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Molecular Epidemiology of *Pseudomonas cepacia* Determined by Polymerase Chain Reaction Ribotyping

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Traditional ribotyping detects genomic restriction fragment length polymorphisms by probing chromosomal DNA with rRNA. Although it is a powerful method for determining the molecular epidemiology of bacterial pathogens, technical difficulties limit its application. As an alternative, polymorphisms were sought in the 16S-23S spacer regions of bacterial rRNA genes by use of the polymerase chain reaction (PCR). Chromosomal DNA from isolates of *Pseudomonas cepacia* was used as a template in the PCR with oligonucleotide primers complementary to highly conserved sequences flanking the spacer regions of the rRNA genes. Length polymorphisms in the amplified DNA distinguished unrelated isolates of *P. cepacia*. Isolates of *P. cepacia* previously implicated in person-to-person transmission were shown to have identical amplification patterns. These data demonstrate the utility of this new PCR ribotyping method for determining the molecular epidemiology of bacterial species.

Determining the relatedness of isolates of microorganisms has become increasingly important as the number and spectrum of nosocomial pathogens continue to expand. Recently, approaches at the molecular level have been used to assess the relatedness of bacterial isolates. DNA analysis, rather than analysis of phenotypic parameters such as outer membrane protein profiles (19), biochemical profiles (1), or antimicrobial susceptibility patterns (24), is preferred, since it provides a more stable determination of isolate identity. However, even genetic analysis methods may have limitations; for example, plasmid analysis can only be used for bacteria that contain plasmids (28).

Recently, rRNA was used as a probe to detect polymorphisms in bacterial chromosomal DNA (33). This ribotyping method distinguished unrelated isolates of *Escherichia coli* from bacteriuric women (18), demonstrated the relatedness of isolates of *Pasteurella multocida* in flocks of turkeys and wildlife when serotyping could not (29), and differentiated most serovars of *Leptospira* species (25). Additionally, traditional ribotyping was used to distinguish isolates of *Pseudomonas cepacia* in cystic fibrosis (CF) patients at different CF centers (17) and documented person-to-person transmission of this organism in one setting (15).

Despite the broad applicability of this method, its use in clinical microbiology laboratories has been limited because of the prolonged time needed for Southern blot analysis and the use of radioisotopes. To circumvent these problems, we have used the polymerase chain reaction (PCR) (27) in conjunction with ribotyping. Our results demonstrate that the PCR can be used to detect polymorphisms in the intergenic spacer regions of bacterial rRNA genes and that it can be a broadly applicable tool in molecular epidemiology.

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MATERIALS AND METHODS

Bacteria and growth conditions. *P. cepacia* isolates were obtained from patients with CF by culturing of respiratory secretions on selective medium (8). Some isolates of *P. cepacia* were randomly obtained from patients at one CF center. Additional isolates were obtained from patients attending a summer (1989) educational program, at which person-to-person transmission of *P. cepacia* had occurred and was documented by traditional ribotyping (15). Several isolates of *P. cepacia* were subcultured in vitro for 80 passages (16). All isolates were stored in skim milk at −80°C until further analysis. Organisms were grown overnight in brain heart infusion broth at 37°C with shaking.

DNA preparation. Whole chromosomal DNA from *P. cepacia* was purified as described previously (33). In brief, pelleted bacteria from 1 ml of an overnight broth culture were washed, suspended in Tris-EDTA buffer (pH 8.0), and lysed with lysozyme (100 μg/ml) and proteinase K (100 μg/ml). After 2 h of incubation with sodium dodecyl sulfate (0.5%), the lysate was sequentially extracted with phenol and chloroform. DNA was precipitated with cold ethanol, recovered by centrifugation, dried, and resuspended in 10 mM Tris-HCl (pH 7.5)–1 mM EDTA at a final concentration of approximately 0.5 μg/μl. A modification of this method was used for some isolates (4). In brief, pelleted bacteria from an overnight culture were suspended in Tris-EDTA buffer, and the mixture was placed in a boiling water bath for 5 min. The cells were exposed to lysozyme (100 μg/ml) for 15 min on ice and then to proteinase K (100 μg/ml) at 55°C for 10 min. The proteinase K was inactivated at 95°C for 15 min, and then the cells were exposed to DNase-free RNase (10 μg/ml) at 37°C for 15 min.

