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Genotypic Analysis of *Burkholderia cepacia* Isolates from 13 French Cystic Fibrosis Centers

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***Burkholderia cepacia* has been involved in outbreaks of pulmonary infection among patients with cystic fibrosis (CF), and the spread of a highly transmissible clone has been reported throughout the United Kingdom and Canada. These data prompted a DNA-based typing study of the strains recovered in French CF centers. Ninety-five isolates recovered from 71 patients attending 13 CF centers in 9 regions of France were characterized by randomly amplified polymorphic DNA (RAPD) analysis and pulsed-field gel electrophoresis (PFGE). Twenty-one genotypes were identified among the 95 isolates, and the results of RAPD and PFGE were concordant for 89 isolates (94%). Cross-colonization was demonstrated in 7 of the 13 CF centers. The investigation of serial isolates showed that most chronically colonized patients harbored a single *B. cepacia* strain. A geographically clustered distribution of *B. cepacia* genotypes was observed, except for one genotype, which was detected in four regions but was proven to be different from the genotype of the British-Canadian highly transmissible strain. The present study confirms the ability of *B. cepacia* to spread among CF communities in France and the importance of epidemiological surveys in the institution of prevention policies.**

Pulmonary colonization with *Burkholderia cepacia* (formerly *Pseudomonas cepacia*), a bacterium which was first described as a phytopathogen, is associated with poor clinical prognosis in some patients with cystic fibrosis (CF) (14, 19, 38). Several outbreaks have been reported in North America (15, 21) and Great Britain (12, 24, 26, 33, 34). Person-to-person transmission was corroborated by genomic analysis of isolates in these outbreaks. Furthermore, the spread of a highly transmissible lineage throughout the United Kingdom and Ontario (Canada) has been demonstrated (12, 15, 26). In France, two successive epidemiological studies, conducted in 1993 and 1994, showed that the annual prevalence of *B. cepacia* in patients with CF is approximately 2% and that cases are unevenly distributed among CF centers (31).

Several typing methods have been used to determine the genetic relatedness of *B. cepacia* isolates. Pulsed-field gel electrophoresis (PFGE) of restricted DNA (1, 3, 33, 35) is usually considered as the reference method, owing to its high discriminatory power. Ribotyping (1, 21, 27, 33, 35) and less-labor-intensive PCR methods, including randomly amplified polymorphic DNA (RAPD) typing (3, 22, 23), length heterogeneity analysis of the 16S-23S intergenic spacer region of the rRNA operon (PCR ribotyping) (8, 17), and enterobacterial repetitive intergenic consensus sequence PCR (7, 22), have also been successfully applied to *B. cepacia*. In the present collaborative study, 95 isolates obtained from 71 patients attending 13 CF centers were analyzed by RAPD and PFGE in order to (i) compare the typing abilities of these techniques, (ii) detect epidemic situations, (iii) investigate whether chronic pulmonary colonization of a given patient involves one or several strains, and (iv) assess the geographical distribution of genotypes in France. The two typing techniques were found concordant for 89 isolates (94%) and demonstrated the frequent occurrence of cross-infections. Most patients harbored genetically similar strains throughout the period of observation. Finally, with the exception of one ubiquitous genotype, all strain types were region or center specific, and no genomic relatedness was shown between the French strains and the British-Canadian transmissible lineage.

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MATERIALS AND METHODS

Bacterial strains. Ninety-five French clinical *B. cepacia* isolates were included in the study (Table 1). They were recovered from the sputa of 71 CF patients attending 13 French care centers, located in nine regions, from April 1988 to April 1995. These isolates were provided by O. Bajolet-Laudinat, Reims; G. Berthelot, Dieppe; E. Bingen, Paris; J. Carrère, Giens; G. Chabanon, Toulouse; C. De Champs, Clermont-Ferrand; P. Honderlick, Suresnes; G. Paul, Paris; D. Tande, Brest; J. Texier-Maugein, Pessac; J. Thubert, Roscoff; H. Vu Thien, Paris; and M. Weber, Nancy. The first isolate of the index case of the British-Canadian outbreak, i.e., strain CF5610 (12), kindly provided by J. Govan (Edinburgh, United Kingdom), and the type strain of the species, ATCC 25416, were also analyzed.

