Beta-Lactamase Activity in Ampicillin-Resistant *Haemophilus influenzae*

W. EDMUND FARRAR, JR., AND NOEL M. O’DELL

*Infectious Diseases and Immunology Division, Department of Medicine, Medical University of South Carolina, Charleston, South Carolina 29401*

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The specific activity, substrate profile, response to inhibitors, inducibility, and cellular localization of the beta-lactamase produced by an ampicillin-resistant strain of *Haemophilus influenzae* type B were investigated. In these properties the enzyme resembles *beta*-lactamases produced by other gram-negative bacilli more closely than those produced by gram-positive organisms. It is quite similar to an enzyme found in strains of *Klebsiella pneumoniae*, and differs significantly from those described in other gram-negative bacilli. Comparison of the substrate profile with the minimal inhibitory concentrations of various *beta*-lactamase antibiotics suggests that the *beta*-lactamase plays an important role in the antibiotic resistance of this organism.

During the past few years, cases of meningitis due to ampicillin-resistant strains of *Haemophilus influenzae* type B have been reported from California (10), Maryland (13), Georgia (15), the District of Columbia (9), Texas, Florida (1), and from Wolverhampton, England (3). Four strains isolated in the District of Columbia were reported to produce a *beta*-lactamase (9). The unexpected occurrence of ampicillin resistance associated with *beta*-lactamase activity in this species, strains of which had previously been uniformly susceptible to this antibiotic, raised the possibility that this organism might have acquired a plasmid mediating production of *beta*-lactamase. This study was therefore undertaken to characterize the *beta*-lactamase activity of an ampicillin-resistant strain of *H. influenzae*, and to compare this enzyme with *beta*-lactamases of other bacterial species which might represent the source of its genetic determinants.

MATERIALS AND METHODS

Source of organisms. The ampicillin-resistant strain of *H. influenzae* type B studied (strain W-2) was isolated from a 4-month-old child with meningitis admitted to Grady Memorial Hospital in December 1973 (14); it was kindly provided to us by André J. Nahmias of Emory University, Atlanta, Ga. Criteria for identification of this organism as *H. influenzae* type B included morphology (gram-negative cocccobacillus), growth on chocolate agar but not on sheep blood agar, satellitizing around *Staphylococcus aureus* on sheep blood agar, and agglutination by commercial type B antiserum but not by antisera of other *H. influenzae* types. For comparison, certain of these studies were also carried out on a typical ampicillin-sensitive strain of *H. influenzae* type B (control strain) isolated at the Medical University Hospital, Charleston, S. C., kindly provided by E. R. Bannister.

Growth conditions. One of the following media was used for growth of the organisms in all experiments described in this paper: (i) Tryptic soy agar supplemented with bovine hemoglobin (1%) and IsoVitaleX (BBL) (1 ml/100 ml of agar)-enriched TSA, (ii) brain-heart infusion broth supplemented with hemin (10 μg/ml), 1-histidine (10 μg/ml) and IsoVitaleX (1 ml/100 ml of broth)-enriched BHI.

 Cultures on solid media were incubated in a CO₂ incubator (CO₂ concentration 3 to 5%); cultures in broth were incubated in air in a shaking water bath.

Preparation of *beta*-lactamase. For preparation of *beta*-lactamase cells were grown by heavily inoculating the surface of enriched TSA in 32-ounce medicine bottles and incubating the cultures for 18 h in a CO₂ incubator. Cells were harvested by flooding the agar surface with 5 ml of Trypticase soy broth and pipetting off the liquid phase after gentle agitation by tutting. Purity of each culture was checked by Gram stain. After centrifugation cells were resuspended to 0.1 of the original volume in phosphate buffer (0.00075 M, pH 7.1), and viable count was determined by plating appropriate dilutions on enriched TSA. Where appropriate, cells were disrupted by sonication for 4 min in a Branson sonifier.

Measurement of *beta*-lactamase activity. *Beta*-lactamase activity was measured by means of the alkalimetric titration method (7, 15) using an automatic recording pH-stat (Metrohm-Herisau Combitrator, Brinkmann Instruments). Phosphate buffer (0.00075 M) and substrate (3 x 10⁻⁴ M mol) were placed in the reaction vessel and brought to standard conditions (pH 7.0, 37 °C). The reaction was started by adding enzyme and the rate of reaction was measured by...
continuous titration of the penicilloic acid formed with 0.01 N NaOH. In determination of substrate profile, the reaction was run to completion with each substrate to determine the number of moles of acid produced per mole of substrate hydrolyzed, and this number was utilized in calculation of enzyme activity. The final volume of all reaction mixtures was 25 ml.

**Inhibition of β-lactamase activity.** For studies of inhibition of β-lactamase, sonically disrupted cell preparations were clarified by centrifuging at 30,000 × g for 30 min at 4°C and discarding the sediment. Phosphate buffer (0.00075 M), enzyme and inhibitor (various anti-staphylococcal penicillins, 3 × 10⁻⁴ mol, or para-chloromercurobenzoate [pCMB], 5 × 10⁻⁴ M) were mixed in the reaction vessel with stirring for 10 min at 37°C. Substrate (penicillin G, 3 × 10⁻⁴ mol) was then added and the rate of reaction was measured as described above. Inhibition was calculated as percentage decrease in rate of hydrolysis as compared with the rate of hydrolysis of substrate without inhibitor.

