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Plasmid-Mediated Chloramphenicol Resistance in *Haemophilus influenzae*

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A clinical isolate of *Haemophilus influenzae* HC234 was found to be resistant to chloramphenicol and tetracycline. It was shown that both resistance markers are tranferable as one unit to other *Haemophilus influenzae* strains and also to *Escherichia coli*. Data are presented which indicate that conjugation is the most likely mechanism of resistance transfer. HC234 was shown to carry a single plasmid species with a molecular weight of 38×10^6.

Recent investigations have shown the existence of plasmid-mediated drug resistance in *Haemophilus influenzae* (2, 3, 5, 17). Transfer of resistance, probably by conjugation, was demonstrated for resistance to ampicillin (13, 15) and for resistance to kanamycin (3). Recently, we reported on a chloramphenicol (Cm)-resistant strain of *H. influenzae* isolated from the pharynx of a patient with lymphatic leukemia (10). The resistance to Cm appeared to be due to the production of an enzyme, presumably an acetyltransferase, inactivating this antibiotic. The strain was also resistant to tetracycline (Tc).

This report describes experiments on the transfer of the resistance determinants to susceptible strains of *H. influenzae* and to a strain of *Escherichia coli*. Also, the isolation and characterization of plasmid deoxyribonucleic acid (DNA) from the resistant strain are reported.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are listed in Table 1. The rifampin- and streptomycin-resistant mutant strains HC215-1, HC217-1, and HC218-1 were obtained by selection on plates containing these antibiotics. All strains of *H. influenzae* investigated in this study were untypable according to Pittman's agglutination method (12). The strain of *E. coli* K-12 (SC181) was kindly provided by E. Lederberg (Stanford University).

Media. Nutrient broth was prepared in this institute from fresh meat and contained in addition 0.5% NaCl, 1% peptone (Difco) and 0.069% Na_2CO_3; the pH was 7.5. Nutrient agar contained, in addition, 2% agar (BBL).

NXV broth was nutrient broth, supplemented with 4% X factor and 4% V factor. X and V factors were freshly prepared from lysed horse blood and lysed yeast cells, respectively. NXV agar was prepared by adding 2% agar to NSV broth.

Stock cultures of *H. influenzae* were maintained and transferred every 2 weeks in semisolid NXV medium containing 1% agar (BBL).

Susceptibility tests were usually carried out on NXV agar. Wellcotest agar, supplemented with X and V factors was used for establishing the susceptibility to cotrimoxazole.

Determination of MICs. The minimum inhibitory concentrations (MICs) were determined by agar dilution, using a Steers replicator. As inocula, undiluted overnight cultures (10^8 to 10^9 colony-forming units/ml) and also the dilutions 10^{-1}, 10^{-2}, and 10^{-3} were used. The antibiotics used were: Cm (Globenicol, Mycofarm), Tc (Tifaco), ampicillin sodium (Amfipen, Mycofarm), erythromycin (Erythrocin, Ab-

TABLE 1. *Bacterial strains used*

Strain	Relevant properties[a]
HC234	Clinical isolate of *H. influenzae*; resistant to Cm and Tc (10)
HC215	Clinical isolate of *H. influenzae*; susceptible to Cm and Tc
HC217	Clinical isolate of *H. influenzae*; susceptible to Cm and Tc
HC218	Clinical isolate of *H. influenzae*; susceptible to Cm and Tc
HC215-1	Mutant of HC215; resistant to rifampin and streptomycin
HC217-1	Mutant of HC217; resistant to rifampin and streptomycin
HC218-1	Mutant of HC218; resistant to rifampin and streptomycin
HC25	Clinical isolate of *H. influenzae*; resistant to ampicillin
SC181	*E. coli* K-12, F^-, *leu thi lac sup-44 tonA21 thr chl hsrB*

[a] The nomenclature employed here conforms to the recommendations of Demerec et al. (4), Taylor and Trotter (14), and Novick et al. (11).

bott), kanamycin (Kanymex, Mycofarm), gentamicin (Garamycin, Schering), trimethoprim (Hoffmann-La Roche), and sulfamethoxazole (Hoffmann-La Roche).

