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Molecular Characterization of "Plasmid-Free" Antibiotic-Resistant *Haemophilus influenzae*

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We examined 14 multiresistant and 8 ampicillin- or tetracycline-resistant *Haemophilus influenzae* isolates and 4 ampicillin-resistant *H. parainfluenzae* isolates for plasmid deoxyribonucleic acid. Sixteen strains carried plasmids. Both "plasmid-free" and plasmid-carrying isolates transferred the antibiotic resistance by conjugation. All transconjugants carried plasmid deoxyribonucleic acid, suggesting that the apparent plasmid-free strains contained R plasmids encoding for antibiotic resistance.

In the last six years, antibiotic resistance to ampicillin, due to beta-lactamase production (8), tetracycline (7), and chloramphenicol (10) in *Haemophilus influenzae* has increased dramatically. Before 1974, *H. influenzae* was regarded as uniformly susceptible to ampicillin, tetracycline, and chloramphenicol. Antibiotic resistance to tetracycline and chloramphenicol and β-lactamase-mediated ampicillin resistance were shown to be plasmid mediated in the first strains examined (3-5, 9, 15).

Subsequently, resistant *H. influenzae* which did not appear to harbor plasmids after being lysed and electrophoresed on agarose gels were reported (12, 13). Some of the hypotheses which have been proposed to explain these "plasmid-free," antibiotic-resistant strains include: (i) the resistant determinants are inserted directly into the chromosome (1, 12), (ii) the entire R plasmid is inserted into the chromosome (13), and (iii) extrachromosomal plasmid DNA does exist but the procedure used to lyse the cells damages the plasmid structure or alters the mobility so that the plasmid comigrates with the chromosomal band.

We examined 14 multiresistant and 8 ampicillin- or tetracycline-resistant *H. influenzae* isolates and 4 ampicillin-resistant *H. parainfluenzae* isolates; 12 of these were sent to us by other investigators as plasmid-free resistant isolates.

Each strain was grown overnight, harvested, and suspended in 1.5 ml of 25% sucrose in 0.05 M Tris, pH 8.0, and 0.001 M EDTA, pH 8.0. A 0.2-ml amount of lysozyme (10 mg/ml in 0.25 M Tris, pH 8.0) was added, and cleared lysates were prepared by sodium dodecyl sulfate (SDS) salt precipitation and treated with ribonuclease (20 μg/ml) for 60 min. The lysates were extracted with 50 mM Tris-saturated phenol, ethanol precipitated, and suspended in 200 μl of TES buffer (50 mM NaCl, 5 mM EDTA, 30 mM Tris pH 8.0) as described by Meyers et al. (11). Growing ampicillin-resistant *H. influenzae* on plates overnight, rather than in broth overnight, yielded more reproducible plasmid patterns in our laboratory (unpublished data). Therefore, each strain was grown overnight on four, G C medium base (Difco Laboratories) agar plates supplemented with 10 μg of hemin, 10 μg of L-histidine (3), and 2 μg of nicotinamide adenine dinucleotide per ml, and the appropriate antibiotic, either 5 μg of tetracycline, 10 μg of ampicillin, or 5 μg of chloramphenicol, each per ml. A sample (20 to 100 μl) from each strain was electrophoresed in a 0.7% agarose gel.

A second lysing method was used to determine whether different procedures would give similar or different plasmid patterns. The Hansen and Olsen (6) lysing method was selected because it has been shown to be superior in visualizing very large (100×10^6 to 300×10^6 dalton) plasmids. In addition, antibiotic-resistant *Serratia* and *Proteus* species which appear plasmid free when lysed by the Myers et al. method demonstrated plasmid bands when lysed by the Hansen and Olsen method (L. Tompkins, D. Schaberg, and S. Falkow, personal communication).

