Interrelationships Between Physical State, Phenotypic Stability and Transferability of β -Lactamase Genes in *Haemophilus influenzae*

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We have evaluated 66 β -lactamase-producing (β -Lac⁺) clinical isolates of *Haemophilus influenzae* for the presence of plasmid DNA, ability to conjugate with an *H. influenzae* type b recipient, and stability of the β -Lac⁺ phenotype. Of the 66 strains, 23 (35%) contained a plasmid demonstrable by either of two methods of cleared cell lysate preparation. The molecular weight of 17 of the plasmids was approximately 30×10^6 . Four strains carried a plasmid of mol. wt 3×10^6 and single strains carried plasmids of mol. wt 36×10^6 or 50×10^6 . Of 23 plasmids that were unstable *in vitro*, 10 (43%) had molecular weights of 30×10^6 and coded only for β -lactamase. Stability of the β -Lac⁺ phenotype was observed in all 43 strains in which plasmid DNA was not demonstrated by physical methods. When β -Lac⁺, 'plasmid-free' strains were conjugated with strain Eagan (*H. influenzae* type b), ampicillin-resistant transconjugants were isolated in 21 out of 43 matings (the lower limit of detection was 10^{-7} per donor cell per 30 min mating). With few exceptions, homologous (type b $\times$ type b) crosses with β -Lac⁺, plasmid-containing donors were more efficient (frequency 10^{-2} – 10^{-3}) than heterologous (untypable $\times$ type b) matings (frequency 10^{-6} – 10^{-7}). β -Lac⁺, 'plasmid-free' strains were inefficient donors (frequency 10^{-6} – 10^{-7}) irrespective of serotype, but all transconjugants from these crosses were efficient donors (frequency 10^{-1} – 10^{-4}) and contained a plasmid of mol. wt 30×10^6 that was unstable in 9 cases out of 26 (35%). The increase in transferability and phenotypic instability of plasmid-bearing β -Lac⁺ strains reinforces the concept of a chromosomal locus for β -lactamase genes in 'plasmid-free' β -Lac⁺ strains. Carriage of β -lactamase genes on 30×10^6 mol. wt R plasmids aids transferability at the expense of genetic stability.

INTRODUCTION

Ampicillin resistance in *Haemophilus influenzae*, first observed by Gunn *et al.* (1974), is usually associated with the constitutive production of a β -lactamase; identified as TEM or type IIIa (Farrar & O'Dell, 1974; Laufs *et al.*, 1979; Medeiros & O'Brien, 1975; Saunders *et al.*, 1978; Sykes *et al.*, 1975; Vega *et al.*, 1976). This enzyme is specified by the transposable genetic element TnA (Hedges & Jacob, 1974; Heffron *et al.*, 1975a; Jahn *et al.*, 1979; Laufs & Fock, 1979; Saunders & Sykes, 1977) and is found on many phylogenetically distinct plasmids which reside in Gram-negative bacteria (Heffron *et al.*, 1975b).

The genes that code for β -lactamase production in *H. influenzae* (phenotype β -Lac⁺) were identified on plasmids of molecular weight 3×10^6 or 30×10^6 (DeGraaff *et al.*, 1976; Elwell *et al.*, 1975, 1977; Laufs & Kaulfers, 1977; Laufs *et al.*, 1979). Ampicillin resistant (Ap^R) *H. influenzae* strains that produced β -lactamase but lacked demonstrable R plasmids were described by Slaney & Albritton (1979); Stuy (1979, 1980); and Roberts & Smith (1980). However, these investigators demonstrated R plasmids in transconjugants from matings between Ap^R 'plasmid-free' donors and a rough, non-iridescent recipient derived from a strain of *H. influenzae* type d (Rd) (Alexander & Leidy, 1953). Roberts & Smith (1980) compared two methods for

the isolation of covalently closed circular DNA (Hansen & Olsen, 1978; Meyers *et al.*, 1976) from *H. influenzae* isolates possessing various resistance patterns. The inclusion of a second method did increase their ability to demonstrate R plasmids, but they were unable to demonstrate plasmid DNA in 41% (9 out of 22) of the strains. Slaney & Albritton (1979) suggested that 'host factors' indigenous to the strain determined the physical state of antibiotic resistance plasmids in *H. influenzae*. More specifically, Stuy (1980) suggested that these 'plasmid-free' strains carried their β -lactamase genes on a chromosomally integrated plasmid.

