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Analysis of Relationships among Isolates of *Citrobacter diversus* by Using DNA Fingerprints Generated by Repetitive Sequence-Based Primers in the Polymerase Chain Reaction

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Oligonucleotide probes which match consensus sequences of the repetitive extragenic palindromic (REP) element hybridize to genomic DNA of diverse bacterial species. Primers based on the REP sequence generate complex band patterns with genomic DNA in the polymerase chain reaction (PCR), a technique named REP-PCR. We used REP-PCR with genomic DNA to fingerprint 47 isolates of *Citrobacter diversus*. Previously, 37 were assigned electrophoretic types (ETs) by multilocus enzyme electrophoresis and 35 were evaluated by using outer membrane protein profiles. Fingerprints were compared by visual inspection and by similarity coefficients (SimCs) based on the number of common bands versus total bands between two given isolates. DNA fingerprints were highly similar visually for patient pairs and outbreak-related sets. SimCs for these were ≥0.952. Fingerprints of isolates with different ETs generally were distinctive. Among 21 unrelated isolates representing 15 ETs, only 6 of 210 comparisons had SimCs of ≥0.952. REP-PCR rapidly generated DNA fingerprints which were highly similar for epidemiologically linked isolates of *C. diversus* and distinct for previously characterized strains within this species. The ability of this method to discriminate between *C. diversus* isolates with the same biotype was similar to that of multilocus enzyme electrophoresis and outer membrane protein profiles. REP-PCR may be useful in evaluation of apparent outbreaks of this or other bacterial species which possess these extragenic, repetitive elements.

Traditionally, once bacterial isolates from an apparent outbreak have been determined to be the same species, further evaluation for similarity or relatedness has been based on phenotypic properties. While often sufficient, these methods are not always adequately sensitive to distinguish unrelated strains with similar phenotypes among the properties assessed (33). Molecular genetic methods, including plasmid profiles, restriction endonuclease digestion patterns of plasmid and chromosomal DNA, and DNA hybridization, have been useful in evaluation of outbreaks of a variety of bacterial pathogens in recent years (1, 5, 15, 26, 33, 34).

We describe the application of a recently developed method, repetitive element sequence-based polymerase chain reaction (rep-PCR) (31), for epidemiologic analysis. This method generates fingerprints from genomic DNA which identify specific strains within a bacterial species (31, 32). The term rep-PCR refers to the general methodology involving the application of oligonucleotide primers based on families of short, extragenic repetitive sequences. These repetitive DNA sequences are dispersed throughout the prokaryotic chromosome and appear to be conserved among many members of the family *Enterobacteriaceae* as well as other bacterial species (8, 13, 21, 28, 29, 31). Consensus sequence probes matching two repetitive sequence families, the 38-bp repetitive extragenic palindromic (REP) element and the 126-bp enterobacterial repetitive intergenic consensus (ERIC) sequence, hybridize to genomic DNA of enteric bacteria, other related gram-negative bacteria, and several distantly related bacterial species (31). The palindromic portions of these repetitive elements enable the formation of stable stem-loop structures, but their precise functional role is unclear (21).

Outwardly directed primer sets based on REP and ERIC consensus sequences can be used to generate clearly resolvable bands by agarose gel electrophoresis after PCR amplification using template genomic DNA from species which contain these sequences (31). The terms REP-PCR and ERIC-PCR refer to the application of specific repetitive sequences (REP and ERIC, respectively) to establish DNA fingerprints by PCR. Bands represent amplification of DNA between adjacent repetitive elements within the approximately 5-kb limitation of the polymerase extension. Band patterns provide DNA fingerprints which allow distinctions between species (31) and between strains within species (3, 31, 32). Differences in band sizes apparently represent polymorphisms in the distances between repetitive sequence elements in different genomes.

Primers of the same base pair length but based on a randomly assorted repetitive consensus sequence do not generate band patterns or genomic fingerprints (32a). Therefore, generation of rep-PCR fingerprints appears to require that the primers match the specific repetitive sequences and does not represent random priming. Fingerprints are reproducible from multiple colonies from the same plate and from daily samples of serial cultures (32). Samples of the same stock strain of *Bacillus subtilis* used in neonatal screening programs at geographically widespread institutions also yield reproducible, highly similar rep-PCR fingerprints (32).

We performed genomic fingerprinting of 47 *Citrobacter diversus* isolates by REP-PCR. *C. diversus* causes sporadic and epidemic neonatal meningitis which is associated with an unusually high frequency of brain abscess formation (11,

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TABLE 1. Characteristics of *C. diversus* isolates evaluated in this study

Isolate	Source and yr	Clinical source	Biotype[a]	ET by MEE	OMP classification
Fig. 1					
4632	Houston 1986	CSF	d	—[b]	—
4637	Houston 1986	CSF	d	—	—
4635	Houston 1986	Wound	d	—	—
4023	Houston 1984	CSF	d	—	—
4036	Houston 1984	CSF	d	6	F+
4583	Maryland 1986	Stool	e	6	A−
4410	Kentucky 1966	CSF	c	6	F+
4511	Kentucky 1976	CSF	d	7	D+
4512	Kentucky 1976	CSF	d	7	D+
4509	Florida 1979	CSF	d	16	B+
4510	Florida 1979	CSF	d	16	B+
4277	Houston 1985	Respiratory tract	d	16	B−
4278	Houston 1985	Respiratory tract	d	16	B+
4577	Maryland 1986	Stool	d	16	B−
4472	Houston 1986	Blood	a	14	G−
4476	Houston 1986	Blood	a	—	—
4576	Maryland 1985	Stool	a	12	D−
4408	Louisiana 1970	CSF	a	11	B+
4407	Washington, D.C., 1969	CSF	a	11	B+
4578	Maryland 1983	Stool	a	11	F−
5595	St. Louis 1991	CSF	a	—	—
Fig. 2					
5596	Delaware 1991	CSF	e	—	—
4749	Houston 1987	Blood	e	—	—
5043	Houston 1988	CSF	d	—	—
5115	Houston 1989	Blood	e	—	—
4397	Houston 1986	Respiratory tract	e	15	C−
4573	Houston 1986	Blood	e	15	B−
4406	Delaware 1966	CSF	e	15	B+
4574	Maryland 1985	Stool	e	15	B−
4580	Maryland 1985	Stool	e	15	B−
4514	Maryland 1983	CSF	e	15	A+
4515	Maryland 1983	CSF	e	15	—
4516	Maryland 1983	CSF	e	15	A+
4428	Alabama 1984	Vagina	e	15	C−
4429	Alabama 1984	CSF	e	15	C−
2988	Tennessee 1983	CSF	e	15	B−
4432	Tennessee 1985	Urine	f	15	B+
4513	Delaware 1975	CSF	e	10	D−
4405	Kentucky 1972	CSF	c	9	D+
4582	Maryland 1985	Stool	c	9	G−
4579	Maryland 1986	Stool	c	8	G−
4409	Dallas 1972	CSF	e	5	E+
4485	Houston 1986	Superficial abscess	c	4	G−
4581	Maryland 1985	Stool	e	3	—
4310	Houston 1985	Respiratory tract	c	2	G−
4431	Tennessee 1985	Peritoneal fluid	c	2	D−
4575	Maryland 1986	Stool	c	1	G−

