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Genetic Relationships of Serologically Nontypable and Serotype b Strains of *Haemophilus influenzae*

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A collection of 242 strains of *Haemophilus influenzae*, including 65 nontypable (unencapsulated) isolates and 177 encapsulated serotype b isolates recovered largely from children with invasive and noninvasive diseases in the United States, was characterized by the electrophoretic mobilities of 15 metabolic enzymes presumably encoded by chromosomal genes. All enzymes were polymorphic for three to seven electromorphs, and 94 distinctive multilocus genotypes (electrophoretic types [ETs]) were distinguished, among which mean genetic (allelic) diversity was 0.500. Isolates recovered from cases of invasive or noninvasive diseases did not differ significantly in level of genetic variation. The observation that 29 ETs were represented exclusively by serotype b isolates and that each of the 65 nontypable isolates was of a unique ET strongly confirmed the hypothesis that unencapsulated clinical isolates are not merely phenotypic variants of the common serotype b cell lines. Rather, the two types of isolates are distinctive subsets of the multilocus chromosomal genotypes of the species as a whole. Serotype b capsule occurred in three groups of isolates that are distantly related in multilocus enzyme genotype. Isolates of four closely related nontypable biotype IV ETs associated with obstetrical infections or neonatal bacteremia were highly divergent from all others examined and may be specifically distinct. A phylogenetic scenario was proposed in which the ancestor of *H. influenzae* was encapsulated and the nontypable clones arose by convergent evolutionary loss of the ability to synthesize or extracellularly express a polysaccharide capsule.

Haemophilus influenzae is a small, gram-negative coccobacillus that causes a variety of diseases in humans, especially children (24, 31). Strains recovered from patients experiencing invasive episodes (cellulitis, epiglottitis, septicemia, and meningitis) usually express the type b capsular polysaccharide, which is one of six structurally and antigenically distinct polysaccharide capsules, designated a through f, produced by *H. influenzae* (14). However, most strains associated with noninvasive diseases (otitis media, conjunctivitis, chronic bronchitis, and pneumonia) are unencapsulated and therefore cannot be typed with antisera specific for any of the recognized capsule types (14, 23, 31).

Some investigators have suggested that serologically nontypable isolates are phenotypic variants of encapsulated strains (11, 27). Recently, however, analyses of restriction modification systems (29), outer membrane protein (OMP) profiles (4, 18), and other characters (9, 15) have indicated that most unencapsulated clinical isolates are genetically differentiated from the common serotype b cell lines and have suggested that nontypable isolates are, as a group, genetically much more diverse than serotype b isolates. Although these studies have contributed to an understanding of nontypable strains of *H. influenzae*, several basic questions concerning this clinically important group of organisms remain unanswered. (i) What is the extent of multilocus genotypic diversity among nontypable isolates? (ii) What are the genetic and evolutionary relationships between unencapsulated and encapsulated strains? (iii) Are nontypable strains with particular multilocus genotypes especially likely to cause invasive disease?

We recently described a multilocus genetic typing system for *H. influenzae* based on electrophoresis of chromosomally encoded enzymes (20). This system was employed to estimate genetic diversity and relationships among serotype b isolates, thereby providing a population genetic framework for the analysis of variation in OMPs, biotypes, and other characters.

The objectives of the research reported here were to examine the genetic diversity and structure of populations of nontypable strains of *H. influenzae* and to determine the relationships of nontypable strains and serotype b strains by assessing allelic variation at a large number of chromosomal enzyme loci. Our analysis demonstrated very extensive genetic diversity among nontypable isolates and confirmed earlier indications that most nontypable isolates recovered from children with clinical disease are genetically distinct from those possessing the serotype b polyribosylribitol-phosphate capsule. Additionally, we present evidence that some isolates currently classified as *H. influenzae* biotype IV are differentiated from other strains of *H. influenzae* to a degree that may warrant specific status.

MATERIALS AND METHODS

Isolates. This study was based on a collection of 242 strains of *H. influenzae* isolated between 1937 and 1985, which included 65 nontypable isolates and 177 serotype b isolates (Table 1).

The nontypable isolates were recovered during episodes of clinical disease in children in Canada, Massachusetts, Maryland, Connecticut, Minnesota, Missouri, Ohio, Texas, Alabama, and the District of Columbia. Of these isolates, 40 were cultured from middle ear effusions collected by tympanocentesis, 20 were from blood, and 5 were from cerebrospinal fluid. Most patients contributed a single isolate. However, the sample included eight pairs of isolates obtained from individuals with recurrent episodes of otitis disease; these pairs were selected for study because they had different OMP profiles and electrophoretic enzyme patterns

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TABLE 1. Allele profile, serotype, biotype, and source for 242 isolates of 94 ETs of *H. influenzae*

