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Mechanism of Resistance of an Ampicillin-Resistant, β-Lactamase-Negative Clinical Isolate of *Haemophilus influenzae* Type b to β-Lactam Antibiotics

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The mechanism of non-β-lactamase-mediated β-lactam resistance in a clinical isolate of *Haemophilus influenzae* type b was studied. This clinical isolate showed up to a 32-fold increase in MICs of a wide variety of β-lactams, including moxalactam and cefotaxime, although no β-lactamase activity was detected, even after attempted induction. Transformation of broad-spectrum β-lactam resistance into ampicillin-susceptible *H. influenzae* RD^{nov} was accomplished. Examination of the outer membrane protein profile of the resistant parent by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of Triton X-100-extracted membranes revealed an unusual major outer membrane protein band at a molecular weight of 45,000. This outer membrane protein profile did not transform with β-lactam resistance. Permeability differences were noted between the resistant strain and the nonisogenic susceptible strain of *H. influenzae*, although these penetration differences were not transformed. Comparison of the penicillin-binding protein profile of a resistant transformant with that of a susceptible parent with both whole-membrane preparations and whole-cell labeling, revealed a major reduction in binding affinity to penicillin-binding proteins 3a and 3b (molecular weights, 68,000 and 65,000, respectively). Thus, alteration in penicillin-binding proteins 3a and 3b correlated with the β-lactam resistance.

Ampicillin has been used widely in the treatment of *Haemophilus influenzae* infection; however, significant resistance caused by β-lactamase production is found in up to 35% of infecting organisms in some areas (21, 27, 33, 34, 37). There have been sporadic reports of β-lactamase-negative, β-lactam-resistant *H. influenzae* (4, 30, 38), but the mechanism(s) of this resistance has not been elucidated. Chloramphenicol is effective against many *H. influenzae* isolates, although resistance, including that to both chloramphenicol and ampicillin, has been reported (6, 18). Further, chloramphenicol therapy has potentially serious toxicity (7). Introduction of new β-lactams, most of which are stable in the presence of the common β-lactamase found in ampicillin-resistant *H. influenzae* and show high activity against the bacterium (9, 10, 12, 15, 16), may provide an effective alternative for the treatment of serious *H. influenzae* infection (20). The use of such antibiotics is likely to increase selective pressure on the organism for mechanisms of resistance other than that mediated by β-lactamase. In other species, slight decreases in β-lactam susceptibility have resulted in significant increases in treatment failures with these drugs (14). We have investigated the mechanism of ampicillin resistance in a β-lactamase-negative isolate of *H. influenzae* type b.

MATERIALS AND METHODS

Strains. Strain UCHI-2 was isolated from an immunocompromised adult with pneumonia. Strain UCHI-7, an ampicillin-susceptible clinical isolate of *H. influenzae*, and strain UCHI-8, a β-lactamase-producing *H. influenzae* strain, were from the Foothills Hospital Microbiology and Infectious Diseases Laboratory, Calgary, Alberta, Canada. Strain UAHF-1, a β-lactamase-producing *H. influenzae* strain containing transferable chloramphenicol and tetracycline resistance, has been previously described (6). Strain RD^{nov} is a novobiocin-resistant, nonencapsulated *H. influenzae* strain obtained from Jane Setlow through William Albritton. Centers for Disease Control, Atlanta, Ga. (1). Strains T-1,3 and T-1,5 are β-lactam-resistant, β-lactamase-negative transformants of strain RD^{nov} with DNA from strain UCHI-2.

Typing. Typing of strains was performed by both slide agglutination and capsular swelling tests (13) with *H. influenzae* typing sera from The Wellcome Foundation Ltd., Beckenham, England; Statens Seruminstitut, Copehhagen, Denmark; and Hyland Labs, Costa Mesa, Calif.