Transfer of drug resistance. Matings in liquid medium were carried out by mixing equal volumes of overnight cultures of the donor and the recipient strain, followed by overnight incubation at 37°C. Matings on filters were performed as follows. One-milliliter portions of 1:1 mixtures of overnight cultures of the donor and the recipient were filtered through 13-mm cellulose nitrate filters (Sartorius; pore size, 0.45 μm). The filters were incubated at 37°C during variable times on the surface of NXV agar plates. The filters then were shaken vigorously in 1 ml of NXV broth. Dilutions were plated on NXV agar, supplemented with the appropriate antibiotics (Cm, 2 μg/ml; Tc, 4 μg/ml; and rifampin, 10 μg/ml). To confirm that suspected transcipient colonies were derivatives of the recipient, and not rifampicin-resistant mutants of the donor, replica plating was carried out on plates supplemented with streptomycin (100 μg/ml).

In transfer experiments using *E. coli* as the recipient, dilutions of the mixture of donor and recipient cells were plated on nutrient agar supplemented with Cm (32 μg/ml) or Tc (32 μg/ml).

Isolation and characterization of plasmid DNA. Plasmid DNA was isolated from *H. influenzae* as covalently closed circular DNA (ccc DNA). Cells were lysed by Triton X-100 and, after sedimenting the bulk chromosomal DNA, ccc DNA was separated and purified by equilibrium centrifugation in cesium chloride in the presence of ethidium bromide, as described by Elwell et al. (5). To convert ccc DNA to the open circular form, preparations of ccc DNA were irradiated with ^{60}Co gamma rays for a dose of 2,000 rads (dose rate, 20,000 rads/h).

Electron microscopy of irradiated plasmid DNA preparations and contour length measurements of the molecules have been described previously (16).

RESULTS

MICs. The results of the MIC determinations against the *H. influenzae* strains are listed in Table 2. Strain HC234 appears to be resistant to both Cm and Tc. In comparison with susceptible strains of *H. Influenzae*, a 32- to 64-fold increase in the resistance to these antibiotics was observed. The MICs of both Cm and Tc were influenced very little by the size of the inoculum. HC234 was found to be susceptible to the other drugs tested.

Stability of drug resistance in strain HC234. To see whether there was any spontaneous loss of Cm and Tc resistance, daily transfers (dilution, 1:10^4) were carried out in drug-free NXV broth. After 6 weeks of repeated transfers, no drug-susceptible variants of HC234 were found among any of the approximately 3,000 colonies tested. An attempt was undertaken to cure the cells of the resistance markers by daily transfers for 3 weeks in NXV broth supplemented with 4 μg of ethidium bromide per ml, the highest concentration permitting growth. No loss of resistance was found in any of the approximately 1,000 colonies tested. Overnight incubations in NXV broth supplemented with 32 μg of acridine orange per ml, however, yielded 1% of cells "cured" of their resistance to Cm and Tc.

Transferability and kinetics of transfer of Cm and Tc resistance. To learn whether the Cm and Tc resistance determinants of strain HC234 were transferable, mixtures of cultures of this strain and each of the susceptible recipient strains HC215-1, HC217-1, and HC218-1 were incubated overnight in liquid medium and on filters. The results are shown in Table 3.

No transfer to HC215-1 was detectable, either in liquid medium or on filters. The frequency of transfer of resistance markers to HC217-1 and HC218-1 in liquid medium was low. However, incubation on filters resulted in higher transfer rates of both Cm and Tc resistance determinants. As strain HC217-1 proved to be the best recipient, it was used in further experiments.

The kinetics of the transfer of both the drug resistance markers from HC234 to HC217-1 was studied by incubation of mixed cultures on filters for different periods of time. The results are shown graphically in Fig. 1. As shown in this figure, it takes about 3 h of mixed culturing to reach the maximum frequency of transfer,

TABLE 2. *Susceptibility of H. influenzae to several antibiotic drugs*

Strain	MIC (μg/ml; inoculum about 10^6 cells/ml)[a]						
	Ap	Cm	Tc	Em	Km	Gm	Cotrim
HC234	0.5	16	32	4	4	2	0.03[b]
HC215-1	0.25	0.5	0.5	2	4	2	0.03
HC217-1	0.25	0.5	0.5	2	2	1	0.03
HC218-1	0.25	0.25	0.5	2	4	2	0.03

[a] Ap, Ampicillin; Cm, chloramphenicol; Tc, tetracycline; Em, erythromycin; Km, kanamycin; Gm, gentamicin; cotrim (cotrimoxazole), sulfamethoxazole + trimethoprim in a ratio of 20:1.

[b] For Cotrim, 0.03 = 0.03 μg of TMP per ml + 0.6 μg of SMZ per ml.

