MECHANISMS OF AMPICILLIN RESISTANCE IN HAEMOPHILUS INFLUENZAE TYPE B

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ROBERT VEGA

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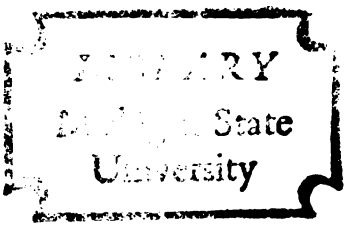

ABSTRACT

MECHANISMS OF AMPICILLIN RESISTANCE IN *HAEMOPHILUS INFLUENZAE* TYPE B

By

Robert Vega

The genetic mechanisms associated with ampicillin resistance in strains of *Haemophilus influenzae* type b were investigated. It was determined that *in vitro* transfer of resistance to susceptible wild type strains occurs at a frequency of approximately 10 percent in total DNA transfer experiments. The minimum inhibitory concentration (MIC) of ampicillin for the transformed strains was similar to that in the resistant donor strains. Resistance in transformants was associated with acquisition of the ability to produce beta lactamase. Exposure to acridine at a concentration of 39 mcg/ml for 18 hours cured resistance at a frequency of 80 percent and there was spontaneous loss of resistance after repeated subculture of some strains, evidence that the resistance factor is plasmid mediated. Cesium chloride - ethidium bromide density gradient analyses were performed to demonstrate the presence and location of the plasmid DNA in the resistant strains.

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Robert Vega

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INTRODUCTION

Acute bacterial meningitis is a medical emergency. With the advances in therapy associated with the antibiotic era and the growing sophistication of clinical laboratory technology, the severe life threatening implications of this infectious disease have been greatly reduced. Preliminary clinical and laboratory data allow immediate institution of antimicrobial therapy aimed at the most common causes of meningitis in each age group.

Among infants and children, the antibiotic of choice in the treatment of suspected bacterial meningitis is ampicillin. The three most frequent pathogens in this age group, Neisseria meningitidis, Haemophilus influenzae type b and Streptococcus pneumoniae, have demonstrated universal susceptibility to this drug. Confidence in this calculated but blind therapeutic approach has been dramatically altered by recent reports of confirmed ampicillin resistant strains of Haemophilus influenzae type b (10,11,12,13,14)

The emergence of these ampicillin resistant strains has been rapid, widespread geographically and associated with the production of a beta lactamase (10, 11, 12). The mechanism of introduction into H. influenzae

of genes mediating ampicillin resistance has been the subject of much current speculation. This study investigates the *in vitro* transfer of functional genetic material from resistant to susceptible strains of *H. influenzae* and the nature of the factors controlling this ampicillin resistance.

REVIEW OF LITERATURE

Haemophilus Influenzae

Haemophilus influenzae, or Pfeiffer's bacillus, as it has also been known, was first isolated and described during the influenza pandemic of 1892, by Richard F. J. Pfeiffer (17). It was then thought to be the sole etiologic agent of influenza, as it was commonly isolated from the sputum of patients with this disease. This belief persisted until the early 1930's, when it was shown that influenza is caused by a virus and that H. influenzae was present as a secondary invader (4).

Haemophilus influenzae belongs to a genera of unknown affiliation and is one of the smallest pathogenic bacilli, rarely exceeding 1.5 microns in length and 0.3 microns in diameter.(7) H. influenzae is predominately a small Gram negative coccobacillus, often growing in short chains resembling pneumococci or streptococci. Occasionally, on direct smear from spinal fluid, this organism will appear as long threads. Bipolar staining is also often observed in Gram stained smears. H. influenzae is nonmotile, nonspore-forming, and grows

best at 37 C in an atmosphere containing 10 percent CO_2 and on a medium supplemented with hemin, 10 mcg/ml, and nicotinamide adenine dinucleotide (NAD), 2 mcg/ml. Under optimum conditons, the generation time is approximately 30 minutes. The G+C ratio is 39.0 moles % (7,25). Genetic mapping of the organism has recently been accomplished (8).

Both capsulated and noncapsulated forms exist, the capsulated forms falling into six immunologic types designated a through f based on specific polysaccharide surface antigens detected by agglutination, precipitation, or quellung tests performed with specific antisera (17). H. influenzae type b is especially virulent, causing severe respiratory tract infections as well as being the most common cause of purulent meningitis in children.

Haemophilus influenzae does not occur naturally in lower animals, but is a human parasite, surviving only a short time away from the host. The organism is found as normal nasopharyngeal flora in a large proportion of the population and is transmitted between humans in droplets by coughing and sneezing.

Bacterial Meningitis

Meningitis, as a general term denoting inflamation of the coverings of the brain, can be further subdivided into two distinct clinical entities based on the anatomical subdivision of the meninges. Inflammation of the inner covering of the brain, the pia-arachnoid, is the more common entity and is what is usually referred to as bacterial meningitis.(33)

Bacterial meningitis is a rapidly fulminating disease which approaches 100 percent mortality in the untreated patient. Eight to ninety percent of cases of bacterial meningitis are caused by Haemophilus influenzae, Neisseria meningitidis, and Streptococcus pneumoniae. The remaining 10 to 20 percent are primarily caused by Staphylococcus aureus, group a streptococci, various Enterobacteriaceae, and rarely by Pseudomonas, Listeria, Clostridium perfringens and Neisseria gonorrheae.(17) The coliform bacilli and group b streptococci are the most common causes of meningitis in neonates.(50)

Most cases of bacterial meningitis are actually secondary to infections elsewhere in the body which may or may not be clinically apparent. Common precursors of meningitis are infections of the middle ear, mastoid, and paranasal sinuses, as well as trama or surgery to the head or face.