Isolates were confirmed to be strains of *B. cepacia* by standard biochemical procedures (API 20NE; bioMérieux, Marcy l'Etoile, France). Same-day isolates exhibiting different colonial morphologies were further compared by determination of their antibiotic susceptibility patterns on Mueller-Hinton agar plates (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France).

RAPD analysis. RAPD analysis was performed as previously described (3), using the primer 5′-AGTCAGCCAC-3′, in a mixture of 100 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, 4 mM $MgCl_2$, 0.4 mM each deoxynucleoside triphosphate, 3 μM each primer, 2.5 U of *Taq* DNA polymerase (Appligene, Gaithersburg, Md.), and 50 ng of bacterial DNA in a final volume of 50 μl. Amplification products were electrophoresed in a 2% agarose gel.

Isolates which differed by two or more major bands were considered sufficiently divergent to warrant separate strain designations. Profiles differing by only one major band or by one or two faint bands were considered to be subtypes of a common strain.

Reproducibility of the random PCR patterns was checked by submitting two bacterial DNAs to 10 independent PCR runs.

PFGE. Genomic DNA from the isolates was prepared according to the method of Godard et al. (10). Briefly, 10^9 exponentially growing cells of *B. cepacia* were chilled on ice, harvested by centrifugation, washed twice in 10 mM Tris-HCl (pH 7.6)–1 M NaCl, and incubated at 42°C. An equal volume of molten

TABLE 1. Genotypic analysis of 95 French *B. cepacia* isolates recovered from 71 patients in 13 CF centers: comparison of results of RAPD and PFGE

