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Amplification of Bacterial 16S Ribosomal DNA with Polymerase Chain Reaction

KENNETH H. WILSON,[1,2]* RHONDA B. BLITCHINGTON,[1,2] AND RONALD C. GREENE[3,4]

Departments of Medicine[1] and Biochemistry,[3] Duke University, and Medical[2] and Research[4] Services, Durham Veterans Affairs Medical Center, Durham, North Carolina 27705

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The sequence of small-subunit rRNA varies in an orderly manner across phylogenetic lines and contains segments that are conserved at the species, genus, or kingdom level. By directing oligonucleotide primers at sequences conserved throughout the eubacterial kingdom, we amplified bacterial 16S ribosomal DNA sequences with the polymerase chain reaction. Priming sites were located at the extreme 5′ end, the extreme 3′ end, and the center of 16S ribosomal DNA. The isolates tested with these primers included members of the genera *Staphylococcus*, *Coxiella*, *Rickettsia*, *Clostridium*, *Neisseria*, *Mycobacterium*, *Bilophila*, *Eubacterium*, *Fusobacterium*, and *Lactobacillus* and the family *Enterobacteriaceae*. Initially, the yields from the reactions were erratic because the primers were self-complementary at the 3′ ends. Revised primers that were not self-complementary gave more reproducible results. With the latter primers, 0.4 pg of *Escherichia coli* DNA consistently gave a visible band after amplification. This method should be useful for increasing the amounts of bacterial 16S ribosomal DNA sequences for the purposes of sequencing and probing. It should have a broad range of applications, including the detection and identification of known pathogens that are difficult to culture. This approach may make it possible to identify new, nonculturable bacterial pathogens.

Because the nucleotide sequences found in 16S rRNAs vary in an orderly fashion throughout the phylogenetic tree, these sequences have been useful for the study of molecular evolution (15). For the same reason, 16S rRNAs are also useful targets for taxon-specific oligonucleotide probes (2, 5), some of which may be useful in clinical microbiology.

There are several reasons why it would be desirable to have the ability to amplify eubacterial (kingdom) 16S rRNA sequences. First, in order to use rRNAs to study molecular phylogeny and to develop probes, most current methods require knowledge of rRNA sequences. The direct sequencing of rRNAs with reverse transcriptase requires microgram amounts of rRNA per reaction. It is difficult to grow adequate numbers of some organisms for this procedure. An alternative approach, cloning ribosomal DNA (rDNA) for sequencing, is time consuming. Amplification of the rDNA followed by double-stranded sequencing could prove to be a more efficient technique. Second, although there are approximately 10,000 copies of rRNA per cell, rRNA probes have thus far not been as sensitive as bacterial cultures. For instance, a ^{32}P-labeled oligonucleotide probe directed at the rRNA of *Clostridium difficile* was able to detect 10^3 cells, making it at least 2 orders of magnitude less sensitive than culturing the organism would be (13). Increasing the amount of 16S rRNA sequence could increase the sensitivity of probes, as well as enable workers to use less sensitive nonradioactive labels. Finally, all known bacterial pathogens of humans belong to the eubacterial kingdom. This fact implies that a method to amplify eubacterial 16S rDNAs could be developed further to allow workers to confirm rapidly the presence or absence of bacterial pathogens at normally sterile body sites.

We approached the problem of amplifying 16S rDNA by using the polymerase chain reaction (PCR) (11). In this method, a segment of DNA between two priming sites can be amplified 10^6-fold or more. Previous work has shown that it is possible to amplify a 200-base segment of 16S rDNA from eubacteria (1). In this study, we chose primers to amplify nearly the entire 16S rDNA sequence (approximately 1,500 bases). Because initial experiments showed that amplification of the entire 16S rDNA molecule at once was not efficient, we decided to amplify the molecule in two segments. The priming sites in this PCR were chosen because they are highly conserved among the phylogenetic group referred to as eubacteria (15) but are not found in eucaryotes, archaebacteria, or mitochondria, so it should be possible to amplify only bacterial 16S rDNA sequences even in the presence of nucleic acids from other types of organisms. The method of amplification described in this paper should be generally applicable to a wide variety of bacteria, including essentially all bacterial pathogens that infect humans. The reaction products can be probed or sequenced.