ET	Reference isolate	No. of isolates	Allele at indicated enzyme locus															Serotype	Biotype	Source[a]
			PGI	MAE	MDH	G6P	GOT	ADK	6PG	PE1	PE2	LAP	PGM	CAT	GLD	G3P	FUM			
1	851	83	1	5	4	3	3	2	2	3	5	1	4	2	4	2	2	b	I	CSF (Mo.)[b]
2	1338	2	1	5	4	3	3	2	4	3	5	1	4	2	4	2	2	b	I	INV (Wis.)[c]
3	3279	1	1	5	4	1	3	2	2	3	5	1	4	2	4	2	2	b	I	BLD (Minn.)
4	3765	1	6	5	4	3	3	2	2	3	5	1	4	2	4	2	2	b	I	INV (Tex.)
5	3752	4	1	5	4	3	5	2	2	3	5	1	4	2	4	2	2	b	I	INV (Tex.)[d]
6	RSM3B	1	1	5	4	3	5	2	4	3	5	1	4	2	4	2	2	b	I	CSF (Mass.)
7	3805	1	4	5	4	3	5	2	2	3	5	1	4	2	4	2	2	b	I	INV (Tex.)
8	3264	2	1	5	4	3	3	2	1	1	5	1	4	2	4	2	2	b	I	CSF (Minn.)[e]
9	3718	1	1	5	4	3	3	2	1	12	5	1	4	2	4	2	2	b	I	INV (Tex.)
10	3438	1	1	5	4	3	1	2	1	3	5	2	4	2	4	2	2	b	I	CSF (Minn.)
11	3219B	1	5	3	4	3	4	2	2	3	5	1	4	2	4	2	2	NT[f]	II	Ear (Tex.)
12	3686	1	1	3	4	3	5	2	2	3	4	1	4	2	4	2	2	NT	III	BLD (Tex.)
13	3242A	1	1	3	4	7	3	2	2	2	4	1	4	2	4	2	2	NT	III	Ear (Ohio)
14	1333	36	4	5	4	3	1	2	2	3	5	3	4	2	4	2	2	b	I	INV (Wis.)[g]
15	1445	1	4	5	4	3	1	2	2	3	5	3	4	2	4	2	3	b	II	CSF (Tex.)
16	808	13	4	5	4	6	1	2	2	3	5	3	4	2	4	2	2	b	II	CSF (Md.)[h]
17	3715	1	4	5	4	3	1	2	2	3	1	3	4	2	4	2	2	b	I	INV (Tex.)
18	3207	1	4	5	4	3	1	2	2	3	5	3	4	2	4	1	2	b	II	INV (Mo.)
19	3516	1	4	5	4	3	1	4	2	3	5	3	4	2	4	2	2	b	I	BLD (Minn.)
20	1059	2	4	5	2	3	1	2	2	3	5	3	4	2	4	2	2	b	I	CSF (La.)[i]
21	1211	2	4	5	4	3	1	2	2	3	5	3	6	2	4	2	2	b	I	INV (Alaska)[j]
22	3254	1	4	5	4	3	8	2	2	3	5	3	4	2	4	2	2	b	II	BLD (Minn.)
23	1993	1	4	5	4	3	1	2	3	3	5	3	4	2	4	2	2	b	I	INV (Mo.)
24	3012	1	4	5	4	6	1	2	3	3	5	3	4	2	4	2	2	b	II	NP (Mo.)
25	1287	1	4	5	3	3	1	2	2	3	5	3	4	2	4	2	1	b	I	CSF (Minn.)
26	1862	1	3	4	4	3	2	2	2	3	5	3	2	3	4	2	2	NT	II	CSF (D.C.)
27	3219C	1	3	5	4	3	2	2	2	1	5	3	2	3	4	2	2	NT	II	Ear (Tex.)
28	3655	1	4	5	4	3	2	2	2	1	5	3	2	2	4	2	2	NT	II	Ear (Mo.)
29	1667	1	1	3	4	3	2	2	2	3	5	1	2	2	4	2	2	NT	III	Ear (Mo.)
30	3246A	1	1	3	4	3	2	2	3	3	2	3	2	2	4	2	2	NT	III	Ear (Ohio)
31	3179B	1	1	5	4	6	2	2	2	1	5	3	4	2	4	2	2	NT	V	Ear (Mass.)
32	3640	1	1	3	4	7	2	2	2	1	5	3	4	2	4	2	2	NT	V	Ear (Mo.)
33	1484A	1	1	5	4	3	2	2	2	4	5	2	4	2	4	2	3	NT	II	BLD (Conn.)
34	1674	1	5	5	4	7	2	2	2	3	5	1	4	2	4	2	2	NT	III	BLD (Mo.)
35	3233A	1	7	5	4	7	2	2	1	3	5	1	4	2	4	2	2	NT	III	Ear (Tex.)
36	3894	1	3	5	2	7	2	2	2	3	5	1	4	2	4	2	2	NT	III	BLD (Mo.)
37	3221B	1	3	5	4	7	2	2	2	3	5	1	3	2	4	2	2	NT	III	Ear (Tex.)
38	3239A	1	3	5	3	7	2	2	2	3	5	1	3	2	9	2	2	NT	III	Ear (Ohio)
39	3179A	1	3	5	4	7	2	2	2	3	5	1	4	2	9	2	3	NT	III	Ear (Mass.)
40	3181B	1	3	5	4	7	2	2	2	3	5	1	4	2	9	2	4	NT	III	Ear (Mass.)
41	1732	1	5	5	2	7	2	2	2	3	5	1	4	2	4	2	1	NT	III	BLD (Md.)
42	3181A	1	4	4	2	7	2	2	2	3	5	1	4	2	4	2	1	NT	III	Ear (Mass.)
43	3A	1	3	5	2	6	2	2	4	3	5	3	4	2	4	2	2	NT	V	Ear (Mo.)
44	3180A	1	6	4	4	7	2	2	2	3	5	3	4	4	4	2	2	NT	III	Ear (Mass.)
45	1161B	1	7	5	4	7	2	2	2	3	5	3	5	4	4	2	2	NT	III	BLD (Mo.)
46	3800	9	5	5	3	6	1	2	2	3	5	2	6	2	4	2	2	b	III	INV (Tex.)[k]
47	1209	2	5	5	3	6	1	2	2	3	5	2	6	2	4	2	1	b	II	INV (Calif.)[l]
48	1971	2	5	5	3	6	1	2	2	3	5	1	6	2	4	2	2	b	II	INV (Mo.)[m]
49	1856	1	1	3	4	7	2	2	2	3	5	1	4	3	4	2	1	NT	II	BLD (Mo.)
50	3245A	1	6	3	4	7	2	2	8	3	5	1	4	3	4	2	3	NT	II	Ear (Ohio)
51	3626	1	1	3	4	8	2	2	2	1	5	1	4	2	4	2	1	NT	II	BLD (Minn.)
52	1855	1	5	3	4	3	2	2	2	1	5	1	4	2	4	2	1	NT	II	BLD (Mo.)
53	3224A	1	3	3	3	3	2	2	2	3	5	1	4	2	4	2	1	NT	II	Ear (Tex.)
54	3220A	1	3	3	4	7	2	2	2	1	5	1	4	2	1	2	2	NT	II	Ear (Tex.)
55	3222A	1	3	3	4	7	2	2	2	1	5	1	4	2	1	2	1	NT	II	Ear (Tex.)
56	3893	1	1	3	2	3	5	2	2	3	5	1	4	2	4	2	1	NT	II	BLD (Mo.)
57	3224B	1	1	3	4	3	5	2	2	3	2	1	3	2	4	2	1	NT	III	Ear (Tex.)
58	1635	1	1	3	3	8	2	2	2	1	5	1	4	2	4	2	2	NT	II	BLD (Mo.)
59	3182A	1	1	3	3	8	2	2	2	2	1	1	4	2	4	2	2	NT	III	Ear (Mass.)
60	3232B	1	3	3	3	8	2	2	1	4	5	1	4	2	4	2	2	NT	II	Ear (Tex.)
61	3675	1	3	3	3	8	3	2	2	3	5	1	4	2	4	2	2	NT	II	BLD (Minn.)
62	3241A	1	5	3	3	8	4	2	4	3	5	1	4	2	4	2	2	NT	II	Ear (Ohio)
63	3243B	1	5	3	3	8	3	2	4	2	5	1	4	2	4	2	2	NT	II	Ear (Ohio)
64	3240A	1	7	3	3	8	3	2	3	1	5	2	4	3	4	2	2	NT	II	Ear (?)
65	1636	1	5	3	4	2	2	2	2	2	5	1	4	2	4	2	2	NT	II	BLD (Mo.)
66	1328	1	5	3	4	2	2	2	2	2	5	2	5	2	4	2	2	NT	II	BLD (Mo.)
67	3247A	1	5	3	4	8	2	2	3	2	5	2	4	4	4	2	2	NT	II	Ear (Ohio)
68	1860	1	5	2	4	3	3	2	4	1	1	1	5	2	1	2	2	NT	I	CSF (Canada)

