Characterization of Non-β-Lactamase-Mediated Ampicillin Resistance in *Haemophilus influenzae*

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Received 19 December 1983/Accepted 10 May 1984

Ampicillin resistance in *Haemophilus influenzae* is most often due to the plasmid-mediated production of TEM β-lactamase. We studied four strains with high-level ampicillin resistance (MIC of 32 μg/ml with an inoculum of 10^5 CFU on solid media) which did not produce detectable β-lactamase activity with two different detection methods. Two of the four strains contained extrachromosomal DNA by agarose gel electrophoresis. Conjugation failed to transfer ampicillin resistance; in contrast, transformation yielded ampicillin-resistant transformants in three of the four strains. These transformants did not contain detectable extrachromosomal DNA. In addition, mobilization of the resistance determinant by transformation, or conjugation with, recombination-deficient strains was unsuccessful. DNA-DNA hybridization experiments revealed no homology of the DNA of these strains with two R plasmids (one coding for ampicillin resistance, the other for chloramphenicol and tetracycline resistance). We conclude that the genetic basis of the non-β-lactamase ampicillin resistance in these strains appears to be chromosomally mediated. We investigated the mechanism of resistance in these strains. Enzymatic modification of penicillin was not detected by autoradiography of a thin-layer chromatogram of cell sonic extracts of three ampicillin-resistant transformant strains incubated with [14C]penicillin. To assess changes in permeability of the cell envelope, a plasmid coding for β-lactamase was conjugated into these strains, and the hydrolysis of penicillin by intact cells and cell sonic extracts was compared. Only one of three transformant strains had significantly diminished permeability. Outer membrane proteins of these strains analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed apparent differences in comparison with the isogenic ampicillin-susceptible recipient strain. Autoradiography of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis of Sarkosyl-solubilized crude membrane (the putative inner membranes) from these ampicillin-resistant transformant strains incubated with [3H]penicillin compared with the isogenic ampicillin-susceptible recipient strain revealed reduced binding to PBP 3 and 6, 3 and 4, or 4. In addition, affinity binding studies revealed decreased affinity of PBP 4 for ampicillin of all four transformants tested. We conclude that the major mechanism of resistance in these strains is altered penicillin-binding proteins; however, other mechanisms, including permeability, may also play a role.

Plasmid-mediated β-lactamase production is the most common mechanism of ampicillin resistance in gram-negative bacteria; however, chromosomal-mediated β-lactamase production has also been previously described (19, 27, 33). The incidence of ampicillin resistance in *Haemophilus influenzae*, first reported in 1974, is increasing (34, 42, 43, 48; W. F. Schlech, J. D. Band, A. W. Hightower, and C. V. Broome, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 22nd, Miami Beach, Fla., abstr. no. 311, 1982). This resistance is due to the R-plasmid-mediated production of β-lactamase and is believed responsible for the increase in ampicillin resistance seen in clinical isolates (3, 5, 35, 44, 45). No chromosomal-mediated β-lactamase production has been reported in *H. influenzae*. There have been several reports of ampicillin-resistant strains of *H. influenzae* lacking detectable β-lactamase activity (1, 11, 17, 25). To date, the genetic basis of this resistance and the mechanism of resistance in these reported strains remain undetermined. We report here four strains of *H. influenzae* with high-level ampicillin resistance which have no detectable β-lactamase production. As the first step in characterization of these isolates, we sought to establish the genetic basis of their resistance. We examined the ampicillin-resistant clinical isolates and their ampicillin-resistant transformants for the presence of plasmid DNA and the transfer of the resistance to recipient strains by conjugation and transformation. DNA-DNA hybridization studies were performed with two well-characterized R plasmids to detect plasmid-specific sequences within the chromosomal DNA of these strains. Although enzymatic degradation of ampicillin by β-lactamase is not involved in the resistance of these isolates, other enzymatic antibiotic modifications and nonenzymatic mechanisms have not yet been elucidated in *H. influenzae*. These may include a permeability barrier, as has been reported previously in *Escherichia coli* (50), or altered penicillin-binding proteins (PBPs), as have been reported in strains of penicillin-resistant, non-penicillinase-producing *Streptococcus pneumoniae* and *Neisseria gonorrhoeae* (4, 49). We examined these ampicillin-resistant transformant strains for enzymatic resistance other than β-lactamase by incubating sonic extracts of these strains with [14C]penicillin and separating the radioactive products by thin-layer chromatography with detection by autoradiography. To investigate permeability, we conjugated a plasmid coding for β-lactamase production into the three ampicillin-resistant transformant strains and the isogenic ampicillin-susceptible recipient strain and determined the rate of hydrolysis of penicillin spectrophotometrically by cell sonic extracts and intact cells. This allowed comparison of the penicillin concentration in the periplasmic space and the relative permeability of
the outer membrane to penicillin of these isolates. To
determine whether a change in the outer membrane protein
(OMP) patterns correlated with ampicillin resistance, we
compared the OMP profiles of these strains by sodium
dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-
PAGE). To investigate the role of PBPs as a mechanism of
resistance, we compared the PBP profiles of these strains as
relative binding at a single concentration of radiolabeled
penicillin and determined the binding affinities of their PBPs
for ampicillin after incubation with increasing concentrations
of unlabeled ampicillin before reaction with radiolabeled
penicillin.

