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# Mechanisms of Ampicillin Resistance in *Haemophilus influenzae* Type B[1]

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The genetic mechanisms associated with ampicillin resistance in strains of *Haemophilus influenzae* type b were investigated. In experiments concerned with transfer of total deoxyribonucleic acid in vitro, expression of resistance by wild-type strains occurred at a frequency of about 10%. The minimum inhibitory concentration of ampicillin for the transformed strains was similar to that of the resistant donor strains, and resistance in transformants was associated with acquisition of the ability to produce beta-lactamase. Exposure to 39 μg of acridine per ml for 18 h cured resistance at a frequency of 80%, and there was spontaneous loss of resistance after repeated subculture of some strains. Analysis by cesium chloride-ethidium bromide density gradient centrifugation demonstrated the presence of extrachromosomal deoxyribonucleic acid in the resistant strains, providing further evidence that the resistance factor is plasmid mediated.

Since 1963, ampicillin has been regarded as the drug of choice in the treatment of meningitis in children. It has proved highly effective against *Streptococcus pneumoniae, Neisseria meningitidis,* and *Haemophilus influenzae* type b, the organisms most often isolated from cases of childhood meningitis. In recent years, however, there has been a steady increase in reports of the failure of ampicillin treatment in cases of *H. influenzae* infections. Although most of these failures have subsequently been attributed to factors other than drug resistance (1, 8, 11, 15), the presence of significant numbers of ampicillin-resistant strains has now been documented (3, 4). The first ampicillin-resistant *H. influenzae* type b confirmed by the Center for Disease Control (CDC) was isolated in May 1972 from a child in Germany (7). Additional strains of ampicillin-resistant *H. influenzae* have been confirmed by the CDC from a broad geographic base in the United States (3, 4). These strains show a mean minimum inhibitory concentration (MIC) of 55 μg/ml (4). Recent work has demonstrated that ampicillin resistance is due to the production of a beta-lactamase by the resistant strains tested (4, 12). The enzyme has been characterized (6, 14), and several procedures for its rapid determination have been presented (2, 18).

There has been speculation that susceptible wild-type strains of *H. influenzae* might have acquired a plasmid-mediated ability to produce beta-lactamase from other gram-negative bacterial species (2, 6) and, further, that ampicillin resistance is transferable among *H. influenzae* strains. Repeated attempts at intergenus transfers (5, 12, 17) and intragenus transfers during mixed incubation in broth (17) have been unsuccessful. Because the mechanism of transfer remains unclear, this study was initiated to investigate resistance transfer among strains of *H. influenzae* type b.

## MATERIALS AND METHODS

**Organisms.** The five ampicillin-resistant strains of *H. influenzae* type b (74-64148, 74-81082, 73-340, 74-90383, and 74-71518) used in this study were obtained from the CDC, Atlanta, Ga.; all exhibited MIC values ranging from 8 to 32 μg/ml. The five ampicillin-susceptible strains were provided by John W. Dyke of Sparrow Hospital, Lansing, Mich. The susceptible strains were all isolates from meningitis cases and were shown to have MIC values of approximately 0.1 μg/ml. Strains were preserved for study by lyophilization or freezing of bacterial suspensions in phosphate-buffered glycerol (0.05 M $KPO_4$, pH 7.0) at −20 C.

**Media.** The bacteria were maintained on chocolate agar with IsoVitaleX supplement obtained from BBL and were transferred every 3 days. Fresh subcultures from the frozen stocks were retrieved monthly. The broth medium used was brain heart infusion (BHI, BBL) with hemin (10 μg/ml, Sigma Chemical Co.) and nicotinamide adenine dinucleotide (2 μg/ml, Sigma). This same medium, when freshly supplemented with deoxyadenosine, (250 μg/ml, Sigma) was used for the radioisotope studies

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or with ampicillin (Bristol Laboratories) for the MIC assays.

