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Quantification of 16S rRNAs in Complex Bacterial Communities by Multiple Competitive Reverse Transcription-PCR in Temperature Gradient Gel Electrophoresis Fingerprints

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A novel approach was developed to quantify rRNA sequences in complex bacterial communities. The main bacterial 16S rRNAs in Drentse A grassland soils (The Netherlands) were amplified by reverse transcription (RT)-PCR with bacterium-specific primers and were separated by temperature gradient gel electrophoresis (TGGE). The primer pair used (primers U968-GC and L1401) was found to amplify with the same efficiency 16S rRNAs from bacterial cultures containing different taxa and cloned 16S ribosomal DNA amplicons from uncultured soil bacteria. The sequence-specific efficiency of amplification was determined by monitoring the amplification kinetics by kinetic PCR. The primer-specific amplification efficiency was assessed by competitive PCR and RT-PCR, and identical input amounts of different 16S rRNAs resulted in identical amplicon yields. The sequence-specific detection system used for competitive amplifications was TGGE, which also has been found to be suitable for simultaneous quantification of more than one sequence. We demonstrate that this approach can be applied to TGGE fingerprints of soil bacteria to estimate the ratios of the bacterial 16S rRNAs.

Since its initial application to environmental 16S ribosomal DNA (rDNA) by Muyzer et al. (20), denaturing gradient gel electrophoresis (DGGE) has been an attractive technique in molecular microbial ecology. Various workers have described microbial diversity as assessed by DGGE for a variety of different ecosystems. In spite of the growing interest in this technique, little attention has been given to the quantitative aspects of the fingerprints of bacterial communities. In most studies the workers investigated uncultured bacteria which were detected in environmental nucleic acid extracts by 16S rDNA fingerprints generated either by temperature gradient gel electrophoresis (TGGE) (25) or DGGE (13). Since such fingerprints were a result of PCR amplification of nucleic acid sequences, quantification of the signals had to be based on the principles of the quantitative PCR approach. In spite of the wide application of PCR, the quantitative use of PCR is not straightforward. Since the DNA molecules are amplified during PCR, the amount of initial target molecules can be estimated only by presuming that amplification efficiency is reproducible. The exponential nature of the amplification process is highly sensitive to any disturbance of amplification efficiency, which can easily result in major PCR bias. The main reason to use PCR for quantification is its sensitivity and specificity in comparison to the sensitivity and specificity of other techniques.

The three main methods used for quantitative analysis by PCR (or reverse transcription [RT]-PCR) are the limiting dilution PCR (23, 29), the kinetic PCR (1, 4, 7, 31), and the competitive PCR (3, 14, 33). The limiting dilution PCR approach is based on simple dilution of the template. For the other two methods a standard template of known concentration is required. This standard must be similar to the target to ensure equal amplification of both templates. The kinetic PCR determines the increase in the number of amplicons with time by measuring the absolute amount of DNA per cycle. On the one hand, this technique monitors the amplification efficiency (i.e., the exponential increase in the amount of PCR product). On the other hand, the time shift in the exponential growth curve between the target and the standard allows calculation of the unknown template DNA concentration in the target sample. An easier and more convenient method is the competitive PCR. In this method the standard and the target have different sequences to distinguish them and are amplified in the same reaction tube. This eliminates bias caused by the thermocycler or the reaction mixture. Defined serial dilutions of the standard template in a couple of parallel PCR mixtures are prepared to compete with the target sequence. The reaction in which the amounts of the PCR products of the standard and target are the same indicates the concentration of the original target template. The crucial point is to design a standard sequence that can be easily distinguished from the target after amplification. Since TGGE and DGGE are tools that are used to separate amplicons on the basis of their sequences, they are also suitable detection tools for quantitative PCR.

Competitive PCR initially was developed and used for mRNA obtained from target cells growing in pure culture (3, 14, 33), not for nucleic acids obtained from uncultured environmental bacteria. Recently, the amounts of particular genes in bacterial genomic DNA retrieved from soil and sediments have been determined (15, 19, 35). The application of kinetic PCR to 16S rDNA sequences (4) and the first attempt to perform a competitive PCR with environmental 16S rDNA (17) have been described only recently. In the latter study, the application of quantitative PCR to 16S rDNA of uncultured bacteria could be disputed, because the amount of 16S rDNA sequences per cell could not be estimated. It has been observed previously that the variable numbers of *rrn* operons and the genome sizes of different species are crucial parameters, and

