

Resistance to fusidic acid in *Escherichia coli* mediated by the type I variant of chloramphenicol acetyltransferase

A plasmid-encoded mechanism involving antibiotic binding

Alan D. BENNETT* and William V. SHAW†

Department of Biochemistry, University of Leicester, Leicester LE1 7RH, U.K.

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Plasmid-encoded fusidic acid resistance in *Escherichia coli* is mediated by a common variant of chloramphenicol acetyltransferase (EC 2.3.1.28), an enzyme which is an effector of chloramphenicol resistance. Resistance to chloramphenicol is a consequence of acetylation of the antibiotic catalysed by the enzyme and the failure of the 3-acetoxy product to bind to bacterial ribosomes. Cell-free coupled transcription and translation studies are in agreement with genetic studies which indicated that the entire structural gene for the type I chloramphenicol acetyltransferase is necessary for the fusidic acid resistance phenotype. The mechanism of resistance does not involve covalent modification of the antibiotic. The other naturally occurring enterobacterial chloramphenicol acetyltransferase variants (types II and III) do not cause fusidic acid resistance. Steady-state kinetic studies with the type I enzyme have shown that the binding of fusidic acid is competitive with respect to chloramphenicol. The inhibition of polypeptide chain elongation *in vitro* which is observed in the presence of fusidic acid is relieved by addition of purified chloramphenicol acetyltransferase, and equilibrium dialysis experiments with [³H]fusidate and the type I enzyme have defined the stoichiometry and apparent affinity of fusidate for the type I enzyme. Further binding studies with fusidate analogues, including bile salts, have shown some of the structural constraints on the steroidal skeleton of the ligand which are necessary for binding to the enzyme. Determinations of antibiotic resistance levels and estimates of intracellular chloramphenicol acetyltransferase concentrations *in vivo* support the data from experiments *in vitro* to give a coherent mechanism for fusidic acid resistance based on reversible binding of the antibiotic to the enzyme.

Fusidic acid is one of a small group of antibiotics which have a steroidal structure. It inhibits polypeptide chain elongation in prokaryotes by stabilizing the intermediary complex of ribosome with elongation factor G and GDP, thus preventing further rounds of GTP hydrolysis and concomitant elongation of the nascent polypeptide chain

Abbreviations used: CAT, chloramphenicol acetyltransferase; CAT_I, CAT_{II}, CAT_{III}, the three naturally occurring enterobacterial enzyme variants; *cat*, gene for CAT; Amp, ampicillin; Tet, tetracycline; Cml, chloramphenicol; Fus, fusidic acid; Kan, kanamycin; Nal, nalidixic acid (all used for antibiotic resistances).

* Present address: Department of Molecular Genetics, Celltech Ltd., 250 Bath Road, Slough, Berks. SL1 4DY, U.K.

† To whom reprint requests should be addressed.

(reviewed by Cundliffe, 1981). Chloramphenicol inhibits elongation but at the preceding peptidyl transfer step. Its structure differs in a number of respects from that of fusidic acid.

Most genera of Gram-negative bacteria are intrinsically tolerant to concentrations of fusidic acid that inhibit Gram-positive bacteria (von Daehne *et al.*, 1979). Thus, to observe the phenotype of plasmid-borne fusidic acid resistance in *Escherichia coli*, it is necessary to use a fusidate-sensitive mutant strain as the plasmid host. Datta *et al.* (1974) used the fusidate-sensitive host strain DB10 in the first reported study. Among 22 naturally occurring R-plasmids examined, seven conferred fusidic acid resistance. Six of these plasmids also encoded chloramphenicol resistance, including five which have been shown to specify the type I variant of

chloramphenicol acetyltransferase, CAT₁ (Foster & Shaw, 1973; A. Bennett, unpublished work).

Genetic mapping studies have been performed on two closely related R-plasmids (Mickel *et al.*, 1977) each of which confer both the fusidic acid and chloramphenicol resistance phenotypes. Both deletion mapping (Dempsey & Willetts, 1976) and restriction endonuclease analysis (Lane & Chandler, 1977; Miki *et al.*, 1978; Timmis *et al.*, 1978) indicated that the chloramphenicol and fusidic acid resistance determinants were closely linked. Both resistance markers were shown subsequently to be present on transposons related to Tn9 (Arber *et al.*, 1978). DNA sequence analysis of Tn9 (Alton & Vapnek, 1979), Tn981 (Marcoli *et al.*, 1980) and plasmid pBR325 (Prentki *et al.*, 1981) demonstrated that a 906 base-pair sequence is sufficient to promote and code for both fusidic acid and chloramphenicol resistance. The only translational reading frame in this nucleotide sequence capable of encoding a polypeptide of more than 65 amino acid residues is that which specifies a protein identical in amino acid sequence to the CAT₁ variant studied by Shaw *et al.* (1979).

