Whole-Cell Repetitive Element Sequence-Based Polymerase Chain Reaction Allows Rapid Assessment of Clonal Relationships of Bacterial Isolates

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Repetitive element sequence-based polymerase chain reaction (rep-PCR) enables the generation of DNA fingerprints which discriminate bacterial species and strains. We describe the application of whole-cell methods which allow specimens from broth cultures or colonies from agar plates to be utilized directly in the PCR mixture. The rep-PCR-generated DNA fingerprints obtained with whole-cell samples match results obtained with genomic DNA templates. Examples with different gram-negative bacteria (e.g., *Citrobacter diversus*, *Escherichia coli*, and *Pseudomonas aeruginosa*) and gram-positive bacteria (e.g., *Staphylococcus aureus* and *Streptococcus pneumoniae*) are demonstrated. Rapid specimen preparation methods enable rep-PCR-based fingerprinting to be completed in several hours and, therefore, allows the timely analysis of epidemiological relationships.

Discriminatory molecular methods such as plasmid profiling, restriction endonuclease digestion of plasmid or chromosomal DNA, ribotyping, and multilocus enzyme electrophoresis (MEE) have been applied retrospectively to study relationships among isolates from apparent clinical outbreaks (5–7, 10, 11, 13, 17, 19, 27, 29, 31). Repetitive element sequence-based polymerase chain reaction (rep-PCR) is a recently described method (24) which generates DNA fingerprints that discriminate bacterial strains (3, 8, 24, 25, 30). However, the preparation of purified samples of genomic DNA from bacterial isolates represents a time-consuming step in rep-PCR and other chromosomal-DNA-based fingerprinting methods. We describe modifications of sample preparation methods for rep-PCR which enable culture plates or broth cultures to be used directly to type bacterial isolates. Modifications which allow the use of whole cells simplify rep-PCR fingerprinting and should facilitate its application in clinical laboratories.

Bacterial isolates. Bacterial isolates used in this study were clinical isolates kindly provided by E. O. Mason from the C.T. Parker Laboratory of Texas Children's Hospital, except for the *Enterobacter* strains obtained from P. Georgiou (Department of Medicine, Baylor College of Medicine), *Escherichia coli* K-12 strain W3110 obtained from Y. Kohara, and *Salmonella typhimurium* LT2 obtained from B. Erickson. The *Citrobacter diversus* isolates have been described previously (30). Bacteria were maintained as stocks frozen at –70°C in Luria-Bertani (LB) broth (16) with 20% glycerol.

Bacterial preparations for rep-PCR. Genomic DNA from each isolate was prepared as previously described (24). Subcultures used for whole-cell preparations were plated on tryptic soy agar with 5% sheep blood (Becton Dickinson)

(*Staphylococcus aureus* and *Streptococcus pneumoniae*) or plated on LB agar (16) and then inoculated into LB broth (all other isolates) for growth overnight. Two bacterial strains, *E. coli* W3110 and *C. diversus* 4514, were selected for initial standardization studies. Scrapings of colonies from LB plates or pellets of 5-ml LB broth cultures of both strains were either (i) suspended immediately in 3-ml Tris-EDTA buffer (10 mM Tris, 1 mM EDTA; pH 8.0) or (ii) washed once in 3 ml of 1.0 M NaCl, washed once in Tris-EDTA, and resuspended in 3 ml of Tris-EDTA. Suspensions of each isolate were kept on ice and diluted with Tris-EDTA to optical densities at 600 nm (A_{600}) of 0.5, 1.0, and 1.5. After samples were spun in a centrifuge and cells were suspended in Tris-EDTA in one-fourth of the diluted volume, 1 μ l of each whole-cell preparation was used to provide template DNA for PCRs. The quantity of purified genomic DNA per PCR was 100 ng. Results were similar for enteric bacteria whether the initial samples were taken from agar plates or broth cultures. The 1.0 M NaCl wash step and resuspensions at A_{600} values of 1.5 were used in all subsequent whole-cell preparations of bacterial species except *S. typhimurium* and *S. typhi*. Colonies of the two *Salmonella* strains were washed once in 1.0 M NaCl, washed once in Tris-EDTA, and resuspended at an A_{600} of 1.5 in Tris-EDTA in 1.5-ml Eppendorf tubes. Tubes were placed in a floating rack in tap water. Cell suspensions were sonicated for 5 min at 40 W by placing the sonication probe into the water surrounding the tubes. Tubes then were incubated at 100°C for 5 min and inverted several times. Two microliters of each sonicated suspension was added to the PCR mixtures.

