JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 1995, p. 3304–3307 Vol. 33, No. 12
0095-1137/95/$04.00+0

Comparison of Different PCR Approaches for Characterization of *Burkholderia* (*Pseudomonas*) *cepacia* Isolates

PETER YUK-FONG LIU,* ZHI-YUAN SHI, YEU-JUN LAU, BOR-SHEN HU, JAINN-MING SHYR, WEN-SHIH TSAI, YU-HUI LIN, AND CHING-YU TSENG

Section of Infectious Diseases, Taichung Veterans General Hospital, Taichung, Taiwan, Republic of China

Received 24 July 1995/Returned for modification 6 September 1995/Accepted 21 September 1995

In this study, we evaluated three PCR methods for epidemiological typing of *Burkholderia* (*Pseudomonas*) *cepacia*—PCR-ribotyping, arbitrarily primed PCR (AP-PCR) and enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR)—and compared them with pulsed-field gel electrophoresis. The analysis was performed with 31 isolates of *B. cepacia*, comprising 23 epidemiologically unrelated isolates and 8 isolates collected from the same patient during two episodes of bacteremia. Pulsed-field gel electrophoresis, ERIC-PCR, and AP-PCR identified 23 distinct types among the 23 unrelated isolates, while PCR-ribotyping only identified 12 strain types, even after *Alu*I digestion of the amplification products. Among the eight isolates collected from the same patient, all typing techniques revealed two clones of strains. The day-to-day reproducibilities of PCR-ribotyping and ERIC-PCR were good, while greater day-to-day variations were noted in the fingerprints obtained by AP-PCR. We conclude that all three PCR techniques are useful for rapid epidemiological typing of *B. cepacia*, but ERIC-PCR seems to be more reproducible and discriminative.

Burkholderia (*Pseudomonas*) *cepacia* is increasingly recognized as an important pathogen in nosocomial infections (2, 7, 8, 14, 17, 18), but epidemiologic investigation of epidemic and endemic *B. cepacia* infections has been limited by the lack of a sensitive and specific typing system that allows determination of isolate relatedness. Conventional methods for strain identification have relied on the analyses of phenotypic characteristics, which may not be stably expressed (16). Recently, approaches at the molecular level have been used to assess the relatedness of bacterial isolates. Analysis of the restriction fragment length polymorphism of total DNA by pulsed-field gel electrophoresis (PFGE) and analysis by ribotyping, though highly discriminative and reproducible for typing *B. cepacia* (1, 16, 19), have labor-intensive and skill-demanding natures that provide an obstacle to their wide use in clinical microbiology laboratories. To circumvent these problems, Kostman and coworkers (4, 10) used a PCR technique in conjunction with ribotyping to amplify the 16S-23S intergenic spacer region of the bacterial rRNA operon. They found that this technique was as discriminative as conventional ribotyping but much faster. Bingen et al. (3) used an arbitrarily primed PCR (AP-PCR) technique for an epidemiological investigation of 23 *B. cepacia* isolates obtained from 11 cystic fibrosis patients. The discriminatory power of this AP-PCR was found to be equivalent to that of ribotyping but less than that of the PFGE method. However, Johnson et al. (9) argue that this AP-PCR technique lacks reproducibility and is not sufficiently reliable to evaluate the clonal diversity of *B. cepacia*. Recently, we have reported the use of an enterobacterial repetitive intergenic consensus sequence-based PCR (ERIC-PCR) technique (13) in classifying the relatedness of *B. cepacia* isolates obtained from bacteremic patients. Using this technique, we have successfully documented a case of reinfection with different strains of *B. cepacia*. Our preliminary data also show that the discriminatory power of this ERIC-PCR is comparable to that of ribotyping.

In the present study, three PCR typing methods—PCR-ribotyping, AP-PCR, and ERIC-PCR—were evaluated. These techniques were also compared with the well-established PFGE technique for the analysis of epidemiologically unrelated isolates of *B. cepacia.*

MATERIALS AND METHODS

Bacterial isolates. Thirty-one clinical isolates of *B. cepacia* were included in this study. All of these isolates were collected between 1993 and 1995 at Taichung Veterans General Hospital. The first group consisted of 23 isolates which were recovered from patients with documented nosocomial infections, and the isolates were completely epidemiologically unrelated. The second group of isolates were recovered from patient A during two episodes of *B. cepacia* bacteremia (each episode produced four isolates) (13). Previous characterization of these isolates by ribotyping demonstrated that the strains isolated from these two episodes of infections were distinct (13). Isolates were identified as *B. cepacia* with the Vitek AutoMicrobic system (Vitek AMS; BioMerieux Vitek, Inc., Hazelwood, Mo.) and their identities were confirmed with the API 20NE system (API-BioMerieux, La Balme les Grottes, France). All isolates were maintained at −70°C in Trypticase soy broth with 10% glycerol until further analysis.