Völker *et al.* (1982) have demonstrated that amber mutations in plasmid pBR325 near the beginning and end of the structural gene for CAT₁ result in the loss of resistance to both chloramphenicol and fusidic acid. Informational suppression by appropriate suppressor tRNA molecules restores both resistance phenotypes. In addition, mutants of CAT₁ which are temperature-sensitive for chloramphenicol resistance are also temperature-sensitive for fusidic acid resistance and simultaneous reversion to temperature-independent resistance is observed. It would appear, therefore, that despite the structural dissimilarity of the two antibiotics, the resistance phenotype in each case is due to the same gene product. This report summarizes the results of experiments with the CAT system which provide functional data that is in agreement with the conclusions of the genetic studies.

Materials and methods

Chemicals

[11β-³H]Fusidic acid diethanolamine salt (47.75 Ci·mol⁻¹) in ethanol (stored at -80°C) and sodium fusidate and its analogues were gifts from Dr. W. von Daehne, Leo Pharmaceutical Products Ltd., Ballerup, Denmark. Cephalosporin P1 and helvolic acid were gifts from Ciba-Geigy Pharmaceuticals Division, Horsham, Sussex, U.K. Cephalosporin P1 was recrystallized at -20°C from an 80% pure sample to over 99% purity (as determined by t.l.c.) using methanol instead of ethanol in the procedure of Carey *et al.* (1975). The non-radio-

active compounds above were used as the sodium salts when supplied; free acids were converted to their corresponding sodium salts by mild treatment with NaOH (Carey *et al.*, 1975).

Spectinomycin as the dihydrochloride pentahydrate was purchased from the Upjohn Company Ltd., Crawley, Sussex, U.K.

Guanosine 5'-[γ-³²P]triphosphate, triethylammonium salt (27.0 Ci·mol⁻¹) and L-[³⁵S]-methionine (1320 Ci·mol⁻¹) were purchased from Amersham International. Complex bacterial growth media were purchased from Difco; all other compounds were purchased from Sigma or Fisons.

Bacterial strains

Host strains. *E. coli* DB10: *lacY1, malA1, mtl2, xyl17, leuB6, thr1, thi1, rpsLA132*(Str^R), *nal^R, fus^S, pnp7, rna19* (Datta *et al.*, 1974). This strain was used as the sensitive host to score the fusidic acid resistance phenotype conferred by the plasmids used in this study. Plasmid-containing strains generally exhibited reduced resistance to sodium nalidixate as previously observed (Werner & Daneck, 1981).

E. coli C600: *lacY1, leuB6, thi1, supE44, tonA21* (reviewed by Bachman, 1972). This strain was used to observe the phenotype of normal fusidate-tolerant *E. coli* on antibiotic-containing agar plates. In addition, because growth rates, plasmid yields, and CAT yields were greater for plasmid-containing C600 strains than when DB10 contained the same plasmids, it was used as the host when purifying CAT enzymes and plasmid DNA.

Plasmids. The plasmids used were as listed in Table 1. Plasmid DNA was transformed into the host strains using the procedure of Kushner (1978).

Bacterial growth media

L broth contained (per litre): 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl and 1 g of D-glucose. 2YT broth contained (per litre): 16 g of tryptone, 10 g of yeast extract and 5 g of NaCl; 2YT agar contained the same plus 1.5% (w/v) agar. M56/CAT medium was 56/2 minimal medium (Willetts *et al.*, 1969) plus 1.5% (w/v) casamino acids, 0.2% (v/v) glycerol, 0.002% (w/v) thiamin hydrochloride and 10 μg of tetracycline/ml.

Preparation of plasmid DNA

Plasmid DNA for transformation and for the coupled transcription and translation system was prepared without ribonuclease treatment from cleared lysates of plasmid-containing strains of *E. coli* C600. Cells were grown in L broth containing either tetracycline (10 μg/ml), or chloramphenicol (15 μg/ml), or kanamycin sulphate (10 μg/ml) depending on the plasmid being studied. Plasmid DNA was amplified by addition of 170 μg of chloramphenicol/ml (Clewell, 1972) or, in instances

where the plasmid specified chloramphenicol resistance, spectinomycin at a concentration of 300 µg/ml (Bolivar, 1978).

Determination of antibiotic-resistance levels

Resistance levels were estimated by the gradient plate method (Meynell & Meynell, 1970) using 50 ml of 2YT agar containing an appropriate concentration of antibiotic and set in a wedge in the bottom of a square Petri dish (10 cm × 10 cm). The Petri dish was then returned to the horizontal and the same volume of antibiotic-free 2YT agar was poured in to form an upper wedge. After 1 h when the agar was set and its surface had been dried, bacteria growing exponentially in 2YT broth (A_{660} 0.4) were applied. The application was performed by dipping 0.5 cm wide strips of sterile Whatman 3MM paper into the bacterial suspension, allowing them to drain until free of excess culture medium, and then laying these momentarily across the surface of the agar. The strips were applied in parallel lines up the concentration gradient which was formed by diffusion of the antibiotic out of the lower wedge. Antibiotic resistance was scored after 16 h incubation at 37°C by distance of visible growth across the surface of the agar.

Protocol for investigation of antibiotic inactivation

Eight conical flasks (250 ml) containing 40 ml of 2YT broth were prepared with and without a sub-inhibitory concentration of sodium fusidate (1 µg/ml). Each was inoculated with single colonies of strains of DB10 and C600 carrying no plasmid, pBR328, pKT205 or pAH1, the plasmids specifying respectively the type I, type II, and type III variants of CAT (see Table 1).