Signs and symptoms of bacterial meningitis may vary, but onset is usually rapid with early malaise, anorexia, and chills which are soon replaced by fever, photophobia, headache, disturbed consciousness, and in infants, a bulging fontanelle. There is a general stiffening of the musculature along the neuraxis. The neck becomes stiff, with the patient resisting any passive flexion. General stiffening has been considered a protective mechanism to reduce tension on the inflammed meninges and the irritated nerve roots. Late in the course of untreated meningitis, the swallowing mechanism may become impaired as the patient goes into a deepening coma and possible death.(33)

A definitive diagnosis of acute bacterial meningitis is based primarily on the examination of the cerebrospinal fluid. In addition, cultures of the blood, nose or throat, and ear may also prove helpful.

At lumbar puncture, the spinal fluid will almost invariably be under increased pressure, with the exception of patients who have become dehydrated due to the diarrhea and vomiting which may accompany the early stages of this disease. The spinal fluid appears cloudy on gross examination and microscopic examination reveals from 5 to 1,000 cells per cubic millimeter, 90 to 95 percent of which are polymorphonuclear neutrophils.

Biochemical analysis of the spinal fluid reveals a protein level elevated from a normal value of 15-45 mg/100 ml to 100-200 mg/100 ml. The glucose level is decreased from a normal value of 50-70 mg/100 ml to 10-30 mg/100 ml (33).

Gram stain and culture of the spinal fluid with subsequent determination of antibiotic sensitivity is by far the most valuable diagnostic aid available to the physician in determining appropriate treatment. The value of the Gram stain cannot be overemphasized. Occasionally an organism will be seen on Gram stain while culture results are negative.

Haemophilus influenzae type b is the most common cause of bacterial meningitis in children, occuring almost exclusively between the ages of 2 months and 6 years. In their classic studies, Fothergill and Wright showed that the increased incidence of H. influenzae meningitis is inversely related to the level of bactericidal substances in the blood (23). From the age of 2 months to the age of 3 years, the amount of maternal antibody acquired at birth is depleted and the amount of actively formed antibody is minimal. Thereafter, the amount of antibody increases and the incidence of disease declines.

Meningitis caused by H. influenzae type b is an especially severe disease in children, with a mortality rate of over 95 percent in untreated cases. Even with prompt treatment, the mortality rate is still as high as 10 percent (17). Although antibiotic therapy has dramatically reduced the severe neurologic sequelae of patients who do recover, 5 percent show permanent residual defects and require institutionalization (17).

Treatment of Haemophilus Influenzae Meningitis

Prior to the early 1930's, an effective therapy for the treatment of Haemophilus influenzae meningitis was non-existent and the mortality rate approached 100 percent. In 1931, Fothergill introduced an immune horse serum which was shown in a study reported in 1937 to have reduced the mortality rate to 85 percent (24). The introduction of sulfonamide treatment for meningitis in the late 1930's, provided a welcome alternative to treatment with immune horse serum, but at best, mortality was only reduced to 75 percent (32,39).

Real progress in effectively treating Haemophilus influenzae meningitis was not made until 1938, when Alexander introduced a specific antiserum produced in rabbits (1). The effectiveness of this antiserum was confirmed in 1942 by a study showing that treatment

with specific rabbit sera in combination with sulfadiazine lowered the mortality rate to 26 percent (2). Later work in 1946 reported a mortality of only 20 percent (20).

In 1946, streptomycin was introduced as a treatment for Haemophilus influenzae meningitis. This drug when used singly, reduced the mortality rate to the level of combined specific rabbit sera and sulfonamides (6). The advantages of using streptomycin over treatment with antisera were overshadowed, however, by reports of streptomycin resistant strains of H. influenzae developing during treatment (3). Treatment with Alexander's specific antisera remained the preferred therapeutic regimen for H. influenzae meningitis into the early 1950's.

In 1950, Drake and his colleagues reviewed the use of aureomycin (chlortetracycline) in the treatment of Haemophilus influenzae meningitis. Aureomycin was well tolerated both orally and intravenously without the toxicity or other disadvantages of previous therapy. Effective blood levels were reached rapidly with good penetration into the cerebrospinal fluid (19). Although Drake reported treatment with aureomycin to be as effective as treatment with a combination of streptomycin, antisera, and sulfonamide drugs, reluctance at changing a proven regimen existed.

Chloramphenico, as an effective agent in the treatment of H. influenzae meningitis, was investigated in 1951 by McCrumb and his co-workers (35). Although McCrumb reported chloramphenicol to be no more effective than aureomycin, later studies showed that when administered by injection, chloramphenicol reduced mortality to less than 10 percent, as well as significantly reducing the incidence of residual neurologic defects (18,35,40). Chloramphenicol was shown to be an especially fast-acting antibiotic which penetrated well into the cerebrospinal fluid. Even after reports that treatment with this drug could result in the patient developing aplastic anemia, (31,38,41,45) chloramphenicol quickly became the drug of choice. The advantages of decreased mortality and morbidity were considered far to outweigh the remote consequence of aplastic anemia.

In 1965, Mathies and his associates published the results of a clinical trial comparing the effectiveness of ampicillin to that of chloramphenicol in the treatment of H. influenzae meningitis (34). Based on Mathies' conclusions as well as supporting work by Ivler, (29) Thrupp, (45) Fleming (22) and others (5,31) ampicillin soon replaced chloramphenicol as the drug of choice in the treatment of bacterial meningitis due to H. influenzae type b. Ampicillin was shown to be

as effective, if not more so, as previous therapy without the dangers of hemotoxicity. Ampicillin also proved to be highly effective in the treatment of pneumococcal as well as meningococcal meningeal infections (22,45). Physicians had a single drug that could be used with confidence against the three most common agents of bacterial meningitis.

