

## A Permeability Barrier as a Mechanism of Chloramphenicol Resistance in *Haemophilus influenzae*

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Chloramphenicol resistance in *Haemophilus influenzae* occurs most frequently via plasmid-mediated chloramphenicol acetyltransferase production. We studied four strains with high-level chloramphenicol resistance (MIC > 20 µg/ml) which did not have detectable chloramphenicol acetyltransferase activity. The chloramphenicol resistance determinant was transformed into a chloramphenicol-susceptible laboratory *H. influenzae* strain from each of the four wild-type strains, enabling isogenic comparisons. By thin-layer chromatography and a bioassay, there was no evidence of non-chloramphenicol acetyltransferase modification of chloramphenicol. In vitro protein synthesis in the presence of chloramphenicol was equivalently inhibited in the chloramphenicol-resistant transformants and in the susceptible recipient. Chloramphenicol uptake by these strains during logarithmic growth was compared by high-pressure liquid chromatographic quantitation; at chloramphenicol concentrations of 5, 10, and 20 µg/ml the four transformants showed a decreased rate of uptake of chloramphenicol compared with the isogenic chloramphenicol-susceptible recipient. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of outer membrane proteins revealed a markedly diminished 40-kilodalton protein in the resistant transformants. We propose that the mechanism of chloramphenicol resistance in these strains is a relative permeability barrier due to the loss of an outer membrane protein.

Chloramphenicol is recommended in the initial therapy of invasive disease caused by *Haemophilus influenzae* due to the increasing incidence of ampicillin resistance (20). However, chloramphenicol resistance in *H. influenzae* has been reported worldwide. Currently, the prevalence in the United States is 1% (53).

The most frequently reported mechanism of chloramphenicol resistance in gram-negative bacteria is the plasmid-mediated production of chloramphenicol acetyltransferase (CAT) (13). CAT catalyzes the diacetylation of chloramphenicol with acetyl coenzyme A (acetyl-CoA). The resultant product does not inhibit procaryotic ribosomal protein synthesis.

We screened 58 clinical isolates of chloramphenicol-resistant *H. influenzae*, identifying four strains with no detectable CAT activity. These strains were investigated for low-level or inducible CAT activity and for alternative enzymatic and nonenzymatic resistance mechanisms. Potential mechanisms of resistance include enzymatic inactivation, ribosomal resistance to inhibition by chloramphenicol, or a relative permeability barrier to influx of the antibiotic (9). The genetics of the resistance mechanism was characterized by conjugation, transformation, and DNA hybridization experiments.

(These data were previously reported in part [Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, A106, p. 18; Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, A90, p. 16].)

### MATERIALS AND METHODS

**Bacterial strains.** The organisms used are shown in Table 1. The clinical isolates were unencapsulated strains of *H.*

*influenzae*, as determined by lack of agglutination in Difco polyvalent (a through f) typing sera and by growth requirements for hemin and βNAD (38). Strains 77-1040, 76-81739, and 76-79268 were isolated from the blood and sputum of a single patient at different times. Strains 77-1040 and 76-79268 were β-lactamase producing; strains 76-81739 and 76-79268 were tetracycline resistant. C435 was a sputum isolate from an immunodeficient pediatric patient. It was also tetracycline resistant. All four strains were susceptible to streptomycin and spectinomycin; all were erythromycin resistant. RdMCR is a streptomycin- and rifampin-resistant derivative of Rd (40). MAP is a well-characterized chloramphenicol-susceptible unencapsulated laboratory strain with multiple chromosomal antibiotic resistance markers (6). E<sub>1</sub> is a chloramphenicol-susceptible *H. influenzae* type b clinical isolate (45). HC234 and R385 are chloramphenicol-resistant strains containing plasmids pRI234 and pMR385, respectively, encoding for CAT production and tetracycline resistance (40, 49).

**Media.** Liquid medium was 3.5% brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with 10 µg of hemin, 10 µg of L-histidine, and 10 µg of βNAD per ml (sBHI). Broth cultures were incubated at 36.5°C and shaken at 200 cycles per min. Solid medium was sBHI agar. Plate cultures were incubated at 37°C in 5% CO<sub>2</sub>. Organisms were stored at -70°C in vials of sterile skim milk. They were inoculated onto fresh sBHI plates and incubated overnight before each experiment.

**Antibiotics and chemicals.** Chloramphenicol and chloramphenicol base were obtained in crystalline form from Sigma Chemical Co., St. Louis, Mo. SCH 24893, the 3-fluoro derivative of chloramphenicol, was supplied by George Miller of the Schering Corp., Bloomfield, N.J. [<sup>14</sup>C]chloramphenicol (specific activity, 14.9 mCi/mM), [<sup>14</sup>C]sodium acetate (specific activity, 57.7 mCi/mM), [<sup>14</sup>C]valine (specific activity, 280 mCi/mM), and [<sup>35</sup>S]dATP

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TABLE 1. Bacterial strains and susceptibility to chloramphenicol and SCH 24893

Strains	MIC ( $\mu\text{g/ml}$ ) <sup>a</sup>		Source <sup>c</sup>	Reference
	Chloramphenicol	SCH 24893 <sup>b</sup>		
77-1040 <sup>d</sup>	40	50	C. Thornsberry	33
76-81739 <sup>d</sup>	50	50	C. Thornsberry	33
76-79268 <sup>d</sup>	40	50	C. Thornsberry	33
C435 <sup>d</sup>	20	50	CHMC <sup>e</sup>	
RdMCR	0.1	0.1	M. Roberts	40
MAP	0.1	0.1	B. Catlin	6
E <sub>1</sub>	1	0.1		
HC234	30	0.1	B. van Klingeren	49
R385	20	0.1	R. Michaels	
Transconjugants				
Rd 77-1040 M5	40	50		
Rd 76-81739 M4	50	50		
MAP 76-79268 TC30	40	50		
Rd C435 M5	20	30		
Transformants <sup>f</sup>				
TF 77-1040-8	10	20		
TF 76-81739-7	10	20		
TF 76-79268-1	10	20		
TF C435-2	10	20		

<sup>a</sup> MIC determinations by agar dilution; 10<sup>5</sup> CFU inoculum.

<sup>b</sup> 3-Fluoro derivative of chloramphenicol.

<sup>c</sup> C. Thornsberry; Centers for Disease Control, Atlanta, Ga.; M. Roberts; University of Washington, Seattle; B. Catlin, Medical College of Wisconsin, Milwaukee; B. van Klingeren, National Institute of Public Health, The Netherlands; R. Michaels, University of Pittsburgh, Pittsburgh, Pa.