This study was presented in part at the 82nd Annual
Meeting of the American Society for Microbiology, Atlanta,
Ga., 7 to 12 March 1982 [P. M. Mendelman, M. C. Roberts,
1982, D45, p. 55] and at the 83rd Annual Meeting of the
American Society for Microbiology, New Orleans, La., 6 to
11 March 1983 [P. M. Mendelman, T. L. Stull, D. O. Chaffin,

MATERIALS AND METHODS

Bacterial strains. Four ampicillin-resistant sputum isolates
of H. influenzae, isolated between 1978 and 1980, were
supplied by D. Anderson of the National Health Institute
of New Zealand. The four strains were isolated from different
patients in different hospitals from the following geographi-
cally distinct cities: Christchurch, Auckland, Dunedin, and
Wanganni. These four strains were not iridescent on translu-
cent media and did not agglutinate with Difco Laboratories
(Detroit, Mich.) typing sera a through f. Table 1 lists the
other strains used. All strains had an obligatory requirement
for β-NAD+ and hemin for growth when incubated at 37°C in
room air.

Media. The medium used for growth and determination of
the MIC was brain heart infusion agar or broth (Difco)
supplemented with 10 μg of hemin, 10 μg of L-histidine, and
10 μg of β-NAD+ per ml (sBHI). Plate cultures were
incubated at 36.5°C in 5% CO2, whereas liquid cultures were
incubated at 37°C in room air and shaken at 200 cycles/min.
The liquid media used for the development of competence
was M IV (10).

Antibiotics and chemicals. Ampicillin and penicillin G were
obtained from Wyeth Laboratories, Randor, Pa., and rifamp-
in, chloramphenicol, streptomycin, and phenol red were
obtained from Sigma Chemical Co., St. Louis, Mo. The β-
lactamase substrate (a chromogenic cephalosporin) and
erythromycin were obtained from Calbiochem-Behring, La
Jolla, Calif. Spectinomycin was obtained from The Upjohn
Co., Kalamazoo, Mich. Tetracycline was obtained from
Pfizer Inc., New York, N.Y. [14C]Penicillin G (58 mCi/mmol)
was obtained from Amersham Corp., Arlington Heights, Ill.
[3H]Penicillin G (61 mCi/mg) was a gift from Merck Sharp &
Dohme, Rahway, N.J. 14C-labeled molecular weight standards
and the nick-translation kit were ob-
tained from Bethesda Research Laboratories, Inc. (Gaithers-
burg, Md.), and unlabeled molecular weight standards were
from LKB Instruments Inc. (Bromma, Sweden). Nitrocellu-
lose filter papers were obtained from Schleicher & Schuell
Inc., Keene, N.H. [35S]dATP was obtained from New
England Nuclear Corp., Boston, Mass. Sarksyol (NL-97)
was obtained from Ciba-Geigy (Basel, Switzerland).

Determination of MIC. The strains were inoculated onto
fresh sBHI agar plates from stored vials of skim milk at
−70°C and incubated overnight. The next day the strains
were grown to mid-log phase at an absorbance at 550 nm of
0.20 (±3 h) in liquid media, diluted, and plated with a Steers
replicator. Colony counts were determined on antibiotic-free
media.

The concentrations of ampicillin in sBHI agar for testing
the parent strains and transformants were 2, 4, 8, 16, 32, 64,
and 128 μg/ml. The inocula tested were 102, 103, 104, 105,
and 106 CFU. The following cephalosporins were tested with
these strains at concentrations of 1, 4, 8, 16, 32, 64, 128, and
256 μg/ml in sBHI agar: cephaloridine, cephalothin, cefazo-
lin, cephalcetile, and cefotaxim. The inocula tested were 105,
106, 107, and 108 CFU. The MIC was defined as the lowest
concentration of antibiotic which inhibited visible growth of
the inoculum in comparison with growth on antibiotic-free
media. Plates were examined after 18 to 24 h of incubation in
5% CO2 at 36.5°C.

β-Lactamase detection. The rapid acidimetric method (ampi-
icillin as substrate and phenol red as indicator) of Scheifele
et al. (35) was modified with a 96-well microtiter plate
and inoculation with a sterile wooden stick. Strains were tested

<table>
<thead>
<tr>
<th>Table 1. H. influenzae strains</th>
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<tbody>
<tr>
<td>Strain</td>
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<tr>
<td>MAP</td>
</tr>
<tr>
<td>A8</td>
</tr>
<tr>
<td>RdMCR</td>
</tr>
<tr>
<td>Rec1</td>
</tr>
<tr>
<td>Rec2</td>
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</tr>
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<td>HC234</td>
</tr>
<tr>
<td>RSF007</td>
</tr>
<tr>
<td>RSF0885</td>
</tr>
<tr>
<td>R385</td>
</tr>
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</table>

* Abbreviations: ', resistant; ', susceptible; Amp, ampicillin; Ery, erythromycin; Cm, chloramphenicol; Rif, rifampin; Sm, streptomycin; Spec, spectinomycin; Tc, tetracycline; Rec, recombination proficiency; CAT, chloramphenicol acetyltransferase.
in duplicate and compared with a reference ampicillin-susceptible strain lacking β-lactamase activity and a β-lactamase-producing, ampicillin-resistant strain as well as uninoculated control wells containing substrate and indicator. Similarly, the β-lactamase substrate (chromogenic cephalosporin) was incubated with whole-cell suspensions at room temperature for 60 min and inspected visually (24).

Aagarose gel electrophoresis. Lysates of the ampicillin-resistant clinical isolates and their transformants were prepared as described by Meyers et al. (20). These strains were also lysed by the procedure of Hansen and Olsen (9) to increase the reported detection of plasmid DNA by using both lysis techniques (29). All were subjected to electrophoresis through a 0.7% agarose gel, and the DNA fragments were visualized by staining with ethidium bromide and UV transillumination (20).

Conjugation. Overnight filter paper matings were performed as described by Roberts et al. (31). Each ampicillin-resistant clinical isolate was mated with the recipient strains MAP and RdMCR. Transconjugants were selected on sBHI agar with 5 μg of ampicillin and 250 μg of streptomycin per ml. The control, strain HC234, was mated with the same recipient separately with selection on sBHI agar containing 5 μg of chloramphenicol and 250 μg of streptomycin per ml. The conjugation frequency was determined by the number of transconjugant colonies divided by the number of donor cells plated. Colony counts were determined on antibiotic-free media.