(*Continued on following page*)

TABLE 1—(*Continued*)

ET	Reference isolate	No. of isolates	Allele at indicated enzyme locus															Serotype	Biotype	Source[a]
			PGI	MAE	MDH	G6P	GOT	ADK	6PG	PE1	PE2	LAP	PGM	CAT	GLD	G3P	FUM			
69	3232A	1	3	5	4	7	3	2	2	1	5	1	5	2	4	2	2	NT	I	Ear (Tex.)
70	3230B	1	1	3	4	6	3	2	2	1	5	1	5	3	4	2	2	NT	IV	Ear (Ala.)
71	3220B	1	5	5	2	3	2	2	2	3	4	3	2	2	4	2	1	NT	II	Ear (Tex.)
72	3183A	1	4	4	2	3	2	2	2	3	4	3	2	2	4	1	1	NT	II	Ear (Mass.)
73	3646	1	4	4	2	3	2	2	2	3	4	3	2	2	4	2	2	NT	II	Ear (Mo.)
74	3178A	1	1	4	4	3	2	2	2	3	4	3	2	2	4	1	2	NT	II	Ear (Mass.)
75	3184A	1	1	4	4	8	5	2	2	3	4	3	2	2	4	1	2	NT	II	Ear (Mass.)
76	3639	1	6	4	3	8	3	2	2	1	4	3	4	2	4	2	2	NT	I	Ear (Mo.)
77	3221A	1	6	3	4	2	5	2	4	1	3	3	5	2	6	2	2	NT	IV	Ear (Tex.)
78	3222B	1	5	3	4	3	5	2	4	4	5	1	4	2	4	2	1	NT	II	Ear (Tex.)
79	1276	1	5	3	4	3	5	2	4	4	5	2	5	2	3	2	2	NT	II	BLD (Mo.)
80	820	1	3	5	2	3	1	2	3	4	4	2	3	3	1	2	2	NT	III	CSF (Mo.)
81	1396A	1	3	3	4	7	2	2	3	4	4	2	5	3	4	2	2	NT	II	CSF (Minn.)
82	1136B	1	3	3	4	6	3	2	3	3	6	3	4	2	5	2	2	NT	III	BLD (Mo.)
83	3248A	1	3	3	4	8	3	2	3	3	6	2	3	3	4	2	2	NT	III	Ear (Ohio)
84	3230A	1	3	3	4	7	1	2	3	2	1	3	7	4	4	2	1	NT	I	Ear (Ala.)
85	3185A	1	3	4	2	3	2	2	1	3	5	1	3	2	1	3	1	NT	III	Ear (Mass.)
86	1396B	1	3	5	3	3	1	1	5	1	1	2	6	3	4	2	2	NT	II	CSF (Minn.)
87	3491	1	4	2	4	1	1	1	4	1	5	1	1	3	4	2	2	b	I	CSF (Minn.)
88	3191	3	4	2	4	1	1	1	3	2	5	1	1	3	4	2	2	b	I	CSF (Md.)[n]
89	3205	1	4	2	4	1	1	1	3	2	5	1	1	3	4	3	2	b	IV	CSF (D.C.)
90	3873	1	4	2	4	1	6	1	4	1	1	2	2	3	4	2	2	b	IV	(N.Y.)
91	1673A	1	6	5	2	3	5	3	6	4	2	3	2	2	1	2	2	NT	IV	BLD (Tex.)
92	1595	1	6	5	2	3	5	3	6	4	2	3	2	3	1	2	2	NT	IV	BLD (Mo.)
93	799	1	6	5	2	3	5	3	6	5	2	3	2	3	1	2	2	NT	IV	BLD (Mo.)
94	1610	1	6	5	2	3	5	3	6	4	2	3	4	2	4	2	2	NT	IV	BLD (Mo.)