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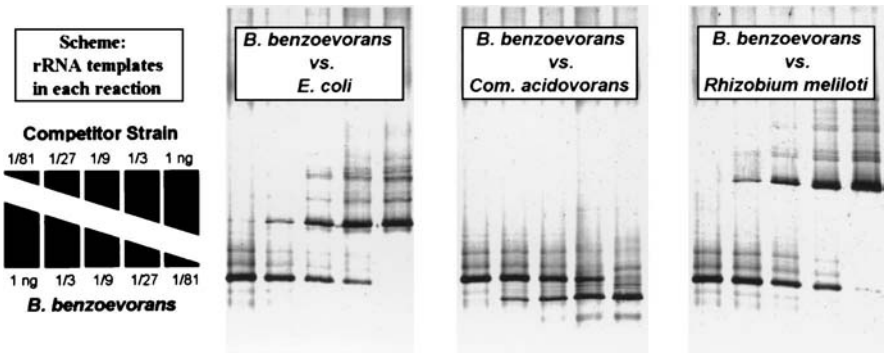

FIG. 1. Competitive RT-PCR performed with rRNA standards from different bacterial taxa. The scheme on the left shows the order of rRNA input. In the third of the five reactions equal amounts of the two competitor rRNAs are present. This ratio is also reflected by band intensities after separation of the amplicons by TGGE and detection by silver staining (2 μl of RT-PCR product per lane). The faint bands accompanying the main bands were RT-PCR side products and were not included in the quantitative analysis. We used more RT-PCR product than necessary to visualize traces of the out-competed sequence. However, the highly sensitive silver staining method also detected some RT-PCR side products, most likely side products representing a DNA polymerization bias.

consequently, 16S rDNA amplification of different bacterial strains reflected neither cell numbers nor ratios of nucleic acid amounts (8). As an alternative approach, we quantified bacterial ribosomes by using their 16S rRNA in order to monitor spatial changes in bacterial activity in soil (9, 10, 12). Ribosomes can be used as a marker for bacterial activity (34), because the amounts of ribosomes (and their rRNA) per cell were found to be roughly proportional to the growth activity of bacteria in pure culture (32).

In a previous study, the predominant 16S rRNAs of a bacterial community in soil were revealed by TGGE, hybridization, cloning, and sequencing (12). This study focused on rRNA to identify the most active bacteria. After direct ribosome isolation from soil, part of the bacterial 16S rRNA was amplified by RT-PCR. Sequence-specific separation of partial 16S rRNA amplicons by TGGE yielded reproducible, soil-specific fingerprints. The predominant bands of these fingerprints were identified by using a clone library of 16S rDNA amplicons, which resulted in characterization by sequence analysis. Here we describe a novel approach to quantify the 16S rRNA of uncultured bacteria by quantitative RT-PCR and evaluation of the amplification step. Careful evaluation of the amplification efficiencies of the sequences concerned was necessary, as demonstrated by different model experiments.

MATERIALS AND METHODS

Soil sampling. We selected a plot with an area of several 100 m^2 in the Drentse A agricultural research area in The Netherlands (06°41′E, 53°03′N) for sample collection. This grassland plot had not been fertilized since 1990 and was described as type A in a previous study (12). Details of the soil properties have been published previously (26). A total of 40 surface samples (depth, <10 cm) were taken in March 1996. Soil cores weighing approximately 50 g were obtained with a drill (depth, 0 to 10 cm) and then were transferred into sterile sample bags and stored at 4°C for a maximum of 48 h before nucleic acid extraction.

Bacterial strains. Several rRNA standards were prepared by extracting rRNA from laboratory cultures of the following strains: *Alcaligenes faecalis* DSM 30030, *Arthrobacter atrocyaneus* DSM 20127, *Azospirillum brasiliense* DSM 1690, *Bacillus benzoevorans* DSM 6385, *Bacillus subtilis* DSM 10, *Comamonas acidovorans* DSM 50251, *Escherichia coli* NM 522, *Pseudomonas fluorescens* DSM 50090, *Rhizobium meliloti* DSM 1981, and *Streptomyces griseus* DSM 773. All of the strains were grown as recommended by the distributors (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; Promega, Madison, Wis.).

Preparation of rRNA standards from pure cultures. Twenty-milliliter bacterial batch cultures at the end of the logarithmic growth phase were harvested by centrifugation for 10 min at 5,000 × g (Sorvall model RC24 superspeed centrifuge equipped with a type SM24 rotor). Each supernatant was discarded, and the bacterial pellet was resuspended in 8 ml of TN150 buffer (10 mM Tris-HCl [pH 8.0], 150 mM sodium chloride). Subsequently, 1 ml of TE-buffered phenol and 1 ml of chloroform-isoamyl alcohol (24:1) were added to a sterilized 12-ml cell homogenizer tube containing 3 g of glass beads (diameter, 110 μm). This tube was closed tightly and treated for 1 min in an MSK cell homogenizer (Braun-Melsungen, Melsungen, Germany) at 4,000 rpm. Then the glass beads, phenol, and precipitated cell debris were separated by centrifugation at 5,000 × *g* for 5 min. The aqueous phase was transferred into a 50-ml centrifuge tube, and after 2 volumes of ice-cold ethanol was added, the nucleic acids were precipitated by incubation for 30 min at −20°C and were collected by centrifugation for 20 min at 10,000 × *g*. The pellet was washed with 5 ml of 70% ethanol, air dried, and then resuspended in 500 μl of TMC buffer (10 mM Tris-HCl [pH 7.5], 5 mM magnesium chloride, 0.1 mM cesium chloride). After transfer into a 1.5-ml microcentrifuge tube, the DNA was digested for 15 min at 37°C with 5 μl of RNase-free DNase (RQ1; Promega). The reaction was terminated by adding 400 μl of water-saturated phenol-chloroform-isoamyl alcohol (25:24:1). The tube was vortexed for 1 min and centrifuged in a microcentrifuge for 1 min at full speed. The extraction procedure was repeated with 400 μl of chloroform-isoamyl alcohol (24:1). Ethanol precipitation was done as described above, and the purified rRNA was resuspended in 500 μl of Tris buffer (10 mM Tris-HCl, pH 8.0). The yields were up to 1 mg per culture, as estimated by UV spectrophotometry. Solutions containing 1 μg of rRNA per ml of Tris buffer-glycerol (1:1, vol/vol) were prepared as standards for subsequent competitive RT-PCR experiments. The glycerol allowed unfrozen storage at −20°C, which is optimal for multiple use (11).