<sup>d</sup> Chloramphenicol-resistant clinical isolates, non-CAT producers by rapid assay.

<sup>e</sup> Children's Hospital Medical Center, Boston, Mass.

<sup>f</sup> Strain MAP was the recipient used in all transformations.

were obtained from New England Nuclear Corp., Boston, Mass. Purified *Escherichia coli* CAT (specific activity, 400 U/ml) was purchased from P-L Biochemicals, Milwaukee, Wis. The nick translation kit was obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Md. Nitrocellulose filters were obtained from Schleicher & Schuell, Inc., Keene, N.H. Chemicals, salts, and buffers were of the purest form available from Mallinckrodt, Inc., St. Louis, Mo., and J. T. Baker Chemical Co., Phillipsburg, N.J.

**Determinations of MICs.** MICs were determined by agar dilutions with a Steers replicator (48). Solid medium was supplemented with 0.1 to 100  $\mu\text{g}$  of chloramphenicol or SCH 24893 per ml. An inoculum of 10<sup>5</sup> CFU was used. Amino acid requirements were determined at the same time as MICs with Catlin agar (5).

**Assays for CAT activity.** Strains were initially screened for CAT production by the rapid visual detection method of Azemun et al. (3). Assays were performed in duplicate; a chloramphenicol-susceptible strain lacking CAT activity and a CAT-producing strain were used as controls. CAT activity was assayed both spectrophotometrically and by a radioenzymatic method in cell sonic extracts of the chloramphenicol-resistant strains identified as lacking CAT activity by the rapid test. In addition to the clinical isolates, other strains assayed included: E<sub>1</sub>, a chloramphenicol-susceptible *H. influenzae* strain; R385, a chloramphenicol-resistant, CAT-producing *H. influenzae* strain; and W677/HCMR5, a chloramphenicol-resistant, CAT-producing *E. coli* strain (44). To determine the sensitivity of each method, commercial *E. coli* CAT was assayed with both systems.

Cell extracts were made from organisms grown to mid-log phase (optical density at 490 nm, 0.2) in 20 ml of broth. The cells were harvested, washed once, and suspended in 10 ml of 0.05 M Tris-hydrochloride (pH 7.8) at 4°C. Each cell suspension was sonified, and the resultant cell extracts were

aliquoted and stored at -70°C; one aliquot was used for a protein determination by the method of Lowry et al. (25), using crystalline bovine serum albumin as a standard.

The spectrophotometric assay quantitates CAT activity by detection of the reaction of reduced CoA with 5,5'-dithiois-2-nitrobenzoic acid yielding free 5-thio-2-nitrobenzoate with a millimolar extinction coefficient of 13.6 at 412 nm (42). Cell sonic extract of each strain (50  $\mu\text{l}$ , containing 5.2 to 12.0  $\mu\text{g}$  of protein per ml) was assayed spectrophotometrically for CAT activity by the method of Shaw (42). To determine the nonenzymatic rate of reaction, control cuvettes lacked cell extract (enzyme source) or chloramphenicol, or both.

An assay for direct measurement of acetylation of chloramphenicol by [<sup>14</sup>C]acetyl-CoA, a highly sensitive method of quantitating CAT activity, was modified from the technique of Shaw (42). The reaction volume contained 8  $\mu\text{l}$  of 100 mM ATP, 50  $\mu\text{l}$  of 20 mM phosphoenolpyruvate, 20  $\mu\text{l}$  of 5 mM acetyl-CoA (reduced, lithium salt), 15  $\mu\text{l}$  of 100 mM MgCl<sub>2</sub>, 25  $\mu\text{l}$  of 1 M Tris-hydrochloride (pH 7.8), 10  $\mu\text{l}$  of 10 mM [<sup>14</sup>C]sodium acetate, 30  $\mu\text{l}$  of acetate kinase (8 U), 20  $\mu\text{l}$  of pyruvate kinase (8.8 U), 20  $\mu\text{l}$  of phosphotransacetylase (10 U), 25  $\mu\text{l}$  of deionized water, and 25  $\mu\text{l}$  of the cell sonic extract to be assayed. The reaction was initiated by the addition of 5  $\mu\text{l}$  of 5 mM chloramphenicol. After 20 min of incubation at 37°C, the reaction was terminated by the addition of 25  $\mu\text{l}$  of 1.1 M sodium arsenate. The product was extracted twice with 2 ml of benzene; the extracts were pooled, combined with 100  $\mu\text{l}$  of glacial acetic acid, and evaporated overnight in a water bath at 50°C. Toluene-based scintillant was added, and <sup>14</sup>C radioactivity was quantitated by scintillation spectrometry with a Packard Tricarb spectrometer. By this method, commercially available *E. coli* CAT was detectable when diluted to 0.0125 U/25  $\mu\text{l}$ . Control reactions did not include the enzyme source or chloramphenicol, or both.

Inducibility of CAT activity was sought by both the spectrophotometric assay and the radioenzymatic assay. Each strain was grown separately in broth culture in the presence of sub-MICs of chloramphenicol (2 µg/ml), chloramphenicol base (10 µg/ml), and SCH 24893 (1 µg/ml for the clinical isolates, 0.2 µg/ml for strain R385, 0.05 µg/ml for *E. coli* W677/HCMR5). Potential induction of CAT activity in strain E<sub>1</sub> with SCH 24893 was not determined.

**Conjugation.** Overnight filter paper matings were performed as described by Roberts et al. (40) in the presence of 1.5 mg of DNase I. Each chloramphenicol-resistant clinical isolate was mated with the recipient strains MAP and RdMCR. Transconjugants were selected on sBHI agar with 2.5 and 5 µg of chloramphenicol and 250 µg of streptomycin or 10 µg of rifampin per ml. In addition, transconjugants of strains 77-1040 and 76-79268 were selected on media containing 5 µg of ampicillin and 10 µg of rifampin per ml. The control strain, HC234, was mated with the same recipients 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 initially plated. Colony counts were determined on antibiotic-free media. Each recipient was tested as described above for the frequency of spontaneous mutation to chloramphenicol resistance.