PFGE. Genomic DNA was prepared as described previously (12). Chromosomal DNA plugs were incubated with *Spe*I (GIBCO-BRL, Life Technologies, Gaithersburg, Md.). Restriction fragments were separated by PFGE through 1.2% SeaKem GTG agarose (FMC Bioproducts, Rockland, Maine) with a CHEF-DRII apparatus (Bio-Rad Laboratories, Richmond, Calif.) at a field strength of 6 V/cm for 24 h at 14°C, with the pulse time being increased from 5 to 40 s. A lambda ladder (Bio-Rad Laboratories) was used as the molecular weight marker. PFGE chromosomal fingerprints were compared according to the criteria of Prevost et al. (15).

PCR typing. Total *B. cepacia* DNA was prepared by guanidinium thiocyanate extraction as previously described (11). Primers used were ERIC1 (5′-GTGAA TCCCCAGGAGCTTACAT-3′) (11), AP-PCR primer (5′-TCACGATGCA-3′) (3), PCR-ribotyping primer 1 (5′-TTGTACACACCGCCCGTCA-3′), and PCR-ribotyping primer 2 (5′-GGTACCTTAGATGTTTCAGTTC-3′ (10). Amplification reactions were performed in a 100-μl final volume with 1 U of *Taq* polymerase (Super Taq; HT Biotechnology Ltd., Cambridge, England), 10 mM Tris (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 250 μM (each) deoxynucleoside triphosphates, and 1 μM each primer. Approximately 50 ng of DNA template was used in each amplification. Amplification was performed in a PHC-3 thermal cycler (Techne, Princeton, N.J.). For ERIC-PCR, the samples were amplified as follows: 95°C for 5 min to denature template; four low-stringency cycles of 94°C for 1 min, 26°C for 1 min, and 72°C for 2 min; 40 cycles of 94°C for 30 s, 40°C for 30 s, and 72°C for 1 min; and finally 72°C for 10 min. For AP-PCR, the temperature was ramped as follows: 95°C for 5 min to denature template; four low-stringency cycles of 94°C for 1 min, 26°C for 1 min, and 72°C

* Corresponding author. Mailing address: Section of Infectious Diseases, Taichung Veterans General Hospital, 160 Taichung Harbour Rd., Section 3, Taichung, Taiwan, Republic of China. Phone and fax: 886-4-4611523.

TABLE 1. Molecular profiles of 23 epidemiologically unrelated *B. cepacia* isolates generated by PFGE and different PCR fingerprintings

Isolate	PFGE profile[a]	ERIC-PCR fingerprint	AP-PCR fingerprint	PCR ribotype[b]
S1	A	A	A	A
S2	B	B	B	B
S3	C	C	C	C
S4	D	D	D	B
S5	E	E	E	B
S6	F	F	F	B
S7	G	G	G	B
S8	H	H	H	D
S9	I	I	I	E
S10	J	J	J	F
S11	K	K	K	B
S12	L	L	L	B
S13	M	M	M	G
S14	N	N	N	F
S15	O	O	O	F
S16	P	P	P	H
S17	Q	Q	Q	I
S18	R	R	R	J
S19	S	S	S	K
S20	T	T	T	E
S21	U	U	U	H
S22	V	V	V	L
S23	W	W	W	F

[a] PFGE profiles were determined after digestion by *Spe*I.
[b] PCR ribotypes were determined after *Alu*I digestion of the amplification products.

