JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1996, p. 2760–2765 Vol. 34, No. 11
0095-1137/96/$04.00+0

Clonality of Multidrug-Resistant Nontypeable Strains of *Haemophilus influenzae*

M. C. FUSTÉ, M. A. PINEDA, J. PALOMAR, M. VIÑAS, AND J. G. LORÉN*

Departament de Microbiologia i Parasitologia Sanitàries, Facultat de Farmàcia, Divisió de Ciències de la Salut, Universitat de Barcelona, 08028 Barcelona, Spain

Received 3 June 1996/Returned for modification 16 July 1996/Accepted 14 August 1996

The genetic structure of a population of multidrug-resistant nontypeable (unencapsulated) *Haemophilus influenzae* strains isolated at a hospital in Barcelona, Spain, was investigated by using multilocus enzyme electrophoresis to determine the allelic variation in 15 structural loci. In our study we have also included some antimicrobial agent-susceptible strains isolated at the same hospital. All enzymes were polymorphic for two to eight electromorphs, and the analysis revealed 43 distinct electrophoretic types among the 44 isolates. The mean genetic diversity of the entire population was 0.55. Multilocus linkage disequilibrium analysis of the isolates revealed a strong association between alleles, suggesting little possibility of recombination. Furthermore, the dendrogram and the allele mismatch distribution are typical of a population with no extensive genetic mixing.

Haemophilus influenzae isolates are commonly found in the upper respiratory tracts of healthy children and adults, and they cause a variety of diseases in humans, especially in children. Most of these organisms are unencapsulated and not typeable with sera specific for the six recognized capsular types. Although these organisms rarely cause invasive infections in healthy children and adults, nontypeable *H. influenzae* isolates are a frequent cause of otitis media, conjunctivitis, pneumonia, and chronic bronchitis, especially in children (17). They are commonly isolated in purulent secretions from the lower respiratory tracts of patients with cystic fibrosis and chronic bronchitis, and they can also occasionally cause bacteremic illnesses in neonates and in older immunocompromised patients. In addition, in the last few years there has been a rapid increase in the frequency of isolation of multidrug-resistant *H. influenzae* strains (15).

The population genetics of *H. influenzae* serotype b have been thoroughly examined by multilocus enzyme electrophoresis (MLEE), restriction modification systems, and outer membrane protein profiles (9, 12), but only some of these studies have included nontypeable strains. The results have revealed that encapsulated *H. influenzae* populations show a clonal structure, but the structures of nontypeable bacteria are unclear since they appear to be more diverse than those of serotype b (8).

The most widely used method of assessing genetic diversity and structure in bacterial populations is MLEE, in which bacterial isolates are characterized by the relative mobilities of a number of enzymes (13). Different mobility variants can be directly equated with alleles at the corresponding structural gene locus. MLEE data have been used to estimate the levels of single-locus and multilocus genotypic variation in populations as well as the extent of genetic exchange within a population (14). MLEE, which indexes the allelic variation in multiple chromosomal genes, has been highly successful in generating large data sets for the statistical analysis of bacterial populations and has shown that many species of bacteria exhibit high levels of linkage disequilibrium (20). Recently, Maynard Smith et al. (6) have developed a statistical test for population genetic data which can unequivocally index the extent of clonality within bacterial populations. This index was first used by Brown et al. (1) to measure population structure in *Hordeum spontaneum.*

In the present study, we used MLEE to assess allelic variation at 15 metabolic enzyme loci in a collection of 44 isolates of nontypeable *H. influenzae* strains isolated in a pediatric hospital in Barcelona, Spain, over a period of 3 years. Most of them are multiresistant, particularly to aminoglycosides, but in our study we have also included some susceptible strains isolated during the same period. The aim of the research was to examine the genetic diversity and structure of a population of multiresistant nontypeable strains of *H. influenzae*. The results of our analysis indicate that the whole population exhibits a significant linkage disequilibrium, which implies a basic clonal population structure. Analyses of population subgroups considering biotype, resistance to antibiotics, and other subsets of data did not affect the main conclusion.

MATERIALS AND METHODS

Bacterial strains. This study was carried out with 44 nontypeable strains of *H. influenzae* isolated during a 3-year period (from 1988 to 1991) in the pediatric hospital Sant Joan de Déu in Barcelona, Spain. Strains were identified as *H. influenzae* on the basis of Gram reaction, cell morphology, lack of β-hemolysis, and NAD and hemin requirements. Strains were assigned to biotypes on the basis of standard tests of indole, urease, and ornithine decarboxylase production. The lack of agglutination with antiserum (Bacto *H. influenzae* polyvalent antiserum [Difco 2237-50-0]; Difco Laboratories, Detroit, Mich.) determined the nontypeable character of the strains. Table 1 shows the source, biotype, antibiotic resistance, electrophoretic type (ET), number of isolates, and allele at the indicated enzyme locus for each of the strains.

