INFECTION AND IMMUNITY, Aug. 1975, p. 404-410

Vol. 12, No. 2
Printed in U.S.A.

Plasmid-Linked Ampicillin Resistance in *Haemophilus influenzae* Type *b*

LYNN P. ELWELL,* JOHANNES DE GRAAFF,[1] DAVID SEIBERT, AND STANLEY FALKOW
Department of Microbiology, University of Washington School of Medicine, Seattle, Washington 98195

Received for publication 31 March 1975

Four ampicillin-resistant, β-lactamase-producing strains of *Haemophilus influenzae* type *b* were examined for the presence of plasmid deoxyribonucleic acid (DNA). Three resistant strains contained a 30 × 10^6-dalton (30 Mdal) plasmid and one resistant strain contained a 3-Mdal plasmid. The ampicillin-sensitive *Haemophilus* strains examined did not contain plasmid DNA. Transformation of a sensitive *H. influenzae* strain to ampicillin resistance with isolated plasmid DNA preparations revealed that the structural gene for β-lactamase resided on both plasmid species. DNA-DNA hybridization studies showed that the 30-Mdal *Haemophilus* plasmid contained the ampicillin translocation DNA segment (TnA) found on some R-factors of enteric origin. The significance of this finding is discussed in relation to the possible origin of the *H. influenzae* plasmids.

Disease due to ampicillin-resistant strains of *Haemophilus influenzae* type *b* has become an increasingly serious and widespread problem (1, 3, 25). Ampicillin-resistant strains have been isolated from cases of epiglottitis, pneumonia, otitis media, and meningitis, including three fatal cases of infant meningitis (15, 17). The primary cause of ampicillin resistance appears to be the production of a β-lactamase shown in one strain to possess a broad-spectrum penicillinase-cephalosporinase activity (9). Thorne and Farrar (J. Infect. Dis., in press) recently have demonstrated transfer of the ampicillin resistance from one strain to an ampicillin-sensitive strain during mixed incubation, presumably by conjugation.

The sudden emergence of ampicillin resistance in *H. influenzae* due to a transferable β-lactamase gene suggested to us, and others (9,15), that an R-factor might be involved. In this study we report the isolation of plasmid deoxyribonucleic acid (DNA) from four ampicillin-resistant strains of *H. influenzae*. Three strains contained a 30 × 10^6-dalton (30 Mdal) plasmid and one strain contained a 3.0-Mdal plasmid, and evidence is presented indicating that the β-lactamase gene is located on those plasmid species. Resistance to ampicillin mediated by the TEM-type β-lactamase in enteric bacteria is found on a segment of DNA that can be translocated from replicon to replicon (13). This same segment is found on the plasmid DNA isolated from resistant *H. influenzae*.

[1] Permanent address: Vakgroep voor Medische Microbiologie en Parasitologie, Free University, Amsterdam, The Netherlands.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study and their sources are as follows. *H. influenzae* HR-7 and HR-9, Clyde Thornsberry, Center for Disease Control, Atlanta, Ga.; *H. influenzae* HR-B and G32, Ann Larson, Clinical Microbiology Department, University of Washington, Seattle; *H. influenzae* HR-885, Stephen Cohen, University of California, San Francisco Medical Center; and *Escherichia coli* RS2, Fred Heffron, Department of Microbiology, University of Washington, Seattle.

Media. The liquid medium contained 3.5% brain heart infusion (Difco) supplemented with 10 μg of hemin, 10 μg of L-histidine, and 3 μg of nicotinamide adenine dinucleotide per ml (22). The solid medium (chocolate agar) consisted of GC agar (Difco) containing 2% defibrinated sheep blood heated at approximately 80 C for 10 to 15 min. Cultures on solid media were incubated at 36 C in a CO_2 incubator (CO_2 concentration, 3 to 8%). The ampicillin-resistant strains were grown on chocolate agar supplemented with ampicillin (20 μg/ml). Cultures in broth were grown in a shaking platform air incubator at 36 C.