When the Hansen and Olsen lysing method was used, each strain was grown in 40 ml of 3.5% brain heart infusion broth supplemented with 10 μg of hemin, 10 μg L-histidine, 2 μg of nicotinamide adenine dinucleotide per ml, and the appropriate antibiotic for 3 or 4 h to a cell density of 10^8 bacteria per ml. The cells were harvested, washed, and suspended in 1.35 ml of 25% sucrose in 0.05 M Tris, pH 8.0, at room temperature. A 0.1-ml amount of lysozyme (10 mg/ml in 0.25 M Tris, pH 8.0) was added and mixed in gently, and the mixture was put in an ice-water bath for

5 min; 0.5 ml of 0.25 M Na_2EDTA, pH 8.0, was added and mixed in, and the mixture was chilled for 5 min. A 0.5-ml amount of 20% (wt/vol) SDS and 40 μg of ribonuclease per ml was added followed by eight cycles of heat pulse at 55°C. A 0.5-ml amount of 3 N NaOH was added and mixed in, then 1.0 ml of 2 M Tris, pH 7.0, was added followed by 0.65 ml of SDS (20% wt/vol) and 1.25 ml of 5 M NaCl. The lysates were chilled and refrigerated in an ice bath overnight. The DNA was precipitated with 1/3 volume polyethylene glycol 6000 (42% wt/vol), refrigerated for 6 h and then centrifuged. The pellets were suspended in 150 μl of TES buffer as described by Hansen and Olsen (6). Samples of 20 to 100 μl from each strain were electrophoresed in 0.7% agarose gels.

The results are summarized in Table 1. Four different patterns were evident when the two lysing methods were used. In 3 of 22 of the *H. influenzae* strains and 2 of 4 of the *H. parainfluenzae* strains, identifiable plasmids were visualized when either lysing method was used. However, five of the *H. influenzae* strains and one *H. parainfluenzae* strain showed plasmids only when lysed by the Meyers et al. (11) method; an additional five *H. influenzae* strains had plasmid bands only when lysed by the method of Hansen and Olsen (6). The remaining *H. influenzae* strains and one *H. parainfluenzae* strain appeared plasmid free when either lysing method was used. Thus, 13 *H. influenzae* and 3 *H. parainfluenzae* strains contained plasmids between 20 and 50 megadaltons which could be visualized by agarose gel electrophoresis when two lysing procedures were used. If one method had been used, only 8 *H. influenzae* strains, rather than 13, would have been identified as

TABLE 1. *Plasmid detection and frequency of resistance transfer*

Strain	Resistance[a]	Capsule[b] Type	Lysing Method		Transconjugants frequency Transfer/donor[c]	Source[d]	Reference
			Myers et al. (11)	Hansen & Olsen (6)			
H. influenzae							
HC234	Tc Cm	–	+	+	2×10^{-3}	B. Van Kilngeren	15
9-80118	Tc Cm	–	+	+	6×10^{-4}	C. Thornsberry	
R328	Tc Cm	non-b[e]	+	+	8×10^{-4}	A. Van	14
R374	Tc Cm Ap	–	+	–	6×10^{-5}	L. Bryan	2
R384	Tc Cm	–	+	–	3×10^{-5}	S. Lerman	
R385	Tc Cm Ap	b	+	–	2×10^{-4}	R. Michaels	
R387	Tc Cm Ap	b	+	–	2×10^{-5}	P. Echverria	
78-2703	Tc Ap	–	+	–	1×10^{-5}	D. Anderson	
C215	Tc Ap	b	–	+	4×10^{-6}	CHMC	
C289	Ap	b	–	+	1×10^{-4}	CHMC	
R375	Tc Cm Ap	b	–	+	6×10^{-5}	M. Glode	
77-36098	Tc Cm	b	–	+	4×10^{-5}	C. Thornsberry	
425	Ap	b	–	+	1×10^{-4}	I. Moberg	
C227	Tc	b	–	–	3×10^{-6}	CHMC	
C226	Ap	b	–	–	5×10^{-4}	CHMC	
C273	Tc Cm	–	–	–	1×10^{-5}	CHMC	
C288	Ap	–	–	–	2×10^{-6}	CHMC	
C599	Tc	b	–	–	8×10^{-4}	COHMC	
C616	Ap	b	–	–	3×10^{-7}	COHMC	
76-79268	Cm Ap	b	–	–	5×10^{-6}	C. Thornsberry	
77-1040	Cm Ap	b	–	–	3×10^{-6}	C. Thornsberry	
78-2264	Ap	–	–	–	2×10^{-5}	D. Anderson	
H. parainfluenzae							
78-2257	Ap	–	+	+	1×10^{-2}	D. Anderson	
394	Ap	–	+	+	3×10^{-3}	I. Moberg	
108	Ap	–	+	–	5×10^{-4}	I. Moberg	
420	Ap	–	–	–	3×10^{-4}	I. Moberg	

[a] Resistance to: Cm, chloramphenicol; Tc, tetracycline, Ap, ampicillin.