Transformation. Whole-cell lysates of the ampicillin-resistant clinical isolates were prepared as described by Roberts et al. (30); 100 μl each of 1/15, 1/150, and 1/1,500 dilutions of crude DNA in 0.15 M NaCl plus 0.015 M sodium citrate (SSC) was added to 2 ml of competent strains MAP, Rec1, Rec2, and A8 separately. Four milliliters of sBHI was added to each flask 30 min after the addition of the DNA. Cells were grown for 4 h, and selection for transformants was performed by plating the mixture on agar containing 5 μg of ampicillin per ml at dilutions of 10−3, 10−2, and 10−1. The bacterial density was determined by plating the recipients on antibiotic-free media. The transformation frequency was determined by the number of transformant CFU divided by the total number of cells plated. Each competent recipient was tested for the frequency of spontaneous mutation to ampicillin resistance (no addition of DNA).

The phenotype of the transformant colonies was confirmed by reisolation on sBHI agar plates containing 5 μg of ampicillin per ml. In addition, the phenotype of the recipient, MAP, was confirmed by plating on media containing 20 μg of erythromycin per ml, 50 μg of spectinomycin per ml, and 250 μg of streptomycin per ml. As a control for chromosomal transformation frequency, DNA from strain MAP was prepared as above, transformed into strain A8, and selected on sBHI agar containing erythromycin (20 μg/ml) or streptomycin (250 μg/ml). The Rec1 and Rec2 strains were made competent as described above and incubated with the DNA of strain MAP under the same conditions to confirm their recombination deficiency. The MIC of ampicillin and the cephalosporins listed were determined for representative transformants, and cleared lysates of these were prepared for agarose gel electrophoresis.

Resistance retransfer by conjugation. Transformants derived from DNA of the three transformable clinical isolates with A8 were mated with two recombination-deficient strains, R850 or Rec1R, and transconjugants were selected on ampicillin-streptomycin or ampicillin-rifampin media, respectively. As a control, R849 DNA was transformed into strain MAP and then mated with Rec1R selecting on media containing 2 μg of tetracycline and 10 μg of rifampin per ml.

Hybridization. Two R plasmids with different resistant determinants, pR1234 and RSF007, were chosen as probes (Table 1). Plasmid isolation was performed by cesium chloride gradient ultracentrifugation (6). The plasmid DNAs were nick translated with [32P]dATP by the guidelines of the kit manufacturer, a modification of the procedure by Rigby et al. (28). The radiolabeled plasmid DNAs were separated from free nucleotides by chromatography on Sephadex G-50 and used as individual probes. The specific activity of the probes was ca. 108 cpm/μg of DNA, and 106 cpm was used for each hybridization. Unlabeled whole-cell DNA from the ampicillin-resistant clinical isolates and their corresponding ampicillin-resistant transformants (in strain MAP) were prepared by the lysis technique of Meyers et al. (20). Control DNA was derived from strains R385 and MAP. The DNA was electrophoresed in a 0.7% agarose gel and transferred to nitrocellulose filters by the method of Southern (38) as modified by Smith and Summers for bidirectional blotting (37). The unlabeled purified DNA of the two plasmid probes was electrophoresed on the same gel before blotting. Probes 35S-labeled RSF007 and 35S-labeled pR1234 were hybridized separately with these identical blots for 17 h at 42°C as described by Wahl et al. (47). The filters were exposed to Kodak XAR-5 X-ray film and incubated at −70°C for 3 weeks, and the film was developed.

Thin-layer chromatography. Strains were inoculated on fresh sBHI agar plates from stored vials of skim milk at −70°C and incubated overnight. Organisms from 6 ml of sBHI were harvested in mid-log phase by centrifugation at 10,000 × g for 10 min at 4°C, washed once in an equal volume of 0.05 M Tris (pH 7.8), and resuspended in 5 ml of the above buffer. The cell suspension was sonicated with a Branson sonicator at 20 W for 4 for 15-s bursts (while immersed in an ice bath) and then centrifuged at 30,000 × g for 30 min at 4°C. The supernatant was separated into 1-ml fractions and stored at −70°C; a sample was used for protein determination by the method of Lowry (26), with crystalline bovine serum albumin used as a standard.

Cell sonic extracts were thawed and kept on ice; 25 μl of [14C]penicillin G (0.25 μCi, 10–6 mM) was placed in a glass tube in a 35°C water bath for 4 min. A total of 25 μl of cell sonic extract (100 μg of protein) was added to the tube; and 10-μl samples were removed at 0, 5, and 10 min and spotted on a silica gel. The three ampicillin-resistant transformants, the isogenic ampicillin-susceptible recipient strain MAP, and a β-lactamase-producing control strain (RSF0885) were tested (Tables 1 and 2). In addition, [14C]penicillin incubated with buffer was used to assess spontaneous decomposition of the antibiotic. The chromatogram was air dried and placed in a solvent system consisting of acetone-acetic acid (19:1) for 60 min. After chromatographic separation, [14C]penicillin was located by exposure of the chromatogram with Kodak XAR-5 film at −70°C for 7 days, and the film was developed.