Ribosome isolation from soil and bacterial rRNA yield estimation. Soil rRNA was obtained by isolating ribosomes from Drentse A soil samples by a previously described protocol (9). Briefly, ribosomes were released from the soil (1 g) by treatment with a bead beater in the presence of ribosome buffer. Subsequent centrifugations removed cell debris and soil particles from the suspension. Then the ribosomes were precipitated by centrifugation for 2 h at 100,000 × *g*. The rRNA was isolated and purified by phenol extraction, ethanol precipitation, and DNase digestion. rRNA solutions were prepared in Tris buffer-glycerol (1:1, vol/vol) for subsequent competitive RT-PCR experiments. The *Bacteria*-specific probe EUB338 (1) was used to estimate the amount of bacterial rRNA per gram of soil by dot blot hybridization. Soil rRNA was blotted and fixed onto a nylon membrane (Hybond-N+; Amersham, Rainham, United Kingdom) as described previously (2). The EUB338 oligonucleotide was 5′ labeled by using phage T4 polynucleotide kinase (Promega) and 30 μCi of [γ-^{32}P]ATP. Prehybridization, hybridization, and stringent washing were performed as described by Manz et al. (18). The signals of the radioactively labeled probe were analyzed with a PhosphorImager SF (Molecular Dynamics, Oakland, Mass.). Soil rRNA signals were related to signals obtained with *E. coli* rRNA standards of known concentrations to calculate the soil rRNA content.

Competitive RT-PCR performed with rRNA and primers U968-GC and L1401. The competitive RT-PCR was performed with an rTth DNA polymerase kit (Perkin-Elmer Cetus, Norwalk, Conn.). The RT reaction mixtures (10 μl) contained 10 mM Tris-HCl (pH 8.3), 90 mM KCl, 1 mM $MnCl_2$, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 200 μM dTTP, 750 nM primer L1401 (22), 2.5 U of rTth DNA polymerase, and 2 μl of rRNA from each competitor. After incubation for 15 min at 68°C, 40 μl of a PCR mixture containing 10 mM Tris-HCl (pH 8.3), 100 mM KCl, 0.75 mM EGTA, 0.05% Tween 20, 3.75 mM $MgCl_2$, 50 μM dATP, 50 μM dCTP, 50 μM dGTP, 50 μM dTTP, and 190 nM primer U968-GC (22) was added. Amplification was performed with a model 2400 GeneAmp PCR System thermocycler by using 35 cycles consisting of 94°C for 10 s, 56°C for 20 s, and 68°C for 40 s. Adjusted rRNA solutions obtained from the 10 bacterial standard strains (see above) were compared with each other in an experiment consisting of 45 competitive RT-PCR assays. Each competitive RT-PCR experiment consisted of five reaction mixtures containing decreasing gradients of competitor rRNA (Fig. 1). For multiple-competitor RT-PCR, the first compet-

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itor was always the *E. coli* rRNA standard, while the second competitor was a defined mixture containing the other bacterial 16S rRNA standards or soil rRNA.

A Diagen TGGE system (Diagen, Düsseldorf, Germany) was used for sequence-specific separation of competitor amplicons after RT-PCR. Electrophoresis was performed in a 0.8-mm polyacrylamide gel (6% [wt/vol] acrylamide, 0.1% [wt/vol] bisacrylamide, 8 M urea, 20% [vol/vol] formamide, 2% [vol/vol] glycerol) with 1× TA buffer (40 mM Tris-acetate, pH 8.0) at a fixed current of 9 mA (about 120 V) for 16 h. A temperature gradient from 37 to 46°C was built up in the direction of electrophoresis. After electrophoresis the gels were silver stained (6). The gels were analyzed with MolecularAnalyst/PC fingerprinting software (Bio-Rad, Hercules, Calif.).