After the flasks had been shaken for 12 h at 37°C, sodium fusidate was added to a final concentration of 40 µg/ml. After a further 3 h of shaking, the total lipids of the culture were extracted by the method of Bligh & Dyer (1959). Aliquots of the trichloromethane/lipid mixture calculated to contain 50 µg of antibiotic were evaporated under vacuum and subjected to t.l.c. The solvent system used was trichloromethane/cyclohexane/methanol/acetic acid (32:4:1:4, by vol.) and the t.l.c. plates were Merck silica gel 60 F₂₅₄ (20 cm × 20 cm). Controls of sodium fusidate extracted from water and extracted from sterile 2YT broth (with and without the initial 1 µg of sodium fusidate/ml) and incubated under the same conditions were included. After chromatography the plates were heated at 110°C for 10 min then submerged in concentrated H₂SO₄ for 1 min. The detection limit of this method is of the order of 0.25 µg of compound. Fusidic acid is observed as a red to purple spot (other analogues

show as red, yellow or brown spots). The sensitivity of this method is such that it can be expected to discern as little as 0.5% modification of the antibiotic.

Transcription and translation studies

Coupled transcription and translation studies *in vitro* were carried out using the method and cell extracts described by Pratt *et al.* (1981) with 5 µg of plasmid DNA per incubation. M_r standards (Sigma MW-SDS-70) were identified by Coomassie Blue stain (Packman & Shaw, 1981) and marked with radioactive ink prior to autoradiography.

Chloramphenicol acetyltransferase purification

The three enzyme variants, CAT_I, CAT_{II} and CAT_{III}, were purified to homogeneity from separate strains of C600 carrying the plasmids pBR328, pKT205 and pAH1 respectively. Plasmid pBR328 was used in preference to plasmid pBR325 since it has the same gene for CAT_I and because its copy number is elevated (Covarrubias *et al.*, 1981), thus providing a significant increase in enzyme yield. Cells were grown in 1–3 litres of M56/CAT medium and harvested in the exponential phase of growth. M56/CAT medium was used because expression of the gene for CAT_I is sensitive to catabolite repression (Le Grice *et al.*, 1982) and CAT_I yields in the absence of glucose or other repressing carbon sources are at least double those obtained in complex media.

Purification of CAT_I and CAT_{III} was by the procedure of Packman & Shaw (1981) with the following modifications. (a) The use of phenylmethanesulphonyl fluoride and deoxyribonuclease was omitted. (b) EDTA (0.1 mM) was included in the 'standard buffer' (50 mM-Tris/HCl, pH 7.8, containing 0.1 mM-2-mercaptoethanol) throughout. (c) CAT_I was eluted from the low substitution affinity resin with standard buffer containing 0.3 M-NaCl and 5 mM-chloramphenicol. (d) CAT_{III} was eluted from the high substitution affinity resin with standard buffer containing 0.6 M-NaCl and 5 mM-chloroamphenicol. CAT_{II} was purified without the 10 min heat step because of its heat lability, but the procedure employed the same affinity resin as that used for the CAT_{III} enzyme but with the low salt wash and elution buffers of the CAT_I purification as outlined above. The specific activity of purified CAT_{II} from strain C600 carrying plasmid pKT205 was approx. 600 units/mg of protein.

Chloramphenicol acetyltransferase activity was assayed by the spectrophotometric method of Shaw (1975). One unit is defined as the amount of enzyme which catalyses the production of 1 µmol of product/min at 37°C.

Protein determinations

Protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Ribosome-dependent GTP hydrolysis assays

Elongation factor G was prepared by Dr. J. Bodley according to the method of Rohrbach *et al.* (1974) and was a gift from Dr. E. Cundliffe. Ribosomes were prepared by a modification of the method of Cundliffe *et al.* (1979).

The reaction mixture (final volume 75 μ l) contained 15 mM-Tris/HCl, pH 7.8, 10 mM-MgSO₄, 80 mM-NH₄Cl, 2 mM-2-mercaptoethanol, 10 nmol of [γ -³²P]GTP (approx. 1.5×10^6 c.p.m.) and 50 pmol of elongation factor G. This was pre-warmed to 37°C for 10 min and the reaction was started by the addition of 1 pmol (10 μ l) of ribosome solution. Sodium fusidate and purified CAT were included when required before addition of the ribosome solution. Three 20 μ l samples were taken from each reaction after incubation at 37°C for 20 min and were each mixed with 20 μ l of ice-cold 1 M-HClO₄ in a 1.5 ml Eppendorf tube. After 10 min on ice, 200 μ l of 5% (w/v) activated charcoal (prepared by the method of Thompson, 1960) was added and each tube was mixed by agitation on a vortex mixer for 30 s to adsorb free ribonucleotides. After centrifugation for 3 min in an Eppendorf microcentrifuge, 100 μ l of the supernatant containing the hydrolysed radioactive inorganic phosphate was removed for scintillation counting. A control incubation lacking elongation factor G was included to estimate the extent of background ribosome-independent GTP hydrolysis.

