R-FACTOR MEDIATED $\beta$-LACTAMASE PRODUCTION
BY HAEMOPHILUS INFLUENZAE

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PLATE XLI

Two recent reports have described $\beta$-lactamase production by antibiotic-resistant strains of *Haemophilus influenzae* (Khan *et al.*, 1974; Williams and Cavanagh, 1974). Fifteen such strains were obtained from this country and the USA, and a study was made of the nature of the $\beta$-lactamase enzyme or enzymes produced by them and, if possible, their genetic location.

**MATERIALS AND METHODS**

**Organisms**

The resistant strains of *H. influenzae* were obtained from four clinical sources: Birmingham Children's Hospital (two strains), Public Health Laboratory, Stafford (four strains), Center for Disease Control, Atlanta, USA (five strains), and the Clinical Center, Md, USA (four strains).

**Minimum inhibitory concentrations**

Dilutions of antibiotics were prepared in nutrient agar with the addition of 5% Bacto-Fildes Enrichment (Difco). Organisms were grown for 18 h in brain-heart infusion broth with 5% Bacto-Fildes enrichment (Fildes broth) in a rotary shaking incubator at 37°C. Plates were seeded from a multipoint inoculator (Denley Instruments Ltd, Sussex), with three sizes of inoculum, $10^3$, $10^5$, and $10^7$ cells. The MIC's were read after incubation for 18, 24, and 36 h at 37°C and recorded as the lowest concentrations of antibiotic preventing growth.

**Detection of $\beta$-lactamase**

The chromogenic cephalosporin 87/312 (O'Callaghan *et al.*, 1972) was spotted on colonies growing on solid media. A red colour indicates the presence of a $\beta$-lactamase.

**Preparation of enzymes**

Organisms in Fildes' broth were grown for 18 h at 37°C in a rotary shaking incubator. Cells deposited by centrifugation were washed in 0.1 M phosphate buffer, pH 7.0, and re-suspended in the same buffer to give a 10-times concentrated cell suspension. This was treated with an ultrasonic disintegrator (MSE Instruments Ltd) 4 A output for 3 min. at 25,000 Hz. The temperature was maintained at 4°C throughout the procedure. The resulting preparation was then centrifuged at 35000g for 30 min. at 4°C to remove cell debris. Protein determinations on the enzyme preparations were made by the method of Lowry *et al.* (1951).

**Determination of substrate profiles**

Substrate profile is defined as the relative rate of hydrolysis of a range of $\beta$-lactam substrates under standard conditions. All assays were performed iodometrically by the

Received 20 Dec. 1974; accepted 3 Mar. 1975.

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method of Perret (1954), as modified by Novick (1962). Substrates were used at a concentration of 6 mM and assays performed at pH 7.0 and 37°C. Enzyme units are defined as µmoles of substrate hydrolysed per min.

**Induction of β-lactamase**

Organisms grown overnight in Fildes' broth at 37°C were diluted 100-fold into fresh warm broth and re-incubated for 4 h. Ampicillin was then added as an inducer at concentrations ranging from 10 to 100 µg per ml. The cultures were re-incubated for another 2 h and β-lactamase activity was determined.

**Analytical isoelectric focusing of β-lactamase**

Crude preparations of sonically disintegrated organisms were focused on thin sheets of polyacrylamide gel containing pH 3.5-10 Ampholine (Awdeh, Williamson and Askonas, 1968; Salaman and Williamson, 1971). Samples were applied near the anode as drops of liquid on the surface of the gel. Focusing was performed at 100-400 V for 40 h. The final pH gradient was read at intervals of 0.5 cm from the cathode to the anode with a miniature

![Graph](image_url)

**Fig. 1.**—MIC of ampicillin for 15 strains of *Haemophilus influenzae* at three inoculum sizes after 18 (○), 24 (●), and 36 (×) hours' incubation.
flat-ended combined glass electrode (Pye Unicam 403.30 M8 E07). Bands with \(\beta\)-lactamase activity were detected by damping the surface of the gel with the chromogenic cephalosporin 87/312 (0.5 mg per ml in 0.1M phosphate, pH 7.0). This substrate is yellow, but becomes pink when the \(\beta\)-lactam bond is broken, so that focused bands with \(\beta\)-lactamase activity appear pink on a yellow background. Photographs were taken on Kodalith Ortho film with transmitted light, and a Wratten green filter. The film was processed in phenidone-hydroquinone developer for 5 min. at 21°C.

**Immunoelectric focusing**

With a perspex former, troughs were set into the sheet of polyacrylamide gel on which the samples were focused. After addition of antiserum to the troughs, the gel was incubated at 4°C in a humid atmosphere for 7 days to allow precipitin arcs to form. These were detected by means of cephalosporin 87/312.

**Transferability of \(\beta\)-lactamase genes**

This was assessed by mating experiments in which the potential donor and recipient cultures were grown together and resistant colonies of the latter were isolated by selection. For the *H. influenzae*-H. *parainfluenzae* mating, 6-h bacterial cultures of both organisms in Fildes' broth were added to fresh broth in a ratio of 1 : 1. Mating mixtures were incubated statically at 37°C for 18 h and plated on nutrient agar containing yeast extract 5% and ampicillin 50 \(\mu\)g per ml. For the *H. influenzae*-Escherichia *coli* mating the same procedure was followed, but this time the organisms were added in the ratio of 10 : 1 (*H. influenzae*/E. *coli*). After incubation, the mating mixtures were plated on to nutrient agar containing ampicillin 50 \(\mu\)g per ml.