Permeability. Filter paper matings were carried out as described by Roberts et al. (31) between the donor strain R385, using the transformant strains, and strain MAP as recipient (Tables 1 and 2). Transconjugants were selected on media containing 5 μg of ampicillin per ml and 250 μg of streptomycin per ml. The conjugation frequency was determined by dividing the number of donor CFU plated. β-Lactamase production was confirmed in the transconjugant strains by two methods of detection: the chromogenic cephalosporin assay (24) and the.
Strain or plasmid & Ampicillin & Cephaloridine & Cephalothin & Cefazolin & Cephradine & Cefotiam  
\hline
Strains & & & & & &  
801107 & 8 & >256 & 256 & >256 & >256 & 128  
781592 & 16 & 256 & 128 & 256 & >256 & 32  
783748 & 8 & >256 & >256 & >256 & >256 & >256  
782704 & 16 & >256 & >256 & >256 & >256 & >256  
RSF0885 & 32 & 128 & 16 & 64 & 64 & 1  
MAP & 0.6 & 8 & 4 & 32 & 64 & 1  
\hline
Transformants & & & & & &  
TF801107-38 & 8 & 256 & 128 & >256 & >256 & 16  
TF801107-14 & 8 & 8 & 128 & 128 & 256 & 32  
TF783748-6 & 8 & >256 & >256 & >256 & >256 & >256  
TF782704-9 & 8 & 256 & 256 & >256 & 256 & 16  
TF782704-3 & 8 & 128 & 16 & 16 & 64 & 4  
\hline
\multicolumn{7}{l}{a MIC determinations were performed at least twice.}  
\multicolumn{7}{l}{b Strains 801107, 781592, 783748, and 782704 all have an MIC of ampicillin of 32 \mu g/ml at an inoculum of 10^3 CFU.}  
\multicolumn{7}{l}{c DNA of strain 781592 did not transform ampicillin resistance.}  
\multicolumn{7}{l}{d Strain RSF0885 produces \beta-lactamase (Table 1).}  
\multicolumn{7}{l}{e Strain MAP was the recipient used for the transformations (Table 1).}  

phenol red detection of ampicillin hydrolysis (35). The transformant strains which contained the plasmid pMR385 were designated with the prefix TC, and strain MAP containing pMR385 was designated TC MAP (Table 3).

The \beta-lactamase assay was performed as follows. Strains TC 801107-38, TC 783748-6, TC 782704-9, and TC MAP were grown separately in 6 ml of sBHI broth as described above and suspended in an equal volume of a buffer containing 0.5 mM Na2HPO4, 0.7 mM NaCl, 1 mM MgCl2, 6H2O, and 30 mM mannitol at pH 7.6. One sample was sonicated as described above and centrifuged for 5 min in a microfuge (12,000 \times g). The supernatant (cell-free sonicated treated material) was used for the assay, and a sample was retained for a protein determination and colony count. The other sample of suspended intact cells was kept on ice until tested.

The absorbance at 558 nm was continuously recorded in a Beckman Acta III spectrophotometer. All reagents were warmed to 30°C before use. The reaction mixture, in a 3-ml glass cuvette, consisted of 2 ml of water, 300 \mu l of 5 mM Na2HPO4 (pH 7.6), 300 \mu l of phenol red solution (0.0125%), and 300 \mu l of penicillin G at the concentrations indicated. The reaction was started by the addition of 100 \mu l (400 \mu g of protein) of intact or sonicated cells, mixed immediately by inversion, and placed in the spectrophotometer. Intact cells were assayed with 5 and 1 mM penicillin G. The \beta-lactamase activity of sonicated cells was determined at six penicillin G concentrations: 5, 1, 0.5, 0.1, 0.007, and 0.05 mM. Each concentration was assayed in duplicate. As controls, each sonic extract and intact cell preparation was assayed without substrate (no penicillin). Also, the nonenzymatic rate of hydrolysis was determined at the beginning and end of a run by substituting water for the enzyme source. In addition, the sBHI broth used for growth was assayed to determine whether enzyme had "leaked" out of the cells. The initial velocity (v) for each substrate concentration, S, was calculated, and 1/v was plotted against 1/S for each strain. An enzyme kinetic program was used to calculate the Kmax and Vmax. Knowing the concentration of penicillin outside the cells (S0), calculating Kmax and Vmax of the \beta-lactamase activity in the sonicated samples, and comparing the activity seen with intact cells allowed us to determine the penicillin concentration inside the cells (Si). The equation of Zimmermann and Rosselet (50) was used to calculate Si as follows:  

\[ S_i = \frac{V_{\text{intact}}}{V_{\text{max}} - V_{\text{intact}}} \cdot K_m. \]

**OMPs.** OMPs of the four ampicillin-resistant transformants, TF 782704-3, TF 782704-9, TF 783748-6, and TF 801107-14, and the isogenic ampicillin-susceptible strain MAP were isolated by lithium chloride extraction, a modification of the procedure of McDade and Johnston (18; T. L. Stull, K. O. Mack, J. E. Haas, J. Smit, and A. L. Smith, submitted for publication). OMP patterns were compared after SDS-PAGE in a 10% separating gel with a 4% stacking gel (13). Protein bands were revealed by silver staining (32), and 1.4 \mu g of protein from each strain was loaded per lane.