[a] CSF, Cerebrospinal fluid; INV, invasive, but site of isolation unrecorded; BLD, blood; NP, nasopharynx.
[b] Plus 46 isolates, CSF; 18, INV; 17, BLD; 1, NP; from Alaska, California, Colorado, District of Columbia, Illinois, Louisiana, New Hampshire, New York, Maryland, Minnesota, Missouri, North Carolina, Ohio, Tennessee, Texas, Washington, Wisconsin.
[c] Plus isolate 1334, INV (Wisconsin).
[d] Plus isolate 3365, CSF (Minnesota); 3745, INV (Texas); 3790, INV (Texas).
[e] Plus isolate 628, BLD (Missouri).
[f] NT, Nontypable.
[g] Plus 14 isolates, CSF; 12, INV; 6, BLD; 2, NP; 1, eye; from California, District of Columbia, Florida, Illinois, Maryland, Minnesota, Missouri, Ohio, Tennessee, Texas, Wisconsin, Australia, the Netherlands, Thailand.
[h] Plus 4 isolates, CSF; 3, BLD; 4, NP; 1, eye; from Missouri, Minnesota, Texas.
[i] Plus isolate 3799, INV (Texas).
[j] Plus isolate 1256, INV (Rhode Island).
[k] Plus 1 isolate, CSF (Minnesota); 6, INV (Texas); 1, BLD (Florida).
[l] Plus isolate 3192, BLD (District of Columbia).
[m] Plus isolate 1481A, BLD (Illinois).
[n] Plus isolate 1206, INV (Alaska); 1148, CSF (Missouri).

and were, therefore, considered to represent separate infections (5). Most of the other nontypable isolates were earlier described and typed for electrophoretic OMP pattern by Barenkamp et al. (4).

The sample of serotype b isolates, consisting of 173 isolates from 19 states and Canada, 2 from the Netherlands, and 1 each from Thailand and Australia, was earlier analyzed for multilocus enzyme variation by Musser et al. (20). Each serotype b isolate was obtained from a separate child; 167 isolates were cultured from blood or cerebrospinal fluid, 8 were from the nasopharynx, and 2 were from the eye.

Preparation of lysates for electrophoresis. Each isolate was grown for 6 h at 35°C in 150 ml of brain heart infusion broth supplemented with NAD and hemin, each at 2 μg/ml. Cells were harvested by centrifugation, suspended in 2 ml of phosphate-buffered saline, and sonicated (Branson Sonifier Cell Disruptor, model 200, with microtip) for 30 s at 50% pulse, with ice-bath cooling. After centrifugation at 20,000 × *g* for 20 min at 4°C, the clear supernatant (lysate) was stored at −70°C.

Electrophoresis and specific enzyme staining. Techniques of horizontal starch-gel electrophoresis and the demonstration of specific enzyme activity were similar to those described by Selander et al. (Appl. Environ. Microbiol., in press). The following 15 enzymes were assayed: phosphoglucose isomerase (PGI), NADP-dependent malic enzyme (MAE), NAD-dependent malate dehydrogenase (MDH), glucose-6-phosphate dehydrogenase (G6P), 6-phosphogluconate dehydrogenase (6PG), glyceraldehyde-3-phosphate dehydrogenase (G3P), glutamate dehydrogenase (GLD), glutamic oxaloacetic transaminase (GOT), adenylate kinase (ADK), two peptidases (PE1 and PE2), leucine aminopeptidase (LAP), phosphoglucomutase (PGM), catalase (CAT), and fumarase (FUM).

GLD and FUM were electrophoresed in a Poulik buffer system (Tris citrate gel buffer, pH 8.7; borate electrode buffer, pH 8.2); G6P, 6PG, PE1, and PE2 were electrophoresed in a Tris maleate buffer (pH 7.4); PGM, MAE, MDH, GOT, and PGI were electrophoresed in a Tris citrate buffer (pH 8.0); and for G3P, ADK, CAT, and LAP, we used a potassium phosphate buffer system (gel buffer, pH 7.0; electrode buffer, pH 6.7).

For each enzyme, distinctive mobility variants (electromorphs), numbered in order of decreasing rate of anodal migration, were equated with alleles at the corresponding structural gene locus, and an absence of enzyme activity was

attributed to a null allele, scored as zero. Because virtually all isolates showed activity for all 15 enzymes, the corresponding structural gene loci are presumed to be located on the chromosome rather than on plasmids.

Each isolate was characterized by its combination of alleles at the 15 enzyme loci (Table 1). Distinctive combinations of alleles, corresponding to unique multilocus genotypes, were designated as electrophoretic types (ETs) (7). (The numerical designations of ETs employed in the present study do not correspond to those used by Musser et al. [20].)

In our earlier study of electrophoretically demonstrable allelic variation in serotype b isolates of *H. influenzae* (20), we analyzed indophenol oxidase in addition to the 15 enzymes listed above. However, indophenol oxidase activity frequently was too diffuse to be scored in the unencapsulated isolates and, therefore, the IPO locus was omitted from the present analysis. Consequently, 3 of the 32 ETs identified by Musser et al. (20) on the basis of distinctive combinations of alleles at the indophenol oxidase and other loci were not recognized in the present study.

Serotyping. Isolates were serotyped, as previously described (4), with commercial type-specific rabbit antisera prepared against *H. influenzae* capsular types a through f (Burroughs Wellcome Co., Research Triangle Park, N.C.) or with reference antisera provided by the Centers for Disease Control, Atlanta, Ga.

Biotyping. Isolates were tested for urease activity, indole production, and ornithine decarboxylase activity (2, 3, 12, 13). Five biotypes (I through V), corresponding to distinctive patterns of positive and negative scores for these tests, were distinguished.

Statistical analyses. Genetic diversity at a locus among ETs or isolates was calculated as $h = (1 - \Sigma x_i^2)(n/n - 1)$, where x_i is the frequency of the ith allele and n is the number of ETs or isolates in a sample (21). Mean genetic diversity ($\bar{h}$) is the arithmetic average of h values for all loci.

