# *Proteus mirabilis* and CAT

by

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A thesis submitted for the degree of Doctor of Philosophy in the University of Leicester.

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ABBREVIATIONS

| | |
|---|---|
| $Ap^r/Ap^s$ | resistance /sensitivity to ampicillin |
| $A_x$ | absorbance at x nm |
| b | base (s) |
| bp | gene encoding β-lactamase |
| BSA | bovine serum albumin |
| cat | gene encoding chloramphenicol acetyltransferase |
| CAT | chloramphenicol acetyltransferase |
| cAMP | cyclic AMP [adenosine 3' 5'-cyclic monophosphate] |
| CAP | catabolite gene-activator protein |
| Ci | curies |
| CoA | coenzyme A |
| $Cm^r/Cm^s$ | resistance/sensitivity to chloramphenicol |
| cpm | counts per minute |
| DEPC | diethylpyrocarbonate |
| DMF | diemthyl formamide |
| DMSO | dimethyl sulphoxide |
| DNA | deoxyribonucleic acid |
| DNase | deoxyribonuclease |
| dsDNA | double stranded DNA |
| DTNB | 5, 5', dithio-bis(2-nitrobenzoic acid) |
| DTT | dithiothreitol |
| EDTA | ethylene diaminetetra-acetic acid |
| $Em^r$ | resistance to erythromycin |
| hrs | hours |
| IgG | immunoglobulin G |
| IPTG | isopropyl-β-D-thiogalactoside |
| kb | kilobases |

| | |
|---|---|
| MCS | multi-cloning site |
| mins | minutes |
| MOPS | morpholinopropanesulphonic acid |
| mRNA | messenger ribonucleic acid |
| NMR | nuclear magnetic resonance |
| $OD_x$ | optical density wavelength in nm |
| O/N | overnight |
| PEG | polyethylene glycol |
| pfu | plaque forming units |
| RNA | ribonucleic acid |
| RNase | ribonuclease |
| rpm | revolutions per minute |
| SDS | sodium dodecylsuphate |
| ssDNA | single stranded DNA |
| $Tc^r/Tc^s$ | resistance/sensitivity to tetracycline |
| TEMED | N, N, N', N' tetramethyl ethylenediamine |
| tet | gene specifying resistance to tetracycline |
| UV. | ultraviolet |
| X-GAL | 5-bromo-4-chloro-indolyl-β-D-galactoside |
| 3-deoxyCm | 1-p-nitrophenyl-2-dichloracetamido-propan-1-ol |
| 3-fluoroCm | 1-p-nitrophenyl-2-dichloroacetamido-3-fluoro-propan-1-ol. |

| | |
|---|---|
| dATP | 2'-deoxyadenosine 5'-triphosphate |
| dCTP | 2'-deoxycytidine 5'-triphosphate |
| dGTP | 2'-deoxyguanosine 5'-triphosphate |
| dTTP | thymidine 5'-triphosphate |

| | |
|---|---|
| ddATP | 2', 3'- dideoxyadenosine 5'-triphosphate |
| ddCTP | 2', 3'- dideoxycytidine 5'-triphosphate |
| ddGTP | 2', 3'- dideoxyguanosine 5'-triphosphate |
| ddTTP | 2', 3'- dideoxythymidine 5'-triphosphate |

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# CHAPTER 1

# GENERAL INTRODUCTION

# CHAPTER 1 : GENERAL INTRODUCTION

## 1.1 Chloramphenicol Resistance

### 1.1.1 Resistance to antibiotics

The strategies by which bacteria can avoid inhibition or killing by antibiotics have been discussed by Davis and Maas (1952), and recently reviewed (Gale *et al.*, 1981). The biochemical basis of resistance may be due to:

1. Modification of the cellular target so that binding of the antibiotic is reduced or abolished;
2. Reduction in physiological importance of the target by adoption of an alternate pathway which bypasses it;
3. Duplication of the target enzyme, the second version being resistant to inhibition;
4. Prevention of access to the target (by decreased rate of entry or increased rate of efflux of the antibiotic);
5. Inactivation of the antibiotic by chemical modification.

All of these mechanisms have been encountered in clinical situations (Gale *et al.*, 1981) and furthermore many of the genes for them are plasmid-encoded, and thus can be propagated by conjugal transfer. This fact has accounted, in large measure, for the striking increase in the incidence and dissemination of antibiotic resistance following the widespread clinical use of antibiotics. Among gram-negative bacteria, transmissible resistance plasmids (R. plasmids) commonly carry drug resistance determinants for as many as six (or more) antibiotics simultaneously, and consequently they

have become a major problem in the treatment of infectious diesases (first reviewed by Watanabe, 1963). Gram-positive bacteria can also exchange antibiotic-resistance genes, but in these cases transfer is generally mediated by phage transduction rather than conjugation (Lacey, 1975).

1.1.2 Chloramphenicol

Chloramphenicol (Cm) was first isolated in 1947 from the soil bacterium *Streptomyces venezuelae* (Ehrlich *et al.*, 1947). Subsequently it has been found to be produced by a number of bacteria (reviewed by Shaw, 1983). The structure of chloramphenicol is shown in Fig. 1.1. Of the four possible stereoisomers only the D(-) *threo* form is bacteriostatic, and indeed this is the only form synthesized by *S. venezuelae* (Rebstock *et al.*, 1949). The mode of action of chloramphenicol involves the inhibition of the peptidyl transferase activity of the 70s ribosomes (reviewed by Gale *et al.*, 1981). Due to its simple structure the antibiotic is currently made on a commercial scale by chemical synthesis (reviewed by Ehrlich, 1982), although it is no longer widely used in clinical situations because of the possible side effect of aplastic anaemia. Various analogues of chloramphenicol have been synthesized; electronegative group substitution at the *para* position of the aromatic ring retains antibacterial activity; the dichloroacetamido group on $C_2$ can be altered and antibacterial activity retained; however little alteration can be tolerated in the propanediol moiety (Gale *et al.*, 1981). If the $C_3$ hydroxyl group is replaced by fluorine, antibacterial activity is retained (Hahn, 1980), and NMR studies have suggested that

this part of the molecule is important in binding to the ribosome (Tritton, 1979).

### 1.1.3 Resistance to Chloramphenicol

Various mechanisms of chloramphenicol resistance have been reported, and have recently been reviewed by Shaw, (1983). Chloramphenicol resistance has been observed to operate by the degradation of the antibiotic in *Flavobacterium* spp. (Sussmuth *et al.*, 1979), while *Clostridium acetobutylicum* can reduce the aryl *nitro* group, which effectively inactivates chloramphenicol (O'Brien and Morris, 1971). Chromosomal loci have been identified in *E.coli* which confer low level chloramphenicol resistance, these include *cmlA* (resistance to chloramphenicol and tetracycline, Reeve, 1968); and *lon* (resistance to chloramphenicol, Reeve, 1968). The *marA* locus (Stuart and Levy, 1983) has been reported to convey high level resistance ( 100µg/ml) to chloramphenicol and tetracycline. Some plasmids have been identified in bacteria which confer chloramphenicol resistance by a mechanism that does not involve drug inactivation or modification (Gaffney *et al.*, 1981; Kono and O'Hara, 1976), wherein resistance is mediated by decreased permeability of the cell envelope to the drug. Such mechanisms may be inducible, and post-transcriptionally regulated (Dorman and Foster, 1985). The most common mechanism of chloramphenicol resistance found in clinical isolates, however, is drug inactivation by a plasmid borne *cat* gene specifying the enzyme chloramphenicol acetyltransferase (CAT, EC 2.3.1.28).

Fig 1.1 Chloramphenicol

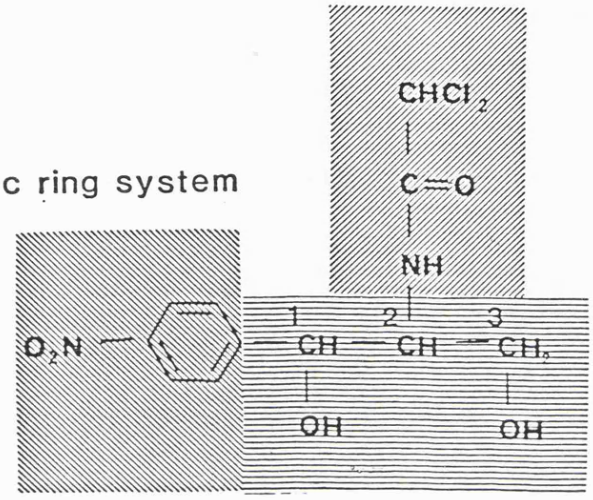
The structure of chloramphenicol is shown (Rebstock *et al.*, 1949). In 3-deoxyCm -H replaces -OH on $C_3$.

Fig 1.2 The acetylation of Chloramphenicol catalysed by CAT

Following the initial acetylation of the hydroxyl group ($C_3$ on the propanediol side chain, see above) a non-enzymic isomerization occurs (yielding 1-acetoxy chloramphenicol) exposing the primary hydroxyl to re-acetylation and formation of the 1,3 diacetyl derivative.

FIG. 1.1

Chloramphenicol

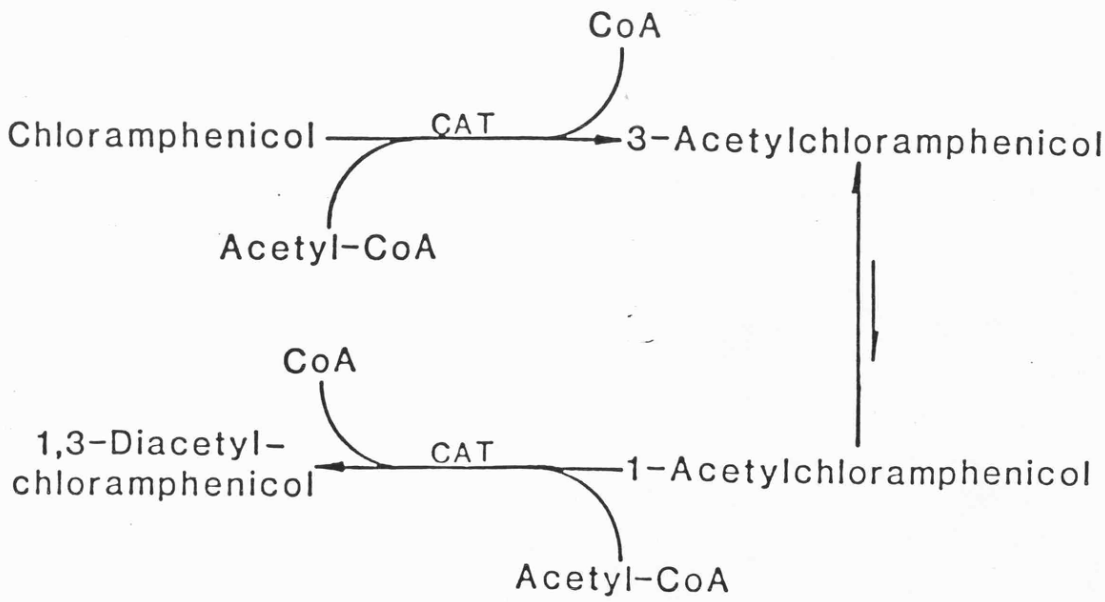



Dichloroacetamido side chain

Aromatic ring system

Propanediol moiety

FIG. 1.2



The first studies on the chloramphenicol resistance determinants specified by such plasmids were made after conjugal transfer of the plasmids from epidemic strains into laboratory strains of E.coli. Miyamura, 1964, reported that E.coli harbouring such transmissible elements for chloramphenicol resistance were able to inactivate the drug. Shaw, 1967, and Suzuki and Okamoto, 1967, showed that this inactivation was due to acetylation of chloramphenicol on the $C_3$-hydroxyl (Figs. 1.1 and 1.2). In the inactivation scheme the formation of the 3-acetoxyCm, which is in non-enzymic equilibrium with 1-acetoxyCm, is further acetylated by CAT to yield the diacetate (1,3-diacetoxyCm). Acetylated chloramphenicol was found to be ineffective as an antibiotic in vivo (Shaw, 1967) while in vitro assays demonstrated that ribosomes from E.coli bound 20-fold less 3-acetoxyCm than chloramphenicol (Shaw and Unowsky, 1968). Until recently the quaternary structure of CAT was believed to be that of a tetramer of four identical sub-units (Packman and Shaw, 1981). However, recent hydrodynamic and crystallographic studies (Shaw et al., 1985 manuscript in preparation) have revealed that CAT must be a trimer.

### 1.1.4 CAT variants: a Survey

The cat genes of gram-positive organisms studied so far encode a family of immunologically cross-reactive variants. They are almost all inducible by chloramphenicol or selected analogues (reviewed by Shaw, 1983). The original observation of 'induction' (Dunsmoor et al., 1964) showed that certain isolates of S.aureus could be 'pre-adapted' to grow in the presence of chloramaphenicol

by prior incubation with the antibiotic. Shaw and Brodsky (1968) showed that resistance was due to CAT; the specific activity of CAT under these conditions was found to increase by as much as 100-fold upon induction with chloramphenicol or close structural analogues (Shaw and Winshell, 1969).

Isolates of S.aureus that are chloramphenicol resistant typically harbour cat genes on small multicopy plasmids (Shaw, 1983). Certain of such plasmids, pC194 and pC221, can also replicate in Bacillus subtilis, and the chloramphenicol-inducible resistance phenotype has been demonstrated in both hosts (Ehrlich, 1977; Horinouchi and Weisblum, 1982). Similarly, strains of Bacillus pumilus can harbour chloramphenicol-inducible cat genes, which are also inducible in B. subtilis (Duvall et al., 1984; Williams et al., 1981). The mechanism of induction by chloramphenicol has not yet been elucidated, however, the available data suggests that control may operate post-transcriptionally, and furthermore, that it may be different in S.aureus and B.pumilus (Mongkolsuk et al., 1984).

The cat genes of gram-negative enteric bacteria have been broadly classified into three variants (type I, II and III) on the basis of a variety of physical, immunological, and biochemical criterian (Zaidenzaig et al., 1979). CAT is produced constitutively by all three variants, although the synthesis of the type I variant is under the positive control by cAMP and the catabolite-gene activator protein (CAP). The phenomenon of catabolite repression was first demonstrated in vivo by Harwood and Smith (1971), and

was subsequently confirmed *in vitro* (de Crombrugghe *et al.*, 1973; Dottin *et al.*, 1973). Positive regulation by the cAMP/CAP complex is discussed in section 1.3.1.

The observation that the type I *cat* is the resistance determinant of transposon Tn*9* (where it is flanked by the direct repeats of the insertion sequence IS*1*, (Alton and Vapnek, 1979), may account for the predominance of the type I sequence among *cat* determinants present in gram-negative bacteria.

Other gram-negative variants have also been examined, some of which show similar properties to the three main variant classifications. The *cat* gene of *Agrobacterium tumefaciens* is unique as it is the only gram-negative gene to be inducible by chloramphenicol or its 3-deoxy analogue (Shaw, 1974). The CAT produced by *Haemophilus influenzae* and *parainfluenzae* are similar to the type II variant (Shaw *et al.*, 1978), while the CAT of *P.mirabilis* is similar, but not identical, to the type I variant (Zaidenzaig *et al.*, 1979). The mechanism by which the expression of the *cat* gene is controlled in the strain *P.mirabilis* PM13 is the subject of the work in this thesis.

## 1.2 Chloramphenicol resistance in *P.mirabilis* PM13: initial observations

Chloramphenicol-sensitive bacteria usually contain no CAT, thus selection for chloramphenicol-resistant mutants from such strains invariably yields cells which have some alternative mechanism of resistance (George and Levy, 1983). The situation is quite different

for the appearance of chloramphenicol resistance in P.mirabilis PM13; strains which show traces of CAT activity can give rise to colonies exhibiting high level resistance with concomitant increase in enzyme production. Thus, when cultures of P.mirabilis PM13 are grown at 37°C in Penassay-broth to the mid-exponential phase, then plated onto Penassay-agar supplemented with chloramphenicol at a concentration of 50μg/ml, resistant colonies appear at a frequency of between $10^{-4}$ to $10^{-5}$ per cell per generation. This phenomenon has been termed enhancement (S. Harford and W.V. Shaw, preliminary unpublished experiments).

The CAT enzyme responsible has been purified from cultures of P.mirabilis PM13 grown in the presence and absence of chloramphenicol, and biochemical parameters compared. This comparison is presented in Table 1.1, and suggests that CAT purified from cultures of P.mirabilis PM13 grown in the presence of chloramphenicol is indistinguishable from the enzyme purified from cells grown in the absence of chloramaphenicol. This suggests that chloramphenicol resistance is mediated by an increase in the amount of CAT, rather than to any alteration in the properties of the protein or the synthesis of a second CAT variant.

Chloramphenicol resistant colonies which have been isolated after growth on chloramphenicol-agar exhibit a novel phenotype with respect to chloramphenicol resistance. When a single colony grown on chloramphenicol-agar (50μg/ml) is transferred to liquid medium and propagated in the absence of antibiotic for 150 generations a

Table 1.1

| | Km (μM) Chloramphenicol | Km (μM) acetyl CoA | Reaction with antibody to type I CAT (a) | Affinity chromatography elution behaviour (b) | polyacrylamide gel electrophoresis (c) |
|---|---|---|---|---|---|
| PM13 $Cm^s$ CAT | 16 ± 1 | 73 ± 5 | + | + | + |
| PM13 $Cm^r$ CAT | 14 ± 1 | 82 ± 6 | + | + | + |

(a)+ indicates line of identity by two dimensional gel diffusion in agar between both samples of CAT purified from P.mirabilis and goat-antiserum to type I CAT. Partial identity of both proteins from P.mirabilis was noted when compared with the immunogen, a homogenous preparation purified from E.coli as described (Method, 2.3).

(b)+ indicates both enzymes elute from highly substituted chloramphenicol base/agarose in 1.0 M NaCl in 5mM chloramphenicol as previously described (Method, 2.3).

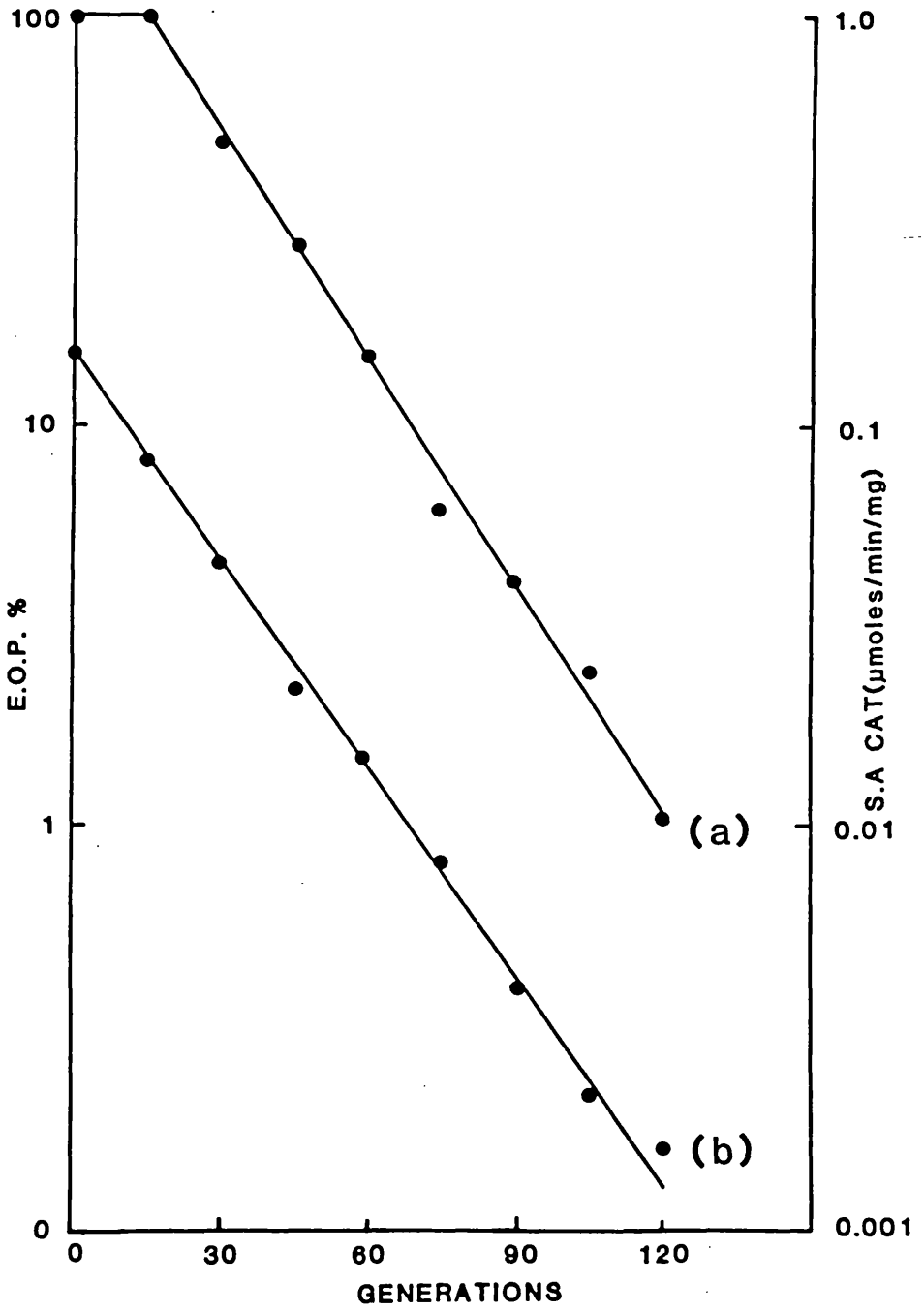
(c)+ indicates identical mobilities (Rf = 0.6) of purified proteins in polyacrylamide (8% w/v) gels under non-denaturing conditions.

population of predominantly sensitive cells arises, i.e. there is a population reversion to the chloramphenicol-sensitive state. This disappearance of chloramphenicol-resistant cells with time is graphically presented in Fig. 1.3. The efficiency of plating of cells on chloramphenicol after growth for n-generations is paralleled by a decrease in CAT activity, suggesting that the loss of CAT activity is responsible for the loss of resistance.