**RESULTS**

**Antibiotic resistance**

All the strains of *H. influenzae* showed high levels of resistance to ampicillin. From fig. 1 it can be seen that the resistance varied considerably with changes in inoculum size and time of incubation. Production of \(\beta\)-lactamase was detected in all the strains when they were tested with the chromogenic substrate.

**Enzyme activity and substrate profiles**

All enzyme preparations produced an identical substrate profile when tested against seven \(\beta\)-lactam substrates (table). The enzymes showed strong hydrolytic activity against penicillin G, ampicillin, and cephaloridine but had

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<th>Substrate profile of (\beta)-lactamases produced by the resistant strains of Haemophilus</th>
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Pen. G = benzylpenicillin; Amp. = ampicillin; Carb. = carbenicillin; Meth. = methicillin; Cet. = cephalothin; Cer. = cephaloridine; Cex. = cephalaxin.

* Arbitrary value of 100 for the rate of hydrolysis of benzylpenicillin.
little effect on methicillin or cephalaxin. The breakdown of cephalothin and of
carbenicillin was intermediate. All strains produced 0.05-0.1 enzyme units
per mg of protein. Induction of enzyme activity was not observed.

The substrate profile of the haemophilus β-lactamase suggested that this
enzyme was the same or similar to the type IIIa β-lactamase often produced by
R-factor-carrying strains of E. coli, e.g., E. coli R_{TEM} (Richmond and Sykes,
1973).

Isoelectric focusing

Isoelectric points of six haemophilus-enzyme preparations and enzymes
from E. coli R_{TEM} (Datta and Richmond, 1966), E. coli R_{GN238} (Egawa,
Sawai and Mitsuhashi, 1967) and E. coli RPI (Sykes et al., 1972), were deter-
mined by analytical isoelectric focusing. Fig. 2 shows the haemophilus
enzymes to have isoelectric points identical with those of the β-lactamase
produced by E. coli R_{TEM}. The immunoisoelectric focusing picture (fig. 3)
shows the two enzyme types to be immunologically identical.

Genetic transfer

Because the E. coli R_{TEM} β-lactamase is always R-factor mediated
(Richmond and Sykes, 1973), experiments were performed to transfer the
β-lactamase gene from the resistant strains of H. influenzae. Three strains of
H. parainfluenzae (fresh clinical isolates) and E. coli strain W3110 (Sykes and
Richmond, 1970) were used as recipient strains in mating experiments with two
H. influenzae donor strains. Transfer of β-lactamase production was achieved
from both donor strains to the H. parainfluenzae recipients (fig. 4). Some
transfer was observed to the E. coli strain but the transferred factor was highly
unstable in it.

DISCUSSION

Until recently, strains of H. influenzae have been regarded as devoid of
β-lactamase and almost universally have been found to be sensitive to ampicillin,
which has established itself as a treatment of choice for many infections due to
the organism. The recent emergence, almost concurrently in several parts of
the world, of strains that produce a high level of β-lactamase has thus aroused
interest. Our investigation has confirmed that in 15 strains examined, resistance
to β-lactam antibiotics was associated with the production of a β-lactamase.
All the strains produced the same type of β-lactamase, and we find that this is of
type IIIa, similar to that from E. coli R_{TEM}.

Immunological identity with the E. coli R_{TEM} enzyme, and its transferability,
suggest that the β-lactamase gene carried by strains of H. influenzae may have
been acquired from R-factor carrying strains of E. coli. The results of DNA
hybridisation studies may furnish evidence of this.

A substance that is not susceptible to attack by β-lactamase of type IIIa
seems to be required for the treatment of infections by H. influenzae that pro-
duce the enzyme.
FIG. 2.—Isoelectric-focusing patterns of β-lactamase from six resistant strains of *H. influenzae* compared with β-lactamases from various R-factor-carrying strains of *Escherichia coli*: (1) *E. coli* strain R<sub>TEM</sub>; (2 and 3) Stafford strains of *H. influenzae*; (4 and 5) Atlanta strains; (5, 6 and 7) Bethesda strains; (8) *E. coli* strain RPI; (9) *E. coli* strain R<sub>GN238</sub>.

FIG. 3.—Immunoelectrofocusing patterns of β-lactamases from three strains of *H. influenzae* against antiserum to *E. coli* strain R<sub>TEM</sub> β-lactamase: (1) *E. coli* strain R<sub>TEM</sub>; (2-6) *H. influenzae* strains from Stafford, Atlanta and Bethesda.

FIG. 4.—Isoelectric-focusing patterns of β-lactamase from (1) *H. influenzae* donor strain and (2, 3 and 4) *H. parainfluenzae* recipient strains.
**Summary**

Production of β-lactamase by 15 strains of *Haemophilus influenzae* has been investigated. All the strains produce a constitutive β-lactamase, which readily hydrolyses penicillin G, ampicillin, and cephaloridine. The β-lactamase produced by these strains is indistinguishable from the type-IIIa enzyme commonly found in strains of *Escherichia coli*. The β-lactamase gene has been transferred from the enzyme-producing strains of *Haemophilus* to strains of *H. parainfluenzae* and a strain of *E. coli*.

We thank Dr P. Cavanagh for the strains of *H. influenzae* and *H. parainfluenzae*.

**References**