Genetic distance (D) between pairs of ETs was calculated as either the proportion or the number of loci at which dissimilar alleles were represented (mismatches), and clustering of ETs from a matrix of pairwise coefficients of genetic distance was performed by the average-linkage method (28).

RESULTS

ETs and genetic diversity. In the collection of 242 isolates of *H. influenzae* (Table 1), all 15 enzymes assayed were polymorphic for from three to seven alleles encoding electrophoretically distinctive variants. The average number of alleles per locus was 5.0.

By comparing the allele profiles of isolates typed for all 15 enzymes, we identified 94 distinctive multilocus combinations or ETs (Table 1), among which mean genetic diversity per locus ($\bar{h}$) was 0.500 (interlocus variance, s^2 = 0.041) (Table 3). There was significantly less diversity among the 242 isolates themselves ($\bar{h}$ = 0.320), reflecting the circumstance that 13 of the ETs were represented by multiple isolates, ranging in number from 2 to 83.

Of the 94 ETs, 29 were represented exclusively by serotype b isolates, and the remaining 65 ETs were represented by isolates that failed to react with antisera specific for all known *H. influenzae* capsular polysaccharides. Each nontypable isolate had a distinctive ET, and there was no case of sharing of multilocus genotype between serotype b and unencapsulated isolates.

Genetic relationships among the 94 ETs are shown in the dendrogram in Fig. 1. The smallest observed genetic distance (0.07) corresponds to a single-locus difference between

TABLE 2. Frequencies of alleles at seven enzyme loci in isolates and ETs of nontypable and serotype b *H. influenzae*

Locus and allele[a]	Frequency of allele			χ^2 test for ETs[b] (df)
	Nontypable isolates or ETs (n = 65)	Serotype b Isolates (n = 177)	Serotype b ETs (n = 29)	
PGI				32.20** (3)
1	0.246	0.537	0.276	
3	0.323	0.000	0.000	
4	0.062	0.384	0.586	
5	0.200	0.073	0.103	
6	0.123	0.006	0.034	
7	0.046	0.000	0.000	
MAE				12.28** (1)
2	0.015	0.034	0.138	
3	0.508	0.000	0.000	
4	0.138	0.000	0.000	
5	0.338	0.966	0.862	
G6P				13.12** (2)
1	0.000	0.040	0.172	
2	0.046	0.000	0.000	
3	0.400	0.808	0.655	
6	0.062	0.153	0.172	
7	0.308	0.000	0.000	
8	0.185	0.000	0.000	
GOT				47.06** (3)
1	0.046	0.446	0.621	
2	0.600	0.000	0.000	
3	0.154	0.508	0.207	
4	0.031	0.000	0.000	
5	0.169	0.034	0.103	
6	0.000	0.006	0.034	
8	0.000	0.006	0.034	
PE1				9.12* (2)
1	0.062	0.011	0.069	
2	0.092	0.000	0.000	
3	0.015	0.000	0.000	
4	0.154	0.000	0.000	
5	0.649	0.989	0.931	
6	0.031	0.000	0.000	
PE2				7.69* (2)
1	0.246	0.023	0.103	
2	0.108	0.023	0.069	
3	0.492	0.949	0.793	
4	0.138	0.000	0.000	
5	0.015	0.000	0.000	
12	0.000	0.006	0.034	
GLD				4.50* (1)
1	0.123	0.000	0.000	
3	0.015	0.000	0.000	
4	0.785	1.000	1.000	
5	0.015	0.000	0.000	
6	0.015	0.000	0.000	
9	0.046	0.000	0.000	

[a] Because there are alleles at some loci in *H. influenzae* that are not represented in the present sample of isolates (J. M. Musser, unpublished data), allele numbers are not consecutive for some loci in this table and in Table 1.

[b] Test of significance of observed differences in allele frequencies between samples of nontypable ETs and serotype b ETs. When required, adjacent cells were pooled to provide adequate allele numbers.

*, $P < 0.05$; **, $P < 0.01$.

TABLE 3. Mean genetic diversity per locus ($\bar{h}$) in *H. influenzae* recovered from invasive and noninvasive infections

Category	Isolates			ETs		
	No.	Diversity	Variance	No.	Diversity	Variance
Total[a]	242	0.320	0.042	94	0.500	0.041
Invasive	192	0.262	0.038	53	0.484	0.039
Noninvasive	50	0.466	0.049	41	0.496	0.044
Nontypable	65	0.519	0.037	65	0.519	0.037
Invasive	25	0.551	0.038	25	0.551	0.038
Noninvasive	40	0.496	0.043	40	0.496	0.043
Serotype b	177	0.194	0.041	29	0.354	0.039

[a] Includes both serotype b and nontypable organisms.

ETs, and the largest distance (0.71) to differences at 13 of the 15 loci assayed. At a genetic distance of about 0.30, there were 34 groups or lineages of ETs; 12 clusters consisted of three or more ETs and 6 clusters included four or more ETs. The 29 ETs to which serotype b isolates were assigned are ET-1 through ET-10, ET-14 through ET-25, ET-46 through ET-48, and ET-87 through ET-90. These four groups of ETs correspond to the clusters A, B, C, and D through F, respectively, identified previously by Musser et al. (20). Except for the three nontypable isolates of ET-11, ET-12, and ET-13, no unencapsulated isolate was related to a serotype b isolate at a genetic distance of less than 0.30.

The most divergent strains were of ETs 86 through 94, which were separated from other ETs at a genetic distance of about 0.67. Isolates of ETs 87 through 90 were serotype b and had the rare type 8 OMP profile (2, 3, 8); and isolates of ETs 91 through 94 were nontypable, biotype IV (12), and shared a unique OMP profile (4).