Antimicrobial susceptibility tests. All strains were tested for antimicrobial susceptibility by disk diffusion techniques (15). The following antibiotics were assayed: amikacin, ampicillin, amoxicillin-clavulanic acid, cefotaxime, chloramphenicol, cotrimoxazole, erythromycin, gentamicin, kanamycin, netilmicin, neomycin, rifampin, sisomicin, spectinomycin, streptomycin, sulfamethoxazole, tetracycline, tobramycin, and trimethoprim.

Preparation of lysates for electrophoresis. Each isolate was grown overnight at 37°C in brain heart infusion medium (bioMérieux) supplemented with hemin (10 $\mu g \cdot ml^{-1}$) and β-NAD (1 $\mu g \cdot ml^{-1}$) in a water bath shaker. The bacteria were harvested by centrifugation (15,000 × *g* for 10 min), suspended in TE buffer (10 mM Tris-HCl and 1 mM EDTA [pH 6.8]), and sonicated at 100 W for two 1-min periods, with cooling in an ice bath in a Labsonic 1510 (Braun) apparatus. After lysis and centrifugation at 15,000 × *g* for 15 min at 4°C, aliquots of lysate

* Corresponding author. Phone: 34-3-4024497. Fax: 34-3-4021886. Electronic mail address: loren@farmacia.far.ub.es.

