JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1994, p. 924–930
0095-1137/94/$04.00+0

Vol. 32, No. 4

Linkage Analysis of Geographic and Clinical Clusters in *Pseudomonas cepacia* Infections by Multilocus Enzyme Electrophoresis and Ribotyping

W. M. JOHNSON,[1,2]* S. D. TYLER,[2] AND K. R. ROZEE[1,3]

Departments of Microbiology[1] and Medicine,[3] Victoria General Hospital, Dalhousie University, Halifax, Nova Scotia, and Laboratory Centre for Disease Control, Health Canada, Ottawa, Ontario,[2] Canada

Received 17 November 1993/Returned for modification 5 January 1994/Accepted 17 January 1994

Multilocus enzyme electrophoresis and ribotyping were used to characterize 83 strains of *Pseudomonas cepacia*, mostly isolated from cystic fibrosis (CF) patients, although a number of isolates from non-CF nosocomial infections and reference environmental strains were represented. Twenty enzyme electrophoretic types (ETs) were determined; of these, one clone (ET12) was associated with six of nine ribotypes (RTs) said to be geographically representative of the United Kingdom and all of the Ontario (Canada) isolates from CF patients. This clone was not associated with nosocomial infections or environmental strains and was never found in CF isolates from British Columbia or Nova Scotia, Canada, or a center in the eastern United States. Individual isolate *Eco*RI RT signatures did not cluster geographically as did the ET signatures by clonal analysis. Frequently RTs occurred in more than a single ET. Known point source focal nosocomial outbreaks were typified by single ETs and stable RTs. Dendrographic analysis of the strains grouped those strains from CF patients, nosocomial outbreaks, and environmental sources into separate ET families, and diversity analysis indicated that, with the exception of ET17, CF isolates clustered in unique and closely related ETs different from those from nosocomial and environmental sources. This study has also shown the potential of multilocus enzyme electrophoresis to monitor the intercontinental spread of *P. cepacia* strains in CF patients, and this may have a significant impact on plans for CF patient summer camps and design of infection control practices. Whether the intercontinental ET12 clone, which predominates in the United Kingdom and the province of Ontario, linked by summer camp acquisition, has increased virulence for CF patients remains to be established.

Recognition by Toronto, Canada, investigators in 1984 (13) that *Pseudomonas cepacia* was an important pathogen in cystic fibrosis (CF) disease has been amply confirmed (7, 9, 11, 16, 28, 30, 31). Early studies (4, 17) of transmission and risk factors incriminated person-to-person transmission and nosocomial acquisition, while later evidence showed that socially and community-acquired infections were of substantial importance (10, 29). At present, there are more studies suggesting person-to-person transmission of *P. cepacia* than of *Pseudomonas aeruginosa* (10, 17, 23). Investigations of transmission and of clonal virulence have been limited because the usually applied techniques of fine identification such as genomic fingerprinting by restriction fragment length polymorphisms, although of considerable value in outbreak investigations, have not in this regard been sufficiently discriminatory (1, 19, 29). Although of value in rapid screening of strains for gross genomic similarities or differences, random amplification fingerprinting (2) is limited in that it lacks reproducibility and is not sufficiently reliable or robust to evaluate clonal diversity. To date, *Eco*RI ribotyping has been generally accepted as the best method available for typing of *P. cepacia* for epidemiological purposes, and length polymorphisms observed after PCR amplification of the 16S-23S intergenic spacer regions have been suggested as an alternative or adjunct to ribotyping (14, 15). We report here the use of multilocus enzyme electrophoresis (MLEE) to determine enzyme profiles for 83 strains of *P. cepacia* that differ widely in their geographic distribution and clinical and environmental origins. MLEE was found to clearly differentiate isolates according to origin and clinical source to a degree not obtained with *Eco*RI ribotyping or other previous methods.

* Corresponding author. Mailing address: National Laboratory for Special Pathogens, Laboratory Centre for Disease Control, Health Canada, Tunney's Pasture, Ottawa, Ontario, Canada K1A 0L2. Phone: (613) 957-1356. Fax: (613) 957-1358. Electronic mail address: WJOHNSON@HPB.HWC.CA.