Preparation of DNA standards for kinetic PCR. The 10 bacterial strains which were used as rRNA standards were checked for equal amplification efficiency by kinetic PCR, and the 20 environmental cloned ribotypes representing the predominant band signals in the TGGE fingerprints from Drentse A soil were also checked (12). Uniform DNA templates were generated by PCR to overcome the problem of different numbers of 16S rDNA target sequences per amount of DNA. This could vary between different bacterial genomes (8), and the plasmid DNA of the transformants provided a much higher 16S rDNA target sequence concentration than genomic DNA provided. After the bacterial standard strains and the transformants containing the environmental sequences were grown on solid medium, single colonies were transferred into 1.5-ml microcentrifuge tubes containing 50 μl of TE buffer. The tubes were heated for 15 min at 95°C to lyse the cells and then chilled on ice. The 16S rDNA sequences were amplified by using 35 cycles consisting of 94°C for 10 s, 48°C for 20 s, and 68°C for 2 min. Each PCR mixture (50 μl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 150 μM dATP, 150 μM dCTP, 150 μM dGTP, 150 μM dTTP, 30 pmol of each primer, 2.5 U of *Taq* DNA polymerase (Life Technologies, Paisley, United Kingdom), and 1 μl of cell lysate. Bacterium-specific primers 8f and 1512r (10) were used for the cultured bacteria, and vector-specific primers T7 and SP6 (16) were used for the cloned sequences. Rough estimates of the DNA amplification yields were obtained by 1.4% agarose gel electrophoresis, and the preparations were diluted to concentrations of approximately 1 ng of DNA μl^{-1}.

Kinetic PCR. The 16S rDNA PCR products obtained from the 10 standard bacteria and the 20 environmental ribotypes (see above) were used as uniform templates for kinetic PCR. Fivefold dilutions (approximately 200 and 40 pg μl^{-1}) were prepared from the template solutions (approximately 1 ng of DNA μl^{-1}) in order to determine the influence of template concentration on amplification efficiency. The preparations containing the three different DNA concentrations were amplified with an Amplitron II thermocycler (Barnstead/Thermolyne, Dubuque, Iowa) by using 10 to 26 cycles consisting of 94°C for 10 s, 56°C for 20 s, and 68°C for 40 s. Each PCR mixture (eight mixtures, 20 μl per mixture) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 50 μM dATP, 50 μM dCTP, 50 μM dGTP, 50 μM dTTP, 100 pmol of labeled primers U968-GC/Biot. and L1401/TBR (4), and 0.5 U of *Taq* DNA polymerase (Life Technologies). After a 160-μl reaction mixture containing 8 μl of template DNA was prepared, the mixture was distributed into eight tubes (20 μl per tube). The eight replicates per sample were removed from the thermocycler one after another when the 10th, 12th, 14th, etc., cycles were completed (see Fig. 3). Ten microliters of PCR product from each reaction mixture was mixed with 40 μl of 1.25× QPCR buffer (12.5 mM Tris-HCl [pH 8.3], 62.5 mM KCl) in a separate QPCR sample tube for measurement of the electrochemiluminescence signal with a QPCR System 5000 instrument (Perkin-Elmer Cetus) as described previously (4). After addition of 15 μl of a 2-mg ml^{-1} preparation of streptavidin-coated paramagnetic beads (Perkin-Elmer Cetus), the biotin-labeled PCR products were captured during 30 min of shaking incubation at 1,400 rpm. After capture, 340 μl of QPCR assay buffer (Perkin-Elmer Cetus) was added, and the mixture was analyzed with the QPCR System 5000 instrument. The slopes of the amplification kinetics lines were calculated by performing a linear regression analysis with the computer software QPCR ANALYSIS V 0.63 (Perkin-Elmer Cetus). In this process the correlation coefficient, r^2, was increased to >0.99 by removing one or two first datum points (if they were below the lower detection limit) and/or one or two last datum points (if they were in the stationary phase of the PCR). Data sets were normalized by considering the last value of each kinetic used as 100% and calculating the previous values as a part of this value. From this slope the multiplication factor (m) per cycle was estimated by using the following formula: $c_n = mc_{n-1}$, where c is the DNA yield and n is the cycle number.

TGGE analysis of soil DNA by limiting dilution PCR. PCR assays performed with diluted template DNA were used to search for sequences that exhibited reduced amplification efficiency. At the detection limit of a template dilution series, the most abundant sequence, not the sequence which exhibits the best amplification efficiency, was predominant. DNA was used instead of rRNA in order to detect lower target concentrations. The *Taq* DNA polymerase required much lower amounts of target DNA sequences than the rTth DNA polymerase used for rRNA targets required (21). Soil DNA was isolated as described previously (11). The concentration was adjusted to approximately 100 pg μl^{-1} after a rough estimate was obtained on an ethidium bromide-stained agarose gel. Then serial twofold dilutions were prepared in 12 steps. The resulting samples were the templates used for PCR and to check the TGGE results as described above.