Assay of β-lactamase activity. β-Lactamase activity was measured by the acidimetric method of Rubin and Smith (21). One unit of β-lactamase activity was defined as the amount of enzyme hydrolyzing 1 μmol of benzyl penicillin in 1 h at 25 C.

Measurement of minimum inhibitory concentration (MIC). The agar dilution method of Steers et al. (24) was used. Inocula were prepared from 4- to 6-h-old broth cultures and diluted to give viable counts of approximately 5 × 10^8 organisms/ml. A replicator delivering 0.001 ml was used to inoculate the agar surfaces of media containing antibiotics of interest.

Preparation of cell lysates and ultracentrifugation procedures. The bacteria were grown to about 10^9 cells/ml in liquid media. The cells were harvested

by centrifugation and lysed by a modification of the method of Clewell and Helinski (2) in which Triton X-100 (0.1% final concentration) was used in place of Brij 58. Covalently closed circular (CCC) plasmid DNA was further purified by isopycnic centrifugation in a CsCl-ethidium bromide gradient as previously described (10). The final density was adjusted to 1.7936 g/cm^3 (refractive index, 1.4080), and the solution was centrifuged for 40 to 48 h at 15 C and 40,000 rpm in a Beckman type 50 rotor.

DNA bands in the gradients were located by using a black-light lamp (UVL-22, Ultraviolet Products Inc., San Gabriel, Calif.). Chromosomal and plasmid DNA bands were collected from the side of the gradient tube by using an 18-gauge needle and a 3-ml syringe.

DNA contour length. Plasmid DNA was spread onto parlodian-coated electron microscope grids according to the Kleinschmidt method (8). Well-isolated molecules were photographed under the electron microscope, and the developed negatives were projected through a photographic enlarger onto paper and traced. The contour length of the tracing was determined by using a map measurer. The true magnification of the electron microscope was determined by calibration with a diffraction grating, and the molecular weights of the plasmids were calculated by assuming 1 μm of DNA to be equivalent to 2.07 $\times$ 10^6 daltons.

Preparation of labeled plasmid DNA. ^{3}H-labeled RSF1030 DNA was prepared from strain 1485 of *E. coli* as previously described by Heffron et al. (13).

Extraction of unlabeled DNA for hybridization. Unlabeled whole-cell DNA from ampicillin-sensitive and -resistant strains of *H. influenzae* was prepared by the method of So et al. (23).

DNA-DNA duplex studies. Labeled and unlabeled DNA were sheared by sonication before hybridization in a Branson sonicator model 140D for 5 min at 4 C (output setting, 3) and then dialyzed against 0.42 M NaCl. Hybridizations were performed as described by Crosa et al. (5). Approximately 0.001 μg of ^{3}H-labeled, sheared RSF1030 DNA (specific activity, 1.5 $\times$ 10^6 counts/min per μg) was mixed in a glass vial with 150 μg of unlabeled whole-cell DNA in a total volume of 1.5 ml of 0.21 M NaCl. The DNA was allowed to reanneal at 70 C for 125 min. Renaturation was stopped by placing the vials into an ice-cold water bath. The S1 endonuclease reaction was carried out as previously described (5). The actual degree of duplex formation for the homologous reaction was 72 to 78%, whereas the actual degree of duplex formation with the chromosomal control was 5 to 6%. Normalized values (recorded in the table in this paper as percentage of homology) were obtained from raw data by subtracting the chromosomal control and dividing by the homologous reaction.

Transformation. The transformation method used was that basically described by Cohen et al. (4). After the 42 C heat pulse, the transformation mixture was diluted 1:5 into L broth and incubated for 4 h at 37 C to allow phenotypic expression. Cells were then plated on chocolate agar supplemented with 1.0 μg of ampicillin per ml. Plates were then incubated in a CO_2 incubator at 36 C for 48 h.