[b] –, untypable using a–f Difco antiserum.

[c] All transconjugants showed plasmids by the Meyers et al. lysing method (11).

[d] CHMC, Children's Hospital and Medical Center, Boston, MA; COHMC, Children's Orthopedic Hospital and Medical Center, Seattle, WA.

[e] R328 was originally an encapsulated strain. The strain has lost its capsule and we were unable to determine type.

carrying plasmids. Therefore, it appears that one cannot assume an antibiotic-resistant *H. influenzae* or *H. parainfluenzae* strain is plasmid free when only a single lysing method is used for screening.

Each of the resistant strains was then used as a donor in mating experiments to determine whether the plasmid-free strains were capable of transferring their resistant determinants. We also questioned whether the frequency of transfer was comparable in resistant strains which appeared to carry or lack plasmids by agarose gel electrophoresis. The donors were sensitive to rifampin, erythromycin, and streptomycin or to rifampin and erythromycin. A laboratory strain, Rd (12), resistant to rifampin, erythromycin, and streptomycin was used as the recipient. Overnight filter mating as described previously by van Klinegren et al. (15) was performed, and transconjugants were selected on ampicillin and rifampin, tetracycline and rifampin, or chloramphenicol and rifampin agar plates. Colonies were streaked three or four times on the same selective medium and then screened for resistance to streptomycin and erythromycin. Only those which were resistant to all three antibiotics were considered true transconjugants. In certain experiments, 1 mg of DNase per ml was added to the filter before the cells were added and an additional 1 mg of DNase per ml was added before the cells were removed from the filter. The frequencies of transfer between the DNase-treated and untreated cells were identical (data not shown). This suggests that the genetic exchange was DNase-resistant conjugation, rather than transformation. The frequency of resistant transfer ranged from 10^{-2} to 3×10^{-7} with the majority of strains having a frequency of transfer between 10^{-4} and 10^{-6} (Table 1). The frequency of antibiotic resistance transfer is the same for both the apparent plasmid-free and plasmid-containing strains.

Stuy (13) similarly found that 18 of 23 antibiotic-resistant plasmid-free *H. influenzae* strains could be used as donors in mating experiments. However, the frequency of transfer found was considerably lower, 10^{-6} to 10^{-8}. The difference in frequency may be due to differences in mating procedures or due to the use of different strains. We found no difference between donors which were unencapsulated and type b donors in frequency of transfer or in recipient (strain Rd) viability.

Representative transconjugants from each mating pair were then grown overnight on antibiotic-containing agar plates, lysed by the Meyers et al. method (11), and electrophoresed on 0.7% agarose gels. Both the apparently plasmid-free and plasmid-containing donors yielded Rd transconjugants carrying a single plasmid band with a molecular weight ranging between 20×10^6 and 50×10^6. The 14 multiresistant strains transferred all the resistance determinants as a unit, and in each case the presence of a large plasmid correlated with the phenotypic antibiotic resistance.

All 26 resistant strains examined appeared to carry their resistant determinants on plasmids. The location of the plasmids in the "plasmid-free" strains is not clear. Bendler (1) and Stuy (12, 13) have suggested that plasmid-free antibiotic-resistant *H. influenzae* have the resistant determinants or the entire plasmid integrated into the chromosome. Both have used transformation experiments to support this hypothesis. We have prepared crude DNA lysates from seven plasmid-mediated antibiotic-resistant strains and used them to perform transformations with both competent cells and $CaCl_2$-treated cells (12, 13). We found that some of the donors could be used to transform competent cells; two of five resulting transformants carried plasmids, whereas three appeared to be plasmid free (data not shown). Whether these transformants carry only the transposons, the entire plasmid integrated into the chromosome, or are extrachromosomal, is presently under investigation. However, since plasmid-free transconjugants were found when donors carrying plasmids were used, transformation experiments may not support the hypothesis that the apparent plasmid-free donors have plasmids integrated into their chromosome. We have also found that certain plasmids can be transformed into $CaCl_2$-treated cells, whereas others cannot.