Drugs. The antibiotics used in MIC determinations were obtained as follows: penicillin G, ampicillin, and carbenicillin, Ayerst Laboratories, Montreal, Quebec, Canada; ticarcillin and amoxicillin, Beecham Pharmaceuticals, Point Claire, Quebec, Canada; cefsulodin, Delbays Research Corp., Florham Park, N.J.; cefotaxime (HR756), Roussel, Montreal, Quebec, Canada; cefamandole and moxalactam, Eli Lilly & Co., Indianapolis, Ind.; cefametazole, Sankyo Centre Research Laboratories, Osaka; piperacillin and tetracycline, Lederle Laboratories, Pearl River, N.Y.; imipenem and cefoxitin, Merck Sharp & Dohme, Rahway, N.J.; chloramphenicol, Parke, Davis & Co., Scarborough, Ontario, Canada; ceftriaxone (Ro-139904), Hoffmann-La Roche, Inc., Vaudrevil, Quebec, Canada; cefoperazone, Pfizer Canada Ltd., Montreal, Quebec, Canada; ceftizoxime, Smith Kline & French Canada Ltd., Mississauga, Ontario, Canada; cefminoxime, Takeda Chemical Industries Co., Ltd., Osaka, Japan; and rifampin, Sigma Chemical Co., St. Louis, Mo. ^{3}H-radiolabeled penicillin (64 mCi/mg) was a gift from Merck Sharp & Dohme, Kirkland, Quebec, Canada.

β-Lactamase assays. β-Lactamase activity was assayed by microiodometric (28), nitrocefin (29), and acidometric (31) methods, with both whole- and ruptured (sonicated)-cell

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preparations. Attempts were made to induce β-lactamase activity by growing cells in subinhibitory concentrations of β-lactams before assay.

Susceptibility testing. MICs of β-lactams were determined by a tube dilution method. Strains were grown overnight in Mueller-Hinton broth (GIBCO Diagnostics, Madison, Wis.) containing 0.01 g of bovine hemin (Sigma) and 10 ml of CVA enrichment (GIBCO) per liter. The final pH of the medium was 7 to 7.2. Overnight cultures were diluted grown to log phase, and subsequently diluted in 0.89% NaCl to 10^4 CFU/ml and inoculated into equal volumes of doubling dilutions of antibiotic in double-strength growth medium. Tubes were incubated for 20 h at 37°C. The MIC was defined as the lowest concentration of antibiotic which inhibited visible growth at 20 h. All determinations were done at least in triplicate. MBCs were determined by examining viable cell numbers from MIC determination tubes. The MBC was defined as the lowest concentration of antibiotic which killed greater than 99.9% of the inoculated cells at 20 h.

Membrane preparation. Log-phase cells aerobically grown on brain heart infusion broth (GIBCO) containing 1% CVA enrichment and 10 μg of hemin per ml were collected by centrifugation, and the pellet was passed three times through a French press (Fred S. Carver, Inc., Summit, N.J.) at 8,000 lb/in^2. Phenylmethylsulfonyl fluoride (Sigma) was added to a final concentration of 1 mM, and the preparation was centrifuged at 1,500 × *g* for 10 min to remove whole cells. The supernatant was collected and spun at 146,000 × *g* for 1 h. The amber-colored pellet was collected and suspended in either phosphate-buffered saline (PBS) (Oxoid Ltd., London) (whole-membrane preparation) or 10 ml of the 0.01 M phosphate buffer containing 2% Triton X-100 (pH 7.5). The suspension was centrifuged at 146,000 × *g* as before for 1 h at 4°C. The supernatant was collected as the inner membrane fraction, and the pellet was resuspended in 10 ml of the phosphate buffer, to which 2% Triton X-100 and 0.005 M EDTA had been added. This suspension constituted the outer membrane fraction. Portions of the membrane preparations were stored at −70°C until needed. The protein concentrations were assayed by the method of Lowry et al. (23), with bovine serum albumin (Sigma) as the standard.

Binding of penicillin G. (i) Isolated membrane. The procedure for binding radiolabeled penicillin was modified from the method described by Spratt (35) and used by Godfrey et al. (11). Prepared whole-membrane fractions were thawed slowly, and 100 μg of protein was combined with 10 μl of [^{3}H]penicillin G at a concentration of 0.625 μg of penicillin per 100 μg of protein, with a final concentration of 12.5 μg/ml.