**Induction of β-lactamase activity.** An overnight culture of strain W-2 in enriched BHI was diluted 1:10 with fresh enriched BHI and grown with shaking to an optical density at 540 nm (Coleman Junior II spectrophotometer) of approximately 0.4. Either penicillin G, 0.5 minimal inhibitory concentration (MIC) (25 μg/ml) or 0.05 MIC (2.5 μg/ml), or cephaloridine, 0.5 MIC (0.5 μg/ml) or 0.05 MIC (0.05 μg/ml), was added, and incubation was continued for another 4 h. Cells were then harvested by centrifugation and assayed for β-lactamase activity.

**Intracellular versus extracellular β-lactamase activity.** Strain W-2 was grown with shaking in enriched BHI to an optical density (540 nm) of 0.6 (late log phase). Cells were removed by centrifugation, and the supernatant was filtered and dialyzed overnight at 4°C against 0.00075 M phosphate buffer. Cells and supernatant were then assayed for β-lactamase activity as described above.

**Measurement of MIC of antibiotics.** MIC of various β-lactam antibiotics for these organisms were determined by a standard 2-fold tube-dilution test in enriched BHI (final test volume 1 ml). Inoculum consisted of approximately 10⁶ organisms from an overnight culture in enriched BHI. The test was read after 24 h of incubation.

**Efforts to detect ampicillin-susceptible variants.** Since it was considered possible that the ampicillin resistance of strain W-2 was determined by genes located on a plasmid, ampicillin-susceptible variants occurring either spontaneously or after exposure to ethidium bromide (2) were sought. To look for spontaneous loss of ampicillin resistance, an overnight culture of strain W-2 in enriched BHI was plated onto enriched TSA and incubated overnight. Isolated colonies were transferred with sterile toothpicks to both drug-free plates of enriched TSA and plates containing ampicillin, 5 μg/ml. The plates were incubated overnight and examined for colonies which grew on drug-free plates but not on ampicillin-containing plates.

In an effort to "cure" organisms of plasmids conferring resistance to ampicillin, strain W-2 cells were exposed to ethidium bromide as described by Bouanchaud et al. (2). An overnight culture in enriched BHI was diluted 1:100 and inoculated into tubes of enriched BHI containing 2-fold dilutions of ethidium bromide. After overnight incubation optical density (at 620 nm) was measured to determine the MIC of ethidium bromide. Cells exposed to this concentration, and to 2-fold higher and lower concentrations of ethidium bromide, were plated onto enriched TSA. After overnight incubation colonies were transferred to drug-free and ampicillin-containing plates which were incubated overnight. These were examined for the presence of drug-susceptible variants as described above.

**RESULTS**

**Production of β-lactamase activity by strain W-2.** β-lactamase activity was found in cultures of the ampicillin-resistant strain W-2, but not in the ampicillin-susceptible control strain. Strain W-2, whether grown on enriched TSA or in enriched BHI, hydrolyzed approximately 20 μmol of penicillin G per h per 10⁶ cells. Exposure to either 0.5 MIC or 0.05 MIC of penicillin G did not significantly increase β-lactamase activity in this organism (Table 1); similar results were obtained when strain W-2 was exposed to cephaloridine. We conclude that β-lactamase is produced constitutively in this organism and the enzyme does not appear to be induced by exposure of the organism to β-lactam antibiotics. In log phase cultures, more than 99% of enzyme activity is cell associated. When cells were disrupted by sonication, β-lactamase activity actually decreased slightly, indicating that significant surface permeability barriers against entry of these substrate antibiotics are not present in this organism, and further, that sonication may have a deleterious effect on β-lactamase activity in this organism.

**Substrate profile.** The rates of hydrolysis of various β-lactam antibiotics by sonically disrupted cells of strain W-2 are shown in Table 2.

<p>| Table 1. β-lactamase activity of strain W-2 grown with or without induction by penicillin G |
|---------------------------------|------|---------|---------|</p>
<table>
<thead>
<tr>
<th><strong>Inducer</strong></th>
<th>Penicillin G</th>
<th>Ampicillin</th>
<th>Cephaloridine</th>
<th>Cephalothin</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>20</td>
<td>25</td>
<td>11</td>
<td>1.8</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>0.5 MIC (25 μg/ml)</td>
<td>24</td>
<td>28</td>
<td>15</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>0.05 MIC (2.5 μg/ml)</td>
<td>25</td>
<td>29</td>
<td>15</td>
</tr>
</tbody>
</table>

* Micromoles of substrate hydrolyzed/hour per 10⁶ cells.
The enzyme of this organism must be classified as a broad-spectrum penicillinase/cephalosporinase (8; unpublished data), since cephaloridine is hydrolyzed almost as rapidly as penicillin G and ampicillin. Other cephalosporins tested were hydrolyzed less rapidly, and the anti-staphylococcal penicillins were hydrolyzed very slowly or not at all. No activity against penicillin G, ampicillin, cephalothin, or cephaloridine was found in the control strain.