Amplification. Oligonucleotide primers were designed to be complementary to conserved regions of the 16S and 23S regions of the rRNA genes (areas 1 and 2 in Fig. 1) (12, 22). The sequences of primers 1 and 2 were 5′-TTGTACACA CCGCCCGTCA-3′ and 5′-GGTACCTTAGATGTTTCAGT TC-3′, respectively. Primers were obtained from Oligos Etc. (Guilford, Conn.).

Amplifications were performed in a final volume of 100 μl

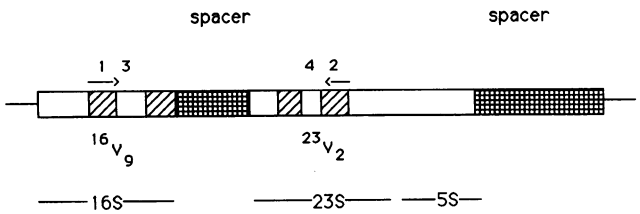

FIG. 1. Organization of an rRNA operon in eubacteria. $^{16}V_9$, variable region 9 in the 16S rRNA subunit. $^{23}V_2$, variable region 2 in the 23S rRNA subunit. 1 and 2, conserved areas used as primers in the PCR (see Materials and Methods for sequences). 3 and 4, Variable regions with potential use as species-specific PCR primers. Arrows indicate the direction and location of PCR primers.

with a reaction mixture containing 10 mM Tris-HCl (pH 8.8), 50 mM potassium chloride, 1.5 mM magnesium chloride, 0.1% Triton X-100, 200 μM deoxynucleoside triphosphates, and 100 pmol of each primer. These conditions were found to be optimal by a series of experiments with different concentrations of each component. One to two microliters of purified DNA or 10 μl of the crude preparation was used as template DNA. Following an initial denaturation for 6 min in a boiling water bath, 2.5 U of *Taq* polymerase (Promega, Madison, Wis.) was added. The DNA was amplified during 30 cycles of PCR consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, except for the last cycle, during which the extension step lasted 4 min. A negative control consisting of reaction buffer, primers, deoxynucleoside triphosphates, and distilled water instead of template DNA was used with each PCR. Twenty microliters of the PCR mixture was analyzed by electrophoresis in 2 or 3% agarose gels (SeaKem GTG; FMC Bioproducts, Rockport, Maine). The PCR patterns obtained were compared after ethidium bromide staining.

RESULTS

Banding patterns of *P. cepacia*. Oligonucleotide primers selected from conserved regions of several bacterial species and whole-cell DNA from *P. cepacia* were used to amplify the 16S-23S spacer regions by PCR. Five unrelated clinical isolates of *P. cepacia* were distinguished by the polymorphisms resulting from amplification of the spacer regions (Fig. 2). The multiple bands seen in lanes C to E represent spacer regions of different lengths among the rRNA operons of *P. cepacia* and illustrate how these three isolates can be separated on the basis of the banding patterns of these regions. The single bands in lanes F and G correspond to uniform spacer regions in the rRNA genes of these two isolates; differentiation can be made on the basis of size variations of these regions (800 versus 1,020 bp).

To investigate the stability and reproducibility of the banding patterns seen after PCR amplification, we amplified chromosomal DNA from a *P. cepacia* isolate that had been passaged in vitro 80 times (Fig. 3). Individual amplifications of subcultures 20, 40, 60, and 80 were performed under identical conditions but at different times. Of note, identical amplification patterns were obtained with all subcultures (lanes B, C, D, and E), illustrating the stability of PCR ribotyping with in vitro passage, as well as the reproducibility of the method with the same isolate. The high-molecular-weight band seen in lane D represents chromosomal DNA, not an amplified spacer region.

Molecular epidemiology of *P. cepacia*. The banding patterns

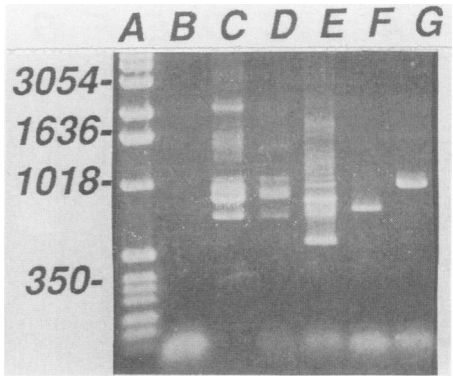

FIG. 2. PCR amplification patterns of five unrelated isolates of *P. cepacia* analyzed by 1% agarose gel electrophoresis (lanes C to G). Lane A represents a 1-kb molecular weight marker (Bethesda Research Laboratories, Gaithersburg, Md.). Lane B represents a negative control. The broad bands at the bottom of the gel in lanes B to G represent nonreacting primers. Numbers at left are in base pairs.

