Restriction Enzyme Analysis of Plasmids from *Haemophilus influenzae*

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Examination of *Haemophilus influenzae* strains isolated in New Orleans revealed ampicillin-resistant strains with plasmids of size classes not previously detected in North America. The molecular weight of plasmids in five ampicillin-resistant strains ranged from 0.8×10^6 daltons (0.8 Mdal) to 36 Mdal. The molecular weights of the plasmids were determined by sucrose gradient centrifugation, electron microscopy, and agarose gel electrophoresis. Plasmids of the previously detected 30-Mdal size class were found in three of the five ampicillin-resistant strains. Restriction enzyme analysis is consistent with a close relationship between these three 30-Mdal plasmids. Of the two remaining ampicillin-resistant strains, one contained a single plasmid of 36 Mdal and the other contained two plasmids of 0.8 and 2.3 Mdal.

The recent emergence of antibiotic resistance in isolates of *Haemophilus influenzae* had aroused interest in searching these organisms for the presence of plasmid deoxyribonucleic acid (DNA). Elwell et al. (4-6, 14) have identified two size classes of *Haemophilus* plasmids from clinical isolates collected in North America before 1975 that bear ampicillin resistance determinants. One of these plasmid classes has a molecular mass of 30×10^6 daltons (30 Mdal) and carries an ampicillin transposon similar or identical to the ampicillin transposon found in plasmids of gram-negative enterics. The other class is approximately 3 Mdal and contains only a portion of the ampicillin transposon. The regularity in size of the plasmids bearing ampicillin resistance suggests a common origin for the plasmids within each group from which little if any evolution has taken place since initial formation.

To study the evolution of these plasmids, we have been monitoring both antibiotic-susceptible and -resistant strains of *H. influenzae* from clinical isolates for plasmid DNA. Twenty-three strains have been examined thus far. The only antibiotic resistance encountered was to ampicillin, and all five ampicillin-resistant strains studied contained at least one plasmid. This report characterizes the plasmid population of these five strains and that of one antibiotic-susceptible strain. The molecular weight and the restriction enzyme digestion pattern were determined for each plasmid.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Table 1 lists the six clinical strains studied. Each strain

represents a clinical isolate from patients living in the New Orleans area, with the exception of strain MH15 which was sent to laboratories as a Microbiology Check Sample (MB-80) by the American Society of Clinical Pathology in May 1976. All strains were grown in a shaking water bath at 37°C in brain heart infusion supplemented (sBHI) with nicotinamide adenine dinucleotide (2 µg/ml) and hemin (10 µg/ml), except when isotopic labeling was desired. Isotopic labeling was achieved by growing cells in MIC-Cit, a defined medium described by Herriott et al. (9), supplemented with 10 µCi of [methyl-³H]thymidine (20 Ci/mmol from New England Nuclear) per ml.

Plasmid transfer. The recipient strain for all plasmid transfers consisted of an Rd strain of *H. influenzae* obtained from S. Goodgal that carries chromosomal markers for resistance to streptomycin (*str*), kanamycin (*kan*), and nalidixic acid (*nal*). Donor and recipient cells were each grown in sBHI in a shaking water bath at 37°C to mid-log phase. Equal volumes of donor and recipient cells were mixed in a tube with fresh sBHI and collected on a sterile 0.2-µm membrane (Millipore) filter. The filter was placed on the surface of an sBHI agar plate, and the plate was incubated overnight at 37°C. The filters were next washed in sBHI, and 0.1 ml of this broth was spread on the surface of an sBHI agar plate containing 25 µg of streptomycin and 10 µg of ampicillin per ml. Plates were incubated for a total of 72 h at 37°C. Colonies appearing on the plates were cloned and tested for susceptibility to ampicillin, streptomycin, kanamycin, and nalidixic acid by using standard susceptibility disks (Difco).