Subsequent to the initial standardization of these whole-cell preparation methods with enteric bacteria, efforts were directed at expanding the utility of this typing method with other gram-negative and gram-positive bacteria. rep-PCR with these organisms required an extra heating step prior to the PCR. Cells were harvested and washed as described above. Reaction tubes containing cells, water, and buffer were preheated at 80°C for 15 min in a DNA thermal cycler (Perkin-Elmer). While the tubes remained in the thermal

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FIG. 1. Comparison of rep-PCR fingerprints generated by ERIC-PCR with different sample preparations of *E. coli* W3110 and *C. diversus* 4514. Lanes DNA, PCR with purified genomic DNA; lanes Plate, whole-cell samples cultivated on semisolid LB media with agar; lanes Plate/1M NaCl, as described above but with an extra 1 M NaCl wash step; lanes Ovnt. culture, whole-cell samples grown overnight in LB liquid media; lanes Ovnt. culture/1M NaCl, as described above but with an extra 1 M NaCl wash step. The numbers above each lane are the A_{600} values of suspensions of whole-cell samples. No template DNA was added to the negative (neg.) control lane. The DNA molecular size marker was a 1-kb ladder (Bethesda Research Laboratories).

cycler at 80°C, oligonucleotide primers, deoxynucleoside triphosphates (Pharmacia), and AmpliTaq DNA polymerase (Perkin-Elmer) were added (24); this was followed by initial denaturation of DNA templates at 95°C for 7 min and by 30 cycles of amplification as described previously (24). This preheating step was necessary with samples of *Bacillus subtilis*, *Klebsiella pneumoniae*, *N. meningitidis*, *Pseudomonas aeruginosa*, *S. aureus*, and *S. pneumoniae*.

rep-PCR. The term “rep-PCR” refers to the general method which utilizes oligonucleotide primers matching interspersed repetitive DNA sequences to yield DNA fingerprints of individual bacterial isolates (24). Primer pairs, REP1R-I plus REP2-I (24) and REP1R-Dt (24) plus REP2-Dt (23), were based on the repetitive extragenic palindromic (REP) consensus and used in the application named REP-PCR. The REP1R-I and REP2-I primers contain the nucleotide inosine at ambiguous positions (24) in the REP consensus. Inosine can form Watson-Crick base pairs with any of the four nucleotide bases (A, C, G, and T). The REP1R-Dt and REP2-Dt primers contain multiple nucleotides (A, G, C, or T), instead of inosine, at the same ambiguous positions (23, 24). The REP1R-Dt and REP2-Dt primers were previously found to be useful with gram-positive bacteria (23). The ERIC primers, ERIC1R and ERIC2 (24), were based on

the enterobacterial repetitive intergenic consensus (ERIC) sequences in the application named ERIC-PCR. The components of the PCR mixtures and PCR amplification conditions were as described previously (24).

DNA fingerprints generated by ERIC-based oligonucleotide primers with genomic DNA and three dilutions of each of four whole-cell preparations of *E. coli* W3110 and *C. diversus* 4514 are shown in Fig. 1. Samples whose fingerprints are shown in Fig. 1 were not preheated at 80°C prior to PCR (this preheating step was not necessary for these enteric bacteria). The whole-cell ERIC-PCR-derived fingerprints had identical band positions and were as distinct as the purified genomic DNA-derived fingerprints. Lower-molecular-mass products were more prominent with the genomic DNA substrate of *E. coli* W3110. The 1.0 M NaCl wash of the whole-cell preparations improved the clarity of DNA fingerprints obtained from *E. coli* W3110. The addition of the NaCl wash step did not affect the appearance of the *C. diversus* whole-cell fingerprints. This distinction may result from differences in cell wall-associated macromolecules (4) between *E. coli* W3110 and *C. diversus* 4514. Fingerprints of suspensions with A_{600} values of 1.5 had bands which were more prominent than those obtained with suspensions with A_{600} values of 0.5. On the basis of the results obtained from