Fig. 1.3

Disappearance of chloramphenicol-resistant cells of *P.mirabilis* PM13 from a uniformly resistant population as a function of time

Cells were collected by centrifugation after growth in Penassay-broth containing chloramphenicol (50μg/ml) and, after washing, were resuspended in antibiotic-free medium. Samples taken at appropriate intervals were divided and taken for simultaneous plating on Penassay-agar containing 50μg of chloramphenicol per ml and on plates lacking the antibiotic, and for lysis before measurement of the specific activity of CAT. The experimental points giving line (a) represent the efficiency of plating (E.O.P.) of cells on chloramphenicol, and line (b) depicts the parallel and exponential fall in CAT from the population.


100
10
1
0
E.O.P. %
1.0
0.1
0.01
0.001
S.A CAT(µmoles/min/mg)
(a)
(b)
0
30
60
90
120
GENERATIONS

### 1.2.1 How widespread is the phenomenon?

Additional representatives of *Proteus* spp., *Morganella* spp., and *Providencia* spp. were screened for the enhancement phenomenon described above, *i.e.* the appearance of chloramphenicol-resistant cells arising from a predominantly chloramphenicol-sensitive population at high frequency (approximately $10^{-5}$ per cell per generation). Thirteen of 32 strains from the National Collection of Type Cultures (United Kingdom) showed the phenomenon (Table 1.2), whereas only two produced CAT at high level without prior exposure to chloramphenicol.

### 1.2.2 Definitions

In this work I refer to enhancement as the phenomenon whereby there is an increase in the level of CAT produced in *P.mirabilis* PM13 cells that have been grown in the presence of chloramphenicol as compared with cells that have not. No mechanism is implied by this term. The phenotype of cells of *P.mirabilis* PM13 grown in the presence of chloramphenicol is referred to as $CAT^+$ whereas that of *P.mirabilis* PM13 cultured in its absence is described as $CAT^-$. It should be noted that the latter do contain CAT activity that can be detected by the radiometric assay (Methods, 2.3), and purified by affinity chromatography, but it is dramatically lower than in $CAT^+$ cells.

Table 1.2

| SPECIES | NCTC NUMBER | PHENOMENON |
|---|---|---|
| Proteus mirabilis | 10374 | + |
| | 9559 | + |
| | 6396 | + |
| | 6197 | - |
| | 3177 | - |
| | 60 | - |
| Proteus vulgaris | 10376 | - |
| | 10015 | - |
| | 4175 | - |
| Morganella morganii | 10375 | + |
| | 10041 | - |
| | 7381 | + |
| | 5845 | + |
| | 2818 | + |
| | 2815 | + |
| | 1707 | - |
| | 235 | - |
| | 232 | + |
| Providencia rettgeri | 10377 | C |
| | 8893 | + |
| | 7481 | + |
| | 7480 | - |
| | 7479 | - |
| | 7477 | - |
| | 7476 | + |
| | 7475 | + |
| Providencia species | 10318 | C |
| | 10286 | - |
| | 8113 | - |
| | 8056 | - |
| | 6345 | - |
| | 2481 | - |

+ and - indicate presence or absence of the phenomenon.

C indicates constitutive CAT production.

### 1.2.3 Aims of this study

The aims of this project were: (i) to examine the mechanism by which chloramphenicol-resistant colonies of *P.mirabilis* PM13 arise from a predominantly chloramphenicol sensitive population, and (ii) to examine the fate of such resistant cells when the population is no longer exposed to the antibiotic. As this project ultimately addressed the molecular mechanism(s) by which the expression of the *P.mirabilis* PM13 *cat* gene is regulated, it may be appropriate to review briefly the mechanisms by which bacteria regulate gene expression.

## 1.3 Regulation of bacterial gene expression

The molecular mechanisms which exist to control bacterial gene expression can be divided broadly into two classes: (i) those which operate at the transcriptional level, controlling the rates at which specific RNA molecules are synthesized from a DNA template, and (ii) those which operate post-transcriptionally, controlling the rate at which mRNA molecules once synthesized are translated into their polypeptide product. The following review is only concerned with genes which give rise to mRNAs, rather than those which are transcribed into tRNA or rRNA; the synthesis of these stable RNAs has been recently reviewed (Lamond, 1985).

### 1.3.1 Transcriptional control

The synthesis of RNA can be regulated at a variety of specific signals encoded in the DNA, some of which specify start sites, and others which specify stop, or pause sites. Regulation of gene expression has been demonstrated to occur at either of these locations.

RNA polymerase initiates RNA synthesis after binding at sites known as promoters, and to date more than 100 of these have been reported (Hawley and McClure, 1983). Homologies between promoter regions have been identified (Rosenberg and Court, 1979) and have been used to compile a so-called consensus sequence.

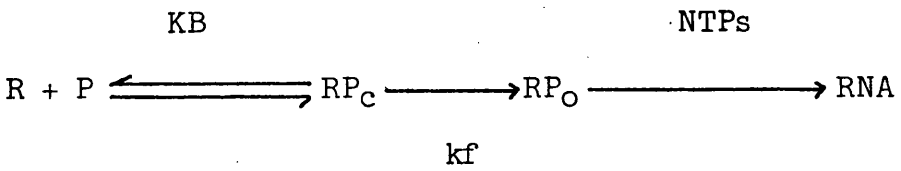
The current sequence (Hawley and McClure, 1983) is:

[-35] [-10]

TTGACA - $N_{15-19}$ - TATAAT - $N_{5-7}$ START

The consensus sequence appears to be maximal in promoter strength, i.e. mutations which decrease homology to the consensus sequence are 'down-promoter' mutations, while mutations which increase homology are 'up-promoter' mutations. The sequences of bona fide promoters in E.coli do not, however, always contain striking homology to the consensus sequence.

The current model for transcription initiation (Walter et al., 1967; Chamberlin, 1974) is outlined below:




1) Binding 2) Isomerization 3) Promoter clearance

In this scheme RNA polymerase (R) binds to the promoter (P) with a binding constant $K_B$, to form an inactive intermediate 'closed' complex ($RP_c$), The latter isomerizes with a rate constant $k_f$ to form the transcriptionally active open complex ($RP_o$) which, in the presence of nucleoside triphosphates (NTPs), immediately catalyses chain elongation. The strongest promoters have the highest values of $K_B$ and $k_f$, a high value of $K_B$ is generally associated with a good -35 consensus sequence, while the value of $k_f$ depends on two parameters; the degree of homology to the -10 (Pribnow box) sequence, and the distance between the -35 and -10 sequences (Stefano and Grulla, 1982).

Changes in the frequency of transcription initiation can be achieved by modulating the efficiency with which RNA polymerase can recognize and interact with the promoter (-35 and -10) recognition sites, or the interaction with other effector molecules can positively, or negatively control transcription.

In their operon model, Jacob and Monod (1961), proposed that the regulation of the lactose operon occurred at the level of

transcription of the mRNA species. Initiation of transcription was found to be inhibited by the tight binding (Kd = $10^{-13}$M) of a repressor protein to an operator site on the DNA near the promoter. If an inducer (alloctose) binds to the repressor, its affinity for the operator is decreased, and transcription can proceed. Many other repressors have now been described, and it has become apparent that there is considerable diversity both in the affinity of the repressor for the operator, and in the location of the operator site with respect to the promoter (Reznikoff and Abelson, 1978). In most cases, however, the DNA sequence comprising the operator has been found to overlap the DNA sequences comprising the promoter, an observation which has led to the proposal that a bound repressor sterically interferes with RNA polymerase binding. Such a mechanism has been observed with the bacteriophage $\lambda$ system where the $\lambda$cI repressor was demonstrated to inhibit the rate of $\lambda$ $P_R$ open complex formation by decreasing $K_B$ (Hawley et al., 1984 as cited in von Hippel et al., 1984).

Protein:DNA interactions can not only decrease transcription rates, but also stimulate them by the binding of an activator protein. The location of activator binding sites distinguishes activators from repressors. All known activators bind near, or upstream from the -35 region. A comparison of positively regulated promoters (Raibaud and Schwartz, 1984) from gram-negative bacteria demonstrated that their sequences diverged significantly from the consensus sequence of typical promoters. This deviation was most notable in the -35 region, and in the first T in the

consensus TATAAT in the -10 region. A variety of genes are positively regulated by CAP, the catabolite activator protein (reviewed by de Crombrugghe et al., 1984). When cyclic AMP (cAMP) levels are high in response to glucose starvation, CAP stimulates initiation of transcription. The lac$P_1$ promoter is positively stimulated by a 20-fold increase in $K_B$, the binding constant, (Malan and Kolb, 1984) while the $\lambda$ -repressor increases the transcription rate of the $\lambda$ $P_{Rm}$ promoter by an 11-fold increase in $k_f$ (the isomerization step). These positive regulatory elements discussed above can also act negatively on transcription; cAMP-CAP binding at the $P_2$ promoter of the gal operon, (Musso et al., 1977) and $\lambda$ repressor preventing the establishment of the lytic phase (reviewed by Brammar and Hadfield, 1984). Repressors and activators typically bind DNA as dimers (or higher multimers). The structure of three control proteins has been determined using X-ray crystallography, and models for the specific complexes between DNA and cI, cro and the CAP-cAMP complex have been proposed (reviewed by de Crombrugghe et al., 1984). All three proteins appear to bind right-handed DNA by the interaction of two $\alpha$-helical polypeptide units in the major groove of the DNA. The bound DNA sequence varies from 14 to 20-bp in length, and often displays a dyad axis of symmetry.

### 1.3.2 Termination of transcription

Control of the termination of transcription, as well as its initiation, can be important in the control of gene expression. The primary function of the termination process is to partition

transcription into units such that genes or operons may be regulated independently of one another. Termination sites may also be located within genes or operons where they are known as attenuators. The expression of the tryptophan biosynthesis operon (trp-operon) in E.coli is controlled not only by a repressor of transcriptional activation (Zubay et al., 1972), requiring tryptophan as a co-repressor, but also by attenuation; that is transcription termination before the start of the structural gene (Morse and Morse, 1976; Bertrand et al., 1976; Yanofsky, 1981).

The regulator site is called the attenuator, and lies within the transcribed leader sequence of 162 nucleotides that precedes the initiation codon of trpE. The structure of this attenuator is shown in Fig. 1.4. When tryptophan is scarce, ribosomes translating the RNA leader sequence stall at the tandem Trp codons due to a shortage of tryptophanyl-tRNA (Bertrand and Yanofsky, 1976). As sequence 1 can then no longer base pair with sequence 2, it is free to base-pair with sequence 3. This disrupts the formation of the terminator structure (3 pairing with 4), and transcription is allowed to proceed through the tryptophan structural genes, generating full length mRNA. This mechanism of antitermination relies upon the physical disruption of the terminator RNA structure; antitermination dependent on the interaction of protein factors has also been demonstrated as a mechanism of transcriptional regulation. The best characterized example of this protein-regulated antitermination is provided by phage $\lambda$. Following $\lambda$ infection, or prophage induction, the immediate early genes N and cro are transcribed

FIG. 1.4

Secondary structure of trp RNA

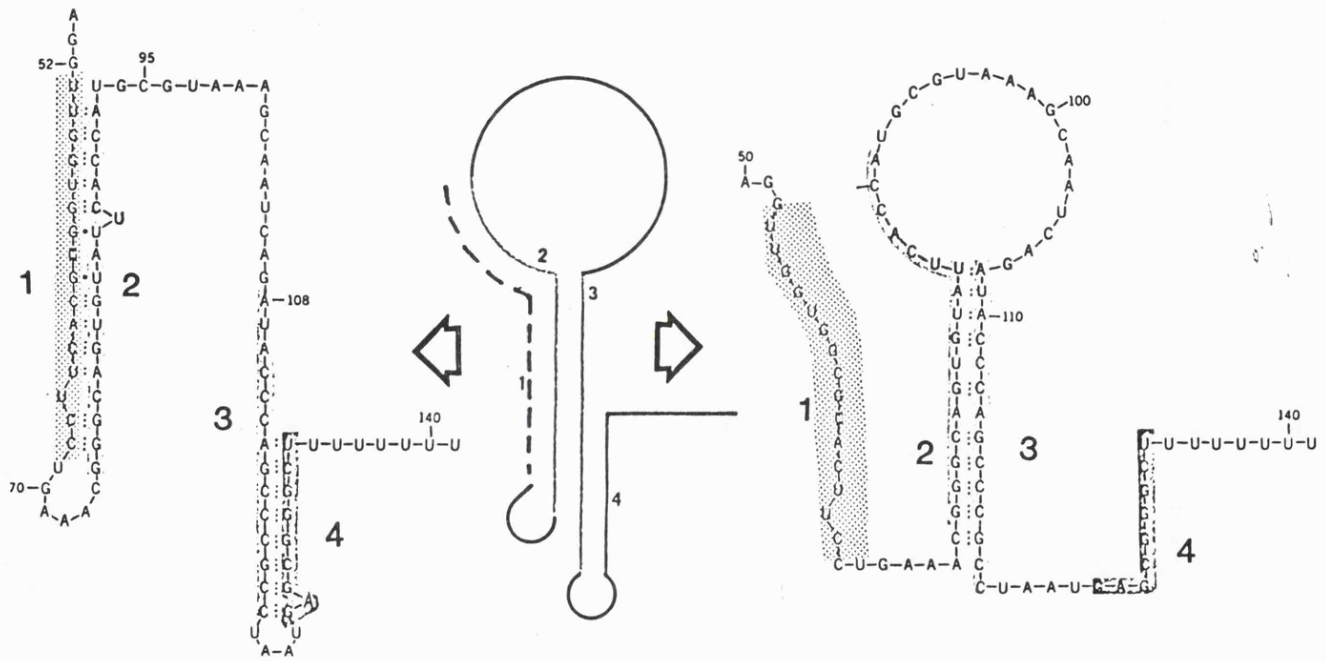
The central figure shows the trp leader RNA with its 2 sets of inverted repeats.

(a) In this configuration 1 pairs with 2, while 3 pairs with 4 forming the stop site (attenuator).

(b) In this configuration hindered ribosomes (Kolter and Yanofsky, 1982) have modified the mRNA structure so that 2 pairs with 3. Note that the terminator structure is no longer formed.

(a)

(b)



respectively to the left and right from the promoters $P_L$ and $P_R$ by RNA polymerase. This transcription stops at the end of genes N and cro at the rho-dependent terminators $t_{L1}$ and $t_{R1}$. The prerequisite for the function of the N protein is the presence of a site, (possessing a dyad axis of symmetry), proximal to the terminator called nut (for N-utilization). Along with these conserved sequences another sequence, known as the 'Box A sequence' is required. A number of bacterial gene products are also required for N-protein function, among which are the products of the nusA and nusB genes (Luk et al., 1982). During elongation, RNA polymerase passes the Box A element of the nut site. At this point the polymerase changes configuration, allowing the binding of the NusA protein, followed by the subsequent binding of N-protein to NusA. This modification renders the polymerase unresponsive to termination signals.

### 1.3.3 Recombinational control of gene expression

Phase variation in Salmonella typhimurium is governed by the alternate expression of two non-allelic genes for flagellin. The gene for H1 flagellin is only transcribed in the absence of transcription of the H2 flagellin gene. This is brought about as a result of H2 forming an operon with the gene rhl (encoding a repressor of H1), thus the co-ordinate expression of the H2-rhl operon expresses H2 and represses H1. The expression of the H2-rhl operon is regulated by a promoter carried on a 995-bp invertible DNA segment (Zieg and Simon, 1980). When the invertible segment is in one orientation H2-rhl is expressed; in the other orientation the operon is switched off. The invertible DNA segment is bounded by two 14-bp

inverted repeats and contains within it a trans-acting gene, hin, the product of which is responsible for catalysing the inversion (Zieg and Simon, 1980). Thus the orientation of the invertible region determines in cis whether the H2-rhl operon is transcribed, and the Rhl protein determines in trans whether the H1 gene is transcribed (Fig. 1.5).

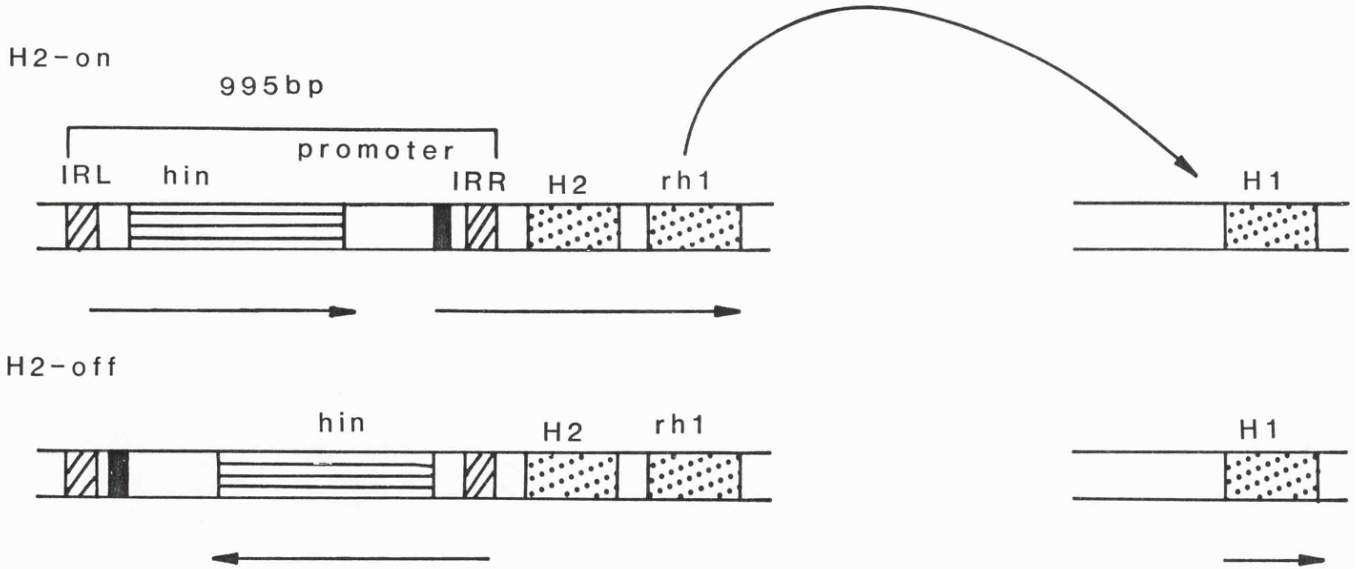
This site-specific recombinational control has been found in a number of organisms wherein it controls a variety of unrelated functions. Thus, inversion of the G-loop, a 3000-bp invertible DNA segment in phage Mu, specifies the host range of the phage. In one phase, the '+' orientation, a 56K protein is encoded which allows phage Mu to infect E.coli K12, while the '-' orientation encodes a 48k protein which allows phage Mu to infect E.coli C and Citrobacter freundii. The G-loop has 50-bp inverted repeats at its ends, but the gene responsible for inversion, gin, lies outside the loop. Phage P1 has an invertible segment, the C-segment, which is highly homologous to the G-loop of phage Mu, and its site-specific inversion is controlled by the cin gene product. Inversion of an 1800-bp region, the P segment of the e14 prophage in the E.coli chromosome has recently been reported to be controlled by the pin gene product.

All these inversion reactions are closely related: the element-encoded recombinases - Gin, Cin, Hin, and Pin - are highly homologous and can complement each other. The recombination sites also share homologous sequences. The in vivo and in vitro

FIG. 1.5

Phase variation in S. typhimurium

The hin region of S. typhimurium showing both orientations of the invertible segment. In one orientation the H2-rhl operon is transcribed, giving rise to the flagellin protein H2, and the repressor of H1, Rh1. When inversion occurs, the H2-rhl operon is switched off, freeing transcription of the flagellin gene H1.