RESULTS

Competitive RT-PCR with primers U968-GC and L1401. Equal amplification of different 16S rRNAs was verified with 10 cultured bacterial strains belonging to diverse taxa. Corresponding rRNA standards containing 1 ng μl^{-1} and subsequent threefold dilutions were prepared for each strain and compared with each other in a competitive RT-PCR experiment. After performing reactions in which both rRNA competitors were present at the same concentration we observed approximately identical band intensities on TGGE gels (Fig. 1). Similar results were obtained when 16S rDNA amplicons were used as competitors in competitive PCR. 16S rDNA amplicon preparations were adjusted to equal concentrations and were used as templates for competitive PCR in order to compare the cloned environmental sequences to each other and to cultured strains (data not shown). Some bacterial sequences exhibited a few minor mismatches with the primer sequence (G-T or A-C mismatches), but we did not observe any amplification bias related to this, even when the annealing temperature was increased from 56 to 60 or 64°C.

Sequence-specific amplification efficiency for cultured and uncultured bacteria. The sequence-specific amplification efficiency was measured by monitoring the amplification kinetics by kinetic PCR. We used only kinetic PCR performed with 16S rDNA amplicons as the targets to directly compare the cloned 16S rDNA sequences of the uncultured soil bacteria and cultured strains. Our comparison of amplicons from cultured strains with amplicons from cloned inserts of environmental 16S rDNA did not reveal any significantly different amplification kinetics (Fig. 2). All of the bacterial strains tested and the cloned 16S rDNAs from soil exhibited the same amplification kinetics. The slope of the exponential DNA increase during PCR allowed us to calculate the average amplification efficiency. For all of the bacterial sequences the measured multiplication factor per PCR cycle was approximately 1.34 (for a primer annealing temperature of 56°C). This indicates that the DNA polymerization process was properly initialized and completed with 34% of all template molecules in each cycle. The multiplication factor varied for different annealing temperatures between approximately 1.5 (48°C) and 1.2 (64°C).

Multiple quantification of rRNAs in TGGE fingerprints. The method described above (competitive RT-PCR and subsequent detection by TGGE) could also be used to quantify each of several different sequences in one sample. In defined artificial rRNA mixtures containing rRNA from four species, the signals of the individual competitors could be quantified by identifying the reaction in which one particular target signal and the standard band had the same intensity. After quantitative image analysis, the values could be used to relate the target concentration to the known template rRNA concentration of the standard. The values obtained with the rRNA standard indeed reflected the theoretical template input (Fig. 3). These results indicated that this approach might also be used with environmental fingerprints. However, we could not check to determine whether the amplification efficiencies of all the sequences present were identical. Important but unknown 16S rRNA sequences could produce faint bands or even be absent from the TGGE band pattern if their amplification efficiencies were much lower than the amplification efficiencies of the other sequences. Abundant sequences which cannot compete with other sequences might be detected if amplification template concentrations were reduced. At the highest dilutions competition is reduced and amplification is limited to only the most abundant sequences. Indeed, for the Drentse A fingerprints no signals other than the strongest bands in the original

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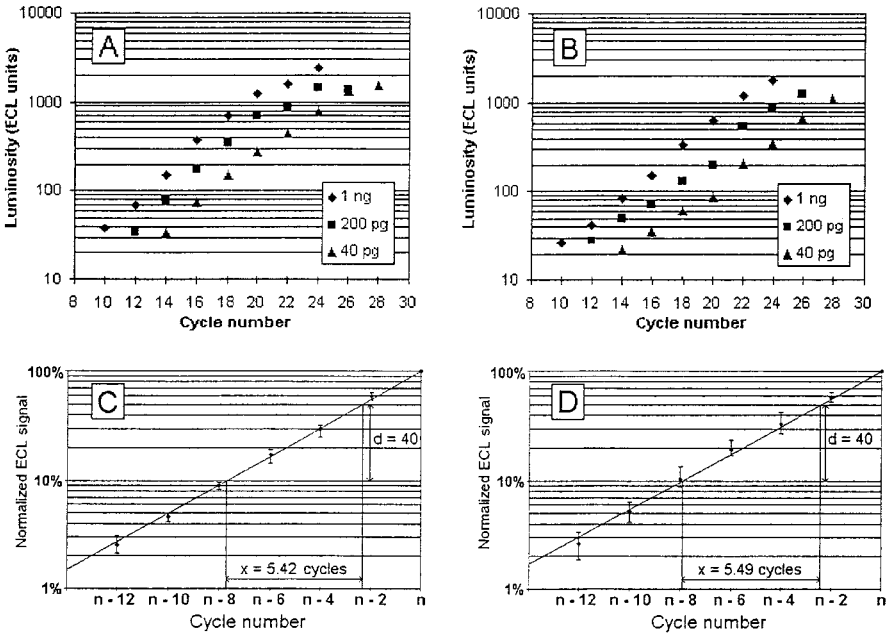