Purification of plasmid DNA. Cells were grown and lysed as previously described (8). Partial purification of plasmid DNA was achieved by the high-salt extraction method of Hirt (10). When further purification of plasmid DNA was not required, supernatants of the Hirt extraction procedure were dialyzed overnight against a TES buffer, containing a solution of 50

TABLE 1. *Plasmid-bearing clinical isolates*

Bacterial strain	Antibiotic pattern ^a	Clinical source ^b	Date of isolation	Clinical laboratory
MH2	S	CSF	11/1/75	Hotel Dieu
MH3	Ap	CSF	12/14/75	Charity Hospital
MH10	Ap	NP	4/15/76	Charity Hospital
MH15	Ap	Eye		Touro Infirmary
MH20	Ap	NP	6/28/76	Touro Infirmary
MH27	Ap	Blood	11/2/76	Charity Hospital

^a S, Susceptibility to all antibiotics tested; Ap, resistance to ampicillin. All strains were tested for susceptibility to: streptomycin, tetracycline, chloramphenicol, kanamycin, ampicillin, cephalothin, carbenicillin, and gentamicin. Susceptibility or resistance was determined by relative zone sizes using standardized antibiotic-susceptibility disks.

^b CSF, Cerebrospinal fluid; NP, nasopharynx.

mM tris(hydroxymethyl)aminomethane (Tris), pH 7.5, 50 mM NaCl, and 5 mM disodium ethylenediaminetetraacetic acid (EDTA), and either used immediately or frozen at -80°C . Further purification of plasmid DNA was achieved by dye-buoyant density gradient centrifugation in a Ti 60 rotor, using propidium diiodide as the intercalating dye (7).

Sucrose gradient sedimentation analysis. The sedimentation coefficient of each plasmid was determined by comparing the rate of sedimentation of the dye-buoyant density-purified DNA to that of a standard, covalently closed circular (CCC) PM2 phage DNA in 5 to 20% neutral or alkaline sucrose gradients as previously described (8). The molecular weight of each plasmid was calculated according to the formula of Bazal and Helinski (2).

Electron microscopy. Plasmid DNA purified by dye-buoyant density gradient centrifugation was nicked by leaving the sample at room temperature for 3 to 4 days. Parlodian-coated electron microscope grids were prepared according to the method of Davis et al. (3) and shadowed with platinum-palladium. From 6 to 10 randomly chosen open circular molecules were photographed by using a Phillips 201 electron microscope. Molecular weight determinations were made by comparing the contour length of each molecule to that of the standard PM2 phage DNA.

Agarose gel electrophoresis. Agarose gel electrophoresis of high-salt-extracted or of dye-buoyant density-purified plasmid DNA was performed as described by Elwell et al. (11), with the exception that Tris-acetate buffer (1) containing 40 mM Tris-hydrochloride (pH 7.9)–20 mM sodium acetate–2 mM disodium EDTA was substituted for Tris-borate buffer. Gels were prepared by melting 0.7% (wt/vol) agarose (Sigma, type) in Tris-acetate buffer in a microwave oven for 6 min. Gels were used 1 to 2 h after being poured. The gels were run for 4 h at 100 V in the slab gel apparatus described by Sugden et al. (15). The gels were stained for 15 min in an aqueous solution containing 5 μg of ethidium bromide per ml and destained for 15 min in distilled water in the dark. Bands were made visible by short-wave ultraviolet light illumination. Pictures were made of each gel by using a Wratten 23A filter and Polaroid type 105 positive/negative film. The molecular weight of each plasmid was determined from its migration in the gel relative to the standard plasmid DNA.

Restriction endonuclease cleavage of plasmid DNA and analysis of DNA fragments by agarose

gel electrophoresis. Duplicate samples of each plasmid DNA and λ standard DNA (Bethesda Research Laboratories) were treated in a similar manner except that one sample was incubated with the restriction endonuclease and the other was incubated without it. The reaction mixture for cleavage by *Hind*III consisted of 1 to 2 μg of DNA, 10 U of *Hind*III endonuclease (Bethesda Research Laboratories), 10 mM Tris-hydrochloride, pH 7.5, 50 mM NaCl, 1 mM EDTA, and 6 mM MgCl_2 (12). The reaction mixture for *Eco*RI cleavage contained 1 to 2 μg of DNA, 15 U of *Eco*RI endonuclease (Miles Laboratories), 50 mM Tris-hydrochloride, pH 7.5, 50 mM NaCl, 2.5 mM disodium EDTA, and 10 mM MgCl_2 (13). Samples were incubated at 37°C for 2 h at which time the reaction was halted by the addition of 5 μl of a dye solution containing bromophenol blue (0.07%), sodium dodecyl sulfate (7%), and glycerol (33%). Samples were immediately subjected to electrophoresis in 0.7 or 1.0% agarose gels as above. The molecular weight of each fragment was determined from its migration in the gel relative to the λ DNA fragments.