Chromosomal integration of an R plasmid might be expected to affect both the stability and dissemination of the phenotype. We have reported spontaneous loss of ampicillin resistance *in vitro* from two *H. influenzae* type b strains, both of which possessed plasmids of mol. wt 30×10^6 , and suggested that in such strains the β -Lac⁺ phenotype was unstable (Daum *et al.*, 1981). In contrast, an Ap^R *H. influenzae* type b strain that uniformly maintained its β -Lac⁺ phenotype *in vitro* was subsequently shown to lack extrachromosomal DNA. Inoculation of infant *Macaca mulatta* with either of the two *H. influenzae* type b Ap^R isolates containing plasmids resulted in the isolation of only β -Lac⁻ bacteria from the blood and cerebrospinal fluid (CSF). In contrast, inoculation with the phenotypically stable strain resulted in isolation of uniformly β -Lac⁺ bacteria from blood and CSF (Daum *et al.*, 1981). The correlation between the presence of plasmid DNA and phenotypic stability *in vitro* and *in vivo* documented in these three Ap^R *H. influenzae* type b strains prompted an investigation into the relationships between these phenomena.

In this report, we (1) assess the prevalence of Ap^R *H. influenzae* isolates that have demonstrable R plasmids, and the stability of the β -Lac⁺ phenotype; (2) compare conjugational transfer frequencies between plasmid-containing and 'plasmid-free' donors and an *H. influenzae* type b recipient; (3) examine transconjugants for plasmid DNA and their phenotypic stability; and (4) consider the evolutionary significance of the mobility of genetic determinants of ampicillin resistance in *H. influenzae*.

METHODS

Bacteria. A total of 66 clinical *H. influenzae* isolates resistant to ampicillin were obtained for study. The source, serotype, phenotype, and site of isolation of these strains are listed in Tables 1 and 2. Strain Eagan (E), a well characterized Ap^S *H. influenzae* type b strain (Anderson *et al.*, 1972), was used in matings. All *H. influenzae* strains were identified by standard criteria (Morse, 1980), serotyped with *H. influenzae* antisera (Difco), and assessed for β -lactamase production by chromogenic cephalosporin hydrolysis (O'Callaghan *et al.*, 1972). Stock cultures were maintained in skim milk at -70°C .

Resistant mutants. For the purposes of conjugation experiments, a streptomycin-resistant (Str^R) mutant of wild-type strain E was obtained as described previously (Moxon *et al.*, 1974). This strain was made rifampin resistant (Rif^R) by growing it overnight in Brain Heart Infusion broth supplemented with horse erythrocyte lysate and β -nicotinamide adenine dinucleotide (BHIs). A sample of approximately 10^{10} c.f.u. was then plated on BHIs agar that contained rifampin ($2\text{ }\mu\text{g ml}^{-1}$). The frequency of spontaneous mutant isolation was 1.2×10^{-9} per E c.f.u. for E Str^R and 3.4×10^{-9} per E Str^R c.f.u. for E Str^R Rif^R. The minimum inhibitory concentration (m.i.c.) of streptomycin exceeded $500\text{ }\mu\text{g ml}^{-1}$ for both strains while the m.i.c. of rifampin was $<0.5\text{ }\mu\text{g ml}^{-1}$ and $>2.0\text{ }\mu\text{g ml}^{-1}$ for E Str^R and E Str^R Rif^R, respectively.