RESULTS

Production of β-lactamase by resistant H. influenzae. The resistant *H. influenzae* strains used in this study were typical type *b* organisms with surprisingly high MICs to ampicillin (Table 1). Since resistances of this magnitude are usually associated with the production of a β-lactamase, we examined sonicates of our resistant *Haemophilus* strains and found that they all possessed enzymatic activity. No β-lactamase activity was detected in the sonicate of the ampicillin-sensitive strain, G32.

The rates of hydrolysis of several β-lactam antibiotics by sonically disrupted cells of *H. influenzae* strain HR-7 and *E. coli* RS2, a strain containing the plasmid R1drd-19, which specifies a TEM-type β-lactamase, are shown in Table 2. The so-called TEM-type β-lactamase exhibits high activity on ampicillin and cephaloridine substrates but low activity against isoazolyl penicillins such as oxacillin and methicillin (11). The β-lactamase produced by strain HR-7 had high activity on ampicillin and peni-

TABLE 1. *MICs of ampicillin for H. influenzae strains*

Strain no.	MIC (μg/ml)
G32	0.25
HR-7	700
HR-885	>1000
HR-B	20
T7-1[a]	>1000
T7-5[a]	>1000
T8-13[b]	700
T8-2[b]	>1000

[a] Transformant strain derived from the exposure of strain G32 to the plasmid DNA isolated from strain HR-7.

[b] Transformant strain derived from the exposure of strain G32 to the plasmid DNA isolated from strain HR-885.

TABLE 2. *Relative rates of hydrolysis of various β-lactam antibiotics by culture sonic fluids of H. influenzae HR-7 and G32 and E. coli R32*

Sonic fluid	Substrate					
	Penicillin G[a]	Ampicillin	Cephalothin	Oxacillin	Methicillin	Dicloxacillin
HR-7	100 (756)[b]	111	20	13	0	0
RS2	100 (150)	95	17	7	2	2
G32	0	0	—[c]	—	—	—

[a] Penicillin G = 100.

[b] Numbers in parentheses indicate rate of hydrolysis relative to benzyl penicillin (specific activity expressed as units per milligram of bacterial protein).

[c] —, Not done.

cillin G and low activity against oxacillin and methicillin and resembled the general substrate profile of the *Haemophilus* β-lactamase characterized by Farrar and O'Dell (9).

Demonstration of plasmids in resistant H. influenzae. On the basis of its substrate specificity, the β-lactamase found in strain HR-7 appeared analogous to the TEM-type class, the most common R-factor-associated β-lactamase (11). Therefore, we next examined our ampicillin-resistant strains to determine whether they contained plasmid DNA. Cleared cell lysates were prepared and centrifuged in CsCl-ethidium bromide. All of the resistant strains examined contained a DNA component characteristic of circular, covalently closed (CCC) molecules. This DNA component was absent in the ampicillin-sensitive strain, G32 (Fig. 1). Material from the CCC region of the gradients was pooled, extracted with isopropyl alcohol, dialyzed, and allowed to stand at 4 C for 1 week. The DNA molecules were then examined in the electron microscope, and well-isolated, open, circular DNA molecules were photographed and measured (Table 3).

Plasmids of two distinct sizes were seen. Strains HR-7, HR-9, and HR-B contained a plasmid approximately 30×10^6 Mdal in mass (Fig. 2). The plasmid fraction of strain HR-7 also contained a CCC DNA species with a mass of 60 Mdal. This larger plasmid species constituted less than 1% of the total plasmid DNA, and we assume that it represents a dimer of the predominant 30-Mdal plasmid.