**Transformation.** Whole-cell lysates containing DNA of each of the four chloramphenicol-resistant, CAT-negative clinical isolates and their transconjugants were prepared as described by Roberts et al. (39). The recipient strain, MAP, was made competent by the method of Herriott et al. (17) with the nongrowth medium M-IV. Chloramphenicol-resistant transformants were selected by plating suspensions diluted 10<sup>-1</sup> and 10<sup>-2</sup> on sBHI agar containing 2.5 or 5 µg of chloramphenicol per ml. 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 mutation frequency to chloramphenicol resistance by plating cells without the addition of DNA.

The phenotype of the transformant colonies was confirmed by reisolation on sBHI agar plates containing 2.5 or 5 µg of chloramphenicol per ml. In addition, the phenotype of the recipient, MAP, was confirmed by plating the putative transformants on media containing 50 µg of spectinomycin per ml and 250 µg of streptomycin per ml. The MIC of chloramphenicol was determined for representative transformants and transconjugants. As a control, known chromosomal antibiotic resistance markers were transformed into an antibiotic-susceptible strain (27).

In the studies which follow, the isogenic comparison of the susceptible recipient with a resistant transformant from each of the four non-CAT-producing clinical isolates was performed.

**Agarose gel electrophoresis.** DNA lysates of the chloramphenicol-resistant clinical isolates and their transconjugants and transformants were prepared as described by Meyers et al. (28). These strains were also lysed by the procedures of Hansen and Olsen (16) and Holmes and Quigley (18) to increase the frequency of detection of plasmid DNA. 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 (28).

**Hybridization.** Two R-plasmids with different resistance determinants were chosen as probes: pRI234, a 38-mega-

dalton conjugative plasmid which codes for tetracycline resistance and CAT production, and RSF0885, a 3.6-megadalton nonconjugative plasmid which codes for β-lactamase production (10, 49). Plasmid isolation was performed by cesium chloride gradient ultracentrifugation (11). The plasmid DNAs were nick translated with [<sup>35</sup>S]dATP according to the manufacturer's guidelines, a modification of the procedure of Rigby et al. (37). 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 approximately 10<sup>7</sup> cpm/µg of DNA, and 10<sup>6</sup> cpm were used for each hybridization. Unlabeled whole-cell DNA from the chloramphenicol-resistant clinical isolates and their corresponding chloramphenicol-resistant transformants (in strain MAP) was prepared by the lysis technique of Meyers et al. (28). 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 (47) as modified by Smith and Summers for bidirectional blotting (46). The unlabeled purified DNA of the two plasmid probes as well as a third plasmid, RSF007, known to encode β-lactamase production (10), was electrophoresed on the same gel before blotting. [<sup>35</sup>S]-labeled probes RSF0885 and pRI234 were hybridized separately with these identical blots for 17 h at 42°C as described by Wahl et al. (52). The filters were exposed to Kodak XAR 5 X-ray film, incubated at -70°C for 3 weeks, and developed.

**Bioassay for enzymatic modification of chloramphenicol.** A technique developed by Slack et al. (43) was modified as an assay for inactivation of chloramphenicol by *H. influenzae*. A lawn of susceptible *E. coli* was streaked on an sBHI plate. Disks of Whatman no. 1 filter paper, 8.5 mm in diameter, were placed on the *E. coli* lawn. A heavy inoculum of the *H. influenzae* strain to be tested was applied evenly over the surface of the filter paper, and a 30-µg chloramphenicol disk was placed centrally on the inoculated filter paper. The plates were incubated overnight, and the zone of inhibition measured. The chloramphenicol must pass unmodified through the filter paper inoculated with the *H. influenzae* to inhibit the growth of the *E. coli*. The zone size is reduced or absent if the chloramphenicol is inactivated by the organism on the filter paper.

**Thin-layer chromatography.** Organisms were grown to mid-log phase (~5 × 10<sup>8</sup> CFU/ml), harvested by centrifugation, washed twice in 2 M Tris-hydrochloride (pH 7.8), and concentrated 20-fold. Cells were sonified in an ice bath with a Heat Systems-Ultrasonic cell disruptor with a tapered microtip in four 20-s bursts at 45 W output. Cell debris was pelleted and discarded; the resultant supernatant was stored at -20°C. Equal volumes of cell sonicates, 2.5 mM [<sup>14</sup>C]chloramphenicol, 2 M Tris-hydrochloride (pH 7.8), and 1 mM S-adenosylmethionine, 1 mM ATP, or 1 mM acetyl-S-CoA were combined and incubated at 37°C for 60 min. A 5-µl sample was spotted on a silica-gel chromatography plate (Kodak) which was developed in a solvent system consisting of chloroform-methanol (4:1). The plates were placed against Kodak XAR 5 X-ray film and incubated for 48 h at -70°C. The autoradiographs were developed, and R<sub>f</sub> values were determined for the radioactive compounds.

**Protein synthesis. (i) Intact organisms.** Organisms were grown to early log phase (optical density at 490 nm, 0.1, with a Lumetron C colorimeter) in 20 ml of sBHI broth, harvested by centrifugation, washed once, and suspended in 0.3 ml of buffer A (10 mM Tris-hydrochloride [pH 8.0], 10 mM magnesium acetate, 60 mM ammonium

chloride, 6 mM 2-mercaptoethanol). Cells (30  $\mu$ l) were preincubated for 15 min at 37°C with chloramphenicol ranging in concentrations from 0 to 500  $\mu$ g/ml. To facilitate comparison with S30 fractions and ether-extracted cells, this was added to a 100- $\mu$ l reaction mixture containing 66 mM Tris, 9 mM ammonium chloride, 2.6 mM ATP, 6  $\mu$ M GTP, 8 mM phosphoenolpyruvate, 2  $\mu$ g of pyruvate kinase, 10 mM 2-mercaptoethanol, 3 mM dithiothreitol, 0.5 mM each of 19 amino acids (excluding valine), and 4 mM [<sup>14</sup>C]valine (specific activity, 50 mCi/mM). After a 30-min incubation at 37°C, the reaction mixture was spotted onto filter papers (Whatman no. 3). The filters were collected in cold 10% trichloroacetic acid (TCA), washed twice in 5% TCA, heated to 90°C for 15 min in 5% TCA, and washed once with ethanol-ether (1:1 at 37°C) and once with 100% diethyl ether. The filters were dried, and the entrapped radioactivity was quantified with a Packard Tricarb scintillation spectrometer.