Plasmid stability. Isolated colonies plated on solid medium from 18 h broth cultures were assayed for β -lactamase production by hydrolysis of penicillin G (Lilly, Indianapolis, Indiana, U.S.A.) as indicated by water-soluble phenol red (Sigma) (Daum *et al.*, 1981). A minimum of 1000 colonies was examined for each strain.

Conjugation experiments. Broth cultures (15 h) of donor (approx. 10^9 c.f.u. ml^{-1}) and recipient bacteria were prepared in BHIs broth. MgSO_4 (1 mM final concentration) was added to 1.0 ml broth containing recipient cells and the mixture vortexed vigorously for 20 s. A 0.1 ml sample of the donor strain suspension (approx. 10^8 c.f.u.) was then added and the mixture was filtered through a Metrical filter (type GA-6, pore size $0.45\text{ }\mu\text{m}$) (Gelman Instrument Co., Ann Arbor, Michigan, U.S.A.). The filter was incubated (30 min, 5% CO_2 , 38°C) on the surface of a fresh, antibiotic-free BHIs agar plate. Cells were recovered by vigorous agitation of the filter in 4.0 ml phosphate-buffered saline and plated on to selective BHIs agar containing appropriate combinations of streptomycin ($500\text{ }\mu\text{g ml}^{-1}$), ampicillin ($2\text{ }\mu\text{g ml}^{-1}$) and/or rifampin ($2\text{ }\mu\text{g ml}^{-1}$). Antibiotic-resistant phenotypes were scored after 18–24 h incubation at 37°C . For overnight matings, the filters were allowed to incubate for about 18 h before bacteria were washed from the filters and plated. The inclusion of DNAase (type I, calf thymus, Sigma) (Laufs & Fock, 1979) during matings did not affect the observed transfer frequencies. The lower limit of

transconjugant detection for 30 min matings was 10^{-7} c.f.u. per donor cell. All 66 Ap^R *H. influenzae* strains were mated with the *H. influenzae* type b E Str^R recipient for 30 min. The 10 Ap^R *H. influenzae* strains that did not mate with E Str^R in 30 min were mated with E Str^R overnight. Transconjugant strains, when isolated, were verified as Ap^R *H. influenzae* type b and mated with the E Str^R Rif^R recipient.

Plasmid DNA preparation and agarose gel electrophoresis. Cleared cell lysates from all 66 Ap^R *H. influenzae* strains were prepared by acid phenol extraction (Daum *et al.*, 1981); 24 of these strains were also lysed with SDS in an alkaline denaturation technique of plasmid isolation (Hansen & Olsen, 1978). Electrophoresis conditions were as previously described (Daum *et al.*, 1981). The approximate molecular weight of each plasmid was determined from its migration distance in agarose gels relative to the migration distance of plasmids of molecular weights ranging from 2.6×10^6 to 36×10^6 (Broda, 1979).

RESULTS

Serotype of Ap^R clinical isolates. Of the 66 *H. influenzae* strains examined, 37 (56%) belonged to serotype b and 26 (39%) were untypable. Two strains were of serotype d and there was one type c isolate (Tables 1 and 2).

Presence of R plasmids in wild-type strains. All 66 clinical *H. influenzae* Ap^R isolates and recipient strain E were screened for the presence of plasmid DNA by agarose gel electrophoresis of cleared lysates prepared by acid phenol extraction. Plasmid DNA was demonstrated in 23 strains, of which 10 were of serotype b (Table 1). Plasmid DNA was not found in the strain E, E Str^R or E Str^R Rif^R.

The molecular weights of the 23 plasmids isolated were approximately 30×10^6 in 17 cases (16 Ap^R phenotype and one Ap^R Tc^R phenotype), 3×10^6 in four cases (all Ap^R phenotype), 36×10^6 in one case (Ap^R Cm^R Tc^R phenotype), and 50×10^6 in one case (Ap^R Cm^R Tc^R phenotype).

