Nucleotide sequence analysis and overexpression of the gene encoding a type III chloramphenicol acetyltransferase

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The gene cat, encoding a type III enterobacterial chloramphenicol acetyltransferase, was cloned from the transmissible plasmid R387 into pBR322 and bacteriophage M13 mp8. Nucleotide sequence analysis of 1160 bp of DNA identified an open reading frame encoding a protein of 213 amino acid residues and a calculated molecular mass of 24965 Da. The predicted N-terminal sequence is identical with that determined by Edman degradation of chloramphenicol acetyltransferase purified from Escherichia coli harbouring R387. Sequences equivalent to the consensus motifs for initiation and rho-factor-independent termination of transcription in E. coli occur 5' and 3' to the cat open reading frame. In contrast with the cat, gene, present on transposon Tn9 and many enterobacterial plasmids, expression of cat is not subject to cyclic AMP-mediated catabolite repression in vivo and there is no sequence in the 5' non-coding DNA that resembles that deduced as the consensus for the binding of cyclic AMP receptor protein. Unique restriction-endonuclease cleavage sites were introduced adjacent to the cat reading frame by using oligonucleotide-directed mutagenesis to facilitate insertion into E. coli expression vectors. Fully active chloramphenicol acetyltransferase represents 30–50 % of the soluble protein component of cell-free extracts of E. coli containing the appropriate plasmids.

INTRODUCTION

Enzymic acetylation catalysed by chloramphenicol acetyltransferase (CAT, EC 2.3.1.28) is the commonest mechanism of bacterial resistance to the antibiotic chloramphenicol, an inhibitor of prokaryotic peptidyltransferase activity (Shaw, 1967). Transfer of the acetyl group of acetyl-CoA to the primary (C-3) hydroxy group of the antibiotic yields 3-acetylchloramphenicol, which fails to bind to bacterial ribosomes (Scheme 1). CAT variants have been isolated from numerous bacterial genera, and in all cases studied the enzyme is a trimer of identical 25 kDa subunits.

Mechanistic studies have in recent times concentrated on the efficient enterobacterial type III variant associated with the transmissible plasmid R387, originally isolated from Shigella flexneri. In addition to its high catalytic turnover, this variant yielded crystals suitable for X-ray-diffraction analysis (Leslie et al., 1986), and the structure has been refined to 0.175 nm resolution in the presence and in the absence of bound ligand (Leslie et al., 1988).

Steady-state kinetic analyses of the forward and reverse...
reactions suggest a ternary-complex (sequential) catalytic mechanism with independent binding of substrates (Kleanthous & Shaw, 1984). Production of the ternary complex proceeds via a rapid-equilibrium random-order mechanism with a preference for acetyl-CoA as the leading substrate. Inactivation of type III CAT with methyl 4-nitrobenzenesulphonate (Nakagawa & Bender, 1970) and the affinity reagent 3-bromoacetylchloramphenicol both identify an essential histidine residue, which is believed to function as a general base during catalysis (Corney, 1983; Kleanthous et al., 1985). The critical imidazole group is thought to abstract a proton from the primary hydroxy group of chloramphenicol, thus promoting nucleophilic attack at the acetyl-CoA thioester bond (Fig. 1).

The present paper reports the cloning, nucleotide sequence analysis and overexpression in *Escherichia coli*, of the gene *cat*$_{III}$, encoding type III CAT, to enable further analysis of the catalytic mechanism and ligand-binding properties of CAT via site-directed mutagenesis.

**MATERIALS AND METHODS**

**Materials**

Restriction endonucleases, T4 DNA ligase, DNA polymerase I (Klenow fragment and holoenzyme) and T4 polynucleotide kinase were purchased from Bethesda Research Laboratories and used under the manufacturer's recommended conditions. Calf intestinal phosphatase and dideoxynucleotide triphosphates were obtained from Beohringer; radiolabelled nucleotides and deoxynucleotides were from Amersham International. Antibiotics and deoxynucleotide triphosphates were purchased from Sigma Chemical Co. All other reagents were of analytical quality.

Plasmid and M13 RF DNAs were isolated by the method of Birnboim & Doly (1979) and purified by centrifugation in CsCl in the presence of ethidium bromide. *E. coli* cells were transformed by the method of Kushner (1978). DNA fragments were labelled with [$\alpha$-$^32$P]dATP by nick translation (Rigby et al., 1977).

**Bacterial strains, plasmids and bacteriophage**

*E. coli* K12 strains J53/R387 (Shaw et al., 1972) and JM101 (Messing, 1979), plasmids pBR322 (Bolivar et al., 1977) and pUC18 (Yanisch-Perron et al., 1985) and bacteriophages M13 mp8 (Messing & Vieira, 1982) and M13 mp18 (Yanisch-Perron et al., 1985) have been described previously. *E. coli* K12 SK3430 (*thi* *aroD6* *leu* *hsdR*) was a gift from S. R. Kushner to A. R. H. Strain J53/R387 was tested for carbon-source-mediated catabolite repression of CAT production in vivo by the method of Harwood & Smith (1971).