Increased reports in the late 1960's of ampicillin failure in the treatment of Haemophilus influenzae meningitis were found to be due to factors other than ampicillin resistance. These failures have been attributed to inadequate dose of the antibiotic, (27,30) improper route of administration, (30) insufficient duration of therapy, (27,30,48) or improper storage and handling of the drug prior to administration (48). All of these factors can result in failure to obtain or to maintain effective antibiotic levels in the cerebrospinal fluid. Other causes of ampicillin failure were ascribed to sequestered foci of infection, extrameningeal sources of infection, or to compromised host defenses (30,42).

In May, 1972, an ampicillin resistant Haemophilus influenzae type b was isolated from a child in Germany (26). The finding of this strain was not immediately published. In December, 1973, the first ampicillin resistant H. influenzae type b isolated in the United

States was recovered from a child with bacterial meningitis in Patuxent, Maryland (10). Since this Maryland case, over 50 additional confirmed ampicillin resistant isolates have been reported from a wide geographic distribution in the United States (11). Resistant strain have also been confirmed in Great Britain (15,45). These reports have had a disturbing effect on the general consensus as to the proper regimen for treatment of bacterial meningitis due to Haemophilus influenzae. The physician can no longer assume that isolates of H. influenzae type b will be uniformly susceptible to ampicillin therapy.

Due to the rapidly fulminating course of this disease, patients presenting with clinical signs of meningitis must be treated immediately. When reports of confirmed ampicillin resistant H. influenzae type b from cases of meningitis appeared in the literature, the security provided by ampicillin treatment of meningitis of unknown origin no longer existed. A means of demonstrating ampicillin resistance sooner than currently possible was needed.

In 1974, research by W. Khan et al. led to the observation that ampicillin resistance in the strains of H. influenzae examined was associated with the presence of beta lactamase activity (30). Subsequent work by

Thornsberry and Kirven (45) and Catlin (9) led to the development of rapid procedures for the demonstration of beta lactamase activity.

To date, all resistant strains of Haemophilus influenzae tested have been positive for beta lactamase activity (11). Rapid determination of beta lactamase activity, will allow a tentative decision about therapy to be made often within an hour of obtaining visible growth on laboratory media. In many instances, this can be within 12 hours of obtaining the sample for initial culture. Assays for beta lactamase production are, however, merely screening procedures for ampicillin resistance. All organisms screened in this manner must also be subjected to standard sensitivity testing either by disc diffusion technique where relative susceptibility is assessed or by serial dilution methods to determine minimal inhibitory concentration (MIC) directly (11).

The acceptance of ampicillin resistant strains of Haemophilus influenzae as a reality has prompted the reconsideration of ampicillin as the drug of choice in the treatment of bacterial meningitis (30,37). Subsequently, optional courses of therapy have been presented. The regimen gaining the most support is initiation of therapy with ampicillin and close monitoring of the patient's response. Upon confirmation of the presence

of ampicillin resistant H. influenzae, the regimen of therapy is changed to treatment with chloramphenicol. In this situation, the risk of aplastic anemic is less of a factor in light of the high mortality in untreated cases. A less favored course of treatment is to initiate therapy with chloramphenicol. If an ampicillin susceptible organism is subsequently isolated, the therapy is changed to treatment with ampicillin. Chloramphenicol should be continued in the treatment of an ampicillin susceptible organism only when the patient presents a history of allergic reaction to penicillin or when continued treatment with ampicillin fails to produce a favorable response.

A third option of initiating treatment with a combination of ampicillin and chloramphenicol, with one drug being discontinued after susceptibility test results have been obtained, has been avoided as unsound and hazardous in light of reports that these drugs may be antagonistic (34,47).

Mechanisms of Ampicillin Resistance

The recent appearance of ampicillin resistant strains of Haemophilus influenzae type b has prompted concern as to the origin of this resistance. Whether the resistant strains have arisen from a single progenitor in one mutation event (36) or from other organisms

by conjugation or transductional, the subsequent selection steps should be identical.

The origninal resistant strain or strains were able to persist and multiply as part of the upper respiratory flora in the healthy human. These strains were then gradually selected by the increasing use of ampicillin. Carrier rate studies show that in the early part of 1974, 10 percent of the randomly sampled population of a Washington, D.C., community were carrying ampicillin resistant stains of Haemophilus influenzae (30). The shift in MIC patterns of Haemophilus influenzae type b can be seen in the surveys by Khan in 1964 to 1966 (30) when all strains tested showed an MIC of 0.78 mcg/ml or less and again in 1974 (30) when only 80 percent of strains were inhibited by 0.78 mcg/ml of ampicillin.

Epidemiological evidence suggests amplification of sporadic resistant strains in closed population after contact with an index case (46) and among people with a likelihood for travel. Low numbers of gradually developing resistant strains might have existed undetected due to several factors. The first of these is that resistant clones might have represented only a small percentage of the total organisms infecting the host, as in the report of Khan et al. showing resistant satellite colonies in a patient successfully treated

with ampicillin (31). The lack of appropriate standardized techniques for assessing antibiotic susceptibilty *in vitro* is another factor responsible for overlooking of resistant strains. It has been shown that length of incubation, size of inoculum, and media and conditions of growth substantially alter results (11,44,49). Finally, Medeiros and O'Brien described the relatively low level resistance found in *Haemophilus influenzae* compared to certain of the coliform bacteria as masking these emerging strains (43). The low level resistance was attributed to increased permeability of penicillin into intact cells of *Haemophilus influenzae* overpowering the penicillin inactivating system.