To determine whether a second technique of lysis might reveal plasmid DNA in any of the 43 remaining isolates, 22 strains designated 'plasmid-free' by acid phenol extraction were examined by the method of Hansen & Olsen (1978). This technique demonstrated plasmid DNA in R724 (Table 1) and in two positive control strains shown to possess plasmid DNA by acid phenol extraction.

Conjugational transfers. Almost all b $\times$ b crosses (E Str^R recipient) in which the donor contained demonstrable plasmid DNA resulted in transconjugant isolation in 30 min matings, the exception being when the donor contained non-conjugative plasmids of mol. wt 3×10^6 . In contrast, transconjugants were isolated from 6 out of 13 non-b $\times$ b matings in which the donors contained demonstrable plasmids. The seven non-b donor strains whose resistance determinants did not transfer in 30 min did transfer resistance determinants following overnight mating (with the exception of two strains with plasmids of mol. wt 3×10^6).

Of 43 'plasmid-free' strains, 21 (49%) transferred their resistance to E Str^R in 30 min matings. Ten strains which did not transfer (two type b, one type c, one type d, and six untypable) were chosen for overnight mating. All ten matings resulted in the isolation of a transconjugant strain.

Transconjugants derived from matings with 'plasmid-free' donors, including those isolated after overnight mating only, were evaluated for the presence of plasmid DNA. All such strains had plasmids demonstrable by acid phenol extraction. The molecular weights of plasmids identified in each transconjugant uniformly approximated 30×10^6 .

Haemophilus influenzae type b strains carrying demonstrable plasmid DNA transferred at significantly higher frequencies (10^{-2} – 10^{-3}) than did 'plasmid-free' donors (10^{-6} – 10^{-7}) ($P < 0.001$, *t*-test of geometric means). Exceptions included three *H. influenzae* type b strains with plasmid DNA that transferred at a frequency of 10^{-5} , two of which were multiply resistant (Ap^R Cm^R Tc^R); two 'plasmid-free' strains also transferred at 10^{-5} . All type b transconjugants, irrespective of the presence of demonstrable plasmid DNA in their donors, were themselves efficient donors and transferred their resistance at frequencies of 10^{-1} – 10^{-4} during 30 min matings with E Str^R Rif^R.

Untypable *H. influenzae* strains, irrespective of demonstrable plasmid presence, transferred to E Str^R at a frequency of 10^{-6} – 10^{-7} . (Two untypable *H. influenzae* strains with 30×10^6 mol.

