Ampicillin Resistance and Penicillin-binding Proteins of *Haemophilus influenzae*

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Penicillin-binding protein (PBP) alterations have been associated with non- β -lactamase-mediated ampicillin resistance in *Haemophilus influenzae*. We evaluated the PBP profiles of several ampicillin-susceptible and -resistant clinical isolates of *H. influenzae* to determine how consistently the described alterations occurred, and to document the reproducibility of the PBP profiles for this species. The MIC of ampicillin ranged from 0.06 to 0.13 $\mu\text{g ml}^{-1}$ for the susceptible isolates at an inoculum of 100000 c.f.u. when tested by broth dilution, and was 0.5 $\mu\text{g ml}^{-1}$ for all four isolates when tested by agar dilution. The MIC for the resistant isolates ranged from 4 to 8 $\mu\text{g ml}^{-1}$ when tested by broth dilution, and from 1.5 to 16 $\mu\text{g ml}^{-1}$ when tested by agar dilution. At least eight distinct PBPs with molecular masses ranging from 27 to 90 kDa were detected both in cell membrane preparations and whole cell (*in vivo*) binding assays done on cells in the exponential growth phase. PBP variability was evident both in the ampicillin-susceptible and -resistant isolates; however, much greater variability existed within the four resistant strains. The differences in PBP patterns included (1) electrophoretic mobility, (2) binding capacity for the antibiotic and (3) the presence of additional PBPs in two of the resistant isolates. However, decreased binding capacity was consistently demonstrated in PBP 5 (56 kDa) of all of the resistant isolates. Saturation curves with both penicillin and ampicillin indicated that PBP 5 had decreased affinity for the antibiotics. These results suggest (a) that care should be taken in interpreting changes in PBP profiles for species that demonstrate variability such as *H. influenzae*, and (b) that the decreased binding affinity of PBP 5 is a consistent finding associated with multiple ampicillin-resistant wild-type isolates.

INTRODUCTION

Penicillin-binding proteins (PBPs) are cell-membrane-bound proteins that covalently bind β -lactam antibiotics; they are present in many Gram-positive and Gram-negative bacteria (Spratt, 1983; Tomasz, 1982). The number of PBPs varies from genus to genus, and their molecular masses range from 25 to 100 kDa. These proteins are associated with cell wall synthesis, and are important in such processes as differentiation and cell division. Alterations in an organism's PBP profile may be responsible for increased resistance to β -lactam antibiotics (Spratt, 1983; Tomasz, 1982). Alterations which have been reported include changes in electrophoretic mobility, in the amount of radiolabelled antibiotic bound, and the absence of a particular PBP. Examples of these changes occurring in conjunction with increased resistance exist for *Escherichia coli* (Spratt, 1978) and various other organisms.

The association of PBP alterations with non- β -lactamase-mediated ampicillin resistance in

Abbreviations: PBP, penicillin-binding protein; MBC, minimum bactericidal concentration.

Haemophilus influenzae has recently been reported. Parr & Bryan (1984) were able to transform broad spectrum β -lactam resistance from a resistant clinical isolate of *H. influenzae* into an ampicillin-susceptible recipient strain and show cotransfer of 68 kDa and 65 kDa PBPs with decreased binding for penicillin. Mendelman *et al.* (1984) subsequently reported the transfer of ampicillin resistance from three of four strains of *H. influenzae* into an ampicillin-sensitive strain. Affinity binding studies on stationary phase cells revealed the presence of a 59 kDa PBP with decreased affinity for ampicillin in all transformants tested.

The purpose of this study was to document the reproducibility of the PBP profile of *H. influenzae* and to examine the frequency with which decreased binding affinity of the two PBPs previously identified occurred in association with ampicillin resistance.

METHODS

Micro-organisms. Four ampicillin-susceptible isolates of *H. influenzae*, designated H flu S, Hi 261, Hi 265 and Hi 272, were isolated from respiratory specimens submitted to the Microbiology Laboratory at Boston University Medical Center, Boston, Mass., USA. Three ampicillin-resistant isolates of *H. influenzae*, CDC 76-039256 (CDC 76), CDC 77-046332 (CDC 77) and CDC 78-054472 (CDC 78), were kindly provided by the Centers for Disease Control, Atlanta, Ga., USA; one ampicillin-resistant isolate, recovered from a blood culture and designated CH Hi 1, was obtained from the Children's Hospital, Boston, Mass., USA. Identification of the isolates was confirmed by conventional methods (Killian, 1981); the serotype was determined using slide agglutination with type-specific antisera (Difco). Biotyping was done using the biochemical scheme described by Killian (1980).