TABLE 3. *Transfer frequencies of Cm and Tc resistance of strain HC234 to various recipient strains of H. influenzae*[a]

Recipient	Matings in liquid medium	Matings on filter
HC215-1	$<10^{-8}$	$<10^{-8}$
HC217-1	10^{-7}	10^{-3}–10^{-4}
HC218-1	10^{-8}	10^{-6}

[a] Matings were carried out overnight as described in Materials and Methods. Transfer frequencies are expressed as the number of drug-resistant transcipient cells per total number of donor cells.

i.e., 10^{-3} to 10^{-4} per donor cell. No drug-resistant transcipients were found at time zero and after 30 min of mixed incubation.

In all experiments, the Cm and Tc resistance of HC234 behaved virtually as a single unit of transfer. Replica plating of transcipient colonies, selected for Cm resistance, showed that only 6 out of 2,912 colonies were susceptible to Tc. No Cm-susceptible colonies were found among approximately 10^4 transcipient colonies selected on plates supplemented with Tc.

Once transferred to HC217-1, the aquired Cm and Tc resistance determinants were found to be retransferable to an ampicillin-resistant clinical isolate of *H. influenzae* (HC25) as well as to *E. coli* SC181.

Mechanism of resistance transfer. To find out whether or not transformation was the mechanism of drug resistance transfer, filter matings of HC234 × HC217-1 were performed in the presence of 100 μg of deoxyribonuclease I per ml and 0.01 M $MgCl_2$. These substances were added to the donor and recipient cultures 5 min before mixing and also to the agar plates, which were used for incubation of the filters.

The frequency of transfer of Cm and Tc resistance markers in this mating was not influenced by the presence of deoxyribonuclease I. Consequently, transformation seems not to be the mechanism of transfer.

Also, transduction does not seem to be a mechanism of transfer, as was demonstrated as follows. Transfer experiments were carried out under the conditions of the filter matings, except that cell-free filtrates of the donor were used instead of cultures of the donor. No Tc- or Cm-resistant recipient colonies could be found. Also, dilutions of cell-free filtrates of the donor strain, spotted on NXV agar plates 20 min after these plates had been inoculated with a confluent layer of a logarithmically growing culture of the recipient strain HC217-1, did not reveal any plaques.

Since these experiments suggest that neither transformation nor transduction is a mechanism of resistance transfer in strain HC234, it follows that resistance to Cm and Tc is probably transferred by conjugation.

Transfer to *E. coli*. After unsuccessful trials to transfer Cm and Tc resistance from HC234 to *E. coli* SC181 in liquid medium, positive results were obtained with overnight matings on filters. In these experiments, a transfer frequency was observed that ranged from 10^{-6} to 10^{-7} per donor cell. Twenty-nine transcipient colonies selected on Tc-containing medium were found to be also resistant to Cm. However, 25 of 44 transcipient colonies selected for Cm resistance appeared to be susceptible to Tc (2 μg/ml). The MICs of both antibiotics toward resistant transcipients were ≥128 μg/ml, whereas the MICs of Cm and Tc towards the recipient strains SC181 were 8 and 2 μg/ml, respectively.

Presence and characterization of plasmid DNA in HC234. After equilibrium centrifugation (in cesium chloride plus ethidium bromide) of cleared lysates of strain HC234, two DNA bands were found, indicating the presence of ccc DNA. Electron photomicrographs of gamma-ray-irradiated preparations of plasmid DNA revealed the presence of only one species of DNA molecules. Twenty molecules were measured,

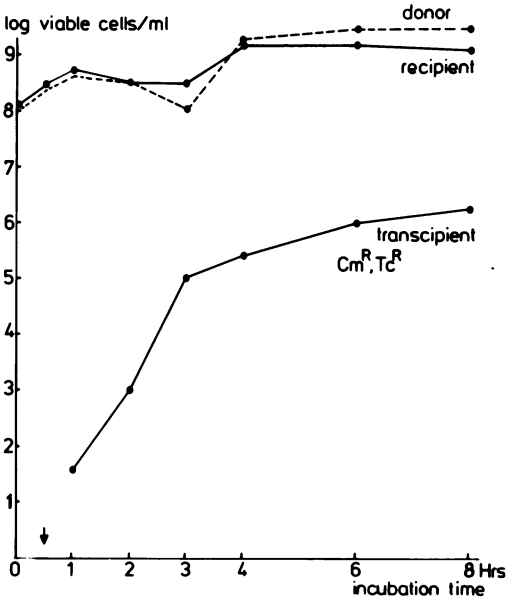

FIG. 1. *Kinetics of transfer of Cm and Tc resistance from H. influenzae HC234 to strain HC217-1 during mixed incubation on membrane filters. At time zero and 30 min, the transfer frequency was below the detection level (arrow).*

and the mean length was found to be 18.4 ± 0.6 μm. This size corresponds to a molecular weight of about 38 × 10^6. This plasmid will be designated as pRI234.