Table 1. *Characteristics of H. influenzae with demonstrable R plasmids*

Strain*	Serotype†	Phenotype†	Site of isolation	Source‡	Conjugational transfer frequency per donor		Plasmid instability (percentage of bacterial population β -Lac ⁻)		10 ⁻⁶ × Approx. mol. wt of plasmid in donor¶
					30 min mating	Overnight mating§	Donor	Transconjugant	
R274	b	Ap ^R	ear	CHMC	1.6 × 10 ⁻²	—	—	—	30
Sb1729 ¹	b	Ap ^R Tc ^R	CSF	J. Stuy	1.6 × 10 ⁻³	—	—	—	30
C306 ²	b	Ap ^R	throat	CHMC	8.0 × 10 ⁻³	—	1.4	1.7	30
C121 ²	b	Ap ^R	nose	CHMC	1.3 × 10 ⁻³	—	3.2	2.9	30
Sb1498 ¹	b	Ap ^R	Nasal pharynx	J. Stuy	7.2 × 10 ⁻³	—	1.8	6.1	30
H119	ut	Ap ^R	ear	JDNM	2.6 × 10 ⁻³	—	3.6	3.7	30
MH3 ³	ut	Ap ^R	CSF	M. Murray	2.8 × 10 ⁻³	—	2.4	0.4	30
G	b	Ap ^R Cm ^R Tc ^R	throat	M. Glode	3.0 × 10 ⁻⁵	—	—	—	50
R374	b	Ap ^R Cm ^R Tc ^R	sputum	A. L. Smith	8.0 × 10 ⁻⁵	—	—	—	36
H511	b	Ap ^R	blood	CHNO	4.4 × 10 ⁻⁵	—	—	—	30
H73	ut	Ap ^R	ear	JDNM	4.0 × 10 ⁻⁶	—	1.6	1.8	30
H398	ut	Ap ^R	bronchial aspirate	TMC	9.2 × 10 ⁻⁶	—	0.1	1.6	30
H399	ut	Ap ^R	sputum	TMC	2.7 × 10 ⁻⁷	—	1.5	2.1	30
H404	ut	Ap ^R	ear	JDNM	<1.0 × 10 ⁻⁷	2.0 × 10 ⁻¹	2.4	1.5	30
MH15 ³	ut	Ap ^R	eye	M. Murray	<1.0 × 10 ⁻⁷	4.8 × 10 ⁻¹	2.2	1.5	30
H318	b	Ap ^R	NA	LSHD	<1.0 × 10 ⁻⁷	—	—	NT	3
H322	b	Ap ^R	blood	LSHD	<1.0 × 10 ⁻⁷	—	—	NT	3
H49	ut	Ap ^R	nose	CHNO	<1.0 × 10 ⁻⁷	—	—	NT	3
H109	ut	Ap ^R	throat	CHNO	<1.0 × 10 ⁻⁷	—	—	NT	3
H63	ut	Ap ^R	ear	JDNM	<1.0 × 10 ⁻⁷	5.4 × 10 ⁻¹	—	—	30
H469	ut	Ap ^R	bronchial aspirate	TMC	<1.0 × 10 ⁻⁷	6.0 × 10 ⁻¹	1.0	16.4	30
H550	ut	Ap ^R	ear	JDNM	<1.0 × 10 ⁻⁷	4.6 × 10 ⁻¹	—	—	30
H199	ut	Ap ^R	nose	CHNO	4.0 × 10 ⁻⁷	—	—	—	30

NA, Not available; NT, transconjugant not isolated.

* References for strains indicated: 1, Stuy (1979, 1980); 2, Daum *et al.* (1981); 3, Harkness & Murray (1978).† Abbreviations: ut, untypable; Ap^R, ampicillin resistant; Cm^R, chloramphenicol resistant; Tc^R, tetracycline resistant.

‡ Abbreviations: CHMC, Children's Hospital Medical Center, Boston; CHNO, Charity Hospital, New Orleans; JDNM, Jefferson Davis Hospital, Natchez; LSHD, Louisiana State Health Department; TMC, Tulane Medical Center, New Orleans.

§ Apparent transfer 'frequencies' are listed (transconjugants per donor quantitated after overnight filter incubation).

|| —, Stable phenotype; no β -Lac⁻ colonies observed.¶ No transfer of the plasmid of mol. wt 3 × 10⁶ was observed. Matings employing donors with plasmids of other mol. wt yielded transconjugants with plasmids of identical mol. wt.

Table 2. *Characteristics of H. influenzae without demonstrable R plasmids*

All strains were resistant to ampicillin. For abbreviations see Table 1.