Ampicillin-resistant strain HR-885 contained

TABLE 3. *Control length measurements of plasmids from ampicillin-resistant H. influenzae strains*

Strain no.	No. of molecules measured	Mean mol wt
HR-7	30	29.9 ± 0.73
T7-1	28	30.2 ± 0.59
HR-885	22	2.94 ± 0.08
T8-2	36	3.19 ± 0.12

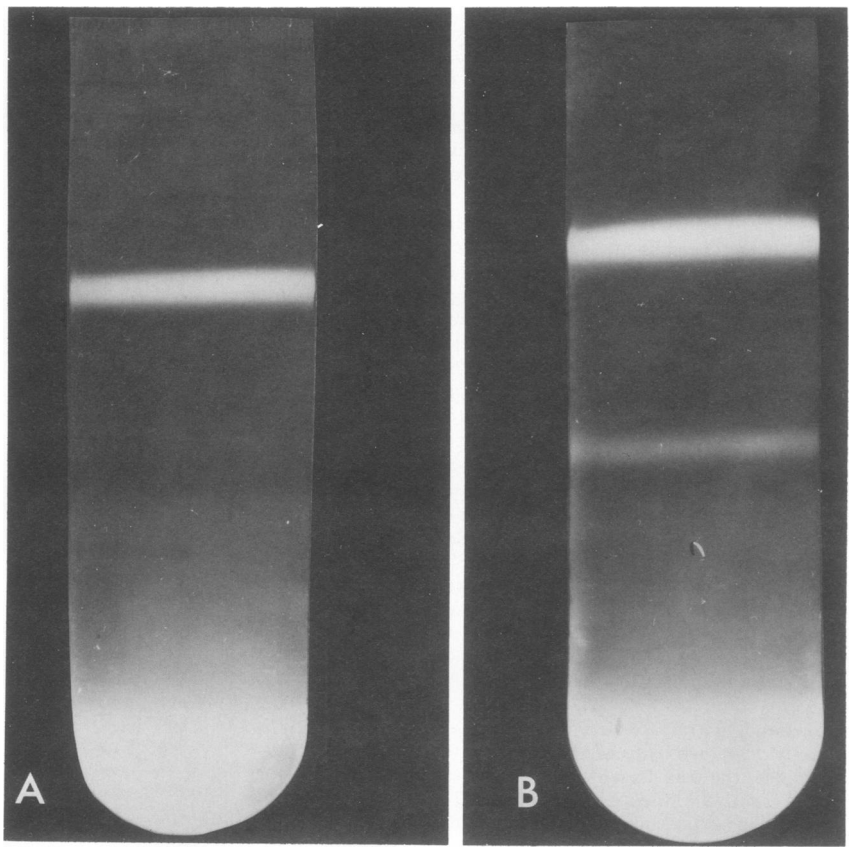

FIG. 1. *CsCl-ethidium bromide gradient tubes containing cleared cell lysates of (A) ampicillin-sensitive H. influenzae strain G32 and (B) ampicillin-resistant H. influenzae strain HR-7.*

a much smaller plasmid with a mass of approximately 3 Mdal (Fig. 2). The presence of some linear DNA in our original plasmid preparations derived from this strain raised the possibility that we were missing a larger plasmid species either by our isolation procedures or by the action of nucleases. Plasmid DNA preparations from strain HR-885, in which meticulous care was taken to avoid chromosomal DNA contamination, contained very little linear DNA, and no plasmids larger than 3 Mdal were seen. We could find no plasmid smaller than 30 Mdal in either the CCC DNA fraction or the chromosomal fraction of the CsCl-ethidium bromide gradients of strain HR-7. In addition, cell lysates of the ampicillin-sensitive strain, G32, were run in CsCl-ethidium bromide gradients and the area of the gradient where CCC normally banded was carefully examined in the electron microscope for circular DNA, but none was found.

Transformation of ampicillin resistance by Haemophilus plasmid DNA. To show definitively that the gene for β-lactamase was located on the *Haemophilus* plasmids, we used plasmid DNA isolated from the resistant strains, HR-7 and HR-885, to transform a sensitive recipient, G32, to ampicillin resistance (Table 4). Chromosomal DNA from these resistant strains lacked transforming ability. Exposure of the sensitive strain, G32, to plasmid DNA isolated from HR-7 and HR-885 resulted in the appearance of a significant number of ampicillin-resistant clones. These transferred clones now showed the same plasmid species as the parental HR-7 and HR-885 strains (Table 3) and also synthesized a potent β-lactamase.