MATERIALS AND METHODS

Bacterial strains. Nucleic acids from *Rickettsia montana* and *Rickettsia conori* were obtained from Greg McDonald, Rocky Mountain Laboratory. Nucleic acid extracts from *Coxiella burnettii* were obtained from Louis Malavia, Washington State University. The other organisms used included *Staphylococcus aureus* ATCC 12600, *Neisseria meningitidis* ATCC 13090, *Escherichia coli* DH-1, *Mycobacterium avium* ATCC 25291, *Mycobacterium intracellulare* ATCC 13950, *Eubacterium aerofaciens* ATCC 25986, *Eubacterium rectale* ATCC 33656, *Clostridium bifermentans* ATCC 638, *Lactobacillus minutus* ATCC 33267, *Fusobacterium necrogenes* ATCC 25556, and *Clostridium sordellii* ATCC 9714. *Bilophila wadsworthii* 130H was donated by Sidney Finegold.

Extraction of nucleic acids. Cells were grown to maximum turbidity and then centrifuged at 2,000 × *g* and 4°C for 20 min, and the resulting pellet was suspended in 0.5 ml of LETS buffer (100 mM LiCl, 10 mM EDTA, 10 mM Tris [pH 7.8], 1% sodium dodecyl sulfate). An equal volume of phenol-chloroform-isoamyl alcohol (50:48:2) and 0.05 vol-

* Corresponding author.

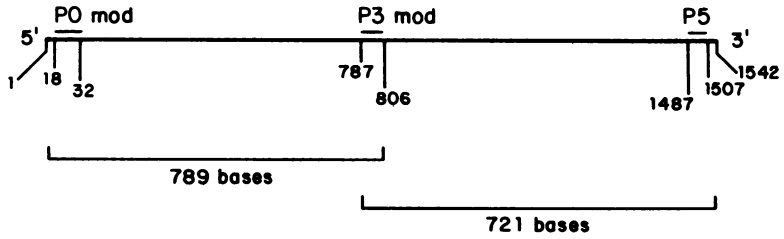

FIG. 1. Diagram of locations of primers on *Escherichia coli* 16S rDNA, an open reading frame that is 1,542 bases long. The locations of the modified priming sites used for the PCR are shown. P0mod primes at site P0 with an extension going to the right. P3mod primes at site P3 with the reaction going to the right; PC3mod primes at the same site with the reaction going to the left. PC5 primes at site P5 with the reaction going to the left. The designations 5′ and 3′ and base numbering refer to the sense strand.

ume of glass beads (diameter, 25 to 50 μm) were added. The mixture was agitated in a 1.5-ml Eppendorf tube with a mini-beadbeater (Biospec Products, Bartlesville, Ind.) for 1 min at room temperature. The sample was centrifuged for 1 min at 16,000 × *g* in an Eppendorf centrifuge, and then the aqueous phase was extracted with phenol-chloroform-isoamyl alcohol and with chloroform-isoamyl alcohol (48:2). The aqueous phase was then transferred to a clean Eppendorf tube, and the nucleic acids were precipitated with 0.1 volume of 2 M LiCl and 2.5 volumes of ethanol for 15 min on ice. The tubes were centrifuged for 20 min at 16,000 × *g* and 4°C in an Eppendorf centrifuge, and the resulting pellet was washed with 95% ethanol. The pellet was dissolved in water and precipitated again with 0.1 volume of 2 M sodium acetate and 3 volumes of ethanol. The precipitated nucleic acids were washed with ethanol, dried in vacuo, and dissolved in 50 μl of water. The concentration of nucleic acids was determined by measuring the A_{260} of the final solution.

