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Species-Specific Oligonucleotide Probes for rRNA of *Clostridium difficile* and Related Species

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The large copy number of rRNA makes it an appealing target for oligonucleotide probes designed to identify microorganisms. Given that nucleotide sequences in rRNA are known to reflect phylogeny, species-specific rRNA probes should be feasible if the sequences found in closely related species are different. We sequenced portions of the 16S rRNA of three closely related clostridia found in the human colonic microflora: *Clostridium bifermentans*, *C. sordellii*, and *C. difficile*. The rRNAs of these three species showed 97 to 98% sequence similarity. Five oligonucleotide probes complementary to unique segments of the sequences were end labeled with ^{32}P and hybridized on a nylon filter to the immobilized rRNA of each clostridium. Each probe efficiently hybridized only to the rRNA of the species to which it was directed. Complementary probes emitted a signal that exceeded by a factor of 100 to 1,000 the signal of probes that mismatched the target rRNA by 2 to 5 bases. Even a 1-base difference in rRNA sequence allowed a clear distinction between species. A systematic approach can efficiently yield taxon-specific oligonucleotide probes directed at rRNA.

Most bacterial isolates are cultivated and identified within 48 h in the clinical laboratory. Some organisms, however, are slow growing or difficult to differentiate from related species. For instance, many species of *Mycobacterium* require weeks to grow and anaerobes are relatively difficult to identify.

By developing species-specific nucleotide probes, a number of workers have attempted to simplify and accelerate the process of identifying bacteria (11, 15). Usually, the probes consist of cloned DNA segments from the organism of interest. Some probes have been derived from genetic elements associated with a characteristic of the species (e.g., pathogenicity); others have been derived by screening random, cloned sequences for specific hybridization. While these methods can produce specific probes, they require either considerable empirical work or knowledge about the molecular basis of unique characteristics of the species of interest.

For several reasons, rRNA is an appealing target for species-specific oligonucleotide probes. Bacterial cells contain about 10,000 ribosomes compared with, at most, 4 copies of chromosomal DNA (16), the target of most previously described probes. Thus, probes to rRNA should be more sensitive by several orders of magnitude than most previously described nucleotide probes. Sequence analysis of 16S and 18S rRNAs has shown that some segments of these molecules are highly conserved in all organisms, while others are free to vary (9). Oligonucleotides complementary to the conserved segments can be used as primers in dideoxy sequencing to determine rapidly the sequence of any 16S or 18S rRNA (12). As Woese (17) and others have shown, changes in the variable portions of rRNAs are stable enough to allow the construction of consistent phylogenetic trees. Thus, it is reasonable to expect that some of the changes in sequence between closely related taxa have led to sequences that are taxon-specific.

Here we report the partial sequencing of the 16S rRNAs of the closely related clostridia *Clostridium bifermentans*, *C. difficile*, and *C. sordellii*. These three clostridia all belong to RNA homology group II described by Johnson and Francis (10) and share at least one common surface antigen (14). Oligonucleotide probes complementary to unique segments of rRNA could differentiate among these species.

MATERIALS AND METHODS

Bacterial strains. The type strains *C. bifermentans* ATCC 638, *C. difficile* ATCC 9689, and *C. sordellii* ATCC 9714 were obtained from the American Type Culture Collection (Rockville, Md.). Four other strains of *C. difficile* were also used: an isolate from a hospital environment (K-302), an isolate from a hamster with antibiotic-induced cecitis (49A), a hypertoxigenic strain isolated from an abdominal abscess (VPI 10463), and a nontoxigenic, nonsporulating strain (VPI 2018). The strains *C. beijerinckii* ATCC 25752, *C. butyricum* ATCC 19398, and *C. innocuum* ATCC 14501 were type strains from the American Type Culture Collection. Strains of *C. septicum*, *C. clostridiiforme*, and *C. perfringens* were isolated in the Clinical Microbiology Laboratory, Duke University Medical Center.

