Demonstration of a functional variant of chloramphenicol acetyltransferase in *Haemophilus influenzae*

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Summary. Seven clinical isolates of chloramphenicol-resistant *Haemophilus influenzae* were studied. The products of chloramphenicol inactivation by chloramphenicol acetyltransferase (CAT) were identified by high performance liquid chromatography. The sole product in *H. influenzae* is a single monoacetyl compound, whereas variants of CAT isolated from other chloramphenicol-resistant bacteria usually produce both monoacetyl and diacetyl chloramphenicol metabolites. The chloramphenicol resistance gene was found to reside on a 65-kb plasmid which, in five of the six cases studied, appeared to be integrated into the host cell chromosome.

Introduction

Haemophilus influenzae is a cause of acute meningitis and epiglottitis in young children. The high incidence of ampicillin resistance in this organism has led to the continued use of chloramphenicol as a treatment of first choice. Chloramphenicol-resistant strains of *H. influenzae* are unusual but well recognised (Margués *et al.*, 1984; Powell *et al.*, 1987). Conjugative transfer of chloramphenicol resistance to both *Escherichia coli* and *H. influenzae* recipient strains is well established (van Klingerén *et al.*, 1977; Roberts *et al.*, 1980), although studies have demonstrated that plasmids are not consistently detected in the donor strains (Shaw *et al.*, 1978; Stuy, 1979).

Resistance to chloramphenicol in *H. influenzae* may be due to a permeability barrier, as suggested by Burns *et al.* (1985), but is more frequently the result of acetylation by chloramphenicol acetyltransferase (CAT) (Roberts *et al.*, 1980). Studies of *E. coli* (Shaw, 1967), *Staphylococcus aureus* (Shaw and Brodsky, 1968) and *Streptococcus faecalis* (Nakagawa *et al.*, 1979) have demonstrated that CAT variants catalyse the acetyl coenzyme A (CoA)-dependent acetylation of chloramphenicol resulting in the initial major product, 3-acetyl chloramphenicol, which rearranges non-enzymically to yield 1-acetyl chloramphenicol, a substrate for the second acetylation to yield 1,3-diacetyl chloramphenicol (Shaw, 1983).

The activity and metabolic products of the enzyme derived from *H. influenzae* are poorly documented. The present study was designed to characterise further some of the properties of CAT derived from *H. influenzae*.

Materials and methods

Bacterial strains

The bacterial strains used in this study are listed in the table. Only *H. influenzae* strain 2035 was Pitman capsular type b. *H. influenzae* strain 1388 is a plasmid recipient strain (RecA) which does not allow the integration of any acquired plasmid into the chromosome. *E. coli* K12 strain 525 is a lactose-fermenting plasmid recipient and *E. coli* strain W677/R5 (NCTC 11186) is a known type-I CAT producer (Shaw W V, personal communication). Although CAT from *H. influenzae* has similarities to type-II CAT (Shaw, 1983), a suitable type-II CAT control was not available for inclusion in this study.

Media

Disk sensitivity tests and minimum inhibitory concentrations (MICs) for *E. coli* were performed on Diagnostic Sensitivity Test (DST) Agar (Oxoid). Sensitivity tests and MICs for *H. influenzae* were performed on DST agar containing “chocolated” horse blood 10%. Fluid cultures were in Digest Broth (Southern Group Laboratories), with the addition of IsoVitaleX (BBL Microbiology Systems) 3% v/v for *H. influenzae*. Chloramphenicol-resistant transconjugant strains were cultured on selective media containing the appropriate antibiotics.

Table . Chloramphenicol acetyltransferase (CAT) activity and susceptibilities of clinical isolates of chloramphenicol-resistant *H. influenzae*, and their *E. coli* and *H. influenzae* transconjugants

		Original isolate		<i>H. influenzae</i> transconjugants		<i>E. coli</i> transconjugants	
		CAT activity (μmol/min/10 ⁹ orgs)	Chloramphenicol MIC (mg/L)	CAT activity (μmol/min/10 ⁹ orgs)	Chloramphenicol MIC (mg/L)	CAT activity (μmol/min/10 ⁹ orgs)	Chloramphenicol MIC (mg/L)
Strain	Resistance pattern						
<i>H. influenzae</i>							
2035	Cm Tc Amp	2.56	4	—	—	—	—
W1	Cm Tc	0.47	8	3.09	16	1.64	>256
W2	Cm Tc	0.70	16	1.52	16	5.76	>256
W3	Cm Tc	0.71	8	1.40	16	3.36	>256
W4	Cm Tc	1.31	4	8.27	16	1.95	>256
RN5	Cm Tc	0.38	8	2.74	16	2.75	64
A6	Cm Tc Amp	0.60	16	0.60	16	—	—
Recipients							
<i>H. influenzae</i> 1388	Str	0	0.5	...	...	...	...
<i>E. coli</i> K12 525	Nal	0	2	...	...	...	...
Control							
<i>E. coli</i> W677/R5	Cm Str	0.93	>256	...	...	...	...