for 2 min; 40 cycles of 94°C for 30 s, 36°C for 1 min, and 72°C for 1 min; and finally 72°C for 10 min. For PCR-ribotyping, an initial denaturing step of 95°C for 5 min was followed by 30 cycles of denaturing 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 10 min during the last cycle. A negative control was run with each experiment. Amplified products (10 μl for ERIC-PCR and AP-PCR, 20 μl for PCR-ribotyping) were analyzed by electrophoresis in a 2.0% agarose gel containing ethidium bromide (1 μg/ml) at 40 V for 6 h and were detected by UV transillumination. The PCR patterns were considered identical on the basis of similar numbers and matching positions of all major bands. Small differences in the intensities of major bands or loss of faint bands was ignored. In order to confirm the banding patterns of PCR-ribotyping, digestion of amplification products by the restrictive enzyme *Alu*I was performed as described previously (5). Analysis of restriction fragments was performed by electrophoresis in 4% agarose gels. The day-to-day reproducibilities of the above-mentioned PCR typing techniques were examined by comparing patterns obtained on three different days.

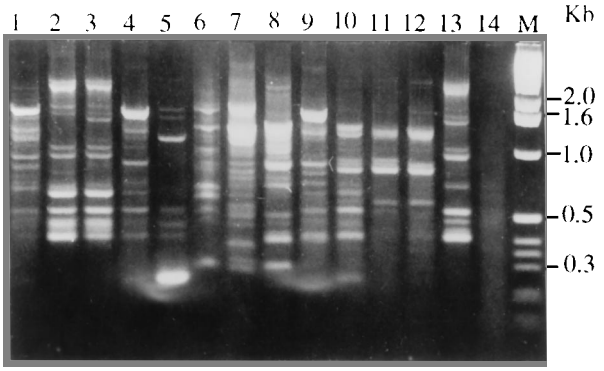

FIG. 1. ERIC-PCR products of epidemiologically unrelated *B. cepacia* isolates analyzed by 2% agarose gel electrophoresis. Lanes 1 to 13, products of isolates S11 to S23, respectively; lane 14, negative control; lane M, 1-kb molecular size marker (GIBCO-BRL).

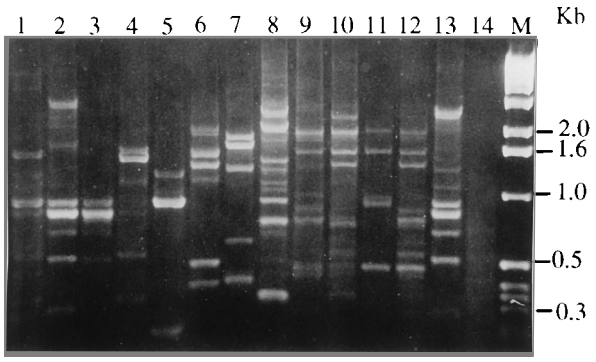

FIG. 2. AP-PCR products of epidemiologically unrelated *B. cepacia* isolates analyzed by 2% agarose gel electrophoresis. Lanes 1 to 13, products of isolates S11 to S23, respectively; lane 14, negative control; lane M, 1-kb molecular size marker (GIBCO-BRL).

RESULTS AND DISCUSSION

Table 1 shows the results of PFGE and different PCR-based fingerprintings of the 23 epidemiologically unrelated isolates of *B. cepacia*. Twenty-three distinct strain types were identified by PFGE, ERIC-PCR, or AP-PCR fingerprinting. Some representative profiles and patterns are illustrated in Fig. 1 and 2. For ERIC-PCR, the banding patterns between isolates S12 and S13 and between isolates S21 and S22, though distinguishable, showed only small differences (Fig. 1). In contrast, AP-PCR generated banding patterns with greater variations (Fig. 2). On the other hand, PCR-ribotyping only distinguished 12 strain types among the 23 isolates, even after *Alu*I digestion of the amplification products (Table 1). Some representative profiles of PCR-ribotyping and restriction patterns of the amplification products are shown in Fig. 3. The banding patterns generated by PCR-ribotyping also were difficult to interpret without running them on the same gel. Among the eight isolates collected from patient A, two distinct clones of strains which were each suspected of causing two episodes of *B. cepacia* bacteremia were successfully identified by PFGE (not