(ii) Whole-cell labeling. Early-log-phase cultures were pelleted and washed twice in PBS. The pellet was resuspended in PBS to the optical density of a McFarland no. 2 Standard. One milliliter was placed in a microfuge tube, and the cells were pelleted for 2 min in a Beckman model B centrifuge. The pellet was resuspended in 100 μl of PBS, and the radiolabeling reaction was performed as with whole membranes; the final concentration of the drug was 5.7 μg/ml. Once the reaction was stopped by the addition of cold penicillin, the cells were repelleted and washed in 1 ml of PBS. After being washed, the pellet was resuspended in 40 μl of lysis buffer consisting of 2% sodium dodecyl sulfate, 4% 2-mercaptoethanol, 10% glycerol, 1 M Tris (pH 6.8), and 0.002% bromphenol blue. The resuspended, lysed cells were heated to 100°C for 3 min and loaded for electrophoresis.

(iii) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A 10% discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described by Laemmli and Favre (19) was used.

(iv) Protein staining and autofluorography. Gels were stained with a solution of 0.1% (wt/vol) Coomassie brilliant blue R (Sigma) in a solution of 50% methanol–10% acetic acid.

Gels radiolabeled with penicillin G were prepared for autofluorography with En^{3}Hance autoradiography enhancer (New England Nuclear Corp., Lachine, Quebec). Fluorographs were exposed by prefogging X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) by the method of Bonner and Laskey (5). Laser densitometry was performed with an LKB 2202 ultrascan densitometer with a Hewlett-Packard 3390A integrator. Binding affinities of β-lactams for penicillin-binding proteins (PBPs) were expressed as ID_{50}s, that is, the concentration of competing β-lactam needed to reduce binding of [^{3}H]penicillin G by 50% in whole-cell labeling experiments. The preincubation time for competing β-lactam before adding [^{3}H]pencillin G was 10 min.

(v) Molecular weight determinations. Molecular weight determinations for both inner and outer membrane fractions were made by plotting the mobility in the gels of standard-molecular-weight proteins versus the log of the molecular weights of the standard proteins. The proteins used, with their molecular weights, were: phosphylase b, 94,000; bovine serum albumin, 68,000; pyruvate kinase, 57,000; lactate dehydrogenase, 35,000; soya bean trypsin inhibitor, 21,000; and lysozyme, 14,300 (Sigma).

(vi) Permeability assay. The penetration of penicillin G through the outer membrane of *H. influenzae* was assessed by the method of Zimmermann and Rosselet (40). The plasmid specifying β-lactamase, chloramphenicol acetyltransferase, and tetracycline resistance was transferred from strain UAHF-1 by conjugation into a streptomycin-rifampin double-resistant derivative of the test strains (resistance to each drug, greater than 500 μg/ml) by a solid-phase mating method.

Ten microliters of cell preparation (whole cell or sonicated) was combined with an equal volume of penicillin B in a time course experiment. The rates of hydrolysis of various concentrations of penicillin G by β-lactamase were compared for both whole and lysed cells by the microiodometric method (28). The concentration of whole and lysed cells used in each experiment varied, depending on the rate at which the sonicated preparation hydrolyzed the β-lactam. However, the concentration used was always identical for each sample of whole and lysed cells. Assays were repeated a minimum of three times at each β-lactam concentration. Results are expressed as the ratio of the initial rates of hydrolysis of the whole cell β-lactamase on penicillin G to the initial rate of hydrolysis for free β-lactamase. Statistical significance was determined by a paired *t* test at all concentrations for which the ratios were not identical.

(vii) Transformation. Transformation of β-lactam resistance was accomplished by the method of Vega et al. (39) modified from Juni and Janik (17). Selection for putative β-lactam-resistant transformants was on chocolate plates containing 4 μg of cefamandole per ml. An additional control was run by adding the lysis solution without DNA to recipient cells. Streptomycin resistance was also transformed into strain RD^{nov} from streptomycin-resistant (MIC, 500 μl/ml) strain UCHI-2. Selection was on chocolate plates with 100 μg of streptomycin per ml. Selection for β-lactam resistance was attempted at cefamandole concentrations of

TABLE 1. MICs of chloramphenicol and β-lactam antibiotics for *H. influenzae* RD^{nov} and UCHI-7 (susceptible), UCHI-2 (resistant), and T-1,3 (transformed resistant)[a]