(b) **Ether-extracted cells.** Bacterial cells were made permeable to macromolecules by a modification of the technique of Vosberg and Hoffman-Berling (51). Organisms were grown to mid-log phase in 300 ml of sBHI broth, harvested by centrifugation, washed once, and suspended in 10 ml of buffer A. The cells were extracted with an equal volume of diethyl ether for 1 min. After separation of aqueous and organic phases, the ether was removed by pipetting. The ether-extracted cells were harvested by centrifugation at 10,000  $\times g$  for 10 min, washed, and resuspended in 2 ml of buffer A. The cells were stored frozen at -70°C in 150- $\mu$ l aliquots. Protein synthesis was measured as described above for intact cells with the exception that there was no preincubation period with chloramphenicol.

(ii) **S30 fraction.** S30 fractions were prepared by a modification of the method of Davies (8). Organisms were grown in 3 liters of sBHI broth, harvested by centrifugation, and washed once. The cell pellet was ground with twice its weight of alumina (Sigma) at 4°C for 5 min and extracted with buffer A (1 ml/g of cells). Supernatants were prepared by two-step centrifugation at 4°C: 10 min at 15,000  $\times g$  and then 30 min at 30,000  $\times g$ . After the first spin, DNase (0.5 mg/ml; Worthington Diagnostics, Freehold, N.J.) was added. The S30 fractions were dialyzed overnight against 100 volumes of buffer A, aliquoted, and stored at -70°C. The protein synthesis system used was identical to that for ether-extracted cells with two exceptions: the specific activity of the [<sup>14</sup>C]valine was 280 mCi/mM, and 50  $\mu$ g of tRNA (*E. coli* K-12; Calbiochem-Behring, La Jolla, Calif.) was added to the reaction mixture.

**Chloramphenicol uptake.** Organisms were grown to mid-log phase ( $\sim 5 \times 10^8$  CFU/ml) in sBHI broth. Organisms in broth (5 ml) were aliquoted to individual Erlenmeyer flasks and chloramphenicol was added to each, resulting in a final concentration of 5, 10, or 20  $\mu$ g/ml. The flasks were incubated with shaking at 37°C, and 500  $\mu$ l was sampled at 0, 15, 30, and 60 min. Colony counts were performed at 0 and 60 min. The samples were immediately placed on ice and then centrifuged at 12,000  $\times g$  at 4°C for 5 min. Chloramphenicol loss from growth medium (i.e., uptake into bacteria) was assayed by high-pressure liquid chromatographic quantitation of the chloramphenicol concentration in the resultant supernatant. The high-pressure liquid chromatography technique was a modification of the method of Weber et al. (54). Supernatant (50  $\mu$ l) was combined with 50  $\mu$ l of internal standard (*p*-nitropropionanilide in acetonitrile, 25  $\mu$ g/ml), 20  $\mu$ l was injected onto a C-18  $\mu$ -Bondapak cartridge (Waters, Milford, Mass.), and the compound was eluted with a mobile phase consisting of 43.5% methanol in 0.02 M acetate buffer

(pH 3.5). The absorbance was monitored at 254 and 280 nm, and chloramphenicol was identified by its 254/280 ratio,  $0.55 \pm 0.06$ . Chloramphenicol concentration was calculated by linear regression from its absorbance at 280 nm compared with the internal standard.

**Outer membrane proteins.** Outer membrane proteins were extracted by a modification of the method of McDade and Johnston (26). Organisms were grown to mid-log phase in 300 ml of sBHI broth and harvested by centrifugation. The cell pellet was suspended in 20 ml of 200 mM lithium chloride-100 mM lithium acetate (pH 6.0). The suspension was incubated with 2 g of glass beads for 2 h at 45°C with shaking at 300 rpm. Cell debris was pelleted, and the supernatant was ultracentrifuged at 225,000  $\times g$  for 2 h. The pellet was washed and suspended in sterile glass-distilled water and stored at -70°C. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a 10% running gel and visualized by ultrasensitive silver staining as described by Sammons et al. (41).

## RESULTS

**MICs.** The MICs for all of the strains used are listed in Table 1. The clinical isolates and their putative transconjugants had similar MICs of chloramphenicol, suggesting that the chloramphenicol resistance marker was fully transferred. However, the MICs for the resistant transformants were lower, suggesting only partial transfer. Strains 77-1040, 76-81739, and 76-79268 are prototrophic; C435 requires methionine for growth. Tetracycline resistance in three of the four clinical isolates could be transferred by transformation. Susceptibility to  $\beta$ -lactams was not examined in the transformants.

**Conjugation.** Strains 77-1040 and 76-79268 effectively transferred the ampicillin resistance phenotype and  $\beta$ -lactamase production to the recipient strains at a frequency of  $10^{-6}$ . All four clinical isolates transferred the chloramphenicol resistance phenotype at a frequency of  $10^{-9}$  per donor cell. There was no detectable mutation frequency to chloramphenicol resistance with the recipient strains ( $<10^{-10}$ ). In addition, all putative transconjugants harbored the other antibiotic resistance genes of the recipient. The control R<sup>+</sup> strain, HC234, had a conjugation frequency of  $10^{-4}$  per donor cell for both recipients.

**Transformation.** DNA from all four clinical isolates was unable to transform the susceptible recipients to chloramphenicol resistance. In contrast, DNA of the putative transconjugant of each strain was capable of transforming the recipient strain but at a frequency of only  $10^{-9}$  per recipient cell. Again, the spontaneous mutation frequency to chloramphenicol resistance for the recipient strain was  $<10^{-10}$ .

**Agarose gel electrophoresis.** None of the four clinical isolates contained visually detectable extrachromosomal DNA when prepared by any of the three lysis techniques. However, transconjugants of 77-1040 and 76-79268, the ampicillin-resistant,  $\beta$ -lactamase-producing strains, did contain visible plasmid DNA when strains were selected on ampicillin-supplemented media. Putative transconjugants and transformants selected for chloramphenicol resistance harbored no detectable extrachromosomal DNA.

**Hybridization.** Figure 1 shows the results of the Southern hybridization of DNA from the clinical isolates and their transformants. Both [<sup>35</sup>S]-labeled plasmid probes, pRI234 and RSF0885, had DNA homology with the positive control strain, R385 (data not shown) (27), and three of the four clinical isolates; only strain 76-81739 had no DNA homology. In contrast, the chloramphenicol-resistant transform-

ants of all four clinical isolates and the recipient strain, MAP (data not shown) (27), had no DNA homology with either probe.