Strain(s)	Serotype	Site of isolation	Source	Conjugational transfer frequency per donor		Plasmid instability (percentage of bacterial population β -Lac ⁻)		10 ⁻⁶ × Approx. mol. wt of plasmid in transconjugant
				30 min mating	Overnight mating*	Donor	Transconjugant	
H401	ut	ear	JDNM	1.3 × 10 ⁻⁵		—	1.4	30
H217	b	ear	JDNM	1.4 × 10 ⁻⁵		—	—	30
H477	b	CSF	CHNO	2.0 × 10 ⁻⁵		—	0.1	30
H328	ut	sputum	CHNO	2.4 × 10 ⁻⁶		—	—	30
H251	b	blood	CHNO	<1.0 × 10 ⁻⁷	1.1 × 10 ⁻⁴	—	—	30
H324	b	blood	LSHD	<1.0 × 10 ⁻⁷	3.6 × 10 ⁻⁴	—	—	30
H308	ut	eye	LSHD	<1.0 × 10 ⁻⁷	2.0 × 10 ⁻⁶	—	—	30
H229	d	throat	CHNO	<1.0 × 10 ⁻⁷	8.0 × 10 ⁻⁴	—	4.1	30
H125	d	ear	JDNM	<1.0 × 10 ⁻⁷		—	NT	NT
H455	c	ear	TMC	<1.0 × 10 ⁻⁷	8.0 × 10 ⁻⁴	—	—	30
6 strains	b	CSF (3) blood (2) nose (1)		10 ⁻⁶ -10 ⁻⁷		—	1.4-3.5	30
11 strains	b	CSF (7) blood (3) pleural fluid (1)		10 ⁻⁶ -10 ⁻⁷		—	—	30
6 strains	b	CSF (2) blood (2) eye (1) throat (1)	Various	<1.0 × 10 ⁻⁷		—	NT	NT
10 strains	ut	nose (3) eye (1) ear (4) bronchial aspirate (1) NA (1)		<1.0 × 10 ⁻⁷		—	NT	NT

* Apparent transfer 'frequencies' are listed (transconjugants per donor after overnight filter incubation).

wt R plasmids that transferred at a frequency of 10^{-3} and one strain lacking a demonstrable plasmid that transferred at a frequency of 10^{-5} , were exceptions.)

The transfer frequencies for the ten matings which were allowed to continue overnight ranged from 10^{-1} to 10^{-6} transconjugants per donor.

Plasmid stability. A mean of 1500 colonies from each strain was examined for the absence of β -lactamase production. The proportion of the bacterial population of a given strain that did not produce β -lactamase ranged from 0 to 16.4% (Tables 1 and 2). Instability of the β -Lac⁺ phenotype was evident in 10 out of 23 strains that possessed demonstrable plasmids. All 10 plasmids were of mol. wt. 30×10^6 and none coded for multiple drug resistance. Significantly, the transconjugants of all 10 unstable strains also exhibited phenotypic instability.

All 43 Ap^R *H. influenzae* strains lacking demonstrable plasmid DNA were phenotypically stable. However, 35% (9 out of 26) of their transconjugants, including one of five isolated from overnight mating, demonstrated phenotypic instability. All strains demonstrating instability, whether clinical isolates or transconjugants, contained plasmid DNA of mol. wt 30×10^6 .

DISCUSSION

We selected the Eagan strain as the recipient because of the clinical importance of R plasmid acquisition by *H. influenzae* type b strains. Plasmid DNA is demonstrable in transconjugants produced by 'plasmid-free' strains crosses with Rd recipients (Slaney & Albritton 1979; Stuy 1979, 1980; Roberts & Smith 1980). Our data confirm that the majority (65%) of Ap^R *H. influenzae* clinical isolates are 'plasmid-free' but transfer their resistance to *H. influenzae* type b strain E Str^R. The resulting transconjugants invariably contain demonstrable plasmid DNA.

Roberts & Smith (1980) performed overnight filter matings with a serotype b or untypable *H. influenzae* donor and an Rd recipient. Identical transfer frequencies were observed irrespective of donor serotype, or the presence of demonstrable plasmid DNA in the donor. The transfer frequencies we observed following 30 min matings were significantly different ($P < 0.001$) if 'plasmid-free' (10^{-6} – 10^{-7}) and plasmid-carrying donors (10^{-1} – 10^{-4}) were compared. Moreover, transfer between strains of identical serotype (b $\times$ b) is more efficient than that between strains of different serotypes (non-b $\times$ b). This difference is not apparent when these matings are allowed to continue overnight (data not shown). Since Rd transconjugants are themselves efficient donors and rapidly pass on plasmid DNA to other susceptible Rd cells (Stuy, 1980), the prolonged mating time increases the apparent 'frequency' of resistance transfer.