**PBPs.** Strains were inoculated from stored vials of skim milk at −70°C onto sBHI agar plates containing 5 \mu g of ampicillin per ml and incubated overnight. The next day organisms were grown in 500 ml of sBHI broth to stationary phase and harvested by centrifugation at 15,000 \times g at 4°C. The pellets were suspended in 0.75 M sucrose–10 mM Tris–5 mM MgCl2 (pH 7.8), sonicated as described in five 35 ml samples, pooled, and centrifuged at 3,000 \times g at 4°C for 10 min; the pellet was discarded. The supernatant was layered over a 15 to 70% discontinuous isocose gradient and centrifuged at 200,000 \times g in a swinging bucket rotor at 4°C for 2 h. The interfaces were collected, diluted with 2 volumes of 0.05 M Tris (pH 7.8), and centrifuged at 122,000 \times g at 4°C for 2 h in a fixed-angle rotor. The crude membranes (the pellet) were resuspended in a minimum volume of the above buffer and stored at −70°C. A sample was taken for Lowry protein determination (26). At the time of the study, the crude membrane preparations were thawed and adjusted to yield a protein concentration of 1 mg in a volume of 100 \mu l with the above buffer. Binding reactions were performed by a modification of the procedures of Makover et al. and Spratt (16, 39). To determine those PBPs which were the most penicillin susceptible, an affinity gel was performed with the crude membranes of the ampicillin-susceptible recipient strain MAP. Increasing concentrations of unlabeled ampicillin from 10−3 to 10−1 \mu g/ml (final concentration) were added in a 10- \mu l volume to separate microtube tubes. A control tube contained 10 \mu l of water (no ampicillin). One milligram of the crude membranes of strain MAP in a 100- \mu l volume was
added to each tube and incubated at 30°C in a water bath for 30 min. [3H]penicillin (5.42 μCi, 0.1 μg) was then added to each tube, and the incubation was continued for an additional 30 min. The reactions were terminated with the addition of 5 μl of unlabeled 1 M penicillin and 12.7 μl of 20% Sarkosyl and incubated for an additional 20 min at room temperature. The samples were then centrifuged at 160,000 × g for 2 h. The supernatants (30 μg of protein) were subjected to an SDS-PAGE in a 10% separating gel with a 4% stacking gel. Lowry protein determinations and scintillation spectrometry were used to monitor the equality of the samples loaded (26). The gels were electrophoresed at a constant current of 10 mA overnight. The gels were fixed for 1 h in 30% methanol–10% glacial acetic acid–10% trichloroacetic acid and placed in the fluor EnHance (New England Nuclear) for 1 h. The fluor was precipitated in the gel with cold water for 1 h, and the gel was dried on a Hoorf model SE540 gel dryer and exposed to Kodak XAR-5 film at −70°C for 10 days. Densitometry readings were recorded with a Hoorf model 300 series. Binding affinities were expressed as K_d values, the amount of unlabeled ampicillin required to decrease the binding by 50% compared with the control sample with no ampicillin added. The K_d values were expressed in micromolars per milliliter. An affinity gel was performed as described above for each transformant. A gel comparing the PBPs of the ampicillin-resistant transformants with the isogenic ampicillin-susceptible recipient strain was performed at a single radiolabeled penicillin concentration. One milligram of crude membrane from each strain in a 100-μl volume was incubated with 5.42 μCi (0.1 μg) of [3H]penicillin as described above. Affinity binding gels with 100 μCi (2 μg) of [3H]penicillin and loading 300 μg of protein per lane were performed for the same representative transformants and isogenic recipient. In addition, a gel comparing these strains at this 20-fold-higher concentration of [3H]penicillin and loading the 10-fold-higher concentration of protein was performed.

RESULTS

Properties of the clinical isolates. (i) MIC. The MIC of ampicillin for all four clinical isolates was 32 μg/ml at an inoculum of 10^5 CFU and 8 to 16 μg/ml at an inoculum of 10^4 CFU (Table 2). The four clinical isolates were resistant to the five cephalosporins tested (Table 2). In contrast, transformants revealed a variable resistance pattern to the cephalosporins tested (Table 2). (ii) β-Lactamase activity. All four clinical isolates were negative for β-lactamase activity by the chromogenic cephalosporin and the ampicillin-phenol red assays when compared with the controls. (iii) Plasmid content. DNA analysis of the four clinical isolates revealed that two strains, 801107 and 782704, harbored a 23-megadalton plasmid, and two had no detectable plasmid DNA when lysed by the method of Meyers et al. (20). Only strain 801107 had a visually detectable plasmid (23 megadaltons) by the method of Hansen and Olsen (9). (iv) Conjugation. There was no detectable frequency of ampicillin resistance transfer with conjugation (i.e., <10^-9) of all four clinical isolates after mating with strains MAP or RdMCR. The conjugation frequency of antibiotic resistance for control strain HC234 was 10^-4 with both of the above recipients. (v) Transformation. The frequency of transformation of the ampicillin resistance marker to recipient strain MAP was 10^-6, 10^-7, and 10^-3 for the DNA of strains 783748, 801107, and 782704, respectively. The DNA of strain 781592 did not transform ampicillin resistance at a detectable frequency (10^-9) to strain MAP. Strain MAP did not have a detectable mutation frequency to ampicillin resistance (10^-9), supporting the transformation data above. DNA analysis of transformants from the three strains capable of transferring the resistance by transformation revealed no detectable plasmid DNA by the lysis method of Meyers et al. (20). As a control, the DNA of strain MAP effectively transferred the chromosomally mediated resistance genes for erythromycin and streptomycin to strain A8 at a frequency of 10^-6. The Rec^- strains, Rec1 and Rec2, were not transformable by the identical DNA preparations, confirming their recombination deficiency and the need for a general recombination pathway for transformation. In addition, strains Rec1 and Rec2 did not have a detectable mutation frequency to ampicillin resistance (10^-9). (vi) Resistance retransfer by conjugation. The ampicillin-resistant transformants into A8, derived with DNA from the three clinical isolates capable of transformation, did not yield transconjugants when mated with Rec^- strains R850 and Rec1R. In contrast, a transformant derived with control DNA of strain R849 when mated with strain Rec1R yielded transconjugants. Strain R849 contains a 36-megadalton conjugative plasmid integrated into the chromosome (Table 1; 40).

