: viable bacterial count = (number of cells labeled with SYTO9/number of cells labeled with both SYTO9 and PI) × (number of cells stained with DAPI). For RT-qPCR, the analytical curve generated with the dilution series of RNA extracted from *C. perfringens* cells at 18 h (Fig. 1D) was used to determine the bacterial number. The CFU count was determined by culturing samples on GAM agar plates for 24 h. The results are the means and standard deviations of triplicate samples.

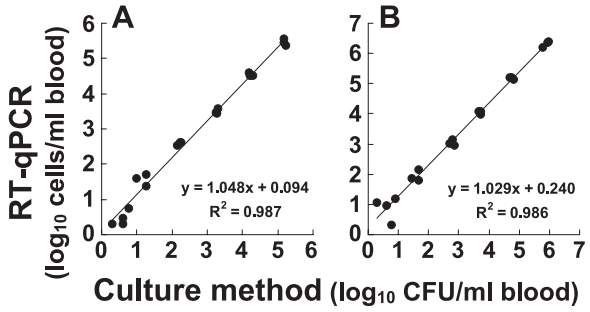

FIG. 6. Comparison of bacterial counts in human peripheral blood determined by RT-qPCR and the culture method. Human peripheral blood samples (0.5 ml) from three individuals were spiked with various amounts of live *S. aureus* ATCC 12600^T (A) or *P. aeruginosa* ATCC 10145^T (B) to obtain final concentrations ranging from 10^0 to 10^6 CFU per ml. RNA fractions extracted from each sample were then assessed by the RT-qPCR assay. The C_T values obtained were applied to the analytical curves for *S. aureus* ATCC 12600^T and *P. aeruginosa* ATCC 10145^T (Fig. 1C and E) to determine the RT-qPCR counts. The CFU counts were determined by culturing the same samples on BHI agar plates and then were plotted against the RT-qPCR counts; data for single samples from the three different donors are shown.

correlation coefficient of 0.981 (Fig. 5B), suggesting that there was a good correlation between the two methods. On the other hand, when *S. aureus* and *P. aeruginosa* were spiked into human peripheral blood, 2 CFU of *S. aureus* and 1 CFU of *P. aeruginosa* in 500 μl of human peripheral blood could be detected by RT-qPCR (Fig. 6). No false-positive results were obtained for the bacterium-free controls, showing that there was neither reagent contamination nor a cross-reaction with human nucleic acids in the determination (data not shown).

DISCUSSION

To develop a sensitive, specific, and convenient quantitative RT-PCR method to detect commensal subdominant bacteria, we focused on rRNA as the target. The sensitivity of the rRNA-targeted RT-qPCR method was approximately 100- to 1,000-fold higher than the sensitivity of the DNA-targeted qPCR (Fig. 1). rRNA is a universal constituent of bacterial ribosomes, and 5S, 16S, and 23S rRNAs are the components of small (30S) and large (50S) subunits that comprise the complete active ribosome (70S). In *E. coli*, the total number of ribosomal particles (30S, 50S, and 70S particles) per cell is known to reach a peak of more than 20,000 (1), with approximately 10^3 copies of each rRNA species per cell, while only seven copies of rRNA operons are present in a cell. Moreover, rRNA constitutes the largest fraction of RNA in the cell, and the proportion of rRNA in the total RNA is more than 80% (17). These aspects of rRNA, the high copy number and the high proportion of molecules, seem to contribute to the higher sensitivity of detection by RT-PCR than by PCR (Fig. 1). Although the expression of the rRNA gene has been considered more constant than the expression of other genes, which has been frequently used in quantitative studies and as an internal reference to analyze other gene expression (45, 47), the control of rRNA synthesis in bacteria has been found to be dependent on the growth rate. In rapidly dividing bacteria, the ribosome content per bacterium is much greater than that in slowly dividing cells (9), which is known as growth rate-dependent control. In the case of *E. coli*, the ribosome content has been reported to vary by more than 10-fold when the growth rate increased from a doubling time of 100 min to a doubling time of 24 min (10), and the rRNA synthesis is repressed by feedback mechanisms that prevent excessive production of more ribosomes than are needed for protein synthesis during balanced or steady-state growth (2, 9). These aspects of rRNA affect whether this molecule can be used as a target for standardizing bacterial populations by RT-qPCR. In this study, although comparisons of RT-qPCR counts and CFU counts during the logarithmic phase showed that the bacterial counts obtained by RT-qPCR tended to be higher than the CFU counts (Fig. 3), there was no significant difference between the values; the differences were at most fourfold. Therefore, we suggest that the rRNA-targeted RT-qPCR method is suitable