**Transfer of resistance.** Resistance transfer experiments were modified from the transformation procedure described by Juni and Janik (10). Deoxyribonucleic acid (DNA) from ampicillin-resistant strains of *H. influenzae* was prepared by suspending one large loopful of cells from an overnight culture on chocolate agar in 0.5 ml of saline citrate buffer (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) containing 0.05% sodium dodecyl sulfate. The suspension was then heated for 1 h at 60 C. Three or four colonies of ampicillin-susceptible cells were mixed with one loopful of DNA on chocolate agar and allowed to grow in a candle jar for 18 to 20 h at 37 C. Each DNA preparation was additionally subcultured to ensure that no viable ampicillin-resistant cells remained, and representative preparations were treated with deoxyribonuclease (Sigma) (200 μg/ml at 37 C for 0.5 h) to demonstrate loss of transforming ability. A loopful of the resulting progeny growth was suspended in 1.0 ml of BHI broth, and this suspension was then streaked onto chocolate agar to yield 100 to 200 isolated colonies.

The frequency of cells transformed to ampicillin resistance was determined by streaking isolated colonies radially away from an ampicillin disk (10 μg, Difco). Colonies were scored as presumptive resistants if they grew within 5 mm of the disk edge.

**MICs.** MIC assays were performed on the original ampicillin-resistant and ampicillin-susceptible strains and on presumptive transformed resistant strains of *H. influenzae*. Twofold serial dilutions of ampicillin (stored as a stock powder at −20 C) were freshly prepared in supplemented BHI broth, 0.5 ml per tube, beginning at a concentration of 250 μg/ml. The inoculum used contained approximately $5 \times 10^5$ colony-forming units and consisted of 0.5 ml of a 100-fold dilution of an 18- to 20-h culture of each organism to be tested. The final volume was 1.0 ml per tube with the ampicillin concentration ranging from 0.097 to 125 μg/ml. The tubes were incubated at 37 C without increased $CO_2$ and examined for turbidity against indirect light after 20 h. Control tubes contained BHI and inoculum without ampicillin at 37 C (positive), BHI and inoculum at 5 C, or BHI with ampicillin at 37 C (negative). The MIC was recorded at the lowest concentration of ampicillin inhibiting visible growth of the organism.

**Detection of beta-lactamase.** All strains of *H. influenzae* and all DNA preparations used in this study were tested for the production of beta-lactamase by the iodometric method of Catlin (2) modified for use with chocolate agar as the growth medium. To test tubes (12 by 75 mm) containing 0.5 ml of a stock solution of penicillin G (6,000 μg/ml, Sigma, stored as a powder at −20 C) was added DNA or sufficient organisms to give an opaque suspension. The tubes were incubated at room temperature for 1 h, and 0.1 ml of starch indicator (1.0% soluble starch, BBL, in distilled water) was added to each. After the contents were mixed, 1 drop of iodine reagent (2.03 g of iodine and 53.2 g of potassium iodide in 100 ml of distilled water) was added to each, and the tubes were gently shaken. Blue color persisting longer than 10 min indicated the absence of the enzyme. Controls included two tubes of penicillin without bacteria assayed, respectively, before and after the incubations to ensure absence of spontaneous hydrolysis of penicillin.

**Acridine cure of ampicillin resistance.** Serial twofold dilutions of acridine orange (3,6-bis-dimethylamino-acridinium chloride, Sigma) in supplemented BHI broth were prepared to give concentrations ranging from 1.0 mg/ml to 1.95 μg/ml, 0.5 ml per tube. To each tube was added 0.5 ml of a cell suspension (equivalent in turbidity to a standard prepared by adding 0.5 ml of 0.048 M $BaCl_2$ to 99.5 ml of 1.0% $H_2SO_4$), and the tubes were incubated at 37 C for 18 to 20 h. A minimal bactericidal concentration was determined by streaking a loopful of each suspension onto chocolate agar and examining resulting growth. A loopful of culture broth from the highest concentration in which growth occurred was suspended in 1.0 ml of broth and this suspension was streaked onto chocolate agar to yield 100 to 200 isolated colonies per plate. The frequency of curing by acridine orange was determined by streaking isolated colonies radially away from an ampicillin disk (10 μg, Difco); curing of ampicillin resistance was recorded as absence of growth within 10 mm of the ampicillin disk edge.