**Effects of inhibitors.** The effects of various potential inhibitors of \( \beta \)-lactamase activity on the hydrolysis of penicillin G by sonically disrupted cells of strain W-2 are shown in Table 3. Among the anti-staphylococcal penicillins, the isoxazolyl penicillins (oxacillin, cloxacillin, and dicloxacillin) were relatively ineffective as inhibitors of \( \beta \)-lactamase activity, whereas methicillin and nafcillin showed considerably more effect. pCMB was not a potent inhibitor of the \( \beta \)-lactamase activity of this organism.

**Susceptibility to \( \beta \)-lactam antibiotics.** The MIC of various \( \beta \)-lactam antibiotics for strain W-2 and the control strain are shown in Table 4. A general correlation was found between the increased resistance of strain W-2 to certain antibiotics, as compared with the control strain, and the susceptibility of the various antibiotics to hydrolysis by the \( \beta \)-lactamase. Strain W-2 was 250 times more resistant than the control strain to penicillin G, and 600 times more resistant to ampicillin. These two drugs were the most rapidly hydrolyzed of those tested. Little difference in susceptibility between W-2 and the control was found with the anti-staphylococcal penicillins and the cephalosporins; except for cephaloridine these agents were hydrolyzed only slowly or not at all.

**Search for ampicillin-susceptible variants.** Examination of approximately 1,500 colonies from organisms grown in drug-free enriched

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**DISCUSSION**

In several respects (intracellular localization, total enzyme activity produced, substrate profile, and lack of inducibility) the \( \beta \)-lactamase found in this ampicillin-resistant strain of *H. influenzae* resembles enzymes found in other species of gram-negative bacilli more closely than those found in gram-positive organisms (4, 11, 12). We have recently investigated the properties of \( \beta \)-lactamase enzymes in several species of gram-negative bacilli (unpublished data). The substrate profile of the \( \beta \)-lactamase of *H. influenzae* W-2 (broad-spectrum penicillinase/cephalosporinase activity) is similar to that which we found in enzymes of *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*. However, unlike the *Haemophilus* enzyme, the enzymes found in *P. mirabilis* were inhibited by cloxacillin, and those found in *E.

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**Table 2. Absolute and relative rates of hydrolysis of various \( \beta \)-lactam antibiotics by the \( \beta \)-lactamase of *H. influenzae* W-2**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Penicillin G</td>
</tr>
<tr>
<td>W-2</td>
<td>Absolute</td>
</tr>
<tr>
<td></td>
<td>Relative</td>
</tr>
<tr>
<td>Control</td>
<td></td>
</tr>
</tbody>
</table>

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* Micromoles of substrate hydrolyzed per hour per 10⁸ cells.

* Penicillin G = 100.

* Not done.
coli were either inhibited by cloxacillin or hydrolyzed this drug at a relatively rapid rate. Thus, this enzyme resembles β-lactamases which we have found in K. pneumoniae more closely than it does any other β-lactamase which we have encountered. This finding is intriguing since K. pneumoniae is a frequent inhabitant of the respiratory tract in individuals working in hospitals or in those receiving antibiotic therapy, and suggests that this species might be the source of the genetic material determining production of β-lactamase in *H. influenzae*.

It seems likely that elaboration of β-lactamase plays a major role in the resistance of strain W-2 to β-lactam antibiotics, since the resistance of this strain to penicillin G and ampicillin, the two drugs most effectively hydrolyzed by the enzyme, was several hundred-fold greater than that of the control strain. That this resistance is significant at the clinical level is evident in the fact that several patients infected with these organisms have exhibited suboptimal responses when treated with large doses of ampicillin (1, 3, 9, 13, 14), and three have died (9, 13).

The finding that this organism was not more resistant than the control strain to cephaloridine, even though this drug was rapidly hydrolyzed by the β-lactamase, was not unexpected to us. Cephaloridine penetrates the cell surface of bacteria more readily than most other β-lactam antibiotics (5, 6), and is lethal in low concentration to many β-lactamase-producing strains of gram-negative bacilli (unpublished data).

Our studies were carried out on only one strain of ampicillin-resistant *H. influenzae*, and it is possible that other strains may differ significantly from ours. However, the limited data on β-lactamase activity in strains of ampicillin-resistant *H. influenzae* isolated in Washington, D. C., reported by Khan et al., are entirely consistent with our findings (9).

Our efforts to “cure” strain W-2 of resistance to ampicillin by exposure to ethidium bromide have been unsuccessful. However, G. M. Thorne, in this laboratory, has obtained evidence of the transfer of ampicillin resistance from strain W-2 to another strain of *H. influenzae* type B (G. M. Thorne, unpublished data). Further studies to elucidate the mechanism of this transfer of resistance are in progress.

ACKNOWLEDGMENTS

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LITERATURE CITED