The MICs of the donor strains, recipient strain, and several of the transformant strains are given in Table 1. It is clear that transformant strains may have either higher or lower MICs to ampicillin relative to the donor strain. This might be due to so-called "intrinsic factors," i.e., altered surface properties of recipient bacteria, which have been shown to play an important role in the resistance of gram-nega-

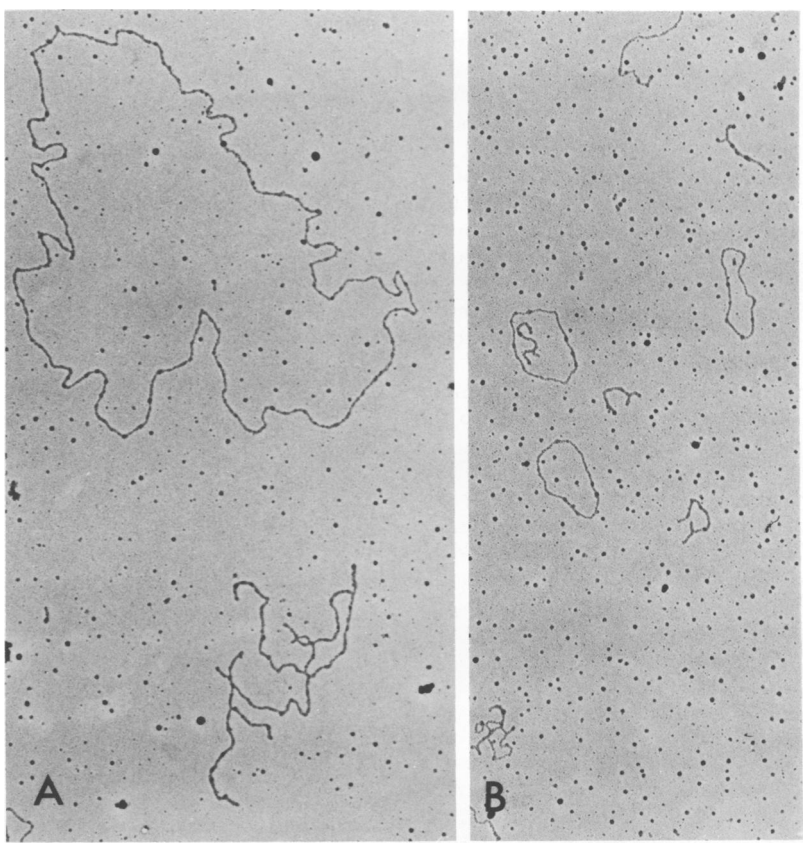

FIG. 2. *Electron micrograph of H. influenzae plasmids isolated by dye-buoyant density centrifugation from (A) strain HR-7 and (B) strain HR-885. Final magnification, ×17,342.*

TABLE 4. *Transforming capacity of plasmid DNA isolated from ampicillin-resistant Haemophilus strains*

Source of transforming DNA	No. of ampicillin-resistant transformants (per 10^8 cells)[a]
HR-885 plasmid DNA	23.1
HR-7 plasmid DNA	3.3
HR-885 chromosomal DNA	0
HR-7 chromosomal DNA	0

[a] Transformations were done according to the method of Cohen et al. (4). After incubation of $CaCl_2$-treated cells with the various DNA preparations (1 μg/ml), bacteria were diluted fivefold into antibiotic-free L broth. After 4 h of incubation at 37 C, samples of the bacterial cultures were spread onto chocolate agar containing ampicillin and incubated for 24 to 48 h for determination of the numbers of transformants. Each value shown is an average of two experiments.

tive bacteria to β-lactam antibiotics (19). Furthermore, it has been shown that the host bacteria cytoplasm may control some R-factor gene functions including β-lactamase production (16).