Comparison of allele frequencies between ETs of nontypable isolates and ETs of serotype b isolates revealed major differences involving unique alleles at moderate to high frequencies at four loci, PGI, MAE, G6P, and GOT, and substantial differences at three other loci, GLD, PE1, and PE2 (Table 2).

Genetic variation in relation to capsular phenotype. Mean genetic diversity per locus ($\bar{h}$) among ETs of the nontypable isolates was 0.519, and because each nontypable ET was represented by only a single isolate, mean genetic diversity among isolates was the same as among ETs (Table 3). The comparable value for ETs of the serotype b isolates was 0.354, or 68% of the diversity of the unencapsulated strains. Among serotype b isolates, mean genetic diversity per locus was only 0.194, reflecting the circumstance that, in striking contrast to ETs of the nontypable isolates, many (45%) serotype b ETs were represented by multiple (but epidemiologically unrelated) isolates (20).

The distributions of pairwise genetic distances (D) between ETs and between isolates were markedly dissimilar for type b and nontypable organisms (Fig. 2). For both nontypable ETs and isolates, the distributions were unimodal and identical, and pairs of ETs differed, on average, at 7.7 loci. In contrast, both serotype b ETs and isolates showed trimodal distributions, and pairs of ETs differed, on average, at 5.1 loci. The trimodality of the serotype b distributions reflects the occurrence of several genotypically distinctive groups of ETs in the sample (Fig. 1): (i) a cluster formed by ETs 1 through 10, all of which were relatively closely related to one another and only moderately differentiated from the ETs of a second group of closely related genotypes consisting of ETs 14 through 25; (ii) a cluster including ETs 46 through 48; and (iii) a cluster composed of ETs 87 through 90, which were relatively heterogeneous and only distantly related to other serotype b ETs.

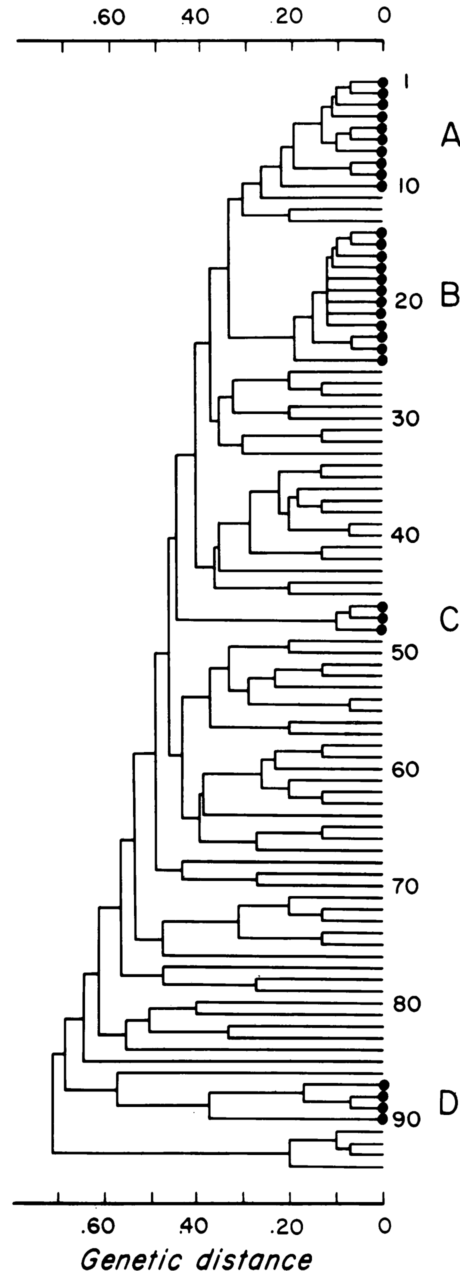

FIG. 1. Genetic relationships among 94 ETs of *H. influenzae*. The dendrogram was generated by the average-linkage method of clustering from a matrix of coefficients of genetic distance, based on 15 enzyme loci. ETs are numbered sequentially from top to bottom, and those represented by serotype b isolates are indicated by dots. Four clusters of ETs represented by serotype b isolates are labeled A through D.

Genetic variation in relation to clinical episode. Within groups of ETs represented by isolates recovered from either invasive or noninvasive disease episodes, there was little if any less genetic diversity per locus (0.484 and 0.496, respectively) than in the total sample of ETs (Table 3), indicating that each of these types of disease is caused by strains of a wide variety of chromosomal enzyme genotypes. Similarly,

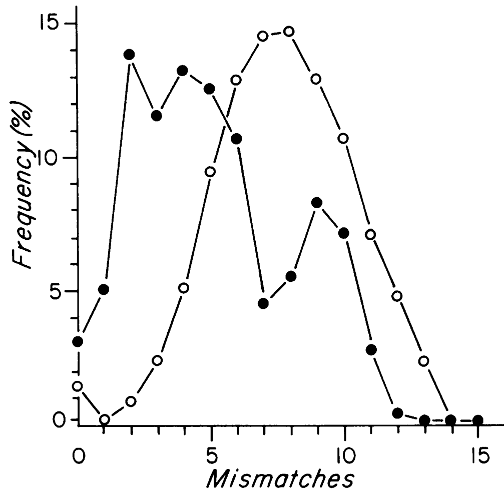

FIG. 2. Distributions of numbers of enzyme loci at which unlike alleles (mismatches) occurred in pairwise comparisons of 65 nontypable ETs and 29 serotype b ETs of *H. influenzae*. Symbols: ○, nontypable; ●, serotype b.

for ETs of nontypable isolates, estimates of mean genetic diversity were high for both the invasive and the noninvasive groups; and for all but one of the loci (ADK), diversity within each source group was closely similar to that for the total sample of nontypable ETs. Hence, there is no evidence that invasive nontypable isolates are, as a group, significantly less variable than those recovered from noninvasive sources.