Hybridization. Plasmid probes 35S-labeled pRI234 and 35S-labeled RSF007 had DNA homology with each other (Fig. 1, lanes I and J), as previously reported (7). Each also had DNA homology at the chromosomal level with the positive control strain R385 (Fig. 1, lane K). There was no detectable DNA homology with these probes and DNA from any of the four clinical isolates or with DNA of transformants of three of these strains (Fig. 1, lanes A to G). The recipient strain MAP also had no DNA homology with these probes, as expected (Fig. 1, lane H). Of note, the two clinical isolates which harbored visually detectable plasmids had no DNA homology with either probe (Fig. 1, lanes A and F). Thin-layer chromatography. Autoradiography of the thin-

### Table 3. β-Lactamase kinetic parameters and relative permeability

<table>
<thead>
<tr>
<th>Strain*</th>
<th>K_m (mM)</th>
<th>V_max (μmol/min)</th>
<th>V_max (μmol/min)</th>
<th>S_t (mM)</th>
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<td>TC MAP</td>
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<td>0.028</td>
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</tbody>
</table>

* Plasmid pMR385 (Table 1) was conjugated into the three ampicillin-resistant transformants listed in Table 2 and into the ampicillin-susceptible recipient strain MAP. These strains are designated with the prefix TC.

* S_t is the calculated penicillin concentration inside the cells; the penicillin concentration outside the cells was 5 mM.

* n, Number of times assay was done.

* NS, Not significant (P > 0.05 by the two-sample t test).
layer chromatogram comparing cell sonic extracts of the three ampicillin-resistant transformants, the ampicillin-susceptible isogenic recipient strain MAP, and buffer alone (no cell sonic extract) revealed no differences in the migration pattern of the radioactive penicillin (Fig. 2). The transformant of strain 801107 (Fig. 2C) was representative of the other transformants tested. In contrast, incubation with cell sonic extract of the β-lactamase-producing strain RSF0885 revealed a change in the migration pattern (Fig. 2D) consistent with the generation of penicilloic acid.

**Permeability.** The conjugation frequency of plasmid pMR385 coding for β-lactamase production was $10^{-3}$ to all three ampicillin-resistant transformants and the recipient strain MAP. All transconjugants produced β-lactamase as detected by the chromogenic cephalosporin substrate and the phenol red detection of ampicillin hydrolysis.

Sonication and centrifugation reduced the viable cell density by 98%. Only strain TC 782704-9 was significantly less permeable than strain TC MAP (Table 3). Strains TC 801107-38 and TC 783748-6 were not significantly less permeable than strain TC MAP. The controls lacking substrate or enzyme had no detectable hydrolysis of penicillin. The sBHI broth tested after growth of the strains did not have detectable β-lactamase activity, confirming the periplasmic location of the enzyme.

OMP. SDS-PAGE of the lithium chloride-extracted outer membranes revealed no apparent differences between two of the four ampicillin-resistant transformants (Fig. 3, lanes A and B) and the ampicillin-susceptible isogenic recipient strain MAP (Fig. 3, lane E). However, the two other transformants revealed differences (Fig. 3, lanes C and D). Of note, the OMPs of the transformant with significantly reduced permeability (strain TF 782704-9; Fig. 3, lane D) revealed the absence of a protein with an apparent molecular weight of 27,000 that was present in the isogenic ampicillin-susceptible recipient strain MAP and in the transformant strain TF 782704-3 derived from the same donor DNA. These two transformants derived from identical DNA revealed different resistance phenotypes to the cephalosporins tested (Table 2).

**PBPs.** The PBP profile of the recipient strain MAP revealed six major PBPs with apparent sizes of 90, 80, 71, 59, 36, and 25.5 kilodaltons (Fig. 4, lane A). PBPs 1 and 2 appeared to have two proteins at a similar molecular weight and were designated additionally as a and b. Incubation of the crude membranes of strain MAP with a 20-fold-greater amount of [3H]penicillin (100 μCi) and loading a 10-fold-greater amount of protein (300 μg) detected 10 PBPs. The PBPs with the highest affinity for ampicillin (lowest $I_{50}$ values) were PBPs 4, 2b, and 6, with $I_{50}$ values of 0.02, 0.06, and 0.14 for strain MAP (Table 4). All four transformants revealed at least a 20-fold-decreased affinity for PBP 4, whereas the affinity for PBPs 1 and 2 remained relatively unchanged (Table 4). $I_{50}$ values for PBPs 3 and 6 for the two transformants that were derived from the same donor DNA and for PBP 3 of strain TF 801107-14 could not be determined. These changes could be due either to a relative loss of the PBPs or a change in the binding affinity. PBPs 5 and 6 of strain TF 783748-6 revealed 12- and 4-fold-less affinity for ampicillin, respectively. All four ampicillin-resistant transformants had different PBP profiles compared with the isogenic ampicillin-susceptible recipient strain MAP (Fig. 5). The two transformant strains derived from the same donor DNA had identical PBP patterns (Fig. 5, lanes B and D). These showed almost no binding of [3H]penicillin to PBPs 3 and 6 and reduced binding to PBP 4. The transformant of strain 801107 also revealed minimal binding of [3H]penicillin to PBP 3 and reduced binding to PBP 4 (Fig. 5, lane E). The transformant of strain 783748 revealed decreased binding to PBP 4 (Fig. 5, lane C). Thus, alterations in PBPs 3, 4, and 6, and possibly 5 appear to correlate with the ampicillin resistance phenotype.

**TABLE 4. Affinity for ampicillin of the PBPs of H. influenzae strains expressed in $I_{50}$ values.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>$I_{50}$ (μg/ml) with the following PBP:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>MAP</td>
<td>0.81</td>
</tr>
<tr>
<td>TF 801107-14</td>
<td>0.98</td>
</tr>
<tr>
<td>TF 783748-6</td>
<td>1.28</td>
</tr>
<tr>
<td>TF 782704-3</td>
<td>0.86</td>
</tr>
<tr>
<td>TF 782704-9</td>
<td>0.98</td>
</tr>
</tbody>
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* $I_{50}$ is the amount of unlabeled ampicillin required to decrease the binding to [3H]penicillin by 50%.