Equilibrium dialysis

Equilibrium dialysis was performed at 4°C in two separate eight-place dialysis modules (Hofer Scientific Instruments, San Francisco, CA, U.S.A.). Each cell in the apparatus is divided into two 0.5 ml chambers by a dialysis membrane with a 12 000–14 000 M_r cut-off. On one side of the membrane was a fixed concentration of each CAT variant (M_r approx. 100 000) in 50 mM-Tris/HCl (pH 7.8)/0.1 mM-2-mercaptoethanol/0.1 mM-EDTA. The opposite side of the cell contained a variable concentration (0–125 μ M) of the diethanolamine salt of [³H]fusidic acid in the same buffer. Two control places were reserved in each experiment to check (a) that equilibrium had been achieved and (b) the activity of CAT after dialysis. The modules were rotated at 10 rev./min for 90 h to allow equilibrium to be achieved for the highest concentration of fusidate. After dialysis five duplicate samples of 50 μ l were withdrawn from both sides of each pair of chambers and counted in 4 ml of Bray's fluid (Fox, 1976). The

mean values of the fusidate present in each chamber was determined and the results were plotted according to Scatchard (1949). A regression line then was fitted to each set of 14 points. The binding strengths of non-radioactive analogues competing with the [³H]fusidate were calculated by using the equation of Edsall & Wyman (1958).

Results

Antibiotic resistance levels

Plasmids which carry the gene for CAT_I confer resistance to fusidate which can be detected in fusidate-sensitive hosts such as DB10. There is no change in the fusidate-sensitivity phenotype for DB10 derivatives harbouring homologous plasmids which lack the gene for CAT_I. This includes plasmids with amber mutations in the CAT_I gene and those which carry the genes for CAT_{II} and CAT_{III} (Table 1). The latter observations are noteworthy in view of the many close similarities which exist among the three enterobacterial CAT proteins (Gaffney *et al.*, 1978; Zaidenzaig *et al.*, 1979, and reviewed by Shaw, 1983).

Experiments to observe possible chemical modification of fusidic acid

Experiments designed to investigate the possible inactivation of fusidate by CAT-containing strains were carried out. No t.l.c. evidence of modification of the antibiotic was seen with any of the plasmid-bearing strains of DB10 and C600 that were used, even after prior incubation of cells in the presence of a sub-inhibitory concentration of fusidate (results not shown).

Studies on expression of fusidate resistance in vitro

Coupled transcription and translation experiments were carried out in a cell-free system, using as template covalently closed plasmid DNA, to observe the number of polypeptides encoded by the 906 base-pair CAT_I nucleotide sequence. In view of the lack of other long or overlapping open reading frames in the sequence it seemed most probable that either (a) the full length CAT_I polypeptide is the effector of both antibiotic resistances or (b) a smaller derivative of the CAT_I monomer might independently mediate fusidate resistance.

Track (f) of the autoradiograph shown in Fig. 1 reveals radioactive proteins synthesized in response to the presence of plasmid pBR322 (Sutcliffe, 1979) as template DNA. The polypeptides of apparent M_r 30 000, 28 000 and 25 000 correspond to the three polypeptides related to β -lactamase which were identified immunologically by Dougan *et al.* (1979) in pBR322-containing minicells. Protein bands of apparent M_r 34 000 and 18 000 which have been reported to be associated with the constitutive

Table 1. Plasmids used in this study (referenced in text)

Plasmid	Antibiotic resistance phenotype in DB10	CAT variant*	Antibiotic resistances ($\mu\text{g/ml}$) determined by the gradient plate method for plasmid-containing DB10 strains	
			Chloramphenicol	Sodium fusidate
pBR322	Amp, Tet - - -	-	2	5
pBR325	Amp, Tet, Cml, Fus -	I	50	80
pBR325 <i>cat</i> am H22	Amp, Tet - - -	-	2	5
pBR325 <i>cat</i> am H32	Amp, - - -	-	2	5
pBR328	Amp, Tet, Cml, Fus -	I	25	105
pKT205†	- Tet, Cml - -	II	90	5
pAH1‡	- Tet, Cml - -	III	110	5
pNJ2004§	Amp - - - Kan	-	2	5
pABO2	- - Cml, Fus, Kan	I	40	100

* Gaffney *et al.* (1978).

† pKT205 was constructed by insertion of a *Pst*I restriction fragment from the R plasmid S-a into the *Pst*I site of the β -lactamase gene of pBR322 (Timmis, 1981).

‡ pAH1 was constructed by insertion of a *Pst*I restriction fragment from the R plasmid R387 into the *Pst*I site of the β -lactamase gene of pBR322.

§ pNJ2004 is an *Eco*RI/*Sal*I deletion of plasmid pMK2004 (Kahn *et al.*, 1978; Grinter, 1978).

|| pABO2 was constructed by insertion of a *Pst*I restriction fragment from the R plasmid EDR51 into the *Pst*I site of the β -lactamase gene of pNJ2004. EDR51 is a deletion derivative of R100 constructed by Dempsey & Willetts (1976).