MATERIALS AND METHODS

Bacterial strains. Sources of *P. cepacia* strains used in this study are summarized in Table 1. We selected strains from CF patients who were geographically widely distributed and also included some strains from both nosocomial outbreaks and the environment. The isolates from CF patients in Vancouver (n = 14), Ottawa (n = 10), and Halifax (n = 18), Canada, were collected over a period of 9, 2, and 7 years and were isolated from 12, 2, and 3 patients, respectively. American Type Culture Collection (ATCC) reference strains were included for reference purposes. The identification of all strains as classical *P. cepacia* was confirmed by fatty acid methyl ester analysis (8, 22) and conventional biochemical phenotypic characterization prior to inclusion in the study. Isolates were frozen in glycerol-peptone at −70°C for prolonged storage and used as required.

Preparation of enzyme extracts. *P. cepacia* isolates from storage were grown overnight at 37°C on heart infusion agar plates (Difco Laboratories). Cultures were harvested by scraping the growth from one petri dish (150 by 15 mm) per strain and suspending the cells in 2 ml of 10 mM Tris–1 mM EDTA–0.5 mM NADP (pH 6.8). Cells were lysed with a Braun-sonic 1510 sonicator (B. Braun Melsungen AG) with three 10-s sonication pulses, each followed by at least 1 min of

TABLE 1. Clinical associations and geographic sources of *P. cepacia* isolates

Clinical association or source	Geographic location	No. of strains	Abbreviation
CF patients	Canada		
	Vancouver, British Columbia	14[a]	CF-VAN
	Toronto, Ontario	4	CF-TOR
	Sudbury, Ontario	1	CF-SUD
	Ottawa, Ontario	10[a]	CF-OTT
	Halifax, Nova Scotia	18[a]	CF-HFX
	United States (Pittsburgh)	4[b]	CF-PITT
	United Kingdom and Ireland[c]		
	Birmingham	1	CF-BIRM
	Belfast	2	CF-BELF
	Edinburgh	1	CF-EDIN
	London	2	CF-LOND
	Manchester	1	CF-MANC
	Dublin	2	CF-DUBL
Strains from CF patients used for serotyping	Canada and United States[d]		
	Toronto (serotype A)	1	CF-STA
	Cleveland (serotype B)	1	CF-STB
	Calgary (serotype C)	1	CF-STC
	Cleveland (serotype D)	1	CF-STD
	Toronto (serotype E)	1	CF-STE
Nosocomial outbreak	Ottawa (neonatal nursery)	6	NS-OTT
	Pittsburgh	9[e]	NS-PITT
ATCC reference strains	Bronchial washings (ATCC 25609)	1	ATCC-HUM
	Onion (ATCC 25416)	1	ENV-ONIO
	Forest soil (ATCC 17759)	1	ENV-SOIL

[a] Some isolates in these groups were collected from the same patients over extended time periods and were obtained courtesy of D. Haase (Halifax), D. Speert (Vancouver), and N. MacDonald (Ottawa).
[b] Strains received from D. Speert, provided by T. Stull and representative of the RTs as previously described (17).
[c] Strains received courtesy of M. Kaufmann, Central Public Health Laboratory, Division of Hospital Infection, Colindale, London, and representative of typical United Kingdom and Ireland RTs.
[d] Strains received from D. Woods, representative of serotypes A to E (21).
[e] Strains received from J. LiPuma, representative of the seven outbreaks previously described by Rabkin et al. (24).

ice bath cooling. Each sample was centrifuged in a 1.5-ml microcentrifuge tube for 20 min at 12,000 × *g* in an Eppendorf refrigerated centrifuge. The supernatant was filtered with 0.2-μm-pore-size sterile Acrodisc low-protein-binding nonpyrogenic membrane filters (Gelman Sciences). Aliquots (1 ml) were stored in vials at −70°C.