That the original strains might have acquired markers from other organisms is particularly interesting. All strains of ampicillin resistant *Haemophilus influenzae* investigated, produce a constitutive beta lactamase (11) which has been preliminarily characterized as a type IIIa enzyme similar to that found in *Klebseilla pneumoniae* (21,36). The gene mediating this type IIIa enzyme in *Klebsiella pneumoniae* is carried on a plasmid and transferred with high frequency (36). *Klebsiella pneumoniae* is commonly isolated from the respiratory tract in the very young, the elderly, and those receiving antibiolic therapy.

Chromosomal or extrachromosomal mechanisms other than beta lactamase production that mediate destruction of penicillins in other organisms include altered permeability of the bacterial membrane, (16) varying concentrations of cell bound penicillinase, (36) inaccessibility of the mucopeptide synthesizing site, and the degree of vulnerability of the bacterial cell integrity (16,36). The extent to which these mechanisms participate in ampicillin resistance of Haemophilus influenzae has not been assessed.

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MECHANISMS OF AMPICILLIN RESISTANCE IN *HAEMOPHILUS INFLUENZAE* TYPE B

By

R. Vega, M.J. Patterson and H.L. Sadoff

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ABSTRACT

The genetic mechanisms associated with ampicillin resistance in strains of Haemophilus influenzae type b were investigated. It was determined that in vitro transfer of resistance to susceptible wild type strains occurs at a frequency of approximately 10 percent in total DNA transfer experiments. The minimum inhibitory concentration (MIC) of ampicillin for the transformed strains was similar to that in the resistant donor strains. Resistance in transformants was associated with acquisition of the ability to produce beta lactamase. Exposure to acridine at a concentration of 39 mcg/ml for 18 hours cured resistance at a frequency of 80 percent and there was spontaneous loss of resistance after repeated subculture of some strains, evidence that the resistance factor is plasmid mediated. Cesium chloride - ethidium bromide density gradient analyses were performed to demonstrate the presence and location of the plasmid DNA in the resistant strains.

INTRODUCTION

Since 1963, ampicillin has been regarded as the drug of choice in the treatment of meningitis in children. It has proven highly effective against Streptococcus pneumoniae, Neisseria meningitidis and Haemophilus influenzae type b, the organisms most often isolated from cases of meningitis in children.

In recent years, however, there has been a steady emergence of ampicillin resistant cases of Haemophilus influenzae infections reported to the Center for Disease Control (CDC) in Atlanta, Georgia. While most of these ampicillin failures have subsequently been attributed to factors other than drug resistance, (11,15) the presence of significant numbers of ampicillin resistant strains has been documented (4,5).

The first ampicillin resistant Haemophilus influenzae type b confirmed by CDC was isolated in May, 1972, from a child in Germany (8). In December, 1973, CDC confirmed the first two isolates of ampicillin resistant H. influenzae type b in the United States (4) from cases of meningitis in Altanta, Georgia and Patuxent,

Maryland. Prevalence studies conducted in Maryland and Washington D.C. revealed that approximately 10 percent of the sampled population carry ampicillin resistant strains of Haemophilus influenzae type b as normal flora in the nasopharynx (12). Additional strains of ampicillin resistant H. influenzae have been confirmed by the CDC from a broad geographic base in the United States (4,5). These strains show a mean MIC of 55 mcg/ml (5). Resistant strains have also been confirmed in Great Britain (6,17,19) and Europe (8).

Recent work has demonstrated that ampicillin resistance is due to the production of a beta lactamase by the resistant strains tested (12). Subsequently, the enzyme has been characterized and several procedures for the rapid determination of beta lactamase activity have been presented (3,18).

There has been speculation that susceptible wild type strains of Haemophilus influenzae might have acquired a plasmid mediating beta lactamase production from other gram negative bacterial species (3,7) and that ampicillin resistance is transferable among H. influenzae strains. This study was initiated to investigate the genetics of ampicillin resistance in H. influenzae type b.

MATERIALS AND METHODS

Organisms

The five ampicillin resistant strains of Haemophilus influenzae type b (74-64148, 74-81082, 73-340, 74-90383, 74-71518) used in this study were obtained from the Center for Disease Control (CDC). These strains were shown at CDC to have Minimum Inhibitory Concentration (MICs) ranging from 8-32 mcg/ml. The five ampicillin susceptible strains were provided by John W. Dyke of Sparrow Hospital, Lansing, Michigan. The susceptible strains were all isolates from spinal meningitis cases and were shown to have MICs of approximately 0.1 mcg/ml.

Strains were preserved for study by lyophilization and by 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 (BBL) and were transferred every three days. Fresh subcultures from the frozen stocks were retrieved monthly. The broth medium used was brain heart infusion (BHI,BBL) with hemin (10 mcg/ml,

SIGMA) and nicotinamide adinine dinucleotide (NAD, 2 mcg/ml, SIGMA). This same broth, when freshly supplemented with deoxyadenosine (250 mcg/ml, SIGMA), was used for the radioisotope studies or with ampicillin (Bristol Laboratories) for the MIC assays.