H2-on
995bp
promoter
IRL
hin
IRR
H2
rh1
H1
H2-off
hin
H2
rh1
H1

analysis of inversion due to Gin (Kahmann et al., 1985), Hin (Johnson and Simon, 1985) and Cin (Huber et al., 1985) has demonstrated the existence of DNA sequences that act as enhancers of site-specific recombination. These regions behave in a fashion formally analogous to eukaryotic transcriptional enhancers, in that they act in cis in a distance and orientation-independent fashion. (Craig, 1985).

There are a number of other examples of DNA rearrangements, dinstinct from inversions, which occur at high frequency, and can affect gene expression. One such well-characterized example is that of gene amplification, a phenomenon which occurs spontaneously over a wide range of frequencies in bacteria, phage, and plasmids (Anderson and Roth, 1979; Stark and Wahl, 1984). Chromosomal cat gene amplification has accounted for increased chloramphenicol resistance in E.coli (Meyer and Iida, 1979) and B. subtilis (Young, 1984). Growth of P.mirabilis harbouring the plasmid NR1 in the presence of chloramphenicol (or any of the antibiotics to which the resistance-determinant (r-det) region of the plasmid conveys resistance) results in an increase in copy number of the resistance genes (Rownd and Michel, 1971; reviewed by Broda, 1979). This selective amplification usually proceeds via tandem duplication of the resistance genes (Fig. 1.6) as a consequence of recombination between direct repeats of IS1, which exists at either end of the r-det region.

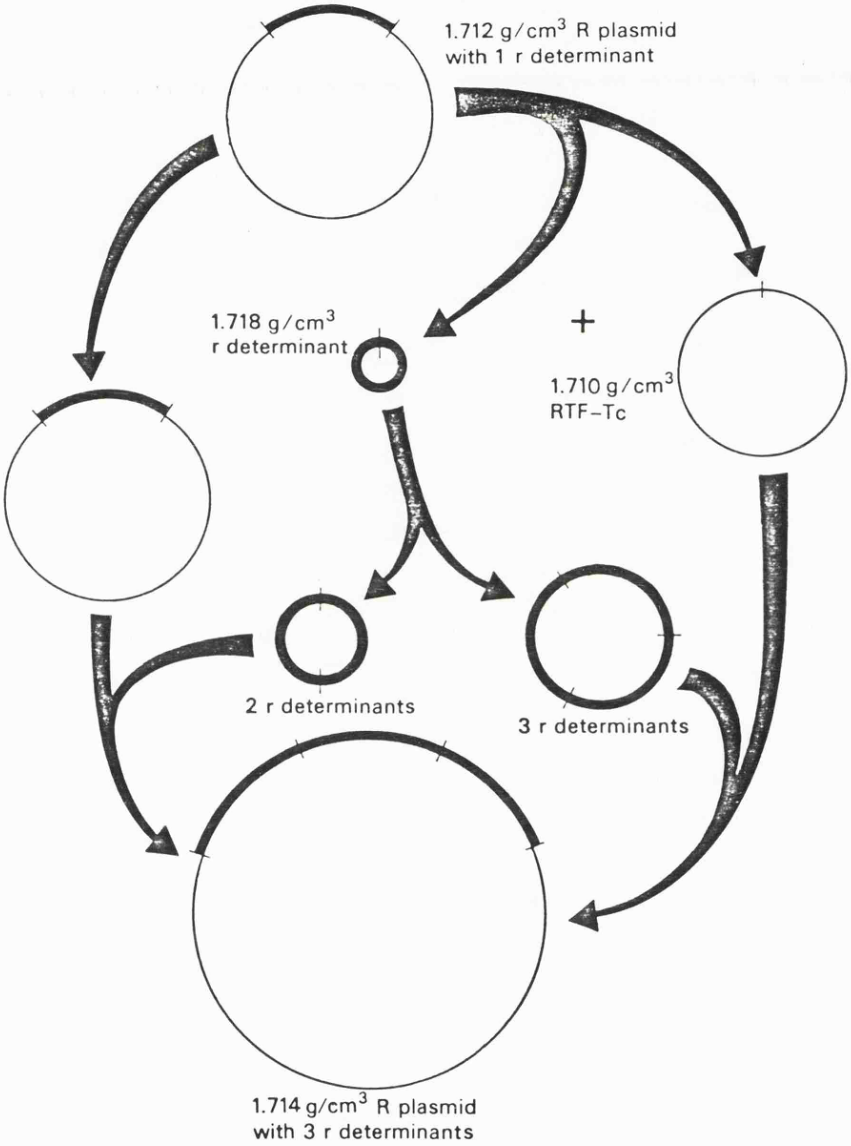
In addition to these relatively well characterized rearrangements that regulate gene expression, there exists a variety

FIG. 1.6

Transitioning of R100 (NR1) in P.mirabilis: a possible scheme

The plasmid R100 comprises a resistance transfer fragment which contains a tetracycline resistance determinant (RTF-Tc) and a resistance determinant region (r-det) flanked by IS1 sequences. Growth of P.mirabilis harbouring NR1 in chloramphenicol results in the formation of poly r-det regions which integrate back into NR1. This gene amplification results in increased antibiotic resistance.

Growth of strains harbouring NR1 (which have undergone 'transitioning'), in the absence of chloramphenicol results in the loss of the poly r-det regions. After Broda, In "Plasmids", (1979), W.H. Freeman.


1.712 g/cm3 R plasmid
with 1 r determinant
1.718 g/cm3
r determinant
+
1.710 g/cm3
RTF–Tc
2 r determinants
3 r determinants
1.714 g/cm3 R plasmid
with 3 r determinants

of examples of more complex changes (Simon and Silverman, 1984). Among these are the mechanisms involved in the regulation of both the pilin (P) and opacity proteins (Op) in N. gonorrhoeae (Stern et al., 1985). Colonies of N. gonorrhoeae can give rise to piliated ($P^+$) and non-piliated ($P^-$) variants; the $P^+$ to $P^-$ conversion occurring at much higher frequency than the reverse $P^-$ to $P^+$ switch. It has been suggested (Meyer et al., 1982; Segal et al., 1985) that the expression of different surface antigens may depend on the movement of a basic silent copy to a new site (expression linked copy) rather like the situation described for antigenic variation in trypanosomes (Pays et al., 1983) or the yeast mating-type interconversion system (Herskowitz, 1983).

Another cell surface component of N. gonorrhoeae whose expression shows a similar variation is that of the opacity proteins (OP); $OP^+$ colonies can give rise to $OP^-$ variants, and vice versa. In this case, however, minor sequence variations rather than gross genomic rearrangements have been detected. Stern and co-workers, 1985, concluded that random point mutations alone could not account for the reversibility and frequency of occurrence of OP variation, and that a more likely explanation was the possibility of recombination and/or gene conversion at the sites of OP expression.

### 1.3.4 Changes in superhelicity can regulate gene expression

In addition to these mechanisms, gene expression may be regulated by the insertion of a transposon (carrying a promoter)

that can direct transcription of adjacent genes (Iida et al., 1983; Kleckner, 1983). Transposon insertion can also modulate gene expression by altering the local conditions of superhelicity, thus IS1 or IS5 insertions near the cryptic bgl genes in E.coli were found to modulate gene expression (Di Nardo et al., 1982), as was the insertion of the transposon gamma-delta near to the ebgA gene (Stokes and Hall, 1984).

### 1.3.5 Post-transcriptional regulation

Post-transcriptional regulation of gene expression has been described for a variety of genes, and has been observed to operate by different mechanisms. The secondary structure of the mRNA is important in the inducible regulation of the erythromycin resistance genes of gram-positive bacteria (reviewed by Weisblum, 1984). A stable mRNA transcript is constitutively produced by the erythromycin resistance gene (Shivakumar et al., 1980), albeit in an inactive form (Fig. 1.7). The 5' end of this mRNA species contains a set of four (numbered 1 to 4 in Fig. 1.7) inverted complementary repeat sequences which are capable of assuming alternative conformations. Thus 1 and 2, and 3 and 4 can pair in one conformation, or alternatively 2 and 3 can pair leaving 1 and 4 free. The 1 + 2, 3 + 4 association is characteristic of the inactive conformation, as the ribosome binding site (Shine-Dalgarno sequence, SD-2) is sequestered in the secondary structure of the 3 + 4 loop. When erythromycin is present, ribosomes translating the 19 amino acid leader sequence stall. This results in the alternative conformation of the mRNA structure forming, in which

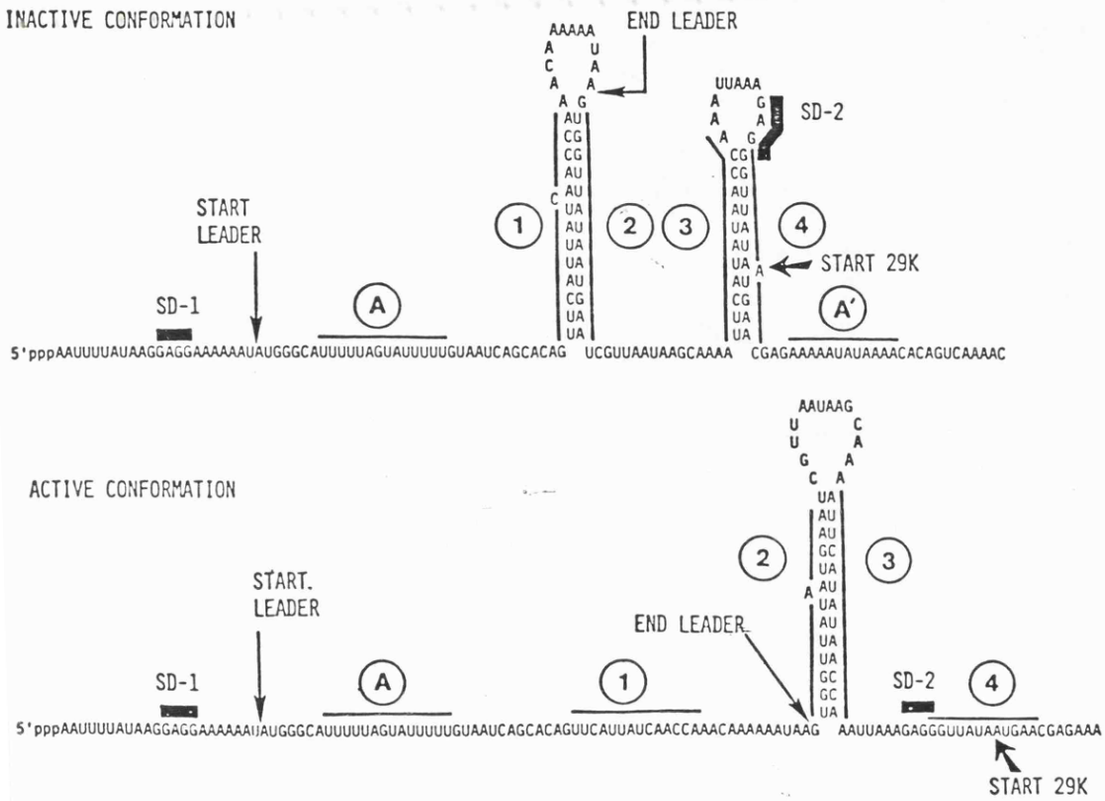
FIG. 1.7

Secondary structures for the $Em^R$ gene RNA

The 5' end of the mRNA encoding the $Em^R$ methylase is shown with the four complementary inverted repeats; 1, 2, 3 and 4 (and an additional set A and A' bearing no sequence similarity to 1, 2, 3 or 4).

(a) shows the 1 + 2, 3 + 4 configuration. The mRNA is inactive because the Shine-Dalgarno sequence for the methylase gene (SD-2) is sequestered in secondary structure.

(b) represents an alternative configuration. The conformational rearrangement results from the hindered translation of a 19 amino acid 'leader' peptide. If the ribosomes stall during translation the 1 + 2 configuration is disrupted, allowing the formation of 2 + 3. The free ribosome binding site (SD-2) is then free to bind ribosomes, and thus translate the message.


INACTIVE CONFORMATION
END LEADER
SD-2
START LEADER
START 29K
SD-1
A
A'
1
2
3
4
5'pppAAUUUUAUAAGGAGGAAAAAAUAUGGGCAUUUUUAGUAUUUUUGUAAUCAGCACAG UCGUUAAUAAGCAAAA CGAGAAAAAUAUAAAACACAGUCAAAAC


ACTIVE CONFORMATION
START. LEADER
END LEADER
SD-1
SD-2
A
1
2
3
4
5'pppAAUUUUAUAAGGAGGAAAAAAUAUGGGCAUUUUUAGUAUUUUUGUAAUCAGCACAGUUCAUUAUCAACCAAACAAAAAAUAAG AAUUAAAGAGGGUUAUAAUGAACGAGAAA
START 29K

the inverted repeats 2 + 3 pair, thus freeing the Shine-Dalgarno sequence (SD-2) which is then consequently free to bind to ribosomes, and thus translate the message. This mechanism for the regulation of erythromycin resistance resembles that involved in the transcriptional attenuation of the trp operon (see above), and consequently has been termed "translational attenuation" by Horinouchi and Weisblum (1981).

Post-transcriptional regulation can also occur as a result of the interaction of anti-sense RNA. This RNA forms a duplex with the complementary mRNA thereby preventing efficient translation. This type of translational control has been reported to play a role in the regulation of the transposition of Tn10 in E.coli (Simons and Kleckner, 1983). A similar mechanism has been proposed (Mizuno et al., 1984) to explain the reciprocal regulation of ompF and ompC in E.coli.

The processing of mRNA, resulting in increased or decreased stability of the message can also result in modulation of gene expression. An example of this is given by cleavage of the bacteriophage λ int gene mRNA by RNase III. This cleavage results in faster degradation of the mRNA relative to the unprocessed transcript and thus lower levels of int specified protein synthesis. (Schmeissner et al., 1984). A reversal of this situation occurs in bacteriophage T7 mRNAs, most of which, when processed by RNase III, result in a more stable RNA species (Panayotatos and Truong, 1985).

Results obtained during the course of investigations into the molecular mechanism(s) for the control of cat expression in P.mirabilis PM13 were reviewed in the light of the likely contributions made by one or more of the mechanisms described above.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 CHEMICALS AND ENZYMES

Most chemicals were purchased from BDH or Fisons Ltd. and, unless otherwise stated, were of analytical grade purity. Agarose was from Miles Labs., except low melting temperature agarose, which was from BRL (Gibco Ltd.). Tris (TRIZMA) base, DTT, lysozyme, ethidium bromide, amino acids, nucleic acids, DNase, RNase, vitamins and antibiotics were purchased from Sigma. Sephadex G50 and G25 were from Pharmacia. Trypticase soya broth was from BBL; Bactotryptone, tryptone, yeast extract, Penassay-broth, and Penassay-agar were from Difco. Restriction enzymes were purchased from BRL (Gibco Ltd.), Amersham or Pharmacia. DNA polymerase I (Klenow fragment) and T4 polynucleotide kinase were from BRL; T4 DNA ligase was from New England Biolabs; calf intestinal phosphatase (CIP) was from Boehringer. Pronase was from Calbiochem. Radioactively labelled nucleotides ($\alpha$[$^{32}$P]- ATP, $\gamma$[$^{32}$P]-dATP, and $\alpha$[$^{35}$S]-dATP were supplied by Amersham International. Nitrocellulose was from Anderman & Co. Ltd.

## 2.2 MATERIALS

L-broth (Lennox, 1955)

| | | |
|---|---|---|
| Bactotryptone | 10g/l | |
| Yeast extract | 5g/l | |
| Sodium Chloride | 10g/l | |
| Glucose/Maltose | 1g/l | |
| Magnesium Sulphate | 2.5g/l | (Phage culture medium only) |
| (Adjust pH to 7.0 with 5M NaOH) | | |

L-agar

As above + 15g/l agar (Difco)

(Autoclave)

BBL-agar

| | | |
|---|---|---|
| Trypticase (Baltimore Biological Labs) | 10g/l | |
| Sodium Chloride | 5g/l | |
| Magnesium Chloride | 2.5g/l | |
| agar (Difco) | 15g/l | (Plates) |
| agar (Difco) | 6.5g/l | (Top layer) |

M9 medium

| | |
|---|---|
| $Na_2HPO_4$ | 6g/l |
| $KH_2PO_4$ | 3g/l |
| NaCl | 0.5g/l |
| $NH_4Cl$ | 1.0g/l |

The pH was adjusted to 7.4 and the solution autoclaved, after cooling the following sterile reagents were added.

| | |
|---|---|
| 1M $MgSO_4.7H_2O$ | 2ml |
| 20% Glucose | 10ml |
| 1M $CaCl_2$ | 0.1ml |

For Proteus mirabilis M9 medium the following was added:

0.1% Nicotinic acid 400µl.

For M9 minimal plates 15g/l Difco agar was added before autoclaving. Proteus mirabilis stains were grown in Difco Penassay-broth at the recommended concentration of 17.5g/l of total solids, and on Penassay nutrient plates.

Antibiotic solutions (in distilled water except as noted)

| | | |
|---|---|---|
| D-threo | chloramphenicol | 50mg/ml in ethanol |
| | tetracycline hydrochloride | 12.5mg/ml in 50% ethanol |
| | ampicillin | 20mg/ml |
| | streptomycin sulphate | 20mg/ml |
| | kanamycin sulphate | 20mg/ml |

All antibiotics were sterilized by membrane filtration (0.2µm pore size) with a Swinnex Millipore filter, and stored at -20°C.

PBS2 buffer

| | |
|---|---|
| NaCl | 3.2g |
| KCl | 0.8g |
| $Na_2HPO_4.7H_2O$ | 4.6g |
| $KH_2PO_4$ | 0.8g |
| Distilled Water | to 400ml |

pH to 7.5

TE Buffer

10mM Tris-HCl, pH 7.5

1mM EDTA, pH 7.5

Tris Acetate (TA) Electrophoresis Buffer

40mM Tris base

20mM $CH_3$ COONa (sodium acetate)

1mM EDTA

pH to 8.2 with glacial acetic acid

Tris Borate (TBE) Electrophoresis Buffer

0.9M Tris base

0.9M boric acid

25mM EDTA

1 x SSC

0.15M NaCl

15mM sodium citrate

pH to 7.0

20 x SSPE

174g NaCl

26.6g $NaH_2$ $PO_4.H_2O$

7.4g EDTA

pH to 7.4. Adjust volume to 1 litre.

50 x Denhardt's solution

5g Ficoll

5g poly vinyl pyrrolidone

5g BSA (Fraction V)

Make up to 500ml with sterile water. Filter sterilize and freeze in aliquots.

10 x Kinase Buffer

0.5M Tris-HCl, pH 7.6

0.1M $MgCl_2$

50mM DTT

1mM spermidine

1mM EDTA

10 x Nick - Translation Buffer

0.5M Tris-HCl, pH 7.5

50mM $MgCl_2$

0.1M 2-mercaptoethanol

Quench Mix

10mM Tris-HCl, pH 7.5

10mM EDTA

0.5% SDS

Potassium Acetate (for plasmid isolation)

| | |
|---|---|
| 5M potassium acetate | 60ml |
| glacial acetic acid | 11.5ml |
| distilled water | 28.5ml |

DEPC Treated Water

0.1% DEPC

(Autoclave)

Heat-treated ribonuclease

20mg/ml ribonuclease A (Sigma)

0.2M NaCl

The solution was placed in a boiling water bath for 5 mins. and then allowed to cool to room temperature slowly.

Self-digested Pronase

20mg/ml Pronase (Calbiochem)

Incubated at 37°C for 60 mins. prior to use.

[$^{35}$S] dATP Klenow mix [for 8 clones]

| | | |
|---|---|---|
| [$^{35}$S] - dATP (Amersham) | 5 µl | |
| Klenow polymerase (BRL) | 2.5 µl | (5 units/ µl) |
| sterile water | 64 µl | |

Sequencing dye mix

| | |
|---|---|
| deionized formamide | 100ml |
| xylene cyanol FF | 0.1g |
| bromophenol blue | 0.1g |
| 0.5 M EDTA | 2ml |

Oligonucleotide dye mix

| | |
|---|---|
| deionized formamide | 90ml |
| xylene cyanol FF | 0.2g |
| 10 x TBE | 10ml |

Agarose - gel loading buffer (10 X)

| | |
|---|---|
| bromophenol blue | 0.25% |
| xylene cyanol FF | 0.25% |
| Ficoll (type 400) | 25.0% |

Acrylamide gel mixes

Stock Acrylamide solution

| | |
|---|---|
| acrylamide | 38g |
| bis acrylamide | 2g |
| distilled water to | 100ml |

De-ionize by the addition of 5g of Monobed Resin-MB1 (BDH Fine Chemicals Ltd.) and stir for 10 minutes. Filter, and store at 4°C.