**CAT assay.** The chloramphenicol-susceptible strain, MAP, and the four resistant clinical isolates, 77-1040, 76-81739, 76-79268, and C435, all lacked detectable CAT activity by the rapid visual assay. Strain HC234 contained CAT activity as previously reported (40).

In the spectrophotometric assay, the four chloramphenicol-resistant strains lacking CAT activity by the rapid assay did not have detectable chloramphenicol-dependent hydrolysis of acetyl-CoA. Both with and without induction, in all four clinical isolates and strain E<sub>1</sub>, activity was less than 0.06  $\mu\text{mol}/\text{min}$  per mg protein. Extracts of strain R385, a known CAT producer, had a specific activity of 0.2  $\mu\text{mol}/\text{min}$  per mg of protein, and extracts of the CAT-producing *E. coli* had a specific activity of 1.9  $\mu\text{mol}/\text{min}$  per mg of protein.

The results of the radioenzymatic CAT assay on induced and uninduced cell extracts are shown in Table 2. Incorporation of [<sup>14</sup>C]acetate into the benzene-extractable chloramphenicol was 3 to 4 logs greater by extracts of *H. influenzae*

TABLE 2. Radioenzymatic assay for CAT activity

Strain	CAT activity (cpm) of strain preincubated with:			
	Uninduced	Chloramphenicol	Chloramphenicol base	SCH 24893
77-1040	456	446	391	408
76-81739	464	430	376	552
76-79268	413	392	400	340
C435	481	501	463	582
E <sub>1</sub>	270	310	ND <sup>a</sup>	ND
R385	217,391	151,515	128,205	114,943
W677/HCMR5	1,789,264	2,027,027	1,761,252	1,768,173

<sup>a</sup> ND, Not done.

R385 and *E. coli* W677/HCMR5 than by strain E<sub>1</sub> and the chloramphenicol-resistant strains under investigation. There was no detectable induction of CAT activity by the addition of sub-MICs of chloramphenicol, chloramphenicol base, or SCH 24893.

**Bioassay.** Strain HC234, the CAT-producing control, inactivated the chloramphenicol from the antibiotic disk, permitting the *E. coli* to grow to the edge of the filter paper (8.5 mm). The susceptible strain, MAP, and the four resistant CAT-negative strains had zones of inhibition equivalent to that seen in the absence of bacteria on the filter paper.

**Thin-layer chromatography.** An autoradiograph of the thin-layer chromatogram for two of the transformants and controls is shown in Fig. 2. The mobility of [<sup>14</sup>C]chloramphenicol was changed only by incubation in cell extracts of the strain producing CAT. The acetylated product seen in HC234, a known CAT-producing organism, had a mobility identical to that produced by purified *E. coli* CAT (data not shown). None of the resistant transformants showed evidence of chemical alteration of chloramphenicol as indicated by mobility in this thin-layer chromatography system (data not shown for TF 77-1040-8 or TF 76-79268-1).

**Whole-cell protein synthesis.** The effect of chloramphenicol on protein synthesis in intact cells, comparing the susceptible recipient with the four resistant transformants, is shown in Fig. 3. All five strains showed a decrease in protein synthesis with increasing antibiotic concentrations; strain MAP appeared to be more sensitive to inhibition by chloramphenicol, which corresponds with its lower MIC.

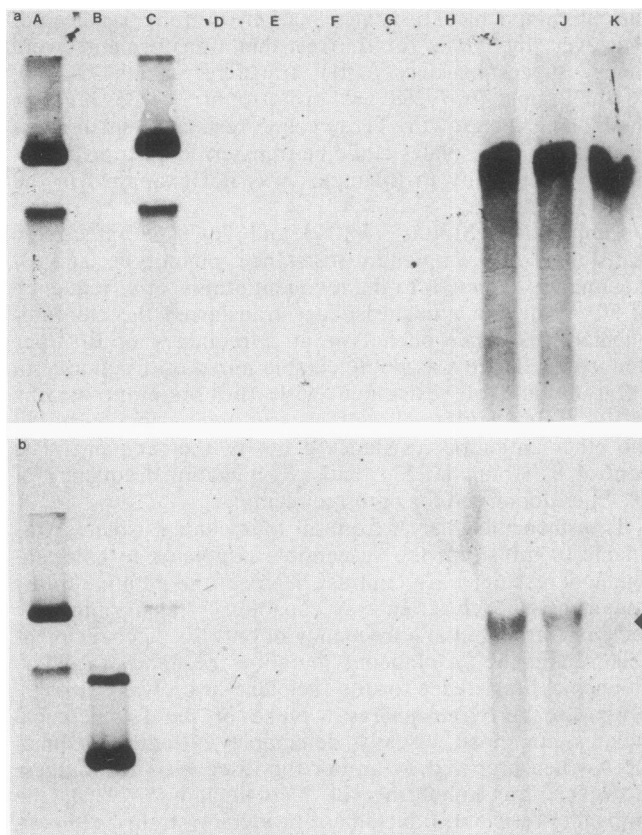


FIG. 1. Southern hybridization of DNA from non-CAT-producing, chloramphenicol-resistant clinical isolates and transformants. (a) Probe used was <sup>35</sup>S-labeled plasmid pRI234; (b) probe used was <sup>35</sup>S-labeled plasmid RSF0885. Lanes represent DNA from: A, plasmid RSF007; B, plasmid RSF0885; C, plasmid pRI234; D, TF 77-1040-8; E, TF C435-2; F, TF 76-79268-1; G, TF 76-81739-7; H, 76-81739; I, 76-79268; J, 77-1040; K, C435. Lanes A, B, and C contain cesium chloride-purified plasmid DNA; the less dense bands represent open circular or linear forms of plasmid DNA. Lanes D through K contain DNA prepared by the method of Meyers et al. (28). The arrow indicates perceptible extrachromosomal DNA. Control hybridizations with strains MAP and R385 are not shown.

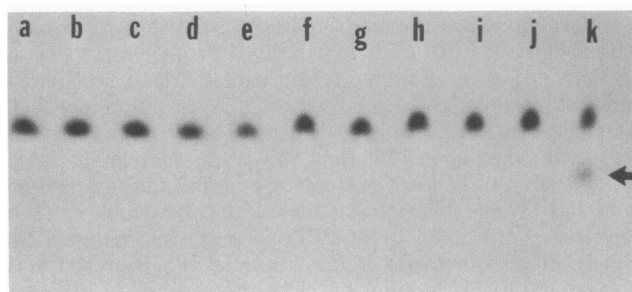


FIG. 2. Autoradiograph of thin-layer chromatogram of [<sup>14</sup>C]chloramphenicol incubated with cell extracts of MAP (lane b), TF 76-81739-7 (lanes c through e), TF C435-2 (lanes f through h), and HC234 (lanes i through k). Lane a is [<sup>14</sup>C]chloramphenicol in buffer. Lanes c, f, and i contain 1 mM S-adenosylmethionine. Lanes d, g, and j contain 1 mM ATP. Lanes e, h, and k contain 1 mM acetyl-CoA. The arrow points to acetylated [<sup>14</sup>C]chloramphenicol seen in the incubation products of the cell extract of HC234 plus acetyl-CoA.