TABLE 1. *H. influenzae* isolates used in this study

ET	No. of strains	Reference isolate	Allele at indicated enzyme locus[a]														Biotype	Source	Antibiotic resistance or susceptibility[b]
			ME	6PG	G6P	GD2	IDH	ADH	FUM	MDH	LYD	LED	ALD	PGM	HEX	GP1	IPO			
1	1	1211	1	3	3	3	0	1	1	1	0	0	0	0	0	2	0	I	Eye	Ct,Su,Tp
2	1	1234	4	2	6	3	0	2	4	4	2	1	2	4	0	4	1	I	Throat	Ct, Su,Tp,Ap,Cm,Te
3	1	1242	5	3	7	3	0	0	3	3	0	0	0	1	0	2	1	I	Trachea	Ct,Su,Tp
4	1	1277	3	3	4	4	0	0	3	3	0	0	0	0	0	2	1	I	Ear	Susceptible
5	1	1279	6	0	6	3	0	0	4	5	2	1	3	0	0	0	1	I	Joint	Ct,Su,Tp
6	1	1294	1	1	4	2	1	2	4	6	2	2	1	0	0	4	0	I	Ear	Ct,Su,Tp,Km,Nm,Sm,Ge,To
7	1	1350	3	3	5	3	0	3	3	1	0	0	0	3	0	3	1	I	Blood	Susceptible
8	1	1369	4	0	7	2	0	0	2	3	3	1	3	2	0	4	0	I	Sputum	Ct,Su,Tp,Nm,Sm
9	1	1373	6	3	4	1	0	1	4	5	2	1	3	2	0	4	1	I		
10	1	1398	3	3	4	3	0	0	3	2	0	0	0	2	0	2	1	I	Sputum	Su,Ap,Km,Nm,Sm
11	1	1419	5	3	7	4	0	1	3	1	0	0	0	5	0	1	1	I	Eye	Ct,Su,Tp,Ap,Km,Nm,Sm
12	1	1205	3	3	4	3	0	0	4	3	0	0	0	0	0	1	1	II	Ear	Ct,Su,Tp,Ap
13	1	1207	2	3	7	3	0	0	5	2	0	0	0	0	0	2	1	II	Nose	Ct,Su,Tp,Ap,Cm,Te
14	1	1217	2	3	4	0	0	0	3	2	0	0	0	0	1	1	0	II	Blood	Susceptible
15	1	1226	3	3	2	3	0	0	5	3	2	1	2	1	4	2	0	II	Ear	Ct,Su,Tp
16	1	1253	2	3	3	2	0	0	0	2	0	0	0	0	2	0	1	II	Vagina	Ct,Su,Tp,Ap,Sm,Cm,Te
17	1	1256	3	0	5	0	0	0	6	4	0	0	0	5	2	2	1	II		Ct,Su,Tp,Ap,Sm,Cm
18	1	1275	4	3	7	4	0	1	5	4	0	3	3	2	2	2	1	II	Ear	Ct,Su,Ap,Sm,Cm,Te
19	1	1276	4	4	7	3	0	0	5	3	0	0	0	0	0	2	1	II	Ear	Susceptible
20	1	1282	2	3	7	3	0	2	3	2	0	1	0	1	0	2	1	II	Ear	Susceptible
21	1	1296	5	2	4	1	0	2	3	3	1	1	3	0	3	4	0	II	Nose	Ct,Su,Tp,Ap,Km,Nm,Sm
22	1	1310	0	0	4	3	0	1	0	1	2	1	3	0	0	4	0	II	Pus	Ct,Su,Tp
23	1	1312	3	3	7	0	0	0	3	3	0	0	0	5	0	4	1	II	Pus	Susceptible
24	1	1321	4	0	1	3	0	0	6	3	1	0	0	1	0	0	1	II	Vagina	Ct,Su,Tp,Ap,Km,Nm,Sm
25	1	1374	3	3	3	2	0	0	3	3	0	0	0	1	0	2	1	II	Abscess	Ct,Su,Tp,Ap
26	1	1407	1	3	0	0	0	1	0	1	0	1	1	1	0	4	1	II	Bronchus	Susceptible
27	1	1424	1	3	5	3	0	0	1	2	0	0	0	0	0	0	0	II	Eye	Ct,Su,Tp,Ap,Km,Sm
28	1	1212	2	0	5	3	0	0	1	2	0	0	0	0	0	2	1	III	Eye	Ct,Su,Tp,Ap,Km,Nm
29	2	1220	3	0	5	3	0	0	2	3	0	0	0	0	0	2	1	III	Nose	Ct,Su,Ap,Cm,Te
		1284	3	0	5	3	0	0	2	3	0	0	0	0	0	2	1	III	Eye	Ct,Su,Tp,Ap,Cm,Te
30	1	1237	3	0	4	3	0	0	2	3	0	0	0	0	0	2	0	III	Eye	Ct,Su,Tp
31	1	1272	2	0	4	2	0	0	1	1	0	0	3	0	0	2	0	III	Blood	Ct,Su,Tp,Ap
32	1	1301	3	0	4	3	0	0	2	3	0	0	3	0	0	2	1	III	Ear	Ct,Su,Tp,Ap
33	1	1336	3	0	4	3	0	0	3	3	0	0	0	0	0	2	0	III	Sputum	Ct,Su,Tp,Ap,Km,Nm,Sm
34	1	1360	3	4	4	0	0	0	3	3	0	0	0	0	0	2	1	III	Nose	Ct,Su,Tp,Ap,Km,Nm
35	1	1227	3	2	6	3	0	1	3	3	2	0	0	0	0	4	1	IV	CSF[c]	Susceptible
36	1	1260	3	3	3	3	0	0	3	3	0	0	0	0	0	2	1	IV	Eye	Susceptible
37	1	1287	3	0	2	3	0	0	3	3	0	0	0	0	0	2	0	IV	Ear	Ct,Su,Tp
38	1	1322	3	0	4	3	0	0	3	3	0	2	0	0	0	2	1	IV	Vagina	Ct,Su,Tp,Ap,Km,Nm
39	1	1371	3	0	5	0	0	0	2	3	0	1	0	0	0	2	0	IV	Mucus	Ct,Su,Tp,Ap
40	1	1246	3	2	7	2	2	0	3	3	0	0	0	0	2	2	0	V	Ear	Ct,Su,Tp,Sm,Cm,Te
41	1	1283	2	0	4	3	0	0	2	2	0	0	0	0	0	2	0	V	Ear	Susceptible
42	1	1291	0	1	5	3	0	1	0	0	1	0	0	0	0	2	0	V	Ear	Susceptible
43	1	1338	6	2	6	0	0	1	3	3	2	0	0	0	0	4	1	V	Blood	Susceptible

[a] Abbreviations for enzymes: ME, malic enzyme; 6PG, 6-phosphogluconate dehydrogenase; G 6P, glucose-6-phosphate dehydrogenase; GD2, NADP-dependent glutamate dehydrogenase; IDH, isocitrate dehydrogenase; ADH, alcohol dehydrogenase; FUM, fumarase; MDH, malate dehydrogenase; LYD, lysine dehydrogenase; LED, leucine dehydrogenase; ALD, alanine dehydrogenase; PGM, phosphoglucommmutase; HEX, hexokinase; GP1, NAD-dependent glyceraldehyde-phosphate dehydrogenase; and IPO, indophenol oxidase.