$^{a}$ MAP is the recipient strain used for the transformations.
FIG. 2. Autoradiograph of a thin-layer chromatogram of cell sonicates incubated with $^{14}$C-penicillin and developed in acetone-acetic acid (19:1). The numbers above columns indicate duration of incubation (in minutes) before spotting on the silica gel. (A) Buffer alone (no cell sonicate added); (B) cell sonicate of strain MAP; (C) cell sonicate of a transformant of strain 801107; (D) cell sonicate of strain RSF0885 (β-lactamase producing).

FIG. 3. Silver strain of 10% SDS-PAGE of lithium chloride-extracted OMPs. Lanes A, B, C, and D represent OMPs of the transformant strains TF 801107-14, TF 782704-3, TF 783748-6, and TF 782704-9, respectively. Lane E represents the OMPs of strain MAP. Lane F contains molecular weight markers; numbers to the right indicate apparent molecular weight in thousands. Each lane contains 1.4 μg of protein. The transformants represented in lanes B and D are derived from the identical donor DNA; however, the strain represented in lane B is resistant to the five cephalosporins tested, whereas the strain represented in lane D is resistant to cephalexin and, less so, to cephalothin (Table 2). The strain represented in lane D was significantly less permeable to penicillin compared with the recipient strain. The arrows indicate apparent protein differences in two resistant transformants compared with the recipient strain MAP. The strain in lane D appears to have a decreased amount of a 27,000-dalton protein. The strain represented in lane C appears to have a 39,700-dalton protein not present in the recipient and an increased amount of a 27,000-dalton protein.

DISCUSSION

Although several strains of non-β-lactamase-producing, ampicillin-resistant *H. influenzae* have been reported, the characterization of these strains has been limited. Genetic characterization of one isolate was previously attempted (17). Markowitz sought physical evidence of a plasmid and performed a liquid mating with a standard *Escherichia coli* recipient, both of which were unsuccessful (17). No further attempt has been made to characterize the genetic basis of the resistance in any reported strains. We present here four naturally occurring sputum isolates of *H. influenzae* (from four different patients from widely spaced geographic locations in New Zealand) with high-level resistance to ampicillin that do not produce detectable β-lactamase. It is of interest that the apparent MIC increases geometrically from an inoculum of $10^3$ to $10^5$ CFU or greater (the so-called "inoculum effect") despite a non-β-lactamase mechanism of resistance (41). In addition, these isolates were resistant to the five cephalosporins tested. Two of these four isolates harbored a 23-megadalton plasmid. However, attempted conjugal transfer of resistance from all four isolates was unsuccessful, suggesting that a conjugative plasmid was not involved. In addition, the use of Rec" recipients in conjugation studies with the ampicillin-resistant transformants, as described by Stuy, to "excise" a chromosomally integrated conjugative R plasmid in *H. influenzae* was unsuccessful. In contrast, the control experiments were successful, confirming Stuy's results (40). These data suggest the lack of an integrated conjugative plasmid in these strains. Transfer of ampicillin resistance by transformation with DNA of three of these four isolates was successful. Lysis and electrophoresis...
This is in contrast to the penicillin-resistant, equivalent to the frequency of transfer of control of representative transformants revealed no detectable lin resistance phenotype in a single transformation (Table resistance gene(s). In addition, the transformants from all tion of multiple stretches of DNA. The use of limiting DNA characterized plasmids, confirm the hypothesis of gene linkage. of somal DNA, ampicillin concentrations revealed the same result-coin lack of a plasmid-mediated resistance, we selected two and their transformants for evidence of R plasmid probes to screen against the DNA of the four clinical isolates DNA homology with these strains and our probes, further These data are consistent with a non-plasmid-mediated non-β-lactamase mechanism of ampicillin resistance in these strains. However, the exact mechanism of resistance remained to be determined. Ampicillin resistance may exist by several mechanisms: (i) enzymatic inactivation of the antibi-otic other than by β-lactamase, (ii) a permeability barrier such that the antibiotic does not reach its target(s), or (iii) a change in the target(s) such that it is no longer affected to the same extent. Transformation of the ampicillin-resistant determinator into an ampicillin-susceptible recipient for three of these four strains allowed us to make the isogenic compari-son. Autoradiographs of the thin-layer chromatograms of cell sonic extracts of the transformants revealed no detect-able change in the migration pattern of the radioactive penicillin. This suggests no structural change in penicillin molecule by an enzymatic mechanism.

To assess permeability, we conjugated a β-lactamase-producing plasmid into the transformant strains and the isogenic ampicillin-susceptible recipient strain and com-pared the hydrolysis of penicillin spectrophotometrically. Several authors have studied the permeability of β-lactam antibiotics into gram-negative bacteria (22, 50). The validity of this technique is based on several assumptions, which were well described by Zimmermann and Rosselet (50). Of of representative transformants revealed no detectable extrachromosomal DNA. The transformation frequency was equivalent to the frequency of transfer of control chromo-somal DNA, supporting a chromosomal location of the resistance gene(s). In addition, the transformants from all three donor DNAs showed complete transfer of the ampicil-lin resistance phenotype in a single transformation (Table 2). This is in contrast to the penicillin-resistant, non-ampicillinase-producing strains of S. pneumoniae and N. gonorrhoeae, which required multiple transformations to approach the high-level resistance of the clinical strains (4, 50). This suggests either linkage of the resistance genes or transformation of multiple stretches of DNA. The use of limiting DNA concentrations and selection on media containing different ampicillin concentrations revealed the same result—complete resistance transfer. These data support the former hypothesis of gene linkage.