[a] Biotypes are based on fermentation pattern of four sugars: dulcitol, rhamnose, sorbose, and sucrose.
[b] —, not determined.

16). *C. diversus* outbreaks have been evaluated with biotypes (6, 9, 10, 20, 25, 34), serotypes (10, 34), and antibiograms (6, 9, 25, 34). These methods, while helpful to identify and track epidemic strains in small outbreaks, correlate poorly with one another when applied on a larger scale (22).

Previously, 35 of these isolates were evaluated by using outer membrane protein (OMP) profiles (18) and 37 were evaluated by multilocus enzyme electrophoresis (MEE) (19). The bank includes four pairs of isolates from the same patients, one set of isolates from a mother-infant pair, and three sets of isolates from outbreaks of meningitis. Distinct geographic regions of the United States are represented with 16 isolates from the Houston, Tex., area during a 5-year period and 17 from the Maryland-Delaware-Washington, D.C., area during a 25-year period. This study demonstrates that genomic fingerprinting by REP-PCR is a rapid, reproducible, and useful method to examine isolates of bacterial species from apparent outbreaks of disease.

MATERIALS AND METHODS

Bacterial isolates. *C. diversus* isolates (Table 1) have been collected in the C. T. Parker Laboratory of Texas Children's Hospital since 1984. Isolates with known OMP or MEE types have been described previously (18, 19). Isolate 5595 was provided by N. Middelkamp, St. Louis, Mo., and 5596 was provided by S. Eppes, Wilmington, Del. Other isolates not previously described were obtained from local Houston

hospitals from 1986 to 1989. The isolates are from diverse clinical sites: cerebrospinal fluid (CSF) (n = 23), stool (n = 10), blood (n = 5), respiratory tract (n = 4), vagina (n = 1), peritoneal fluid (n = 1), urine (n = 1), a wound (n = 1), and an abscess (n = 1).

Related sets of isolates are 4023 and 4036 (CSF cultures from the same infant 5 weeks apart); 4277 and 4278 (tracheal aspirate cultures from the same patient several hours apart on the same day); 4472 and 4476 (blood cultures from the same infant 3 days apart); 4632 and 4637 (CSF cultures from the same infant 10 days apart) and 4635 (wound culture registered the same day as 4637 from a different patient in the same hospital); 4428 and 4429 (maternal vaginal culture and her infant's CSF culture); 4509 and 4510 (CSF cultures from different infants in the same hospital 5 weeks apart) (10); 4511 and 4512 (CSF cultures from different infants in the same location in the same year); and 4514, 4515, and 4516 (CSF cultures from three different infants in the same hospital over a 4-month period) (20).

All isolates were maintained as −70°C frozen glycerol stocks. Biotypes of *C. diversus* isolates not previously reported (17) were assigned according to the scheme of Richard et al. (27).

REP-PCR fingerprints. Genomic DNA samples from isolates were prepared and quantitated as previously described (31). REP-based primer sequences REP1R-I (5′-IIIICGICG ICATCIGGC-3′) and REP2-I (5′-ICGICTTATCIGGCCT AC-3′) were previously described (31). PCR amplifications were performed in an automated thermal cycler (first-generation Perkin-Elmer Cetus DNA thermal cycler) with an initial denaturation (95°C, 7 min) followed by 30 cycles of denaturation (90°C, 30 s), annealing (40°C, 1 min), and extension (65°C, 8 min) with a single final extension (65°C, 16 min). Five-microliter samples of each PCR mixture were electrophoresed directly in 1% agarose gels containing 40 mM Tris acetate–1 mM EDTA buffer and 0.5 μg of ethidium bromide per ml. Gels were photographed with a 60-s exposure to Polaroid type 55 film.

Analysis of band patterns. Sizes of bands generated by electrophoresis of the PCR amplifications were assigned by direct comparison to concurrently run 1-kb DNA ladder standards (BRL Life Technologies, Inc.). The presence or absence of bands within a gel lane was determined by two observers (C.R.W. and J.V.) with one fully blinded to exact gel lane locations of epidemiologically linked isolates.

DNA fingerprints of isolates first were compared for similarity by visual inspection of band patterns. Unlike the SimC analyses below, entire lanes were compared without size ranges by visual inspection. Fingerprints were considered highly similar when all visible bands represented in each isolate had the same apparent migration distance. Variations in intensity and shape did not represent differences. The absence of up to two bands from one isolate when all other visible bands in the fingerprints had matching positions was allowed before isolates were considered different by visual inspection.

Bands in the 900- to 3,200-bp range of selected *C. diversus* isolates were analyzed more rigorously by the method of van Soolingen et al. (30), which determines a coefficient of similarity (SimC) between fingerprints of two isolates as follows:

$$\mathrm{SimC}_{AB} = \sum_{i=1}^{k} (a_i + b_i - [a_i - b_i]) / \sum_{i=1}^{k} (a_i + b_i),$$

where a_i and b_i, the intensities of band i in patterns A and B, are either 0 (not present) or 1 (present) and k is the number of distinct bands present among patterns A and B. This coefficient ranges from 0 to 1.0, where 1.0 represents the identical presence and position of all bands in the two fingerprints being compared.