[b] Abbreviations for antibiotics: Ap, ampicillin; Cm, chloramphenicol; Ct, cotrimoxazole; Ge, gentamicin; Km, kanamycin; Nm, neomycin; Sm, streptomycin; Su, sulfamethoxazole; Te, tetracycline; To, tobramycin; and Tp, trimethoprim. Susceptible, nonresistant to all antibiotics tested.

[c] CSF, cerebrospinal fluid.

TABLE 2. Allele frequencies and genetic diversities at 15 enzyme loci in 43 ETs of *H. influenzae*

Enzyme[a]	No. of electromorphs	No. of strains	Frequency	H^b
ME	0	2	0.045	0.74
	1	4	0.091	
	2	7	0.159	
	3	20	0.455	
	4	5	0.113	
	5	3	0.068	
	6	3	0.068	
6PG	0	16	0.363	0.66
	1	2	0.045	
	2	5	0.113	
	3	19	0.431	
	4	2	0.045	
G6P	0	1	0.023	0.78
	1	1	0.023	
	2	2	0.045	
	3	4	0.091	
	4	15	0.340	
	5	8	0.181	
	6	4	0.091	
	7	9	0.204	
GD2	0	7	0.159	0.60
	1	2	0.045	
	2	6	0.136	
	3	26	0.590	
	4	3	0.068	
IDH	0	42	0.954	0.09
	1	1	0.023	
	2	1	0.023	
ADH	0	30	0.681	0.47
	1	9	0.204	
	2	4	0.091	
	3	1	0.023	
FUM	0	4	0.091	0.78
	1	4	0.091	
	2	7	0.159	
	3	18	0.409	
	4	5	0.113	
	5	4	0.091	
	6	2	0.045	
MDH	0	1	0.023	0.67
	1	6	0.136	
	2	8	0.182	
	3	23	0.523	
	4	3	0.068	
	5	2	0.045	
	6	1	0.023	
LYD	0	32	0.727	0.45
	1	3	0.068	
	2	8	0.182	
	3	1	0.023	
LED	0	31	0.704	0.46
	1	10	0.227	
	2	2	0.045	
	3	1	0.023	
ALD	0	32	0.727	0.41
	1	2	0.045	
	2	2	0.045	
	3	8	0.182	
PGM	0	29	0.659	0.55
	1	6	0.136	
	2	4	0.091	
	3	1	0.023	
	4	1	0.023	
	5	3	0.068	
HEX	0	37	0.840	0.29
	1	1	0.023	
	2	4	0.091	
	3	1	0.023	
	4	1	0.023	
GP1	0	4	0.091	0.59
	1	3	0.068	
	2	26	0.591	
	3	1	0.023	
	4	10	0.227	
IPO	0	16	0.363	0.47
	1	28	0.636	

[a] See footnote *a*, Table 1, for abbreviations.
[b] *H*, genetic diversity.

(supernatant) were transferred to Eppendorf tubes and stored at −45°C until use.

Electrophoresis and specific enzyme staining. Continuous nondenaturing vertical polyacrylamide gel electrophoresis was used for all the enzymes. An acrylamide concentration of 12% in 0.8 M Tris-hydrochloride (pH 8.8) buffer was used in all gels. Tris-glycine buffer (pH 8.3) was used for the electrode compartments. Gels were used within 24 h of preparation and run at 4°C. A constant voltage of 75 V was applied until the bromophenol blue band reached the bottom of the gel (3). The staining of the gels to reveal specific enzyme activity was performed according to the method of Selander et al. (13). Electrophoresis under these conditions produces narrow well-defined bands, and relative electrophoretic mobilities are reproducible on single gels and on gels run at different times.

The following 15 enzymes were assayed: malic enzyme (EC 1.1.1.40), 6-phosphogluconate dehydrogenase (EC 1.1.1.44), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), NADP-dependent glutamate dehydrogenase (EC 1.4.1.4), isocitrate dehydrogenase (EC 1.1.1.42), alcohol dehydrogenase (EC 1.1.1.1), fumarase (EC 4.2.1.2), malate dehydrogenase (EC 1.1.1.37), lysine dehydrogenase (EC 1.4.3.x), leucine dehydrogenase (EC 1.4.3.2), alanine dehydrogenase (EC 1.4.1.1), phosphoglucomutase (EC 2.7.5.1), hexokinase (EC 2.7.1.1), NAD-dependent glyceraldehyde-phosphate dehydrogenase (EC 1.2.1.12), and indophenol oxidase (EC 1.15.1.1). For each enzyme, distinct mobility variants were designated as electromorphs and numbered in order of increasing migration towards the anode. Displacement of the electromorphs was expressed in terms of relative electrophoretic mobility with respect to the bromophenol blue band. The electromorphs of an enzyme were equated with alleles at the corresponding structural gene locus. The absence of enzyme activity was attributed to a null allele and assigned a value of zero. Distinct combinations of alleles over the 15 loci assayed were assigned as ETs.