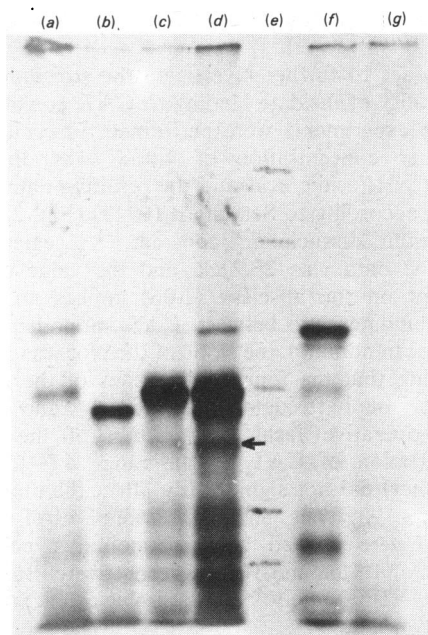


Fig. 1. Autoradiograph of the results of coupled transcription and translation experiments

Tracks (a)–(d) and (f) are identified in the text. Track (e) contains M_r standards (66000, 48000, 24000, 18400 and 14300). Track (g) is the control incubation that has no added template DNA.

tetracycline-resistance phenotype (Tait & Boyer, 1978), were not observed. Track (c) shows the polypeptides expressed from pBR325. As well as the pBR322-directed proteins, a protein of apparent M_r 24000 is expressed which is the CAT_I monomer (the actual M_r of the CAT_I monomer from the amino acid sequence is 25668 but a lower value is often obtained with sodium dodecyl sulphate/polyacrylamide-gel electrophoresis; Shaw *et al.*, 1979). In addition to these bands, the track contains a less intense band (apparent M_r 20000) indicated by the arrow. The minor band is always co-synthesized with CAT_I , irrespective of orientation, when the CAT_I fragment is inserted into the *Pst*I restriction site in the β -lactamase gene at pBR322 (results not shown) and must, therefore, represent a segment of the CAT_I polypeptide. (A lack of fidelity of gene expression peculiar to this system is made unlikely by the detection of the same minor band in minicells; Schröder *et al.*, 1981.)

To separate fusidate resistance from the M_r 20000 product would be to demonstrate the dual role of CAT_I as effector of resistance to both fusidate and chloramphenicol. Conversely, to dissociate the fusidate resistance phenotype from the M_r 24000 band attributed to CAT_I would relate fusidate resistance to the presence of the M_r 20000 polypeptide. Such a distinction was achieved by using two derivatives of pBR325 constructed by Völker *et al.* (1982) which carry different chain-

terminating amber mutations within the CAT_I structural gene. Track (a) contains the translation products of plasmid pBR325 *cat* am H22, the derivative carrying an amber mutation 'early' in the CAT_I coding sequence. No products related to CAT_I are observed either due to the instability of the transcript or of the truncated polypeptide.

Track (b) contains polypeptides expressed by plasmid pBR325 *cat* am H32, the derivative that carries an amber mutation 'late' in the CAT_I structural gene. The resulting synthesis of a protein of apparent M_r 22000 corresponds to the exact size of the truncated CAT_I polypeptide that is predicted from the DNA sequence. The polypeptides observed in track (d) are a mixture of the translation products which were loaded in tracks (b) and (c) and confirm the observed size difference for the prematurely terminated product. Both of the amber mutations in the gene for CAT_I result in the simultaneous loss of the chloramphenicol and fusidate resistance phenotypes. However, the 'late' amber mutation (track b) still expresses the M_r 20000 band. It can be concluded, therefore, that the CAT_I enzyme is sufficient, by itself, to confer both the chloramphenicol and fusidic acid resistances.

Steady-state kinetic analysis of fusidate binding to CAT_I

The steady-state kinetics of the interaction between CAT_I and fusidate was observed by the effect of the presence of fusidate on the rate of acetylation of chloramphenicol in the spectrophotometric assay (Shaw, 1975). Since the presence of plasmids specifying the enzyme variants CAT_{II} and CAT_{III} have not been associated with fusidate resistance, the effects of fusidate on the rates of chloramphenicol acetylation catalysed by these proteins were examined as controls. Each of the three CAT variants was purified to homogeneity for such studies and for the experiments described subsequently.

Fusidic acid is not an acetyl acceptor from acetyl-CoA in the spectrophotometric assay with any of the three enterobacterial CAT variants. Inhibition of the rate of chloramphenicol acetylation by fusidate was only observed with the type I variant of CAT . The inhibition observed was judged to be competitive with respect to chloramphenicol and mixed uncompetitive–noncompetitive with acetyl-CoA. [The Michaelis constants (K_m) for chloramphenicol and acetyl-CoA were $7.2 \pm 1.2 \mu M$ and $46 \pm 5 \mu M$, respectively, for CAT_I in the absence of fusidate.] The inhibition constant (K_i) from the variable chloramphenicol concentration data was $1.7 \pm 0.3 \mu M$. Fusidate did not inhibit the CAT_{II} - and CAT_{III} -catalysed acetylation of chloramphenicol even when present at a concentration of $50 \mu M$ (results not shown).