RESULTS

Dye-buoyant density analysis. Supercoiled circular DNA was isolated from each strain by dye-buoyant density gradient centrifugation. Typical results for strain MH3 are shown in Fig. 1. Although no peak of radioactivity corresponding to CCC DNA was apparent in MH20, fractions from the appropriate area of the gradient were pooled and dialyzed as with the other strains. Subsequent analysis of this material revealed the presence of CCC DNA in all strains.

Molecular weight determinations. The molecular weight of each plasmid was determined by at least two independent methods (Table 2).

Rate zonal sedimentation of dye-buoyant density-purified plasmid DNA was performed on all isolates except MH20. All strains tested revealed a single plasmid peak, with the exception of MH27 which showed two plasmid peaks. Further characterization of the plasmid DNA by electron microscopy and agarose gel electropho-

resis revealed differences in the molecular weights of the plasmids that were not always evident from analysis of the plasmids in sucrose velocity gradients. Although pMH4 and pMH5 have similar *S* values in neutral sucrose gradients, pMH5 was found to be slightly smaller than pMH4 in electron micrograph measurements. This difference in molecular weight was also evident from analysis of the plasmids in agarose gels. The data indicate that plasmids pMH4, pMH5, and pMH6 have identical or similar molecular masses of 28 or 30 Mdal. Strain MH20 was found to contain a single plasmid (pMH7) of 36 Mdal. Plasmids pMH8 and pMH9, which have molecular masses of 2.3 and 0.9 Mdal, respectively, were both detected in strain MH27.

Plasmid transfer. Transcipients were isolated from crosses using MH3, MH10, and MH15 as donor strains. Selection of transcipients was made on the basis of resistance to ampicillin and streptomycin. Subsequent studies of the antibiotic resistance pattern of all transcipients indicated that each also carries resistance to kanamycin and nalidixic acid, which are unselected

markers of the recipient strain. The phenotypes of the parents and transcipients are reported in Table 3. In all cases, transfer was very inefficient and erratic. Repeated attempts to isolate transcipients from strain MH20 and MH27 failed to indicate that the ampicillin resistance observed in these strains was plasmid borne, and plasmids from these strains may be considered cryptic in this report.

The recipient strain was shown by dye-buoyant density gradient analysis and agarose gel electrophoresis to be plasmid-free. Each transcipient was shown by agarose gel electrophoresis to contain a plasmid of the same size as the donor parent.

Restriction enzyme analysis. Agarose gel electrophoresis patterns of plasmid DNA that had been digested with the restriction endonuclease *Hind*III are shown in Fig. 2. The circular forms of plasmids pMH4, pMH5, and pMH6 were each cleaved once by *Hind*III to produce a single linear molecule (Fig. 2F, J, and L). Plasmid pMH1 was not cleaved by *Hind*III (Fig.

FIG. 1. Dye-buoyant density analysis of a lysate of strain MH3.

TABLE 3. Phenotype of parents and transcipients

Strain designation	Plasmid	Antibiotic resistance pattern ^a			
		Ap	Kan	Str	Nal
Donor strains					
MH3	pMH4	R	S	S	S
MH10	pMH5	R	S	S	S
MH15	pMH6	R	S	S	S
Recipient strain					
MH12		S	R	R	R
Transcipient strain					
MH14	pMH4	R	R	R	R
MH29	pMH6	R	R	R	R
MH30	pMH5	R	R	R	R

^a Ap, Ampicillin; Kan, kanamycin; Str, streptomycin; Nal, nalidixic acid. S, Susceptibility; R, resistance. Susceptibility or resistance was determined by relative zone sizes using standardized antibiotic-susceptibility disks.

TABLE 2. Properties of plasmids from clinical isolates

Strain	Phenotype ^a	Plasmid	<i>S</i> value	Molecular mass (Mdal)		
				Neutral sucrose	Electron microscopy	Agarose gels
MH2	S	pMH1	45	19.7	20.5	18.5
MH3	Ap	pMH4	54	30.1	31.0	30.1
MH10	Ap	pMH5	54	30.1	28.9	28.0
MH15	Ap	pMH6	54	30.1		30.1
MH20	Ap	pMH7			35.8	38.0
MH27	Ap	pMH8	18	2.3		2.2
		pMH9	12	0.9		0.8

^a S, Susceptible; Ap, resistance to ampicillin.