In contrast, mean genetic diversity among all noninvasive isolates was 0.466, whereas among invasive isolates it was 0.262, or only 56% of that of the invasive strains (Table 3). Furthermore, as noted above, the distributions of pairwise genetic distances (*D*) between isolates were dissimilar in the two groups (Fig. 2) because all noninvasive isolates had unique genotypes, whereas many invasive isolates were of identical multilocus genotype.

Genetic variation in relation to biotype. The frequencies of the various biotypes differed in the serotype b and nontypable samples (Table 1). For example, 72% of the serotype b isolates but only 6% of the nontypable isolates were biotype I. Conversely, 48% of the nontypable isolates but only 21% of the serotype b isolates were biotype II; and the frequencies of biotype III among the nontypable and serotype b isolates were 32 and 3%, respectively. Biotype IV was confined to nontypable isolates of ETs 89 through 94, and biotype V was limited to four isolates representing ETs 14, 31, 32, and 43. For the comparison of serotype b and nontypable isolates biotyped as I, II, and III, $\chi^2_{(2)} = 98.18$, $P < 0.001$.

Estimates of genetic diversity among isolates and ETs of each of five biotypes are presented in Table 4. Disregarding the values for biotype V, which are unreliable because of small sample size, we note that mean genetic diversity per locus among ETs of the same biotype varied from 0.377 in the case of biotype I to 0.607 for biotype IV, with a mean of 0.479, which is 96% of the value (0.500) for the total sample of ETs (Table 3).

DISCUSSION

The multilocus electrophoretic technique attempts to determine overall genetic relatedness among bacterial isolates from examination of a small fraction of the structural gene loci of the genome. Hence, it is noteworthy that estimates of genetic relatedness based on multilocus enzyme electrophoresis for strains of both *Legionella* spp. (26) and *Escherichia coli* (22) have been shown to be positively correlated with measures of similarity in total nucleotide sequences derived from DNA hybridization experiments.

TABLE 4. Mean genetic diversity per locus ($\bar{h}$) in *H. influenzae* classified by biotype

Biotype	Isolates			ETs		
	No.	Diversity	Variance	No.	Diversity	Variance
I	131	0.153	0.023	23	0.377	0.045
II	69	0.385	0.051	39	0.480	0.050
III	26	0.460	0.055	21	0.453	0.041
IV	12	0.562	0.049	8	0.607	0.046
V	4	0.289	0.113	3	0.267	0.114

Limitations of the sample studied. In interpreting the results of our study and evaluating their implications, the composition and character of the collection of isolates examined must be considered. The 177 serotype b isolates were originally chosen for analysis of enzyme variation because they represented a wide variety of geographic areas, predominantly in the continental United States, and had been typed for OMP pattern (20). An analysis of electrophoretic variation in enzymes in approximately 200 additional serotype b isolates (J. M. Musser, unpublished data) indicates that the sample examined in the present study adequately represents the population of invasive serotype b genotypes in the continental United States.

Although the 65 serologically nontypable isolates also were derived from diverse geographic areas in North America, they may not be an adequately representative sample of the nontypable population because of the limited variety of host-site sources from which they were recovered. All but a few of the isolates were obtained from cases of otitis media and bacteremia, and there are no isolates in the sample from cases of conjunctivitis or from the nasopharynx of carriers. For this reason, we suspect that the magnitude of the genotypic variation within the nontypable population of *H. influenzae* in North America is even greater than shown here. Although the somewhat restricted character of the collection may limit the quantitative conclusions drawn from our study, it probably does not affect the qualitative ones.

Variation in capsular phenotype in relation to genetic population structure. On the basis of results obtained from analyses of electrophoretically demonstrable enzyme variation (20), sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of OMPs (4, 18), and other characters (15, 29), it has been suggested that most serologically nontypable isolates of *H. influenzae* recovered from cases of clinical disease in children are not merely common serotype b cell lines that have lost the polysaccharide capsule. The results of the present study of allelic variation at 15 enzyme loci in 65 nontypable isolates recovered from children with various diseases strongly confirms this hypothesis. Indeed, our analysis failed to detect sharing of electrophoretic type between serotype b and nontypable isolates. Taken together, the data indicate that cell lines of serotype b that are frequently recovered from patients in the continental United States and elsewhere (J. M. Musser, unpublished data) are a restricted subset of the multilocus genotypes of the species *H. influenzae* as a whole. However, this finding should not be interpreted to mean that some serologically nontypable

strains are not closely related to serotype b isolates or that clusters of genotypically similar nontypable isolates do not occur. The dendrogram of genetic relationships shown in Fig. 1 includes examples of both situations, most notably a relatively close relationship between ETs 11 through 13 (nontypable isolates) and ETs 1 through 10 and 14 through 25 (serotype b isolates), together with several clusters of relatively closely related nontypable isolates (e.g., ETs 34 through 42 and ETs 91 through 94).

From both medical and evolutionary perspectives, it is important to understand the molecular bases for the genetic differences between serotype b and nontypable isolates. Using molecular probes, Moxon et al. (19) and Hoiseth et al. (10) found evidence that many if not most (exact numbers not specified) nontypable strains apparently lack DNA sequences necessary for capsule synthesis. This finding is consistent with our discovery of large genetic differences between most nontypable and serotype b isolates. Because Hoiseth et al. (10) also demonstrated that serotype b strains may give rise to capsule-deficient variants at frequencies of 0.1 to 0.3% during in vitro cultivation or passage through nonhuman mammalian hosts, one would expect to find cases of sharing or close similarity of multilocus enzyme genotypes among serotype b and nontypable isolates in natural populations. However, the data presented by Hoiseth et al. (10) suggest that in vivo loss of the ability to synthesize capsule occurs at a sufficiently low rate that examination of a relatively small number of isolates, such as reported here, would fail to detect a rare, spontaneous, capsule-negative variant. Because a large amount of genetic material apparently is required for synthesis and extracellular expression of the capsular polysaccharide (6, 10), in theory a large number of independent mutations could result in a capsuleless phenotype without necessarily altering the probe hybridization pattern. It is of interest that each of the three serologically nontypable strains containing DNA sequences with probe homology possessed patterns of hybridization which were unique and distinct from those described for serotype b isolates (19). A corollary of this hypothesis is that there is a class of mutants which are capsuleless but produce polyribosylribitolphosphate, and apparently such strains are known (M. Menegus, personal communication).

