Characterization of PCR-Ribotyping for *Burkholderia* (*Pseudomonas*) *cepacia*

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Ribotyping, a method of genotyping bacterial isolates for epidemiologic study, uses rRNA as a probe to detect chromosomal restriction fragment length polymorphisms. Although ribotyping is accurate, its utility is limited by the labor and time necessary for Southern blot analysis. PCR-ribotyping uses PCR to amplify the 16S-23S intergenic spacer region of the bacterial rRNA operon. Length heterogeneity in the spacer region has previously been found to be useful as an alternative to standard ribotyping in a study of *Burkholderia* (*Pseudomonas*) *cepacia*. To further analyze the accuracy of PCR-ribotyping, three groups of previously characterized isolates of *B. cepacia* were investigated. PCR-ribotyping grouped 90 isolates recovered from seven well-defined epidemics into the correct outbreak group with a mean concordance of 93%. Both standard ribotyping and PCR-ribotyping separated 15 unrelated isolates into 14 types. In an analysis of 83 *B. cepacia* isolates from chronically colonized cystic fibrosis patients, the concordance of PCR-ribotyping with standard ribotyping ranged from 83 to 100%, with a mean of 98%. One isolate from a chronically colonized patient had a different type by standard ribotyping but was identical to the other isolates from this patient by PCR-ribotyping. Thus, PCR-ribotyping is a rapid and accurate method for typing *B. cepacia* and is less labor intensive than standard ribotyping.

Because cystic fibrosis (CF) patients colonized with *Burkholderia* (*Pseudomonas*) *cepacia* are hospitalized longer and have a higher mortality rate than noncolonized patients matched for severity of illness, accurate characterization of the epidemiology of *B. cepacia* is important (3). A wide variety of methods, including serotyping, biotyping, antibiograms, and bacteriocin susceptibility and production, have been used to distinguish strains of *B. cepacia*. In a multicenter, blinded study to assess *B. cepacia* typing systems, ribotyping was the most accurate method for placing isolates into outbreak-related groups (14). Ribotyping has since proved useful in a number of investigations of the epidemiology of *B. cepacia* (10–12). However, the time and labor required for Southern blot analysis is a significant obstacle to the wide use of this method.

The sources of the polymorphisms detected by ribotyping have not been characterized but probably result largely from sequence heterogeneity of the DNA flanking bacterial rRNA operons. In addition, the 16S-23S spacer region of the rRNA operon is a potential source of polymorphisms because it contains a variable number of tRNA genes and DNA unrelated to ribosome structure (1, 15). We previously reported the use of primers specific for conserved regions in the 16S and 23S rRNA genes in the development of PCR-ribotyping (8). This method identifies length polymorphisms in the intergenic spacer regions of *B. cepacia* rDNA as an alternative genotyping method.

In the current study, the accuracy of PCR-ribotyping for epidemiological investigations was determined by testing the ability of PCR-ribotyping to correctly group epidemiologically defined strains of *B. cepacia*. PCR-ribotyping was then compared with standard ribotyping in analyses of unrelated isolates and isolates recovered from patients with CF.

MATERIALS AND METHODS

Bacteria. Three previously described groups of *B. cepacia* were used to evaluate the potential utility of PCR-ribotyping. The first group consisted of 90 *B. cepacia* isolates obtained during seven nosocomial outbreaks investigated by the Centers for Disease Control and Prevention (14). Investigation of each of these outbreaks revealed a common source of contamination, indicating that the isolates were the same strain. The isolates were previously examined by standard ribotyping and several other typing systems in a multicenter study (14). The two strains which were not correctly grouped by any typing system were excluded from the present study. Ribotyping grouped the remaining 90 isolates into their outbreak-related groups with a concordance of 100%. The second group of *B. cepacia* isolates consisted of epidemiologically unrelated isolates obtained from the American Type Culture Collection (ATCC). These isolates, also previously analyzed by ribotyping (12), were selected on the basis of differences in time and location of isolation in order to examine the discriminatory power of PCR-ribotyping within the species. The third group of *B. cepacia* consisted of 83 isolates serially recovered from 12 chronically colonized CF patients. Isolates were cultured from individual patients during periods of time ranging from 2 to 54 months. Previous characterization of these isolates by ribotyping demonstrated that most patients remain colonized by the same strain of *B. cepacia* for long periods of time (11).