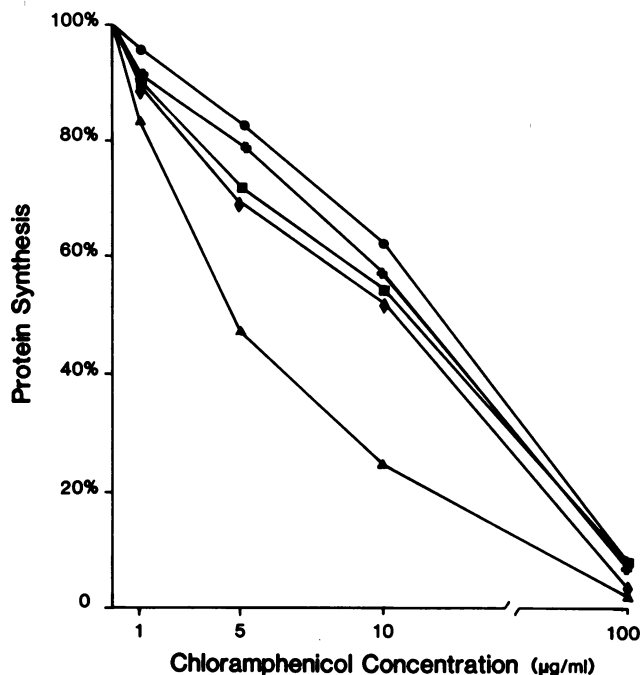


FIG. 3. Effect of chloramphenicol on protein synthesis in intact cells. Symbols: ▲, MAP; ■, TF 77-1040-8; ◆, TF 76-81739-7; ◐, TF 76-79268-1; ●, TF C435-2. Chloramphenicol concentration was increased from 0 to 100 µg/ml. Protein synthesis was quantitated by the incorporation of [<sup>14</sup>C]valine into TCA-precipitable material. One hundred percent protein synthesis (after subtracting background counts) represented 2,825 to 10,644 cpm.

**In vitro protein synthesis.** Low incorporation of [<sup>14</sup>C]valine was found with S30 fractions from *H. influenzae* in the absence of an antibiotic: maximum incorporation was 165 to 1,315 cpm. This made unreliable the comparison of the inhibition of cell-free protein synthesis by chloramphenicol in strain MAP and the resistant transformants.

A second technique for in vitro protein synthesis was employed with nonviable ether-extracted cells. The results of this technique are shown in Fig. 4. Again, the chloramphenicol-resistant transformants are compared with the isogenic susceptible strain, MAP; there was no significant difference in the amount of inhibition of incorporation of [<sup>14</sup>C]valine by increasing concentrations of chloramphenicol.

**Chloramphenicol uptake.** The uptake of chloramphenicol by strain MAP and the four resistant transformants is shown in Fig. 5. The number of CFU per ml remained constant over the 60-min uptake period independent of chloramphenicol concentration. To allow for slight variations in the initial antibiotic concentration and to enable direct comparison between strains, the initial chloramphenicol concentration in the culture medium was standardized to equal 100%. At all three chloramphenicol concentrations used, the resistant strains had a decreased rate of uptake.

**Outer membrane proteins.** Figure 6 is a silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel of outer membrane proteins obtained by lithium extraction of MAP and the isogenic chloramphenicol-resistant transformants. The only detectable difference between the CAT-negative transformants and the susceptible strain is markedly diminished quantities of a major outer membrane protein with an apparent molecular weight of 40,000.

## DISCUSSION

Chloramphenicol resistance in *H. influenzae*, other than plasmid-mediated CAT production, has not been previously described. Alternative mechanisms of chloramphenicol resistance that have been reported in other bacterial strains include alterations of the target site and a permeability barrier to the antibiotic (9).

Chloramphenicol inhibits bacterial protein synthesis by binding to the 50S ribosomal subunit (32). Alterations of ribosomal proteins and a decrease in their ability to bind chloramphenicol have been described by Osawa et al. (34) in chloramphenicol-resistant mutants of *Bacillus subtilis*. In both *B. subtilis* and *E. coli*, chloramphenicol resistance mutations that map in the ribosomal protein region have been described, although neither strain had a detectable alteration of ribosomal sensitivity to chloramphenicol (2, 4).

A permeability barrier to chloramphenicol is a more frequently reported mechanism of resistance. Both R-plasmid- and chromosomally-mediated resistance described in *E. coli* and in *Pseudomonas aeruginosa* is thought to operate via decreased uptake of chloramphenicol.

A plasmid-mediated barrier to chloramphenicol uptake was hypothesized by Nagai and Mitsuhashi (30) in a strain which did not inactivate chloramphenicol and in which cell-free protein synthesis was inhibited by the antibiotic.

Gaffney et al. (15) reported two plasmids from incompatibility groups P-1 and C which conferred chloramphenicol resistance in *E. coli*; a cytoplasmic membrane-located barrier was proposed. There was no evidence of inactivation of the antibiotic, in vitro protein synthesis was inhibited by chloramphenicol, and spheroplasts expressed the same level of resistance as whole cells. In addition, cells were con-