Relief of inhibition of polypeptide chain elongation in vitro

The rate of polypeptide chain elongation *in vitro* was monitored on the basis of the rate of ribosome-dependent GTP hydrolysis. The latter occurs in the absence of both exogenous mRNA and amino-acyl tRNA molecules but is dependent on the presence of elongation factor G. Maximal rates of GTP hydrolysis under the conditions described were observed only when fusidate was absent. The mean value of maximal GTP hydrolysis was 101 pmol/min during the 20 min incubation. At a final fusidate concentration of $1.0 \mu M$ ($0.54 \mu g/ml$), ribosome-dependent GTP hydrolysis is 55% of the maximal rate, an observation in close agreement with the findings of Okura *et al.* 1970. The effect of increasing concentrations of CAT_I on the elongation was measured. Whereas the addition of CAT_I only slightly depresses GTPase activity, it relieves the inhibition caused by the presence of $1.0 \mu M$ -fusidate. Reversal of the inhibition was judged to be consistent with an equimolar and independent binding of fusidate by each of the four CAT_I subunits. The other enzyme variants, CAT_{II} and CAT_{III} , did not relieve fusidate inhibition even when present at a final concentration of $10 \mu M$ enzyme monomer (results not shown).

Equilibrium dialysis

In order to further investigate the stoichiometry and avidity of fusidate binding to CAT_I equilibrium dialysis experiments were performed. Enzyme at a monomer concentration of $4.6 \mu M$ was dialysed against [3H]fusidic acid and the resulting data were plotted according to Scatchard (1949) (Fig. 2). The equilibrium dissociation constant, K_d , calculated from the data was $25.2 \mu M$, and the value of the intercept on the abscissa (1.06) implies an equimolar binding ratio between CAT_I monomers and fusidate. In addition, the slope of the plot was linear, indicating that the four binding sites of the CAT_I tetramer bind fusidate independently and in a non-co-operative fashion. Variation of the fixed concentration of CAT_I in the range 4.0 – $15.0 \mu M$ (monomer) did not significantly affect the observed value for K_d . The enzyme variants CAT_{II} and CAT_{III} were studied at fixed monomer concentrations of $1.8 \mu M$ and $10.0 \mu M$ respectively. Both fail to bind [3H]fusidate to any detectable extent (results not shown).

During 90 h of dialysis at $4^\circ C$ each of the three CAT variants had only a small loss in catalytic specific activity (an average of 7% with a maximum of 10%). Thus, in all determinations of fusidate binding at equilibrium, the protein was in each case predominantly in an enzymically active conformation. Calculations of the ligand– CAT binding

properties were based upon the value observed for the concentration of CAT at equilibrium.

Competition binding experiments were carried out to learn whether fusidate binding might be due

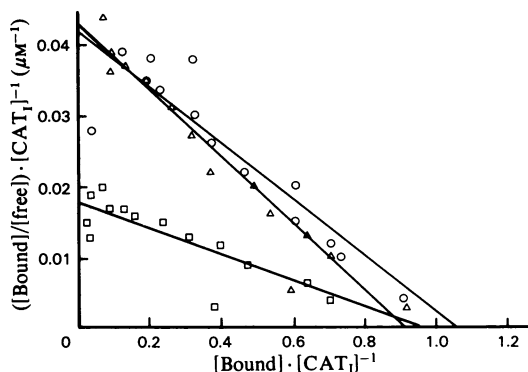


Fig. 2. Analysis of the binding of $[11\beta\text{-}^3\text{H}]$ fusidate to the type I variant of chloramphenicol acetyltransferase by equilibrium dialysis

The experiments were performed as described in the Materials and methods section and were analysed according to Scatchard (1949). ○, $[^3\text{H}]$ fusidate binding in the absence of other ligands; △, $[^3\text{H}]$ fusidate binding in the presence of non-radioactive glycocholate ($40\mu\text{M}$); □, $[^3\text{H}]$ fusidate binding in the presence of 24,25-dihydrofusidate ($40\mu\text{M}$). Extension of the method to other ligands yielded the apparent dissociation constants (K_d) listed in Table 2.

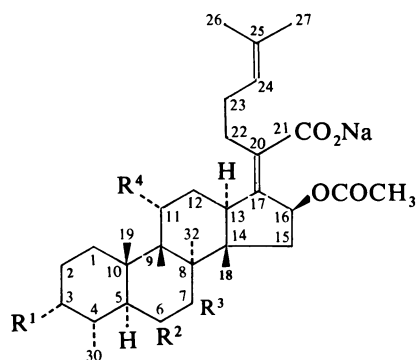
merely to the general hydrophobicity of the antibiotic or be a consequence of more specific interactions. Such experiments were performed as described above, but in each case a non-radioactive analogue of fusidate was included at a fixed concentration. The dissociation constants of the competing analogues were calculated from the resulting data using the equation of Edsall & Wyman (1958) which can be rearranged thus:

$$K_{dc} = \frac{[c]}{\left(\frac{K_{da}}{K_d}\right) - 1}$$

where K_d is the dissociation constant of the radioactive ligand at equilibrium ($25.2\mu\text{M}$ for $[^3\text{H}]$ fusidate in these experiments), K_{da} is the apparent dissociation constant in the competition experiment at equilibrium, K_{dc} is the dissociation constant of the competing ligand at equilibrium, and $[c]$ is the final concentration of competing ligand at equilibrium in the absence of binding protein ($20\mu\text{M}$ for each non-radioactive ligand in these experiments).