Growth conditions. Organisms were grown overnight in Mueller Hinton (MH) broth at 30 or 37°C. Slow-growing isolates were plated on MH agar and grown at 30 or 37°C and then subcultured into MH broth.

DNA preparation. Whole chromosomal DNA was purified

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TABLE 1. PCR-ribotypes of outbreak-related *B. cepacia* isolates

Outbreak group (no. of isolates)	PCR-ribotype (no. of isolates)	% Concordance[a]
I (9)	P1 (9)	100
II (6)	P2 (6)	100
III (9)	P1 (9)	100
IV (27)	P3 (27)	100
V (17)	P4 (16), P5 (1)	94
VI (7)	P6 (4), P7 (1), P8 (1), P9 (1)	57
VII (15)	P10 (15)	100
Total (90)		93 (mean)

[a] Percent concordance between outbreak group and PCR-ribotype.

FIG. 1. PCR-ribotyping of ATCC isolates of *B. cepacia*. PCR products were electrophoresed in agarose, stained with ethidium bromide, and visualized by UV light to determine the PCR-ribotype. The samples in lanes E and F have the same PCR-ribotype. The samples in lanes A to D and G to O have unique PCR-ribotypes. Lane mw, molecular size markers (*Hae*III-digested φX174 RF DNA); lane P, negative control.

as previously described (16). Briefly, pelleted bacteria were washed in 0.5 ml of Tris-EDTA (pH 8.0) and lysed with lysozyme (100 μg/μl), proteinase K (100 μg/μl), and sodium dodecyl sulfate (0.5%). After incubation at 37°C until cleared, the lysate was extracted with phenol and chloroform, and DNA was precipitated with 95% cold ethanol. The recovered DNA was resuspended to a final concentration of 0.5 μg/ml.

PCR-ribotyping. Oligonucleotide primers were designed to complement conserved regions of the 16S and 23S regions of the rRNA operon (5, 13), as previously reported (8). The sequences of the primers used were: 16S, 5'-TTGTACACACC GCCCGTCA-3', and 23S, 5'-GGTACCTTAGATGTTTCA GTTC-3'. Amplification was performed in a mixture of 10 mM Tris-HCl (pH 8.8), 50 mM potassium chloride, 2.0 mM magnesium chloride, 0.1% Triton X-100, 200 μM deoxynucleoside triphosphates, and 100 pmol of each primer. Approximately 20 ng of template DNA was used in each amplification. The total volume of the reaction mixture was brought up to 100 μl with distilled H$_2$O, and 2.5 U of *Taq* DNA polymerase (Promega, Madison, Wis.) was added. An initial denaturing step of 95°C for 3 min was followed by 30 cycles 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 an extension step of 4 min during the last cycle. The samples were then maintained at 4°C until analyzed by electrophoresis. A negative control contained all reagents except template DNA. After amplification, 10 to 20 μl of the PCR product was electrophoresed in a 2% Seakem (FMC BioProducts, Rockland, Maine) agarose gel in Tris-borate buffer. Isolates with identical banding patterns were grouped into the same PCR-ribotype. Nonidentical banding patterns were considered unique PCR-ribotypes.

Study design. Within each of the three groups of *B. cepacia*, isolates were coded, and PCR-ribotyping was performed in a blinded fashion. For the first group, the results of PCR-ribotyping were compared with the known epidemiologic relatedness of the isolates by calculating the concordance as the percentage of isolates within the outbreak type, as previously described (14). For the second and third groups of isolates, the results of PCR-ribotyping were compared with the results previously obtained by standard ribotyping (11, 12).

RESULTS

Table 1 shows the results of PCR-ribotyping of 90 isolates from seven outbreaks. Ten different PCR-ribotypes, designated P1 through P10, were identified. Among isolates within five of the seven outbreak groups, the concordance between PCR-ribotype and outbreak grouping was 100%. In outbreak group V, one isolate was of a different PCR-ribotype than the other 16 isolates (94% concordance), and in outbreak group VI, seven isolates were separated into four different PCR

ribotypes (57% concordance). The mean concordance for all seven groups was 93%.