A B C D E F G H I J K L

FIG. 2. *Hind*III restriction endonuclease digestion pattern. Undigested and digested samples are arranged pairwise from right to left: (A and B) bacteriophage λ DNA, (C and D) pMH1, (E and F) pMH4, (G and H) pMH4 from strain MH13, (I and J) pMH5, and (K and L) pMH6.

2D). *Hind*III also showed no activity against pMH4 from strain MH13, a transcripient of MH3 (Fig. 2H). An explanation for this apparent discrepancy is that strain MH13 is a derivative of strain Rd, the source of the *Hind*III enzyme. DNA from strain MH13 was thus protected from *Hind*III restriction, presumably as a result of *in vivo* modification by the endogenous *Hind*III methylase.

The plasmids were also digested with the restriction endonuclease *Eco*RI. Agarose gel pat-

terns of *Eco*RI digestion products of each plasmid are shown in Fig. 3. The size of each fragment generated by *Eco*RI cleavage, as determined from migration distances of fragments and standards, is depicted in Fig. 4.

The plasmids that we have shown to carry the ampicillin resistance determinants (pMH4, pMH5, and pMH6) had similar, but not identical, *Eco*RI digestion patterns (Fig. 3F, H, and O). Both pMH4 and pMH5 had five *Eco*RI sites and pMH6 had six sites. It is likely, but not

FIG. 3. *EcoRI* restriction endonuclease digestion pattern. Undigested and digested samples are arranged pairwise from right to left: (A and B) bacteriophage λ DNA, (C and D) pMH1, (E and F) pMH4, (G and H) pMH6, (I and J) pMH7, (K and L) pMH8 and pMH9, (M) λ digest, and (N and O) pMH5. Wells A through L are 0.7% agarose.

FIG. 4. Relative migration distance of *EcoRI* or *HindIII* digestion fragments after electrophoresis through 0.7% agarose. The symbols are designated as follows: ●, bacteriophage λ (*EcoRI*); ○, pMH6 (*EcoRI*); X, pMH4 (*EcoRI*); □, pMH5 (*EcoRI*); ■, pMH1 (*HindIII*); □, pMH4 and pMH6 (*HindIII*).

rigorously proven, that all five sites seen in pMH4 are similar or identical to five of the six sites seen in pMH6. Plasmid pMH5 appears to lack two of four fragments seen in both pMH4 and pMH6. Examination of the molecular weights of the plasmid fragments reveals that one of the fragments seen only in pMH5 could result from the deletion of the *EcoRI* site that produces the two fragments not seen in pMH5. Further support for this hypothesis is that a fragment appears in partial digestion mixtures of pMH4 and of pMH6 (e.g., Fig. 3H) at a position similar to the unique fragment seen in pMH5. It is thus likely that pMH5 shares all of its five *EcoRI* sites with pMH6 and four sites with pMH4. Plasmids pMH7, pMH8, and pMH9 seem quite different from pMH4, pMH5, and pMH6 since the restriction patterns of the two groups were quite different. Neither pMH8 nor pMH9 from strain MH27 exhibited *EcoRI* restriction sites.

DISCUSSION

Restriction enzyme analysis indicates that plasmids pMH4, pMH5, and pMH6 are probably related to each other, and that pMH4 and pMH5 have evolved by a series of deletions from (and possibly insertions into) the primordial plasmid pMH6. It is unlikely that the similarity in restriction enzyme digestion patterns we have described would have arisen independently in each of the three plasmids. The data suggest four *EcoRI* restriction sites common to plasmids pMH4, pMH5, and pMH6. Plasmids pMH4 and pMH5 each have a fifth site, different in each; and pMH6 has all six of these sites (Fig. 5). Restriction enzyme analysis also suggests that plasmids pMH1, pMH7, pMH8, and pMH9 are not related to pMH4, pMH5, and pMH6.

FIG. 5. Schematic representation of the relationship between plasmid pMH6, pMH4, and pMH5, as determined by *EcoRI* digestion analysis.

Plasmids bearing ampicillin resistance determinants have apparently conserved their identity since their emergence several years ago. The three related plasmids we identified fall into one of the classes described by Elwell et al. (5) in 1975. This conservatism may be due to the paucity of indigenous plasmids in the genus *Haemophilus* (8). However, the plasmid pool is likely to increase due to the selective advantage conferred by the newly acquired genes for antibiotic resistance. If so, we may expect to see an increase in the rate of plasmid diversification in *Haemophilus*. The plasmids in the ampicillin-resistant strains MH20 and MH27 may represent such a trend.

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