Region	CF center	Patient	Isolate[a]	Date of collection[b]	RAPD fingerprint	PFGE profile	Genotype
Aquitaine	I	1	1.1	05/03/94	R1	P1	1
	I	1	1.2	10/94	R2	P1	21
	I	2	2.1	11/07/95	R3	P2i	3
Auvergne	II	3	3.1	07/07/93	R4a	P3a	2
	II	3	3.2	20/07/93	R4a	P3a	2
	II	4	4.1	14/09/93	R4b	P3a	2
	II	4	4.2	04/10/93	R4b	P3c	2
	II	5	5.1	15/10/93	R4a	P3b	2
	II	5	5.2	26/11/93	R4a	P3a	2
	II	6	6.1	29/10/93	R4a	P3a	2
	II	7	7.1	01/09/94	R4a	P3a	2
Brittany	III	8	8.1	24/11/93	R3f	P2i	3
	III	9	9.1	07/02/94	R3f	P2c	3
	III	10	10.1	15/02/94	R3f	P2a	3
	IV	8	8.2	16/09/94	R3f	P2g	3
	IV	11	11.1	22/07/94	R3f	P2a	3
	IV	12	12.1	29/10/94	R3g	P2g	3
	IV	13	13.1	04/11/94	R3g	P2a	3
	IV	14	14.1	24/10/94	R3g	P2h	3
	IV	15	15.1	21/12/94	R5a	P2a	4
	IV	16	16.1	14/11/94	R5b	P4	5
	IV	17	17.1	17/09/94	R6a	P5	6
	IV	17	17.2	24/10/94	R6b	P5	6
Champagne	V	18	18.1	05/10/94	R7	P6	7
Île-de-France	VI	19	19.1	13/03/95	R8c	P7g	8
	VII	20	20.1	30/05/94	R9	P8	9
	VIII	21	21.1	16/12/93	R8d	P7d	8
	VIII	22	22.1	10/10/94	R3c	P2a	3
	IX	23	23.1	10/01/94	R8b	P7c	8
	IX	24	24.1	26/10/94	R8a	P7a	8
	IX	25	25.1	17/08/94	R8a	P7e	8
	IX	26	26.1	28/11/94	R8a	P7c	8
	IX	27	27.1	19/04/94	R8a	P7c	8
	IX	28	28.1	07/09/94	R8a	P7a	8
	IX	29	29.1	16/11/94	R8a	P7b	8
	IX	30	30.1	24/11/94	R8a	P7d	8
	IX	31	31.1	28/10/94	R8a	P7a	8
	IX	32	32.1	19/10/94	R8a	P7b	8
	IX	33	33.1	04/07/94	R8a	P7f	8
	IX	34	34.1	19/12/94	R8a	P7b	8
	IX	35	35.1	09/11/94	R8a	P7d	8
	IX	36	36.1	08/11/94	R10	P9	10
Lorraine	X	37	37.1	16/08/89	R3g	P2b	3
	X	38	38.1	23/10/92	R3f	P2e	3
	X	39	39.1	19/04/88	R3c	P2a	3
	X	39	39.2	19/12/90	R3a	P2a	3
	X	39	39.3	17/04/91	R3d	P2a	3
	X	39	39.4	27/08/92	R3e	P2c	3
	X	40	40.1	31/08/89	R3a	P2d	3
	X	41	41.1	19/01/90	R3b	P2f	3
	X	42	42.1	08/02/90	R3b	P2d	3
	X	43	43.1	10/93	R3a	P2c	3
	X	44	44.1	20/05/92	R11a	P10a	11
	X	45	45.1	29/10/92	R11a	P10b	11
	X	46	46.1	04/09/92	R11b	P10b	11
	X	47	47.1	10/93	R11a	P10a	11
	X	48	48.1	10/93	R11a	P10b	11
	X	49	49.1	30/10/91	R11a	P10b	11
	X	49	49.2	04/12/92	R11a	P10b	11
	X	49	49.3	16/12/92	R11a	P10b	11
	X	50	50.1	08/07/92	R12	P11	12
South-Pyrenees	XI	51	51.1a	13/04/94	R13a	P12a	13
	XI	51	51.1b	13/04/94	R13a	P12a	13
	XI	51	51.2	29/06/94	R13e	P12a	13
	XI	51	51.3	21/09/94	R13c	P13c	20
	XI	51	51.4a	06/01/95	R13b	P13a	20
	XI	51	51.4b	06/01/95	R13d	P13b	20
	XI	52	52.1	15/07/94	R13b	P12b	13
	XI	52	52.2	13/02/95	R13b	P12b	13
	XI	53	53.1	03/10/94	R14	P14	14
	XI	53	53.2	28/10/94	R14	P14	14
	XI	53	53.3	07/11/94	R14	P14	14
	XI	53	53.4a	28/12/94	R14	P14	14
	XI	53	53.4b	28/12/94	R14	P14	14
Normandy	XII	54	54.1	28/07/94	R15	P15	15
Provence-Alps-Côte d'Azur	XIII	55	55.1	10/94	R16a	P16a	16
	XIII	56	56.1	11/05/94	R16b	P16b	16
	XIII	57	57.1	28/07/93	R16a	P16a	16
	XIII	58	58.1	23/08/93	R16a	P16b	16
	XIII	59	59.1	04/92	R16a	P16a	16
	XIII	59	59.2	17/06/93	R16a	P16a	16
	XIII	60	60.1	10/93	R16a	P16b	16
	XIII	61	61.1	11/93	R16a	P16b	16
	XIII	62	62.1	18/05/94	R16b	P16b	16
	XIII	63	63.1	17/06/94	R16a	P16a	16
	XIII	64	64.1	06/94	R16a	P16a	16
	XIII	65	65.1	20/09/94	R16b	P16b	16
	XIII	66	66.1a	27/02/95	R16a	P16b	16
	XIII	66	66.1b	27/02/95	R16a	P16b	16
	XIII	67	67.1	17/02/94	R16a	P16a	16
	XIII	68	68.1	13/04/95	R16a	P16a	16
	XIII	69	69.1	08/06/94	R17	P17	17
	XIII	70	70.1	17/02/93	R18	P18	18
	XIII	71	71.1a	24/11/94	R19	P19	19
	XIII	71	71.1b	24/11/94	R19	P19	19