β -Lactamase activity and susceptibility testing. β -Lactamase production was determined by the chromogenic cephalosporin assay (O'Callaghan, 1972) using both sonicated and whole cell suspensions. In addition, a previously described bioassay system was used to test for biological inactivation of ampicillin (Needham & Smith, 1980).

Broth dilution susceptibility testing was done in 2.0 ml Fildes (Difco) enriched Mueller-Hinton broth according to the standard broth dilution method described by Thornsberry *et al.* (1977). The final inoculum tested contained 10^5 c.f.u. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of antibiotic to inhibit visible growth after 24 h incubation. The minimum bactericidal concentration (MBC) was determined by quantitatively subculturing macroscopically clear tubes. The MBC was defined as the concentration of antibiotic which reduced the original inoculum by 1000-fold. Agar dilution assays were done as described by Mendelman *et al.* (1984) using an inoculum of 10^3 to 10^5 c.f.u.

Cell membrane preparation. *H. influenzae* isolates were grown in brain heart infusion broth (500 ml volumes) supplemented with NAD^+ ($2 \mu\text{g ml}^{-1}$, Sigma) and haemin ($10 \mu\text{g ml}^{-1}$, Sigma) in shaker flasks (200 r.p.m.) at 35°C . The cells were harvested at the mid to late exponential phase of growth by centrifugation (15000 g , 4°C , 25 min). Cell pellets were washed twice with cold $0.01 \text{ M-KH}_2\text{PO}_4$ buffer, pH 7.5, and stored at -70°C .

Cell membrane fractions were prepared according to the procedure of Makover *et al.* (1981) with the following modification: cell pellets were thawed slowly, resuspended in cold $0.01 \text{ M-KH}_2\text{PO}_4$ buffer, and sonicated on ice 10 to 12 times with 2 min pulses followed by 2 min cooling. Protein concentrations were determined with Coomassie Brilliant Blue G-250 (Eastman Kodak), using bovine serum albumin (Sigma) as the standard (Bradford, 1976). The final cell membrane preparations were stored in $200 \mu\text{l}$ samples at -70°C .

Saturation curves. Cell membrane samples were thawed at room temperature and adjusted to a concentration of $5.0 \text{ mg protein ml}^{-1}$ with $0.05 \text{ M-KH}_2\text{PO}_4$ buffer containing 1 mM-MgCl_2 , pH 7.5. Saturation assays were done according to the method of Spratt (1977). Penicillin saturation studies were done by adding $20 \mu\text{l}$ of various concentrations of [^3H]penicillin G [ethylpiperidinium salt; 25 Ci mmol^{-1} ($925 \text{ GBq mmol}^{-1}$); Merck] to $200 \mu\text{l}$ of the cell membrane fraction. The concentration of penicillin tested ranged from 0.001 to $24 \mu\text{g ml}^{-1}$. Ampicillin saturation studies were done by incubating $20 \mu\text{l}$ of various concentrations (0.001 to $24 \mu\text{g ml}^{-1}$) of ampicillin (Bristol Laboratories) with $200 \mu\text{l}$ of the membrane fraction for 10 min at 35°C , and then adding $10 \mu\text{l}$ [^3H]penicillin (final concentration $3.0 \mu\text{g ml}^{-1}$). The reactions were terminated by the addition of $5 \mu\text{l}$ of 1.0 M unlabelled benzylpenicillin, and the membrane fractions solubilized in 20% (w/v) *N*-lauroylsarcosine (Sigma). The reaction mixtures were then prepared for SDS-PAGE as described by Mendelman *et al.* (1984). Samples containing $27.3 \mu\text{g protein}$ were subjected to SDS-PAGE in a 10% (w/v) separating gel with a 6% (w/v) stacking gel at a constant current of 30 mA per gel . Gels were stained, destained and prepared for fluorography according to the method of Bonner & Laskey (1974). Gels were dried, placed against prefogged Kodak XAR-5 film (Eastman Kodak) and stored at -70°C for 6 d. The fluorograms were developed in a Kodak automated processor and further analysed with a Transidyne scanning densitometer coupled to a computing integrator (Transidyne General Corp.). The 50% saturation value (S_{50}) was defined as that concentration of antibiotic required to saturate 50% of the binding capacity of a particular PBP. As controls for non-specific binding, outer membrane preparations, cell

membrane preparations pre-incubated with excess unlabelled penicillin, and cell preparations boiled for 3 min before incubation with [^3H]penicillin were assayed for PBPs in a similar manner.