Datum treatment. Genetic diversity for a locus was calculated according to the method of Nei (11). Genotypic diversity (G) was calculated as follows: $G = 1 - \Sigma\, p_j^2$ where p_j is the frequency of jth genotype (ET). Clustering of the values obtained by isoenzyme electrophoresis was performed with a matrix of coefficients of genetic distances by the unweighted pair-group method by using the simple matching coefficient (NTSYS-pc, version 1.80; Applied Biostatistics, Inc). The genetic distances between pairs of ETs were calculated as the proportions of loci at which dissimilar electromorphs occurred. Multilocus linkage disequilibrium was calculated on the basis of the distribution of allelic mismatches between pairs of bacterial isolates among all the loci examined. The ratio of the variance observed in mismatches (V_O) to the variance expected at linkage equilibrium (V_E) provides a measure of multilocus linkage disequilibrium that can be expressed as the index of association (I_A) as follows: $I_A = V_O/V_E - 1$ (6). For populations in linkage equilibrium, V_O equals V_E and I_A is not significantly different from zero, whereas values of I_A of greater than zero indicate that

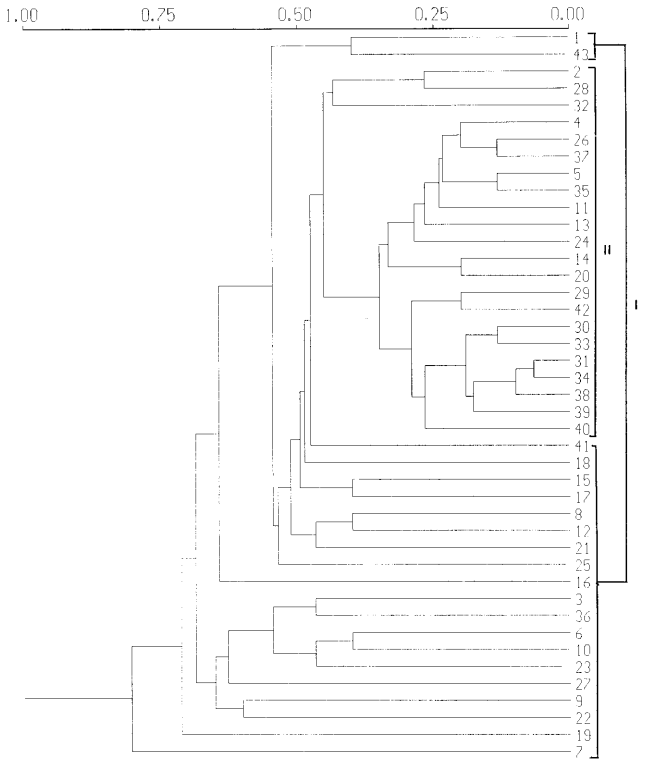

FIG. 1. Dendrogram showing genetic relationships among 43 ETs of nontypeable *H. influenzae* strains. The dendrogram shown is based on allelic profiles at 15 enzyme loci (numbers indicate genetic distances).

recombination has been rare or absent. To determine whether V_O is significantly different from V_E in any sample, a Monte Carlo procedure was iterated randomly, scrambling the alleles to eliminate any effect of linkage disequilibrium (16). A preliminary study of population linkage disequilibrium was kindly carried out by S. Strain at Tufts University (Medford, Mass.). Computer programs written by J. G. Lorén were used afterwards to calculate V_O and V_E and to perform the Monte Carlo simulations.

RESULTS

ETs and genetic diversity. All the enzyme loci studied were polymorphic, and the number of alleles ranged from two (indophenol oxidase) to eight (glucose-6-phosphate dehydrogenase). The average number of alleles per locus was 5.06. Among the 44 *H. influenzae* isolates examined, 43 ETs were identified. The most diverse locus was glucose-6-phosphate dehydrogenase ($H = 0.78$), and the least diverse was isocitrate dehydrogenase ($H = 0.09$) (where H is genetic diversity) (Table 2). The mean genetic diversity of all samples was 0.55, and that of the resistant subset was 0.57. The genotypic diversity was 0.93.