RESULTS

Evaluation of fingerprints of *C. diversus* isolates from the same patient or outbreak. Isolates epidemiologically linked by recovery from the same patient or outbreak were used as the standard of similarity to assess REP-PCR-based DNA fingerprinting. Isolates of each pair or set have the same biotype. When available for both members of a pair (Table 1), electrophoretic types and OMP profiles are identical within pairs, except for 4277 and 4278, which have OMP profiles of B− and B+, respectively.

For each of the four pairs of isolates from the same patient and the isolates from the one mother-infant pair, band patterns were highly similar on visual inspection (Fig. 1 and 2, bracketed lanes). In several instances one band was absent from the analysis range of the pattern in one member of a pair. In the mother-infant isolate pair (4428 and 4429; Fig. 2), one lane had a smear correlating with two bands in the other member of the pair. Otherwise, the remaining bands were present in both members of each of these five pairs. When differences in band intensity were present in closely related isolates, these generally were evident throughout the fingerprint, suggesting differences in amounts of amplified DNA in the samples. Bands were considered present or absent; differences in intensity were ignored.

Among the three outbreak-related sets, patterns again were highly similar on visual inspection. Among the triplet of 4514, 4515, and 4516 (Fig. 2), the last differed from the other two by the absence of one band in analysis range. The pair of 4511 and 4512 (Fig. 1) differed by the absence of one band from the fingerprint of 4511. The pair of 4509 and 4510 (Fig. 1) showed the most apparent differences among the related strains: two bands greater than 3,000 bp were absent in 4509, and two other bands in the 2,300- to 2,700-bp range also were of low intensity. The remainder of their bands in each fingerprint were in matching positions. ERIC-PCR-based DNA fingerprints of 4509 and 4510 were very similar (data not shown).

SimCs for the related isolates (seven comparisons among pairs and three comparisons among the outbreak triplet) ranged from 0.952 to 1.00 with one exception: the SimC for 4509 and 4510 was 0.889. SimCs of >0.952 represent no more than one band difference between two fingerprints in the analysis range. We considered this degree of similarity between two isolates to be sufficient and necessary to identify them as the same strain, or clone, of *C. diversus*. Analysis of DNA fingerprints generated by ERIC-PCR supported the high degree of similarity among epidemiologically related isolates (data not shown).

Comparison of rep-PCR fingerprints of *C. diversus* isolates with MEE and OMP classifications. Visual inspection of rep-PCR fingerprints of the 47 *C. diversus* isolates suggested 18 different patterns (Fig. 1 and 2). Among the 29 epidemiologically unrelated isolates for which both ET and OMP classifications are known (18, 19), REP-PCR provided a similar degree of discrimination within biotype groups versus MEE and OMP profiles (Table 2). Visual inspection of fingerprints of these 29 isolates delineated 16 REP-PCR

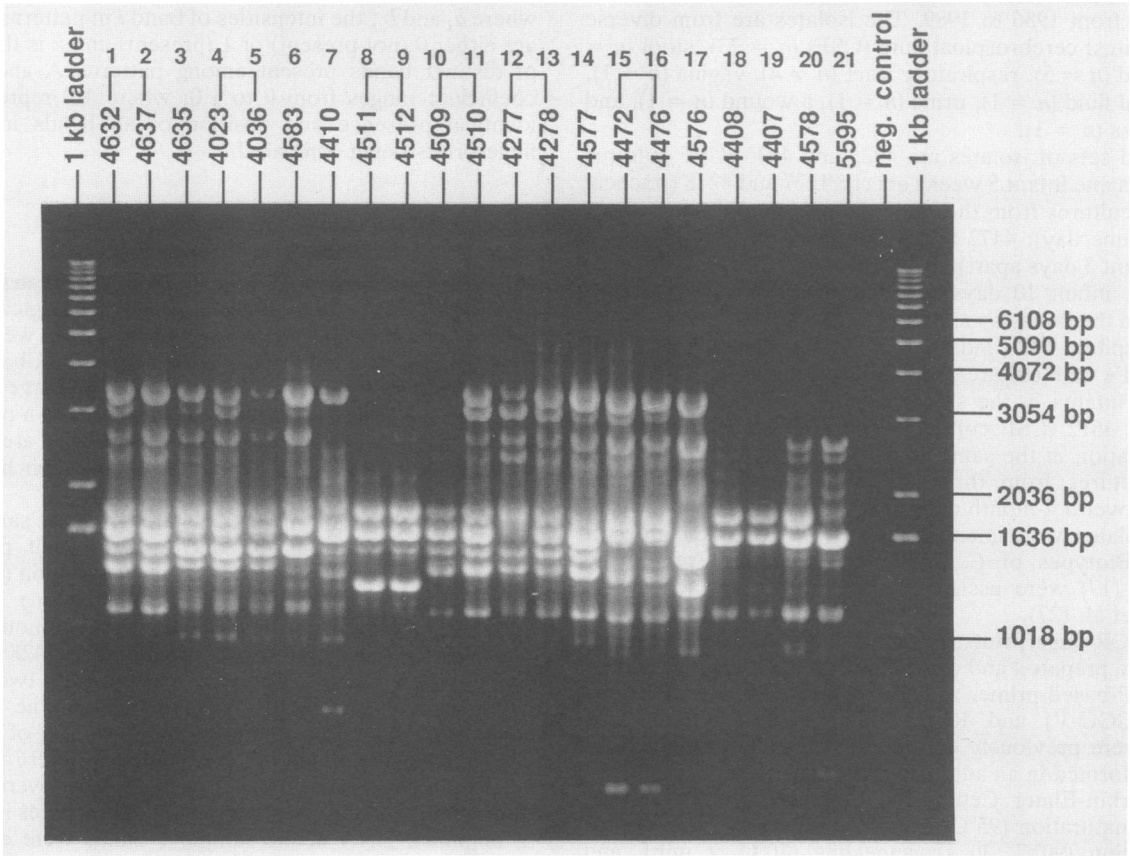

FIG. 1. REP-PCR-based DNA fingerprints of 21 *C. diversus* isolates. Bracketed isolates represent pairs from the same patient (4632 and 4637; 4023 and 4036; 4277 and 4278; 4472 and 4476) or from the same meningitis outbreak (4511 and 4512; 4509 and 4510). Isolates 4036 to 4410 (lanes 5 to 7) are ET 6; isolates 4511 and 4512 are ET 7; isolates 4509 to 4577 (lanes 10 to 14) are ET 16; isolate 4472 is ET 14; isolate 4576 is ET 12; and isolates 4408 to 4578 (lanes 18 to 20) are ET 11.