Cm, chloramphenicol; Tc, tetracycline; Amp, ampicillin; Str, streptomycin; Nal, nalidixic acid.

— = No transfer obtained; ... = not tested.

Determination of MICs

MICs for all donor, recipient and transconjugant strains were determined by an agar dilution method. The strains were applied with a multipoint inoculator at inocula of 10^5 cfu/spot. The MICs of chloramphenicol (Parke-Davis), tetracycline (Lederle) and ampicillin (Beecham) were determined.

Transfer of drug resistance

Each donor strain of *H. influenzae* was mated with the *E. coli* recipient strain 525 and also with the *H. influenzae* recipient strain 1388. Filter matings were performed by a modification of the method of van Klingeren *et al.* (1977). Donor and recipient strains were grown overnight (18 h) in the appropriate broth. Samples (1 ml) of a 1:1 mixture of donor and recipient cultures were filtered through cellulose filters (25 mm diameter, 0.22 μm pore size). The filter was removed and incubated for 18 h on chocolate blood agar in CO_2 5%. The resulting growth was rinsed from the filters by agitation in 1 ml of digest broth and samples of the suspension were plated on to the appropriate selective media. MacConkey Agar (Oxoid) containing chloramphenicol 20 mg/L and nalidixic acid 50 mg/L was used for the *E. coli* transconjugants and chocolate DST agar containing chloramphenicol 2 mg/L and streptomycin 20 mg/L for the *H. influenzae* transconjugants. Single colonies of the resultant transconjugants were re-cultured on selective media for confirmation before MICs were determined.

Preparation and characterisation of plasmid DNA

The isolation and detection of plasmids was performed by agarose gel electrophoresis, by the method of Kado and Liu (1981).

Preparation of cell-free extracts

Overnight (18 h) broth cultures were prepared (20 ml) and viable counts were performed before harvesting the cells by centrifugation (6000 *g* for 30 min). The cell pellet was resuspended in 2 ml of 0.1 M Tris-HCl buffer (pH 7.8). This step was repeated and then the cells were disrupted by ultrasonication. The clear cell-free supernate obtained after high speed centrifugation (38 000 *g* for 60 min at 4°C) was used for the assay of CAT activity.

Assay of CAT activity and characterisation of metabolites

Chloramphenicol acetyltransferase activity was assayed by the technique of Lovering *et al.* (1986), in which high performance liquid chromatography (HPLC) is used to detect chloramphenicol and its metabolites. Single estimations were performed for each enzyme extract.

The enzyme extracts were incubated with chloramphenicol in the presence of acetyl CoA generated by a mixture of coenzyme A, acetyl phosphate and phosphotransacetylase as described by Breeze and Simpson (1982). The final reaction mixture contained Tris-HCl buffer (pH 7.8) (Sigma) 100 μmol , MgCl_2 (BDH) 15 μmol , chloramphenicol (Parke-Davis) 800 nmol, coenzyme A

(Sigma) 0.25 mg, acetyl phosphate (Sigma) 5 mg, and phosphotransacetylase (Sigma) 25 units in a volume of 800 μ l, to which was added 100 μ l of the crude enzyme extract, giving a final volume of 900 μ l. The reaction mixture was incubated at 37°C and at timed intervals 50- μ l samples were removed and the acetylation reaction was stopped by the addition of an equal volume of acetonitrile (Rathburn Chemicals). These samples were then injected into the chromatograph within 8 h of sampling. CAT activity remains stable under these conditions (Shaw W V, personal communication). The chromatography was performed with a PU 4010 pump (Pye Unicam), a Partisil 10 ODS-3 C₁₈ column (Whatman Ltd) and a PU 4020 UV-absorbance detector (Pye Unicam) set at 254 nm. The mobile phase consisted of methanol:water:phosphoric acid (20:79:1) at a flow rate of 3 ml/min.

Results

Resistance phenotypes and their transfer

During the filter-mating procedures, chloramphenicol resistance was transferred from donor to both *H. influenzae* and *E. coli* recipient strains in nearly all cases. The exceptions were *H. influenzae* strain 2035 which failed to transfer resistance to either recipient strain and *H. influenzae* strain A6 which only transferred resistance to the *H. influenzae* recipient. The MICs of chloramphenicol for donor, recipient and transconjugant strains are given in the table.