Electrophoretic enzyme typing. Electrophoretic analysis of enzymes and subsequent staining procedures were performed as described by Selander et al. (26) and Carson et al. (5). Starch was purchased from Connaught Laboratories Ltd., Willowdale, Ontario, Canada. The following enzymes were assayed: adenylate kinase (ADK; EC 2.7.4.3), esterases (EST; EC 3.1.1.1), fumarase (FUM; EC 4.2.1.2), glutamic-oxalacetic transaminase (GOT; EC 2.6.1.1), glucose-6-phosphate dehydrogenase (G6P; EC 1.1.1.49), 3-hydroxybutyrate dehydrogenase (HBD; EC 1.1.1.30), isocitrate dehydrogenase (IDH; EC 1.1.1.42), 6-phosphogluconate dehydrogenase (6PG; EC 1.1.1.44), phosphoglucose isomerase (PGI; EC 5.3.1.9), and malate dehydrogenase (MDH; EC 1.1.1.37). Electromorphs (alloenzymes) of each enzyme were numbered by decreasing anodal mobility and were equated with alleles at the corresponding structural gene locus. Distinctive combinations of alleles over the 11 enzyme loci (multilocus genotypes) were designated electrophoretic types (ETs). Simultaneous double-allele banding patterns observed for HBD and IDH were scored independently and designated HBT (top), HBB (bottom), and IDH (top).

Ribotype (RT) analysis. *P. cepacia* was cultured for DNA extraction as described above. Cells were harvested into TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) and lysed by the addition of sodium dodecyl sulfate and proteinase K (Boehringer Mannheim) to 0.5% and 50 μg/ml, respectively, and incubation at 37°C for 30 min. DNA was extracted and quantified by standard techniques (25) and resuspended in TE buffer plus 10 mg of RNase (Boehringer Mannheim) per ml. DNA (5 μg) was digested with 50 U of *Eco*RI in accordance with the manufacturer's specifications (Boehringer Mannheim) and analyzed by electrophoresis using 0.8% agarose gels in TAE buffer (40 mM Tris–20 mM acetic acid–1 mM EDTA) with ethidium bromide (10 μg/ml). In each gel, *Hin*dIII DNA fragments of λ (GIBCO BRL) were used as migration references. Size-separated restriction fragments were transferred to a Hybond-N+ membrane (Amersham) with a PosiBlot pressure blotter (Stratagene) and then fixed to the membrane by cross-linking using a UV Stratalinker (Stratagene). The 7.5-kb *Bam*HI fragment of plasmid pKK3535 containing the *E. coli rrnB* gene was used as a probe. The *E. coli* strain harboring pKK3535 was received through the courtesy of M. Altwegg, Department of Medical Microbiology, University of Zurich (20). The probe was randomly labelled with digoxigenin-11-dUTP using the DIG DNA Labelling and Detection Kit (Boehringer Mannheim). Hybridization was performed at 65°C following methods recommended for Hybond-N+ using approximately 50 ng of labelled probe. Chemiluminescence detection procedures were performed as described in the DIG DNA Labelling and Detection Kit. The rRNA gene fingerprints of isolates were visually scored and given numerical designations for each unique RT.

Statistical analysis. Genetic diversity (h) at a given enzyme locus among either ETs or isolates was calculated from the allele frequencies among ETs or isolates as $h = (1 - \Sigma x_i^2)(n/n - 1)$, where x_i is the frequency of the ith allele and n is the

TABLE 2. Clonal allelic profiles and frequency of isolation of strains belonging to 20 ETs of *P. cepacia*