**Isolation, purification and assay of CAT**

Cell-free extracts of *E. coli* were prepared by sonication or passage through a French pressure cell. CAT was purified by affinity chromatography on chloramphenicol immobilized on Sepharose 4B as previously described (Packman & Shaw, 1981). Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard, and purity was determined by polyacrylamide-gel electrophoresis in the presence of SDS (Laemmli, 1970). The spectrophotometric assay method of Kleanthous & Shaw (1984) was used to monitor CAT activity of crude extracts and purified enzyme; 1 unit of activity is defined as 1 µmol of chloramphenicol acetylated/min.

**Oligonucleotide synthesis**

Oligonucleotides were synthesized manually by using phosphotriester chemistry with Whatman 3MM paper (Brenner & Shaw, 1985) or controlled-pore glass (Sproat & Bannwarth, 1983) supports and purified by electrophoresis through 50% urea/20% polyacrylamide gels.

**Cloning**

Restriction-endonuclease digestion products were analysed by horizontal agarose-gel electrophoresis

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![Fig. 2. Strategy for determination of the nucleotide sequence of cat$_{III}$](image)


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(Maniatis et al., 1982). All ligation reactions included a 2–5-fold molar excess of target DNA fragments over cut and dephosphorylated vector DNA. Recombinant M13 plaques containing cat\textsubscript{III} sequences were identified by DNA–DNA hybridization (Benton & Davis, 1977).

### Sequencing

The Sanger chain-terminator protocol (Sanger et al., 1977) with buffer gradient gels and [α\textsuperscript{32P}ATP (Biggin et al., 1983) was used throughout. Autoradiography was carried out with Fuji RX film.

### Oligonucleotide site-directed mutagenesis

The dual-primer protocol (Zoller & Smith, 1984) was employed with the oligonucleotides 5'-GCCATGGATCCAACCTG-3' (two mismatches) and 5'-TAGTTCAAAATCGATCTTG-3' (three mismatches). The oligonucleotide 5'-AGGCTATACGTCATT-3' was synthesized as the secondary primer and is complementary to nucleotides 6915–6930 of the M13 mp8 (+)-strand (6894–6909 of M13 mp8).

### RESULTS AND DISCUSSION

**Absence of carbon-source-mediated catabolite repression of cat expression in vivo**

*E. coli* J53 harbouring plasmid R387 (Cm\textsuperscript{r}, Sm\textsuperscript{r}) was cultured in M9 minimal medium supplemented with l-proline and l-methionine (each 0.02%, w/v), streptomycin sulphate (25μg/ml) and glucose or glycerol (0.2%, w/v) in the presence and in the absence of either 5 mM S\textsuperscript{3}-AMP or 5 mM L-methionine. In all cases the specific activity of CAT measured in crude cell-free extracts was the same (1.7 ± 0.2 units/mg).

### Cloning of cat\textsubscript{III}

Plasmid R387 DNA restricted with *Pst*I was ligated to equivalently cut dephosphorylated pBR322 and the products were used to transform *E. coli* SK3430. Chloramphenicol-resistant transformants were analysed and found to contain recombinant plasmids with a 5.5 kb *Pst*I-digestion fragment derived from R387. The cat determinant was localized further via the introduction of Sau3A partial-digestion products of one such recombinant plasmid (designated pMH1) into *Bam*H1-cleaved pBR322 and screening SK3430 transformants for chloramphenicol-resistance. Plasmid pJK111, containing approx. 1.5 kb of DNA originally present in R387 including the cat gene, served as the source of DNA for subcloning in M13 mp8. The specific activity of CAT purified to apparent homogeneity from *E. coli* harbouring plasmid R387, pMH1 or pJK111 was identical at 800 ± 50 units/mg.

### Nucleotide sequence analysis

A single HindIII, two *Hinc*II and multiple *Ahu*I and Sau3A restriction sites were identified within the R387-derived DNA of pJK111 (results not shown). These restriction endonucleases were therefore used to generate DNA fragments for subcloning in M13 mp8 before nucleotide sequence determination. Potential M13 recombinants containing cat\textsubscript{III} DNA were identified, in the first instance, as bacteriophage plaques that gave positive signals when hybridized against a ‘nick-translated’ pJK111 probe but failed to bind \textsuperscript{32P}-labelled pBR322 DNA. Nucleotide sequence data obtained from individual recombinants by use of the M13 ‘universal’ primer were extended, where appropriate, by using synthetic oligonucleotide primers designed according to the derived sequence (Fig. 2).

The nucleotide sequence of 1160 bp of pJK111 DNA was determined from both DNA strands without ambiguities and predicts a single cat open reading frame encoding 213 amino acid residues with a calculated molecular mass of 24965 Da (Fig. 3). The deduced N-terminal sequence, Met-Asn-Tyr-Thr-Lys-Asp, corresponds to that determined by Edman degradation of type III CAT purified from *E. coli* J53 containing R387 (Fitto et al., 1978), and the overall deduced amino acid sequence is in agreement with the partial protein sequence determined by Packman (1978).