types. MEE and OMP profile classifications delineated 14 and 11 types, respectively. Each of the three typing methods occasionally discriminated between isolates identified as the same strain by both of the other methods.

REP-PCR types determined by visual inspection correlated directly with ETs with the following exceptions: (i) fingerprints of ETs 1 and 2 were the same, (ii) fingerprints of each of the three ET 6 isolates were different, (iii) fingerprints of ETs 8 and 9 were the same, and (iv) there were three fingerprints among the 12 ET 15 isolates. When more than one ET was represented by a single REP-PCR type, the ETs always had the same biotype but not always the same OMP profile. ETs 1 and 2 and ETs 8 and 9 differ by allelic polymorphism of only 1 of the 20 enzymes used in the MEE analysis (19). REP-PCR corresponded better with MEE than did either method with the OMP profile classification: multiple ETs and REP-PCR types were present in each of the four OMP profile groups with more than two isolates.

Relationships among *C. diversus* isolates with different ETs. *C. diversus* isolates with different ETs by MEE (19) were used as the standard of difference to assess REP-PCR-based fingerprinting within this one species. The results of the visual inspection described above suggested that REP-PCR was able to distinguish between most MEE-defined strains of *C. diversus*. SimCs were determined for the 210 possible comparisons among a 21-isolate subset, which represented the 15 ETs available to us (we did not have an ET 13 isolate in our strain bank). All three ET 6 isolates were included since these had different biotypes and a SimC of <0.950 among themselves. Two unrelated isolates from each of ETs 11, 15, and 16 and both ET 9 isolates were included. Only one of two ET 2 isolates was included since these had highly similar fingerprints on visual inspection and a SimC of 1.0 relative to each other.

Only 6 of 210 relationships had SimCs of ≥0.952 (Table 3). One was between isolates with the same ET. Four others were between isolates with the same biotype. ETs of two of these pairs (4575 and 4431; 4579 and 4582) again are polymorphic at only 1 of 20 enzymes used in the MEE classification (19). This confirmed the visual impression that REP-PCR was able to distinguish most MEE-defined strains of *C. diversus*.

Relationships of *C. diversus* isolates with the same ET. As described above, isolates with the same ET generally had similar DNA fingerprints on visual inspection (Fig. 1 and 2; see Table 1 for ETs of isolates). Among the ET 15 group (Fig. 2), all isolates had similar REP-PCR-based fingerprints except for isolate 4406 (from 1966; the oldest member of the group). The fingerprints of four isolates, 4428, 4429, 2988, and 4432 (from Alabama and Tennessee during a 3-year period), also appeared slightly different relative to the other ET 15 isolates. These had SimCs of ≥0.956 among themselves and had SimCs of ≤0.917 with all other ET 15 isolates except 4574 (Table 4). SimC analysis suggested two strains