ET	No. of isolates (total = 83)	Strain(s) clustering in ET group (no. of isolates)[a]	Electrophoretic position of allele at enzyme locus:											Strain signature *Eco*RI RT(s) (no. of isolates)
			HBT	HBB	IDH	MDH	6PG	G6P	GOT	ADK	EST	FUM	PGI	
1	1	CF-STB	0	2	5	1	2	1	3	3	6	1	3	17
2	1	CF-VAN	0	2	7	1	2	3	4	1	0	1	1	24
3	1	NS-PITT	0	2	7	1	3	3	5	1	0	1	1	24
4	2	CF-VAN, CF-BIRM	1	2	1	1	4	1	3	5	0	2	5	18, 20
5	1	CF-VAN	1	2	1	2	4	1	3	5	0	2	5	26
6	3	CF-VAN	2	1	1	1	4	1	3	5	0	2	5	19 (3)
7	1	CF-LOND	2	2	1	1	2	1	3	5	0	2	5	20
8	2	NS-PITT	2	3	3	1	2	2	1	4	6	1	2	30, 31
9	1	ENV-SOIL	3	2	2	1	3	1	1	3	4	2	3	10
10	1	NS-PITT	3	2	5	1	2	1	3	3	6	3	1	34
11	5	NS-OTT (2)	3	3	6	1	4	3	3	4	3	1	3	7 (2)
		NS-PITT (2)												32, 33
		CF-STC (1)												7
12	22	CF-ONT (15)	4	2	2	1	2	1	3	3	2	2	3	1 (2), 2, 4 (10), 15, 23
		CF-UK (6)												1, 2, 3, 4 (2), 5
		CF-STA (1)												5
13	1	CF-VAN	4	2	2	1	2	1	3	3	6	2	3	22
14	1	CF-DUBL	4	2	5	1	1	1	2	3	1	2	3	25
15	1	NS-PITT	4	2	5	1	2	1	3	1	1	2	1	12
16	18	CF-HFX	4	2	5	1	2	1	3	2	1	2	1	12 (14), 27, 28, 29 (2)
17	12	CF-VAN (7)	4	2	5	1	2	1	3	3	1	2	1	11, 12 (4), 13, 14
		CF-STD and CF-STE (2)												12, 16
		NS-PITT (2)												12 (2)
		ATCC-HUM												8
18	4	NS-OTT	4	2	5	1	2	1	3	3	1	2	3	6 (4)
19	1	ENV-ONIO	4	4	2	1	2	1	2	3	5	2	3	9
20	4	CF-PITT	5	4	4	1	2	1	4	5	0	2	4	21 (4)

[a] See Table 1 for abbreviations.

number of ETs or isolates (26). Mean genetic diversity (H) is the arithmetic average of the h values for all of the loci. Genetic distance was expressed as the proportion of mismatched loci between pairs of ETs, and null alleles were excluded from the calculation of pairwise distances. The multilocus diversity analysis and dendrogram based on the average-linkage algorithm were generated by using ETDIV and ETCLUS programs (version 2.3) provided through the courtesy of T. S. Whittam, Institute of Molecular Evolutionary Genetics, Pennsylvania State University. Linkage disequilibrium analysis was performed by using the ETLINK component of this analysis software.

RESULTS

In the collection of 83 strains of *P. cepacia* described in Table 1 and analyzed by MLEE and *Eco*RI ribotyping, 66 were isolates from sputum samples from chronically colonized CF patients and included 5 isolates used to develop a differential serotyping scheme (21). Of the other 18 strains, 15 came from point source nosocomial infections and 3 were ATCC strains, with 2 originating from the environment and 1 from a human source. To our knowledge, only the CF isolates used for serotyping had been previously examined by MLEE (5). Some of the isolates which were kindly provided by other laboratories had been categorized by these sources as having RTs that were representative of the strains isolated in their geographic area.

Of the 14 enzymes originally described for *P. cepacia* MLEE analysis (5), 10 showed differences in electrophoretic mobility that were readily interpretable and polymorphic for a range of two to seven alleles. Although single alleles were observed for most enzymes in this MLEE analysis, two alleles were detected simultaneously for both HBD and IDH in almost all isolates and double-allele banding patterns were scored for HBD (HBT and HBB). Only one of the IDH alleles was sufficiently polymorphic to warrant statistical analysis. Initially aconitase, phosphoglucomutase, alkaline phosphatase, and acid phosphatase were included in the enzymes analyzed, but these were eliminated on the basis of banding patterns that were poorly defined and difficult to interpret. Peptidases, malic enzyme, and aspartate dehydrogenase were chosen as possible replacements but failed to produce consistent recordable banding patterns for the majority of isolates. Thus, 11 enzyme loci were scored for 10 polymorphic enzymes as specified earlier.