Roberts & Smith (1980) showed that preparation of cleared cell lysates by an alternative method (Hansen & Olsen, 1978) allowed the isolation of plasmid DNA from some 'plasmid-free' strains. However, we identified an R plasmid in only one strain (R274) designated 'plasmid-free' by acid phenol extraction. We had anticipated an extrachromosomal resistance determinant in this strain since, among those Ap^R isolates classed as 'plasmid-free' by the acid phenol technique, it uniquely transferred its resistance to E Str^R at high frequency (10^{-2}) and could be cured by acridine orange (data not shown).

Hypotheses advanced by others to explain 'plasmid-free' antibiotic resistant *H. influenzae* include the following: (1) damage of extrachromosomal DNA during lysing procedures resulting in the loss of plasmid DNA, or alterations in plasmid mobility resulting in co-migration of plasmid DNA with chromosomal DNA (Roberts & Smith, 1980); (2) insertion of resistance determinants directly into the chromosome (Bendler, 1976); and (3) integration of the entire R plasmid into the chromosome (Stuy, 1980).

Our data support the concept of chromosomally integrated R plasmids (Stuy, 1980) rather than differences in lysing techniques (Roberts & Smith, 1980), for the absence of demonstrable R plasmids in most Ap^R *H. influenzae* 'plasmid-free' isolates. Evidence for this conclusion includes: (1) the fact that an increase in transferability of resistance is observed when the 'plasmid-free' donor and its plasmid containing transconjugant are compared; (2) transconjugants of R plasmid-bearing donors mimic the plasmid instability of their donor, indicating that loss of ampicillin resistance *in vitro* is a plasmid function rather than a cell function; (3) 'plasmid-

free' donors are 100% (43 out of 43) phenotypically homogeneous, while only 65% (17 out of 26) of their respective plasmid-containing transconjugants are phenotypically homogeneous; and (4) all transconjugants have demonstrable R plasmids. We do not know whether transfer of the integrated plasmid occurs by chromosome mobilization or by excision followed by transfer. However, the former seems less likely since Stuy (1980) has provided evidence that transformants from strains without demonstrable R plasmids transfer resistance to an Rd *recA* recipient. This suggests a low frequency excision rate resulting in extrachromosomal DNA capable of conjugal transfer.

The existence of two modes of R plasmid carriage, chromosomal and extrachromosomal, confers definite evolutionary advantages to *H. influenzae*. Extrachromosomal R plasmids are efficient in disseminating resistance genes throughout the species but are easily lost in the absence of selective pressure. We previously observed that when either of two Ap^R *H. influenzae* isolates carrying 30×10^6 mol. wt. R plasmids were inoculated into infant *M. mulatta*, the Ap^R resistance determinants were quickly lost (Daum *et al.*, 1981). Moreover, Ap^R *H. influenzae* type b strains carrying 30×10^6 mol. wt R plasmids are less efficient pathogens in the infant rat model of experimental *H. influenzae* type b infection than their Ap^S derivatives (Daum *et al.*, 1982). These data suggest that the host may place negative selective pressure on Ap^R *H. influenzae* carrying R plasmids, especially those strains demonstrating instability of the β -Lac⁺ phenotype. Further support for this was found among the serotype b isolates from blood or CSF, none of which contained unstable 30×10^6 mol. wt plasmid DNA. Conversely, 22 out of 27 (81%) 'plasmid-free' type b isolates were isolated from patients with systemic infections. 'Plasmid-free' *H. influenzae* with chromosomally integrated R plasmids are inefficient at the dissemination of resistance genes; however, they are uniformly stable and perpetuate resistance determinants in the *H. influenzae* gene pool. We conclude that the existence of two modes of R determinant carriage in *H. influenzae* confers the evolutionary advantage of high frequency β -lactamase gene dissemination while retaining phenotype immortalization.

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