Amplification procedure. Various concentrations of nucleic acid extracts were suspended in 50-μl portions of water. Then 10 μl of 10× PCR buffer (100 mM Tris [pH 8.3], 500 mM KCl, 9 mM $MgCl_2$, 0.1% gelatin), PCR primers (final concentration, 1 μM each; i.e., 100 pmol of each primer was added), deoxynucleoside triphosphates (final concentration, 200 μM; in a total volume of 100 μl), and 2.5 U of *Taq* polymerase (Cetus Corp.) were added. Water was added to bring the volume to 100 μl. The mixture was placed in a thermal cycler (Coy Laboratory Products, Ann Arbor, Mich.), and the temperature was cycled to 94°C for 70 s, to 55°C for 2.5 min, and then to 72°C for 3 min. The cycle was repeated once, and then the denaturing temperature was changed to 90°C for 70 s, with the rest of the cycle remaining the same for 23 additional cycles. The rationale for the change in denaturing temperature was that genomic DNA would require a higher temperature to denature than a 700- to 800-base segment would and that lowering the denaturing temperature to 90°C would prolong the life of the *Taq* polymerase. As discussed below, the primers were modified and retested. The temperature profile used for the modified primers was the same except that the hybridization temperature was 33°C and the extension phase was done at 75°C for 4 min. In addition, 25% dimethyl sulfoxide was added to the reaction mixtures for the latter primers to increase the specificity of the hybridization. PCRs were optimized by varying the final $MgCl_2$ concentration from 0.5 to 9 mM. A blank control tube containing no added nucleic acids was run with every set of reaction mixtures to control for the inadvertent introduction of exogenous nucleic acids. The oligonucleotide primers used for the PCR were chosen because they were theoretically not highly involved in secondary structure and because a computer search of GenBank and European Molecular Biology Laboratory data bases and a previously published data base of small-subunit rRNAs (7) showed that these primers are directed at sites that are highly conserved throughout the eubacterial kingdom but not in mitochondria, eucaryotes, or archaebacteria. These primers corresponded to sites P5, P3, and P0 on 16S rDNA (Fig. 1). The sequences of the primers are shown in Table 1.

Southern blot hybridization. Portions (5 μl) of the reaction products were subjected to electrophoresis on 0.7% agarose gels containing 0.5 μg of ethidium bromide per ml, and the bands were transferred to a nylon membrane (Gene Screen; New England Nuclear Corp., Boston, Mass.) in a Vacublot apparatus (American Bionetics, Emoryville, Calif.). Polynucleotide kinase and [^{32}P]ATP were used to 5′ end label oligonucleotides that were complementary to conserved 16S rRNA sequences that were found within the amplified segments. Each membrane was then probed with a ^{32}P-labeled oligonucleotide.

RESULTS

Range of species amplified. Using primer PC3 with primer P0 and primer PC5 with primer P3, we were able to amplify nucleic acids from a variety of genera belonging to the eubacterial group. Members of the genera *Clostridium*, *Rickettsia*, *Mycobacterium*, *Neisseria*, *Staphylococcus*, *Coxiella*, and *Eubacterium* and the family *Enterobacteriaceae*, all gave bands of the expected sizes (789 base pairs for primers PC3 and P0 and 721 base pairs for primers PC5 and P3) when they were amplified with either set of primers.

Sensitivity. We attempted to determine the sensitivity of our method by serially diluting DNA prepared from *Esche-*

TABLE 1. Sequences of primers used in amplification of 16S rDNA[a]

Primer	Sequence
PC5	5′ TACCTTGTTACGACTT 3′
P3	5′ AGGATTAGATACCCTDTAG 3′
PC3	5′ CTAHAGGGTATCTAATCCT 3′
P0	5′ GAGTTTGATCMTGGCTCAG 3′
P3mod	5′ ATTAGATACCCTDTAGTCC 3′
PC3mod	5′ GGACTAHAGGGTATCTAAT 3′
P0mod	5′ AGAGTTTGATCMTGG 3′

[a] Primers PC5, PC3, and PC3mod were complementary to rRNA. Primers P3, P0, P3mod, and P0mod contained sequences homologous to rRNA. D = A, T or G; H = C, T, or A; M = A or C. Primers P3mod, PC3mod, and P0mod are revised versions of primers PC5, P3, PC3, and P0; revised versions of these primers were necessary because of complementarity at the 3′ ends (Fig. 2).

```
PC3-PC3 self priming
      CTAHAGGGTATCTAATCCT
          |||:|     |:|||
          TCCTAATCTATGGGAHATC


P0 -- P0

GAGTTTGATCCTGGCTCAG
          |||: :|||
          GACTCGGTCCTAGTTTGAG


P3 -- P3

AGGATTAGATACCCTDTAG
              ||||||
              GATDTCCCATAGATTAGGA
```