Enzyme profiles of the 83 *P. cepacia* isolates allowed the identification of 20 ETs, for which the strain source, *Eco*RI RT, and individual allelic profiles are shown in Table 2. The average number of alleles per locus for all isolates and the three categories of strains or populations is presented in Table 3 and varies from 4.64 for the total number of isolates to 1.45 for the environmental strains. The reference strains used for serotyping were analyzed as CF isolates for assignment of population category but have been designated CF-STA to CF-STE to assist in the interpretation of results. ET diversity for the total population and within each major category ranged from 0.780 for CF samples to 0.795 for nosocomial samples and 0.848 for all ETs as indicated in Table 3. The mean genetic diversity within 20 ETs and within 83 isolates (H) also appears in Table 3 for the total population of strains and the three categories. Among the ETs in which CF isolates clustered, six were favored by multiple isolates (2 to 22); consequently, there was less genetic diversity, on average, among isolates (H = 0.343) than among ETs (H = 0.558). This observation was consistent with the greater genetic diversity among ETs in the total population (H = 0.581) compared with that among

TABLE 3. Analysis of the genetic diversity among multilocus ETs and isolates of *P. cepacia* by using allelic differences at 11 enzyme loci

Population	No. of ETs	No. of isolates	Polymorphic loci	Mean no. of alleles	ET diversity[a]	Genetic diversity among:			
						ETs		Isolates	
						H^b	SE	H^b	SE
Total	20	83	1.00	4.64	0.848	0.581	0.069	0.408	0.072
CF	13	66	1.00	3.82	0.780	0.558	0.072	0.343	0.079
Nosocomial	7	15	0.91	3.00	0.795	0.606	0.070	0.543	0.062
Environmental	2	2	0.45	1.45	0.000	0.455	0.157	0.455	0.157

[a] Estimates probability that two isolates chosen at random from within a population are of different ETs.
[b] Mean genetic diversity (H) is the arithmetic average of h values for all loci calculated by $h = (1 - \Sigma x_i^2)(n/n - 1)$. Details of h values for individual enzyme loci are given in Table 4.

isolates ($H = 0.408$). Since most of the ETs which represented nosocomial and environmental isolates contained only single isolates, there was less difference in genetic diversity between ETs and isolates for these two population subsets. For the total population, single-locus diversity (h) was high for IDH (0.821), EST (0.805), HBT (0.800), PGI (0.742), and ADK (0.737). This pattern was similar for isolates from CF patients, and some differences were observed in frequencies for G6P and FUM for nosocomial isolates (Table 4). Linkage disequilibrium (D') calculated for each pair of alleles at two loci and the frequency tabulated over all pairs of loci resulted in a U-shaped distribution from -1.0 ($D' = 0.606$) to $+1.0$ ($D' = 0.218$). This distribution of D' values is typical for *Escherichia coli* populations and is the consequence if most pairs of alleles are in complete association (3, 12).

ETs 12, 16, and 17 were most represented among our ET groups, containing 22, 18, and 12 isolates, respectively (Table 2). An analysis of a much larger number of isolates would have to be examined to establish whether these ETs predominate in all clinical isolates of *P. cepacia*. Without exception, however, all of the isolates found to cluster by MLEE in ETs 12 and 16 originated from CF patients and included the serotype A reference strain originally isolated from sputum of a CF patient in Toronto. The serotype A strain shared a common RT (RT5) with one of the representative strains from London, United Kingdom. ET17 was more diverse and clustered isolates from CF patients in Vancouver with nosocomial strains from the United States, two of the serotyping strains, and the ATCC reference strain from bronchial washings. Serotyping strains D and E clustered in ET17; originated from Cleveland, Ohio, and from Toronto; and were RT16 and RT12, respectively, in this study.

Three genetically similar families of ETs were observed for CF isolates with a genetic distance of ≤0.200 (Fig. 1) and included ETs 4, 5, 6, and 7 with 7 isolates from Vancouver, Birmingham (United Kingdom), and London; ETs 12 and 13 with 23 isolates from Canada, the United Kingdom, and Ireland; and ETs 15, 16, and 17 with 31 isolates from Halifax, Vancouver, Cleveland (serotype D), and Toronto (serotype E). All strains in this study from Ontario (Canada) clustered in ET12 in addition to the representative strains from Manchester, London, and Edinburgh in the United Kingdom; two strains from Belfast, United Kingdom; and one from Dublin, Ireland. This ET is characterized by a unique EST-2 allele which may be a marker for the predominant clone in the United Kingdom-Eire and Ontario.