obtained with whole-cell DNA from isolates of *P. cepacia* as a template for PCR were used to test the potential of PCR ribotyping for molecular epidemiology. PCR ribotyping was applied to isolates that had been characterized previously by traditional ribotyping (15). The three identical isolates (Fig. 4, lanes F to H) originated from two patients involved in person-to-person transmission of *P. cepacia* (lanes G and H) and from the same CF center as that of the patient isolate represented by lane H (lane F). The relatively increased intensity of the band in lane G represents a different quantity of the same uniform spacer regions as those in lanes F and H. Six of eight other isolates from this center had the same banding patterns (data not shown). The four other *P. cepacia*

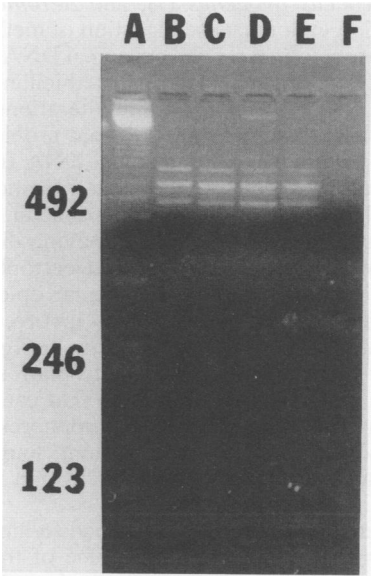

FIG. 3. PCR amplifications of repeated subcultures of one isolate of *P. cepacia*. Lane A represents a 123-bp molecular weight marker (Bethesda Research Laboratories). Lanes B, C, D, and E represent the amplification of a *P. cepacia* isolate subcultured 20, 40, 60, and 80 times in vitro, respectively. Lane F represents a negative control. Numbers at left are in base pairs.

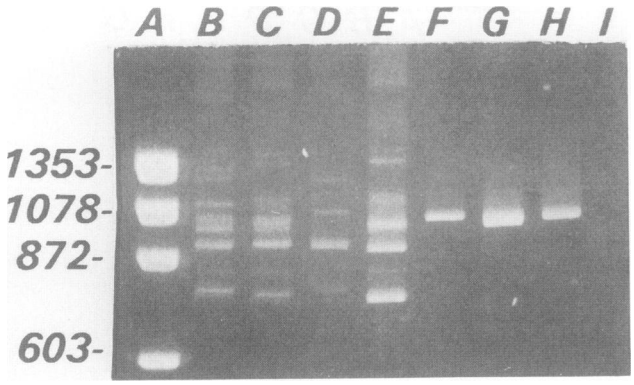

FIG. 4. PCR amplification of seven isolates of *P. cepacia* analyzed by 3% agarose gel electrophoresis. Lanes G and H represent isolates from two patients previously implicated in person-to-person transmission. Lanes B to F represent five additional *P. cepacia* isolates. Lane I represents a negative control. φX-174 DNA digested with *Hae*III was used as a molecular weight marker (lane A). Numbers at left are in base pairs.

isolates originated from the same CF center as that of the patient isolate represented by lane G and were genetically different from the isolates implicated in person-to-person transmission, as illustrated by the different banding patterns obtained by PCR of the 16S-23S spacer regions. Five additional isolates from this center had banding patterns similar to those of the isolate represented in lane E (data not shown).

DISCUSSION

Analysis of restriction fragment length polymorphisms has been applied to the differentiation of bacterial isolates. Restriction endonuclease digestion of chromosomal DNA has been used to differentiate isolates of *Candida albicans* (34), *Streptococcus pyogenes* (7), and *Aeromonas* species (14). Restriction endonuclease digestion of methicillin-resistant *Staphylococcus aureus* chromosomal DNA revealed the persistence of a single isolate of methicillin-resistant *S. aureus* at a London hospital despite alterations in the antimicrobial resistance pattern and plasmid profile (13). However, a large number of chromosomal DNA fragments are usually produced by restriction enzyme digestion, and comparison of a large number of bands is difficult. Large DNA fragments resulting from restriction enzyme digestion have been separated by field inversion gel electrophoresis. This method has been used for the molecular epidemiology of staphylococci (9), *P. cepacia* isolates involved in a nosocomial outbreak (2), and *Pseudomonas aeruginosa* in CF siblings (11). Field inversion gel electrophoresis is able to resolve large chromosomal fragments that cannot be separated by conventional agarose gel electrophoresis; however, it requires more sophisticated equipment, long electrophoresis times, and considerable standardization to determine truly related isolates of bacteria.