Purification of rRNA. Bacteria were grown overnight anaerobically in 30 to 100 ml of brain heart infusion broth (Difco Laboratories, Detroit, Mich.) and were centrifuged at 1,500 × *g* for 15 min at 4°C. Our method of obtaining rRNA was similar to that used by Aiba et al. (1) to obtain mRNA. The pellets were suspended in 0.5 to 1 ml of acetate buffer (0.02 M sodium acetate [pH 5.5], 0.5% sodium dodecyl sulfate, 1 mM EDTA) and extracted 3 times at 60°C with an equal volume of redistilled phenol equilibrated with buffer. The aqueous phase was then extracted with an equal volume of chloroform-isoamyl alcohol (24:1). Three volumes of absolute ethanol were added, and the RNA was precipitated on ice for 15 min. The material was spun at 12,000 rpm for 20 min in an Eppendorf centrifuge at 4°C, and the pellet was suspended in 300 μl of acetate buffer. RNA was then reprecipitated for 15 min on ice and centrifuged at 12,000 rpm and 4°C for 20 min in an Eppendorf centrifuge. The

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pellet was suspended in 100 to 300 μl of TE buffer (10 mM Tris [pH 7.4], 1.0 mM sodium EDTA), and the A_{260} was determined. Assuming that an A_{260} of 1 represented 50 μg of RNA per ml, the RNA concentration was adjusted to 5 mg/ml. An equal volume of 4 M NaCl was added, and the high-molecular-weight fraction was precipitated overnight on ice. After another centrifugation at 4°C, the precipitate was suspended in TE buffer. Residual salt was then removed when a 1/10 volume of 2 M sodium acetate and 2.5 volumes of ethanol were added to precipitate the RNA at 20°C for 4 h. Samples were then recentrifuged, and pellets were washed in 95% ethanol and dried in vacuo. RNA was then redissolved at a concentration of 2 mg/ml in 10 mM Tris buffer (pH 8.3) and stored at −70°C.

Sequencing reaction. The 16S rRNA was sequenced by using reverse transcriptase in an adaptation of the methods of Lane et al. (12) and Huibregtse and Engelke (8). The three oligonucleotide primers, which were designated A, B, and C by Lane et al. (12), were complementary to highly conserved regions of rRNA. In a microfuge tube, 4.0 μl of template rRNA was added to 1 to 2 μl of ^{32}P-labeled primer (7 to 13 ng) and H_2O was added to 65 μl. The mixture was heated to 90°C for 2 min and then slow-cooled to room temperature over 10 min. Ten microliters of reverse transcriptase buffer (500 mM Tris [pH 8.3], 60 mM $MgCl_2$, 400 mM KCl) was added. The reaction mixture was divided into 5 equal portions (15 μl per tube). Avian myeloblastosis virus reverse transcriptase (Seikagaku America, St. Petersburg, Fla.) was diluted to 2.1 U/μl in reverse transcriptase buffer, and 1.2 μl was added to each tube. Four microliters of a mixture containing deoxyadenosine, deoxythymidine, deoxycytosine, and deoxyguanosine (deoxynucleotide triphosphates) plus one dideoxynucleotide triphosphate (ddNTP) was then added. Four reaction tubes each received a different ddNTP; the fifth tube served as a control and contained no ddNTP. The final concentration of the nucleotide triphosphates was 600 μM, except for the nucleotide triphosphate corresponding to the ddNTP added to the reaction tube (60 μM). ddNTPs were added at a final concentration of 20 μM, except for ddCTP, which was added to 13 μM. The reaction tubes were incubated for 5 min at room temperature and then for 30 min at 55°C. EDTA was added to stop the reaction, and the reaction products were precipitated with 3 volumes of ethanol at room temperature for 30 min and then centrifuged for 30 min in a microcentrifuge at 12,000 rpm. The pellet was dried and redissolved in dye mix (90% formamide, 1 mM EDTA, 10 mM NaOH, 0.1% xylene cyanol, 0.1% bromphenol blue). Two microliters of each sample was electrophoresed on an 8% polyacrylamide sequencing gel containing 7 M urea at 60 W and 50°C. Gels were dried and autoradiographs were made by standard techniques.

Synthesis of oligonucleotide probes. A DNA synthesizer (model 380; Applied Biosystems) was used to synthesize oligonucleotides by the phosphoamidite method (2), as described by the manufacturer.