Strain R387 has a very fragile 42-megadalton plasmid. When the DNA preparations from this strain were stored, they spontaneously formed open circles and linears. When the samples were run on agarose gels, the plasmid band smeared across the chromosomal band (data not shown); therefore, the chromosomal band used by Stuy (13) for transformation may contain contaminating plasmid fragments. Similarly, linear plasmid DNA fragments will comigrate with the chromosomal fragments in a sucrose gradient. Therefore, using the chromosomal fractions obtained from agarose gels or sucrose gradients for transformation and obtaining resistant transformants does not rule out the possibility of transformation with extrachromosomal plasmid DNA; plasmid contamination of the chromosomal fractions must be ruled out.

All 26 donor strains could transfer plasmids to the recipient strain Rd. In each case, a plasmid could be visualized by the Meyers et al. method

(11). In addition, the frequency of antibiotic resistance transfer between the plasmid-carrying and plasmid-free groups was identical, suggesting that all of the original strains examined carry their plasmids as extrachromosomal elements and that strain variation, rather than a variation in plasmid characteristic, determines which lysing method is required for visualization in agarose gels. Therefore, one can hypothesize that the present lysing methods used were not adequate to visualize the plasmid in the so-called plasmid-free strains.

The data presented indicate that with the use of two lysing methods a number of plasmid-carrying strains can still be missed; only when mating experiments were performed using the Rd strain as a recipient were all 26 strains shown to carry conjugative R plasmids.

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LITERATURE CITED

1. **Bendler, J. W. III.** 1976. Physical size of the donor locus and transmission of *Haemophilus influenzae* ampicillin resistance genes by deoxyribonucleic acid-mediated transformation. J. Bacteriol. **125**:197-204.
2. **Bryan, L. E.** 1978. Transferable chloramphenicol and ampicillin resistance in a strain of *Haemophilus influenzae*. Antimicrob. Agents Chemother. **14**:154-156.
3. **DeGraaff, J., L. P. Elwell, and S. Falkow.** 1976. Molecular nature of two beta-lactamase-specifying plasmids isolated from *Haemophilus influenzae* type b. J. Bacteriol. **126**:439-446.
4. **Elwell, L. P., J. DeGraaff, D. Seibert, and S. Falkow.** 1975. Plasmid-linked ampicillin resistance in *Haemophilus influenzae* type b. Infect. Immun. **12**:404-410.
5. **Elwell, L. P., J. R. Saunders, M. H. Richmond, and S. Falkow.** 1977. Relationships among some R plasmids found in *Haemophilus influenzae*. J. Bacteriol. **131**: 356-362.
6. **Hansen, J. B., and R. H. Olsen.** 1978. Isolation of large bacterial plasmids and characterization of the P2 incompatibility group plasmids pMG1 and pMG5. J. Bacteriol. **135**:227-238.
7. **Hansman, D.** 1975. *Haemophilus influenzae* type b resistance to tetracycline isolated from children with meningitis. Lancet **i**:893-896.
8. **Kahn, W., S. Ross, W. Rodriguez, G. Controni, and A. K. Saz.** 1974. *Haemophilus influenzae* type b resistant to ampicillin. J. Am. Med. Assoc. **229**:298-301.
9. **Laufs, R., and P. M. Kaulfers.** 1977. Molecular characterization of a plasmid specifying ampicillin resistance and its relationship to other R factors from *Haemophilus influenzae*. J. Gen. Microbiol. **103**:227-286.
10. **Manten, A., B. van Klingeren, and M. Dessens-Kroon.** 1976. Chloramphenicol resistance in *Haemophilus influenzae*. Lancet **i**:702.
11. **Meyers, J. A., D. Sanches, L. P. Elwell, and S. Falkow.** 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. **127**:1529-1537.
12. **Stuy, J. H.** 1979. Plasmid transfer in *Haemophilus influenzae*. J. Bacteriol. **139**:520-529.
13. **Stuy, J. H.** 1980. Chromosomally integrated conjugative plasmids are common in antibiotic-resistant *Haemophilus influenzae*. J. Bacteriol. **142**:925-930.
14. **Van, A. D., G. Bieth, and D. H. Bouanchaud.** 1975. Résistance plasmidique à la tetracycline chez *Haemophilus influenzae*. C. R. Acad. Sci. Ser. D **208**:1321-1323.
15. **van Klinegren, B., J. D. A. van Embden, and M. Dessens-Kroon.** 1977. Plasmid-mediated chloramphenicol resistance in *Haemophilus influenzae*. Antimicrob. Agents Chemother. **11**:510-516.