Hybridization of H. influenzae plasmids with RSF1030. RSF1030 (referred to hereafter as R-AMP) is a 5.5- to 5.6-Mdal plasmid present in its host *E. coli* as a multi-copy pool of about 30 copies per chromosome equivalent (6). R-AMP contains a DNA sequence (TnA) of 2.7 to 3.3 Mdal in size that includes the structural gene for the TEM β-lactamase. Current evidence suggests that a diverse group of R-factors, isolated from enteric bacteria containing the TEM-type β-lactamase gene, arose as the result of the translocation of this 2.7-Mdal sequence of DNA from plasmid to plasmid (12, 13).

To determine whether *H. influenzae* plasmids contained a DNA sequence homologous to the TnA sequences found in enteric bacteria, we hybridized ^{3}H-labeled R-AMP with cold DNA extracted from selected *Haemophilus* strains (Table 5). Unlabeled DNA extracted from strains HR-7 and T7-1, containing the 30-Mdal plasmid, hybridized to 42 to 48% with the R-AMP plasmid. The size of the homologous region calculated from those hybridization values is 2.3 to 2.6 Mdal, which is in good agreement with the measured 2.7 to 3.3 Mdal of the TnA translocation sequence (13).

DNA extracted from strains HR-885 and T8-8, harboring the 3-Mdal plasmid, shared a lower level of sequence homology, 21 to 26%, with the R-AMP plasmid. In terms of molecular weight the region of homology is 1.2 to 1.4 Mdal in mass, which is close to the estimated mass of the structural gene for β-lactamase, 0.9 Mdal (11). DNA extracted from the ampicillin-sensitive *H. influenzae* strain, G32, shared only 1% of its sequences with R-AMP.

DISCUSSION

Ampicillin has been extensively used to treat *Haemophilus* infections for over 10 years without the appearance of resistant strains. The recent alarming increase in high-level ampicillin resistance among strains of *H. influenzae* type *b* suggested to a number of investigators that genetic elements analogous to the R plasmids of enteric bacteria might be involved. This view was strengthened by the observation that ampicillin resistance in *Haemophilus* was mediated by a β-lactamase. We have now shown by physical and genetic means that this speculation is correct by demonstrating plasmid DNA in ampicillin-resistant *H. influenzae* strains. The majority of the resistant strains we examined contained a 30-Mdal plasmid. Transformation of an ampicillin-sensitive *H. influenzae* strain with isolated plasmid DNA revealed that the structural gene for β-lactamase resided upon the plasmid genome. One strain, HR-885, contained only a 3-Mdal plasmid species. Like its larger counterpart, this plasmid also specified a TEM-type β-lactamase.

The origin of the plasmids in *H. influenzae* is of critical epidemiological importance. If we had observed only a single plasmid species within the resistant *Haemophilus* strains, a case might have been made that we were dealing essentially with a single "clone" of drug-resistant *H. influenzae* that had become widely

TABLE 5. *DNA-DNA hybridizations between RSF1030 and whole-cell H. influenzae DNA*

Source of unlabeled DNA	Relative DNA sequence homology with RSF1030 (%)[a]
C600 (RSF1030)	100
G32	1
HR-7	48
T7-1	42
HR-885	21
T8-8	26

[a] The degree of DNA-DNA duplex formation was assayed by the S1 endonuclease method (5). In every case, the degree of duplex formation was calculated relative to the homologous reaction. The actual extent of binding of [^{3}H]RSF1030 purified plasmid DNA with whole-cell *E. coli* C600 (RSF1030) was approximately 80%, which was corrected for a 5% reaction between [^{3}H]RSF1030 DNA and whole-cell DNA extracted from an *E. coli* F^- strain. Each value shown is an average of three reactions.

disseminated throughout the world. The finding of the much smaller HR-885 plasmid indicates that either there have been several independent genetic events leading to the development of resistant *Haemophilus* strains or that a single plasmid has already undergone a series of recombinational interactions. The best clue that we presently have as to the origin of the plasmids of *H. influenzae* comes from two observations. The first is that the 30-Mdal plasmid is conjugative. We have shown that the conjugative *H. influenzae* plasmid described by Thorne and Farrar (J. Infect. Dis., in press) is 30 Mdal in mass (Elwell and Falkow, unpublished observations), whereas Thorne and Farrar (personal communication) have demonstrated that the HR-7 strain examined by us is capable of transferring ampicillin resistance to a *Haemophilus* recipient by conjugation. Not unexpectedly, the HR-885 strain containing the 3-Mdal plasmid was not transmissible by their methods. Hence, at least some of the plasmids found in *Haemophilus* apparently possess a transfer mechanism similar to that found in the R plasmids of enteric bacteria.