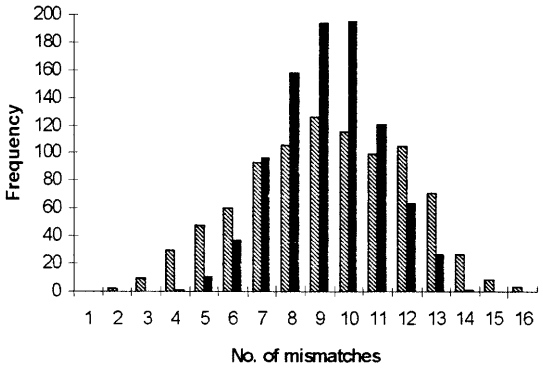

FIG. 2. Allele mismatch distribution among 44 nontypeable *H. influenzae* strains (hatched bars). Mismatch allele distribution of the scrambled population (black bars) is also included for the purpose of comparison.

Dendrogram. The genetic relationship among the isolates is shown in Fig. 1. The dendrogram, generated by the average-linkage method of clustering, shows that isolates are distributed through most of the dendrogram with no conspicuous pattern. No significant clustering among isolates was detected when we considered origin, biotype, or antibiotic resistance. Instead, there is a random distribution of isolates. Considering the shape of the figure, we can distinguish two different population subgroups, the first of which (I) exhibits an extensive branching pattern and the second of which (II) exhibits a tree-like structure.

Linkage disequilibrium analysis. The complete set of isolates and population subsets was analyzed for multilocus linkage disequilibrium (Table 3). Figure 2 shows the allele mismatch distribution, which is bimodal in shape. Among the 43 ETs, 903 pairwise comparisons were possible. On average, the isolates differed at 8.25 of the 15 loci examined. The ratio of V_O to V_E was 2.27; therefore, the I_A was 1.27.

The Monte Carlo procedure indicated that the differences between the observed and expected variances were significant ($P < 0.001$) on the basis of 10^4 iterations. This indicates a significant degree of linkage disequilibrium among the 15 loci studied. When we analyzed different population subgroups, the results were similar (Table 3), with each of them, except the

TABLE 3. Multilocus linkage disequilibrium analysis of *H. influenzae*

Isolate group[a]	No. of ETs	No. of pairs of ETs	X^b	V_O	V_E	95% confidence limits of V_E	I_A	P^c
I	21	210	8.99	7.43	3.22	1.28–5.15	1.31	<0.001
II	22	231	7.58	7.72	3.20	1.32–5.08	1.41	<0.001
BIOI	11	55	9.95	7.48	2.94	0.50–5.39	1.54	<0.001
BIOII	16	120	8.94	5.57	3.12	0.98–5.26	0.79	<0.001
BIOIII	7	21	4.10	2.56	1.88	−0.01–3.77	0.36	<0.2
Resistant	32	496	8.57	7.97	3.24	1.67–4.82	1.46	<0.001
NonResistant	11	55	7.16	3.88	2.83	0.49–5.17	0.37	<0.001
Noninvasive	38	703	8.11	8.56	3.24	1.81–4.67	1.64	<0.001
All	43	903	8.25	7.33	3.23	1.88–4.60	1.27	<0.001

[a] I and II, groups I and II of the dendrogram, respectively; BIOI, -II, and -III, biotypes I, II, and III, respectively.
[b] Mean number of allelic mismatches between pairs of ETs among 15 loci.
[c] Probability of rejecting by chance alone the null hypothesis that $V_O = V_E$ (V_O based on 10,000 iterations of the Monte Carlo procedure).

biotype III subset, showing a significant degree of linkage disequilibrium.

DISCUSSION

Although bacteria are microorganisms that reproduce asexually and therefore a clonal structure would be anticipated, recent studies consider that bacterial population structures range from strictly clonal to panmictic (6). Bacterial species in which recombination appears to be common show a high degree of variability, but the frequency of horizontal gene transfer in nature is usually too low to destroy linkage disequilibrium (19). However, some bacterial species such as *Neisseria gonorrhoeae*, *Bacillus subtilis*, and *Rhizobium meliloti* show a nonclonal structure (4, 6). Most of these are naturally transformable, but transformation per se, like other natural mechanisms of genetic interchange, does not imply extensive recombination in nature, since a naturally transformable bacterium such as *H. influenzae* b (9) is clonal (10).

The time of maintenance of specific multilocus gene combinations, the clone persistence time, can vary among species. Some evidence suggests that collectively the persistence time of *H. influenzae* is less than that of *Escherichia coli* clones (12), but even though the distribution of alloenzyme pairs appears to be closer to random for *H. influenzae* than it is for *E. coli*, evidence for clonal structure remains.

The study of allelic variation by MLEE has yielded significant insights into the genetic diversity, systematics, and population structure of bacteria. MLEE provides a rapid analysis of a large number of individuals and is an excellent method for assessing the genetic structure of a population. Although this method is able to detect no less than 80 to 90% of the amino acid substitutions responsible for allelic variations at the different loci (13), some authors have suggested that MLEE underestimates polymorphism, since apparently the deduced amino acid sequences encoded by some genes showed more variation than MLEE finds (2, 5).