Dot blot hybridizations. Oligonucleotide probes were labeled at the 5′ ends with ^{32}P with polynucleotide kinase (13). rRNA was denatured in a heated formaldehyde solution and applied to a nylon membrane (GeneScreen; Dupont, NEN Research Products, Boston, Mass.) in a standard dot blot manifold (Schleicher & Schuell, Inc., Keene, N.H.), as described by Berent et al. (3). The rRNA was then bonded to the membrane by exposure to UV light, as recommended by the manufacturer. The membrane was then prehybridized for 4 h at 42°C in 5× SET (20× SET is 0.5 M NaCl, 0.03 M Tris [pH 7.4], and 2 mM EDTA)–0.1% sodium PP_i–0.2% sodium dodecyl sulfate–2 mg of heparin per ml. Labeled probe, at a concentration of 5×10^6 to 10×10^6 cpm, was then added to the reaction mixture, which was incubated with shaking overnight at 42°C. The membrane was washed 4 times in 5× SET–0.2% sodium dodecyl sulfate at 42°C for 30 min. The resulting dot blot was autoradiographed on SB-5 film (Eastman Kodak Co., Rochester, N.Y.).

TABLE 1. Sequences in three segments of 16S rRNA of *C. difficile*, *C. sordellii*, and *C. bifermentans*[a]

Segment and organism	Sequence
Segment I	
C. difficile	CUcUUGaAACUgggagACUUGA
C. sordellii	CUuUUGgAACUguaggACUUGA
C. bifermentans	CUuUUGgAACUagagaACUUGA
Segment II	
C. difficile	UAAAGCuAcUCUCAGU
C. sordellii	UAAAGCcAuUCUCAGU
C. bifermentans	UAAAGCcAuUCUCAGU
Segment III	
C. difficile	GUCCACGCuGUAAACG
C. sordellii	GUCCACGCcGUAAACG
C. bifermentans	GUCCACGCcGUAAACG

[a] The segments served as sites at which oligonucleotide probes were directed and are designated segments I, II, and III. Differences between clostridia are indicated with lowercase letters.

RESULTS

Sequence data. Comparison of sequences obtained from *C. fermentans*, *C. sordellii*, and *C. difficile* confirmed their close phylogenetic relationship. The sequences of *C. sordellii* and *C. bifermentans* were 98% similar, those of *C. sordellii* and *C. difficile* were 97% similar, and those of *C. bifermentans* and *C. difficile* were 97% similar. Despite this high degree of similarity, there were 16 loci in which *C. difficile* differed by 1 or more bases from the other two clostridia. To help locate the most promising sites for probes, we eliminated loci that differed from one another in the clostridial sequences but that happened to be identical to the sequence of one or more unrelated organisms for which 16S rRNA sequences were known. To eliminate these loci, the Nucleic Acid Query program (National Biomedical Research Foundation, Washington, D.C.) was used on a VAX model 11750 computer to search GenBank for sequences that were identical to the segments of interest in the rRNA of *C. difficile*. A hypothetical 16- to 21-mer probe site was chosen for each locus in which the sequence of *C. difficile* differed from those of the other two clostridia. The site was designed to place the mismatches between clostridial sequences as near the center of the segment as possible. By using the Nucleic Acid Query match routine, GenBank was searched for all sequences that were identical to the probe sites, allowing for up to two mismatches. The segments were identical or nearly identical only to sequences found in 16S rRNA. Of the 16 segments, 4 were identical to segments of 16S rRNA found in unrelated organisms; 4 were within 1 base of being identical to unrelated rRNAs, and 3 mismatched unrelated rRNAs by 2 bases. There remained five segments of rRNA of *C. difficile* that were not within even 2 bases of matching the rRNA of any unrelated organism found in GenBank. Two of these segments were chosen as sites at which to direct probes; the sequences at these sites