FIG. 2. Amplification kinetics of 30 different 16S rDNA sequences: detection signal value versus PCR cycle number. The templates used were different 16S rDNA amplicon samples, and three different amounts (1 ng, 200 pg, and 40 pg) were tested. (A) The target was a 16S rDNA amplicon from *E. coli*. (B) The target was a 16S rDNA amplicon of clone DA001. (C) Normalized results of all experiments and parallel experiments performed with 10 pure-culture organisms (30 kinetics experiments). (D) Normalized results of all experiments and parallel experiments performed with 20 environmental sequences (60 kinetics experiments). The slopes were used to calculate the amplification factor per cycle (1.341 in panel C and 1.346 in panel D). The error bars indicate the minimal and maximal deviations in the data sets.

band pattern remained at the highest dilutions (Fig. 4). This possibility was checked by performing PCR with soil DNA, because the RT-PCR product began to disappear at rRNA levels below approximately 10 pg. Since this level corresponded to approximately 10^6 target sequences, the band pattern shifts could not be observed or anticipated. On the basis of all of this evidence for equal amplification of the different sequences, we used an rRNA standard for the soil rRNA to perform multiple-competitor RT-PCR. In order to find a suitable rRNA standard for the fingerprints, we had to select a bacterial strain that produced a TGGE signal somewhere in a bandless gap in the environmental fingerprints. For the Drentse A fingerprints *E. coli* rRNA was a suitable choice (Fig. 5). First, the 20 most prominent sequences were quantified absolutely by using the principles of conventional competitive PCR, and values of about 20 to 200 ng per ribotype were obtained (Fig. 6A). Then the specific rRNA yields were related to the corresponding total rRNA yield from the soil sample as estimated by quantitative dot blot hybridization with *Bacteria*-specific probe EUB338 (Fig. 6B). The average yield from test plot A was 2.5 ± 0.6 μg of rRNA g of soil^{-1}; the minimum and maximum yields were 1.8 and 3.2 μg g^{-1}, respectively. The sum of all of the values estimated for the 20 predominant sequences accounted for 48% ± 16% of the total rRNA yield.

DISCUSSION

General problems of quantitative PCR. Since PCR is a process that involves exponential amplification, correct calculation of the original number of target sequences on the basis of the amount of the final PCR product can be massively distorted by experimental bias. The first obvious problem with PCR-based

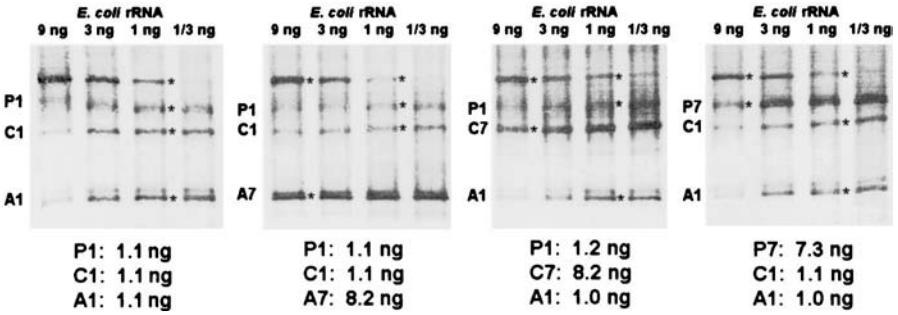

FIG. 3. Four multiple-competitor RT-PCR of rRNA with four competing rRNAs resolved by TGGE and detected by silver staining (2 μl of RT-PCR product per lane). *E. coli* rRNA was applied at different dilutions, as indicated. Signals A1 and A7 represented 1 and 7 ng of rRNA from *Arthrobacter atrocyaneus*. Signals C1 and C7 represented *Comamonas acidovorans*, and signals P1 and P7 represented *Pseudomonas fluorescens*. Each rRNA was quantified in the lane in which the intensity of the corresponding *E. coli* signal (indicated by an asterisk) was most similar in relation to the amount of rRNA represented by the *E. coli* signal. Results are given below the fingerprints.

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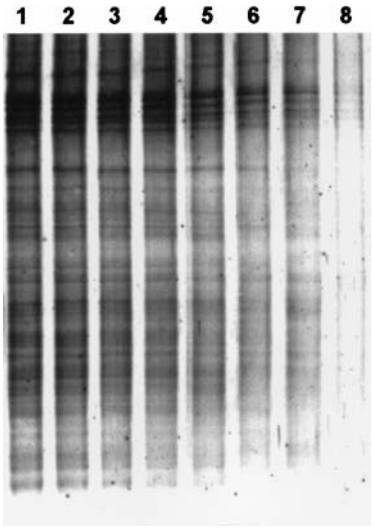

FIG. 4. Silver-stained TGGE gel with PCR products from soil DNA from sample A1 (12 μl of RT-PCR product per lane). Lane 1 contained the PCR product generated from 100 pg of template DNA. Lanes 2 through 8 contained twofold serial dilutions of template DNA. Lane 8 contained approximately 0.8 pg of template DNA, which might represent a few hundred genomic units of soil bacteria.