Antimicrobial agent	MICs (μg/ml) for strain:			
	UCHI-2	T-1,3	RD^{nov}	UCHI-7
Penicillin G	1.56 (16)[b]	1.56 (16)	0.09	0.39
Ampicillin	1.56 (8)	1.56 (8)	0.185	0.185
Carbenicillin	1.56 (8)	1.56 (8)	0.185	0.39
Ticarcillin	1.56 (16)	1.56 (16)	0.09	0.185
Amoxicillin	0.78 (4)	0.78 (4)	0.185	0.39
Azlocillin	0.09 (16)	0.09 (16)	0.0056	<0.023
Piperacillin	0.045 (16)	0.023 (8)	0.0028	<0.045
Cefsulodin	250 (16)	250 (16)	15.6	12.5
Cefmetazole	6.25 (4)	6.25 (4)	1.56	1.56
Cefoxitin	1.56 (1)	1.56 (1)	1.56	ND[c]
Cefamandole	6.25 (16)	3.125 (8)	0.185	0.39
Cefmenoxime	0.06 (8)	0.03 (4)	0.008	ND
Cefoperazone	0.045 (16)	0.023 (8)	0.0028	ND
Ceftriaxone	0.023 (16)	0.011 (8)	0.0014	ND
Cefotaxime	0.023 (32)	0.045 (64)	0.00078	<0.09
Moxalactam	0.39 (16)	0.39 (16)	0.023	0.045
Imipenem	0.09 (1)	0.09 (1)	0.09	ND
Chloramphenicol	0.39 (1)	0.39 (1)	0.39	0.185

[a] MICs of drugs were determined by tube dilution. Supplemented Mueller-Hinton broth inoculated with 10^4 CFU of test strain per ml were incubated for 20 h at 37°C without increased CO_2 tension.
[b] Ratio of MIC for indicated strain to MIC for strain RD^{nov}.
[c] ND, Not determined.

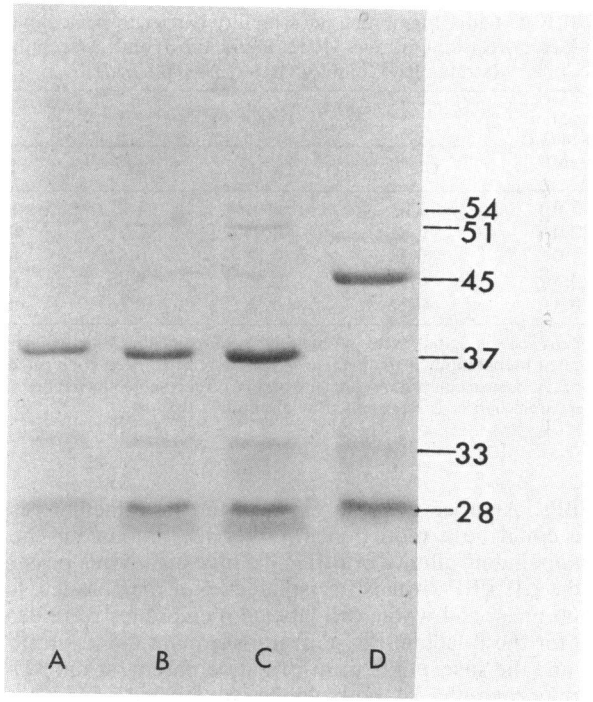

FIG. 1. Outer membrane protein profile of 2% Triton X-100-insoluble fraction of *H. influenzae* membranes. Lanes A and B, β-Lactam-resistant transformant strains T-1,5 and T-1,3; lane C, susceptible parent strain RD^{nov}; lane D, resistant parent strain UCHI-2. Apparent molecular weights ($\times 10^{-3}$) are indicated on the right.

0.39, 0.78, and 1.56 μg/ml (two, four, and eight times the MIC of cefamandole for strain RD^{nov}, respectively), as well as at 4 μg of cefamandole per ml.