FIG. 2. Base pairing of the first set of primers at the 3′ ends. Primers PC3, P0, and P3 were self-complementary.

richia coli DH-1 cells and found that the sensitivity of detection varied from 4 pg to 40 ng. Investigations to find a cause for this lack of consistency included a search for primer complementarity; i.e., it was possible that one or more of the primers were self-priming, leading to formation of dimers during the extension phase of the PCR and rapidly depleting the involved primers. A computer search for self-complementarity showed that there was a potential for self-priming with primers PC3, P3, and P0 (Fig. 2). When run on a 3% agarose gel, the products of the reactions described above showed the presence of low-molecular-weight bands typical of the reaction products of self-priming (Fig. 3).

Modification of primers. Modifications of the primers eliminated the self-complementary regions but still allowed priming at essentially the same sites (Table 1). The new primers also gave bands of the appropriate molecular weights when they were used to amplify bacterial nucleic acids, but not when they were used with nucleic acids from human cell lines (Fig. 4). Control reaction mixtures that did not contain nucleic acids gave no bands. Thus, amplification of segments from the organisms described above was not due to contamination from ubiquitous bacteria. The bands were transferred to a nylon membrane and probed with ^{32}P-labeled oligonucleotides A and B of Lane et al. (9). These oligonucleotides are complementary to highly conserved regions of the 16S rRNA molecule. All bands hybridized with these probes, confirming their origin (Fig. 5). Nucleic acids from *B. wadsworthii*, *L. minutus*, and *F. necrogenes* also gave the appropriate bands in the amplification reactions (data not shown). Studies were then performed to determine the sensitivity of the PCR for amplifying *Escherichia coli* DH-1. DNA was prepared as described above and then treated with RNase. The limit of detection after 30 cycles of amplification was consistently in the range of 0.4 pg of chromosomal DNA. As Fig. 5 shows, a weak second band with a lower molecular weight appeared in some of the lanes and hybridized with the 16S rRNA probes. This band probably represented single-stranded DNA produced by a slight excess of one of the primers. It was possible to accentuate this band by using a large excess of one primer, a strategy of asymmetric amplification that was previously described as producing single strands that are useful for sequencing PCR products (8).

DISCUSSION

Because some segments of rRNA are conserved while others are variable to different degrees (6), it is possible to generate oligonucleotides which are complementary to segments that are specific for any level of the phylogenetic tree from kingdom to species (4, 5). Using genus-specific oligonucleotides, we previously performed PCRs to amplify 16S rRNA sequences from rickettsiae by a factor of 10^6- to 10^7-fold (14). The amplification reaction appeared to be

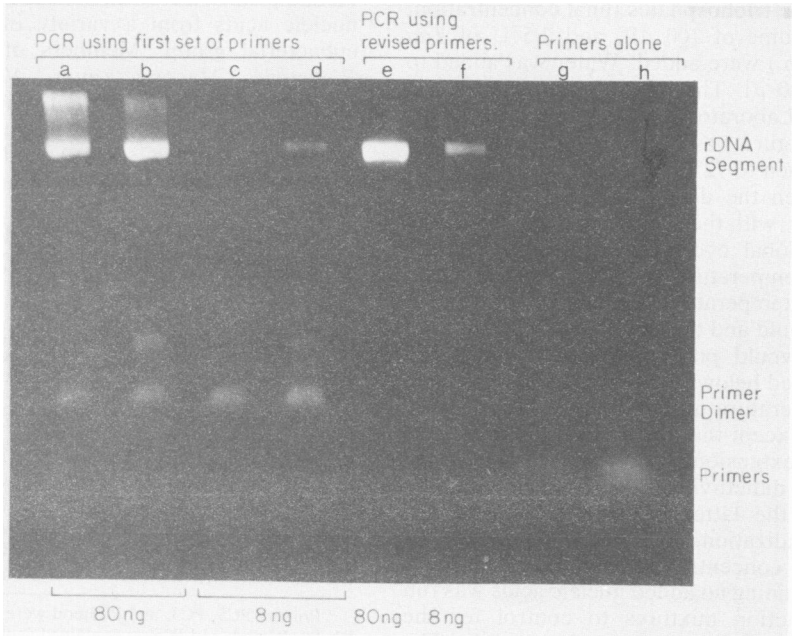

FIG. 3. Effect of revised primers on self-priming in the PC3-P0 reaction. A PCR was performed by using 80 ng (lanes a, b, and e) or 8 ng (lanes c, d, and f) of *Escherichia coli* DNA; reaction products were run on a 3% agarose gel and stained with ethidium bromide. Dimethyl sulfoxide was not used with the revised primers in this experiment. The low-molecular-weight bands in lanes a through d are typical of concatamers which are formed from primers that are self-complementary at the 3′ ends.