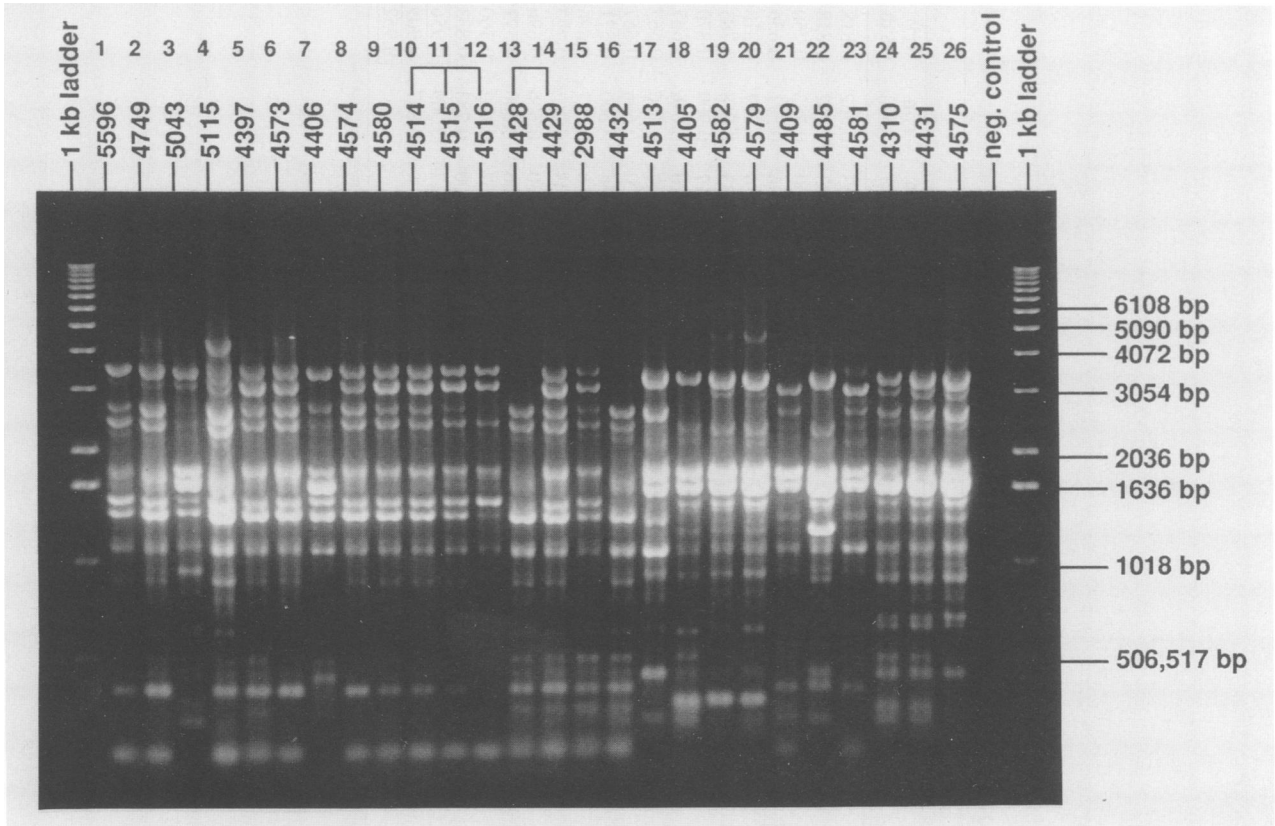

FIG. 2. REP-PCR-based DNA fingerprints of 26 *C. diversus* isolates. Bracketed isolates represent a set from a meningitis outbreak (4514, 4515, and 4516) or a mother-infant pair (4428 and 4429). Isolates 4397 to 4432 (lanes 5 to 16) are ET 15; isolates 4513, 4405, 4582, and 4579 are ETs 10, 9, 9, and 8, respectively; and isolates 4409 to 4575 (lanes 21 to 26) are ETs 5, 4, 3, 2, 2, and 1, respectively.

among the other seven isolates, correlating with a geographic source of Houston or Maryland. Thus, visual inspection suggested three fingerprints, and SimC analysis suggested a fourth strain (Table 4). All of the ET 15 isolates were biotype e except 4432, which was biotype f. Four different OMP profiles were represented by these isolates.

SimCs of the unrelated isolates of ETs 16, 11, 9, and 2 were 0.909 to 0.952, 0.909 to 0.956, 0.909, and 1.00, respectively. These SimCs confirmed the impression from visual inspection that fingerprints within these ETs were similar, although not always to the same degree as those of the epidemiologically linked pairs. The members within each of these ET groups had the same biotype.

The three ET 6 isolates are unrelated epidemiologically and have different biotypes, although two have the same OMP profile (Table 1). Visually, their fingerprints were different (Fig. 2). SimCs among these three isolates failed to reach the level of similarity observed among the epidemiologically related isolates (Table 3).

Analysis of Houston-area isolates and isolates without known ETs or OMP profiles. Four pairs of unrelated Houston isolates with the same biotype had fingerprints which were highly similar visually and had SimCs of ≥0.952. These were 4036 and 4635, 4278 and 4635, 4397 and 4573, and 4749 and 5115. Fingerprints of biotype d isolates 4632 and 4635, which were obtained from different patients in the same hospital 10 days apart, were slightly different visually and had a SimC of 0.917. This comparison suggested that these temporally related isolates were not the same strain and arose from different epidemiologic sources.

Isolate 5595, a biotype a strain (unknown ET) from St. Louis in 1991, had a SimC of 1.0 with isolate 4407, an ET 11 biotype a strain from Washington, D.C., in 1969. The entire fingerprints of these two isolates were highly similar on visual inspection. Isolate 5596, a biotype e strain (unknown ET) from Delaware in 1991, had a REP-PCR-based SimC of

TABLE 2. Ability of REP-PCR versus MEE and OMP profiles to discriminate among *C. diversus* isolates

Biotype group	No. of unrelated isolates[a]	No. of strains identified by:		
		ET by MEE	OMP profile	REP-PCR type[b]
a	5	3	4	3
c	8	6	4	4
d	5	3	4	3
e	11	4	7	6
Total	29	14[c]	11	16

[a] This analysis is based only on isolates for which both ET and OMP profile are known.

[b] REP-PCR types were based on differences in fingerprints apparent on visual inspection.

[c] The ET 3 isolate 4581 was not included because its OMP profile is not known.

TABLE 3. SimCs based on REP-PCR-generated fingerprints of 21 unrelated *C. diversus* isolates representing 15 ETs

Isolate (ET and biotype)	SimC of isolate (ET and biotype)[a]:																		
	4431 (2 c)	4581 (3 e)	4485 (4 c)	4409 (5 e)	4036 (6 d)	4583 (6 e)	4410 (6 c)	4512 (7 d)	4579 (8 c)	4405 (9 c)	4582 (9 c)	4513 (10 e)	4578 (11 a)	4407 (11 a)	4576 (12 a)	4472 (14 a)	4514 (15 e)	4429 (15 e)	4510 (16 d)	4277 (16 d)
4575 (1 c)	1.00	0.667	0.636	0.636	0.609	0.696	0.500	0.583	0.667	0.571	0.696	0.727	0.696	0.667	0.500	0.667	0.696	0.783	0.762	0.818
4431 (2 c)[b]		0.667	0.636	0.636	0.609	0.696	0.500	0.583	0.667	0.571	0.696	0.727	0.696	0.667	0.500	0.667	0.696	0.783	0.762	0.818
4581 (3 e)			0.583	0.917	0.800	0.880	0.692	0.615	0.692	0.609	0.720	0.750	0.720	0.609	0.545	0.769	0.720	0.800	0.783	0.750
4485 (4 c)				0.636	0.696	0.696	0.750	0.750	0.833	0.952	0.870	0.818	0.609	0.667	0.600	0.667	0.609	0.696	0.571	0.636
4409 (5 e)					0.870	0.956	0.750	0.667	0.750	0.667	0.783	0.818	0.696	0.571	0.600	0.833	0.783	0.870	0.857	0.818
4036 (6 d)						0.917	0.880	0.800	0.720	0.727	0.750	0.870	0.750	0.636	0.667	0.720	0.833	0.833	0.818	0.783
4583 (6 e)							0.800	0.720	0.800	0.727	0.833	0.870	0.750	0.636	0.667	0.800	0.833	0.917	0.909	0.870
4410 (6 c)								0.846	0.769	0.783	0.800	0.750	0.640	0.522	0.636	0.692	0.720	0.720	0.696	0.667
4512 (7 d)									0.692	0.783	0.720	0.750	0.560	0.522	0.727	0.692	0.640	0.640	0.696	0.667
4579 (8 c)										0.870	0.960	0.833	0.560	0.522	0.454	0.692	0.640	0.720	0.696	0.667
4405 (9 c)											0.909	0.857	0.545	0.600	0.526	0.609	0.545	0.636	0.600	0.571
4582 (9 c)												0.857	0.583	0.545	0.454	0.720	0.667	0.750	0.727	0.696
4513 (10 e)													0.696	0.667	0.600	0.667	0.696	0.783	0.762	0.727
4578 (11 a)[c]														0.909	0.667	0.640	0.750	0.750	0.636	0.696
4407 (11 a)															0.632	0.522	0.636	0.636	0.500	0.571
4576 (12 a)																0.636	0.667	0.667	0.632	0.700
4472 (14 a)																	0.720	0.800	0.783	0.833
4514 (15 e)[d]																		0.917	0.909	0.956
4429 (15 e)																			0.857	0.909
4510 (16 d)[e]																				0.952