FIG. 2. Comparison of purified genomic-DNA-based (lanes A) and whole-cell-based (lanes B) fingerprints generated by REP-PCR with different bacterial species. Whole cell-based fingerprints (lanes B) were obtained with cells cultivated on agar plates. The REP1R-Dt (24) plus REP2-Dt (23) primer pair was used with *P. aeruginosa*, *S. aureus*, and *S. pneumoniae*. The REP1R-I plus REP2-I primer pair (24) was used with the remainder of the isolates. No template DNA was added to the negative (neg.) control lanes. The DNA molecular size marker was a 1-kb ladder (Bethesda Research Laboratories).

these pilot experiments, subsequent reactions included a 1.0 M NaCl wash of whole-cell preparations with a cell density (A_{600}) of 1.5.

Comparisons of REP-PCR-generated DNA fingerprints obtained with either purified genomic DNA or washed whole-cell samples of selected isolates of gram-negative and gram-positive bacteria are shown in Fig. 2. To ensure uniformity, all samples in Fig. 2 were preheated at 80°C prior to REP-PCR. The REP1R-I and REP2-I primers were used with the enteric bacteria (Fig. 2). The REP1R-Dt and REP2-Dt primers were found to yield more complex DNA fingerprints with nonenteric bacteria such as *P. aeruginosa*, *S. aureus*, and *S. pneumoniae* (23) (Fig. 2). REP-PCR-based fingerprints obtained with paired genomic DNA and with whole-cell samples have similar band patterns (Fig. 2). Whole-cell-based fingerprints were as distinct as genomic-DNA-based fingerprints, with the exception of the *Salmonella* fingerprints, which had brighter, or more intensely staining, backgrounds when whole cells were used. Interestingly, these *Salmonella* isolates required sonication for effective cell lysis, and therefore, this more intensely staining background may represent sheared genomic DNA. Whole-cell preparations of *Salmonella* strains without sonication yielded only partial DNA fingerprints compared with genomic-DNA-based fingerprints. When bands were present in both paired samples, band positions in the whole-cell fingerprints were the same as those obtained in paired genomic-DNA fingerprints. Whole-cell-based rep-PCR fingerprints with quality similar to that of fingerprints generated from genomic DNA also were obtained with *B. subtilis* (25),

FIG. 3. Whole-cell-based DNA fingerprints of a mock outbreak of seven biotype e isolates of *C. diversus* generated by REP-PCR and ERIC-PCR. No template DNA was added to the negative (neg.) control lane. The DNA molecular size marker was a 1-kb ladder (Bethesda Research Laboratories).

Citrobacter freundii, *Enterobacter sakazakii*, and *N. meningitidis* (data not shown).

Occasional differences in the presence or absence of bands may be due to the presence of inhibitory cell surface polysaccharides in whole-cell samples (4), high-molecular-weight nonspecific PCR products (2), and nuclease-mediated DNA degradation. Since all differences in presence or absence of bands occur close to the upper limit of *Taq* polymerase extension, a size limit (e.g., approximately 3 kb [30]) which eliminates artifactual inconsistencies between matched whole-cell- and genomic-DNA-based fingerprints can be arbitrarily assigned. Differences in fingerprint band intensities were at least partly attributable to differences in DNA quantities among whole-cell and genomic-DNA preparations added to the PCR mixtures.

In order to examine the utility of these whole-cell rep-PCR methods in epidemiologic analysis, seven *C. diversus* isolates were selected from a bank of isolates of this species (12, 30) to simulate conditions of an apparent outbreak. Previously, *C. diversus* isolates from different geographical regions with identical biotypes were distinguished by rep-PCR (30). The seven *C. diversus* isolates in the present study were all biotype e, but three patterns could be distinguished by MEE (12, 13) and rep-PCR (30). Five isolates (4514, 4515, 4516, 4574, and 4580) have the same electrophoretic type as determined by MEE, were recovered originally from the same geographic region, and have highly similar rep-PCR fingerprints with purified genomic-DNA preparations (30). Three of the five isolates were originally isolated from a single outbreak (14). The other two isolates (4513 and 4583) are unrelated to these five and to each other as determined by epidemiology, MEE, and genomic-DNA-based rep-PCR fingerprints (12, 13, 30).