Figure 1 shows the results of PCR-ribotyping of strains from the ATCC selected on the basis of differences in the time and location of isolation. Both ribotyping (12) and PCR-ribotyping distinguished 14 different types among the 15 strains. The two isolates which were of the same ribotype, represented in lanes E and F, also had the same PCR-ribotype.

PCR-ribotyping of the 83 isolates serially recovered from 12 different CF patients revealed 100% concordance with the results of standard ribotyping for each patient except one. For that patient, one isolate which was identified by standard ribotyping as unique was of the same PCR-ribotyping as the other isolates from that patient.

DISCUSSION

Ribotyping, which detects restriction fragment length polymorphism of whole chromosomal DNA (16), has been useful in characterizing the distribution of *B. cepacia* strains in CF treatment centers and in demonstrating person-to-person transmission of *B. cepacia* between patients with CF (10, 12). However, the time and labor required for Southern blotting limit the wide use of ribotyping. By using primers specific for the 16S and 23S regions of the rRNA operon, PCR-ribotyping detects length heterogeneity in the intergenic spacer region as an alternative means of typing *B. cepacia* isolates. Because the method requires only ethidium bromide staining of DNA fragments separated by agarose gel electrophoresis, it circumvents the need for Southern blotting and hybridization.

The data from the present study demonstrate that PCR-ribotyping, in addition to being technically simpler, is also an accurate and reliable alternative to standard ribotyping of *B. cepacia*. In a blinded multicenter study which assessed 10 different typing systems, ribotyping was the most accurate in placing isolates into the correct outbreak-related groups (14). In fact, ribotyping was 100% concordant with the known epidemiology of the 90 isolates examined. The mean concordance of PCR-ribotyping and the known epidemiology of the same isolates in the present study was 93%. Because of the sensitivity of PCR-ribotyping for detecting minor differences in DNA sequence, it has the potential to reveal genotype differences that are not epidemiologically significant.

Epidemiologically unrelated *B. cepacia* isolates from the ATCC were investigated to determine the discriminatory power of PCR-ribotyping. The separation of 15 isolates into 14 different types demonstrates that PCR-ribotyping has a discriminatory power similar to that of standard ribotyping for typing *B. cepacia*.

In addition to examining isolates from outbreaks not related

to patients with CF, we also examined isolates recovered serially from patients with CF which had previously been typed by standard ribotyping. The two methods were 100% concordant except for a single isolate. Ribotyping identified this isolate as different from the other isolates recovered from a patient, whereas PCR-ribotyping grouped it with all other isolates from this patient. Larsen et al. examined 14 *B. cepacia* isolates recovered from a CF patient during 2.5 years of colonization (9). Although antibiotic susceptibilities, outer membrane protein profiles, and plasmid analysis indicated marked differences among the isolates, PCR-ribotyping demonstrated chronic colonization with a single strain. Thus, based on epidemiological relatedness, both standard ribotyping and PCR-ribotyping are accurate systems for grouping CF- and non-CF-related isolates.

The requirements for PCR are simple, and the method can be used in a variety of approaches to characterizing infections. The primers proposed by Kostman et al. and used in the present study amplify DNA from a wide range of bacterial species, including spirochetes and mycobacteria (8). In contrast, species-specific primers not related to the rRNA operon have been used to detect organisms such as *Yersinia enterocolitica* (6) and *Clostridium difficile* (4) from human feces, *Listeria monocytogenes* (7) from cerebrospinal fluid, and *Mycobacterium tuberculosis* (2) from sputum. The polymorphisms detected in the rDNA intergenic spacer region suggest that the use of species-specific primers designed from the nucleotide sequence of the variable regions of the 16S and 23S rRNA genes may allow the simultaneous detection and typing of *B. cepacia* and *Pseudomonas* species directly from clinical specimens by PCR-ribotyping. Studies to investigate this possibility are under way.

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