RESULTS

Susceptibility testing and transformation. Strain UCHI-2 did not produce detectable β-lactamase by the microiodometric (28), nitrocefin (29), or acidometric (31) method. Although control strains UAHF-1 (6) and UCHI-8 had the expected β-lactamase activity, no enzymatic activity could be detected in strain RD^{nov}, UCHI-2, T-1,3, or T-1,5 with penicillin G, nitrocefin, or ampicillin. Assays were performed with both whole- and sonically disrupted-cell preparations. Strain UCHI-2 was grown in a subinhibitory concentration (one-half the MIC) of penicillin or ampicillin in an attempt to induce β-lactamse activity, but none could be demonstrated. The control β-lactamase-producing strains showed a much greater effect of inoculum on the MIC of β-lactamase-susceptible β-lactams than did either strain UCHI-2 or T-1,3 (data not shown). MBCs of ampicillin, carbenicillin, imipenem, and ceftriaxone for strains UCHI-2 and T-1,3 ranged from two to eight times the corresponding MIC.

Transformation of the β-lactam resistance of strain UCHI-2 into the susceptible background of strain RD^{nov} was accomplished. The rate of transformation was 1 in 2.5×10^5 cells. MICs of a wide range of β-lactams (Table 1) were nearly identical for the resistant parent (strain UCHI-2) and a transformed clone (strain T-1,3). Both the β-lactamase-negative, resistant parent (strain UCHI-2) and its transformant (strain T-1,3) showed increased MICs of a wide variety of β-lactams compared with susceptible control organisms (Table 1). The maximum ratio increases of strains UCHI-2 and T-1,3 over the susceptible strain RD^{nov} were 32 and 64, respectively, for the β-lactam cefotaxime. The most frequent ratio noted was 8 or 16. There were no increases in the MIC of cefoxitin, imipenem, or chloramphenicol for strain T-1,3 or UCHI-2.

Outer membrane proteins and permeability assessment. The type b capsule of strain UCHI-2 was not cotransformed with β-lactam resistance. The outer membrane protein profiles of transformed clones and susceptible and resistant parents were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1). The banding patterns for the transformants and the susceptible recipient were identical. The major 45,000-molecular-weight band in strain UCHI-2 did not transfer with the β-lactam resistance.

To assess the permeability of the outer membrane to β-lactams, the method of Zimmerman and Rosselet (40) was employed. This method involves comparison of the rates of hydrolysis of the same β-lactam by a β-lactamase in whole and sonicated cells. The ratios of the initial rates of hydrolysis of penicillin G at concentrations of 0.3 to 2.0 mM of whole-cell to sonicated preparations were higher in another susceptible clinical isolate, strain UCHI-7, than in the resistant UCHI-2 strain (Table 2). By the paired *t* test, this difference was significant at all concentrations less than 3 mM ($P < 0.05$). At less than 0.3 mM, the rate of hydrolysis was too slow to accurately measure. At 3.0 ml, all strains tested had overcome the apparent permeability barrier of the outer membrane in that a consistent ratio of 1.0 was achieved for all strains. The apparent permeability barrier difference noted between the resistant strain UCHI-2 and the susceptible strain UCHI-7 was not noted when the β-lactam-resistant transformant strain T-1,3 or the susceptible strain RD^{nov} transformation parent was compared with strain UCHI-2 (Table 2).

TABLE 2. Outer membrane permeability barrier to penicillin G in β-lactam-resistant (strains UCHI-2 and T-1,3) and -susceptible (strains RD^{nov} and UCHI-7) *H. influenzae*

Concn of penicillin G (mM)	Ratio for strain[a]:			
	UCHI-2	UCHI-7	RD^{nov}	T-1,3
3.0	1.0	1.0	1.0	1.0
2.0	0.81	0.94	0.77	0.81
1.0	0.54	0.78	0.62	0.65
0.5	0.30	0.45	0.37	0.35
0.3	0.22	0.38	0.24	0.32

[a] Ratio of the initial rates of hydrolysis of penicillin G in whole and sonicated preparations of *H. influenzae* into which had been conjugated the same β-lactamase plasmid. A complete barrier is represented by a ratio of 0.0. No apparent barrier is represented by a ratio of 1.0.