1. 20% Acrylamide / urea (for Oligonucleotide purification)

| | |
|---|---|
| 38:2 acrylamide:bis acrylamide | 25ml |
| urea | 21.5g |
| 10 x TBE | 5ml |
| distilled water | to 50ml |
| 10% ammonium persulphate | 0.3ml |
| TEMED | 15µl |

2. 6% Acrylamide / urea (for gradient sequencing gels)

A) 0.5 x TBE Acrylamide - urea mix

| | |
|---|---|
| 38:2 acrylamide:bis acrylamide | 150ml |
| 10 x TBE | 50ml |
| urea | 430g |
| distilled water | to 1 litre |

B) 2.5 x TBE Acrylamide - urea mix

| | |
|---|---|
| 38:2 acrylamide:bis acrylamide | 150ml |
| 10 x TBE | 250ml |
| urea | 430g |
| sucrose | 50g |
| bromophenol blue | 50mg |
| distilled water | to 1 litre |

Solutions (A) and (B) are mixed (see Chapter 7) to form the gradient sequencing gel.

3. 15% Polyacrylamide Gels (Protein Gels)

A) Running-Gel

| | |
|---|---|
| 30% (w/v) acrylamide, 0.8% (w/v) bis acrylamide | 9.2ml |
| 0.75M Tris-HCl, 0.2% (w/v) SDS pH 8.0 | 13.5ml |
| distilled water | 3.3ml |

B) Stacking-gel

| | |
|---|---|
| 30% (w/v) acrylamide, 0.8% (w/v) bis acrylamide | 0.8ml |
| 0.25M Tris-HCl, 0.2% (w/v) SDS pH 6.8 | 5.0ml |
| Distilled water | 4.2ml |

Running Buffer (for 15% polyacrylamide gels)

0.025M Tris-HCl

0.2M Glycine

0.1% (w/v) SDS

pH to 8.5

10 x Protein-gel loading buffer

0.125M Tris-HCl pH 6.8

1.4M 2-mercaptoethanol

20% (w/v) bromophenol blue

4% (w/v) SDS

4. Restriction Endonuclease Buffers

High: 100mM NaCl; 50mM Tris-HCl, pH 7.5; 10mM $MgCl_2$; 1mM DTT

Medium: 50mM NaCl; 10mM Tris-HCl, pH 7.5; 10mM $MgCl_2$; 1mM DTT

Low: 10mM Tris-HCl, pH 7.5 10mM $MgCl_2$; 1mM DTT

SmaI: 20mM KCl, 6mM Tris-HCl, pH 7.8; 6mM $MgCl_2$
100µg/ml BSA; 6mM 2-mercaptcethanol.

DNase Buffer

50mM $CH_3COONa$ pH 5.6

10mM $MgCl_2$

2mM $CaCl_2$

Coomassie Stain (for Ouchterlony plates)

16ml glacial acetic acid

134ml $H_2O$

50ml 0.1% (w/v) Coomassie Brilliant Blue R250 dye (in ethanol)

Destain solution

7% (v/v) acetic acid

5% (v/v) methanol

Prehybridization mix

6 x SSC

0.5% (w/v) SDS

5 x Denhardt's solution

100 µg/ml denatured salmon sperm DNA

Hybridization mix

6 x SSC

0.01 M EDTA

5 x Denhardt's solution

0.5% (w/v) SDS

100 µg/ml denatured salmon sperm DNA

Packaging extract buffer A

6mM Tris-HCl (pH 8.0)

20mM $MgCl_2$

0.1mM EDTA

30mM 2-mercaptoethanol

Packaging extract buffer B

6mM Tris-HCl (pH 8.0)

50mM spermidine

50mM putrescine

20mM $MgCl_2$

30mM ATP

30mM 2-mercaptoethanol

## 2.3 METHODS

### Glycerol Storage Cultures

L-broth (5ml) was seeded with a single E.coli colony from a culture which had been checked previously for its genetic markers, and incubated overnight at the appropriate temperature (37°C for most strains and 31°C for temperature-sensitive strains). After centrifugation at 3000rpm in an MSE Centaur 1 bench-top centrifuge for 10 minutes, the pellet was resuspended in 850ul of 10mM $MgSO_4$ in 15% glycerol and stored at -20°C.

### Phage Storage

Bacteriophage $\lambda$ stocks were stored in CsCl at 4°C after removal from the block-gradient-purification centrifugation step. No fall in titre of viable phage was detected after 2 years' storage. M13 phage solutions were stored in L-broth at 4°C.

### Plating Cells

A single colony was picked into 5ml L-broth (containing appropriate antibiotics if required for selection) and grown to stationary phase. The cells were collected by centrifugation at 3000rpm for 10 minutes in a bench-top centrifuge as above and were resuspended in 5ml of 10mM $MgSO_4$. The suspension was stored at 4°C and remained viable for up to a month.

Plate Matings

Plate matings were the method of choice for the inter or intrageneric transfer of conjugative plasmids such as R100. An antibiotic-plate (containing such antibiotics so as to select for the presence of the plasmid, and counter select the donor cell), was spread over one half with a thick suspension ($10^9$ cells/ml) of either parent. When dry, a wire loop innoculated with the other parent was streaked from the cell-free half of the plate into the lawn of cells on the other half. After overnight growth at the appropriate temperature (usually 37°C) transconjugants were evident only at the intersection of the 2 strains. Lack of growth of either donor or recipient acts as a control indicating that no parent alone can grow on the plate. Individual transconjugants were picked from the plate, and re-streaked twice for single colonies on the appropriate antibiotic plates.

Preparation of Crude Extracts from Bacteria for CAT assays

Generally, small-scale cultures were sufficient for CAT assays. Normally cultures (10ml) of bacteria in the exponential phase of growth ($A_{660}$ = 0.8) were harvested in an MSE Centaur 1 centrifuge at 4,000 rpm for 10 minutes. The cell pellet was washed, and resuspended in 1ml of a solution of 10mM Tris-HCl containing 0.1mM 2-mercaptoethanol (pH 7.8). The cell suspension was then transferred to a 1.5ml Eppendorf tube. Cells were disrupted by sonication using an MSE 100 watt ultrasonic disintegrator set at maximum amplitude, and fitted with a microtip. The samples were sonicated with 4 x 15 second pulses with an interval of 30 seconds between each pulse to allow for cooling, or until the sample had cleared. The cells were kept on ice throughout the procedure. Cell debris was removed from the samples by centrifugation in a microfuge for 10 minutes at 4°C. The supernatant was transferred to a sterile 1.5ml Eppendorf tube, and kept on ice until assayed. Samples could be frozen at -20°C and retain their activity for several months.

Ouchterlony Double Diffusion in Agar

Double diffusion tests were based on the method of Ouchterlony (1949). A sterile plastic 9cm Petri dish was filled with 20ml of 0.7% (w/v) agar in PBS2 buffer (Methods, 2.2). When set, 3mm diameter holes 7mm apart, were cut in the agarose in the form of a hexagon using a cork borer. Plugs of agarose were removed to form the wells by using a Pasteur pipette attached to a vacuum source.

Cell free extract or purified CAT was used as antigen, and 10µl samples (containing from between 10 to 100µg of protein) were added to the outer wells. Antibody to $CAT_I$ was obtained from W.V. Shaw in the form of a crude immunoglobulin (mostly IgG) fraction of a goat anti-serum. 100µl of this IgG was centrifuged in a microfuge for 2 minutes, the supernatant removed to a clean tube, and diluted 10-fold in PBS2 buffer. 10µl of this diluted IgG was applied to the central wells.

The Petri dish was incubated overnight, or until a precipitin line was visible. Unprecipitated protein was washed out of the gel by 5 x 1 hour changes in PBS2 buffer. The Petri dish was then drained, and a stack of 10 9cm Whatman No. 1 filters placed on top, weighted down with a brick. After 2 hours blotting, the now thin agarose gel was removed from the Petri dish, and the precipitin band visualized by staining in a Coomassie dye solution (Methods, 2.2).

Determinations of Protein Concentration

(Lowry *et al.*, 1951)

Three solutions were made up and stored separately at 4°C; solution A, 2% (w/v) sodium tartrate; solution B, 1% (w/v) cupric sulphate pentahydrate, and solution C, 2% (w/v) sodium carbonate, 0.1M sodium hydroxide.

A standard curve (range 10µg/ml to 500µg/ml) was made from a 1mg/ml stock solution of bovine serum albumin (BSA) fraction V stored at -20°C. Each standard was made up to a final volume of 200µl. Three ten-fold serial dulutions of each protein sample were taken each with a final volume of 200µl.

Solutions A, B, and C were mixed in the proportions 1:1:100 respectively, 1ml of the mixture was then added to each protein sample. After ten minutes at room temperature 100µl of a freshly diluted 50% (v/v) aqueous solution of Folin and Ciocalteu's phenol reagent was added to the mixture and left for a further 30 minutes at room temperature or overnight at 4°C.

Protein concentration was determined at $A_{750}$ in a Pye Unicam SP1800 dual beam spectrophotometer against a protein-free reagent blank.

Radiometric Detection of Chloramphenicol Acetyltransferase

The radiometric assay described by Shaw (1975) was used when the amounts of CAT produced by bacterial lysates were too low to be detected by the spectrophotometric assay (see below).

A sample (usually 0.2ml) of an overnight culture was added to 2ml of L-broth, and 20µl of a solution containing [$^{14}$C]-chloramphenicol (100µCi/ml) and unlabelled chloramphenicol (5µg/ml) was added in a final volume of 0.5ml. After overnight growth at 37°C the samples were transferred to 1.5ml Eppendorf tubes and centrifuged for 1 minute in a microfuge. 750µl of supernatant was transferred to a new large Eppendorf tube, and extracted with an equal volume of ethyl acetate. The extraction process was repeated twice and the ethyl acetate supernatants pooled, and evaporated to dryness in an air stream. The sample was then resuspended in 30µl of ethyl acetate, and spotted onto a silica gel chromatography plate along with a control of 1-acetate, 3-acetate and 1,3-diacetate standards. The samples were then subjected to ascending chromatography in chloroform - methanol (85:15) and the radioactive acetylation products identified by their RF value relative to known standards.

Spectrophotometric Assay of Chloramphenicol Acetyltransferase

CAT activity was routinely assayed using the spectrophotometric method of Shaw (1975).

The acetylation of chloramphenicol to chloramphenicol-3-acetate, uses acetyl CoA, and generates CoASH. It is the reaction of this reduced CoA with the compound 5,5'-dithiobis (2 nitrobenzoic acid) (DTNB) which is the basis of the spectrophotomeric assay. In the above reaction the mixed disulphate of thionitrobenzoic acid and CoA, and a molar equivalent of free 5-thio-2-nitrobenzoate are produced. The latter has a molar extinction coefficient of 13600 litre/mol cm at pH 7.8 and its concentration can be followed at 412nm., using a Pye Unicam SP1800 dual-beam spectrophotometer fitted with a chart recorder, and a water bath set at 30°C.

For CAT assays, a fresh solution of DTNB was made by dissolving 4mg of DTNB in 9.6ml of 50mM Tris-HCl, 1mM EDTA pH 7.8. Cuvettes containing 960µl of this solution were prewarmed to 30°C in the spectrophotometer chamber for 10 minutes. Next 10µl of 10mM acetyl CoA was added, and the solution mixed. 10µl of crude cell extract was added, the solution mixed, and the chart recorder switched on to determine the background rate of acetylation. Finally, 20µl of 5mM Cm (in 50mM Tris-HCl, 1mM EDTA, pH 7.8) was added, the solution mixed, and the initial rate of chloramphenicol acetylation followed on the chart recorder. Rates were measured for between 30 to 60 seconds, and the CAT activity calculated.

Preparation of Acetyl CoA

Acetyl CoA was prepared in the following way: 100mg of coenzyme A, (Boehringer) was dissolved in 7ml cold (4°C) distilled water, and was mixed with 1ml of cold potassium bicarbonate. A total volume of 16µl of acetic anhydride was then added over a period of five minutes. After 10 minutes on ice the level of reduced CoA was estimated by adding 10µl of the solution to 990µl of freshly prepared 1mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in 50mM Tris-HCl (pH 7.8), 0.1mM disodium EDTA. The $A_{412}$ was then read against a DTNB solution blank using a Pye Unicam SP1800 dual beam spectrophotometer. If the solution gave an absorbance of greater than 0.02 O.D units further acetic anhydride was added. When the $A_{412}$ reading had dropped to 0.02 O.D units the concentration of acetyl CoA was determined.

Small Scale Purification of Chloramphenicol Acetyltransferase

Purification of P.mirabilis PM13 CAT was carried out using an affinity-column protocol (Zaidenzaig et al., 1979; Packman and Shaw, 1981). The affinity resin "K2", (Packman and Shaw, 1981) was pre-treated by soaking a sample (5ml) in a solution of 6M guanidine hydrochloride at 4°C for 1 hour. The resin was then washed with water followed by washing with several volumes of 'CAT buffer': 50mM Tris-HCl, 0.1mM 2-mercaptoethanol, 0.1mM $Na_2$ EDTA pH 7.8. The washed resin was then used to pour a column in a 1ml short-form Pasteur pipette plugged with siliconized glass wool.

A 50ml culture of P.mirabilis PM13, grown in the presence of chloramphenicol (50μg/ml) to $A_{660}$ = 0.8, was disrupted by sonication as previously described. The resulting extract was heat-shocked (by holding the sample in a 60°C water bath for 10 minutes), then cooled on ice. The sample was then centrifuged in a microfuge for 10 minutes and the cleared lysate dialysed against 3 changes of 'CAT buffer' (described above) for 1 hour. The CAT in the sample was assayed for activity by the spectrophotometric method. The cleared lysate was then added to the top of the affinity column, and unbound material washed through by the addition of 5 column volumes of 0.3M NaCl, 50mM Tris-HCl, 0.1mM 2-mercaptoethanol, 0.1mM $Na_2$ EDTA (pH 7.8). The resulting column eluent was monitored for the presence of protein by following the absorbance at 280nm. When the absorbance had fallen to less than $A_{280}$ = 0.02 the CAT was eluted from the column in a buffer containing 1.0M NaCl, 5mM Cm. The eluent was collected in fractions and assayed for CAT activity by the spectrophotometric assay. Those fractions containing CAT were then dialysed against an excess of 'CAT buffer' (see above) to remove the salt and chloramphenicol. Purified CAT was frozen at -20°C.

Polyacrylamide Gel Electrophoresis of Protein Samples

Polyacrylamide gel electrophoresis was carried out under the conditions described by Laemmli, (1970). Typically 15% (w/v) acrylamide gels were poured between upright glass gel-formers (150mm x 140mm x 1.5mm). The 'running-gel' (Methods, 2.2) was

polymerized by the addition of 750µl of a freshly prepared solution of 10% (w/v) ammonium persulphate followed by 50µl of TEMED, and the gel poured within 3cm of the top. A buffer saturated n-butanol solution was carefully layered on top of the gel while it was setting in order to generate a flat surface. After the gel had polymerized the n-butanol layer was poured off and the 'stacking-gel' (Methods, 2.2), polymerized by the addition of 250µl of the above ammonium persulphate solution and 15µl of TEMED, poured on top. The gel-forming comb was immediately inserted, and the gel allowed to set. Protein samples, in a maximum volume of 50µl, were mixed with 10 x loading buffer (Methods, 2.2) in a 1.5ml Eppendorf tube, and were placed in a boiling water bath for 10 minutes prior to loading. Electrophoresis was carried out in a glycine buffer (Methods, 2.2) at 100V until the sample had entered the 'running-gel' at which point the voltage was reduced, and the sample run at between 3-4V/cm overnight. After electrophoresis the gel was stained in a solution of 50% methanol, 10% acetic acid, containing 0.1% Coomassie Blue Stain R250. The stained gel was then destained (in a solution of 10% methanol, 10% acetic acid) until the desired amount of unbound dye had been removed.

Phenol Extraction of DNA

Solid phenol (Fisons) was purified by distillation, equilibrated with 0.5M Tris-HCl, pH 7.4, and made 0.1% with respect to 8-hydroxy-quinoline. The phenol was aliquoted, and stored at -20°C.

For phenol extraction of DNA the Tris-equilibrated phenol was mixed with equal volumes of chloroform/isoamyl alcohol (24:1). An equal volume of this phenol/chloroform/isoamyl alcohol mix was added to the DNA solution, and the two phases mixed by vortexing for 2 bursts of 10 seconds duration. These extractions were normally performed in large Eppendorf tubes, and the organic and inorganic phases were conveniently separated by centrifugation in a microfuge for 2 minutes. The upper aqueous phase was removed, taking care not to disturb any material at the interface, and transferred to a sterile Eppendorf tube. Normally only one phenol extraction was carried out on a DNA sample, but when samples had been subjected to the action of the enzyme alkaline phosphatase, 2 phenol extractions were employed. Traces of phenol in the aqueous phase were removed by extraction with chloroform, followed by a diethyl ether extraction.

Precipitation of DNA in Cold Ethanol Solution

Ethanol precipitation was the routine method employed to precipitate DNA from solution. One tenth of a volume of 2.5M sodium acetate (pH 5.6) was added to the DNA solution, followed by 2.5 volumes of ethanol. The mixture was incubated at -70°C for 10 minutes in a dry ice/ethanol bath, or overnight in a -70°C freezer, and centrifuged for 10 minutes in a microfuge. The supernatant was removed carefully so as not to disturb the pellet which was then washed with 500µl of a 70% ethanol (v/v) solution. The DNA pellet was recovered by centrifugation in a microfuge for 5 minutes, dried under vacuum, and resuspended in the desired buffer.

Purification of DNA on Sephadex G-50 and G-25 Columns

Sephadex G-50 (fine) and G-25 (fine) were equilibrated with water (following manufacturers recommendations on swelling), and then autoclaved. Two types of 'mini' Sephadex column were employed to purify DNA, G-50 columns were used with DNA fragments which had been cut from gels, whereas G-25 columns were used to purify oligonucleotides. The slurry was poured into a 1ml sterile disposable syringe, which had been plugged with sterile siliconized glass wool. The mini-column was placed in a 10ml disposable tube, so that the lugs at the top of the syringe were supported by the lip of the tube.

The columns were centrifuged at 2000rpm for 2 minutes in an MSE bench-top centrifuge. It is important to equilibrate the column such that the volume of sample loaded on top of the column is the

same as volume collected after centrifugation. To do this, 100µl of sterile $H_2O$ was loaded, and the column centrifuged under identical conditions. When the volume loaded = volume collected (usually 2 to 3 steps) the DNA sample to be purified was loaded.

Denatured Salmon Sperm DNA

The salmon sperm DNA was purchased from Sigma (Sigma Type - III sodium salt) and was dissolved in water at a concentration of 10mg/ml. The DNA was sheared by passing it three times through an 18-gauge hypodermic needle. The solution was then boiled for 10 minutes, and stored at -20°C in 500µl aliquots. Just prior to use the DNA was boiled for 5 minutes and quenched on ice.

Ligation

The ability of $T_4$ DNA ligase to join covalently lengths of DNA *in vitro* is of primary importance in the construction of recombinant DNA molecules. The DNA substrates may either have complementary single-stranded extensions (so called "sticky-ends") or blunt ends, although for blunt-ended ligation approximately 10 fold more ligase is required. Ligations were carried out in reaction volumes of between 10µl and 50µl containing 1 x ligation buffer (50mM Tris-HCl, 10mM $MgCl_2$, 10mM DTT, 10mM ATP (pH 7.0)). The total concentration and relative amount of each DNA species in the reaction mixture is critical, and conditions which maximize the final yield of recombinants can be calculated from theoretical analysis (Dugaiczyk *et al.*, (1975). In practical terms however, a normal plasmid ligation reaction consisted of a three fold molar excess of the DNA fragment to be inserted over the chosen vector. The reaction was incubated at 12°C for 8-20 hours, the enzyme inactivated with heat (70°C, 5 mins) and the recombinant DNA molecules recovered by transformation or *in vitro* packaging.

Phosphatase Treatment

In any cloning experiment construction of recombinants is aided by the avoidance of self ligation of the vector. This can be accomplished by treating the vector with alkaline phosphatase which removes 5'- terminal phosphate groups. Circularization of the vector can then occur only by insertion of un-phosphatased foreign DNA, which provides one 5'-terminal phosphate at each join.

Calf intestinal phosphatase (CIP) (Chaconas and Van de Sande 1980) purchased from BCL as an ammonium sulphate precipitate, was pelleted, and resuspended in sterile water before use. 0.01 units of CIP are required to remove the terminal phosphate groups from 1pmole of 5'-ends of DNA. Routinely 0.5 units of CIP were used per microgram of DNA, and was added at the start of a restriction digest. The phosphatase was then inactivated and removed along with the restriction endonuclease during phenol extraction.