Transfer of Resistance

Genetic transfer experiments were modified from the method described by Juni and Janik (10). DNA from ampicillin resistant strains of Haemophilus 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 (SDS). The suspension was then heated for 1 hour at 60 C. Three or four colonies of ampicillin susceptible cells were mixed with one loopful of ampicillin resistant DNA on chocolate agar and allowed to grow in a candle jar for 18-20 hours at 37 C. Each DNA preparation was additionally subcultured to insure that no viable cells remained, and representative preparations were treated with DNAse (200 mcg/ml, 37 C, 0.5 hours SIGMA) to demonstrate loss of transforming ability. A loopful of the resulting progeny growth was suspended in 1.0 ml BHI broth to give a slight turbidity. A loopful of this suspension was then streaked onto

chocolate agar in such a way as to yield 100-200 isolated colonies (Fig. 1) to select subsequently for acquisition of ampicillin resistance.

Frequency of transformed cells was determined by streaking isolated colonies radially away from an ampicillin disk (10 mcg, Difco). Colonies were preliminarily scored as resistant if they grew within 5 mm of the disk edge.

MICs

MICs were performed on the original ampicillin resistant and ampicillin susceptible strains and on transformed resistant strains of Haemophilus 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 mcg/ml. The inoculum used contained approximately 5×10^5 colony forming units and consisted of 0.5 ml of a 1:100 dilution of an 18-20 hour 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 mcg/ml. The tubes were incubated at 37 C without increased CO_2 and examined against indirect light at 20 hours for turbidity. Control tubes consisted of BHI and inoculum without ampicillin at 37 C (positive), BHI and inoculum at 5 C, and BHI with

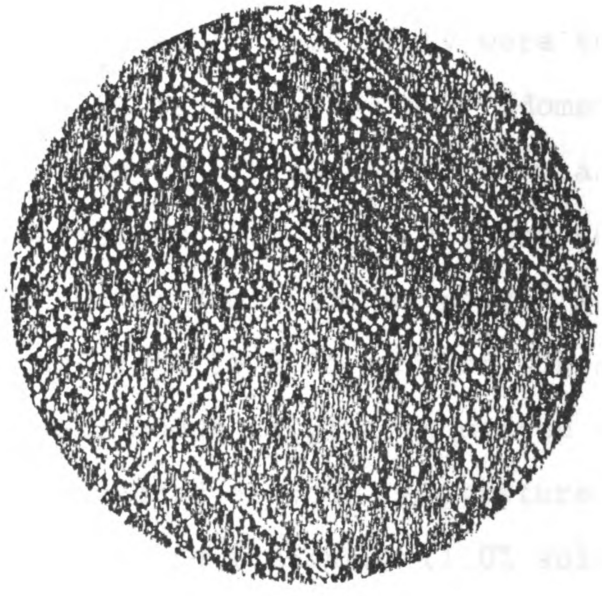


Fig. 1. Streaking pattern used to obtain 100 to 200 isolated colonies.

ampicillin at 37 C (negative). The MIC was recorded as the lowest concentration of ampicillin inhibiting visible growth of the organism.

Detection of Beta-Lactamase Production

All strains of Haemophilus influenzae as well as all DNA preparations used in this study were tested for the production of beta lactamase by the iodometric method of Catlin (3) modified to use chocolate agar as the growth medium. Basically, 0.5 ml of a stock solution of penicillin G (10,000 Units/ml, SIGMA, powder stored at -20 C) was dispensed into 12 x 75 mm test tubes and the orgnnisms or DNA were added to give an opaque suspension. These tubes were incubated at room temperature for 1.0 hour. Two drops of starch indicator (1.0% soluble starch, BBL, in distilled water) were added to each tube. After the tubes were mixed, one drop of iodine reagent (2.03 g iodine, 53.2 g potassium iodide in 100 ml distilled water) was added to each and the tubes gently shaken. Blue color persisting longer than 10 minutes indicated the absence of the enzyme. Controls included two tubes of penicillin without bacteria assayed respectively before and after the incubations to insure absense of spontaneous penicillin hydrolysis.

Acridine Cure of Ampicillin Resistance

Serial twofold dilutions of acridine (SIGMA) were prepared to give concentrations ranging fom 1.0 mg/ml to 1.95 mcg/ml in supplemented BHI broth, 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 percent H_2SO_4. The tubes were incubated at 37 C for 18-20 hours. A minimal bactericidal concentration was determined by streaking a loopful of each suspension onto chocolate agar and observing for growth. A loopful of culture broth from the first tube demonstrating visible growth was suspended in 1.0 ml of broth and a loopful of this suspension streaked onto chocolate agar in such a way as to yield 100 to 200 isolated colonies. The frequency of curing was determined by streaking isolated colonies radially away from an ampicillin disk (10 mcg Difco). Cure 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 use supplemented BHI as the culture medium and to employ a double volume of lysate. A 20 ml

amount of supplemented BHI with deoxyadenosine (250 mcg/ml) and ^{3}H-thymidine (0.1 mCi), both from 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 TES buffer and suspended in 2 ml of TES containing 20 percent sucrose. Lysozyme (200 mcg/ml, SIGMA) and RNAse (50 mcg/ml, SIGMA) were added and the suspension was incubated at 37 C for 40 minutes. TES buffer (2 ml), Sarkosyl NL-30 (to 0.8%), and predigested pronase (to 500 mcg/ml) were added, and incubation was continued for 30 minutes. The lysate was adjusted to 5 ml with TES buffer, and 6.9 grams 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"). The tubes were topped with paraffin oil and centrifuged in a type 50 rotor at 40,000 rpm and 15 C for 40 hours. Eight drop fractions were collected and precipitated with 5 percent trichloroacetic acid. The precipitates were collected on 24 mm glass fiber filters, dried, and placed into scintillation vials containing 5 ml toluene based scintillation fluid (25 mg/liter POPOP, 1.25 g/liter PPO, Packard Instrument Co.) and mixed well. The radioactivity was measured on a Packard Tricarb liquid scintilation counter.