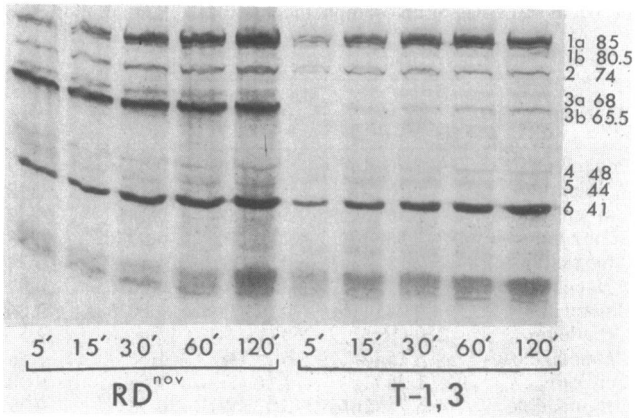

FIG. 2. Fluorograph of whole-cell labeling time course strains RD^{nov} and T-1,3. One milliliter of early-log-phase cells suspended in PBS to an optical density of a no. 2 McFarland Standard were pelleted and resuspended in 100 μl of PBS. [^{3}H]penicillin G (10 μl) was added and incubated for the times indicated (5 to 120 min). Final concentrations were 12.5 μg/ml. The binding reaction was stopped with cold penicillin G, and the cells were solubilized in lysis buffer. The samples were heated to 100°C for 3 min and electrophoresed in a 10% Laemmli gel system. The autofluorograph was developed for 48 h. PBP numbers and apparent molecular weights ($\times 10^{-3}$) are indicated on the right.

PBPs. Another possible explanation for β-lactam resistance could be a reduction in either the amount or the β-lactam binding affinity of PBPs. To investigate this possibility, the [^{3}H]PBP profiles for isolated inner membranes, total membranes, and whole-cell-labeled membranes were examined for the β-lactam-resistant transformant clone, strain T-1,3, and the susceptible transformation parent, strain RD^{nov}. Autofluorographs of both whole-cell-labeled (Fig. 2) and isolated total-membrane fractions (Fig. 3) showed the same number of PBPs for each strain, but a significant difference in profile existed between strain RD^{nov} and its β-lactam-resistant transformant. PBP-3a and -3b, which have molecular weights of 68,000 and 65,500, respectively, and which are prominent in the susceptible RD^{nov} strain, bound radiolabeled penicillin G with higher affinity than did the equivalent proteins in the resistant transformant (Fig. 2 and 3). This reduction in penicillin-binding affinity of these PBPs was also seen in the resistant parent, strain UCHI-2 (data not shown). The binding of [^{3}H]penicillin G by PBP-3a and -3b was possible over a long period of time and at a relatively low concentration (5.7 μg/ml), but it never achieved the prominence exhibited by strain RD^{nov} (Fig. 2). Examination of Coomassie blue-stained whole-cell and membrane preparations showed no decrease in the quantity of the 68,000- and 65,500-molecular-weight proteins. The intensity of the fluorographs for other PBPs was equal for strains RD^{nov} and T-1,3.

The ID_{50}s of cefoxitin, imipenem, and piperacillin for PBP-1, -2, and -3 are shown in Table 3. The primary target of cefoxitin is the PBP-1 group (ID_{50}, 0.72 μg/ml). For imipenem, the first PBP hit is PBP-2 (ID_{50}, 0.09 μg/ml), and for piperacillin, the primary target is PBP-3 (ID_{50}, <0.009 μg/ml). Prelabeling for 10 min with piperacillin resulted in filamentation of strain RD^{nov} at subinhibitory concentrations, but such was not noted for the transformant strain T-1,3. This morphological change associated with PBP-3 as the primary target is the same as that described previously for other species (8, 36).

DISCUSSION

β-Lactam resistance in *H. influenzae* type b has, since it was noted in 1974, been associated with the production of β-lactamase. This mechanism of resistance is still by far the most common found in clinical isolates of resistant *H. influenzae*. The use of β-lactamase-stable β-lactams such as cefotaxime and moxalactam may select for *H. influenzae* with increased resistance to such antibiotics. This resistance is unlikely to be mediated by β-lactam-hydrolyzing enzymes. The emergence of isolates of *H. influenzae* which are associated with failure of treatment and do not possess β-lactamase has already been reported (4, 25, 30).

In this paper we have characterized a clinically isolated strain of *H. influenzae* type b which was found to be resistant to a wide variety of β-lactam antibiotics but which utilized a mechanism of resistance other than the production of β-lactam-degrading enzymes. The non-β-lactamase-producing, β-lactam-resistant strain UCHI-2 had its β-lactam resistance transformed into the susceptible background of strain RD^{nov}. A β-lactam-resistant transformant, strain T-1,3, exhibited levels of β-lactam resistance that were the same as those of strain UCHI-2. Capsule transformation was not found after the β-lactam resistance was transformed and, hence, does not account for any of the β-lactam resistance noted in strain UCHI-2.