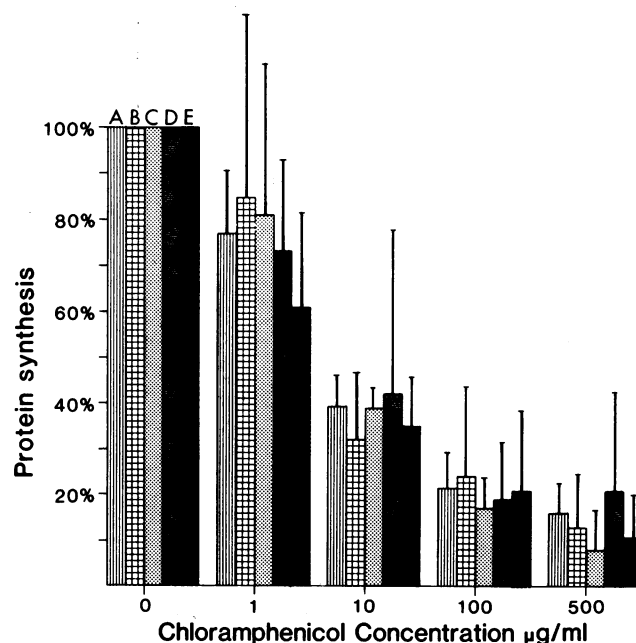


FIG. 4. Effect of chloramphenicol on protein synthesis with ether-extracted cells. A, MAP; B, TF 77-1040-8; C, TF 76-81739-7; D, TF 76-79268-1; E, TF C435-2. Chloramphenicol concentration ranged from 0 to 500 µg/ml. Protein synthesis was quantitated by the incorporation of [<sup>14</sup>C]valine into material precipitated by cold 10% TCA. One hundred percent protein synthesis (after subtraction of background counts) represented 1,000 to 5,010 cpm.



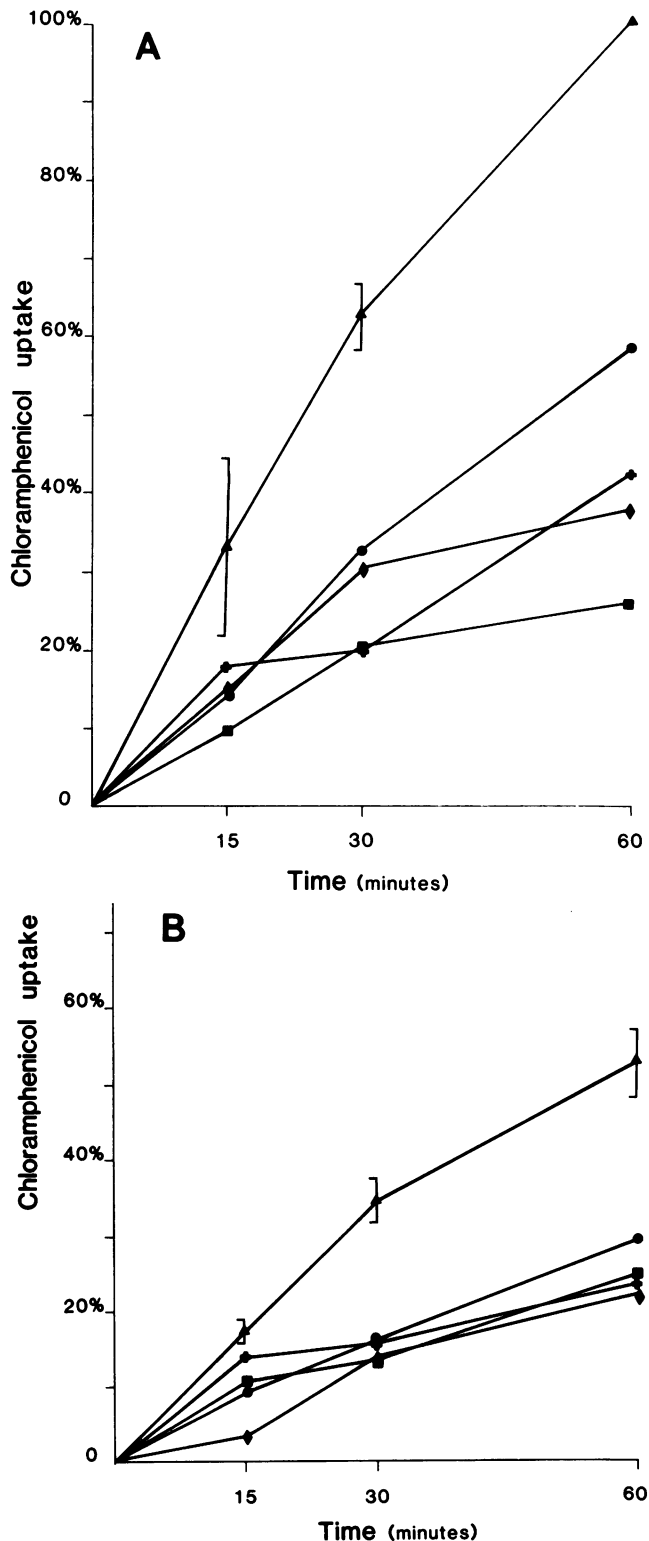


FIG. 5. Uptake of chloramphenicol by bacterial cells. Symbols: ▲, MAP; ■, TF 77-1040-8; ◆, TF 76-81739-7; ◆, TF 76-79268-1; ●, TF C435-2. The amount of uptake was calculated by high-pressure liquid chromatographic quantitation of loss from culture medium. Initial chloramphenicol concentration in sBHI broth equals 100%. For strain MAP the graph shows the mean  $\pm$  the standard deviation. (A) Initial chloramphenicol concentration, 5  $\mu$ g/ml. (B) Initial chloramphenicol concentration, 20  $\mu$ g/ml. Data for initial chloramphenicol concentration of 10  $\mu$ g/ml is not shown.

structed which encoded for intracellular CAT production; chloramphenicol in the culture media was not inactivated by these cells.

An R-factor-mediated permeability barrier to chloramphenicol has also been described in *P. aeruginosa*. Mitsuhashi et al. (29) have reported decreased penetration of [ $^{14}$ C]chloramphenicol into strains of *P. aeruginosa* containing four different plasmids. An R-factor (kR102) derived from a clinical *P. aeruginosa* isolate and encoding resistance to chloramphenicol and four other antibiotics has been described by Kono and O'Hara (22); the plasmid did not encode for CAT production and had no effect on the incorporation of [ $^{14}$ C]valine into TCA-precipitable material.

Reeve (35, 36) described two single-step *E. coli* K-12 mutants to chloramphenicol resistance (*cmlA* and *cmlB*) which are also resistant to tetracycline. The *cmlB* mutant was further characterized by Foster (14) who proposed an altered permeability to both chloramphenicol and tetracycline because of a threefold-lower rate of accumulation of tetracycline. Chopra and Eccles (7) found the *cmlB* mutant to have a major change in outer membrane proteins with the loss of a 38,000-dalton protein (*ompF*) which has subsequently been shown to be a porin protein (31).