Restriction Digests

Type II restriction enzymes are of greater practical use for Molecular Biologists than the type I or type III enzymes. They have an absolute requirement for $Mg^{2+}$ ions, and other factors, such as NaCl concentration, pH, and temperature can affect the rate of reaction. Assay buffers which optimize the rates of reaction are described by the manufacturers, and are often different for each enzyme. In most cases standard restriction enzyme buffers containing either low, medium, or high concentrations of NaCl (Method, 2.2) were used successfully. Digests were incubated for between 1 to 4 hours at 37°C, except BstEII (60°C) TaqI (65°C) and BclII (50°C). Typically 5 units of restriction enzyme (where 1 unit is defined as the amount of enzyme required to cleave 1 µg of phage λ DNA to completion in 1 hour) were added per microgram of DNA. Digests were carried out in reaction volumes of from 10µl to 50µl and reactions were routinely stopped by phenol extraction when subsequent enzymatic manipulations were to be performed, or by

addition of a gel loading buffer (Methods, 2.2) containing EDTA. Digests containing λ phage DNA were always heated to 65°C for 10 minutes prior to gel electrophoresis in order to dissociate the sticky cos ends.

DNA Size Markers

The size of DNA fragments can be deduced from their mobilities in agarose gels when compared with standards of known molecular weight. To facilitate this procedure a computer programme (GELFIT, C. Boyd Ph.D. Thesis, University of Leicester, 1983) was used. The programme incorporates the reciprocal plot of Schaffer and Sederoff (1981), and experience showed an error of from 1 to 2% could be expected using this method. The size of the λ -phage DNA bands used as standards were derived from the published sequence (Sanger et al., 1983).

Agarose Gel Electrophoresis

Both vertical and horizontal slab gels were used in this work, varying in size and agarose concentration depending on the nature of the samples to be electrophoresed. Agarose concentrations varied from 0.6% (for high molecular weight DNA) to 1.5% (for low molecular weight DNA). All agarose gel electrophoresis was carried out in TAE electrophoresis buffer (Methods, 2.2).

Vertical gels (14cm wide, 10cm high, 3mm thick) were supported by a 10ml plug of cyanogum 41 (Sigma) polymerized with 50µl of freshly prepared 10% (w/v) ammonium persulphate and 20ul of TEMED. These gels were used for Southern transfer experiments where the gel thickness (3mm) aided rapid transfer. Vertical gels were run overnight at 15V.

Horizontal submarine gels were used for the bulk of electrophoresis experiments, and were of two varieties. Mini gels (30ml volume) were used for rapid verification of DNA samples, whereas larger gels (200ml volume) were used for restriction analysis. Both large and mini-gels were sealed at the ends with autoclave tape, and sample wells were formed by inserting a perspex comb at one end of the horizontal gel former.

Agarose (in 1 x TAE buffer) was routinely melted in a microwave oven set at medium-low (250W) for 15 minutes. The Agarose-TAE buffer was allowed to cool to 60°C before pouring into the gel former.

When the gel had set, the comb and autoclave tape were removed, and the gel placed in the appropriate electrophoresis tank. Horizontal gels were run just submerged in 1 x TAE buffer at either 150V for an hour, or at 20V overnight. Samples were loaded into gels in loading buffer (Methods, 2.2). After electrophoresis, the gel was stained in 2.5µg/ml ethidium bromide for 15 minutes, followed by destaining in water for 15 minutes. Alternatively, ethidium bromide was added to the gel and electrophoresis buffer (at a concentration of 0.5µg/ml) prior to a run, so that only destaining was required. Gels were viewed under long wave UV. light provided by a Fotodyne 3-3000 transilluminator, and were photographed on Polaroid type 55 or 57 4"x5" land film using a Polaroid MP-4 camera fitted with a Kodak Wratten no. 9 orange filter.

Polyacrylamide Gel Purification of Oligonucleotides

For purification of oligonucleotides a 20% acrylamide/urea gel was used, and pre-run for 30 minutes in 1 x TBE (Method, 2.2) at 45 watts prior to loading the samples. The gel was run until the bromophenol blue dye (Methods, 2.2) was within 2 inches of the bottom of the gel. This typically took from between 3 to 4 hours, (under these conditions a 17-mer oligonucleotide was located in the middle of the gel). The plates were separated (the gel normally adhered to one of them), and the gel transferred to a layer of clingfilm. A second sheet of clingfilm was placed over the gel, and the oligonucleotides visualized by UV. shadowing. The desired band was cut from the gel, and placed in an Eppendorf tube with

100μl of water, and left for between 2 and 12 hours at room temperature. The oligonucleotides were further purified by spun column chromatography prior to use as sequencing primers or as specific hybridization probes.

Induction of a Thermosensitive Lysogen

(Haseltine and Muller - Hill, 1972)

An overnight culture of the thermosensitive lysogen (grown at 30°C) was used to inoculate 250ml of L-broth in a 2 litre baffled flask. After growth at 30°C to an $A_{650}$ = 0.5 the culture was transferred to an orbital shaking water bath set at 45°C for 20 minutes, then to a shaking incubator set at 38°C where it was grown with rapid shaking. The $A_{650}$ was monitored, and was observed to rise, then drop dramatically as the culture lysed about 1 hour after growth at 38°C. 1ml of chloroform was added and the lysate cleared by centrifugation at 10,000rpm for 5 minutes at 4°C.

Phage Liquid Lysates

Bacteriophage lambda was propagated on E.coli in liquid culture, comprising L-broth supplemented with 10mM $MgSO_4$. The inoculum of phage was derived from a single plaque grown on the appropriate host, and was picked (with a region of surrounding E.coli cells) and blown into 200ml of L-broth, 10mM $MgSO_4$. The culture was shaken vigorously to give good aeration (in a rotary incubator) at 37°C for approximately 18 hours. At the end of this period cell lysis was visually apparent. Prior to harvesting the phage, 1ml of chloroform was added and the culture shaken at 37°C for a further 15 minutes. After the cells had been pelleted the phage suspension was serially diluted, and the number of phage calculated by "spotting" onto indicator plating cells. If at least $10^{10}$ pfu/ml were present, DNA was prepared.

λ Phage DNA Preparation

A 200 ml cleared liquid lysate of at least $10^{10}$ pfu/ml ($10^{12}$ phage gives approximately 50μg of DNA) was made 2% with respect to sodium chloride, and incubated on ice for 1 hour. Polyethylene glycol 8000 (Sigma) was added to 10% and the suspension left for another hour or overnight at 4°C. Phage were recovered from the suspension by centrifugation at 5000rpm for 4 minutes at 4°C. The supernatant was removed, and the pellet resuspended in 1ml of phage buffer.

A stock caesium chloride solution was made by dissolving 65g of CsCl in 35g of $H_2O$. This stock was used to prepare the following solutions.

| Density | CsCl | Phage Buffer |
|---|---|---|
| 1.7g/cm$^3$ | 14.0ml | 6.0ml |
| 1.5g/cm$^3$ | 11.1ml | 8.9ml |
| 1.3g/cm$^3$ | 7.7ml | 12.3ml |

A caesium chloride step gradient was prepared in a 14ml poly-allomer tube by overlaying 4ml of each solution (containing 1.7g/cm$^3$, 1.5g/cm$^3$, and 1.3g/cm$^3$ CsCl).

The resuspended phage suspension was layered on top of this, and the step gradient centrifuged at 24,000 rpm for 4 hours at 4°C in an MSE SW 6x14 Ti rotor. The phage bands were found at

the interface of the 1.3g/cm$^3$ and 1.5g/cm$^3$ CsCl blocks, and were removed with a 21-gauge needle inserted through the tube.

The concentrated phage were dialysed against TE buffer (Methods, 2.2) for 2 hours to remove the CsCl, then treated with RNase (20μg/ml final concentration) for 1 hour at room temperature, with further dialysis against TE. Pre-digested pronase (Methods, 2.2) was added, and dialysis continued at 37°C for another hour, against pronase buffer (10mM Tris-HCl, 0.1mM EDTA, 0.1% SDS, pH 7.4). These steps were facilitated by using a dialysis clip at one end of the tubing so all additions could be conveniently made into the same piece of tubing. The phage DNA was removed from the bag, and subjected to phenol extraction, and the DNA recovered from the solution by ethanol precipitation.

λ $CI_{26}$ prepared in this fashion was digested separately with HindIII and double digested with HindIII and EcoRI for use as size markers in gel electrophoresis.

λ DB286, (Brammar, 1982) was prepared in this fashion and was used to generate λ gene banks.

Isolation of High Molecular Weight Bacterial DNA

(Chow et al., 1977)

A 200ml culture of bacteria was grown to the stationary phase in L-broth or Penassay-broth (Difco) and the cells pelleted by centrifugation at 10,000rpm for 5 minutes at 4°C. The cells were washed twice with an equal volume of ice-cold 10mM Tris-HCl pH 7.9, 1mM EDTA, 100mM NaCl, (resuspension buffer) and then resuspended in 25ml of the same buffer. 5ml of a 10mg/ml lysozyme solution was added, and the cells incubated in a water bath set at 42°C for 10 minutes. Cells were then lysed gently by the addition of 30ml of the resuspension buffer made 2% with respect to Sarcosyl NL97 and containing 20µg/ml RNase A. The solution was incubated in a water bath set at 42°C for 1 hour after which time pronase (Methods, 2.2) was added to a final concentration of 1mg/ml, and the 42°C incubation continued for 4 hours. The lysate by this time had cleared, and was phenol extracted 3 times. The DNA was recovered from the aqueous phase by ethanol precipitation, (in this instance the chromosomal DNA was not pelleted by centrifugation, but was "spooled" out of solution on the end of a glass pipette). The DNA was vacuum dried, and resuspended in a TE buffer (Methods, 2.2), a step which took up to 3 days at 4°C.

In some cases, DNA produced in this fashion could not be digested with restriction endonucleases, and was thus further purified by buoyant density CsCl centrifugation. The typical yield was 2mg from 200ml of cells.

Large Scale Isolation of Plasmid DNA

(Birnboim and Doly, 1979)

10ml of an overnight-stationary phase culture harbouring the desired plasmid (grown in L-broth supplemented with the appropriate antibiotic) was inoculated into 200ml of L-broth plus antibiotic in a baffled 2 litre flask. The culture was shaken vigorously at 37°C overnight, and the cells pelleted by centrifugation at 10,000rpm for 5 minutes at 4°C. Cells were resuspended in 8ml of ice cold 25mM Tris-HCl, pH 8.0, 10mM EDTA, pH 8.0, 50mM glucose, 1mg/ml lysozyme (which was added just before use), and left on ice for 5 minutes. Lysis and denaturation of the bacterial chromosomal DNA was achieved by the addition of 16ml of a solution of 0.2M NaOH, 1% SDS at room temperature, which was swirled gently into the cell suspension. The tube was kept on ice for 5 minutes, and after the addition of 12ml of ice cold 3M KAc, pH 4.8 (Methods, 2.2) the mixture was centrifuged at 16,000rpm for 20 minutes at 4°C with the centrifuge brake off.

The cleared lysate was poured through a sterile siliconized glass wool plug to trap most of the flocular contaminating chromosomal DNA, and the plasmid DNA was then recovered from solution by precipitation with the addition of 0.6 volumes of propan-2-ol. The sample was centrifuged at 10,000rpm for 20 minutes at room temperature, the supernatant removed, and the pellet washed with 70% ethanol then dried under vacuum. Once dried the DNA was dissolved in 7.4ml of TE buffer, and 8.4g of CsCl were added. 100µl of 10mg/ml Ethidium Bromide was added, and the

refractive index of the solution measured (a value of between 1.392 to 1.398 was expected). The final solution was placed in a 10ml polycarbonate tube, topped up with heavy paraffin oil and centrifuged at 40,000rpm for 40 hours at 20°C in an MSE 10 x 10 Ti rotor. Alternatively, the CsCl/DNA solution was pipetted into a 5ml heat-sealable Beckman tube, and centrifuged in an upright MSE 62.5 rotor at 50,000rpm for between 8 and 16 hours. The plasmid band (the lower, or more usually the only band) was visualized by irradiation with short-wave UV. light, and removed from the CsCl gradient with a sterile syringe and needle. DNA banded in the 10ml polycarbonate tubes could be removed from the top of the tube, while DNA banded in the 5ml heat-sealable tubes was removed through a hole punctured in the side. The ethidium bromide was removed by repeated extractions with an equal volume of CsCl saturated isopropanol, then the solution was dialysed against 2 litres of TE (3 changes) at 4°C to remove the caesium chloride. This procedure produced from 200 to 400µg of DNA per 200ml culture for a multi-copy plasmid.

Purification of DNA Fragments

It was often necessary to purify a specific DNA fragment prior to subsequent manipulation. The method of choice depended upon several variables such as the size of the DNA fragment to be purified, the quantity of material to be purified, and the next step itself. It was found that DNA could be recovered quantitatively by most techniques, however, the recovered DNA was often not of sufficient quality for ligation. Ligation in the presence of spermidine was sometimes found to increase the frequency of recombinants.

Recovery of DNA by Electro-elution

The DNA fragment to be purified was cut from the agarose gel and placed in a tube of sterile dialysis tubing containing just enough 0.5 x TAE buffer to cover the fragment. The ends of the tubing were clamped with dialysis clips, and the assembly placed in a mini-geltank containing 0.5 x TAE buffer and 0.5μg/ml ethidium bromide. 150V was applied across the dialysis tubing, and migration of the ethidium bromide stained DNA fragment monitored using a hand held UV. lamp (UVGL-58, UV Products). After 5 minutes the DNA could be visualized as a narrow fluorescent band against the wall of the dialysis tubing. The polarity of the power pack was then reversed for 30 seconds to reduce the adhesion of the DNA to the tubing, and the TAE buffer containing the DNA was removed with a sterile plastic pasteur pipette. Typically 1ml of TAE buffer/DNA was recovered from the dialysis tubing. This

volume was reduced by repeated iso-butanol extractions, which also removed the ethidium bromide. The DNA was then ethanol precipitated, and in cases where ligation was the next step, subjected to purification through a Sephadex G-50 column.

Recovery of DNA by Compression

The agarose gel matrix is easily compressed after freezing. This observation makes it possible to recover the DNA and interstitial buffer from a gel fragment by freezing the fragment, then subjecting it to a centrifugal force. The conditions for this "freeze-squeeze" method have been optimized (Tautz and Renz, 1983), and is useful in recovering fragments of up to 10-kb. The section of agarose containing the DNA fragment was cut from the gel, trimmed to its smallest dimensions, and placed in an Eppendorf tube containing 10 volumes of 0.3M sodium acetate pH 7.0 and 1mM EDTA. After 30 minutes the fragment was transferred to a small Eppendorf tube which had been punctured in the bottom with a 21-gauge needle, and plugged with sterile siliconized glass wool. This assembly was then frozen in liquid nitrogen for 5 minutes. Once frozen the small tube was placed in a large decapped Eppendorf tube, and centrifuged in a microfuge for 10 minutes at room temperature. The eluant was phenol extracted, and the DNA recovered by ethanol precipitation.

Recovery of DNA from low-melting temperature agarose

The substitution of hydroxyethyl groups into the agarose molecule causes the agarose to gel at 30°C and melt at 65°C - well below the melting temperature for most DNAs. These properties are exploited in this technique for the recovery of DNA from gels (Weislander, 1979). The fragment of DNA to be purified was cut from the gel, and placed in an Eppendorf tube containing 5 volumes of 20mM Tris-HCl, 1mM EDTA (pH 8.0). The tube was then transferred to a water bath set at 65°C for 5 minutes to melt the agarose. The solution was then subjected to repeated phenol extractions and the DNA recovered from the aqueous phase by ethanol precipitation.

Sucrose Density Centrifugation

Sucrose gradients are the method of choice when preparing large quantities of fragment. The method was used to prepare bacteriophage λ DB286 arms, and to size fractionate partially digested chromosomal DNA. Sucrose gradients were poured into 17ml Beckman polyallomer centrifuge tubes. The two stock sucrose solutions (10% and 40%) were prepared with BRL ultrapure sucrose dissolved in a buffer containing 10mM Tris-HCl, 10mM $Na_2$ EDTA, and 200mM sodium acetate (pH 8.0). Up to 200μg of the DNA to be fractionated was carefully layered onto the top of the gradient, and the sample centrifuged at 24K rpm for 24 hours at 4°C in a Beckman SW27 rotor. After the run had finished 15-drop fractions were collected through a 21-gauge needle inserted into the

bottom of the tube. 10μl of every third fraction was subjected to gel electrophoresis, and the fractions containing the size range of interest were pooled and dialysed. The dialysed sample was concentrated with isobutanol, and the DNA recovered by ethanol precipitation.

Bacterial RNA Preparation

Any method of RNA preparation must minimize the ribonuclease activity during the initial stages of isolation and avoid the accidental introduction of trace amounts of ribonuclease during subsequent procedures.

To ensure that these requirements were met, a lysis method was chosen that disrupted the cells and inactivated nucleases (Miller, 1972). To ensure that no ribonuclease was introduced during the extraction procedure several precautions were taken:

(a) Sterile disposable plastiware was used for the preparation and storage of RNA whenever possible.

(b) Glassware was baked at 250°C for 4 hours.

(c) Solutions were treated with 0.1% diethylpyrocarbonate (DEPC) for 12 hours, and then autoclaved. (Tris-buffer solutions cannot be treated like this, but can be made in water which has been DEPC-treated).

Cells were lysed by the method described by Miller (1972). 50µl of an overnight culture of cells were inoculated into 10ml of M9 medium (Methods, 2.2) and grown to $A_{550}$ in the range of 0.1 to 0.3. An equal volume of ice-cold M9 medium containing 0.02M $NaN_3$ was added, and the cells pelleted by centrifugation at 3000rpm at 4°C for 5 minutes. The cells were washed with an equal volume of M9/azide buffer, and the cell pellet resuspended in 2ml of a solution of 0.02M Na acetate 0.5% SDS and 0.001M $Na_2$ EDTA, pH 5.5. The cells were then added to 4ml of phenol-chloroform, vortexed, and held in a water bath at 60°C for 5 minutes. The

sample was centrifuged at 3000rpm for 5 minutes in an MSE bench top centrifuge to separate the phases, and the upper aqueous phase removed with a plastic sterile Pasteur pipette. The sample was re-extracted with phenol-chloroform as described above, and the nucleic acid recovered from solution by ethanol precipitation.

RNA was purified away from DNA by two methods described below.

1. After phenol extraction, the cell lysate was layered over 0.2 volumes of 5.7M caesium chloride, 0.1M $Na_2$ EDTA, pH 7.5 in a polyallomer centrifuge tube, and topped up with liquid paraffin. The tube was centrifuged at 25,000rpm, 20°C for 18 hours in a Beckman SW27.1 rotor. The paraffin and supernatant were removed with a sterile plastic disposable pipette, care being taken to remove all the DNA band just above the CsCl pad. The remaining CsCl solution was poured away, and the opalescent RNA pellet resuspended in 1ml of DEPC-treated water (Methods, 2.2). The RNA was recovered from solution by ethanol precipitation, and resuspended in 0.01M Tris-HCl, pH 7.6; 0.4M NaCl, 0.02% SDS.

2. Alternatively DNA was removed from the RNA solution by incubation with RNase-free DNase (BRL) in a buffer recommended by the manufacturers (Methods, 2.2).

Purified RNA, prepared by either method was stored at -70°C in ethanol.

Transformation cf E.coli

Most methods for E.coli transformation are based on an observation of Mandel and Higa (1970), who demonstrated that the uptake of plasmid DNA by cells was enhanced by chemical induced competence brought about by treatment with calcium chloride. Many variations of this basic technique have since been described, all directed toward optimizing the efficiency of transformation. Two methods are detailed in this thesis, and the choice depended on whether either (a) a very high transformation efficiency was required (e.g. for the construction of gene banks) in which case the MOPS/RbCl method was preferred, or (b) a large number of transformations were to be performed simultaneously, in which case the $CaCl_2$ technique was preferred. (N. Grinter personal communication).

(a) MOPS/RbCl Method

2ml of a stationary phase culture of the cells to be transformed were inoculated into 50ml of L-broth and incubated with rapid aeration at 37°C to a cell density of approximately $5 \times 10^7$ cells/ml ($A_{600} = 0.4$). This usually took from between 90 minutes to 2 hours. The culture was chilled on ice, and 1ml samples were dispensed into large Eppendorf tubes, and centrifuged for 1 minute in a microfuge. The cells were washed with 1ml of cold MOPS pH 7.0; 10mM $RbCl_2$, then resuspended in 500ul of cold 100mM MOPS pH 6.5; 50mM $CaCl_2$; 10mM RbCl (competence buffer), and the suspension left on ice for 30-60 minutes. The cells were pelleted

by centrifugation in a microfuge for 1 minute, and gently resuspended in 200µl of competence buffer. 3µl of DMSO and 10-200 ng of DNA (in a volume of 10ul or less) was added, and the cells allowed to stand on ice for 30 minutes. Following a heat-shock in a water bath at 55°C for 45 seconds (to induce uptake of DNA) the cells were immediately removed onto ice, and either plated out immediately, or incubated at 37°C for 1-2 hours in 1ml of L-broth (for expression). After this the cells were pelleted by centrifugation, and plated onto agar plates containing the appropriate antibiotic. Routinely $10^7$ transformants per µg of supercoiled DNA were obtained using this technique.