PBP profiles. Whole cells were grown to exponential phase in 10 ml supplemented brain heart infusion broth. When the OD_{660} reached 0.60, a 1 ml sample was removed and the cells were harvested by centrifugation. Cell pellets were washed twice with buffer containing 50 mM-Tris/HCl, pH 7.8, and 1 mM- MgCl_2 , and resuspended in 100 μl of the same buffer. Ten μl (0.1 μg) [^3H]penicillin [5.42 μCi (200 kBq)] was added to each sample; the samples were then incubated at 37 °C for 45 min. We previously used a tenfold higher concentration of [^3H]penicillin (10 $\mu\text{g ml}^{-1}$) which would saturate the binding sites of the PBPs. However, this concentration resulted in high, non-specific background binding which interfered with resolution, interpretive comparisons and densitometry readings. To circumvent the latter problem we used the lower concentration of 1 $\mu\text{g ml}^{-1}$ and did a kinetic study which showed that increasing the binding reaction time from 10 to 45 min gave the maximum detail, allowing PBPs with less affinity to bind. The reactions were terminated as described above and the samples prepared for SDS-PAGE. Samples containing 40 μg protein were subjected to electrophoresis (10%, w/v, separating gel; 4%, w/v, stacking gel; constant current of 10 mA overnight). The gels were fixed, enhanced and dried, then exposed against Kodak XAR-5 film for 9 and 30 d at -80 °C and developed.

Binding of [^3H]penicillin to whole cells. To assess permeability, whole cell binding was determined by a procedure modified from that described by Hakenbeck *et al.* (1980). Cells were grown to exponential phase in supplemented brain heart infusion broth and 35 ml samples were removed for analysis. Cells were removed by centrifugation at 12000 *g* for 10 min and resuspended in 3.0 ml sterile 0.01 M- KH_2PO_4 buffer, pH 7.5. The suspensions were standardized to an OD_{540} of 1.0 and viable cell counts were determined. Standardized cell suspensions (200 μl) were incubated for 10 min at 35 °C after the addition of 20 μl [^3H]penicillin [final concentration 3.3 $\mu\text{g ml}^{-1}$; specific activity 250 $\mu\text{Ci mmol}^{-1}$ (9.25 MBq mmol^{-1})]. Excess unlabelled penicillin (5.0 μl , 120 mg ml^{-1}) was added to each tube and the cells were collected on polycarbonate filters (0.4 μm , Nucleopore membrane filters, VWR) that had been pre-soaked in buffered penicillin (250000 U ml^{-1}). Filters were washed twice, dried overnight, and placed in 4 ml Bray's Solution (National Diagnostics) for determination of radioactivity. Five separate determinations were made for each strain and the average c.p.m. were normalized to represent 1.0×10^9 cells.

RESULTS

Characteristics of the isolates

No morphological abnormalities were evident when isolates were Gram stained and examined microscopically. None of the isolates were serologically typable. Three biotypes (I, II and III) were represented among the isolates. No β -lactamase activity and no evidence of biological degradation of ampicillin were detected. When tested by broth dilution, the MIC of ampicillin for the sensitive isolates ranged from 0.06 to 0.13 $\mu\text{g ml}^{-1}$, and the MIC for the resistant isolates ranged from 4.0 to 8.0 $\mu\text{g ml}^{-1}$. The MBC was similar in all instances to the respective MIC, and no evidence of tolerance was found. When tested by agar dilution using an inoculum of 10^3 c.f.u. ml^{-1} , the MIC of ampicillin and penicillin for all the susceptible isolates was similar: 0.5 $\mu\text{g ml}^{-1}$ and 1 $\mu\text{g ml}^{-1}$, respectively. Similarly, the MIC of ampicillin and penicillin for the resistant strains ranged from 1.5 to 16.0 $\mu\text{g ml}^{-1}$ and from 4 to 16 $\mu\text{g ml}^{-1}$, respectively.