Our data are consistent with the notion that most nontypable *H. influenzae* strains isolated from clinical episodes represent a population of organisms possessing one or more as-yet-unidentified virulence factors that may or may not be unique to these strains. The observation (17) that unencapsulated isolates adhere to epithelial cells better than encapsulated strains is consistent with this hypothesis.

Although the formal possibility exists that nontypable isolates are recently derived phenotypic variants of encapsulated forms of serotypes other than b, we will show elsewhere that this is not the case (J. M. Musser, unpublished data).

Diversity of genotypes causing disease. Our analysis has demonstrated that a large variety of genotypes may cause disease. Indeed, genetic diversity at enzyme loci in the present sample of isolates is fully equivalent to that recorded in the highly polymorphic species *E. coli* (22, 25). The number of isolates examined is too small to permit us to determine whether the four genetically distinct assemblages of clones carrying the b capsule vary in frequency of association with various types of disease. Nontypable isolates from otitis media and bacteremia are distributed through most of the dendrogram, with no conspicuous pattern, but the very divergent isolates of ETs 91 through 94

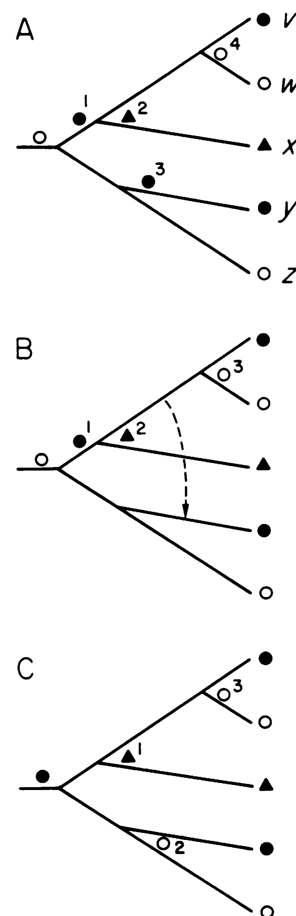

FIG. 3. Three evolutionary scenarios for capsulated (● and ▲) and unencapsulated (○) clones of *H. influenzae*. See text for explanation.

(biotype IV) were associated with disease syndromes that are most unusual for *H. influenzae*: they were recovered from the blood of women with obstetrical infections or from neonates with bacteremia (32). This group of four ETs is sufficiently different in chromosomal genotype from other *H. influenzae* isolates to suggest that DNA hybridization experiments would show a degree of nucleotide sequence similarity well below the 70% level now conventionally taken as the criterion of species limits in many groups of bacteria (16). However, recommendations or action with respect to species taxonomy should await the results of analyses, now in progress (J. M. Musser, manuscript in preparation), of genetic variation in other serotypes and nontypable isolates recovered from the nasopharynx and other sources not represented in the present collection.

Evolutionary relationships between encapsulated and nonencapsulated clones. How can we account for the occurrence of serotype b capsule in several groups of clones that are phylogenetically only distantly related, as, for example, in ETs 1 through 10 and ETs 87 through 90 (Fig. 1)? Several evolutionary scenarios may be proposed, three of which are illustrated by the hypothetical phylogenetic trees in Fig. 3. In scenario A, the ancestor of *H. influenzae* is unencapsulated (open circle), and the genes required for capsule synthesis and extracellular expression evolve independently at points 1 and 3 (dots), presumably by mutations of preexisting genes having other functions. A mutation at point 2 establishes a

lineage (x) with a slightly different capsule type (triangle); and at point 4, a null mutation produces a second unencapsulated line, w. The net result is five extant cell lines (v through z in Fig. 3), two of which (v, y) have the same capsule type as a result of convergent evolution. Similarly, cell lines w and z resemble one another in lacking capsule but are very different in overall genotype.

In scenario B, the capsule is acquired by cell line y through a chromosomal recombination event from the line leading to clone v. Another possibility is that clone y acquired the ability to produce capsule by gene transfer from a species other than *H. influenzae*.

In scenario C, the encapsulated condition is primitive, and the unencapsulated condition is achieved independently in cell lines w and z, at points 2 and 3, by null mutations (point mutations or deletions).

Scenario A is most unlikely because it involves the convergent evolution of a complex of several genes. Scenario B is more plausible because of the ability of *H. influenzae* to undergo transformation and conjugative transfer of chromosomal genes (1, 30), although it requires the transfer and integration into the chromosome of a very large piece of DNA (molecular size, 33×10^6 daltons) containing the genes for capsule synthesis and expression (6). However, the simplest and most probable scenario is C, which derives support from the fact that several other species presently classified as *Haemophilus* also have polysaccharide capsules (14). Scenario C requires long-term conservation of the chemical structure of the polysaccharides but otherwise involves only multiple independent losses of the ability to synthesize or express the capsule.

The major finding of our research is that the population of nontypable isolates, insofar as it is represented by our collection of 65 isolates, is not merely an array of serotype b capsular genotypes that have recently lost the capsule. Nontypable strains are genetically not the same population as the serotype b capsule strains.

It will be interesting to see where strains of capsular types other than b fall in the dendrogram and to determine the genetic relationships of nontypable carrier (nasopharyngeal) isolates to the disease-causing nontypable isolates examined in the present study. Because the genetic structure of populations of *H. influenzae* varies geographically (20), it is likely that further sampling will also uncover additional ETs of serogroup b isolates in Asia, South America, and other parts of the world from which we have yet to examine samples. Finally, studies of additional samples may contribute to a better understanding of the evolutionary relationships between the capsulated and unencapsulated strains.

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