Whole-cell rep-PCR fingerprints of these selected *C. diversus* isolates generated with both REP- and ERIC-based primers are shown in Fig. 3. These samples were not

preheated at 80°C prior to PCR. The quality, in terms of number and intensity of bands, within fingerprints generated by each primer set is similar to the quality observed previously with genomic-DNA preparations (30). The five isolates known to be related demonstrated highly similar fingerprints which were distinct from the fingerprints of the other two isolates. The conspicuous absence of an approximately 3,200-bp band from the REP-PCR-based fingerprint of isolate 4516 is a stable and previously observed phenomenon within the range of accepted dissimilarity in rep-PCR fingerprints among clonally related isolates (30).

Fingerprinting by rep-PCR is a useful tool for DNA-based epidemiologic assessment of clonal relationships among bacterial isolates (8, 23, 30). The results of this study show that whole-cell preparations of bacterial isolates from culture plates or broth cultures can be used to generate fingerprints with quality similar to that of fingerprints obtained with purified preparations of genomic DNA. *S. pneumoniae* isolates yielded more reproducible fingerprints when the colonies were allowed to remain on agar plates for 2 days at room temperature. Spectrophotometric quantitation of cell mass in whole-cell samples allowed sufficient standardization of DNA quantities in rep-PCRs for the generation of comparable DNA fingerprints.

Ribotyping involves restriction endonuclease digestion, Southern blotting, and autoradiography of hybridized ribosomal DNA (10) and requires several days. Pulsed-field gel electrophoresis requires microbial cultivation, rare restriction enzymes, tedious cell preparation in agarose-embedded plugs, and lengthy electrophoretic separations. Restriction endonuclease digestion of chromosomal DNA with conventional agarose gel electrophoresis requires microbial cultivation and often generates complex fingerprint patterns which are difficult to interpret. Plasmid profiling requires microbial cultivation, and some bacterial species have a significant percentage of nontypeable isolates which lack plasmids (27). Also, isolates of the same strain may have different plasmid profiles (9). MEE has been used to assess the clonality of bacterial populations (21) but requires microbial cultivation and specialized reagents, and although it has been used for many years, it has been limited to research laboratories. Arbitrary-priming PCR (28), or randomly amplified polymorphic DNA (1), empirically examines random oligonucleotide primer sets to establish species- and strain-specific DNA fingerprints, but it may require the initial synthesis of many individual primers (1, 28). PCR-based typing schemes such as rep-PCR may enable the characterization of isolates directly from samples in several hours without microbial cultivation. When one isolate of a given bacterial species has yielded rep-PCR-generated DNA fingerprints of significant complexity, all isolates of such a species have yielded rep-PCR-generated DNA fingerprints (3, 8, 23, 24, 25, 30). In the species examined to date, 100% of isolates are typeable by rep-PCR.

REP- and ERIC-based primers have been used for evaluating isolates of *B. subtilis* (25), *C. diversus* (30), *Enterobacter aerogenes* (8), *Rhizobium meliloti* (3), *S. aureus* (22), and *S. pneumoniae* (23). Primers based on repetitive DNA elements in *Neisseria* strains appear to be useful for evaluation of gonococcal and meningococcal isolates (26). A highly repeated DNA element also has been isolated from *Mycobacterium tuberculosis* (20). Other interspersed repetitive elements have been described or will likely be discovered, and these will enable the generation of DNA fingerprints of other gram-positive organisms, nonenteric gram-negative bacteria, and fungi (15). rep-PCR-based DNA

fingerprinting with intact cells and multiple repetitive sequence primers should enable rapid epidemiological analysis of many microorganisms directly from clinical samples. rep-PCR may enable epidemiological typing for fastidious or difficult-to-culture organisms. Rapid typing methodologies will facilitate the prompt and sensitive evaluation of disease outbreaks (5, 18) so that effective preventive measures can be instituted if common sources are identified.

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