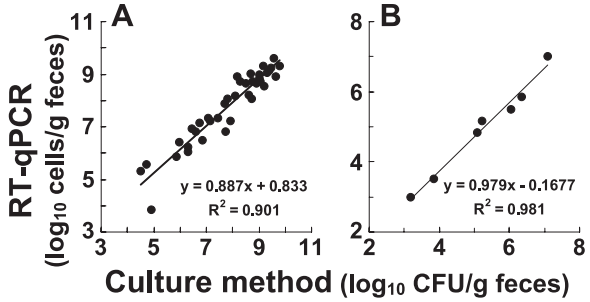

FIG. 5. Correlation between RT-qPCR counts and cultural counts in human feces. Total RNA fractions extracted from 38 human fecal homogenates were assessed by the RT-qPCR assay to determine the indigenous population levels of *Enterobacteriaceae* (A) and *P. aeruginosa* (B). The C_T values obtained were applied to the analytical curves for *E. coli* ATCC 11775^T and *P. aeruginosa* ATCC 10145^T (Fig. 1A and E) to determine the RT-qPCR counts. The CFU counts were determined by culturing the same fecal samples on DHL (A) or NAC (B) agar plates and then were plotted against the RT-qPCR counts.

for quantification of a bacterial population irrespective of the growth phase. However, it is important to consider the changeable metabolic activity of bacteria in order to obtain correct data by this procedure.

Recently, RNA molecules have been used as an indicator of bacterial cell viability as an alternative to colony-forming ability or DNA molecules (6, 11, 14, 32). rRNA has been recognized as more labile and is more susceptible to degradation caused by adverse treatment than DNA, and its level is positively correlated with viability under some bacterial killing regimens (34). In *E. coli*, the decrease in the level of rRNA during 60 h of incubation showed a good correlation with the decrease in the number of CFU (Fig. 3A). In addition, the RT-qPCR counts for *Enterobacteriaceae* and *P. aeruginosa* in human feces were highly correlated with the CFU counts (Fig. 5). However, as it has been reported that severe stress, such as heat shock (33), UV irradiation (34), or ethanol treatment (39), increases the dissociation between the CFU count and the rRNA content, further analysis of whether rRNA can be used as an accurate indicator of bacterial viability under any possible biological conditions is still needed. In any case, the results suggest that the amount of rRNA can be used as an indicator of viable bacterial population size at least in physiologic circumstances such as in gastrointestinal tracts.

The CFU counts of *C. perfringens* were significantly lower than the RT-qPCR counts after 42 h of incubation, and the viable cell counts determined with a LIVE/DEAD *Bac*Light bacterial viability kit were nearly equal to the RT-qPCR counts rather than the CFU counts (Fig. 4). The viable but not culturable state of bacteria has been reported to be a survival mechanism for bacteria that allows them to face environmentally stressful conditions, such as starvation, incubation outside the temperature range for growth, elevated osmotic concentrations, or exposure to white light (37). When in such a state, bacteria often do not grow on conventional culture media but still have metabolic activity, maintain pathogenicity (24), and, in some cases, may return to active growth when optimal conditions are restored (7, 25). The viability of nonculturable cells is typically determined by the substrate responsive assay (direct viable count assay) (22), by detection of respiratory activity (CTC assay) (19), by monitoring the membrane potential (26), or by determining the presence of an intact cytoplasmic membrane (3, 4, 15, 27). Although approximately 60 species have now been reported to demonstrate this physiological response, there have been no descriptions of clostridia (37). Therefore, for this phenomenon in *C. perfringens*, further analysis of the bacterial cell status from several perspectives, such as metabolic activity and membrane potential, in addition to the presence of nucleic acids, membrane integrity, and cultivability, is still needed.