**Cesium chloride-ethidium bromide density gradients.** Gradients were prepared as described by Lovett (13) and modified to employ a double volume of lysate. A 20-ml amount of supplemented BHI with deoxyadenosine (250 μg/ml) and [$^3$H]thymidine (0.1 mCi, New England Nuclear Corp.) was inoculated with approximately $10^5$ cells. The culture was grown to early stationary phase and chilled. The cells were concentrated by centrifugation and washed twice with cold buffer containing 0.02 M tris(hydroxymethyl)aminomethane, 0.1 M NaCl, and 0.005 M ethylenediaminetetraacetic acid (pH 7.5) (TES) and suspended in 2 ml of TES containing 20% sucrose. Lysozyme (200 μg/ml, Sigma) and ribonuclease (50 μg/ml, Sigma) were added and the suspension was incubated at 37 C for 40 min. TES buffer (2 ml), Sarkosyl NL-30 (to 0.8%, vol/vol), and predigested Pronase (to 500 μg/ml) were added, and incubation was continued for 30 min. The lysate was adjusted to 5 ml with TES buffer, and 6.9 g of cesium chloride (Sigma) was added. The resulting solution was mixed with 3 ml of ethidium bromide (Sigma, 4 mg/ml in 0.1 M sodium phosphate buffer, pH 7.0) and placed into a polyallomer tube (2 1/2 by 5/8 inch [6.4 by 1.6 cm]). The tubes were topped with paraffin oil and centrifuged in a type 50 rotor at 40,000 rpm and 15 C for 40 h. Eight-drop fractions were collected and precipitated with 5% trichloroacetic acid. The precipitates were collected on 24-mm glass fiber filters, washed, dried, and placed in vials containing 5 ml of toluene-based scintillation fluid [25 mg of 1-4-bis-(5-phenyloxazolyl)-benzene per liter, 1.25 g of 2,5-diphenyloxazole per liter, Packard Instrument Co.] and mixed well. Radioactivity was measured on a Packard Tri-Carb liquid scintillation counter.

## RESULTS

Transfer of resistance occurred at frequencies of 2, 8, 2, and 14% in four separate determinations of 100 isolated colonies each (Fig. 1). Treatment of crude transforming factor with deoxyribonuclease or by physical shearing by multiple passage through a Pasteur pipette destroyed the ability to transfer ampicillin resistance; control cultures of DNA preparations were negative for viable organisms.

Resistance of a few colonies selected as transformants in each of nine transfer experiments was assayed by tube dilution. Results of MIC determinations are illustrated in Table 1. All transformants uniformly produced beta-lactamase. Transforming DNA preparations were beta-lactamase negative.

The minimum bactericidal concentration of acridine orange was 78 μg/ml; exposure to concentrations of 39 and 18.5 μg/ml resulted in cure of ampicillin resistance at frequencies of 80 and 45%, respectively (Fig. 2).

The presence of plasmid nucleic acid was detected by cesium chloride-ethidium bromide density gradients. A typical gradient obtained from susceptible strain W-3 showed a single peak of chromosomal DNA (Fig. 3), whereas a similar preparation from strain 74-90383 showed the presence of extrachromosomal DNA in addition to the main chromosomal material.

## DISCUSSION

Until recently, strains of *H. influenzae* type b have been uniformly susceptible to treatment with ampicillin (1). The sudden appearance of ampicillin-resistant strains has prompted much concern over possible public health consequences as well as speculation about the mechanism of resistance involved (2, 6, 12, 14).