In the total population of 83 strains, 34 *Eco*RI RTs were identified as indicated in the last column of Table 2. An observation that may have significance is that the ETs that contained strains from a variety of CF patients consistently demonstrated a greater diversity of RTs than did ETs containing predominantly nosocomial isolates. In addition, the same RT was frequently observed to occur in more than a single ET (Fig. 1) as follows: RT24 in ETs 2 and 3, RT20 in ETs 4 and 7, and RT12 in ETs 15, 16, and 17. These data suggest that isolates from ETs 3 and 15 have high potential for nosocomial spread to CF patients. Of all the CF isolates characterized by MLEE, only ET20, comprising four strains from the CF center in Pittsburgh, Pa., diverged from the other clusters at a genetic distance of more than 0.60. These results may be indicative of nosocomial or environmental acquisition in this particular patient population. In this study, the majority of ET12 isolates from Ontario CF patients were of RT4 and identical to the RTs for Manchester and Dublin, and the single Edinburgh isolate, although ET12, was identified as RT3.

TABLE 4. Genetic diversity at 11 enzyme loci for all *P. cepacia* strains and three populations

Allele	Single-locus diversity (h) of:			
	Total population	CF samples	Nosocomial samples	Environmental samples
HBT	0.800	0.833	0.810	1.000
HBB	0.437	0.423	0.476	1.000
IDH	0.821	0.833	0.714	0.000
MDH	0.100	0.154	0.000	0.000
6PG	0.553	0.564	0.524	1.000
G6P	0.353	0.282	0.667	0.000
GOT	0.574	0.410	0.524	1.000
ADK	0.737	0.744	0.762	0.000
EST	0.805	0.756	0.810	1.000
FUM	0.468	0.385	0.714	0.000
PGI	0.742	0.756	0.667	0.000
H^a (SE)	0.581 (0.069)	0.558 (0.072)	0.606 (0.070)	0.455 (0.157)

[a] Mean genetic diversity (H) is the arithmetic average of h values for all loci calculated by $h = (1 - \Sigma x_i^2)(n/n - 1)$.

DISCUSSION

Analysis of the data from MLEE and *Eco*RI ribotyping of our *P. cepacia* isolates demonstrated three main features. First, by MLEE, *P. cepacia* found in the environment is demonstrably quite different genomically from pulmonary and nosocomial isolates and unlikely to be responsible for infections in CF patients. Since the number of environmental strains we examined is underrepresented in this study, additional strains would need to be studied to validate this conclusion. Second, isolates of *P. cepacia* colonizing CF patients attending the same clinic almost always have the same ET and frequently different RTs. This strongly suggests that this pathogen is readily transferred