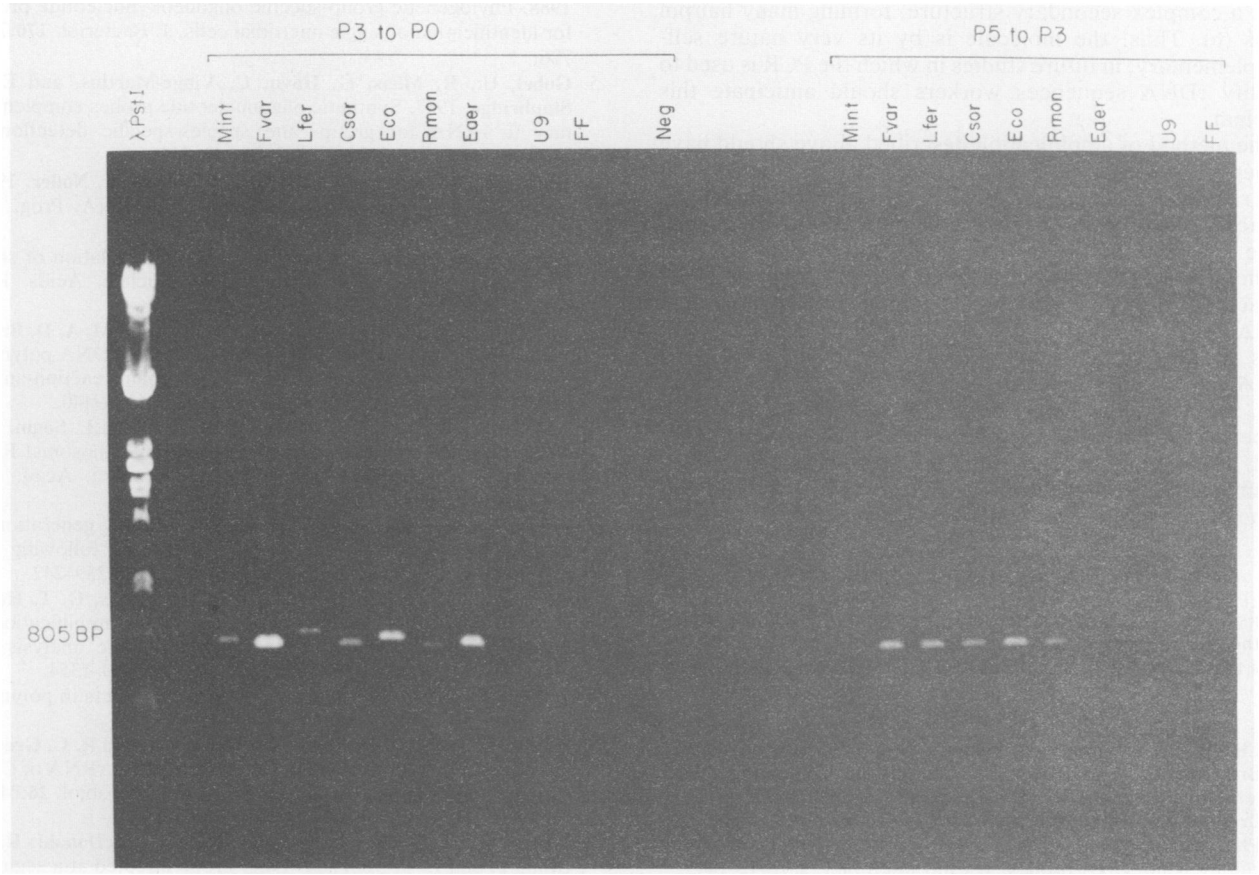

FIG. 4. Amplification of bacterial 16S rDNAs. The bands on the left are PCR products generated from primers PC3mod and P0mod; the bands on the right were generated from primers PC5 and P3mod. Reaction products were run on a 0.7% agarose gel and stained with ethidium bromide. Abbreviations: Fvar, *Fusobacterium varium*; Lfer, *Lactobacillus fermentum*; Eaer, *Eubacterium aerofaciens*; Eco, *Escherichia coli*; Mint, *Mycobacterium intracellulare*; Rmon, *Rickettsia montana*; Csor, *Clostridium sordellii*; U9, human leukemia cell line U9; FF, foreskin fibroblasts; Neg, no nucleic acids except primers added; BP, base pairs.

specific for the genus *Rickettsia*. The amplified segments could then be probed with species-specific probes.

In this study we synthesized oligonucleotides that are specific for the eubacterial kingdom and used these oligonucleotides to amplify practically the entire 16S rDNA molecule. This approach was not as straightforward as expected because primers were depleted as they primed themselves. Self-priming occurs when the 3′ end of a primer is complementary to itself (Fig. 2); a similar reaction can occur when a primer is complementary to the 3′ end of the other primer that is being used in the PCR. The complementarity allows enough base pairing to occur for the Taq polymerase to use each primer molecule as a template. Once this event occurs, both ends of the reaction product are of course complementary to a primer, and primer is consumed rapidly as dimer becomes the favored reaction product (12). In retrospect, this difficulty is not surprising because rRNA is known to

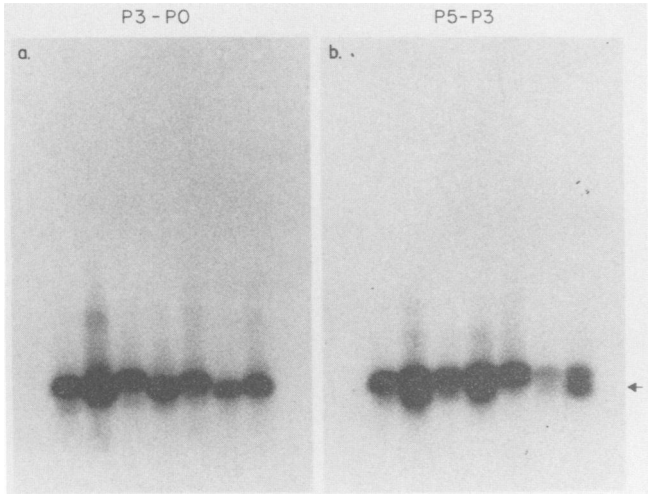