RESULTS

Transfer of resistance occurred at frequencies of 2, 8, 2, and 14 percent in 4 separate determinations of 100 isolated colonies each (see Fig. 2). Treatment of crude genetic material with DNAse destroyed the ability to transfer resistance and cultures of DNA preparations were negative for viable organisms.

Three colonies selected as transformants in each of nine transfer experiments were assayed by tube dilution MIC testing. Results of MIC determinations are illustrated in Table I.

All transformants uniformly produced beta lactamase. Transforming DNA preparations were beta lactamase negative.

Exposure to acridine for 18 hours at concentrations 1 and 2 twofold dilutions beyond the minimum bactericidal concentration (MBC) yielded cures of 80 and 45 percent respectively. The results from an assay where the MBC was 78 mcg/ml are illustrated in Fig. 3.

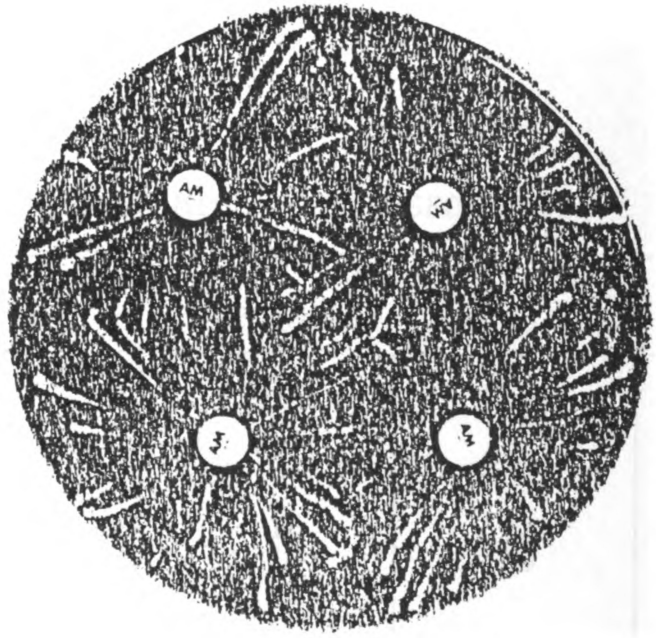


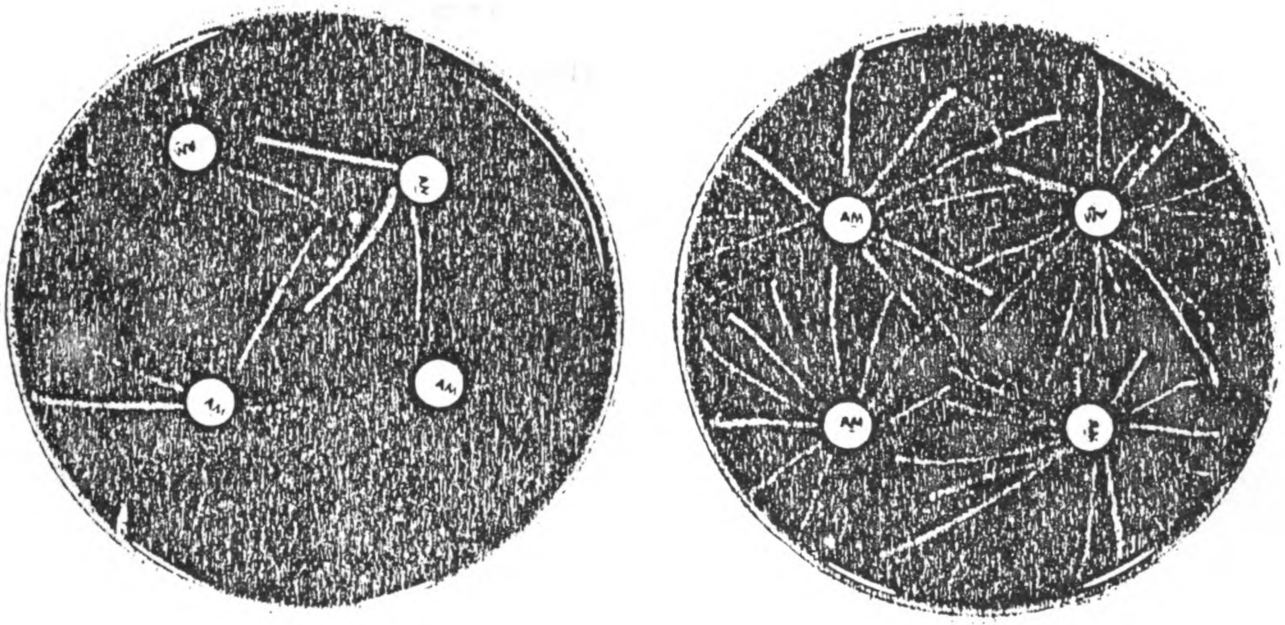
Fig. 2. Radial streaking method used for determination of resistance frequency in transformants.

A total of 100 colonies were streaked for testing. Colonies demonstrating growth within 5 mm of the disk edge were scored as resistnat. This representative assay shows 14 percent resistance transfer.

Table 1. AMPICILLIN MIC* IN TRANSFORMANTS.

Resistant Donor	Susceptible Recipient W-2 (0.12)	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	

* = mcg/ml



3a 3b

Fig. 3. Acridine cure determined by radial streaking technique.