quantitative assays is inherent to amplification itself. In the early cycles of the PCR the amount of product increases exponentially, but due to the depletion of substrates the amount might level off during the last cycles. It has been demonstrated that this change in amplification efficiency results in preferential amplification of less abundant sequences (28). This can be explained by an increased annealing competition effect (24). During the annealing phase the primer target sites could be found by the primers or could rehybridize with their complements on the complementary DNA strand. In the early cycles of PCR this annealing competition is dominated by the huge excess of primer molecules, and proper DNA polymerization can be initialized. Since the primers become part of the PCR product, the number of free primers is significantly reduced in the late cycles of PCR. In contrast, the competitor, the complementary strand of the PCR product, is amplified exponentially. Therefore, the template DNA rehybridization process might become a serious competitor for primer annealing and prevent initialization of DNA polymerization. This inhibition should be most efficient for the abundant sequences, because their amplicon/primer ratio is less favorable than the ratio for the less abundant sequences. Therefore, specialized PCR procedures are needed to determine the amount of the original DNA template. In competitive PCR this bias is eliminated by analyzing only reaction mixtures in which the standard and target are present in similar amounts and are amplified almost equally. In kinetic PCR the breakdown of exponential amplification can be identified and the resulting data points can be neglected. The latter approach is also useful for directly detecting amplification efficiency. In this case sequence-specific factors, such as G+C content, secondary structures, and, especially, the size of the amplicon, might cause some bias which increases exponentially during the PCR. Another significant factor is primer annealing efficiency. In the first cycles of PCR the primers must anneal to the original template. The efficiency of this process might be reduced by some sequence mismatches in the primer target site. Since the primer becomes part of the amplicon and introduces its own sequence, this

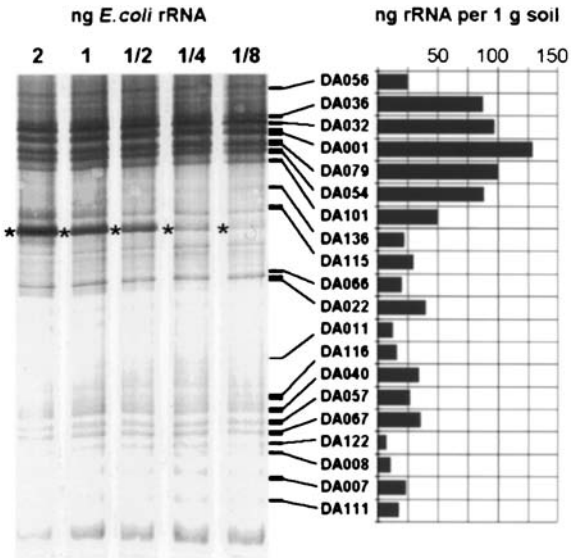

FIG. 5. Multiple-competitor RT-PCR of rRNA from soil sample A1 resolved by TGGE and detected by silver staining (12 μl of RT-PCR product per lane). The 20 signals selected for quantification are indicated; the designations have been described previously (12). The 20 sequences were quantified with image analysis software. Each sequence was quantified in the lane in which the intensity of the corresponding *E. coli* signal (indicated with an asterisk) was most similar in relation to the amount of rRNA represented by the *E. coli* signal and the amount of soil (10 mg) represented by the soil rRNA template.

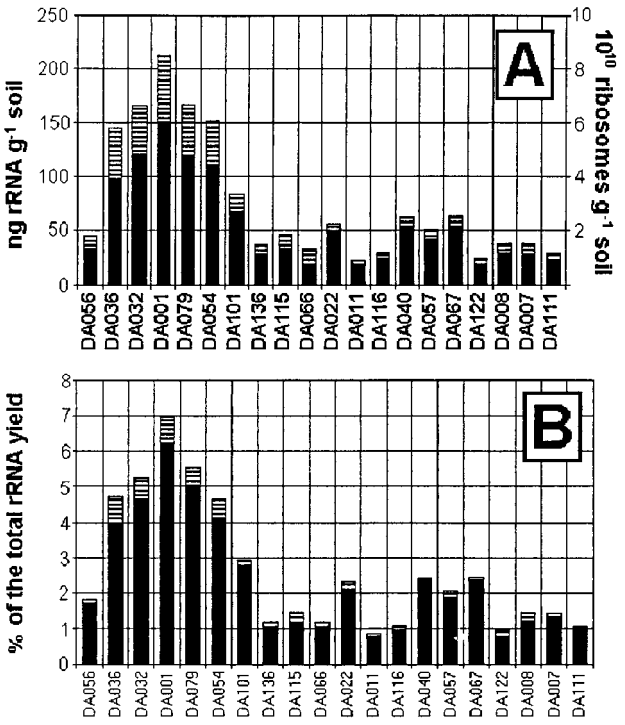

FIG. 6. Average rRNA yields for the 20 sequences in Fig. 5, based on 40 soil samples. The striped columns indicate the standard deviations. (A) Total amounts of rRNA. (B) Relative amounts as part of the total rRNA yield as estimated by quantitative dot blot hybridization with *Bacteria*-specific probe EUB338.