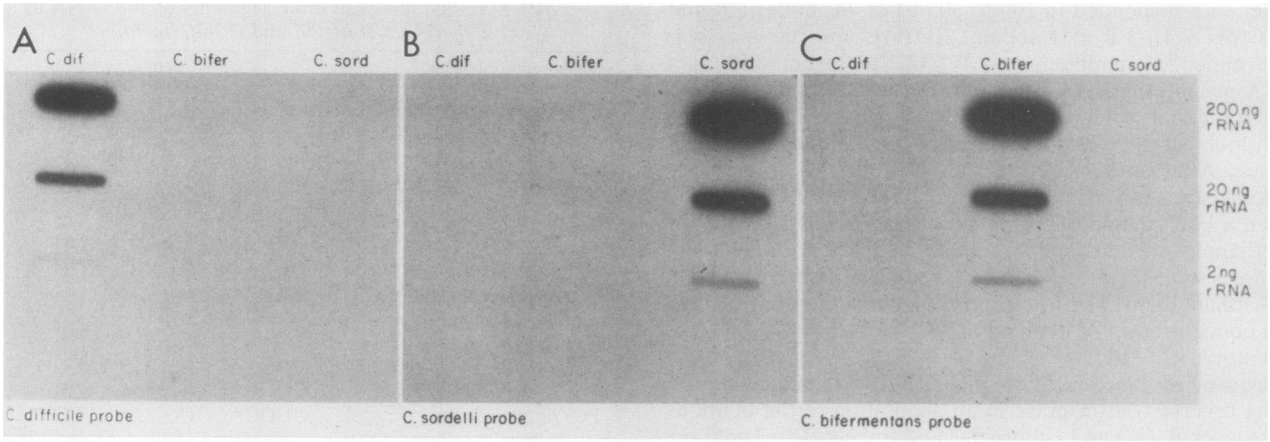

FIG. 1. Slot blot hybridization of oligonucleotide probes complementary to segment I (Table 1) of 16S rRNA from *C. difficile* (C. dif) (A), *C. sordellii* (C. sord) (B), or *C. bifermentans* (C. bifer) (C). Each probe hybridized specifically to the rRNA of the species to which it was directed.

are given in Table 1. Because we were more interested in *C. difficile* than in the other two clostridia, the second site did not differentiate *C. sordellii* from *C. bifermentans*. Results with a third probe site, in which the segment from *C. difficile* matched the sequence of an unrelated organism, are included to demonstrate that these probes were able to differentiate sequences that differed by as little as 1 base.

Hybridizations. From 0.2 to 200 ng of rRNA from each species was applied to a nylon membrane. All probes complementary to segment I (Table 1) hybridized in a species-specific fashion (Fig. 1). In more heavily exposed autoradiographs (data not shown), each probe gave a signal when it was hybridized to 0.2 ng of the matching rRNA that was as intense as the signal given when it was hybridized to 200 ng of the mismatching rRNA. Thus, the probes directed at segment I were 1,000 times more efficient at binding to their matching rRNAs than to their mismatching rRNAs. Probes directed at segment II hybridized about 100 times as efficiently with the perfectly matching rRNA (Fig. 2). Even a 1-base mismatch allowed for a greater-than-10-fold difference in efficiency of hybridization (Fig. 3). Because the isolation and purification of rRNA was the rate-limiting step in the hybridization technique, this step was abbreviated by suspending various numbers of cells in acetate-sodium dodecyl sulfate buffer and extracting them once with hot phenol and then once with chloroform-isoamyl alcohol. The aqueous phase was blotted as described above and hybridized with probes directed at the sequence of *C. difficile* at segments I and II. This abbreviated technique did not decrease the specificity of the hybridization and allowed detection of as little as 10^3 CFU of *C. difficile* if a freshly radiolabeled probe was used. We tested the specificity of both of these *C. difficile*-specific probes by hybridizing them to the rRNAs of *C. beijerinckii*, *C. butyricum*, *C. clostridiiforme*, *C. innocuum*, *C. perfringens*, and *C. septicum*. They did not hybridize efficiently to rRNAs of any of these organisms (data not shown). One might wonder whether the regions probed were so variable that they varied even among strains of *C. difficile*. To rule out that possibility, the first two probes were hybridized to four other strains of *C. difficile* from divergent sources (see above). They hybridized well to all the strains tested (data not shown), indicating that the

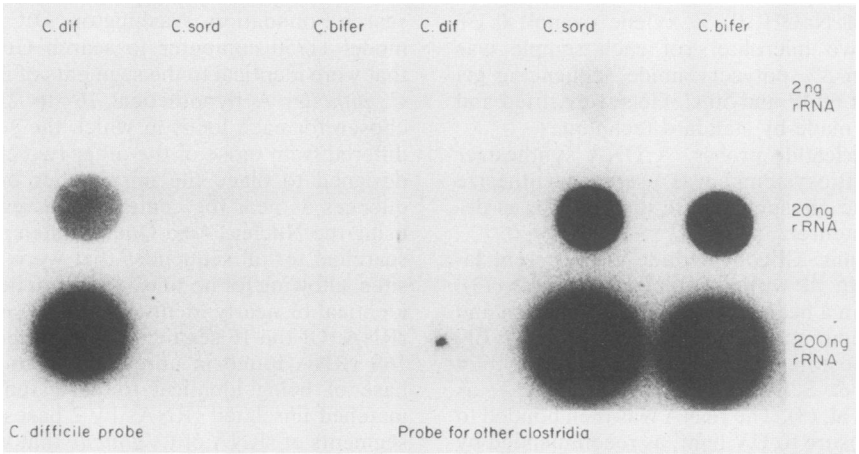

FIG. 2. Dot blot hybridization of probes complementary to segment II of 16S rRNA. The sequences of *C. sordellii* (C. sord) and *C. bifermentans* (C. bifer) were identical at this location and differed from the sequence of *C. difficile* (C. dif) by 2 bases. Again, each probe hybridized specifically to the complementary rRNA.