(b) $CaCl_2$ Method

50ml of log phase cells were grown as above, and pelleted in 25ml Sterilin universals at 3,000rpm, for 10 minutes in a bench top centrifuge set at 4°C. Each pellet was washed with 25ml of 50mM $CaCl_2$, pelleted, and resuspended in one tenth volume of 50mM $CaCl_2$. After 30 minutes on ice 200ul aliquots of cells were placed into large Eppendorf tubes, and were quick-frozen in liquid nitrogen. Competent cells made in this fashion were kept in a -70°C freezer. When required, the cells were slowly thawed on ice, and the DNA (10-200ng) added in a volume of 10µl or less. Cells were then heat-shocked as described above. The method gave approximately $10^5$ transformants per ug of supercoiled vector DNA. Although less efficient than the MOPS/RbCl method, competent cells could be frozen, and thus was the method of choice for M13 transformations.

For M13 phage transformation of JM101, immediately after the heat shock, transformed cells (sufficient to give approximately 100 plaques per plate) were added to 100µl of stationery phase JM101 in a Sterilin tube containing 30µl IPTG (24mg/ml in $H_2O$), 30µl X-GAL (24mg/ml in DMF), and 3ml of molten (50°C) top agar (0.6% L-agar), mixed thoroughly, and poured over the surface of a L-agar plate. For all transformations, positive and negative controls were included, to estimate the efficiency of transformation and check for contamination.

Transformation of P.mirabilis

P.mirabilis cells could not be efficiently or reproducibly transformed by either of the methods employed to transform E. coli. A method used to transform Pseudomonas sp. strains, however, was employed (R. Jeenes, University of Bangor, Personal communication) more successfully.

A 50ml culture of P.mirabilis cells was grown as described above for E. coli transformations. Cells were pelleted by centrifugation at 3,000rpm, 4°C, for 10 minutes in a bench top centrifuge. The pellet was resuspended in 25ml of sterile 0.1M $MgCl_2$, and centrifuged at 3,000 rpm, 4°C, for 10 minutes. The supernatant was removed, and the cell pellet resuspended in 10ml of sterile 0.1M $CaCl_2$. After a 20 minute incubation on ice, cells were re-pelleted by centrifugation (3,000rpm, 4°C, 10 minutes) and resuspended in 2ml of 0.1M $CaCl_2$. At this stage the DNA was added, the cells incubated on ice for 30 minutes, and then heat-shocked as described above. This method produced between 10 and 20 transformants per microgram of supercoiled vector DNA. Plasmid DNA produced by the mini-prep method was found to be unsuitable for transforming P.mirabilis, only DNA that was purified from a CsCl gradient could be used successfully.

Transformation of S.typhimurium

S.typhimurium cells could be transformed using the methods outlined for the transformation of E.coli, the level of transformation being in the order of $10^4$ per µg of supercoiled vector DNA.

DNA Packaging Extracts

*In vitro* packaging of recombinant lambda genomes into mature phage particles was carried out using the method described by Scalenghe *et al.*, (1977). Cell extracts were made from two lysogenic strains harbouring prophages with complementing capsid defects. These prophages have mutations in their *cI* genes which make them heat-inducible and mutations in their *S* genes, so that induced lysogens fail to lyse spontaneously and thus accumulate phage products after induction. The *b2* deletions present in both prophages inactivates the phage attachment site and blocks phage excision after induction. Recombination between exogenous and endogenous phage DNAs is largely eliminated by a *recA* mutation in the host, and by a *redB* lesion in the prophage. The complete genotypes of both packaging strains is listed in Section 2.4.

(a) Preparation of Freeze-Thaw Lysate (FTL)

5ml of an overnight culture of BHB2688 was added to each of 3 x 2-litre flasks containing 250ml of L-broth and the cultures grown at 30°C to an $A_{630}$ of 0.6. 1ml of cells were removed, and stored on ice for the induction test. The flasks were then transferred to a shaking incubator set at 40°C, and 250ml of L-broth, pre-warmed to 62°C was added to each one. After 1 hour induction, a 1ml sample was removed for the induction test. A drop of chloroform was added to each sample of induced, and non-induced cells, (cell clearing in 5 minutes indicated induction). Induced cells were harvested by centrifugation at 10,000rpm at 4°C for 10 minutes.

The supernatant was removed, and each pellet was resuspended in 3ml of a solution of 50mM Tris-HCl pH 7.5, 10% (w/v) sucrose, the cell suspensions were then pooled, and transferred to 2 x 10ml polypropylene tubes. 75µl of a 2mg/ml lysozyme solution was added to each tube, and was gently mixed into solution with the end of a 3ml disposable sterile plastic pipette. The samples were then quick-frozen in liquid nitrogen, for 5 minutes, and allowed to thaw on ice (1 hour). 75µl of packaging buffer B (Methods, 2.2) was mixed into each tube as described above, and the samples centrifuged at 35,000rpm at 4°C for 35 minutes in an MSE 10x10 Ti rotor. 75µl aliquots of the supernatant were then transferred to 2ml Nunc cryotubes, and quick-frozen in liquid nitrogen. This method produced enough FTL for 14 reactions, which were stored in a -70°C freezer.

(b) Preparation of Sonic Extract (SE)

3 x 250ml cultures of the packaging strain BHB2690 were grown, induced, and pelleted as described above for BHB2688. The cell pellets were resuspended in 1ml of packaging buffer A (Methods, 2.2), the samples pooled in a single polypropylene tube, and a further 2.6ml of buffer A added. The cell suspensison was then sonicated with 15 bursts of 5 second duration (with a 30 second pause between each burst) at medium amplitude in an MSE 100 watt ultrasonic disintegrator. Cell debris was pelleted by centrifugion at 10,000rpm at 4°C for 10minutes, and 55µl aliquots of the supernatant placed into Nunc cryotubes and quick-frozen in liquid nitrogen. This method produced enough SE for 14 reactions, which were stored in a -70°C freezer.

Test Packaging Extracts

After each batch of packaging extracts were made they were tested by packaging a lambda DNA sample of known concentration. The FTL and SE extracts were removed from the -70°C freezer, thawed on ice for 10 minutes, and mixed in the bottom of a 1.5ml Eppendorf tube with the following solutions:

| | |
|---|---|
| Buffer A | 7µl |
| λ DNA | 1µl (5µg/ml stock) |
| Buffer B | 1µl |
| SE | 3.5µl |
| FTL | 5.0µl |

The components of the packaging reactions were incubated at room temperature for 90 minutes, and then diluted by the addition of 250µl of phage buffer (Methods, 2.2). A serial dilution of the phage preparation was made in phage buffer, and spotted onto a lawn of C600 plating cells. After overnight incubation at 37°C the efficiency of in vitro packaging was calculated, and expressed as pfu/µg of lambda DNA. Typically packaging extracts produced in this way gave 1-2.5 x $10^8$ phage/µg of λ DNA.

Small-Scale Plasmid DNA Preparation (1)

It is unnecessary for most purposes, to purify large quantities of plasmid DNA to homogeneity. For example, cleavage with restriction endonucleases, ligation, transformation and even DNA sequencing (Wallace et al., 1984) can be carried out on relatively crude preparations of plasmid DNA obtained from small scale cultures. Therefore, a modification (Maniatis et al., 1982) of the Birnboim and Doly method (1979) was found to be sufficient for most purposes. 1.5ml of an overnight culture of the plasmid containing strain was placed into a 1.5ml Eppendorf tube, and centrifuged in a microfuge for 1 minute. The supernatant was removed, and the pellet resuspended in 100µl of 25mM Tris-HCl, pH8.0, 50mM glucose, 10mM EDTA, pH 8.0, 5mg/ml lysozyme. The tube was incubated at room temperature for 5 minutes, after which, 200µl of a freshly prepared lysis solution (0.2M sodium hydroxide, 1% (w/v) SDS) was added. The contents were mixed by inverting the Eppendorf tube 2 to 3 times, and the mixture chilled on ice for 5 minutes. Next, 150µl of an ice cold solution of 3M potassium acetate was added, and the contents mixed by flicking the tube. The sample was then centrifuged in a microfuge for 5 minutes, and the supernatant transferred to a new Eppendorf tube. The solution was phenol extracted, and the nucleic acid removed from the solution by ethanol precipitation.

Plasmid Preparation (2)

Many different methods have been described for plasmid isolation, and it has been noted that a method that works well for one species or strain does not necessarily work well for others.

Notwithstanding this observation, a general method for the isolation of large and small plasmids was used as described by Kado and Liu (1981). 1.5ml of an overnight culture of the plasmid containing strain was placed into a 1.5ml Eppendorf tube and centrifuged in a microfuge for 1 minute. The pellet was resuspended in 500µl of E buffer, (40mM Tris-acetate, 2mM $Na_2$ EDTA (pH 7.9)) and 1ml of lysing solution (3% SDS, 5mM Tris (pH 12.6)). The solution was then incubated in a water bath set at 65°C. After 20 minutes the solution was phenol extracted, and the DNA recovered from solution by ethanol precipitation.

Construction of Genomic DNA Libraries

Genomic libraries were constructed in the plasmid vector pAT153, and the λ replacement vector λDB286, (Brammar, 1982).

For λ gene banks, bacterial chromosomal DNA was prepared as previously described, and partially digested with Sau3A such that the highest yield of fragments was in the 10-15-kb size range. This size range was further purified by centrifugation of the digest through a 10% to 40% sucrose gradient.

The DNA of the replacement vector λDB286 was digested to completion with BamHI and SalI. The BamHI digest removes the central fragment of λDB286 DNA which is replaced by genomic DNA in recombinant molecules. The SalI enzyme cuts within this central fragment, making it easier to purify these small fragments away from the left and right λ-arms on a sucrose gradient.

Purified vector arms, and genomic DNA were separately ligated in the molar ratios 1:3, 1:1, and 3:1. 1μg of vector DNA was used for all three ligations in a total volume of 10μl. Recombinant lambda particles were recovered by in vitro packaging using λ$cI_{26}$, and packaging extracts alone, as positive and negative packaging controls. The titre was estimated by serial dilution followed by spotting onto plating cells of E. coli 5K and E.coli Q359 (a P2 lysogen of 5K which only allows growth of spi⁻ recombinant phage).

Hybridization Techniques

Many protocols have been developed for the detection of nucleic acid bound to solid supports using a variety of radioactive, and non-radioactive probes. Only radioactively labelled DNA probes were used for work in this thesis. The bewildering array of procedures available can now often be simplified (Meinkoth and Wahl, 1984), and the methods described below, while not necessarily optimized, represent the most efficient techniques available at the time.

Nick-translation

Nick-translation was carried out as described by Jeffreys *et al.*, 1980.

When one strand of a double stranded DNA molecule is nicked, *E.coli* DNA polymerase I will eliminate nucleotides from the 5' side of the nick, and sequentially add nucleotides to the 3' hydroxyl side (thus resulting in movement of the nick, hence nick-translation). If $\alpha[^{32}P]$-labelled nucleotide triphosphates are included in the reaction mix, they can be incorporated into the DNA template, generating $\alpha[^{32}P]$-labelled DNA.

The standard reaction mix was set up as follows:

| | |
|---|---|
| DNA (100-200ng) | 5.0µl |
| 10x Nick-mix (2.2) | 2.5µl |
| 50µM dCTP | 2.0µl |
| 50µM dGTP | 2.0µl |
| 50µM dTTP | 2.0µl |

| | |
|---|---|
| $H_2O$ | 8.5µl |
| 8ng/ml DNase I | 1.0µl |
| E.coli DNA Polymerase I(4 units/µl) | 1.5µl |

These constituents were mixed together on ice, 20µCi ( 2.0µl) of $\alpha[^{32}P]$-dATP added and the mixture incubated at room temperature for 60 minutes. The reaction was stopped by the addition of quench mix (Methods, 2.2). Enzymes were removed by phenol extraction and the labelled DNA was recovered by ethanol precipitation in the presence of 100µg of denatured salmon sperm DNA (as carrier). After a second ethanol precipitation, the DNA was dissolved in 500µl of TE buffer (Methods, 2.2). The specific activity of probes labelled in this way was in the order of $10^8$ cpm/µg DNA. Probes could be stored for up to two weeks at -20°C.

End Labelling of DNA

T4 polynucleotide kinase catalyses the transfer of the $\gamma$-phosphate group from ATP to a 5'-hydroxyl terminus in DNA or RNA (Richardson, 1971). When $\gamma[^{32}P]$-ATP is used, radioactive label is incorporated into the substrate DNA molecule. The following protocol was used in this work to make oligonucleotides radioactive:

| | |
|---|---|
| DNA (200ng) +$H_2O$ | 42µl |
| 10x kinase buffer | 5µl |
| $\gamma[^{32}P]$-dATP (20uCi) | 2µl |
| T4 polynucleotide kinase | 1µl (17 units/µl) |

The mixture was incubated at 37°C for 30 minutes and then 50µl of 0.6M sodium acetate was added to stop the reaction. The labelled oligonucleotide was purified away from the unincorporated $\gamma[^{32}P]$-ATP by passage through a mini G-50 column which had been equilibrated with 0.5% SDS, 10mM Tris-HCl pH 8.0, 1mM EDTA.

Single Stranded Probes

Single stranded probes were made by the oligonucleotide-primed synthesis method described by Burke, 1984.

A DNA sequence for use as a specific hybrization probe was first cloned into M13mp8 or M13mp9, and single stranded DNA isolated. M13 sequencing primer was annealed (as described in the sequencing section) and unlabelled deoxynucleoside triphosphates dCTP, dGTP and dTTP added to a final concentration of 100µM in a reaction volume of 10µl. 10pmol (30µCi) of $\alpha[^{32}P]$-ATP (Amersham 3000Ci/mmol, 10mCi/ml) was added along with 1.0 unit of DNA polymerase I Klenow fragment (BRL). Oligonucleotide-primed synthesis was carried out at room temperature for 20 minutes, and the reaction "chased" for a further 20 minutes with "cold" dATP added to a final concentration of 1mM. The reaction was stopped by heating to 65°C for 15 minutes, and then cooling to 4°C. 10 x restriction enzyme buffer was added, and a restriction enzyme which cut in the "multicloning site" 3' to the insert added, and the solution made up to 15µl with sterile $H_2O$. The reaction was incubated at the appropriate temperature for 30 minutes, and stopped by the addition of 5µl of 0.5M EDTA. The sample was

denatured by the addition of 1M NaOH to 0.2M final concentration followed by 15µl of 1M Tris-HCl, pH 4.6. The sample was loaded onto a 1.2% agarose gel and run for 1 hour at 150V. The gel was stained, and the labelled single stranded DNA fragment detected by autoradiography. The probe fragment was rescued from the gel (by one of the methods described earlier) and used in hybridization experiments.

Hybridization Techniques

Recombinant plasmids and phage can be identified by *in situ* hybridization with specific probes of radioactively labelled DNA.

Screening of phage by Hybridization

(a) Transfer of phage

The method of Benton and Davis (1977) was used to probe λ and M13 recombinants. BBL plates containing phage in a soft-agar (0.6% w/v) overlay were placed at 4°C for 1 hour to harden the top agar, then nitrocellulose filters (Sartorius SM 41) were placed in contact with the phage. For λ phage, the filter was left in contact for 10 minutes at 4°C, while for M13 phage, 2 minutes at room temperature was sufficient for transfer. The filters were then given alignment marks, and carefully lifted from the agar surface. Denaturation and neutralization was achieved by passage of filters, phage side up, over wetted pad assemblies which consisted of a single Whatman No.1 filter paper placed in the inverted lid of a petri-dish, and containing 3ml of each solution. The filters were passed over the following pads:

| | |
|---|---|
| 0.5M NaOH | 10 mins |
| 1.0M Tris-HCl pH 7.4 | 2 mins |
| 1.0M Tris-HCl pH 7.4 | 2 mins |
| 1.0M Tris-HCL pH 7.4/ 1.5M NaCl | 15 mins |

Screening of Bacterial Colonies by Hybridization

Recombinant plasmids were identified by *in situ* hybridization to radioactively labelled probes (Grunstein and Hogness, 1975). Colonies to be screened were patch-plated in duplicate. After overnight incubation one of each duplicate was stored at 4°C whilst the other was used for screening. Nitrocellulose filters (Sartorius SM41) were gently lowered onto the surface of the agar plate, and after 3 minutes were removed. The filters were then treated as described for the phage screening protocol. The filters were then dried on a sheet of Whatman 3MM paper at room temperature, and baked for at least 2 hours at 80°C in Whatman 3MM paper envelopes.

(b) Hybridization

Hybridizations were carried out in heat sealed plastic bags, with a volume of between 2 to 3 ml per filter. The initial step of a hybridization experiment is pre-hybridization, where sites on the nitrocellulose filter that bind DNA non-specifically become saturated with unlabelled salmon sperm DNA (a component of the pre-hybridization mix). The filters were washed at 65°C for a minimum of 2 hours in a solution containing 5 x Denhardt's

solution, SDS, and denatured salmon sperm DNA, and then transferred to a new heat-sealable bag containing the hybridization mix (Methods, 2.2) to which the radioactive probe had been added. (The probe was boiled for 10 minutes, then quenched on ice when double stranded DNA was used). Hybridizations were carried out at 65°C overnight.

For end labelled oligonucleotide probes, different conditions were required, and prehybridization, and hybridization steps were carried out at 30°C.

(c) Washing

After hybridization was completed, the filters were washed 3-4 times for 5-15 minutes in a large volume (200-500ml) of 2x SSC (Methods, 2.2) and 0.1% SDS, at room temperature. Filters were then washed twice for 2 hours each, in 200-500ml of 1x SSC, 0.1% (w/v) SDS at 65°C. If the background was too high, or washing at higher stringencies was required, then the filters were further washed for one hour at 65°C in a less concentrated SSC solution (0.1x SSC, 0.1% (w/v) SDS was suitable for completely homologous probes). Once no more probe was eluted by washing, the filters were blotted, dried, and subjected to autoradiography.

For oligonucleotide probes, filters were washed at room temperature in 2 changes of 3x SSC, 0.1% (w/v) SDS, followed by washing in the same solution at 30°C for 30 minutes. The filter was then dried and autoradiographed.

DNA transfer by the method of Southern

Specific DNA fragments can be identified after electrophoresis through an agarose gel and transfer to a nitrocellulose filter, by hybridization to an $\alpha$[$^{32}$P]-labelled DNA probe (Southern, 1975).

After electrophoresis the gel was stained and photographed, and transferred to a plastic tray. Unused portions of the gel were trimmed away, and the section of interest immersed in a solution of 0.25M HCl at room temperature for 20 minutes. This depurination step (Wahl et al., 1979) cleaves the DNA into short fragments which are more readily transferred. The DNA was then denatured by soaking the gel in two changes of 200ml of 1.5M NaCl, 0.5M NaOH, for 1 hour (nitrocellulose binds only to single-stranded nucleic acids). The gel was then neutralised by soaking in 1M Tris-HCl pH 8.0, 1.5M NaCl with several changes over 1 hour. Finally, the gel was soaked for 5 mins in 20x SSC.

A glass plate was supported over a plastic tray containing 500ml of 10x SSC. A double piece of Whatman 3MM paper which had been wetted with 10x SSC was placed over the glass plate so that the ends of the paper dipped in the 10x SSC, thus serving as a wick. The gel was inverted, placed on the damp 3MM paper and the surrounding border or 3MM paper was completely covered with nescofilm. A piece of nitrocellulose filter (Sartorius) was cut to the same dimensions as the gel, and then wetted by immersing it in a solution of 2x SSC.

The wetted nitrocellulose filter was placed onto the gel and all air bubbles trapped between the gel and the filter were removed by rolling a pipette along the surface. Two pieces of 3MM, cut to the same size as the gel and soaked in 2x SSC, were placed on top of the nitrocellulose again removing all air bubbles. A stack of paper towels (10cm high) was placed on top of the 3MM paper and weighted down with a brick. The transfer was allowed to proceed for 12-24 hours at 4°C.

The filter was removed from the apparatus, washed in 6x SSC at room temperature for 5 mins, then dried and baked at 80°C for 2 hours. Filters could be stored at room temperature in a 3MM envelope.

Hybridization to a $\alpha$[$^{32}$P]-labelled DNA probe was carried out as previously described.