Tetracycline and ampicillin resistance did not always transfer along with chloramphenicol resistance. Donor strain *H. influenzae* A6 transferred chloramphenicol, tetracycline and ampicillin resistance to the *H. influenzae* 1388 recipient but no resistance was transferred to the *E. coli* 525 recipient. Tetracycline resistance transferred with chloramphenicol resistance to the *H. influenzae* 1388 recipient in 4 of 6 cases and to the *E. coli* 525 recipient in only 2 of 5 cases.

Spontaneous resistance could not be produced in the recipient strains by serial subculture on media containing subinhibitory levels of chloramphenicol.

Demonstration of plasmids

Only one of the donor *H. influenzae* strains (W4) had a demonstrable plasmid (65 kb). However, all six *H. influenzae* 1388 transconjugants had plasmids of a similar size (65 kb), which were not present in the original *H. influenzae* 1388 recipient strain. No plasmid could be demonstrated in any of the *E. coli* 525 transconjugant strains.

Characterisation of CAT

Standard curves were constructed for known concentrations of chloramphenicol and its metabolite, 1,3-diacetyl chloramphenicol. In the absence of standard material of 3-acetyl chloramphenicol, the concentration was calculated on the assumption of equimolar conversion from chloramphenicol. Graphs were constructed for each enzyme extract plotting concentrations of chloramphenicol and metabolites against time. The activity of CAT in the extracts was then calculated from the initial linear section of the reaction plots. The results are shown in the table.

In reactions performed with a commercial CAT preparation (type I, derived from *E. coli*; Sigma) or an extract of *E. coli* W677/R5, a known type-I CAT producer, both the monoacetyl and diacetyl metabolites were readily demonstrated (fig. 1).

None of the extracts from the original *H. influenzae* isolates produced 1,3-diacetyl chloramphenicol under these conditions (pH 7.8). All had demonstrable CAT but the level of activity varied between strains. In all of these cases, the only product was the monoacetyl metabolite. Varying the pH of the reaction mixture (pH 7.8, 8.2, 8.6, 9.2) and lengthening the reaction time to 10 h did not alter the production of metabolites. Similarly, extracts from all *H. influenzae* 1388 and *E. coli* 525 transconjugants demonstrated CAT activity but failed to produce 1,3-diacetyl chloramphenicol.

Extracts of all donor and transconjugant strains were left to react for 5 h to ensure complete conversion of chloramphenicol to the monoacetyl metabolite, with no demonstrable 1,3-diacetyl product; 30 units of the commercial type-I CAT (Sigma) were then added to 300 μ l of the remaining

Fig. 1. The concentration/time relationship of chloramphenicol (C) and its metabolites, monoacetyl chloramphenicol (MAC) and diacetyl chloramphenicol (DAC), in a reaction mixture containing chloramphenicol, acetyl coenzyme A and type-I CAT buffered at pH 7.8.

Fig. 2. The concentration/time relationship of chloramphenicol (C) and its metabolites, monoacetyl chloramphenicol (MAC) and diacetyl chloramphenicol (DAC), in a reaction mixture containing chloramphenicol, acetyl coenzyme A and an extract of chloramphenicol-resistant *H. influenzae* (W1), buffered at pH 7.8. After 300 min, type-I CAT was added to the reaction.

mixture and 50- μ l samples were taken at further intervals. In every case 1,3-diacetyl chloramphenicol was produced from the monoacetyl precursor (fig. 2).

Discussion

We have confirmed that chloramphenicol resistance may be transferred *in vitro*, presumably by conjugation, from *H. influenzae* into other strains of the same species and also into *E. coli*. All further studies were made on single transconjugants picked after mating. We were unable to demonstrate spontaneous resistance to chloramphenicol in the recipient *H. influenzae* or *E. coli* strains, but it is difficult to be sure that the strains studied were really transconjugants and not spontaneous mutants.

Although plasmid DNA could be detected in only one of the original donor strains of *H. influenzae*, it was evident in each of the *H. influenzae* 1388 transconjugants. Other workers (Shaw *et al.*, 1978) have experienced similar difficulties and the use of different methods for plasmid extraction may have demonstrated plasmids in more of the donor strains. However the results are in agreement with the observations made by Stuy (1980), who concluded that most clinical strains of antibiotic-resistant *H. influenzae* carry a conjugative R plasmid which is normally integrated in the chromosome, but which may be excised to yield a few cells in each population harbouring a free plasmid. Such cells may have been responsible for the transfer of resistance observed in the mating procedure.