To validate the competition dialysis procedure as described above, non-radioactive fusidate was employed as the competing ligand. The K_{dc} value obtained was $31.1\mu\text{M}$ as compared with a directly measured K_d of $25.2\mu\text{M}$ (see Table 2).

Seven fusidate analogues were used in separate competition experiments (Fig. 3). In addition to compounds having an obvious structural similarity

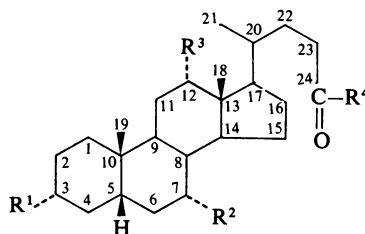


Nature of 1,2 bond	R ¹	R ²	R ³	R ⁴	Nature of 24,25 bond	Trivial name
Single	-OH	-H, -H	-H, -H	-OH	Double	Sodium fusidate
Single	β -OH	-H, -H	-H, -H	-OH	Double	Sodium 3-epifusidate
Single	=O	-H, -H	-H, -H	-OH	Double	Sodium 3-oxofusidate
Single	-OH	-H, -H	α -OH	-OH	Double	Sodium 7 α -hydroxyfusidate
Single	-OH	-H, -H	-H, -H	=O	Double	Sodium 11-oxofusidate
Single	-OH	-H, -H	-H, -H	-OH	Single	Sodium 24,25-dihydrofusidate
Double	=O	β -OCOCH ₃	=O	-H	Double	Helvolic acid sodium salt
Single	-OH	α -OCOCH ₃	β -OH	-H	Double	Cephalosporin P1 sodium salt

Fig. 3. Fusidic acid analogues and isomers used in this study

Table 2. *Antibacterial activity and CAT_I-binding affinities of sodium salts of fusidic acid analogues used in this study*
The minimum inhibitory concentrations were determined by the gradient plate method. Equilibrium dissociation constants (K_d) were determined using equilibrium dialysis by competition with [³H]fusidate as described in the Materials and methods section and shown in Fig. 2.

Fusidate analogues and bile salts	Bacterial strains ...	Minimum inhibitory concentration ($\mu\text{g/ml}$)							Equilibrium dissociation constant (K_d) with CAT _I (μM)
		DB10 + no plasmid	DB10 + pBR322	DB10 + pBR325 (CAT _I)	DB10 + pBR328 (CAT _I)	DB10 + pKT205 (CAT _{II})	DB10 + pAH1 (CAT _{III})	C600 + no plasmid	
Sodium fusidate		5	5	80	105	5	5	>500	31.1
3-Epifusidate		>450	>450	>450	>450	>450	>450	>450	>500
3-Oxofusidate		100	100	100	100	100	100	>1000	142.3
7 α -Hydroxyfusidate		15	15	15	15	15	15	>400	79.5
11-Oxofusidate		100	100	>400	>400	100	100	>400	20.5
24,25-Dihydrofusidate		20	20	>100	>100	20	20	>100	16.8
Helvolic acid		45	45	45	45	45	45	>150	83.0
Cephalosporin P1		40	40	40	40	40	40	>100	>500
Glycocholate	}								>500
Taurocholate									>500
Glycochenodeoxycholate									>500
Taurochenodeoxycholate		>400	>400	>400	>400	>400	>400	>400	>500
Glycodeoxycholate									56.5
Taurodeoxycholate									27.6
Dehydrocholate									>500



R ¹	R ²	R ³	R ⁴	Trivial name
-OH	-OH	-OH	-NHCH ₂ CONa	Sodium glycocholate
-OH	-OH	-OH	-NH[CH ₂] ₂ SO ₃ Na	Sodium taurocholate
-OH	-OH	H	-NHCH ₂ CONa	Sodium glycochenodeoxycholate
-OH	-OH	-H	-NH[CH ₂] ₂ SO ₃ Na	Sodium taurochenodeoxycholate
-OH	-H	-OH	-NHCH ₂ CONa	Sodium glycodeoxycholate
-OH	-H	-OH	-NH[CH ₂] ₂ SO ₃ Na	Sodium taurodeoxycholate
=O	=O	=O	-ONa	Sodium dehydrocholate

Fig. 4. *Bile salts used in this study*

to fusidate, a study was made of the binding properties of some steroidal bile salts (Fig. 4). Such experiments were prompted by observations of Proctor & Rownd (1982) which pointed to an interaction of bile salts with CAT_I.