M13 Phage DNA Preparation

The male specific coli phage M13 (Denhardt et al., 1978) has been modified for use as a cloning vehicle (Messing et al., 1977) by insertion of lac promoter-operator region, and the first 145 codons of the N-terminus of the β-galactosidase gene which can be translated to yield the α peptide. This sequence can complement certain β-galactosidase mutants such as the lac ZΔM15 deletion, (α-complementation), and has been further modified (Messing et al., 1981) by the introduction of in-phase "multi-cloning site (MCS) oligonucleotides". Recombinant phage, generated by insertion of foreign DNA into this MCS can be detected by loss of α-complementation, conveniently followed by a colour change. On plates containing the lac-operon inducer IPTG and the chromogenic substrate 5-bromo-4-chloro-indolyl-β-D-galactoside (X-GAL) blue plaques will be produced due to the production of chloro-indole. Cloning into the MCS abolishes α-complementation and gives the blue plaque to white plaque penotypic change.

During infection of a suitable E.coli host (e.g. JM101) the infecting single stranded (ss) DNA is converted into double stranded (ds) circular replicative-form (RF) DNA and amplified to 100-200 copies per cell. The RF DNA serve as intermediates in the production of progeny ssDNA which are then packaged and extruded from the cell. Both ssDNA phage and dsDNA RF can be prepared from transfected E.coli as described below.

Phage particles were picked from a single plaque (an area of slow growing infected E.coli) into 1ml of phage buffer (Methods, 2.2).

10ul of this phage suspension was then used to transfect E.coli strain JM101 in 1.5ml of L-broth, and grown overnight at 37°C (50µl of a stationary phase culture of JM101 was sufficient inoculum for 50ml of L-broth). The cells were pelleted by centrifugation for 1 min in a microfuge and used in the small scale preparation of dsDNA circular RF, as described for plasmids (Method 1). Extruded phage particles (containing ssDNA) were precipitated from the supernant by addition of 0.25 volumes of 10% PEG8000 (Sigma) in 2.5M sodium chloride. After incubation at room temperature for 30 mins, phage were pelleted by centrifugation for 10 minutes in a microfuge and used either for transfection, or in the preparation of ssDNA template for use in DNA sequencing.

M13 ssDNA sequencing template was prepared by carefully removing the PEG solution from the pelleted phage, and recentrifuging the sample in a microfuge. Residual PEG solution was removed with a drawn-out pasteur pipette. (It is important to remove all traces of PEG, as the slightest contamination interferes with subsequent sequencing reactions). The pellet was resuspended in 200µl of sterile $H_2O$, phenol extracted, and the ssDNA recovered by ethanol precipitation. A 1.5ml culture of infected E.coli JM101 yielded from 3 to 5µg of ssDNA, which was sufficient for 4 sequencing reactions.

For large scale preparation of RF DNA, 10ml of an overnight culture of JM101 was used to inoculate 200ml of L-broth in a 2 litre conical flask and shaken vigorously at 37°C for approximately 2 hours. When the $O.D_{550}$ measured 0.5 ($10^8$cells/ml) phage particles, which had been resuspended in 5ml of L-broth, were added to the culture at a multiplicity of infection of approximately 20:1, and incubation continued for 90 minutes to allow infection and conversion to RF. Chloramphenicol was then added (200µg/ml) and amplification of the RF M13 phage occurred during a further 90 minute period of growth. The cells were lysed, and the DNA extracted and purified as described above. Typically, yields were 0.5-2mg of RF per litre of transfected E.coli.

DNA Sequencing

DNA sequencing was carried out by the chain-termination method (Sanger et al., 1977) using $\alpha[^{35}S]$-ATP, and buffer gradient gels (Biggin et al., 1983).

The 'Klenow' fragment, or large fragment of E.coli DNA polymerase I, lacks the 5' → 3' exonuclease activity of the intact enzyme and will, in the presence of deoxynucleoside triphosphates (dNTPs), extend DNA from the 3'-hydroxyl terminus of an oligonucleotide primer annealed to a single stranded DNA template. If, during this extension, 2,3-dideoxynucleoside triphosphates (ddNTPs), which lack 3'-hydroxyl group, are included in the reaction mix, chain termination will occur. This termination results from the incorporation of a ddNTP, which cannot participate in formation of the next phosphodiester bond. During sequence analysis 4 reactions are carried out. Each reaction is supplied with all four dNTPs (one of which is $\alpha[^{35}S]$-labelled), but only one of the four ddNTPs. By controlling the ratio of dNTP to ddNTP, chain termination will be random, resulting in a 'nested set' of DNA fragments of different length. These fragments can be size-separated on a poly-acrylamide gel to produce a 'ladder'. DNA sequence can be read from an autoradiograph of the gel by recording the position of bands across the four tracks, as each band represents fragments differing in size by a single nucleotide.

Sequencing Reactions

Single stranded recombinant M13 phage was used as template. The primer was the 17-mer 'universal primer' 5'-GTAAAACGACGGCCAGT-3' and was supplied by Celltech, and J.F. Keyte. (University of Leicester).

For the priming reaction 5µl of the single stranded DNA template solution was mixed with 1µl of TM buffer, 2.5ng of the primer, and made up to a volume of 10µl with sterile water. Priming was carried out by incubating the reaction mix in a 65°C incubator for 30 minutes, and then the samples were allowed to anneal by cooling to room temperature. Each primed DNA template was then split into 4 x 2µl samples to which the appropriate di-deoxynucleotide mix (see below) was added. After the addition of 2µl of an $\alpha$[$^{35}$S]-dATP: Klenow polymerase mix (1:2:17; $^{35}$S-dATP: Klenow: $H_2O$) to each tube the mixture was left at room temperature for 20 minutes, and then 2µl of chase mix (0.25mM of each dNTP) was added and the reactions left for a further 20 minutes. At this stage the reactions could be stored at -20°C for subsequent analysis, or alternatively 2µl of sequencing dye (Methods, 2.2) was added to each tube, and the solutions incubated in a boiling water bath for 3 minutes. After the samples had been centrifuged for 30 seconds they were loaded onto a polyacrylamide gel and electrophoresed at 45W constant power until the bromophenol-blue dye reached the bottom.

Di-deoxynucleotide Sequencing Mix

| | | volumes in µl | | | |
|---|---|---|---|---|---|
| | | T | C | G | A |
| 0.5mM | dTTP | 25 | 500 | 500 | 500 |
| 0.5mM | dCTP | 500 | 25 | 500 | 500 |
| 0.5mM | dGTP | 500 | 500 | 25 | 500 |
| 10mM | ddTTP | 50 | - | - | - |
| 10mM | ddCTP | - | 8 | - | - |
| 10mM | ddGTP | - | - | 16 | - |
| 10mM | ddATP | - | - | - | 1 |
| TE | | 1000 | 1000 | 1000 | 1000 |

Buffer-gradient Polyacrylamide Gel Electrophoresis

Two sizes of sequencing gel were routinely used, the choice depending on the number of clones to be sequenced at a given time. A large format gel (30cm x 40cm) had a maximum capacity for 8 clones and a small format gel (18cm x 40cm) had a capacity for 4 clones. The following conditions are described for the large format gel.

Two glass plates (30 x 40cm) were set up with 0.4mm separation, and sealed around the sides and bottom with yellow 1.5" Sellotape. To increase the amount of useful data obtained from each run, an ionic gradient was constructed across the gel. 15ml of 2.5 x TBE acrylamide mix (Methods, 2.2) and 60ml of 0.5 x TBE acylamide mix (Methods, 2.2) were poured separately into Buchner

flasks, and 90µl and 360µl of freshly prepared 10% ammonium persulphate added to each flask respectively. The solutions were then swirled to mix, and degassed for 5 minutes. 5µl and 15µl of TEMED were then added separately to each flask, and the solutions again swirled to mix. The gradient was made in a 25ml pipette by drawing up 8ml of the 0.5 x TBE-acrylamide mix followed by 12ml of the 2.5 x TBE-acrylamide mix in the same pipette. The solutions were mixed by introducing 4 air bubbles into the pipette, and the resulting mix was poured slowly between the gel plates. The gel was filled with the remaining 0.5 x TBE acrylamide mix, and the flat side of the sharks tooth comb (BRL Ltd) inserted. When the gel had polymerized (= 1 hr) the shark's tooth comb and the Sellotape covering the bottom of the gel were removed, and the gel was clamped into a vertical gel tank with bulldog clips. The top reservoir of the tank was filled with 0.5 x TBE buffer (Methods, 2.2) and the bottom tank was filled with 1 x TBE buffer. The gel was heated by pre-running under a constant power constraint of 45W for 30 minutes.

Just prior to loading the sequencing reactions, the sharks tooth comb was inserted into the top of the gel to generate the wells. These wells were then flushed out with buffer, and the samples loaded using a drawn out glass capillary tube.

After the gel had run, the plates were separated, and the gel, which remained on the larger plate, was fixed by immersion in a solution of 10% methanol, 10% acetic acid for 20 minutes. After

excess solution was removed the gel was transferred to Whatman 3MM paper, and dried down using a BioRad gel drier set at 80°C for 40 minutes. The gel was then subjected to autoradiography.

## 2.4 Bacterial Strains

| E.coli strain | Genotype or Comment | Source or Reference |
|---|---|---|
| BHB2688 | recA ; sup° ; ($\lambda i^4$ ; cIts ; b2; redB Dam15; Sam7 | Hohn & Murray, 1977 |
| BHB2690 | recA ; sup° ; ($\lambda i^4$ ; cIts ; b2; redB Eam4; Sam7) | Hohn & Murray, 1977 |
| C600 | supE44; tonA; thr1; leuB6; thi1; lacY1 | Appleyard, 1954 |
| 5K | C600, hsdR | Hubacek & Glover, 1970 |
| J53 | $F^-$; pro; met | Datta et al., 1974 |
| JM83 | strA; thi1; ara; (lac-pro); (Ø80 $\Delta$lacZM15) | Vieira & Messing, 1982 |
| JM101 | $\Delta$(lac-pro); supE; thi1; F'traD36; proA; lacI; $\Delta$lacZM15 | via A.R. Hawkins |
| JM103 | $\Delta$(lac-pro); supE; thi1; F'traD36; proA; endA; sbcB; rpsL; hsdR; lacI; $\Delta$lacZM15 | via A.R. Hawkins |
| Q359 | supE; tonA; hsdR; (P2) | Karn et al., 1980 |
| WJB 910 | recB; recC; hsdR; supE; supF; metB; lacY1; galk; galT | Loenen & Brammar, 1980 |
| SK3430 | thi1 aroD6, leuC | A.R. Hawkins |
| **S. typhimurium** | | |
| LT2 | galE; (transformation strain) | via R. Badawi |
| **P.mirabilis** | | |
| PM13 | Wild type, $tet^+$; $cat^+$ | Coetzee & Sacks, 1960 |
| PM2 | Wild type, $tet^+$; $cat^-$ | NCTC 3177 and this study |
| **Providencia stuarti P6** | Wild type, $tet^+$; $cat^-$ | NCTC 8113 and this study |

| Strain | Genotype or Comment | Source or Reference |
|---|---|---|
| *Pseudomonas aeruginosa* | | |
| PAO1162 | *leu-38* (transformation strain) | Bagdasarian *et al.*, 1981 |
| PAO1161 | *leu-38* (transformation strain) | Bagdasarian *et al.*, 1981 |
| PAO2440 | Wild type (transformation strain) | Bagdasarian *et al.*, 1981 |
| *Methylophilus methylotrophus* (AS1) | | |
| CB1 | *mmeA* | C. Boyd Ph.D. thesis, 1983 |
| *Flavobacterium* | | |
| CB60 | Wild type, $cat^+$ | Süssmuth *et al.*, 1979 |
| CB61 | Wild type, $cat^-$ | Süssmuth *et al.*, 1979 |
| CB67 | Wild type, $cat^-$ | Süssmuth *et al.*, 1979 |

| Plasmid | Relevant Genotype or Comment | Source or Reference |
|---|---|---|
| pAT153 | tet ; bla | Twigg & Sherratt, 1980 |
| pBR328 | tet; bla; cat | Soberon et al., 1980 |
| pUC13 | bla | Vieira & Messing, 1982 |
| pIC100 | pAT153 with P.mirabilis cat in Pst I site | This study |
| pIC101 | pAT153 with P.mirabilis cat in Pst I site | This study |
| pIC200 )<br>pIC201 )<br>pIC202 )<br>pIC203 )<br>pIC204 ) | pAT153 with P.mirabilis cat Pst I site | This study |
| pIC075 | pUC13 with P.mirabilis cat in Hind III site | This study |
| pIC025 | M13mp9 with pBR328 cat in AccI site | This study |
| p Sa | type II cat | via J. Keyte |
| NR1 (R100) | type I cat | via J. Keyte |
| R387 | type III cat | via J. Keyte |
| R994 | type I cat | via J. Keyte |
| pCB60 | 15.6-kb plasmid | This study |

Bacteriophage strains

| | | |
|---|---|---|
| M13mp8 | Cloning/sequencing vehicle | Messing, 1983 |
| M13mp9 | Cloning/sequencing vehicle | Messing, 1983 |
| λDB286 | derivative of λL47.1. | Loenen & Brammar, 1981 |

# CHAPTER 3

## CHLORAMPHENICOL RESISTANCE IN *P.MIRABILIS* PM13

# CHAPTER 3 : Chloramphenicol Resistance in P.mirabilis PM13

## 3.1 INTRODUCTION

In Chapter 1, the observations which constitute the phenomenon of the enhancement of chloramphenicol resistance in P.mirabilis PM13 were introduced. Briefly, the initial observations were that chloramphenicol resistant cells arose spontaneously from a population of predominantly sensitive cells at high frequency (from $10^{-4}$ to $10^{-5}$ per cell per generation). When a single colony from chloramphenicol agar was transferred to liquid medium and grown in the absence of the antibiotic for 150 generations, a population of predominantly sensitive cells arose. Resistance was demonstrated to be mediated by the concomitant appearance of high levels of chloramphenicol acetyltransferase (CAT), and the fall off of resistance with time in the absence of selection was paralleled by a fall in CAT levels.

The work in this chapter was undertaken to clarify the initial observations on the appearance and disappearance of chloramphenicol resistance in P.mirabilis PM13, and to see how these observations fitted in with the current paradigms of gene regulation.

## 3.2 RESULTS

### 3.2.1 Appearance of chloramphenicol resistant cells

In Chapter 1, the mechanisms by which the expression of gram-positive and gram-negative cat genes can be controlled were discussed, and ranged from gene amplification to induction. Two situations can be envisaged to account for the origin of the enhancement of chloramphenicol resistance in P.mirabilis PM13:

(i) The adaptation hypothesis, according to which, every cell may be induced by chloramphenicol to give the resistant phenotype. (A situation which exists for the induction of gram-positive cat genes (see Chapter 1 for references)).

(ii) The spontaneous mutation hypothesis, according to which, every cell has a finite probability of mutating from chloramphenicol-sensitivity to resistance.

Luria and Delbrück (1943) devised the "fluctuation test" to decide between these two alternatives for the appearance of $T_1$ phage resistance among E.coli B cells, and the test is equally applicable for the appearance of chloramphenicol resistance in P.mirabilis PM13.

An important difference between the two situations described above is, that according to the adaptive hypothesis, the population of P.mirabilis PM13 is homogenous with respect to chloramphenicol resistance prior to exposure to the antibiotic. According to the mutation hypothesis, however, the population is not homogenous since mutation to resistance can occur at any stage prior to the addition of the drug. As a consequence of this, the number of P.mirabilis PM13 cells resistant to chloramphenicol will depend on whether the first mutation to resistance occurred at an early or late stage of growth of the culture. According to the adaptation hypothesis, the probability of any P.mirabilis PM13 cell becoming resistant after contact with chloramphenicol should be equal for any

of the bacteria in the culture. The natural corollary of this is that there will be no large fluctuations in the numbers of resistant bacteria from culture to culture in a parallel series of cultures to which chloramphenicol is added. In fact, the fluctuation of resistant colonies arising should be no greater than that encountered in a series of samples all taken from the same culture. Alternatively, if mutation is responsible for the appearance of chloramphenicol resistance, then the time of occurrence of a mutation in a series of parallel cultures will be subject to random variation. Those cultures in which a mutation to chloramphenicol resistance occurred at an early stage of growth will contain large numbers of resistant colonies, while those which contain chloramphenicol-resistant mutants, which arose at a later stage of growth, will contain few resistant cells.

The mutation hypothesis predicts that there will be large variance in the numbers of chloramphenicol-resistant mutants from culture to culture in a parallel series of cultures, than from a series of samples all taken from the same culture. The experimental protocol for the fluctuation test involves determining the number of chloramphenicol resistant bacteria present in two series of samples, one series from parallel independent cultures, and the other series taken from the same bulk culture.

Table 3.1 shows that when the Luria-Delbrück test is applied to the appearance of chloramphenicol-resistant cells of P.mirabilis PM13 from a sensitive population, the variance between parallel and

independent cultures is greater than the variance between samples taken from the same culture. This strongly suggests that chloramphenicol-resistant cells of P.mirabilis PM13 arise from mutation rather than as a consequence of induction. Experiments to discover the possible nature of this mutation event are described in Chapter 6.

Table 3.1 shows the result of a typical Luria-Delbrück fluctuation test applied to the appearance of chloramphenicol-resistant cells from a population of P.mirabilis PM13 cells.

The variance was calculated by:

$$\text{Variance} = \frac{(x - \bar{x})^2}{n - 1}$$

where $\bar{x}$ = the mean of the observed number of chloramphenicol resistant colonies.

$x$ = the observed number of chloramphenicol-resistant colonies in each sample.

$n$ = the number of observations, i.e. 10

The large variance is indicative of a mutation event being responsible for the appearance of chloramphenicol resistance.

Table 3.1 (Luria-Delbrück fluctuation test)

| | Sample set 1 (chloramphenicol-resistant colonies from 10 independent cultures) | Sample set 2 (Chloramphenicol-resistant colonies from 10 samples from the same culture) |
|---|---|---|
| 1 | 0 | 200 |
| 2 | 0 | 210 |
| 3 | 0 | 211 |
| 4 | 0 | 168 |
| 5 | 0 | 214 |
| 6 | 1 2 | 233 |
| 7 | 2 6 7 | 190 |
| 8 | 0 | 193 |
| 9 | 6 | 167 |
| 10 | 5 | 204 |
| Variance | 7010 | 328 |

### 3.2.2 Disappearance of resistance

The appearance of chloramphenicol-resistant cells from a population of sensitive cells is only one aspect of the 'resistance phenomenon' in P.mirabilis PM13. Another feature is highlighted by the observation that a population of P.mirabilis PM13 in which 100% of the cells are phenotypically resistant to chloramphenicol may undergo reversion to the chloramphenicol-sensitive state during growth in the absence of the antibiotic. Plotting the loss of antibiotic resistance as a function of time reveals the rate of loss of resistance to be in the order of $10^{-2}$ per cell per generation.

The rate of this decrease in the efficiency of plating of cells on antibiotic agar is too low to correspond with a model wherein a putative intracellular inducer is progressively diluted by cell division. The alternative explanation, a progressive reduction in the frequency of resistant cells in the population, can be seen to operate by mutation or selection or some contribution of both factors.

Back mutation at a very high rate (from resistant to sensitive cells) would yield an increased proportion of cells which were sensitive with a concomitant reduction in the efficiency of plating on antibiotic-containing media. Equally, were sensitive cells to have a selective advantage over resistant cells under conditions wherein antibiotic was absent, they would be expected to replace resistant cells in the population, and the efficiency of plating would also decline with time.

With back mutation, in any short time period, $\delta t$, a small proportion $u\delta t$, (where u is the back mutation rate per generation) of resistant cells would mutate to sensitive. Thus, if q is the frequency of resistant cells in a population after any number of generations (t), then the integrated expression describing the phenomenon is

$$q_t = q_o e^{-ut} \quad .....(1)$$

where $q_o$ is the initial frequency of resistant cells in the population and $q_t$ is their frequency after t generations. The equation predicts that a plot of ln ($q_t$) or indeed ln (efficiency of plating) against time should yield a straight line, its slope being equal to the back mutation rate.

The second alternative is selection against resistant cells. If s is the selective coefficient against resistant cells then such a process can be quantified by imagining that a proportion of the cells ($s\delta t$) is selectively eliminated in the short time period corresponding to t generations. Quantitatively, the change in q in a time period t is given by the formula

$$\delta q = -s\delta t\ q(1-q)$$

which when integrated yields

$$qt = \frac{q_o}{(1-q_o)\, e^{st} + q_o} \quad \ldots\ldots(2)$$

The equation predicts that a plot of ln (efficiency of plating) against time will show an initial very slow reduction in the efficiency of plating, with the time of this lag being dependent upon how close $q_o$ is to 1. At the point where nearly half of the cells in the population are sensitive, the plot becomes linear, after which ln (efficiency of plating) decreases with a slope of $-s$.

In reality it is likely that both selection and back mutation play a part in the phenomenon under discussion and that resistant cells are not only under the pressure of a selection coefficient s, but also mutate to sensitivity at rate u. The compound expression, corresponding to (1) and (2) in such a case is

$$qt = \frac{q_o\,(u + s)}{(u + s\,(1-q_o))e^{(u+s)t} + sq_o} \quad \ldots (3)$$

The latter reduced to produce (1) and (2) as special cases when s and u are zero respectively.