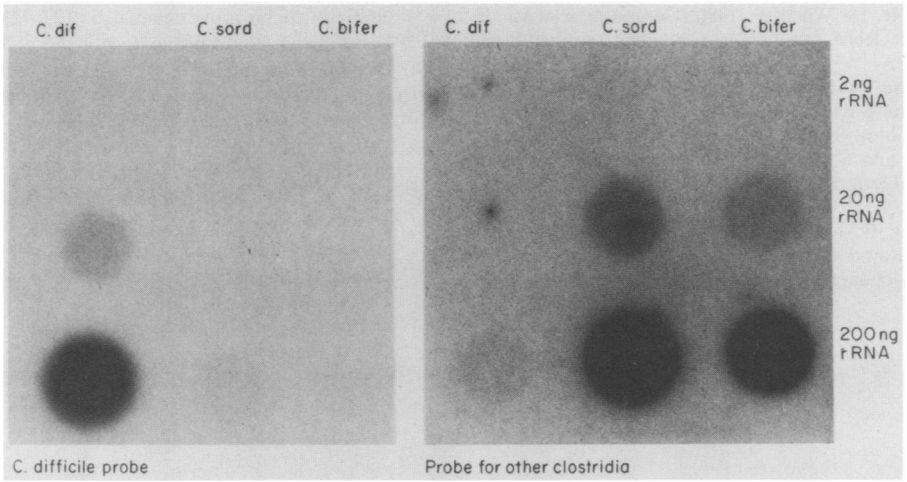

FIG. 3. Hybridization of probes complementary to segment III. At this location there was only a 1-base mismatch between the 16S rRNA of *C. difficile* (C. dif) and the RNA of the other two clostridia. C. sord, *C. sordellii*; C. bifer, *C. bifermentans*.

rRNA sequences in these segments did not vary among these strains.

DISCUSSION

Oligonucleotide probes directed at specific sequences in bacterial rRNA promise to be widely useful for the rapid identification of bacteria. Gobel et al. (7) have reported species- and group-specific oligonucleotide probes directed at the rRNAs of mycoplasmas. However, mycoplasma rRNA sequences seem to have changed more rapidly than the rRNAs of other bacteria (17). Thus, it is not at all clear that the success with mycoplasmas can be generalized to other groups of organisms. Workers at Gen-probe have reported the development of probes for the rRNA of several species of the genus *Mycobacterium* (5), but no experimental details concerning the method of development of these probes or their composition have been published. The results presented above with three clostridia suggest that a systematic approach has a high likelihood of success at yielding probes that are able to differentiate even closely related bacterial species. Oligonucleotides are particularly useful for this purpose, because they are able to differentiate sequences that differ by only 1 base (4). The demonstration by Giovannoni et al. (6) that oligonucleotide probes for rRNA can be used for in situ hybridization suggests that similar probes could be used for the direct detection of organisms as well as the identification of isolates.

Although the GenBank collection of rRNA sequences is limited, a computerized search of this collection located sequences in other organisms that matched or nearly matched potential probes for *C. difficile*. Not all potential probe sites found by comparing the rRNA sequences of these clostridia served to discriminate the organisms even at the genus level. Thus, the computer search was useful in eliminating certain probe sites from further consideration without the necessity of extended empirical studies at the laboratory bench. As more 16S rRNA sequences become available, this computerized approach should become more powerful and it should become possible to predict rapidly which segments of rRNA will be useful for probes of desired specificities, e.g., genus or species specific. Although there are too few data thus far to establish absolutely the specificity of the probes for these clostridia, this problem should also be overcome as more data are compiled. The computer search showed that at least five regions in the rRNA of *C. difficile* failed to come within even 2 bases of matching any other known rRNA sequence. At the very least, then, it appears that by using multiple probes on the same specimen, one could resolve any ambiguities found in testing with a single probe.

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