3a Shows single colonies selected after exposure to 39 mcg/ml acridine demonstrating 80 percent cure.

3b Illustrates 45 percent cure of colonies taken from broth containing an acridine concentration of 18.5 mcg/ml.

Presence of plasmid nucleic acid was detected by cesium chloride-ethidium bromide density gradients. Gradient results of susceptible strain W-3 show a single peak of chromosomal DNA (Fig. 4) while a profile of strain 74-90383 genetic material shows the presence of extra-chromosomal DNA (Fig. 5), in addition to the main chromosomal material.

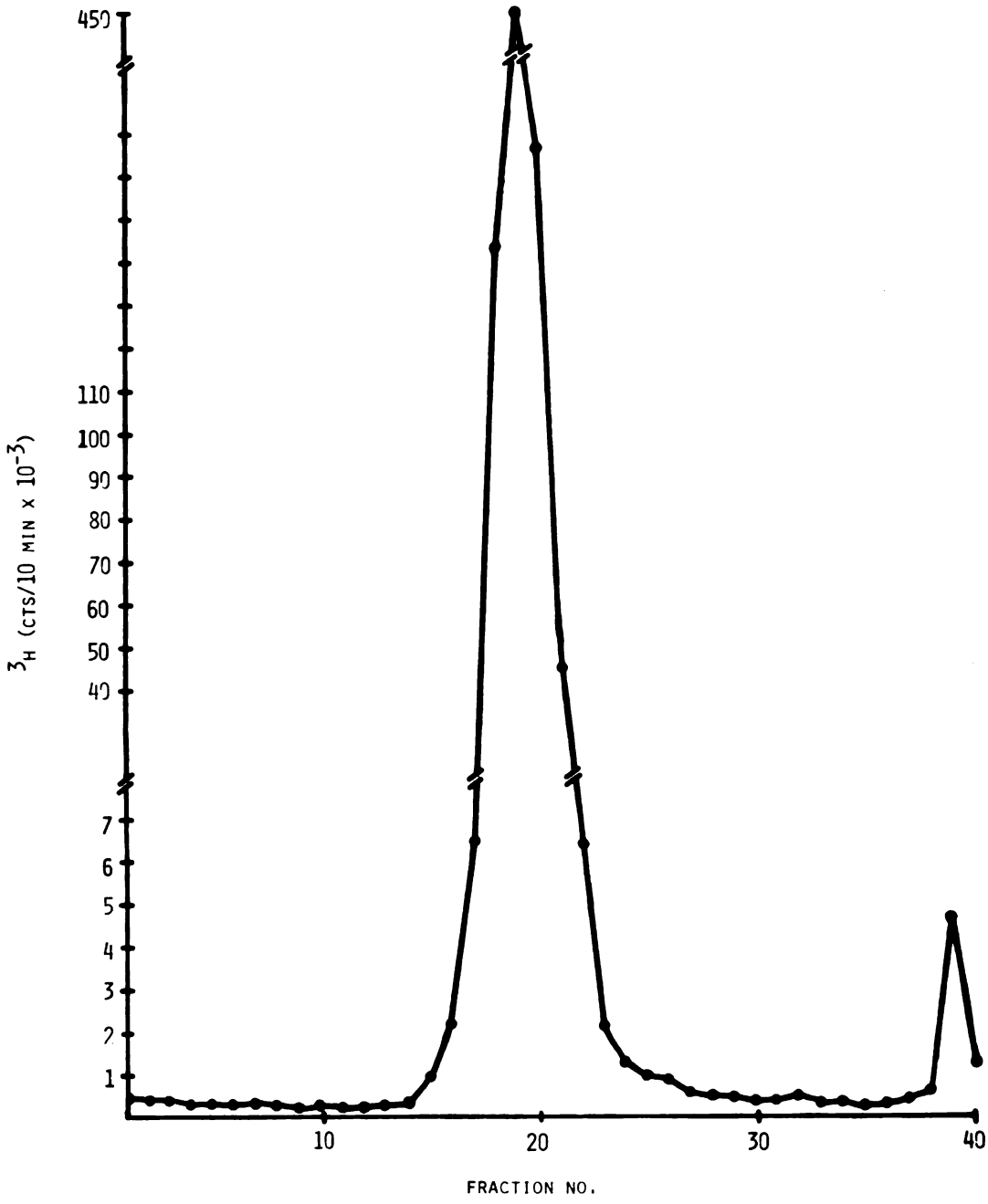


Fig. 4. Profile of genetic material from susceptible strain W-3.