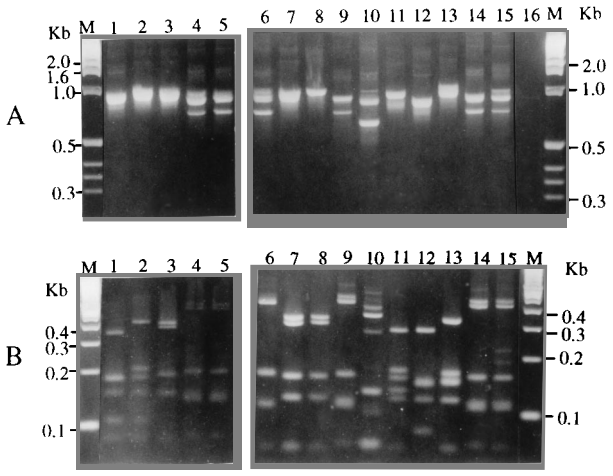

FIG. 3. PCR-ribotyping of epidemiologically unrelated *B. cepacia* isolates. (A) PCR products were analyzed by 2% agarose gel electrophoresis. Lanes 1 to 15, products of isolates S8 to S22, respectively; lane 14, negative control; lane M, 1-kb molecular size marker (GIBCO-BRL). (B) *Alu*I restriction patterns of the amplification products (electrophoresis in a 4% agarose gel). Lanes 1 to 15, digests of PCR products generated from isolates S8 to S22, respectively; lane M, 100-bp-ladder molecular size marker (GIBCO-BRL).

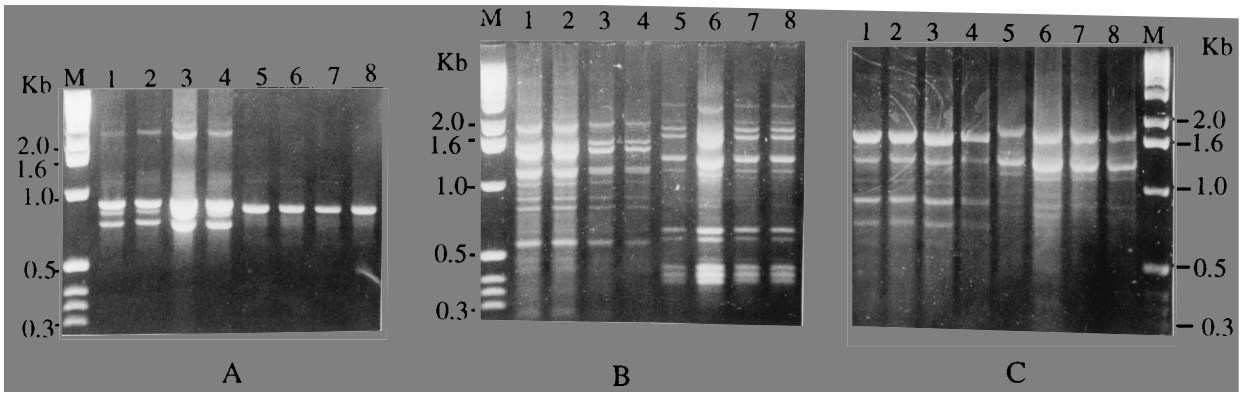

FIG. 4. DNA fingerprints of repeated isolates of *B. cepacia* obtained from patient A during recurrent bacteremia. (A) PCR-ribotyping products. (B) ERIC-PCR products. (C) AP-PCR products. Lanes for all panels: 1 to 4, products of four isolates collected from the first episode of bacteremia; 5 to 8, products of four isolates collected from the second episode of bacteremia; M, 1-kb molecular size marker (GIBCO-BRL).

shown) and all PCR-based typing techniques (Fig. 4). The stabilities of these techniques were also confirmed by the identical patterns produced when the isolates had undergone multiple passages.