[a] Serial isolates from the same patient are indicated by arabic numbers, and phenotypically different isolates from the same sputum sample are indicated by lowercase letters.

[b] Dates are day/month/year or month/year.

1% agarose (FMC Bioproducts, Rockland, Maine) was introduced. The cells in agarose blocks were lysed with 1% sodium lauryl sarcosine and 1 mg of proteinase K per ml for 48 h at 55°C. After several washes, the DNA was digested with the enzyme *Spe*I (Gibco BRL).

PFGE with a contour-clamped homogeneous electric field (CHEF) was performed in a CHEF-DR II system (Bio-Rad, Richmond, Calif.). Gels were run at 150 V, with 20-s pulses for 12 h and then with 5- to 15-s ramping for 17 h at 10°C, and stained with 0.1% ethidium bromide.

Restriction patterns were compared according to the guidelines published by Tenover et al. (39). *Sma*I-restricted DNA from *Staphylococcus aureus* NCTC 8325 was used as a standard ladder for intergel comparison, and isolates with banding patterns differing by less than four bands were considered to belong to the same genotype. Those with minor differences from a common DNA profile were classified as subtypes.

The reproducibility of the technique was confirmed by examining the stability of banding patterns for 20 strains repeatedly subcultured on Mueller-Hinton agar plates.

Data analysis. Reproducibility tests did not show any significant change in the RAPD and PFGE banding patterns of repeatedly studied isolates. The following nomenclature was used to designate the DNA profiles obtained by each method (Table 1): the letters R and P were used to identify RAPD and PFGE profiles, respectively, while arabic numbers and lowercase letters were used to identify types and subtypes, respectively. The results of the two DNA typing methods were combined to define a genotype for each isolate. Isolates were considered to be genetically related if they exhibited the same genotype and genetically divergent if the two methods yielded different patterns. Interpretation of other cases will be discussed elsewhere.

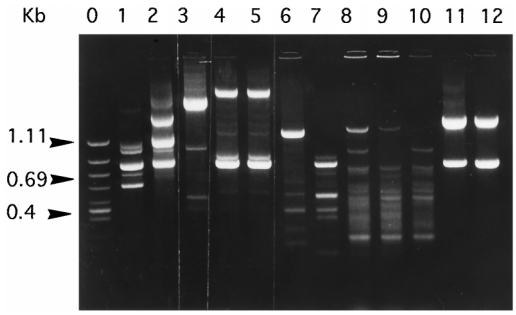

FIG. 1. Typing of some representative *B. cepacia* isolates by RAPD. Lanes: 1, strain ATCC 25416; 2 to 12, French clinical isolates 51.1a, 69.1, 71.1a, 71.1b, 70.1, 23.1, 37.1, 39.1, 40.1, 45.1, and 48.1, respectively; 0, molecular size markers.

RESULTS

Typing results. Nineteen RAPD types and 19 PFGE types, all different from the ATCC 25416 type strain profiles, could be distinguished among the 95 French isolates, which were classified into 21 genotypes after combination of the two methods (Table 1). Figures 1 and 2 illustrate the fingerprints obtained by the two typing methods for the type strain ATCC 25416 and 11 clinical isolates. RAPD and PFGE results were in complete agreement for 89 isolates (94%). Discrepancies were observed for six isolates (6%) and concerned the comparison of DNA fingerprints either between two isolates recovered from two patients within the same center (patients 15 and 16, center IV) or between several isolates from the same patients (patient 1, center I; patient 51, center XI).