Ampicillin resistance in all strains of *H. influenzae* tested is due to the production of a beta-lactamase (2, 4, 18) and, to date, no ampicillin-resistant, beta-lactamase-negative organisms have been described. The beta-lactamase produced by ampicillin-resistant strains of *H. influenzae* is a constitutive enzyme (6, 14, 18) similar to that produced by other gram-negative organisms (16). This enzyme has been characterized by Medeiros and O'Brien (14) and Farrar and O'Dell (6) as a class IIIa beta-lactamase closely resembling the beta-lactamase produced by enteric bacteria.

The present study demonstrates that ampicillin resistance can be transferred in vitro to susceptible strains at frequencies ranging from 2 to 14%. This result stands in sharp contrast to that recently obtained by Thorne and Farrar (17), who achieved a low frequency of transfer (about $10^{-5}$) by specialized conjugation methods. Although the rate of transformation reported here may seem high, data from Hotchkiss and Gabor (9) indicate that unmodified cells of *H. influenzae* can readily take up DNA molecules and be transformed. MIC values determined for transformed cells showed levels of ampicillin resistance similar to those demonstrated by DNA donor resistant strains.

Although attempts by other researchers to cure ampicillin resistance in *H. influenzae* by exposure to ethidium bromide have been unsuccessful (6), an 80% cure of resistance was achieved by exposure to acridine orange, a

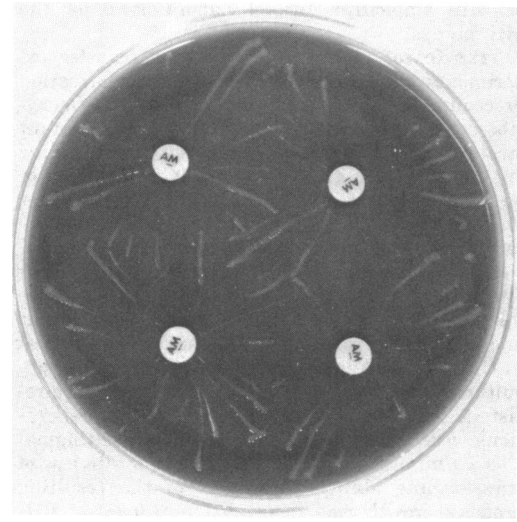

FIG. 1. *Radial streaking method used for determination of resistance frequency in transformants. A total of 100 colonies was streaked for testing. Colonies demonstrating growth within 5 mm of the disk edge were scored as resistant.*

TABLE 1. *Ampicillin MIC[a] in transformants*

| Resistant donor | MIC of susceptible recipient | | |
|---|---|---|---|
| | W-2 (0.12)[b] | W-3 (0.24) | W-5 (0.24) |
| 74-90383 (125) | 125 | 125 | 62 |
| | 62 | 62 | 125 |
| | 31 | 125 | 62 |
| 74-64148 (16) | 16 | 8 | 1.9 |
| | 16 | 125 | 7.8 |
| | | | 16 |
| 73-340 (62) | 62 | 62 | 16 |
| | 125 | 125 | 62 |
| | 62 | 125 | |

[a] MICs expressed as micrograms per milliliter.
[b] Number in parentheses is the MIC of the particular strain listed.