The day-to-day reproducibilities of the PCRs were examined by comparing patterns amplified on three different days (not shown). Good reproducibilities were obtained with PCR-ribotyping and ERIC-PCR. Though sometimes the major bands produced were less intense and the minor bands were difficult to visualize, their overall position and whether they were present or absent were highly consistent. The reproducibility of AP-PCR was also acceptable, though greater day-to-day variations and sometimes even loss of major bands were noted. The reproducibility of AP-PCR is vulnerable to subtle changes in annealing temperature, template and primer concentrations, and Mg^{2+} concentration (6). VanCouwenberghe et al. (20) suggested running reactions in triplicate in order to reduce the possibility of missing similar strains. In contrast, PCR-ribotyping and ERIC-PCR are less affected by changes in the abovementioned PCR conditions, though differences in template concentration may affect the intensities of appearing bands. Both PCR-ribotyping and ERIC-PCR use primers specific for conserved regions of the bacterial genome: either 16S and 23S rRNA genes in PCR-ribotyping (10) or palindromic repeated sequences described for enterobacteria in ERIC-PCR (21). Polymorphisms arose because of the amplification of the heterogenous sequence of the DNA flanking rRNA operons and the 16S-23S spacer region of these operons in PCR-ribotyping or the amplification of variable intergenic regions between the repetitive successive sequences in ERIC-PCR rather than because of the level of mismatch between the primer and the template, as in AP-PCR. The advantage of both PCR-ribotyping and ERIC-PCR is good reproducibility, but the discriminatory powers of these techniques for typing some bacterial species may be less than that of AP-PCR.

It is unquestionable that the PFGE method is the "golden standard" for typing most bacterial species, including *B. cepacia*. Its reproducibility and discriminatory power are excellent (1, 3, 19). Bingen et al. (3) found that the PFGE technique is more discriminative than AP-PCR for typing *B. cepacia* isolates. Our results showed that the discriminatory power of PFGE is equivalent to those of both ERIC-PCR and AP-PCR, while being greater than that of PCR-ribotyping. However, the latter technique is more labor-intensive and skill-demanding than are PCR-based fingerprintings and this hinders its use in most clinical laboratories.

In conclusion, all these PCR-based approaches represent useful tools for the epidemiological typing of nosocomial *B. cepacia* because of their simplicity and speed compared with those of PFGE. ERIC-PCR has the advantages of being more reproducible than AP-PCR and more discriminative than PCR-ribotyping. Its discriminatory power was equivalent to that of PFGE.

ACKNOWLEDGMENTS

The authors thank Meei-Fang Liu for her technical assistance. We also thank Hsin-Jyur Rehn, Meei-Rurng Liu, and Yu-Mei Huang for their help in outbreak investigation.

REFERENCES

1. **Anderson, D. J., J. S. Kuhns, M. L. Vasil, D. N. Gerding, and E. N. Janoff.** 1991. DNA fingerprinting by pulsed-field gel electrophoresis and ribotyping to distinguish *Pseudomonas cepacia* isolates from a nosocomial outbreak. J. Clin. Microbiol. **29:**648–649.
2. **Berkelman, R. L., J. Godley, J. A. Weber, R. L. Anderson, A. M. Lerner, N. J. Petersen, and J. R. Allen.** 1982. *Pseudomonas cepacia* peritonitis associated with contamination of automatic peritoneal dialysis machines. Ann. Intern. Med. **96:**456–458.
3. **Bingen, E. H., M. Weber, J. Derelle, N. Brahimi, N. Y. Lambert-Zechovsky, M. Vidailhet, J. Navarro, and J. Elion.** 1993. Arbitrarily primed polymerase chain reaction as a rapid method to differentiate crossed from independent *Pseudomonas cepacia* infections in cystic fibrosis patients. J. Clin. Microbiol. **31:**2589–2593.
4. **Dasen, S. E., J. J. LiPuma, J. R. Kostman, and T. L. Stull.** 1994. Characterization of PCR-ribotyping for *Burkholderia* (*Pseudomonas*) *cepacia*. J. Clin. Microbiol. **32:**2422–2424.
5. **Dolzani, L., E. Tonin, C. Lagatolla, L. Prandin, and C. Monti-Bragadin.** 1995. Identification of *Acinetobacter* isolates in the *A. calcoaceticus-A. baumannii* complex by restriction analysis of the 16S-23S rRNA intergenic spacer sequences. J. Clin. Microbiol. **33:**1108–1113.
6. **Ellsworth, D. I., K. D. Rittenhouse, and R. L. Honeycutt.** 1993. Artifactual variation in randomly amplified polymorphic DNA banding patterns. BioTechniques **14:**214–217.
7. **Henderson, D. K., R. Baptiste, J. Parrillo, and V. J. Gill.** 1988. Indolent epidemic of *Pseudomonas cepacia* bacteremia and pseudobacteremia in an intensive care unit traced to a contaminated blood gas analyzer. Am. J. Med. **84:**75–81.
8. **Jarvis, W. R., D. Olson, O. Tablan, and W. J. Martone.** 1987. The epidemiology of nosocomial *Pseudomonas cepacia* infections: endemic infections. Eur. J. Epidemiol. **3:**233–236.
9. **Johnson, W. M., S. D. Tyler, and K. R. Rozee.** 1994. Linkage analysis of geographic and clinical clusters in *Pseudomonas cepacia* infections by multilocus enzyme electrophoresis and ribotyping. J. Clin. Microbiol. **32:**924–930.
10. **Kostman, J. R., T. D. Edlind, J. J. LiPuma, and T. L. Stull.** 1992. Molecular epidemiology of *Pseudomonas cepacia* determined by polymerase chain reaction ribotyping. J. Clin. Microbiol. **30:**2084–2087.
11. **Liu, P. Y. F., Y.-J. Lau, B.-S. Hu, J.-M. Shyr, M.-H. Cheung, Z.-Y. Shi, and**