Fusidate analogues and bile salts were also tested for antibiotic activity against strain DB10 and a number of plasmid-bearing derivatives. The data in Table 2 summarize the results of such experiments as well as the data from competition equilibrium dialysis studies (Fig. 2). As expected, none of the bile

salts showed significant antibiotic activity against either a conventional (fusidate-tolerant) strain of *E. coli* (C600) or strain DB10. However, fusidate analogues which inhibited the growth of DB10 and which were observed to be bound avidly by CAT_I (11-oxofusidate and 24,25-dihydrofusidate) were significantly less active *in vivo* against DB10 containing a plasmid specifying CAT_I. An increase in the observed minimum concentration required for inhibition was not observed in the case of DB10 derivatives lacking CAT (pBR322) or in strains with

plasmids specifying CAT_{II} (pKT205) or CAT_{III} (pAH1).

Discussion

Fusidate resistance and phospholipid levels

The absence of any chromatographically detectable change in the fusidate molecule after incubation with CAT_I-containing *E. coli* is in keeping with the findings of bioassay studies performed by Werner & Daneck (1981) and by S. Scannell & W. V. Shaw (unpublished work). Both groups failed to demonstrate a decrease in the level of anti-staphylococcal growth inhibition caused by pre-exposure to fusidate of *E. coli* DB10 strains which contained plasmids conferring fusidate resistance. Werner & Daneck (1981) postulated that DB10 was fusidate-sensitive because of reduced levels of cyclopropane fatty acids and phosphatidylethanolamine. They speculated that plasmid-borne fusidate resistance might be due to an alteration in the synthesis of such phospholipids which in turn was controlled by plasmid genes. However, the observation that DB10 is low in cyclopropane fatty acids may have no immediate or obvious relevance to fusidate sensitivity, since the *E. coli* mutants isolated by Taylor & Cronan (1976) are defective in cyclopropane fatty acid synthetase but are as resistant to fusidate as are wild type *E. coli* strains (results not shown).

Specificity of CAT_I for fusidic acid

It has been reported that CAT_I fails to mediate resistance to the antibiotics helvolinic acid (6-deacetylhelvolic acid) and cephalosporin P1 (Völker *et al.*, 1982). Although interesting, these observations fail to give a clear insight as to the nature of the specificity of CAT_I for fusidane compounds, since both analogues differ from fusidic acid at several positions (Fig. 3). The results of the competition equilibrium dialysis experiments described above on several fusidic acid analogues and bile salts (Fig. 4) show the CAT_I binding specificity (Table 2).

In addition to position 3, the only site tested which fails to tolerate even a small change is position 7. Minor changes in the nature of the groups at positions 4, 5, 9, 11, 12, 13, 14 and 16 are accepted and major changes to the side chain at position 17 are also permitted.

A coherent mechanism for CAT_I-mediated fusidate resistance in vivo

The experiments described represent an extension of the genetic proof offered by Völker *et al.* (1982) that the structural gene for CAT_I is required for both

the chloramphenicol and fusidate resistance phenotypes. For the CAT_I enzyme to represent a plausible resistance mechanism based solely on reversible fusidate binding it ought to satisfy two criteria. Firstly, the concentration of CAT_I within the cell must be sufficient to bind and sequester the incoming antibiotic and, secondly, the affinity of fusidate for the enzyme must approximate or exceed that of fusidate for the complex of ribosome GDP and elongation factor G. (The results indicate that there is a 1:1 binding stoichiometry between each of the CAT_I subunits and fusidate.)

The extent of increase in the observed fusidate resistance of DB10 strains carrying mini-plasmids over the plasmid-free host strain was determined on 2YT agar gradient plates (Table 1). For DB10 carrying two of the plasmids specifying CAT_I (pBR325 and pBR328) this increase is 75 and 100 µg/ml respectively, corresponding to an increase in fusidate concentrations of 138 and 185 µM. The specific activity of CAT_I in crude extracts of DB10 carrying pBR325 and of DB10 with pBR328 was determined from cells grown in 2YT broth. Under such conditions CAT_I is present as 2.8% and 3.7% of the total intracellular protein respectively. If 15% of the wet weight of *E. coli* is presumed to be protein this would imply intracellular CAT_I monomer concentrations of 175 and 230 µM respectively.

The apparent affinity of CAT_I for fusidate was determined in two ways which might reasonably be expected to yield similar values. Equilibrium dialysis gave a K_d value of approx. 30 µM whereas steady-state kinetics gave an apparent K_i value 20-fold lower. Although both experiments were performed in nearly identical buffers, the steady-state kinetic experiments were performed in the presence of saturating levels of acetyl-CoA at 37°C whereas the equilibrium dialysis in the absence of coenzyme took place at 4°C. The latter conditions were dictated by the known ability of CAT_I to hydrolyse acetyl-CoA slowly in the absence of chloramphenicol (Shaw, 1983). If the observed K_i value (1.7 µM) is a more realistic reflection of the situation *in vivo* wherein acetyl-CoA is present, then it is worth noting that this value approaches that of the reported dissociation constants for interactions of the ribosome-GDP-elongation factor G complex with fusidate (0.4 µM observed by Okura *et al.*, 1971) and 24,25-dihydrofusidate (0.39 µM noted by Willie *et al.*, 1975).

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