Comparison of isolates within each CF center. In 4 of the 13 centers (V, VI, VII, and XII), a single patient was colonized by *B. cepacia*, and in 2 centers (I and VIII), two patients were colonized with genetically different strains. In the other seven centers, where 3 to 17 patients were colonized with *B. cepacia*, identical genotypes were detected in several patients and therefore were considered outbreak types. Six genotypes (genotypes 2, 3, 8, 11, 13, and 16) were involved in cross-colonizations. In six CF centers (II, III, IV, IX, XI, and XIII), a single outbreak type was identified, involving 2 to 14 patients, while in center X, two distinct genotypes were recovered in 7 and 6 patients, respectively.

Comparison of multiple isolates from the same patient. Thirty-one serial isolates recovered from 12 patients during periods ranging from 2 weeks to 52 months were investigated. Ten of the 12 patients harbored genetically indistinguishable isolates throughout the study. In contrast, for the two remaining patients, genotypic differences between *B. cepacia* isolates were demonstrated, once by RAPD alone (patient 1) and once by PFGE alone (patient 51). Furthermore, for four patients (patients 51, 53, 66, and 71), we investigated the genotypic relatedness of simultaneous isolates exhibiting differences in colonial morphology and biochemical features or antibiotic susceptibility. These phenotypically different isolates demonstrated the same genotype.

Geographical distribution of genotypes. Genotypes were center specific, except for genotype 3, which was traced in five CF centers located in four different regions (Brittany, Lorraine, Aquitaine, and Île-de-France), and genotype 8, which was traced in three CF centers located in Île-de-France. Two patient transfers within the same region were documented: patient 8, who harbored a genotype 3 strain, attended both centers III and IV (Brittany), and patient 19, who harbored a genotype 8 strain, was transferred from center IX to center VI (Île-de-France). No other epidemiological relationship between centers was documented. Finally, the French isolates studied were all genetically different from the British epidemic strain, CF5610.

DISCUSSION

B. cepacia is recognized as an important pathogen in CF, owing to the occurrence in approximately 20% of infected patients of a marked acceleration of the clinical course and to the transmissibility of this organism within CF communities. Therefore, accurate diagnosis of *B. cepacia* colonization and epidemiological typing of isolates are essential to the adoption of preventive measures.

The difficulties of an unequivocal identification of *B. cepacia* need to be emphasized. There is increasing evidence that organisms identified as *B. cepacia* by standard laboratory proce-

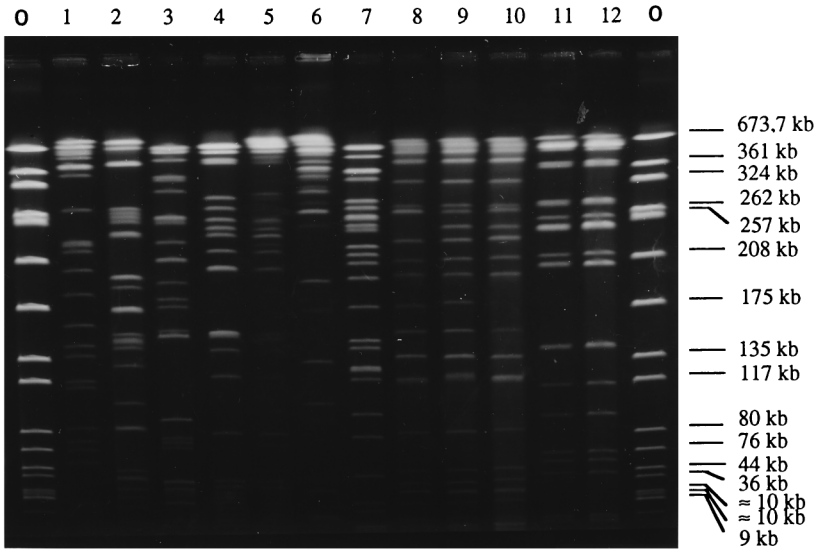

FIG. 2. Typing of some representative *B. cepacia* isolates by PFGE. Lanes: 1, strain ATCC 25416; lanes 2 to 12, French clinical isolates 51.1a, 69.1, 71.1a, 71.1b, 70.1, 23.1, 37.1, 39.1, 40.1, 45.1, and 48.1, respectively; 0, molecular size markers.