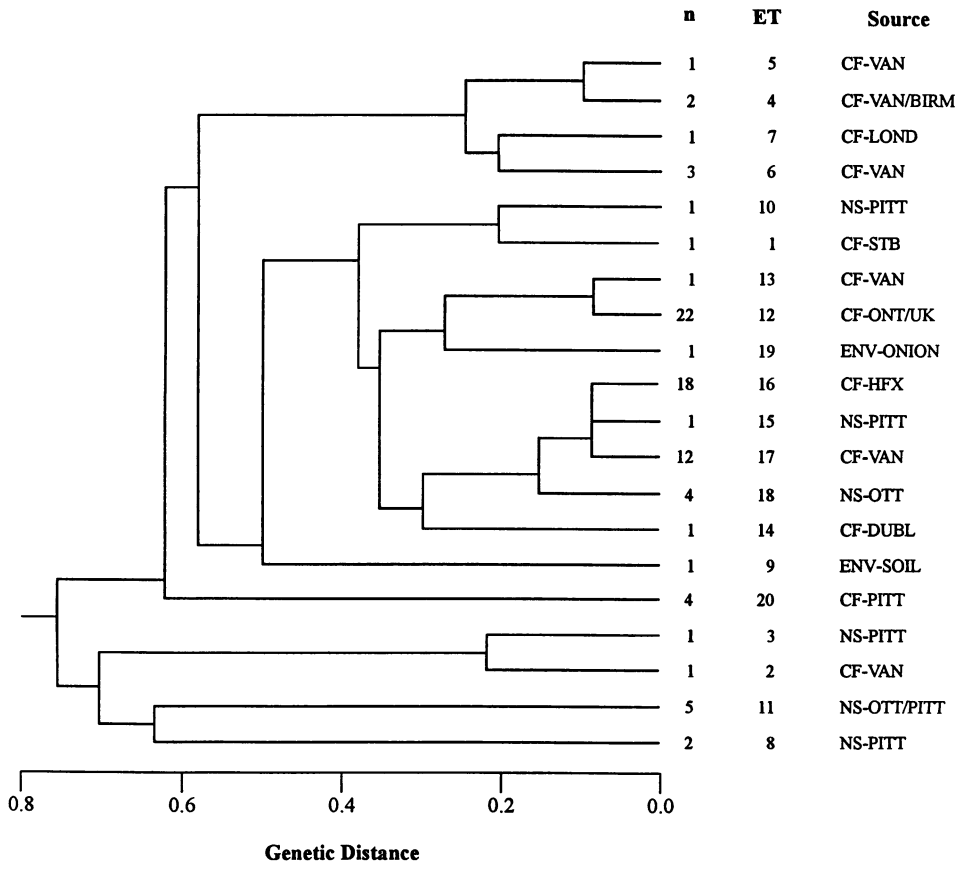

FIG. 1. Genetic relationships among 83 strains and 20 ETs of *P. cepacia* isolated from CF patients, nosocomial infections, and environmental sources. ETs are numbered as assigned after multilocus diversity analysis (ETDIV) based on 11 enzyme loci, and the dendrogram resulted from a cluster analysis (ETCLUS) of genetic distances by the average linkage algorithm using statistical analysis programs (version 2.3) provided by T. S. Whittam. The number (n) of isolates is indicated for each ET. See Table 1 for source abbreviations.

patient to patient, especially in social settings as recently documented (10, 29). Third, cluster analysis of isolates from particular geographic areas groups them into the same ETs, even when their RT profiles are widely different. Geographic areas appear to nurture unique clones of *P. cepacia*, and ET analysis clearly groups these strains.

The stable characteristics of MLEE allowed the clear demonstration of intercontintental spread. The Ontario isolates from CF patients which we grouped as ET12 were identical to many isolates from the United Kingdom but different from the representative strains from the eastern United States (ET20) and both the Atlantic and Pacific coasts of Canada. This was, in part, not unexpected since a recorded epidemic of a highly transmissible strain in the United Kingdom (10) included two colonized patients who were said to have spent time in an Ontario summer camp in September 1990. In our study, both the Manchester isolate and the majority of the Ontario CF isolates were not only of identical ET but also of identical RT (RT4). The Edinburgh isolate, although ET12 and considered part of the same endemic cluster, was RT3. Of some interest is the fact that a few of the ET12 strains from Ontario pediatric CF patients were isolated in 1987 and 1988 and therefore appear to have been established in Ontario prior to 1990. Intercontinental spread of this putative virulent clone may predate the camp of 1990 that is said to have been the source of the United Kingdom outbreak (10). Although we have no additional information on this feature, we surmise that there may have been summer camps in earlier years at which contact and transmission occurred. Additional investigations will be required to establish the initial date and direction of spread of *P. cepacia* (ET12) between the United Kingdom and Ontario. These circumstances suggest that camps attended by CF patients in regions far removed from their own communities represent a risk of infection with different *P. cepacia* strains, some of which may have enhanced virulence.

It is our general conclusion that MLEE provides data on clonality which are stable and capable of delineating families of strains typical of geographic and regional *P. cepacia* populations, uninfluenced by trivial and ephemeral factors. In this respect RT analysis is unsatisfactory, being sensitive to selective pressures from the patient and the intimate environment. Used together, however, these techniques provide the degree of discrimination required of serious demographic and epidemiological studies. This approach has been previously recommended (6) for the analysis of *P. aeruginosa* isolates from chronic lung infections in CF patients.

Finally, with respect to available techniques, in our experience with *P. cepacia*, the technique which identifies most sensitively the highest level of genetic drift is neither ET nor RT analysis but is restriction fragment analysis using pulsed-field gel electrophoresis after restriction endonuclease digestion with specific enzymes which cleave infrequently. Choice of these enzymes is critical to ensure adequate pattern complexity of restriction fragments and permit strain discrimination.