Chloramphenicol resistance in our clinical isolates of *H. influenzae* was observed, in each case, to be associated with the presence of CAT activity. However, there was no correlation between the level of resistance to chloramphenicol and the measured CAT activity in cell extracts. The transfer experiments also demonstrated the presence of CAT in *H. influenzae* and *E. coli* transconjugants. Levels of CAT activity were significantly higher in the *E. coli* transconjugants (Student's *t* test, $p < 0.05$), an observation probably due to a greater number of gene copies per cell in the transconjugants. Levels of CAT activity were slightly higher in the *H. influenzae* transconjugants than in the donor strains, a result which may be due either to an increased gene copy number, or to enhanced expression when the resistance gene is transcribed from a plasmid locus rather than in the integrated (chromosomal) state (Iyobe *et al.*, 1974). The higher CAT activity in cell extracts was associated with greatly enhanced resistance to chloramphenicol for the *E. coli* transconjugants but no such correlation was observed for the *H. influenzae* transconjugants. However, these results are based on single estimates of CAT activity so the apparent lack of correlation between MIC and CAT activity, for individual organisms, may be incorrect.

Early studies of CAT variants from different organisms have shown the production of both monoacetyl and diacetyl chloramphenicol metabolites (Shaw, 1967; Shaw and Brodsky, 1968, Nakagawa, 1981). It has been suggested that chloramphenicol is initially acetylated at the C-3 hydroxyl group to give 3-acetyl chloramphenicol, a fraction of which then undergoes spontaneous non-enzymic rearrangement at an alkaline pH to yield 1-acetyl chloramphenicol. This is thought to be analogous to the spontaneous rearrangement of chloramphenicol-3-monosuccinate (Brent *et al.*, 1980); the equilibrium favours a ratio of 3-acetyl to 1-acetyl derivatives of 3:1 (Kleanthous and Shaw, 1984). A second acetylation at the C-3 hydroxyl group then yields 1,3-diacetyl chloramphenicol. This is summarised as follows:

Previous studies with *H. influenzae* have used indirect methods of determining CAT activity, which do not identify metabolites but which employ a spectrophotometric technique to measure the chloramphenicol-dependent appearance of free coenzyme A (Roberts *et al.*, 1980). The chromatographic approach (HPLC) enabled us to measure directly the disappearance of chloramphenicol and the appearance of each metabolite. The donor strains of *H. influenzae* and all their transconjugants produced only monoacetyl compounds and not the diacetyl compound. We believe that this is the first occasion in which the metabolites produced by CAT have been assayed directly in *H. influenzae*.

The production of 1,3-diacetyl chloramphenicol is known to be pH dependent, observed readily at pH 7.8 but not at pH 6.8 (Lovering *et al.*, 1986). However our experiments were all performed at pH 7.8, and the addition of purified type-I CAT from *E. coli* to the reaction mixtures resulted in the subsequent production of 1,3-diacetyl chloramphenicol. Neither increasing the pH of the reaction mixture nor the duration of the experiment produced any detectable diacetyl metabolite in the absence of the added type-I (*E. coli*) CAT.

Two early studies called attention to the likelihood that some bacteria containing CAT, such as staphylococci and pseudomonads, might be unable to produce 1,3-diacetyl chloramphenicol (Suzuki *et al.*, 1966; Okamoto *et al.*, 1967). It was suggested

subsequently that these experiments may have been performed under sub-optimal conditions for the production of the diacetyl metabolite (Nakagawa *et al.*, 1979). A variant of CAT from *Bacteroides fragilis* was also reported to produce only the 1-acetyl and 3-acetyl metabolites (Britz and Wilkinson, 1978). However the reaction may not have been left for an adequate duration because the second acetylation is known to occur at a much slower rate than that of the initial enzymic step (Shaw, 1983). It should be noted, however, that bacteria need not diacetylate chloramphenicol in order to gain resistance, because the monoacetyl metabolites are inactive as antibiotics. Viewed in such a light the second acetylation may be seen as wasteful of energy, using two moles of thioester to modify chloramphenicol rather than only the one necessary for inactivation of the antibiotic.

It is possible that those CAT variants which do not make 1,3-diacetyl chloramphenicol, including that from *H. influenzae*, may not be able to accommodate 1-acetyl chloramphenicol as a substrate because of a difference in the structural configuration of the active site (Leslie *et al.*, 1988).

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