[a] SimCs greater than or equal to 0.952 are boxed.
[b] The one other ET 2 isolate gave identical SimCs.
[c] The least similar of three ET 11 isolates were used in this comparison.
[d] Two ET 15 isolates representing the two major subgroups of ET 15 were used in this comparison.
[e] Two of the three unrelated ET 16 isolates were used in this comparison.

TABLE 4. REP-PCR-based SimCs among *C. diversus* isolates with ET 15[a]

Strain	SimC of[b]:										
	4573	4406	4574	4580	4514	4515	4516	4428	4429	2988	4432
4397	1.00	0.750	0.960	0.917	0.917	0.917	0.870	0.917	0.917	0.917	0.870
4573		0.750	0.960	0.917	0.917	0.917	0.870	0.917	0.917	0.917	0.870
4406			0.720	0.667	0.667	0.667	0.696	0.750	0.750	0.750	0.696
4574				0.960	0.960	0.960	0.917	0.960	0.960	0.960	0.917
4580					1.00	1.00	0.956	0.917	0.917	0.917	0.870
4514						1.00	0.956	0.917	0.917	0.917	0.870
4515							0.956	0.917	0.917	0.917	0.870
4516								0.956	0.956	0.956	0.909
4428									0.956	1.00	0.956
4429										1.00	0.956
2988											0.956

[a] Visual inspection suggested three fingerprints: 4406, 4428 to 4432, and the others. SimC evaluation suggests that 4573 and 4397 may represent a fourth strain in this group.

[b] Double boxes indicate closely related groups of strains; single boxes indicate groups of strains with overlapping similarities.

1.0 with 4516, an ET 15 biotype e isolate from the same geographic area in 1983.

DISCUSSION

C. diversus isolates which are closely linked epidemiologically and have the same ET and OMP profiles had fingerprints generated by REP-PCR which appeared highly similar by visual inspection. This visual impression was confirmed by quantitative analysis using SimCs. Conversely, isolates of *C. diversus* with different ETs generally had REP-PCR-based fingerprints which differed significantly by visual inspection and by analysis using SimCs. REP-PCR provided a degree of discrimination among *C. diversus* isolates with the same biotype similar to that provided by MEE (19) and OMP profiles (18). REP-PCR types correlated well with ETs with few exceptions.

Highly similar fingerprints from *C. diversus* isolates obtained from distinct geographic regions and separated in time by more than 20 years (5595 and 4407) suggest that the locations of the REP elements within the genome can be very stable over time. A similar observation with respect to the stability of REP-PCR fingerprints over time has been made with *B. subtilis* strains used in newborn screening (32). DNA fingerprints based on a repetitive sequence from *Mycobacterium tuberculosis*, IS*986*, were identical in strains passaged for 6 months in culture and in a single patient over a period of 1 year, indicating the stability of repetitive DNA sequence-based patterns (30).

Fingerprints of unrelated *C. diversus* isolates with the same ET and same biotype were very similar in all cases, although often less so than fingerprints of the epidemiologically linked pairs. Most of the highly similar pairs of isolates were recovered from the same geographic area within a span of weeks to months. Isolates from different geographic areas with the same ET which have SimCs from 0.870 to 0.950 may represent the gradual divergence of progeny of the parent clone.

Biotypes, while simple to determine for this species, provide too little diversity for use as an independent system for epidemiologic assessment of *C. diversus* isolates (18). Outbreak-related isolates express the same biotype (18), but the differences in the REP-PCR fingerprints of isolates 4632 and 4635 from Houston support previous observations (9) that isolates of the same biotype may be recovered during a short time period in the same institution yet represent different strains.

Plasmid profiles have been useful in four outbreaks with small numbers of isolates (6, 9, 20, 34). Plasmid profiles may change within a given strain over time (9, 34) and do not correlate well with biotypes of serotypes or *C. diversus* (22). Different strains from the same locale with different biotypes also may have the same plasmid profile (9). Chromosomal restriction endonuclease digestion may correlate with serotype but appears to be too cumbersome for use with a large number of *C. diversus* isolates (22).

Epidemiologically related *C. diversus* isolates usually have identical profiles among a group of seven OMPs used in the classification scheme of Kline et al. (18). In our experience detection of the three minor OMPs used in this scheme is variable, which may affect reproducibility of this method. An example of this may be the respiratory isolates 4277 and 4278, which were recovered from the same patient on the same day and have OMP profiles of B− and B+, respectively. The difference between these OMP profiles is the presence of the 32-kDa minor OMP which is associated with isolates recovered from the central nervous system (17).

MEE showed that *C. diversus* exists as a polyclonal population with at least 16 distinct strains (19). MEE confirmed the clonal relatedness of the sets of epidemiologically linked isolates used in this study to which it was applied. MEE is a powerful research tool but because of its complexity is not likely to become widely available for study of local outbreaks of bacterial disease in a timely manner.