The second line of evidence for the origin of the plasmids of *Haemophilus* comes from our demonstration that the segment of DNA containing the structural gene for the TEM β-lactamase in R-factors of enteric origin is similarly found on the plasmids in *Haemophilus*. It is now clear that many of the drug resistance determinants found on R plasmids are carried on DNA sequences capable of being translocated from replicon to replicon (13; Berg and Davies, personal communication). For example, this translocation sequence carrying the TEM β-lactamase gene (TnA) is found on R-factors of enteric origin regardless of their incompatibility class, overall guanine plus cytosine content, strain, or geographical source (13). This observation, taken together with the conjugative nature of the *Haemophilus* plasmid, is consistent with the view that the 30-Mdal plasmid found in *H. influenzae* may have originated from one of the established R-factor incompatibility classes of enteric plasmids. However, our attempts to demonstrate the transfer of the 30-Mdal *Haemophilus* plasmids to *E. coli* K-12 have not been successful, nor have Thorne and Farrar (J. Infect. Dis., in press) been able to demonstrate the conjugal transfer of ampicillin resistance from *Haemophilus* to either *E. coli* or *Klebsiella pneumonia*. Although these observations may be interpreted as evidence against an enteric species as the origin of the plasmids of *Haemophilus*, it is worth noting that several of the R-plasmids of *Pseudomonas aeruginosa* are promiscuously transmitted to a variety of species, including soil saprophytes, *Neisseria perflava*, and photosynthetic bacteria (18), but in some cases are not capable of intergeneric transfer to *E. coli* (14). In addition, the entire plasmid or the Ampicillin translocation sequence may be capable of insertion into the bacterial chromosome (20). Thus, some ampicillin-resistant strains of *H. influenzae* may exist without demonstrable cytoplasmic DNA. This possibility and others are being investigated by means of DNA-DNA heteroduplex analysis.

The 3-Mdal *Haemophilus* plasmid is nontransmissible by conjugation, although it easily transformed ampicillin-sensitive *Haemophilus* strains. This smaller plasmid species appears to contain the equivalent of 1.2 to 1.4 Mdal of the 2.7-Mdal TnA sequence. Perhaps this plasmid is a derivative of the 30-Mdal plasmid, being selected out of its larger progenitor by a series of recombinational events. If this was the case, it argues for a very specific reduction of plasmid DNA that resulted in reduction in size by a factor of 10, leaving the essential plasmid genes and the structural gene for β-lactamase intact. An alternative possibility is that the 30-Mdal plasmid was co-resident in a cell containing a small indigenous plasmid of *Haemophilus* and that a portion of TnA segment was translocated to the indigenous plasmid species. Eventually, the larger plasmid might have been eliminated from its host bacterium, leaving only the small plasmid containing a portion of the TnA translocation sequence. Heteroduplex analysis and further physical characterization of the 30-Mdal plasmid should clarify the question of its origin. In any event, the smaller plasmid is a model of efficiency, capable of replicating itself and specifying a β-lactamase enzyme in the short space of 3 Mdal of DNA.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grants AI10885-03 and AI12192-01 from the National Institute of Allergy and Infectious Diseases and grant GB32416 A#1 from the National Science Foundation.

We wish to thank John Sherris and Fritz Schoenknecht for their cooperation and interest in this study. The excellent technical assistance of Julie Sasewich is gratefully acknowledged. We also thank Leonard Mayer for his contribution to the earlier phase of this study.

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