In our study, 33 of the 44 nontypeable *H. influenzae* isolates were chosen because they represent a group of multiresistant strains. We wanted to see if the selective pressure of multiresistance could break the apparently clonal structure of *H. influenzae*. It must be considered that the 44 serologically nontypeable isolates, despite being isolated from different clinical sources, constitute a small sample, although the restricted character of the collection probably does not affect the qualitative conclusions.

The average genetic diversity, the number of alleles per locus, and the genotypic diversity of multiresistant isolates were similar to those given by other authors for the total population of *H. influenzae* (8, 12). The slight differences in these parameters shown by Porras et al. (12) could be due to the fact that they used only six enzymes. Genotypic diversity was very high ($G = 0.93$), with values approaching the theoretical maximum ($G = 0.98$), although isolates were derived from the same geographic area. High local genotypic diversity in a bacterial population such as we observed might result from the coexistence of a large number of independent clones of similar abundance, and the presence of extensive linkage disequilibrium observed is consistent with this explanation.

The dendrogram derived in the present study shows no association among isolates, with regard to source or biotype. Similarly, susceptible strains are distributed randomly (Fig. 1). The fact that the dendrogram presents two patterns suggests that there are two populations with different genetic structures (18). Some authors have suggested that a typical clonal population with limited genetic exchanges is characterized by a deep but limited branching (tree-like) pattern, whereas a freely recombining population typically results in a dendrogram that shows an extensive branching (bush-like) pattern (19). Nevertheless, in our results the independent analyses of subgroup I and II did not reveal significant differences in their genetic structure.

The bimodal shape and the extensive variance exhibited by the allele mismatch distribution for all the 43 ETs of nontypeable *H. influenzae* isolates are typical of a clonal population. However, other authors have reported a unimodal distribution among nontypeable *H. influenzae* mismatches (8). Evidence for clonal proliferation is also provided by the multilocus linkage disequilibrium calculations, which reveal a significant level of association between alleles when population samples are subjected to analysis. Our results indicated that values for I_A were significantly greater than zero, the value expected for absolute equilibrium. Nevertheless, when we analyzed biotype subsets, there was some doubt as to the strict clonality of nontypeable *H. influenzae* isolates. Although isolates belonging to biotypes I and II seem to be clonal, the biotype III subgroup showed a V_O (2.56) that falls within the confidence limits of the test (0.14 and 3.78), and although I_A was different from zero (0.36), we can assume that there is little linkage disequilibrium.

These results would agree with the previous hypothesis that although the population of nontypeable *H. influenzae* might have great variability, when it is subjected to a strong selective pressure, as in the case of a multiresistance that makes the population more adapted to a specific habitat, the most effective individuals are selected and that these seem to have a clonal spread. Highly fit genotypes should not be broken by recombination when they are clonally preserved in the population. Furthermore, the relatively extensive genetic diversity among nontypeable *H. influenzae* isolates might be due to the fact that these strains probably belong to a larger population of nonpathogenic *H. influenzae*. Similar conclusions have been reported by Musser for *H. influenzae* biogroup aegyptius (7). Occasionally, some of these nonpathogenic strains acquire the ability to produce an infectious process or injury to the host allows them to reach areas that are not usually accessible. When we study pathogenic *H. influenzae* we are selecting a population; thus, the sampling is not random.

One challenge now is to understand when, how, and under what conditions the mechanisms of genetic interchange among bacteria have influenced this course of evolution.

ACKNOWLEDGMENTS

We thank Josep Campos (Centro Nacional de Microbiología, Virología e Inmunología Sanitarias, Madrid, Spain) for kindly supplying the strains. We are also grateful to Steve Strain (Tufts University) for his help on data analysis and to Robin Rycroft for his help on preparing the manuscript.

This work was supported by grants DGCYT PM91-0179-C03-02 of the Ministerio de Educación y Ciencia and GRC-UB-02564 of the University of Barcelona.