An ideal method of subtyping bacterial isolates from a given species would be simple, rapid, sensitive, and highly discriminatory (4, 24). These features rarely are encountered in a single method. The DNA-based fingerprints generated by REP-PCR, especially as PCR technology improves and sample preparation becomes simpler, may be able to fulfill these tenets. PCR-based techniques also forgo the necessity for cultivation which may be crucial with fastidious pathogens and obligate intracellular bacteria.

Ribotyping also appears useful to distinguish strains within a species (14, 23, 35) and shows greater discriminatory power among isolates of the same species than MEE when compared directly (2, 35), although the epidemiologic relevance of this observation is unknown. Ribotyping requires restriction endonuclease digestion, Southern blotting, and hybridization of labeled ribosomal DNA (12), which may

make this method less amenable than rep-PCR for routine clinical use in epidemiologic evaluations.

Biotyping remains an appropriate first step for evaluation of apparent outbreaks of *C. diversus* with the caveat that different strains with the same biotype may exist concurrently within the same locale. REP-PCR appears to be a useful adjunct in terms of its ability to discriminate among unrelated isolates with the same biotype while demonstrating a high degree of similarity between strains which are epidemiologically linked. REP-PCR may be an appropriate next level of analysis to evaluate relatedness of *C. diversus* isolates when a series of isolates with the same biotype is encountered. ERIC-PCR has also been useful for distinguishing strains of *C. diversus* (data not shown). REP- and ERIC-PCR methods which use whole cells directly from broth cultures or colonies on plates allow discrimination of *C. diversus* isolates within several hours (data not shown).

The quantitative analysis using SimC was undertaken to provide a more objective analysis than visual inspection alone. These results confirmed that a high degree of visual similarity of the DNA fingerprints of two isolates generated by REP-PCR, especially when these have the same biotype, is sufficient to consider them the same strain. The visual distinctions of fingerprints of different strains also were confirmed by SimC. Therefore, visual inspection alone, without the more rigorous mathematical comparisons, may be adequate for routine epidemiologic use of rep-PCR, at least for *C. diversus*.

rep-PCR, based on REP and ERIC sequences, has been applied successfully to assess relatedness of isolates within the species *B. subtilis* (32), *Rhizobium meliloti* (3), and *Enterobacter aerogenes* (7). To date, these evaluations have involved a limited number of isolates or lacked a comparative standard. Results of this study with *C. diversus* isolates add further evidence that rep-PCR may be broadly applicable to fingerprinting bacterial genomes and for epidemiologic evaluations of outbreaks of bacterial species which possess these extragenic repetitive elements. This method should be especially useful among members of the *Enterobacteriaceae* as both ERIC- and REP-based oligonucleotide primers are able to generate sufficiently complex DNA band patterns from most genera of this family (31).

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REFERENCES

1. **Blake, P. A., D. T. Allegra, J. D. Snyder, T. J. Barrett, L. McFarland, C. T. Caraway, J. C. Feeley, J. P. Craig, J. V. Lee, N. D. Puhr, and R. A. Feldman.** 1980. Cholera—a possible endemic focus in the United States. N. Engl. J. Med. **302:**305–309.
2. **Brenner, D. J., L. W. Mayer, G. M. Carlone, L. H. Harrison, W. F. Bibb, M. C. de Cunto Brandileone, F. O. Sottnek, K. Irino, M. W. Reeves, J. M. Swenson, K. A. Birkness, R. S. Weyant, S. F. Berkley, T. C. Woods, A. G. Steigerwalt, P. A. D. Grimont, R. M. McKinney, D. W. Fleming, L. L. Gheesling, R. C. Cooksey, R. J. Arko, C. V. Broome, and the Brazilian Purpuric Fever Study Group.** 1988. Biochemical, genetic, and epidemiologic characterization of *Haemophilus influenzae* biogroup aegyptius (*Haemophilus aegyptius*) strains associated with Brazilian purpuric fever. J. Clin. Microbiol. **26:**1524–1534.
3. **de Bruijn, F. J.** 1992. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergenic consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl. Environ. Microbiol. **58:**2180–2187.
4. **Falkiner, F. R.** 1988. Epidemiological typing: a user's view. J. Hosp. Infect. **11:**303–309.
5. **Farrar, W. E.** 1983. Molecular analysis of plasmids in epidemiologic investigations. J. Infect. Dis. **148:**1–6.
6. **Finn, A., G. H. Talbot, E. Anday, M. Skros, M. Provencher, and C. Hoegg.** 1988. Vertical transmission of *Citrobacter diversus* from mother to infant. Pediatr. Infect. Dis. J. **7:**293–294.
7. **Georghiou, P., J. Versalovic, and J. R. Lupski.** Unpublished data.
8. **Gilson, E., J.-M. Clement, D. Brutlag, and M. Hofnung.** 1984. A family of dispersed repetitive extragenic palindromic DNA sequences in *E. coli*. EMBO J. **3:**1417–1421.
9. **Goering, R. V., N. J. Ehrenkranz, C. C. Sanders, and W. E. Sanders.** 1992. Long term epidemiological analysis of *Citrobacter diversus* in a neonatal intensive care unit. Pediatr. Infect. Dis. J. **11:**99–104.
10. **Graham, D. R., R. L. Anderson, F. E. Ariel, N. J. Ehrenkranz, B. Rowe, H. R. Boer, and R. E. Dixon.** 1981. Epidemic nosocomial meningitis due to *Citrobacter diversus* in neonates. J. Infect. Dis. **144:**203–209.
11. **Graham, D. R., and J. D. Band.** 1981. *Citrobacter diversus* brain abscess and meningitis in neonates. JAMA **245:**1923–1925.
12. **Grimont, F., and P. A. D. Grimont.** 1986. Ribosomal ribonucleic acid gene restriction patterns as potential taxonomic tools. Ann. Inst. Pasteur/Microbiol. **137B:**165–175.
13. **Hulton, C. S. J., C. F. Higgins, and P. M. Sharp.** 1991. ERIC sequences: a novel family of repetitive elements in the genomes of *Escherichia coli*, *Salmonella typhimurium* and other enterobacteria. Mol. Microbiol. **5:**825–834.
14. **Irino, K., F. Grimont, I. Casin, P. A. D. Grimont, and the Brazilian Purpuric Fever Study Group.** 1988. rRNA gene restriction patterns of *Haemophilus influenzae* biogroup aegyptius strains associated with Brazilian purpuric fever. J. Clin. Microbiol. **26:**1535–1538.
15. **John, J. F., and J. A. Twitty.** 1986. Plasmids as epidemiologic markers in nosocomial gram-negative bacilli: experience at a university and review of the literature. Rev. Infect. Dis. **8:**693–704.
16. **Kline, M. W.** 1988. *Citrobacter* meningitis and brain abscess in infancy: epidemiology, pathogenesis and treatment. J. Pediatr. **113:**430–434.
17. **Kline, M. W., E. O. Mason, and S. L. Kaplan.** 1988. Characterization of *Citrobacter diversus* strains causing neonatal meningitis. J. Infect. Dis. **157:**101–105.
18. **Kline, M. W., E. O. Mason, and S. L. Kaplan.** 1989. Epidemiologic marker system for *Citrobacter diversus* using outer membrane protein profiles. J. Clin. Microbiol. **27:**1793–1796.
19. **Li, J., J. M. Musser, P. Beltran, M. W. Kline, and R. K. Selander.** 1990. Genotypic heterogeneity of strains of *Citrobacter diversus* expressing a 32-kilodalton outer membrane protein associated with neonatal meningitis. J. Clin. Microbiol. **28:**1760–1765.
20. **Lin, F. C., W. F. Devoe, C. Morrison, J. Libonati, P. Powers, R. J. Gross, B. Rowe, E. Israel, and J. G. Morris.** 1987. Outbreak of neonatal *Citrobacter diversus* meningitis in a suburban hospital. Pediatr. Infect. Dis. J. **6:**50–55.
21. **Lupski, J. R., and G. M. Weinstock.** 1992. Short, interspersed repetitive DNA sequences in prokaryotic genomes. J. Bacteriol. **174:**4525–4529.
22. **Morris, J. G., F. C. Lin, C. B. Morrison, R. J. Gross, R. Khabbaz, K. O. Maher, B. Rowe, E. Israel, and J. P. Libonati.** 1986. Molecular epidemiology of neonatal meningitis due to *Citrobacter diversus*: a study of isolates from hospitals in Maryland. J. Infect. Dis. **154:**409–414.