Several methods have been developed to identify genetic polymorphisms by PCR. Although some of these methods rely on very specific sequence information (20), PCR has also been combined with restriction enzyme digestion to produce restriction fragment length polymorphisms. An example is amplification of specific DNA sequences from the rickettsial citrate synthase gene and subsequent cleavage of these sequences with restriction endonucleases to differentiate recognized rickettsial species (26). PCR coupled to restriction enzyme digestion requires only a fraction of the chromosomal DNA needed for Southern blotting analysis (30) and can be completed in a much shorter time because of the elimination of hybridization.

rRNA genes have been particularly useful in detecting genetic polymorphisms among bacteria. The organization of eubacterial transcription units is 5′-16S-23S-5S-3′ (Fig. 1). There are 2 to 11 rRNA gene copies per bacterial cell (10, 32). The intergenic spacer regions between the 16S and 23S rRNA genes encodes several tRNAs and contains several direct repeat sequences in noncoding regions of the gene clusters (3). In *E. coli*, which contains seven transcriptional units for rRNA, there are two classes of rRNA (5). One of these contains tRNA genes for alanine and isoleucine within the 16S-23S spacer regions, while the other contains the tRNA gene for glutamic acid (21). Other heterogeneities have been demonstrated in the intergenic spacer regions of *Bacillus* species (10), *Aspergillus* species (31), and the oomycetous fungus *Pythium ultimum* (6). In this study, we used PCR to demonstrate polymorphisms in the 16S-23S spacer regions of *P. cepacia*. These polymorphisms, which represent size variations of the spacer regions in the multiple rRNA operons, are reproducible and stable in a given isolate over time. The banding patterns produced by the amplification of these regions can be compared for epidemiologic investigations. We subsequently used PCR ribotyping to differentiate unrelated isolates of *E. coli* from clinical specimens (data not shown).

With certain species, PCR ribotyping may not be able to detect polymorphisms in the spacer regions without some modification of the method. For example, the sequential application of PCR and restriction endonuclease digestion with an enzyme that has a 4-base recognition sequence would increase the number of bands for comparison. Another modification might include the use of oligonucleotide primers complementary to different areas of the rRNA genes to produce different amplification products that may contain polymorphisms. Finally, in certain species, there might be bands of equal size that are present in various ratios in different isolates. In this situation, densitometry could be used to determine the band ratios produced by PCR, representing the ratios of different spacer regions in the rRNA genes. The future application of these strategies provides promise for extending PCR ribotyping to a large number of bacterial species.

A final advantage of PCR ribotyping is its potential expansion to include diagnostic capability. Since the oligonucleotide primers used are complementary to highly conserved regions of genes, a single set of primers can be used to type a broad spectrum of species (23). The amplified products can then be analyzed with species-specific probes to identify specific products. Alternatively, species-specific sequences designed from the variable regions of the 16S and 23S rRNAs (areas 3 and 4 in Fig. 1) can be used as PCR primers, detecting the presence of a specific species in a clinical specimen and also providing additional material for typing. A method for detecting such species-specific regions has been described for 16S rRNA genes (4).

Traditional ribotyping has detected polymorphisms in isolates of *P. cepacia*, *E. coli*, and *Haemophilus influenzae* (33). However, the length of time required for blotting and the use of radioactivity in detection have limited the clinical application of traditional ribotyping. The data presented here illustrate the utility of PCR ribotyping in molecular epidemiology. We selected oligonucleotide primers from highly

conserved regions of rRNA genes by alignment with cataloged sequences. These primers were used to amplify 16S-23S intergenic spacer regions in a PCR to detect polymorphisms in this area. We tested the validity of our system with an epidemiologically well-defined situation, using *P. cepacia* from a previous study that documented person-to-person transmission by traditional ribotyping (15). The advantages of PCR ribotyping, including speed, safer detection methods, universal primers for eubacteria, and the use of only small amounts of DNA extracts as starting material, demonstrate its potential as a widely useful tool in molecular epidemiology.

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