W.-S. Tsai. 1994. Use of PCR to study epidemiology of *Serratia marcescens* isolates in nosocomial infection. J. Clin. Microbiol. **32:**1935–1938.

12. **Liu, P. Y. F., Y.-J. Lau, B.-S. Hu, J. M.-Shyr, Z.-Y. Shi, W.-S. Tsai, Y.-H. Lin, and C.-Y. Tseng.** 1995. Analysis of clonal relationships among isolates of *Shigella sonnei* by different molecular typing methods. J. Clin. Microbiol. **33:**1779–1783.
13. **Liu, P. Y. F., Z. Y. Shi, Y. J. Lau, B. S. Hu, J. M. Shyr, W. S. Tsai, Y. H. Lin, and C. Y. Tseng.** Molecular analysis of *Burkholderia cepacia*: differentiation of relapse or reinfection in a case of recurrent bacteremia. Clin. Infect. Dis., in press.
14. **Martone, W. J., O. C. Tablan, W. R. Jarvis.** 1987. The epidemiology of nosocomial epidemic *Pseudomonas cepacia* infections. Eur. J. Epidemiol. **3:**322–332.
15. **Prevost, G., B. Jaulhac, and Y. Piemont.** 1992. DNA fingerprinting by pulsed-field gel electrophoresis is more effective than ribotyping in distinguishing among methicillin-resistant *Staphylococcus aureus* isolates. J. Clin. Microbiol. **30:**967–973.
16. **Rabkin, C. S., W. R. Jarvis, R. L. Anderson, J. Govan, J. Klinger, J. LiPuma, W. J. Martone, H. Monteil, C. Richard, S. Shigeta, A. Sosa, T. Stull, J. Swenson, and D. Woods.** 1989. *Pseudomonas cepacia* typing systems: collaborative study to assess their potential in epidemiologic investigations. Rev. Infect. Dis. **11:**600–607.
17. **Rutala, W. A., D. J. Weber, C. A. Thomann, J. F. John, S. M. Saviteer, and F. A. Sarubbi.** 1988. An outbreak of *Pseudomonas cepacia* bacteremia associated with a contaminated intra-aortic balloon pump. J. Thorac. Cardiovasc. Surg. **96:**157–161.
18. **Steere, A. C., J. H. Tenney, D. C. Mackel, M. J. Snyder, S. Polakavetz, M. E. Dunne, and R. Dixon.** 1977. *Pseudomonas* species bacteremia caused by contaminated normal human serum albumin. J. Infect. Dis. **135:**729–735.
19. **Steinbach, S., L. S. Sun, R. Z. Jiang, P. Flume, P. Gilligan, T. M. Egan, and R. Goldstein.** 1994. Transmissibility of *Pseudomonas cepacia* infection in clinic patients and lung-transplant recipients with cystic fibrosis. N. Engl. J. Med. **331:**981–987.
20. **VanCouwenberghe, C. J., S. H. Cohen, Y. J. Tang, P. H. Gumerlock, and J. Silva, Jr.** 1995. Genomic fingerprinting of epidemic and endemic strains of *Stenotrophomonas maltophilia* (formerly *Xanthomonas maltophilia*) by arbitrarily primed PCR. J. Clin. Microbiol. **33:**1289–1291.
21. **Versalovic, J., T. Koeuth, and J. R. Lupski.** 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. **19:**6823–6831.