PBP profiles

Eight distinct PBPs were detected in the four ampicillin-susceptible strains examined in exponential phase (Fig. 1). This observation is in agreement with other investigators (Makover *et al.*, 1981; Mendelman *et al.*, 1984; Mendelman & Chaffin, 1985; Parr & Bryan, 1984), and the proteins have been numbered to agree with those described by Makover *et al.*, (1981). Approximate molecular mass assignments for the eight PBPs were as follows: PBP 1, 90 kDa; PBP 2, 80 kDa; PBP 3, 67 kDa; PBP 4, 59 kDa; PBP 5, 56 kDa; PBP 6, 43 kDa; PBP 7, 38 kDa; and PBP 8, 27 kDa. In contrast, six to ten PBPs were evident in the four resistant strains (Fig. 1). Comparison of the PBP profiles indicates that inter-strain variability exists even among the susceptible isolates. For example, PBPs 2 and 3 in Hi 261 (lane B) have different electrophoretic mobilities from the same PBPs in the other susceptible strains. Reduced binding capacities associated with PBPs 7 and 8 in Hi 272 (lane D) were noted, as well as an apparent increased binding capacity associated with PBP 3 in H flu S (lane A). However, there was much

Fig. 1. Detection of the PBPs of ampicillin-susceptible and -resistant, non- β -lactamase-producing strains of *H. influenzae*. The numbers (1 to 8) to the left of lane A indicate the major PBPs. Lanes A to D are ampicillin-susceptible strains (H flu S, Hi 261, Hi 265 and Hi 272, respectively); lanes E to H are ampicillin-resistant strains (CDC 76, CDC 77, CDC 78 and CH Hi 1, respectively). The lane to the left of lane A contains ^{14}C -labelled standards; their apparent molecular mass is indicated.

greater variability apparent in the four resistant isolates. Electrophoretic mobility differences were observed in PBPs 2 and 7 in CDC 76 (lane E). Reduced binding capacity could be demonstrated for PBP 5 in all four resistant strains (lanes E–H). In addition, the following reductions in binding were noted: PBPs 1, 3, 4 and 8 in CDC 76, (lane E); PBPs 2, 4 and 8 in CH Hi 1 (lane H) and PBP 6 in CDC 77 (lane F). Of interest, two of the ampicillin-resistant strains contained additional lower molecular mass PBPs. Strain CDC 77 (lane F) contained two additional PBPs with molecular masses of 35 kDa and 33 kDa, respectively. CDC 78 (lane G) contained an additional PBP with a molecular mass of 34 kDa.

Saturation studies

Penicillin and ampicillin saturation assays were done on a minimum of three separate cell membrane preparations. Saturation curves were constructed for each of the PBPs when possible. PBPs 4 and 5 did not always separate sufficiently as individual proteins to allow densitometric resolution and consequently they were analysed as a single protein. The penicillin saturation curve for PBP 3 (Fig. 2a) is representative of the results observed for PBPs 1, 2, 6, 7 and 8. No consistent finding with these PBPs was associated with the resistant strains that could not be demonstrated in one or more of the susceptible strains. In contrast, a distinct difference was evident between the ampicillin-susceptible and -resistant isolates in the saturation curve of PBPs 4 and 5 (Fig. 2b). When S_{50} values were calculated for each of the PBPs (Table 1), significantly more antibiotic was necessary to achieve 50% saturation of PBPs 4 and 5 from the resistant strains. In most cases, the difference was 10 to 100-fold in magnitude.

Whole cell binding capacity

The amount of [^3H]penicillin bound by 10^9 cells of each strain varied from 2059 ± 347 c.p.m. to 4604 ± 694 c.p.m. The whole cell binding capacity of the strains did not correlate with their susceptibility to ampicillin: three of the four resistant strains bound more penicillin than did the susceptible strains.

DISCUSSION

The PBP profiles of four ampicillin-susceptible and four ampicillin-resistant isolates of *H. influenzae* were examined in this study using cells grown to exponential phase. The PBP pattern of the ampicillin-susceptible strains was similar to that originally reported by Makover *et al.*

Fig. 2. [³H]Penicillin G saturation curves. (a) PBP 3; (b) PBPs 4 and 5 analysed as a single protein. Each point represents the mean of three separate determinations. The curves were used for the derivation of S_{50} values (concentration of penicillin required to saturate 50% of the binding capacity of a given PBP). —, Ampicillin-sensitive strains (■, H flu S; ▼, Hi 261; ◆, Hi 265; ▲, Hi 272); ·····, ampicillin-resistant strains (□, CDC 76; ▽, CDC 77; ◇, CDC 78; △, CH Hi 1).