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effect disappears as the cycle number increases. Therefore, the bias in the initial cycles might be not detectable by kinetic PCR. Only preliminary quantitative PCR experiments performed with known template concentrations could reveal this deviation.

Competitive RT-PCR on TGGE. Primers U968-GC and L1401 have been used successfully for equal amplification of 16S rRNAs from bacterial cultures of different taxa and also cloned 16S rDNA amplicons from uncultured Drentse A bacteria. Sequence-specific amplification efficiency was assessed by monitoring the amplification kinetics by kinetic PCR. Primer-specific amplification efficiency was checked by competitive PCR and RT-PCR in which different templates and annealing temperatures were used. TGGE with subsequent silver staining and image analysis proved to be the optimal detection system for competitive amplification. The ability of this system to clearly separate sequences that differed by as little as one nucleotide (22) meant that it was possible to use standards having the same molecule length and almost identical sequences as targets. Such standards were the best competitors for equal coamplification with the target sequence. In contrast, the common approach of using standards of different lengths (14) introduces the danger of bias due to unequal amplification efficiencies (27). In the case of rRNA there is also no need to artificially construct a standard; the natural rRNA of another bacterial strain could meet all demands. This was experimentally confirmed by the equal sequence-specific amplification efficiencies of all of the different target sequences and the *E. coli* rRNA standard used.

Multiple-competitor RT-PCR for environmental 16S rRNAs. We found that the TGGE detection approach was also suitable for simultaneous quantification of several different sequences. We could quantify with one competitive RT-PCR assay numerous predominant bacterial rRNA sequences from complex bacterial communities. In the resulting complex TGGE fingerprints (Fig. 5) the clear signals were the most reliable signals and the many faint signals were less reliable. It should also be verified that one band indeed represents only one sequence and not several different sequences with the same migration speed (10).

Absolute quantification of rRNA sequences (Fig. 6A) is of questionable value, because it cannot be expected that all target molecules can be released from complex environments like soil. As estimated for inoculated sterilized soils, the ribosome isolation method which we used might result in a loss of about 50% of all ribosomes to the soil matrix (9). Much greater losses were estimated for other methods of nucleic acid extraction (17). Therefore, we preferred to use the ratio PCR proposed by Raeymaekers (24). This strategy was first used to analyze the expression of the $GABA_A$ receptor gene family on the basis of its mRNA (5). In this approach the variable expression of a gene can be related to constant mRNA levels of housekeeping genes. In this way uncertain absolute quantification can be replaced by a relative estimate of the change in gene expression. This reasoning may be applied to 16S rRNAs from bacterial communities. Bacteria which react to environmental changes in space or time by changing their ribosome levels can be related to the average or total amount of ribosomes for all bacteria. For the individual predominant ribotypes in soil we calculated values of 20 to 200 ng of rRNA g of $soil^{-1}$, which correspond to approximately 10^{10} to 10^{11} ribosomes g^{-1} (Fig. 6A) since one ribosome contains approximately 2.5×10^{-12} μg of rRNA (approximately 4,500 nucleotides). Of course, we did not expect that the 20 predominant sequences quantified represent all of the rRNA types present in complex soil environments (30). Therefore, we related the values obtained to the total rRNA yield estimated by another method (Fig. 6B). We found that the 20 predominant sequences represented approximately one-half of all of the rRNA extracted from the soil. On the one hand, this demonstrated that a considerable amount of bacterial ribosomes did not give strong signals in the TGGE fingerprints. This should have been due to a huge number of less active species which contributed a high total amount of ribosomes, but the individual different 16S rRNA sequences were too rare to compete successfully during RT-PCR. On the other hand, the major part of the total rRNA represented by the 20 sequences selected indicated that these sequences indeed originated from (at least most of) the predominant members of the bacterial community.

Relative quantification of multiple-competitor RT-PCR mixtures separated on high-resolution TGGE gels meets the demands of molecular microbial ecology for studying numerous species. Moreover, detection by TGGE allows workers to use quantification standards with optimal properties. The possibility of PCR amplification bias was investigated and eliminated for primers U968-GC and L1401 by performing kinetic PCR with the sequences concerned, limiting dilution PCR with soil DNA, and finally simulations of (multiple) competitive RT-PCR assays with defined rRNA standards and artificial rRNA mixtures. In addition, particular uncertainties must always be considered when complex, mainly unknown environmental microbial communities are the subject of investigation. In natural samples we might encounter extended lysis resistance of cells, adsorption and loss of nucleic acids to the environmental matrix, and previously unknown types of 16S rRNA sequences. Therefore, caution is required when conclusions are drawn from competitive PCR performed with environmental samples. At the moment we recommend limiting this approach to rRNA, mRNA, or plasmid DNA target molecules and following the cautious approach of ratio PCR.

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