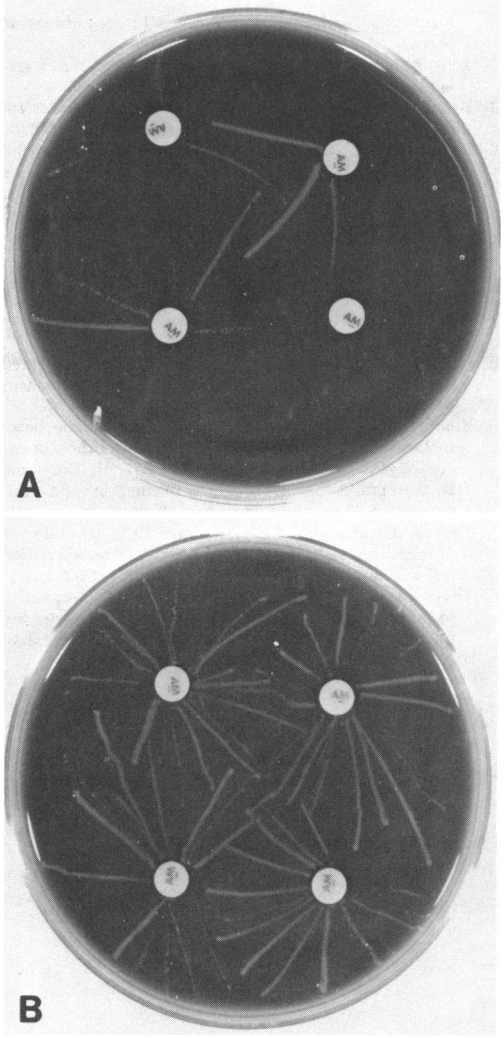


FIG. 2. *Acridine orange cure determined by radial streaking technique. (A) Single colonies selected after exposure to 39 μg of acridine orange per ml, and (B) colonies taken from broth containing an acridine orange concentration of 18.5 μg/ml.*

similar agent capable of complexing DNA. As reported here and previously (2), strain 74-71518 showed high spontaneous loss of its resistance factor. The observation that exposure to one-half of that concentration of acridine orange which yielded 80% cure resulted in only 45% cure is consistent with a conventional dose response curve. Furthermore, the zone criteria used for scoring both transfer of resistance and acridine cure in this study are more stringent than the interpretive zone diameters recommended in the disk diffusion method for ampicillin susceptibility testing of *H. influenzae* (4).

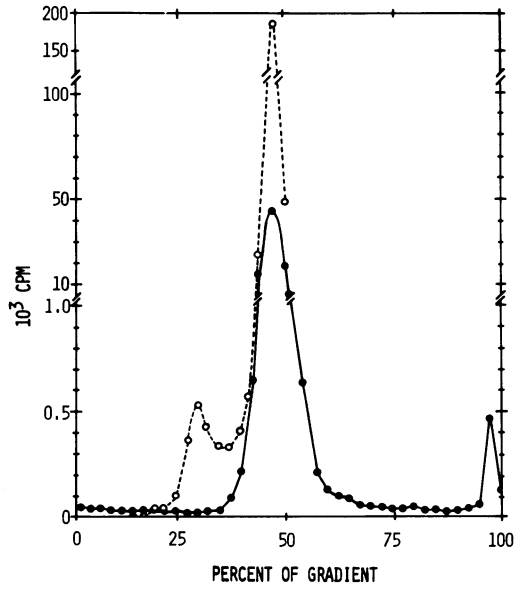


FIG. 3. *Profile of radioactive DNA from [$^3$H]-thymidine-labeled cells in a cesium chloride-ethidium bromide gradient. Symbols: ●, ampicillin-susceptible cells, strain W-3; ○, ampicillin-resistant cells, strain 74-90383. Bottom of gradient is at left.*

The technique described for the culturing, labeling, and lysing of cells of *H. influenzae* must be closely adhered to if the plasmid is to be demonstrated in the cesium chloride-ethidium bromide density gradient. The major difficulty of incorporating sufficient radioactive precursor ([$^3$H]thymidine) into cells grown in the rich BHI medium was circumvented by supplementing the medium with deoxyadenosine (13).

The presence of a second small peak separated from the large peak representing chromosomal DNA is definitive evidence of covalently closed circular DNA characteristic of a plasmid. The wild-type strain shows no such peak in the profile of its genetic material. A similar plasmid-containing fraction of *H. influenzae* DNA has been isolated by Elwell et al. (5). In their concurrent, independent investigation, these investigators were able to isolate two discrete plasmids associated with ampicillin-resistant cells, both of which transform susceptible cells to resistance.

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