The new rRNA-targeted RT-qPCR technique developed in the present study enables detection of minor bacterial species, such as members of the *Enterobacteriaceae*, *Enterococcus*, *Staphylococcus*, and *C. perfringens*, with sensitivity equal to that of the culture method (10^2 to 10^3 CFU per g feces), as well as detection of the predominant populations in the intestines. Because of its high sensitivity and convenience, the RT-qPCR assay targeting rRNA may be useful for a wide variety of bacteriological examinations. It can be used for detection of opportunistic infections in clinical settings. Quick evaluation of contamination is essential in clinical examinations, but even the molecular methods previously reported require a cultivation step to increase the number of cells several days prior to the assay (23). The entire RT-qPCR assay, including the RNA extraction step developed in this study, can be completed in 5 h, and its sensitivity may allow omission of the cultivation step and eliminate the risk of false-positive results. In addition to assessment of specific bacterial counts in feces and peripheral blood, it can be used for rapid detection of potential bacterial contamination in tissue specimens and smaller bacterial populations, such as oral and vaginal microfloras. Moreover, the RT-qPCR assay might also be an effective tool for examining environmental microbial populations, such as those in water and soil, and for quick evaluation of food contamination. The method may be especially valuable for detecting noncultivable, subdominant members of bacterial communities or for examining samples that have been frozen and therefore are not suitable for culture-based examination. On the other hand, identification of certain functions of bacteria is the next objective after determination of the exact population levels by RT-qPCR, leading to information about what commensal bacteria do in the corresponding environments. By using the same RNA specimens used for rRNA quantification, various information concerning bacterial functions should be available from the viewpoint of mRNA. For example, specific pathogens have unique virulence factors, such as the production of toxins, an apparatus for invasion, and drug resistance, which can also be targets for quantitative analysis of the corresponding mRNA expression.

In conclusion, we developed an RT-qPCR detection method targeting rRNA to enumerate bacteria in human feces and peripheral blood. Specific primers for rRNA sequences of *Enterobacteriaceae*, *Enterococcus*, *Staphylococcus*, *Pseudomonas*, and *C. perfringens* were used in conjunction with RT-qPCR, which allowed sensitive and accurate quantification of the target bacteria. The sensitivity was approximately 100-fold higher than that of the existing PCR methods and nearly equivalent to that of conventional culture methods. This RT-qPCR method should be an effective tool for sensitive quantification of viable bacterial populations.

ACKNOWLEDGMENTS

We thank Toshihiko Takada and Takahiro Matsuki for their technical advice. We also thank Rie Fujioka for her assistance with this research.

REFERENCES

1. **Arfvidsson, C., and K. G. Wahlund.** 2003. Time-minimized determination of ribosome and tRNA levels in bacterial cells using flow field-flow fractionation. Anal. Biochem. **313:**76–85.
2. **Asato, Y.** 2005. Control of ribosome synthesis during the cell division cycles of E. coli and Synechococcus. Curr. Issues Mol. Biol. **7:**109–117.
3. **Auty, M. A., G. E. Gardiner, S. J. McBrearty, E. O. O'Sullivan, D. M. Mulvihill, J. K. Collins, G. F. Fitzgerald, C. Stanton, and R. P. Ross.** 2001. Direct in situ viability assessment of bacteria in probiotic dairy products using viability staining in conjunction with confocal scanning laser microscopy. Appl. Environ. Microbiol. **67:**420–425.
4. **Banning, N., S. Toze, and B. J. Mee.** 2002. Escherichia coli survival in groundwater and effluent measured using a combination of propidium iodide and the green fluorescent protein. J Appl. Microbiol. **93:**69–76.
5. **Bartosch, S., A. Fite, G. T. Macfarlane, and M. E. McMurdo.** 2004. Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. Appl. Environ Microbiol. **70:** 3575–3581.