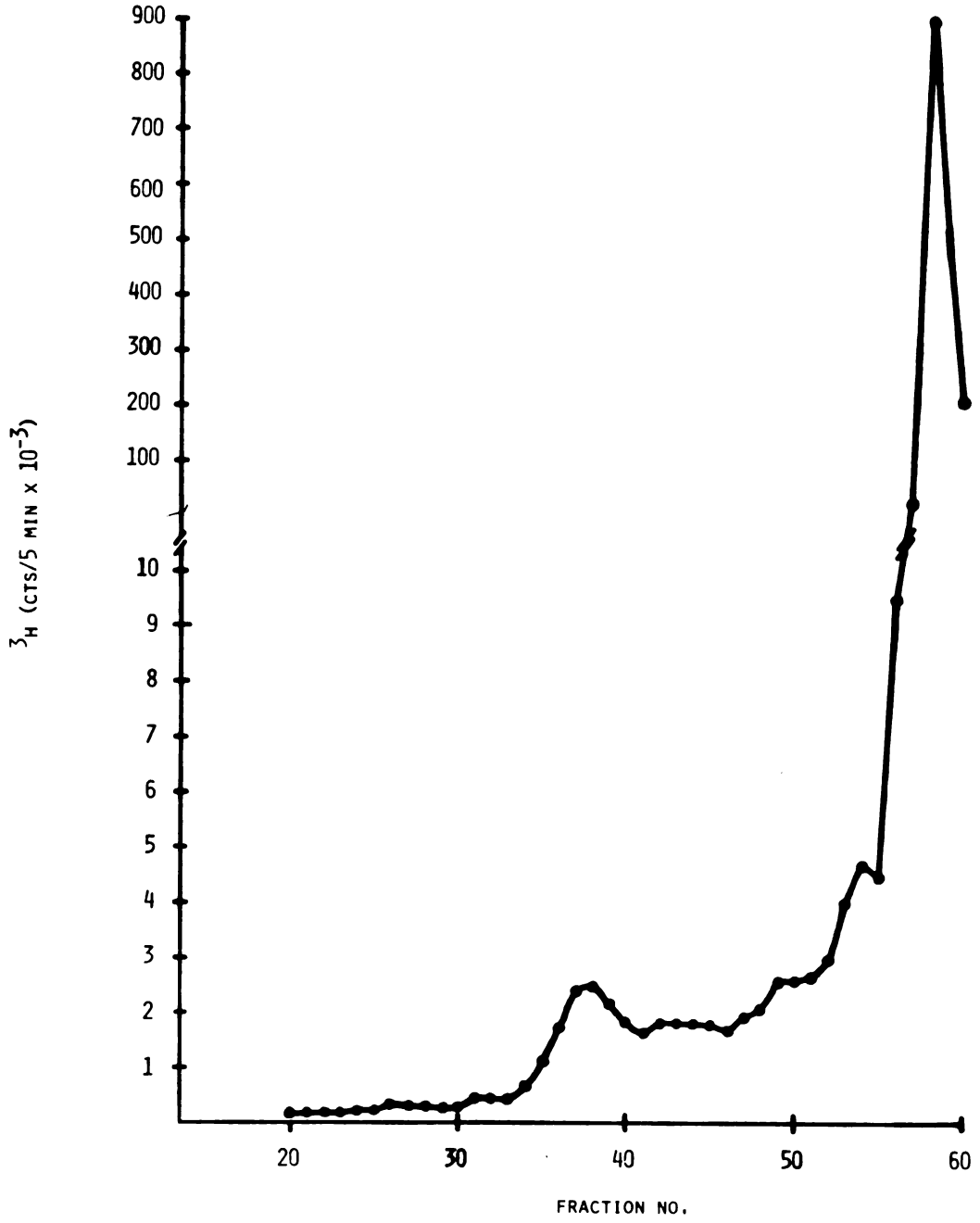


Fig. 5. Profile of genetic material from resistant strain 74-90383.

DISCUSSION

Until recently, strains of Haemophilus influenzae type b have been uniformly susceptible to treatment with ampicillin (1). The sudden appearance of ampicillin resistant strains has prompted much concern as well as speculation as to the mechanism of resistance involved (12,15).

Ampicillin resistance in all strains of Haemophilus influenzae tested is due to the production of a beta lactamase (3,18). To date, no ampicillin resistant, beta lactamase negative organisms have been described.

The beta lactamase produced by ampicillin resistant strains (7,14,18) of Haemophilus influenzae is a constitutive enzyme (7,14,18) 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 (7) as a class IIIa beta lactamase closely resembling the beta lactamase produced by certain strains of Klebsiella pneumoniae.

Since Klebsiella pneumoniae is often isolated from hospital personnel as well as patients receiving antibiotic therapy, it is speculated that strains of

this organism may be the source of genetic material responsible for resistance in strains of Haemophilus influenzae (18). In vivo interspecies transfer of genetic material has been documented but it is also possible that present ampicillin resistant strains of H. influenzae are the result of spontaneous mutant(s) which have only recently been recognized due to the greatly increased use of ampicillin.

Results of prevalence studies carried out in Washington, D.C., and Patuxent, Maryland, (12) indicated that approximately 10 percent of the randomly sampled population carried ampicillin resistant strains of Haemophilus influenzae type b. The rapid emergence of this organism is demonstrated by the fact that one year prior to these surveys, confirmed ampicillin resistant strains of H. influenzae had not been documented (4).

The present study demonstrates that ampicillin resistance can be transferred in vitro to susceptible strains at frequencies ranging from 2 to 14 percent. Although this rate of transfer may seem high in light of the crude total genetic transfer procedures used, data from Hotchkiss and Gabor (9) indicate that unmodified cells of H. influenzae can readily take up DNA molecules and be transformed. MICs performed on 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 Haemophilus influenzae by exposure to ethidium bromide have been unsuccessful (7) an 80 percent cure of resistance was achieved by exposure to acridine orange, a similar agent capable of complexing DNA. As was reported previously, (3) and also in our hands, strain 74-71518 showed high spontaneous loss of its resistance factor. It is possible that the ampicillin plasmid in Haemophilus is refractory to cure with ethidium bromide. The observation that exposure to one half of that concentration of acridine orange which yielded 80 percent cure resulted in only 45 percent cure is consistent with a conventional dose response curve. 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 disc diffusion method for ampicillin susceptibility testing of Haemophilus influenzae (5).

The technique described for the culturing, labeling and lysing of cells of Haemophilus 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 (tritiated thymidine) into cells grown in a rich medium like BHI 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.

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