Table 1. Fifty percent saturation value (S_{50}) of [³H]penicillin G for PBPs in isolates of *H. influenzae*

PBP (Molecular mass)	S_{50} (µg antibiotic ml ⁻¹)							
	Strain:							
	H flu S	Hi 261	Hi 265	Hi 272	CDC 76	CDC 77	CDC 78	CH Hi 1
1 (90 kDa)								
Pen	1.1	1.5	0.7	8.2	1.7	1.5	7.3	0.9
Amp	5.2	7.8	2.5	3.3	6.8	4.7	2.3	2.7
2 (80 kDa)								
Pen	1.9	2.5	0.3	*	0.2	*	*	4.1
Amp	0.06	0.2	0.04	*	0.07	0.03	0.08	0.01
3 (67 kDa)								
Pen	1.8	2.1	1.9	1.8	3.2	2.2	3.7	1.4
Amp	0.2	0.7	0.2	0.3	0.7	0.1	0.1	0.02
4 (59 kDa)								
Pen	†	†	†	†	†	3.3	†	3.5
Amp	0.01	†	0.08	0.01	†	0.5	0.2	0.5
5 (56 kDa)								
Pen	0.1	0.1	0.1	0.1	3.5	3.5	6.0	2.4
Amp	0.1	0.1	0.1	0.03	3.4	0.6	0.2	0.9
6 (43 kDa)								
Pen	0.1	6.2	10.5	ND	7.8	ND	11.5	6.8
Amp	0.04	0.4	0.2	ND	0.03	ND	0.1	<0.01
7 (38 kDa)								
Pen	4.0	3.8	3.8	3.5	2.5	7.6	5.6	5.4
Amp	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
8 (27 kDa)								
Pen	4.4	5.4	7.2	12.5	7.8	12.0	7.4	11.5
Amp	2.7	ND	1.0	>12.0	0.8	8.0	0.5	1.0

ND, Not detected by the densitometer.

* PBPs 1 and 2 evaluated as a single protein.

† PBPs 4 and 5 evaluated as a single protein.

(1981) for *H. influenzae* ATCC 19148 and to four additional ampicillin-susceptible isolates studied by Mendelman & Chaffin (1985). Eight PBPs were detected. To date, the PBP profiles of only four ampicillin-resistant isolates have been determined. Parr & Bryan (1984) reported reduced binding of penicillin with two PBPs in the single isolate tested. Mendelman *et al.* (1984) examined three strains and showed uniformly reduced binding to penicillin and binding affinity for ampicillin with a single PBP of 59 kDa and, in addition, that other PBPs might be involved. Thus, several PBPs of *H. influenzae* appeared to be associated with the resistant phenotype.

Although some variation in PBP profiles is evident among all eight of the wild-type strains tested in this study, it is striking that much greater variability occurs among resistant than among susceptible strains. However, we were consistently able to demonstrate decreased binding capacity associated with PBP 5 (56 kDa) in the resistant strains. Further, the decreased binding capacity was related to decreased affinity in all four strains. Our data support the observations of Mendelman *et al.* (1984) and Parr & Bryan (1984) for this particular PBP. Of interest is the fact that PBP 4 (59 kDa) in two of the four resistant isolates appears to have similar binding capacity for penicillin when compared to the four sensitive strains (Fig. 2). This is in contrast to the previous reports and suggests that PBP 4 may be subject to inter-strain variability independent of ampicillin resistance. The PBPs designated 3A and 3B (68 and 65 kDa, respectively) by Parr & Bryan (1984), and PBPs designated 4 and 5 (62 and 59 kDa, respectively) by Mendelman & Chaffin (1985) appear to correspond to our PBPs 4 and 5 (59 and 56 kDa, respectively). The differences in reported molecular mass presumably reflect minor differences in technique.

Also of interest is the presence of two PBPs with lower molecular masses not yet described in CDC 77. One of these two PBPs (35 kDa) appears to have a relatively high binding capacity for penicillin. Strain CDC 78 also has an additional PBP of approximately 34 kDa. Whether these additional PBPs are simply another example of inter-strain variability or are related to ampicillin resistance must be confirmed by isogenic comparisons. However, it is noteworthy that neither of these two PBPs has been detected in the 10 susceptible strains for which PBP profiles have been examined and reported (Makover *et al.*, 1981; Mendelman & Chaffin, 1985; Parr & Bryan, 1984). Since multiple alterations are very likely to involve several genetic steps, transformation studies could identify which PBPs change along with resistance.

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