6. **Bleve, G., L. Rizzotti, F. Dellaglio, and S. Torriani.** 2003. Development of reverse transcription (RT)-PCR and real-time RT-PCR assays for rapid detection and quantification of viable yeasts and molds contaminating yogurts and pasteurized food products. Appl. Environ Microbiol. **69:**4116–4122.
7. **Chaveerach, P., A. A. ter Huurne, L. J. Lipman, and F. van Knapen.** 2003. Survival and resuscitation of ten strains of *Campylobacter jejuni* and *Campylobacter coli* under acid conditions. Appl. Environ. Microbiol. **69**:711–714.
8. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
9. **Condon, C., C. Squires, and C. L. Squires.** 1995. Control of rRNA transcription in *Escherichia coli*. Microbiol. Rev. **59:**623–645.
10. **Dennis, P. P., M. Ehrenberg, and H. Bremer.** 2004. Control of rRNA synthesis in *Escherichia coli*: a systems biology approach. Microbiol. Mol. Biol. Rev. **68:**639–668.
11. **Dreier, J., M. Stormer, and K. Kleesiek.** 2004. Two novel real-time reverse transcriptase PCR assays for rapid detection of bacterial contamination in platelet concentrates. J. Clin. Microbiol. **42:**4759–4764.
12. **Eckburg, P. B., E. M. Bik, C. N. Bernstein, E. Purdom, L. Dethlefsen, M. Sargent, S. R. Gill, K. E. Nelson, and D. A. Relman.** 2005. Diversity of the human intestinal microbial flora. Science **308:**1635–1638.
13. **Favier, C. F., E. E. Vaughan, W. M. De Vos, and A. D. Akkermans.** 2002. Molecular monitoring of succession of bacterial communities in human neonates. Appl. Environ. Microbiol. **68:**219–226.
14. **Felek, S., A. Unver, R. W. Stich, and Y. Rikihisa.** 2001. Sensitive detection of *Ehrlichia chaffeensis* in cell culture, blood, and tick specimens by reverse transcription-PCR. J. Clin. Microbiol. **39:**460–463.
15. **Gardiner, G. E., E. O'Sullivan, J. Kelly, M. A. Auty, G. F. Fitzgerald, J. K. Collins, R. P. Ross, and C. Stanton.** 2000. Comparative survival rates of human-derived probiotic *Lactobacillus paracasei* and *L. salivarius* strains during heat treatment and spray drying. Appl. Environ. Microbiol. **66:**2605–2612.
16. **Gueimonde, M., S. Tolkko, T. Korpimaki, and S. Salminen.** 2004. New real-time quantitative PCR procedure for quantification of bifidobacteria in human fecal samples. Appl. Environ. Microbiol. **70:**4165–4169.
17. **Hansen, M. C., A. K. Nielsen, S. Molin, K. Hammer, and M. Kilstrup.** 2001. Changes in rRNA levels during stress invalidates results from mRNA blotting: fluorescence in situ rRNA hybridization permits renormalization for estimation of cellular mRNA levels. J. Bacteriol. **183:**4747–4751.
18. **Harmsen, H. J., G. C. Raangs, T. He, J. E. Degener, and G. W. Welling.** 2002. Extensive set of 16S rRNA-based probes for detection of bacteria in human feces. Appl. Environ. Microbiol. **68:**2982–2990.
19. **Hatzinger, P. B., P. Palmer, R. L. Smith, C. T. Penarrieta, and T. Yoshinari.** 2003. Applicability of tetrazolium salts for the measurement of respiratory activity and viability of groundwater bacteria. J Microbiol. Methods **52:**47–58.
20. **Jansen, G. J., A. C. Wildeboer-Veloo, R. H. Tonk, A. H. Franks, and G. W. Welling.** 1999. Development and validation of an automated, microscopy-based method for enumeration of groups of intestinal bacteria. J. Microbiol. Methods **37:**215–221.
21. **Kikuchi, E., Y. Miyamoto, S. Narushima, and K. Itoh.** 2002. Design of species-specific primers to identify 13 species of Clostridium harbored in human intestinal tracts. Microbiol. Immunol. **46:**353–358.
22. **Kogure, K., U. Simidu, and N. Taga.** 1979. A tentative direct microscopic method for counting living marine bacteria. Can. J. Microbiol. **25:**415–420.
23. **Kurupati, P., C. Chow, G. Kumarasinghe, and C. L. Poh.** 2004. Rapid detection of *Klebsiella pneumoniae* from blood culture bottles by real-time PCR. J. Clin. Microbiol. **42:**1337–1340.
24. **Lleo, M. M., B. Bonato, C. Signoretto, and P. Canepari.** 2003. Vancomycin resistance is maintained in enterococci in the viable but nonculturable state and after division is resumed. Antimicrob. Agents Chemother. **47:**1154–1156.
25. **Lleo, M. M., B. Bonato, M. C. Tafi, C. Signoretto, M. Boaretti, and P. Canepari.** 2001. Resuscitation rate in different enterococcal species in the viable but non-culturable state. J. Appl. Microbiol. **91:**1095–1102.
26. **Lopez-Amoros, R., S. Castel, J. Comas-Riu, and J. Vives-Rego.** 1997. Assessment of E. coli and Salmonella viability and starvation by confocal laser microscopy and flow cytometry using rhodamine 123, DiBAC4(3), propidium iodide, and CTC. Cytometry **29:**298–305.