dures, such as the API 20NE system, are heterogeneous and may comprise different genomovars of *B. cepacia* and even other *Burkholderia* species, such as the closely related *B. gladioli*, whose clinical significance in CF patients is unclear (6, 13, 16, 32). The phylogenetic study that we conducted on clinical isolates of gram-negative nonfermenters recovered from CF patients, involving PCR-restriction fragment analysis of 16S ribosomal DNA (30), showed that presumed *B. cepacia* isolates, including those analyzed in the present study, belonged to two major subgroups and allowed easy discrimination of *B. gladioli* isolates.

Many comparative studies of typing methods for clinical isolates of *B. cepacia* have been previously published, differing with regard to (i) the source of the isolates studied, i.e., recovered either from nosocomial infections, usually with a known common source of contamination (1, 8, 22, 25), or from CF colonizations with an unclear mode of acquisition (3, 12, 17, 26, 33, 35); and (ii) the technical conditions and the criteria for analyzing DNA fingerprints. Nevertheless, the PFGE procedures for *B. cepacia* typing are similar from one study to another, since restriction is usually performed with *Spe*I (1, 3, 22, 26, 35), although the use of *Xba*I has also been reported (25, 26), and electrophoresis is usually performed with a CHEF pulsed-field system. Guidelines for interpreting restriction patterns produced by PFGE were recently published (39), which is an important step toward standardization. PFGE is generally considered as a reference typing method for *B. cepacia*, because it is highly reproducible and more discriminative than ribotyping (26, 33, 35); however, PFGE is both labor-intensive and time-consuming, which restricts its use in clinical microbiology laboratories. Therefore, PCR techniques that require smaller amounts of DNA, lack purification steps, and involve much less time have been developed. RAPD fingerprinting for *B. cepacia* typing was already assessed versus ribotyping, PFGE, and other PCR techniques (3, 22, 23). In these studies, the annealing temperature used (36°C) was the same, but other parameters known to affect the reproducibility and/or the discriminatory power of RAPD, especially the nature and concentration of the primers and the $MgCl_2$ concentration (23, 41), differed. The reproducibility of RAPD patterns was found to be insufficient in the study performed by Liu et al. (22), whereas it proved satisfactory in the study performed by Mahenthiralingam et al. (23) and in the present study. It has been emphasized that the optimization of the primer-to-template ratio is essential for good reproducibility of this technique (23). In contrast, all studies show that RAPD analysis exhibits a resolving power similar to that of PFGE. Consensus interpretation rules for RAPD-produced patterns are difficult to elaborate, given the lack of interlaboratory standardization. Therefore, Struelens et al. (36) recommend the use of such techniques only in laboratories experienced in both typing and PCR.

A previous French epidemiological survey showed that the prevalence rates of *B. cepacia* colonization varied from 0 to 40% within CF centers (31). These data strongly suggested the possibility of epidemic situations in some centers, as previously described in North American (21) and British (12, 24, 33, 34) CF centers. The present DNA-based study clearly demonstrates the occurrence of cross-contamination in 7 of the 13 French CF centers investigated. Six different genotypes (genotypes 2, 3, 8, 11, 13, and 16) were found to be involved in French outbreaks, and two of them (genotypes 8 and 16) were observed in a large number of patients (13 and 14 patients, respectively).