REFERENCES

1. **Brown, A. H. D., M. W. Feldman, and E. Nevo.** 1980. Multilocus structure of natural populations of *Hordeum spontaneum*. Genetics **96:**523–536.
2. **Desjardins, P., B. Picard, B. Kaltenböck, J. Elion, and E. Denamur.** 1995. Sex in *Escherichia coli* does not disrupt the clonal structure of the population: evidence from random amplified polymorphic DNA and restriction-fragment-length polymorphism. J. Mol. Evol. **41:**440–448.
3. **Gargallo, D., J. G. Lorén, J. Guinea, and M. Viñas.** 1987. Glucose-6-phosphate dehydrogenase alloenzymes and their relationship to pigmentation in *Serratia marcescens*. Appl. Environ. Microbiol. **53:**1983–1986.
4. **Leung, K., S. R. Strain, F. J. de Bruijn, and P. J. Bottomley.** 1994. Genotypic and phenotypic comparisons of chromosomal types within an indigenous soil population of *Rhizobium leguminosarum* bv. trifolii. Appl. Environ. Microbiol. **60:**416–426.

5. **Maynard Smith, J., and C. G. Dowson.** 1991. Localized sex in bacteria. Nature (London) **349:**29–31.
6. **Maynard Smith, J., N. H. Smith, M. O'Rourke, and B. G. Spratt.** 1993. How clonal are bacteria? Proc. Natl. Acad. Sci. USA **90:**4384–4388.
7. **Musser, J. M.** 1996. Molecular population genetic analysis of emerged bacterial pathogens: selected insights. Emerging Infect. Dis. **2:**1–17.
8. **Musser, J. M., J. S. Barenkamp, D. M. Granoff, and R. K. Selander.** 1986. Genetic relationships of serologically nontypable and serotype b strains of *Haemophilus influenzae*. Infect. Immun. **52:**183–191.
9. **Musser, J. M., J. S. Kroll, D. M. Granoff, E. F. Moxon, B. R. Brodeur, J. Campos, H. Dabernat, W. Frederiksen, J. Hamel, G. Hammond, E. A. Høiby, K. E. Jonsdottir, M. Kabeer, I. Kallings, W. N. Khan, M. Killian, K. Knowles, H. J. Koornhof, B. Law, K. I. Li, J. Montgomery, P. E. Pattison, J. C. Piffaretti, A. K. Takala, M. L. Thong, R. A. Wall, J. I. Ward, and R. K. Selander.** 1990. Global genetic structure and molecular epidemiology of encapsulated *Haemophilus influenzae*. Rev. Infect. Dis. **12:**75–111.
10. **Musser, J. M., J. S. Kroll, E. F. Moxon, and R. K. Selander.** 1988. Clonal population structure of encapsulated *Haemophilus influenzae*. Infect. Immun. **56:**1837–1845.
11. **Nei, M.** 1978. Estimation of average heterozygosity and genetic distance from a small sample of individuals. Genetics **89:**583–590.
12. **Porras, O., D. A. Caugant, B. Gray, T. Lagergård, B. R. Levin, and C. Svanborg-Edén.** 1986. Difference in structure between type b and nontypable *Haemophilus influenzae* populations. Infect. Immun. **53:**79–89.
13. **Selander, R. K., D. A. Caugant, H. Ochman, J. M. Musser, M. N. Gilmour, and T. S. Whitmann.** 1986. Method of multilocus enzyme electrophoresis for bacterial population genetics and systematics. Appl. Environ. Microbiol. **51:**873–878.
14. **Selander, R. K., D. A. Caugant, H. Ochman, J. M. Musser, M. N. Gilmour, and T. S. Whitmann.** 1987. Population genetics of pathogenic bacteria. Microb. Pathog. **3:**1–7.
15. **Seuba, T.** 1994. Estructura poblacional i mecanisme enzimàtic de la resistència als antibiòtics aminoglicòsids en *Haemophilus influenzae*. Ph.D. thesis. Universitat de Barcelona, Barcelona, Spain.
16. **Souza, V., T. T. Neguyeu, R. R. Hudson, D. Pinero, and R. E. Lemski.** 1992. Hierarchical analysis of linkage disequilibrium in *Rhizobium* populations: evidence for sex? Proc. Natl. Acad. Sci. USA **89:**8389–8393.
17. **Turk, D. C.** 1982. Clinical importance of *Haemophilus influenzae*, p. 1–9. *In* S. H. Shell and P. F. Wright (ed.), *Haemophilus influenzae*: epidemiology, immunology and prevention of disease. Elsevier, Amsterdam.
18. **Whittam, T. S.** 1992. Sex in the soil. Curr. Biol. **2:**676–678.
19. **Wise, M. G., L. J. Shimkets, and J. V. McArthur.** 1995. Genetic structure of a lotic population of *Burkholderia* (*Pseudomonas*) *cepacia*. Appl. Environ. Microbiol. **61:**1791–1798.
20. **Zouros, E., G. B. Golding, and T. F. C. MacKay.** 1975. The effect of combining alleles into electrophoretic classes on detecting linkage disequilibrium. Genetics **85:**543–550.