27. **Lowder, M., A. Unge, N. Maraha, J. K. Jansson, J. Swiggett, and J. D. Oliver.** 2000. Effect of starvation and the viable-but-nonculturable state on green fluorescent protein (GFP) fluorescence in GFP-tagged *Pseudomonas fluorescens* A506. Appl. Environ. Microbiol. **66:**3160–3165.
28. **Maidak, B. L., J. R. Cole, T. G. Lilburn, C. T. Parker, Jr., P. R. Saxman, R. J. Farris, G. M. Garrity, G. J. Olsen, T. M. Schmidt, and J. M. Tiedje.** 2001. The RDP-II (Ribosomal Database Project). Nucleic Acids Res. **29:**173–174.
29. **Matsuki, T., K. Watanabe, J. Fujimoto, Y. Kado, T. Takada, K. Matsumoto, and R. Tanaka.** 2004. Quantitative PCR with 16S rRNA-gene-targeted species-specific primers for analysis of human intestinal bifidobacteria. Appl. Environ. Microbiol. **70:**167–173.
30. **Matsuki, T., K. Watanabe, J. Fujimoto, T. Takada, and R. Tanaka.** 2004. Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in human feces. Appl. Environ. Microbiol. **70:**7220–7228.
31. **Matsumoto, M., M. Sakamoto, H. Hayashi, and Y. Benno.** 2005. Novel phylogenetic assignment database for terminal-restriction fragment length polymorphism analysis of human colonic microbiota. J Microbiol. Methods **61:**305–319.
32. **McKillip, J. L., and M. Drake.** 2004. Real-time nucleic acid-based detection methods for pathogenic bacteria in food. J. Food Prot. **67:**823–832.
33. **McKillip, J. L., L. A. Jaykus, and M. Drake.** 1999. Nucleic acid persistence in heat-killed Escherichia coli O157:H7 from contaminated skim milk. J. Food Prot. **62:**839–844.
34. **McKillip, J. L., L. A. Jaykus, and M. Drake.** 1998. rRNA stability in heat-killed and UV-irradiated enterotoxigenic *Staphylococcus aureus* and *Escherichia coli* O157:H7. Appl. Environ. Microbiol. **64:**4264–4268.
35. **Moore, W. E., and L. V. Holdeman.** 1974. Human fecal flora: the normal flora of 20 Japanese-Hawaiians. Appl. Microbiol. **27:**961–979.
36. **Nagashima, K., T. Hisada, M. Sato, and J. Mochizuki.** 2003. Application of new primer-enzyme combinations to terminal restriction fragment length polymorphism profiling of bacterial populations in human feces. Appl. Environ. Microbiol. **69:**1251–1262.
37. **Oliver, J. D.** 2005. The viable but nonculturable state in bacteria. J. Microbiol. **43**(Spec. No.)**:**93–100.
38. **Sen, K.** 2000. Rapid identification of *Yersinia enterocolitica* in blood by the 5′ nuclease PCR assay. J. Clin. Microbiol. **38:**1953–1958.
39. **Sheridan, G. E., C. I. Masters, J. A. Shallcross, and B. M. MacKey.** 1998. Detection of mRNA by reverse transcription-PCR as an indicator of viability in *Escherichia coli* cells. Appl. Environ. Microbiol. **64:**1313–1318.
40. **Stocks, S. M.** 2004. Mechanism and use of the commercially available viability stain, BacLight. Cytometry **61A:**189–195.
41. **Suau, A.** 2003. Molecular tools to investigate intestinal bacterial communities. J. Pediatr. Gastroenterol. Nutr. **37:**222–224.
42. **Suau, A., R. Bonnet, M. Sutren, J. J. Godon, G. R. Gibson, M. D. Collins, and J. Dore.** 1999. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Appl. Environ. Microbiol. **65:**4799–4807.
43. **Takada, T., K. Matsumoto, and K. Nomoto.** 2004. Development of multicolor FISH method for analysis of seven Bifidobacterium species in human feces. J Microbiol. Methods **58:**413–421.
44. **Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins.** 1997. The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **25:**4876–4882.
45. **Vaudaux, P., P. Francois, C. Bisognano, W. L. Kelley, D. P. Lew, J. Schrenzel, R. A. Proctor, P. J. McNamara, G. Peters, and C. Von Eiff.** 2002. Increased expression of clumping factor and fibronectin-binding proteins by *hemB* mutants of *Staphylococcus aureus* expressing small-colony variant phenotypes. Infect. Immun. **70:**5428–5437.
46. **Wilson, K. H., and R. B. Blitchington.** 1996. Human colonic biota studied by ribosomal DNA sequence analysis. Appl. Environ. Microbiol. **62:**2273–2278.
47. **Yarwood, J. M., J. K. McCormick, M. L. Paustian, V. Kapur, and P. M. Schlievert.** 2002. Repression of the *Staphylococcus aureus* accessory gene regulator in serum and in vivo. J. Bacteriol. **184:**1095–1101.
48. **Zoetendal, E. G., C. T. Collier, S. Koike, R. I. Mackie, and H. R. Gaskins.** 2004. Molecular ecological analysis of the gastrointestinal microbiota: a review. J. Nutr. **134:**465–472.