23. **Owen, R. J., A. Beck, P. A. Dayal, and C. Dawson.** 1988. Detection of genomic variation in *Providencia stuartii* clinical isolates by analysis of DNA restriction fragment length polymorphisms containing rRNA cistrons. J. Clin. Microbiol. **26:** 2161–2166.
24. **Parker, M. T.** 1978. Hospital-acquired infections: guidelines to laboratory methods, European series no. 4, p. 35–39. World Health Organization Regional Publications, Copenhagen.
25. **Parry, M. F., J. H. Hutchinson, N. A. Brown, C.-H. Wu, and L. Estreller.** 1980. Gram-negative sepsis in neonates: a nursery outbreak due to hand carriage of *Citrobacter diversus*. Pediatrics **65:**1105–1109.
26. **Penner, J. L., J. N. Hennessy, S. D. Mills, and W. C. Bradbury.** 1983. Application of serotyping and chromosomal restriction endonuclease digest analysis in investigating a laboratory-acquired case of *Campylobacter jejuni* enteritis. J. Clin. Microbiol. **18:**1427–1428.
27. **Richard, C., B. Brisou, and J. Lioult.** 1972. Etude taxonomique de "Levinea" nouveau genre de la famille des enterobacteries. Ann. Inst. Pastuer **122:**1137–1146.
28. **Sharples, G. J., and R. G. Lloyd.** 1990. A novel repeated DNA sequence located in the intergenic regions of bacterial chromosomes. Nucleic Acids Res. **18:**6503–6508.
29. **Stern, M. J., G. F. L. Ames, N. H. Smith, E. C. Robinson, and C. F. Higgins.** 1984. Repetitive extragenic palindromic sequences: a major component of the bacterial genome. Cell **37:**1015–1026.
30. **van Soolingen, D., P. W. M. Hermans, P. E. W. de Haas, D. R. Soll, and J. D. A. van Embden.** 1991. Occurrence and stability of insertion sequences in *Mycobacterium tuberculosis* complex strains: evaluation of an insertion sequence-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. J. Clin. Microbiol. **29:**2578–2586.
31. **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.
32. **Versalovic, J., T. Koeuth, Y.-H. Zhang, E. R. B. McCabe, and J. R. Lupski.** 1992. Quality control for bacterial inhibition assays: DNA fingerprinting of microorganisms by rep-PCR. Screening **1:**175–183.
32a. **Versalovic, J., and J. R. Lupski.** Unpublished data.
33. **Wachsmuth, K.** 1986. Molecular epidemiology of bacterial infections: examples of methodology and of investigations of outbreaks. Rev. Infect. Dis. **8:**682–692.
34. **Williams, W. W., J. Mariano, M. Spurrier, H. D. Donnell, R. L. Breckenridge, R. L. Anderson, I. K. Wachsmuth, C. Thornsberry, D. R. Graham, D. W. Thibeault, and J. R. Allen.** 1984. Nosocomial meningitis due to *Citrobacter diversus* in neonates: new aspects of the epidemiology. J. Infect. Dis. **150:**229–235.
35. **Woods, T. C., L. O. Helsel, B. Swaminathan, W. F. Bibb, R. W. Pinner, B. G. Gellin, S. F. Collin, S. H. Waterman, M. W. Reeves, D. J. Brenner, and C. V. Broome.** 1992. Characterization of *Neisseria meningitidis* serogroup C by multilocus enzyme electrophoresis and ribosomal DNA restriction profiles (ribotyping). J. Clin. Microbiol. **30:**132–137.