The first base of the hexanucleotide AGAAGG, an approximation of the consensus sequence for prokaryotic ribosome binding (AGGAGG; Shine & Dalgarno, 1975), occurs 14 nucleotide residues before the ATG initiation codon. Sequences equivalent to the −10 (TATAAT) and −35 (TTGACA) *E. coli* transcriptional initiation consensus motifs are represented 5' to the cat reading frame by TAAAT and TTG CCT, respectively (Prifhow, 1975; Siebenlist, 1979). The 3' non-coding sequence includes an inverted repeat region followed by several thymidine residues characteristic of the proposed secondary structural unit required for rho-factor-independent termination of transcription in *E. coli* (Rosenberg & Court, 1975). The 5' non-coding DNA of cat\textsubscript{III} does not contain sequences equivalent to the consensus for the binding of cyclic AMP receptor protein observed within the cat\textsubscript{III} determinant present on Tn9, pACYC184 and a number of large enterobacterial plasmids (LeGrice et al., 1982).

The cat\textsubscript{III} open reading frame shows 46% amino acid identity with the type I enzyme encoded by Tn9 (Alton & Vapnek, 1979; Fig. 4). Maximum homology occurs in the region immediately adjacent to the active-site histidine residue (residue 189 in type III CAT). Type III CAT lacks the first five amino acid residues present at the N-terminus of the type I and *Proteus mirabilis* enzymes (Charles et al., 1985), a feature previously believed to be typical of CAT variants isolated from Gram-positive bacteria (Horinouchi & Weisblum, 1982; Harwood et al., 1983; Shaw et al., 1985).

### Site-directed mutagenesis and overexpression of cat\textsubscript{III}

Analyses of the effects of site-directed mutations within the cat\textsubscript{III} structural gene (Murray et al., 1986) have been hampered by the low level of gene expression in *E. coli* from plasmids derived from pBR322 and bacteriophage M13 (results not shown). In addition, although cat\textsubscript{III} has a unique HindIII restriction site immediately 3' to the proposed transcriptional termination point, there are no unique restriction sites in the 5' non-coding DNA that permit insertion into, and excision from, the multiple cloning site of M13 mp8 vectors. To overcome these limitations we have introduced novel restriction sites into the 5' non-coding region of cat\textsubscript{III} by using oligonucleotide-directed mutagenesis.

Plasmid pJK111 has a unique *Bgl*II site approx. 100 bp 5' to the region for which sequence data were determined. The cat gene was excised by cleavage with *Bgl*II and HindIII and ligated to *Bam*H1–HindIII-cut and dephosphorylated RF DNA of M13 mp8 yielding the
construct mp8:IM2 (Fig. 5). The oligonucleotide 5'-GCGATGGATCCACTG-3', complementary to nucleotides 116–131 of cat 

introduced into M13 mp18 RF and plasmid pUC18 to produce mp18:IM3 and pUC18:IM3 (Fig. 5). Constitutive expression of cat in E. coli JM101 harbouring pUC18:IM3 is highly efficient, fully active CAT representing 25–40% of the soluble protein in crude cell-free extracts (Fig. 7).

A second cycle of mutagenesis, using the oligo-
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**Fig. 4.** Sequence homology between type III and type I variants of CAT

*, Conserved amino acid residues; +, conservative amino acid residue substitutions.

**Fig. 5.** Construction of plasmids permitting overexpression of cat, in E. coli

SDM, oligonucleotide site-directed mutagenesis.

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nucleotide 5'-TAGTTCAATAATCGATCCCTG-3' and single-stranded mp18:IM3 DNA, was employed to introduce a Clal restriction site adjacent to the ATG initiation codon of cat\textsubscript{111} (Fig. 6b). This mutation facilitates the in-frame insertion of the cat\textsubscript{111} structural gene into the temperature-inducible expression vector pHD, a dual-origin plasmid conferring ampicillin-resistance that contains the promoter, operator and ribosome-binding site of the trp attenuator peptide followed by unique Clal and EcoRI restriction sites (Thomas, 1987). When present in E. coli grown at 30 °C plasmid pHD replicates via a pSC101 origin (three or four copies per chromosome) and transcription from the trp promoter is inhibited by the chromosomal trp repressor. However, at 37 °C replication is via a ColE1 origin under the control of the P\textsubscript{8} promoter of bacteriophage \(\lambda\) initiated by the inactivation of the temperature-sensitive \(\lambda\) cl857 repressor. The result is a rapid amplification of the plasmid up to several hundred copies per chromosome. At high plasmid copy number the chromosomal trp repressor is titrated out, resulting in very efficient transcription from the trp promoter and through any appropriately positioned exogenous coding sequence. Expression of cat\textsubscript{111} from the pHD-derivative pIM5 (Fig. 5) in E. coli JM101 produces cell-free extracts in which active CAT constitutes 30–50% of the soluble protein component (Fig. 7). Cultures of E. coli harbouring pIM5 can be grown to mid-exponential phase before heat-induction and produce very high yields of CAT within 90–120 min, making this vector the system of choice for the production of mutant CATs that demonstrate enhanced susceptibility to degradation and/or inactivation in vivo.

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