R plasmids, both conjugative and nonconjugative, in H. influenzae are known to share significant DNA homology with one another (3, 7, 12, 14, 15, 31). Therefore, to further confirm the hypothesis of a chromosomal location and the lack of a plasmid-mediated resistance, we selected two well-characterized plasmids, RSF007 and pRI234 (Table 1), as probes to screen against the DNA of the four clinical isolates and their transformants for evidence of R plasmid sequences. Our hybridization data showed that there was no DNA homology with these strains and our probes, further supporting a non-plasmid-mediated mechanism of resistance. Interestingly, there was no homology with the plasmids found in two of the clinical isolates. Laufs et al. screened 699 H. influenzae strains and found only one cryptic plasmid, which had 82% homology with an ampicil-lin-resistant R plasmid (pKRE5367) (14). pKRE5367 has been shown to have ~100% homology with RSF007 and 65% homology with pRI234 (15), our two probes.

These data are consistent with a non-plasmid-mediated non-β-lactamase mechanism of ampicillin resistance in these strains. Our hybridization data showed that there was no homology with the plasmids found in two of the clinical isolates. To assess permeability, we conjugated a β-lactamase-producing plasmid into the transformant strains and the isogenic ampicillin-susceptible recipient strain and compared the hydrolysis of penicillin spectrophotometrically. Several authors have studied the permeability of β-lactam antibiotics into gram-negative bacteria (22, 50). The validity of this technique is based on several assumptions, which were well described by Zimmermann and Rosselet (50).

FIG. 4. Competition of unlabeled ampicillin for the binding of [3H]penicillin to the PBPs of the ampicillin-susceptible recipient strain MAP. The unmarked lane to the left of lane A contains the 3C-labeled molecular weight standards; numbers to the left indicate apparent molecular weight in thousands. Lane A represents crude membranes preincubated with 10 μl of water before the addition of [3H]penicillin. The numbers to the left indicate the major PBPs. Lanes B through H represent membranes preincubated with increasing concentrations of unlabeled ampicillin as follows: B, 0.01; C, 0.1; D, 0.5; E, 1; F, 5.0; G, 10; and H, 100 μg/ml final concentration before the addition of [3H]penicillin.

FIG. 5. Detection of the PBPs of ampicillin-resistant, non-β-lactamase-producing transformants and the isogenic ampicillin-susceptible recipient. [3H]Penicillin was incubated with the crude membrane fractions of the strains, Sarkosyl solubilized, and coelectrophoresed on the same polyacrylamide gel. Lanes A through E represent the PBPs of the following strains: A, ampicillin-susceptible recipient strain MAP; B and D, transformants TF 782704-3 and TF 782704-9, respectively; C, transformant TF 783748-6; and E, transformant TF 801107-14. The numbers to the left of lane A indicate the major PBPs (see Fig. 4, lane A). The unmarked lane to the right of lane E contains numbers indicating the apparent molecular weight in thousands.
crucial importance, the β-lactamase enzyme must be contained within the periplasmic space. *H. influenzae* appears to conform to this criterion, as we could not detect β-lactamase activity in the supernatant of the broth culture. Only the transformant strain TF 782704-9 was significantly less permeable (P < 0.05, two sample t test). This transformant had an OMP profile different from the isogenic ampicillin-susceptible recipient strain. These data agree with the observation of Parr and Bryan (Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 22nd, Miami Beach, Fla., abstr. no. 339, 1982). They reported a strain of *H. influenzae* with decreased permeability to penicillin and an OMP profile by SDS-PAGE that was different from their control strain. However, 21 distinctive subtypes of OMP profiles in *H. influenzae* have been previously reported (8), and comparison of single nonisogenic ampicillin-resistant and -susceptible strains may not be valid. In contrast, we made the isogenic comparison. Of further interest, the transformant strain TF 782704-9 differed phenotypically from the transformant TF 782704-3, as it was resistant to all five cephalosporins and TF 782704-3 was resistant to cephalexin and pradofloxacin. This is the complete speculation that the contributing mechanism of resistance of the transformant TF 782704-9 to ampicillin and the cephalosporins may be a permeability barrier with a corresponding change in the OMPs as noted. The 27,000-dalton OMP may function as a periplasmic protein. However, the transformants TF 783748-6 and TF 801107-38 were resistant to all five cephalosporins, and strain TF 783748-6 did not reveal decreased permeability.

The presence of PBPs was first reported for an ampicillin-susceptible *H. influenzae* strain in 1981 (36). Two reports of non-β-lactamase-producing ampicillin-resistant *H. influenzae* have shown no differences in the PBP profiles of their strain and control strains (Parr and Bryan, 22nd ICAAC, 1982; D. A. Serfass and C. A. Needham, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, A104, p. 18). In contrast to these reports, mobilizing the resistance gene by transformation from three of our four strains enabled us to perform comparisons with the isogenic ampicillin-susceptible recipient. We found differences in the PBP profiles of all transformants tested; two (derived from the same donor DNA) had a relative decrease in PBPs 3 and 6, one had a decrease in binding to PBP 3, and all four had a decrease in PBP 4. In addition, the binding affinity of PBP 4 was decreased for all of the transformants and of PBP 5 for one transformant strain. I0 values for PBPs 3 and 6 could not be determined in the transformants apparently lacking these proteins. Thus, affinity for the β-lactamase as well as relative amount of protein may play a role in resistance.

The mechanism(s) of resistance to ampicillin in three clinical isolates which do not produce detectable β-lactamase appears to be multifactorial. Permeability appears to have a role with one strain, and there was an associated change in its OMP profile. The DNA from the three clinical isolates transferred the ampicillin resistance phenotype and coded for altered PBPs. Several changes in PBPs were transferred. This fact and the complete transfer of the resistance phenotype in a single transformation suggest that these genes are closely linked in *H. influenzae*. We conclude that the primary mechanism of resistance in these three strains is altered PBPs, and the genetic basis of the resistance is chromosomally determined.

**ACKNOWLEDGMENTS**

This work was supported in part by Public Health Service grants AI07044 and T32-HD07233 from the National Institutes of Health. We appreciate the excellent secretarial assistance of Kae Pierce.

**LITERATURE CITED**