FIG. 5. Southern blot of bands shown in Fig. 4. Both sets of bands were probed with ^{32}P-labeled oligonucleotides that were complementary to highly conserved 16S rRNA sequences found between the priming sites. The band produced by PC3mod and P0mod was probed with primer A (GWATTACCGCGGCKGCTG) of Lane et al. (9), and the band produced by PC5 and P0mod was probed with primer B (CCGTCAATTCMTTTRAGTTT) (W = A or T; K = G, T, or M; M = A or C; R = A or G). The arrow indicates a second band consisting of single-stranded reaction product produced by an excess of primer P3mod in panel b. This band was observed only in the second, fourth, and seventh lanes that give signals. The signal from this band overlapped with the signal from the double-stranded reaction product.

have a complex secondary structure, forming many hairpin loops (6). Thus, the molecule is by its very nature self-complementary; in future studies in which the PCR is used to amplify rDNA sequences workers should anticipate this problem.

The method of amplification described above should have numerous applications. It should facilitate the sequencing of 16S rDNA, which can be performed either by direct sequencing of the amplified segments (3, 8, 10) or by cloning these segments into sequencing vectors. In addition, this method should allow investigators to detect smaller numbers of bacteria when they use oligonucleotide probes directed at rRNA sequences and to use nonradioactive detection systems. It also should allow workers to separate bacterial rRNA sequences from eucaryotic sequences in mixed samples. It should not be necessary to culture organisms to probe them or even to sequence them when this method is used. This important attribute of the method should make it possible to detect nonculturable bacteria and is likely to lead to the discovery of new bacterial pathogens.

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