In addition, Irvin and Ingram (19) reported high-level-chloramphenicol-resistant variants of *P. aeruginosa* which

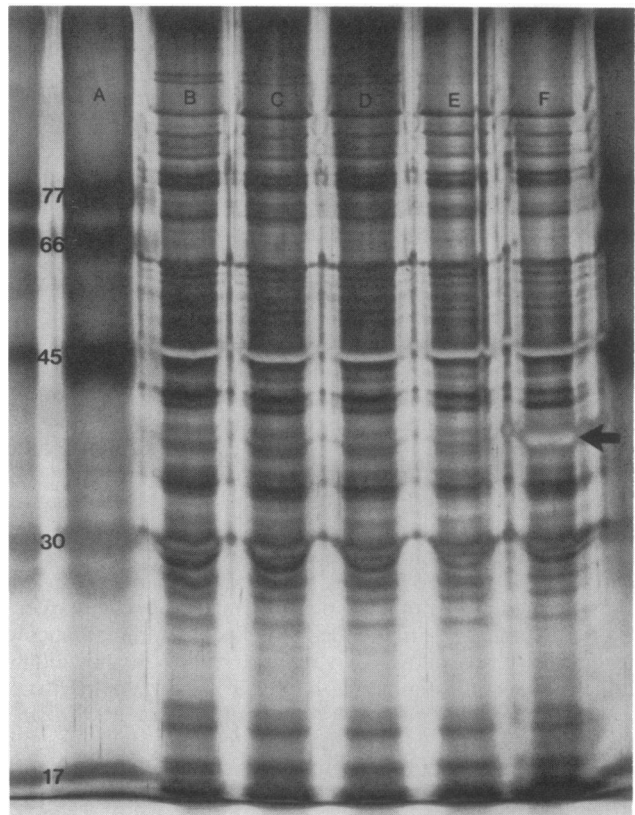


FIG. 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of outer membrane proteins from lithium-treated cells. The protein bands are visualized by silver staining. Lanes: A, molecular weight standards (LKB Instruments, Inc., Bromma, Sweden); numbers to the left indicate apparent molecular weight in thousands; B, TF 77-1040-8; C, TF 76-81739-7; D, TF 76-79268-1; E, TF C435-2; F, MAP. Arrow designates apparent 40,000-dalton protein visualized in MAP and not evident in the isogenic transformants.

exhibited a reduced ability to accumulate certain amino acids, suggesting an alteration in membrane permeability or a resultant transport defect.

We described four clinical isolates of chloramphenicol-resistant *H. influenzae* in which the resistance determinant appeared to be chromosomally mediated. None of the four clinical strains contained visibly detectable extrachromosomal DNA. In addition, after conjugation experiments between these strains and a chloramphenicol-susceptible recipient, the resultant chloramphenicol-resistant transconjugants contained no detectable extrachromosomal DNA. The transfer of chromosomal genes by cell-to-cell contact has recently been demonstrated in *H. influenzae* by Albritton et al. (1). They demonstrated a frequency of transfer of chromosomal markers during filter matings on agar of between  $10^{-6}$  and  $10^{-9}$ . Our transfer frequency of the chloramphenicol resistance marker of  $10^{-9}$  per donor cell is consistent with this mode of nonclassical transformation.

Transformation with donor DNA from the transconjugant strains resulted in chloramphenicol-resistant transformants which did not contain visibly detectable extrachromosomal DNA. Although this is an unusual method of obtaining isogenic transformants, it was necessitated by the apparent low frequency of transformation ( $<10^{-10}$ ) between these clinical isolates and the susceptible recipient strains perhaps due to restriction modification. Once the chloramphenicol resistance marker had been transferred to RdMCR or MAP by cell-to-cell contact, transformation into MAP (a genetically related strain) was possible at higher frequency.

Significant DNA homology is known to exist between R-plasmids in *H. influenzae* (10, 12, 21, 23, 24). To further confirm our hypothesis of a chromosomal location for the chloramphenicol resistance marker in these strains, we selected two well-characterized plasmids, pRI234 and RSF0885, as DNA probes for R-plasmid sequences in the four clinical isolates and their transformants. Data from hybridization experiments showed significant DNA homology with three of the four clinical isolates (including the two  $\beta$ -lactamase-producing strains) but no homology with any of the four chloramphenicol-resistant transformants. These data are consistent with a non-plasmid-mediated mechanism of chloramphenicol resistance in these strains.

During investigation of the resistance mechanism in these strains, we found no evidence of either constitutive or inducible acetylating enzymes and demonstrated, using thin-layer chromatography and a bioassay, that these strains did not inactivate chloramphenicol by mechanisms requiring ATP, S-adenosylmethionine, or acetyl-CoA.

We found no alteration of ribosomal susceptibility to inhibition by chloramphenicol. Because the standard S30 ribosomal preparation used in *E. coli* is inefficient in *H. influenzae* with minimal incorporation of [ $^{14}$ C]valine into TCA-insoluble material, we used a second technique for in vitro protein synthesis that yielded much greater incorporation of  $^{14}$ C-amino acids. Both of these methods showed the ribosomes of the resistant transformants to be equally inhibited by chloramphenicol compared with an isogenic susceptible strain.

Chloramphenicol uptake by microorganisms is difficult to assay because the antibiotic is weakly bound to bacterial ribosomes and readily removed by washing procedures (50). We developed a technique to quantify uptake by measuring chloramphenicol loss from culture media over time. Using high-pressure liquid chromatographic quantitation, we demonstrated a decreased uptake of chloramphenicol in the resistant transformants. In addition, these strains appear to lack a 40,000-dalton major outer membrane protein.

We propose that the mechanism of chloramphenicol resistance in these strains is a chromosomally mediated permeability barrier related to the loss of a porin protein. Thus, the resistance mechanism in these strains of *H. influenzae* closely parallels that described by Chopra and Eccles (7) in the *cmlB* mutant of *E. coli* with the exception that it arose in vivo.

The finding that the clinical isolates are more resistant to chloramphenicol than the transformant strains suggests that resistance transfer was incomplete. This may indicate that several genes which are not closely linked are involved in chloramphenicol resistance in these strains. The absence of a purported porin protein seen in the transformants might represent only a portion of the permeability barrier in the wild-type strains. We are pursuing experiments to more fully characterize chloramphenicol uptake in *H. influenzae*, to determine whether it is an active or passive process, and to determine the role of porins in the transport of chloramphenicol.

#### ACKNOWLEDGMENTS

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