The observation that, in the same center, some genotypes remain unique to single patients while other genotypes infect several patients suggests that some strains have a higher degree of transmissibility. However, genotype 3 and 8 strains, which were shown to be transmissible in some centers, did not spread in other centers, and similar data were reported for the British epidemic strain (26). This may be due to uneven efficiency of hygiene policies among CF centers or particular host susceptibilities as well as to different bacterial characteristics, since gene expression may be regulated by the numerous insertion sequences identified within the *B. cepacia* genome (2, 5, 28, 42). The clone involved in epidemics in Toronto and Edinburgh CF centers has been extensively studied with the aim of finding genetic markers associated with transmissibility which could provide rational arguments for the segregation of patients. Two markers have been identified to date: (i) the *cblA* gene, which encodes a pilin that binds to CF mucin and thus is likely to promote transmission in the CF population (11, 29, 37); and (ii) the peculiar association of two insertion sequences, IS*402* and IS*1356* (40). However, other transmissible lineages were shown to lack the *cblA* gene, and the identification of more genes involved in pathogenicity is obviously required.

Genotyping of serially recovered isolates showed that most patients are chronically colonized with a single strain, as reported in previous studies (3, 8, 18, 20). When differences were observed between serial isolates, they involved only one of the two techniques. Thus, it is not clear whether these observations indicate that these patients harbored two different strains successively or that genomic variations of the colonizing strain, whose detection varies according to the technique, occurred in the course of time. Therefore, Johnson et al. (15), emphasizing the difficulty of interpreting variations in PFGE patterns and ribotypes of serial isolates from the same patients, suggest the use of multilocus enzyme electrophoresis as an alternative to DNA-based techniques for the characterization of *B. cepacia* isolates.

This study also allowed the assessment of the distribution of strains throughout France. Strain genomic diversity is similar to that observed in the United Kingdom, where more than 50 ribotypes were identified from 178 CF patients attending 17 CF centers in Great Britain and Ireland (26).

In most cases, the spread of the genotypes detected in several patients is limited to one center or one region. Person-to-person transmission within a single CF center, or via patients moving from one center to another within the same region, is highly probable. However, possible acquisition of *B. cepacia* from environmental sources also has to be considered, as the distribution of genotypes among clinical isolates may reflect the distribution of *B. cepacia* in local natural environments. Fisher et al. (9) showed that in Philadelphia the same ribotype predominated among clinical isolates recovered from children with CF and among environmental isolates obtained from homes, salad bars, and food stores. They concluded that in some cases, patients may acquire *B. cepacia* from environmental sources. However, Butler et al. (4) did not find any relationship between PFGE profiles of British clinical isolates and natural environmental isolates recovered from soil, water, or vegetation. Further studies are therefore required to determine the sources and modes of transmission of *B. cepacia* in order to set up prevention strategies.

Strains of genotype 3 were detected in four different regions of France, but no epidemiological link via patient transfer between these regions was evident, although it cannot be absolutely excluded. Thus, this observation may suggest either a high frequency of this type among clinical isolates or the spread of a particularly transmissible strain, as reported by Pitt et al. in the United Kingdom (26), where the strain initially

found in the CF centers of Edinburgh and Manchester (12) was further identified in eight other centers. In the present study, genetic comparison of the major British epidemic strain with French isolates, including genotype 3, seems to indicate that the British strain is not present in France to date.

In conclusion, the present typing study of *B. cepacia* isolates recovered from CF patients attending various CF centers has enabled us to assess the distribution of strain types throughout France and to identify potentially epidemic lineages, which are different from the British-Canadian highly transmissible clone. Given the confirmed ability of *B. cepacia* to spread from one patient to another, a continuous epidemiological survey in CF centers appears necessary. From a practical standpoint, RAPD seems to be a very useful and reliable tool for investigating local outbreaks, owing to its technical simplicity, rapidity, and satisfactory discriminatory power. Nevertheless, PFGE is a more standardized technique that remains a "gold standard" for large-scale surveillance. The combination of two methods, which may differ in sensitivity to genetic rearrangements, allows a finer survey of the spread and evolution of bacterial clones.

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