The transformation frequency was quite low; the average for four transformations was 2.5 transformants per 10^5 cells (2.5×10^{-5}). Selection on doubling concentrations of cefamandole with from 2 to 16 times the MIC of the recipient strain RD^{nov} did not alter this frequency significantly. Transformation of high-level (ribosomal) streptomycin resistance was done concurrently with the selection for β-lactam resistance in some transformations. The frequency of transformation of streptomycin resistance was in the same range as β-lactam transformation (4.6×10^{-6}). These data suggest that the genetic materials controlling the expression of the β-lactam resistance described here are located on one gene or on very closely linked genes.

To explore the possibility of reduced permeability leading to β-lactam resistance, the method of Zimmermann and Rosselet was employed (40). A β-lactamase-specifying plasmid (6) was conjugated into the β-lactam-resistant strains UCHI-2 and T-1,3, as well as into two susceptible strains of *H. influenzae*, strains UCHI-7 and RD^{nov}. A significant permeability barrier could be shown between 0.3 and 2 mM

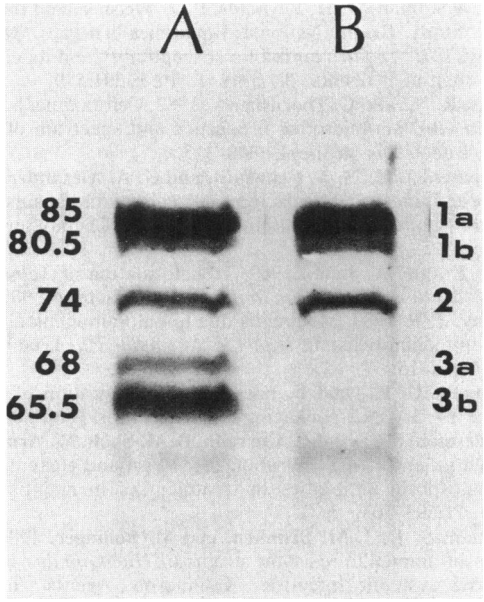

FIG. 3. Total membrane fluorograph of *H. influenzae* RD^{nov} (A) and resistant transformant strain T-1,3 (B). [^{3}H]penicillin G (6.25 μg) was combined with 100 μg of total membrane fraction protein for 15 min at 20°C before being stopped with cold penicillin G and solubilized with Sarkosyl NL-97. The mixture was combined with 60 μl of gel sample buffer and 10 μl of 10% β-mercaptoethanol heated to 100°C for 2 min and electrophoresed in a 10% Laemmeli gel system. Autofluorography was for 48 h. Apparent molecular weights ($\times 10^{-3}$) are indicated. Lower-molecular-weight PBPs are not shown on this figure, but patterns were identical to those of whole-cell labeling (Fig. 2).

with the substrate penicillin G for all strains. Differences in permeability to penicillin G could be demonstrated between strains UCHI-2 and UCHI-7. However, these differences were not noted in the comparison of strain T-1,3 with its susceptible parent strain, RD^{nov}. The barrier to penetration of β-lactams provided by the outer membrane in *H. influenzae* is, as has been previously speculated (26), considerably less that that of enteric gram-negative bacteria. At 3.0 mM, the hindrance to penicillin G provided by the outer membrane is apparently overcome in all strains of *H. influenzae* tested. In *Escherichia coli*, the ratio of the rates of hydrolysis for penicillin G is 0.2 at 5.0 mM (40).

The outer membrane protein profiles of the test strains were examined after extraction with 2% Triton X-100. There was a gross difference noted between strain UCHI-2 and the other strains tested. Strain UCHI-2 has a major outer membrane protein band at a molecular weight of 45,000, whereas the other strains examined have an apparently similar density band at a molecular weight near 37,000. This 45,000-molecular-weight band has not been found in other examinations of outer membranes of *H. influenzae* (2, 3, 22). Transformation of β-lactam resistance did not produce cotransformation of this outer membrane protein. From these experiments we concluded that the resistance noted in strain UCHI-2 and its transformant, strain T-1,3, is not caused by a decrease in the access of β-lactams brought about by alterations in the outer membrane of the resistant strains.