Pulsed-field gel electrophoresis is the most sensitive to changes caused by selective environmental pressures in the host and is most useful if a method at the extremity of sensitivity is required but is limited as a typing method by this very sensitivity.

Of particular interest is the family of ET clusters obtained by MLEE which indicate the close genetic relatedness of the clinical CF isolates, inclusive of even the archived ATCC reference strain from a bronchial washing. Although isolates in these clusters represent many different RTs by conventional *Eco*RI analysis (Table 2) (and more by a discriminatory extended ribotyping using three additional endonucleases [13b]), their close genetic relationship is apparent in MLEE linkage analysis. In addition, and perhaps just as significantly, 7 of 15 strains representative of nosocomial infections unrelated to CF formed a single, closely related family of ET8 and ET11 while other nosocomial isolates were generally distant from each other genetically. This analysis of strain relationships is not possible when conventional or even extended ribotyping is used as the discriminator. When dendrographic analysis was attempted using RT data, the resulting dendrogram appeared more as a lawn rake than as a tree, indicative of the lack of information in this method with which to analyze stable genetic relationships. MLEE, however, appears to be a robust technology ideally suited to analysis of genetic relatedness which ignores changes in genotype identified by ribotyping, which provides individual strain signatures suitable for identifying their patient origins rather than that of their family. Both are, of course, equally valuable in their place when the complete characterization of isolates is desirable.

In the cases of point source nosocomial outbreaks in this study, the isolates clustered in unique ETs and were typified by a single, stable RT per outbreak.

When the mutational evolution of a strain occurs in the same CF patient over years with little evidence for outside acquisition, we have observed in several cases that selective in vivo pressures may frequently alter the pulsed-field gel electrophoresis pattern and also the RT but that the ET does not change (13a). This is in contradiction to previous reports which have documented RT stability of serial pulmonary isolates (15, 18). Discrepant observations such as these need clarification by a definitive evaluation of archived strains from both pediatric and adult CF patients acutely and chronically infected with *P. cepacia*. Such studies will establish whether genomic variation of the same colonizing strain is occurring in vivo or whether the patients are suprainfected with multiple or distinct strains and perhaps, if these changes are associated with clinical deterioration, may identify strains of special virulence.

It seems apparent from these and other observations that the minor genomic base changes that alter the number and location of specific restriction recognition sequences and hence give rise to altered restriction fragment length polymorphism and RT patterns do not frequently result in amino acid alteration compatible with enzyme functions that are the source of allelic (electrophoretic) changes in MLEE. These changes are unselective of virulence or transmission characteristics, being initiated by background mutational events characteristic of bacterial DNA (27), but we suggest that the ET profile better describes strains that have been selected for virulence by the in vivo milieu of the CF patient. CF patients and those compromised by nosocomial experiences may also select the more virulent representatives from the general *P. cepacia* population of all possible ETs. MLEE analysis, because of its characteristics, will be especially useful in these circumstances for the investigation of pathogenesis, carrier state, and transmission.

ACKNOWLEDGMENTS

We are indebted to D. Haase (Halifax, Nova Scotia, Canada), J. LiPuma and T. Stull (Pittsburgh, Pa.), D. Speert (Vancouver, British Columbia, Canada), N. MacDonald (Ottawa, Ontario, Canada), D. Woods (Calgary, Alberta, Canada), M. Kaufmann (London, England), M. Altwegg (Zurich, Switzerland), and T. Whittam (University Park, Pa.) for their generous help in providing strains and statistical analysis software used in this study. We also thank F. E. Ashton and J. Alan Ryan for MLEE technology transfer and Kathy Bernard, Rosemary Kuehn, and Susan Tessier for their expert technical assistance. We appreciate the dedication and valued assistance of Andrea Vandenberg, a biology student on workterm assignment to the LCDC laboratory as part of the cooperative education program at the University of Waterloo.

This work was supported by the Canadian Cystic Fibrosis Foundation and the Laboratory Centre for Disease Control, Health Canada.

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