No ccc DNA could be detected in the recipient strain HC217-1. However, in a transcipient (HC217-2), carrying the Cm and Tc resistance markers from HC234, circular DNA was found. The mean contour length of 21 circular molecules was 18.9 ± 0.7 μm.

DISCUSSION

The data presented suggest that the resistance to Cm and Tc in *H. influenzae* HC234 is mediated by a single plasmid. (i) Acridine orange treatment of HC234 results in simultaneous loss of resistance to Cm and Tc. (ii) The drug resistance markers of HC234 are transferred to other *H. influenzae* strains as one single unit of transfer, although occasionally the Tc resistance determinant was lost as a consequence of transfer to the recipient. Furthermore, the acquired resistance of the *H. influenzae* transcipients was retransferable. (iii) Strain HC234 harbors one single plasmid, pRI234, with a molecular weight of 38 × 10^6. A plasmid species of this size was also found in the transcipient HC217-2.

Curing of the resistance markers in HC234 was obtained with acridine orange but not with ethidium bromide. This finding is in accordance with the observations of other investigators, who found that the ampicillin resistance in *H. influenzae* could be cured with acridine orange, but not with ethidium bromide (6, 17).

The most likely mechanism of drug resistance transfer of HC234 is conjugation, since deoxyribonuclease I in the mating mixture did not influence the transfer frequency significantly, and no evidence could be obtained for transduction. Conjugal transfer of ampicillin resistance between *Haemophilus* strains was demonstrated by Thorne and Farrar (15, and cited in reference 8). Conjugation might be the mechanism of transfer of ampicillin resistance as well as was observed by Sykes et al. (13) and in our laboratory (van Klingeren, unpublished observations). The resistance markers of strain HC234 were transferable to *E. coli* K-12, as was found also for a kanamycin resistance plasmid by Dang Van et al. (3). This is in contrast to the findings of de Graaff et al. (8), who attempted unsuccessfully to transfer the conjugative ampicillin resistance plasmid RSF007 to *E. coli* K-12.

Unlike Dang Van et al. (3), we were not able to retransfer the required resistance markers of HC234 once transferred to *E. coli*. Moreover, no ccc DNA could be demonstrated in Cm- and Tc-resistant *E. coli* transconjugants (unpublished observations). Therefore, the resistance determinants of pRI234 are likely to be integrated into the *E. coli* chromosome. A similar phenomenon was observed by Saunders (personal communication), who showed that the ampicillin resistance determinant of *H. influenzae* is integrated into the chromosome of *Pseudomonas aeruginosa* or *E. coli* after conjugal transfer.

The plasmid pRI234 does not seem to be maintained stably in *E. coli* as a plasmid. However, its transferability to an unrelated species like *E. coli* might point to a broad host range like that of the plasmids belonging to compatibility group P (N. Datta, *In Microbiology—1974*, p. 9–15, American Society for Microbiology, Washington D.C., 1974). Therefore, one might speculate that pRI234 is derived from a P-like plasmid. If so, *H. influenzae* HC234 might have acquired its drug resistance by conjugal transfer of such a plasmid from an unrelated organism like *Pseudomonas* or *E. coli*, which are known to carry conjugative plasmids mediating resistance to Cm and Tc. Alternatively, the origin of the drug resistance in HC234 might be similar to that proposed by Falkow and co-workers (5, 8) for the recently observed ampicillin resistance in *H. influenzae:* a conjugative plasmid, indigenous to *H. influenzae*, might have been hooked up with the translocatable sequence TnA, which codes for the widespread TEM-like β-lactamase (9). In analogy, such a plasmid might have acquired a translocatable sequence coding for Cm and Tc resistance. The existance of such translocatable sequences has been demonstrated in some R factors from *Enterobacteriaceae* (1, 7). Further investigations will be carried out to elucidate the origin of the R factor in HC234 and its relatedness to other *Haemophilus* plasmids.

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