Alterations in the PBPs have, in other species, been shown to correspond to increased resistance to β-lactam antibiotics (32). Changes associated with increased β-lactam resistance have included loss of PBPs, decreased affinity of PBPs found in equal quantity in the membrane for β-lactams, and more rapid turnover of the β-lactam is altered PBPs (more rapid deacylation).

TABLE 3. ID_{50}s of cefoxitin, imipenem, and piperacillin for strain RD^{nov} [a]

Drug	MIC (μg/ml)	ID_{50} for PBP:		
		1a and b	2	3a and b
Cefoxitin	1.56	0.72	>0.90	5.2
Imipenem	0.09	0.18	0.09	4.5
Piperacillin	0.0028	0.09	0.03	<0.009

[a] Various concentrations of the specified β-lactams were incubated for 10 min with whole-cell preparations of strain RD^{nov}, followed by the addition of [^{3}H]penicillin G to saturate unoccupied binding sites. MICs were produced as described in footnote *a* of Table 1.

The PBP profile of the susceptible RD^{nov} strain, as examined by whole-membrane and whole-cell labeling techniques, was similar to, but not identical with, that previously reported (24). The PBP profile of the β-lactam-resistant transformant strain T-1,3 was identical to that of the β-lactam-resistant strain UCHI-2 (data not shown). The PBP-3s of these two strains showed less binding of [^{3}H]penicillin G than did the susceptible transformation parent, strain RD^{nov}. The differences noted between the resistant strains and the susceptible RD^{nov} strain were confined to PBP-3a and -3b. These decreases in binding to the PBP-3s were not caused by a loss of the proteins or a decrease in the cellular concentration, for examination of stained gels revealed equivalent amounts of the proteins corresponding to the PBP-3a and -3b in strains T-1,3 and RD^{nov}. Deacylation did not account for the decrease in binding (Fig. 2). Prolonged incubation periods allowed some binding to the PBP-3s, although the level did not reach that of the susceptible parent.

Triton X-100-extracted inner membrane fractions of the *H. influenzae* membranes examined failed to show differences in PBP profiles (data not shown). The consistency of the PBP profiles noted with whole-membrane preparations and in whole-cell labeling experiments, along with the similarity of these results to those reported in the literature, suggest that the Triton X-100 results are not reliable. Apparently, this detergent interacts in some way with the important PBPs to obliterate actual differences between strains. The consistency of the PBP profiles seen with Triton X-100-extracted inner membrane preparations initially suggested that permeability differences between the susceptible nonisogenic strain UCHI-7 and the β-lactam-resistant strain UCHI-2 may have been the cause of β-lactam resistance (T. R. Parr and L. E. Bryan, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 22nd, Miami, Fla., abstr. no. 339, 1982). Such speculation has been reversed in light of isogenic permeability data and the use of whole-membrane and whole-cell PBP labeling procedures. Similar findings have also apparently been made with another isolate (P. M. Mendelman, T. L. Stull, D. O. Chaffin, C. E. Rubens, and A. L. Smith, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, A105, p. 18).

Reduction in binding affinity for the important PBP-3a and -3b in strain UCHI-2 strongly correlates with the β-lactam resistance noted in this clinical isolate of *H. influenzae* type b. The observation that cefoxitin and imipenem, the only β-lactams tested with primary targets other than PBP-3a and

-3b, showed no increase in MIC (Table 1) further supports this conclusion.

The increases in resistance noted in strain UCHI-2 and its transformant range up to 64-fold. Although MICs of the β-lactams most active for these strains remain low, the clinical significance of such increases could be important. Infection with *H. influenzae* often occurs in the meninges, and β-lactam antibiotic entry into the cerebrospinal fluid is generally poor. Resistance levels of 1 μg of ampicillin per ml, for example, can be associated with treatment failure (14). If such strains were widespread, second-step mutations affecting entry of antibiotics or producing even lower β-lactam affinity for PBP-3a and -3b could produce much higher resistance to even new cephalosporins and penicillins. β-lactamase-resistant β-lactams would be of no value to counteract these forms of resistance.

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