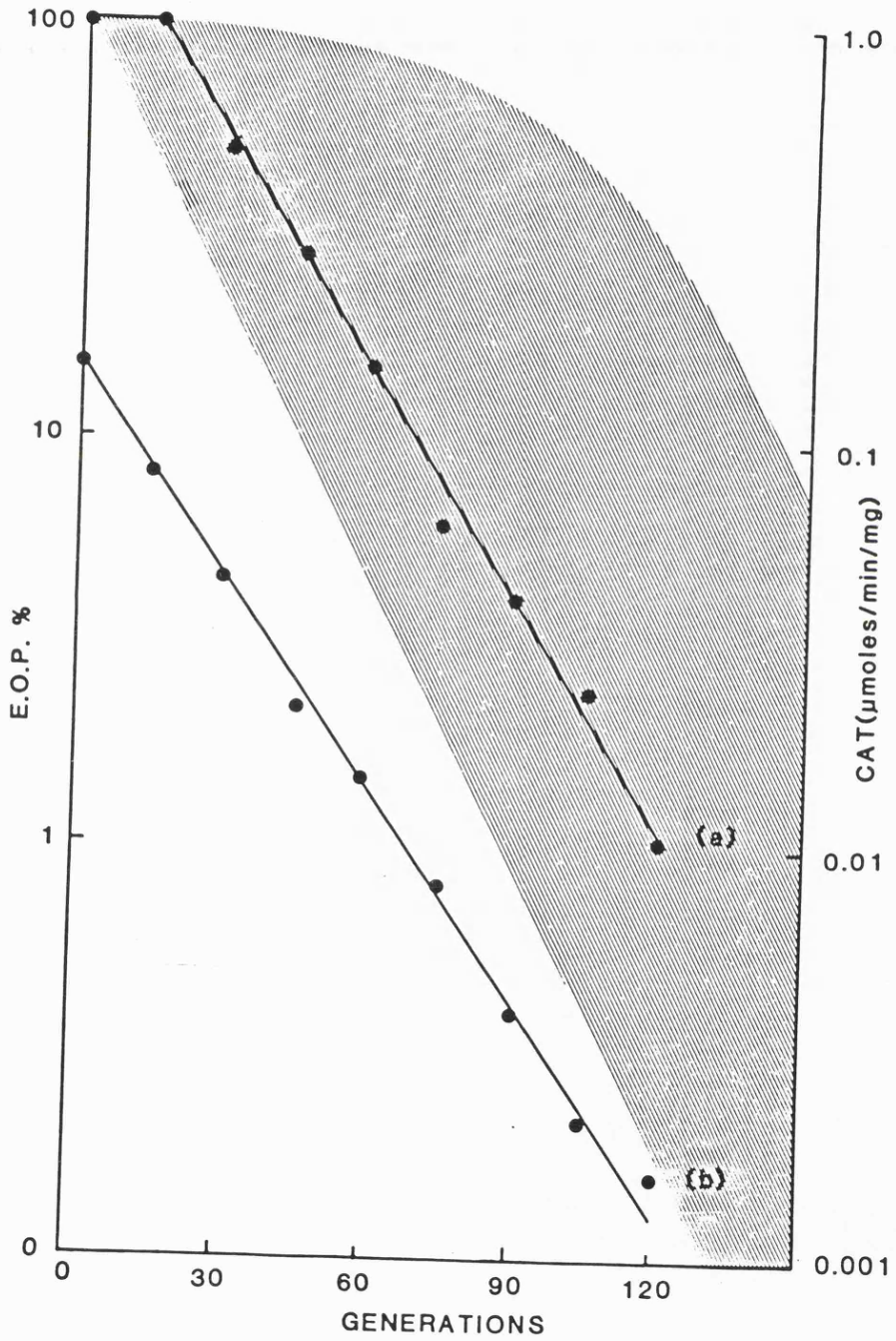
Irrespective of the relative contributions of mutation and selection as t becomes large, the slope of ln (efficiency of plating) against t goes to $-(u + s)$. The amount of selection determines the time of the lag before the slope becomes linear.

The shaded region in Fig 3.1 describes the graphical limits of predictions from equation 4 when (u + s) is equal to the experimentally derived slope of -0.044. The position of the experimental points between the two boundary conditions of back-mutation (lower boundary) and counter-selection (upper boundary) suggests that both back-mutation to sensitivity and selection against resistant cells are likely to play a role in the loss of chloramphenicol resistant cells with time.

I would like to thank J. Brookfield (University of Leicester) for providing the above mathematical analysis.

FIG 3.1

Disappearance of chloramphenicol-resistant cells of P.mirabilis PM13 from a uniformly resistant population as a function of time. The experimental points giving line (a) represent the efficiency of plating (E.O.P.) of cells on chloramphenicol, and line (b) depicts the parallel and exponential fall in CAT for the population. Growth in the absence of chloramphenicol was followed for 120 generations by means of repeated dilutions ($10^{-2}$) of late-exponential-phase cells into fresh medium. The shaded area represents an envelope containing a range of theoretical curves for the fall in efficiency of plating as a function of growth, such plots being generated by solutions to the equation (refer to text) which relates the efficiency of plating to parameters for both selection and mutation. For the case wherein the disappearance of resistant cells from the population is solely due to selection against their persistence, the initial rate of reduction in the efficiency of plating should be low. For the other extreme, wherein the rate of back-mutation to sensitivity is the sole determinant of disappearance of cells, an immediate and exponential fall in the efficiency of plating is predicted. The position of the experimental points between the two extremes described by the boundaries of the shaded area suggests that both back mutation to sensitivy and selection against resistant cells are likely to play a role.


100
10
1
0
E.O.P. %
1.0
0.1
0.01
0.001
CAT(µmoles/min/mg)
(a)
(b)
0
30
60
90
120
GENERATIONS

3.2.3 Are CAT levels related to chloramphenicol concentration?

Chloramphenicol resistance in P.mirabilis PM13 is mediated by the enzyme chloramphenicol acetyltransferase (CAT). When cultures of P.mirabilis PM13 were grown in Penassay broth at 37°C to the mid-exponential phase and then spread onto Penassay agar plates containing chloramphenicol, resistant cells containing CAT appeared at a frequency of between $10^{-4}$ to $10^{-5}$ per cell per generation (see Chapter 1). Initially, chloramphenicol-resistant colonies were selected on Penassay agar plates containing chloramphenicol at a concentration of 50µg/ml. In order to test whether or not increased chloramphenicol concentration resulted in increased CAT levels, the resulting colonies were transferred to agar plates containing 100µg of chloramphenicol per ml, and thence stepwise to 200 and 500µg of chloramphenicol per ml. Resistant colonies were picked into Penassay broth supplemented with the appropriate concentration of chloramphenicol, grown to the mid-exponential phase, harvested, and crude cell extracts containing CAT prepared as described in methods (2.3). Table 3.2 compares the specific activity (in nmoles/min/mg) of CAT prepared from crude cell extracts with the concentration of chloramphenicol in the growth medium. There is no apparent increase in the specific activity of CAT isolated from cells grown in progressively higher concentrations of chloramphenicol. This result supports the view that the appearance of the resistance phenotype, i.e. the enhancement phenomenon, is independent of the concentration of chloramphenicol.

Table 3.2

| Growth conditions (μg/ml chloramphenicol) | Specific Activity CAT (nmole $min^{-1}$ $mg^{-1}$) |
|---|---|
| 0 | 0 |
| 50 | 124 |
| 100 | 150 |
| 200 | 134 |
| 500 | 118 |

Table 3.2 compares the specific activity of CAT (in crude cell extracts) prepared from P.mirabilis PM13 cultures grown in different concentrations of chloramphenicol.

### 3.2.4 Absence of an induction phenomenon

Experiments were carried out to attempt to induce CAT synthesis in P.mirabilis PM13 under conditions which induce synthesis in Staphylococcus aureus, (Winshell and Shaw, 1969), and Bacillus pumulis (Duvall et al., 1983). Incubation of P.mirabilis PM13 cell suspensions with 3-deoxy-chloramphenicol, or 3-fluoro-chloramphenicol (the kind gift of S. Skinner, University of Leicester) was not accompanied by significant increases in the specific activity of CAT. Such results, although negative, strongly suggest that induction by chloramphenicol or its analogues is unlikely to play a role in the enhancement of chloramphenicol resistance observed in P.mirabilis PM13.

### 3.2.5 Absence of concomitant increase in resistance to other antibiotics

Chloramphenicol-resistant isolates of P.mirabilis PM13 were tested for increased resistance to other antibiotics. One hundred independently-isolated chloramphenicol-resistant colonies, and 100 chloramphenicol-sensitive isolates were patch-plated onto L-agar plates containing 50µg/ml chloramphenicol and 20µg/ml tetracycline respectively. After overnight growth at 37°C the resulting colonies were replica-plated onto L-agar plates containing the following antibiotics:- ampicillin (50µg/ml); kanamycin (20µg/ml); nalidixic acid (10µg/ml); spectinomycin (100µg/ml); and streptomycin (20µg/ml). After overnight growth at 37°C the plates were scored for resistance. Chloramphenicol-resistant colonies and chloramphenicol-sensitive colonies remained equally as sensitive to all the other antibiotics tested.

## 3.3 SUMMARY

The results presented in this chapter suggest that the mechanism for the phenomenon of the enhancement of chloramphenicol resistance in P.mirabilis PM13 involves the selection of pre-existing spontaneous mutants in the population. Such mutant cells arise at a frequency of between $10^{-4}$ and $10^{-5}$ per cell per generation and are selected by exposure to chloramphenicol. The resistant mutants persist in the population for as long as they are subjected to chloramphenicol selection. The mutation appears to be an "all or nothing" one, in that growth in the presence of higher concentrations of chloramphenicol does not lead to a further increase in CAT levels.

Populations of P.mirabilis PM13 in which 100% of the cells are phenotypically resistant to chloramphenicol undergo a reversion to the chloramphenicol-sensitive state during growth in the absence of antibiotic, the rate of the process being in the order of $10^{-2}$ per cell per generation. Analysis of the shape of the curve produced by plotting the fall-off of resistant cells in the population against time revealed that back-mutation, combined with counter-selection against resistant cells was the likely mechanism. The appearance of chloramphenicol resistant cells in P.mirabilis PM13 was not paralleled by any increase to other antibiotics tested.

# CHAPTER 4

# HOMOLOGY OF THE *P.MIRABILIS* *CAT* WITH THE TYPE I (Tn*9*) VARIANT

# CHAPTER 4 : HOMOLOGY OF THE P.MIRABILIS CAT WITH THE TYPE I (Tn9) VARIANT

## 4.1 INTRODUCTION

In Chapter 1 the phenomenon of chloramphenicol resistance in P.mirabilis PM13 was introduced and CAT, the enzyme responsible for resistance, was shown to have properties in common with the well-studied type I (Tn 9) variant. The subject of the experiments in this chapter was to pursue the likely homology, and extend the comparison to the DNA level by hybridization experiments.

The ultimate plan was to isolate the cat gene of P.mirabilis PM13 by in vitro genetic manipulation (see Chapter 5), and as a first step to determine whether the cat gene was plasmid-linked or located on the chromosome. The strategy assumed any DNA:DNA homology revealed by such experiments would be of central importance in any subsequent cloning experiments for identification of the P.mirabilis PM13 cat gene.

## 4.2 RESULTS

### 4.2.1 Comparison of the CAT of P.mirabilis with other CAT variants by Ouchterlony immunodiffusion

P.mirabilis PM13 CAT was purified on affinity columns as described in methods (2.3), and used in Ouchterlony immunodiffusion experiments. Three different CAT variants, purified from cells harbouring the type I, II, and III enzymes (the kind gift of A.D. Bennett, University of Leicester) were used as controls. The photographs in Fig. 4a shows the purified P.mirabilis PM13 CAT

used in the immunodiffusion experiment Fig. 4b. Antibody raised against the type I CAT (central well) cross-reacted with purified type I CAT, and the P.mirabilis PM13 CAT variant. The junction of the two precipitin bands produced a 'spur', indicative of partial homology; i.e. an antigenic determinant present in the type I CAT which is not present in the CAT of P.mirabilis PM13. None of the other CAT variants show substantial cross-reactivity against the type I antibody.

4.2.2 Binding of triphenylmethane dyes by CAT of P.mirabilis PM13

Proctor and Rownd (1982) have described a protocol for the detection of chloramphenicol-resistant colonies which discriminates between the CAT+ and CAT− phenotype on the basis of CAT binding triphenylmethane dyes, including crystal violet, competitively with respect to chloramphenicol. Colonies producing CAT intracellularly are stained purple as a result of dye-binding, whereas CAT− colonies remain colourless. Of the commonly observed CAT variants found in enteric bacteria, only the type I enzyme binds the dye (A.D. Bennett Ph.D thesis, 1984, University of Leicester). The CAT of P.mirabilis PM13 was tested for its ability to bind the dye crystal violet. Penassay-agar plates containing crystal violet, and either 50µg/ml chloramphenicol or 20µg/ml tetracycline were prepared as described in methods (2.3). A single P.mirabilis PM13 colony growing on Penassay-agar, supplemented with 20µg/ml tetracycline, was picked into 200µl of Penassay broth. Half of this sample was then spread onto a Penassay-crystalviolet-tetracycline plate, while the other half was spread onto a Penasay-crystalviolet-chloramphenicol plate.

Growth was scored after incubation at 37°C for 48 hours. Colonies growing on the Penassay-chloramphenicol plate had become purple, presumably as a result of CAT binding to the dye crystal violet, while the colonies growing on the Penassay-tetracycline plate remained uncoloured.

In conclusion, the CAT of P.mirabilis PM13 shows immunological cross-reactivity and similar dye-binding characteristics to the type I CAT variant, suggesting that the enzymes are likely to share structural features at the enzyme surface and near the catalytic centre, properties which support a high degree of homology.

FIG. 4.1

Purification and Ouchterlony immunodiffusion of P.mirabilis PM13 CAT.

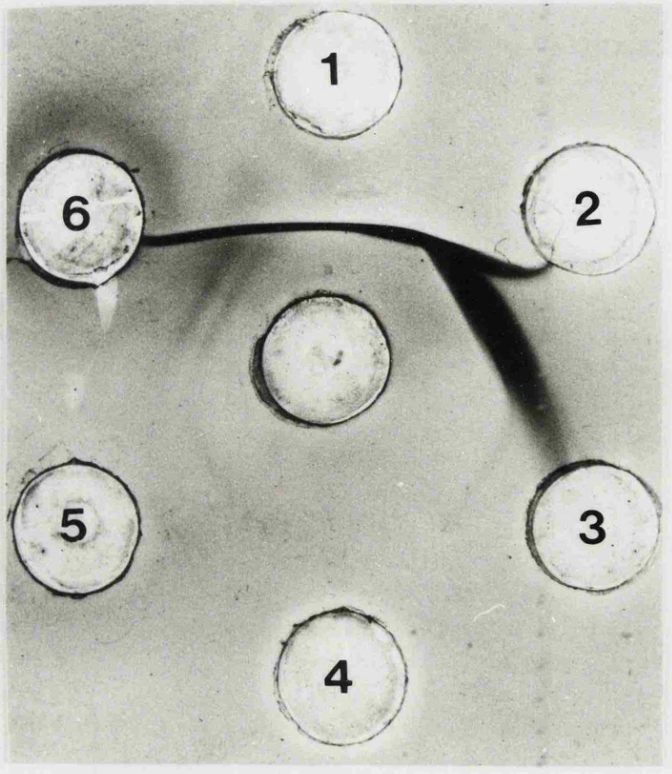
Fig. 4.1a shows the purified P.mirabilis PM13 CAT track (C) after purification on an affinity column. Tracks (A), and (B) show total protein from P.mirabilis PM13 grown in the presence and absence of chloramphenicol. An attempt was made to load the same amount of total protein onto the gel, so as to compare the increase in CAT levels in total-protein preparations.

Fig. 4.1b shows the purified P.mirabilis PM13 CAT used in an Ouchterlony double-diffusion experiment. Goat IgG raised against the type I CAT was placed in the central well. The following proteins were loaded in the outer wells. (1) Purified type I CAT (50μg), (2) Purified P.mirabilis CAT (50μg), (3) Purified type II CAT (100μg), (4) Purified type II CAT (50μg), (5) Purified type III CAT (50μg), (6) Purified type III CAT (100μg).

(a)

A B C

(b)



### 4.2.3 Homology at the DNA level between the *P.mirabilis* *cat* and the type I *cat*

The plasmid pBR328 is a 4.9kb replicon containing genes specifying resistance to ampicillin, tetracycline, and chloramphenicol (Soberon *et al.*, 1980). The *cat* gene harboured by this replicon is a type I variant, and the complete sequence has been determined (Alton and Vapnek, 1979). The entire structural gene (not including the Shine-Dalgarno sequence) can be isolated from pBR328 as a 773-bp *Taq*I fragment, and it was proposed to use this fragment as a labelled probe in hybridization experiments against the *P.mirabilis* PM13 *cat* gene. The strategy required preliminary hybridization experiments to ascertain whether or not the 773-bp *cat* probe might detect *cat* genes known to differ from the type I variant. Genes in this category are the type II and type III *cat* determinants carried by plasmids pSa and R387 respectively. Overnight cultures of cells harbouring the plasmids (i) R994, (a type I variant); (ii) pSA, (a type II variant); and (iii) R387, (a type III variant) were subjected to the plasmid lysis method of Kado and Liu (Methods, 2.3). Plasmids were recovered from the cleared lysates by ethanol precipitation, loaded onto a 0.6% agarose gel, and subjected to electrophoresis (Methods, 2.3). Fig. 4.2 shows (a) a photograph of the gel after ethidium bromide staining and, (b) an autoradiograph of the tracks in (a) after transfer to nitrocellulose and hybridization to a [$^{32}$P]-labelled *cat* I probe. Under the hybridization and wash conditions (3 x SSC, 65°C and 0.1 x SSC, 65°C respectively) hybridization can only be detected to the track containing the type I *cat* bearing plasmid.

4.2.4 Subcloning the type I cat into phage M13 mp9

Two difficulties arose from the isolation of the cat probe as a 773-bp TaqI fragment. (i) Digestion with TaqI often yielded partial digest products which confused isolation of the 773-bp band, and (ii) the banding pattern of pBR328 digested to completion with TaqI produced many bands in the same size range after electrophoresis.

To facilitate isolation of the 773-bp fragment for subsequent Southern hybridization experiments it was decided to sub-clone the fragment into the AccI site of the phage vector M13mp9. 1µg of the M13mp9 replicative form (RF) DNA was digested with AccI in the presence of calf intestinal phosphatase as detailed in Methods (2.3). 20ng of cut and phosphased vector was then ligated with the 773-bp TaqI fragment (isolated from a total TaqI digest of pBR328 by the 'freeze-squeeze' method of Taut & Rentz, (1983)). After transformation (Methods, 2.3) recombinants, identified as white plaques, were screened for the presence of the 773-bp TaqI fragment by restriction digests of their respective RFs.

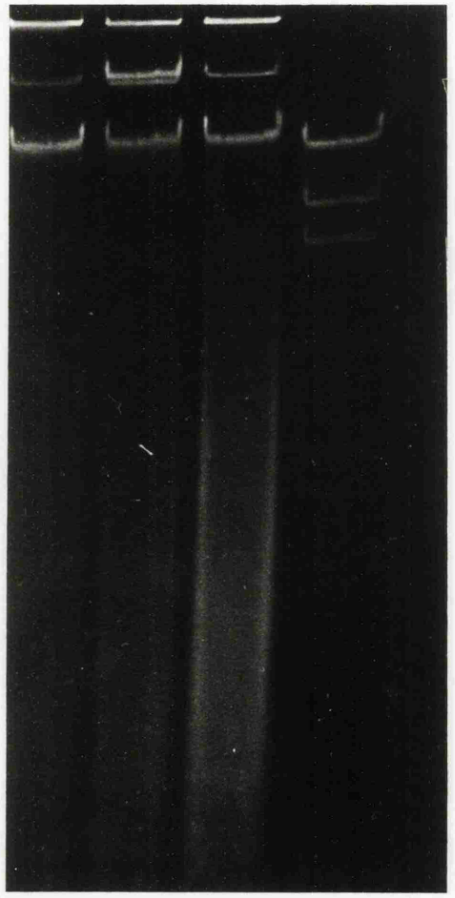
One such recombinant, designated pIC025, was chosen for large-scale RF preparation (Methods, 2.3), and a PstI-BamHI double digest used to isolate the type I cat gene as a 773-bp fragment, which could easily be separated from the 7.6-kb M13mp9 fragment (Fig. 4.3).

FIG. 4.2

Hybridization of a 773-bp cat I probe against plasmids harbouring the other enteric cat genes

(a) Ethidium bromide stained agarose gel (0.6% w/v) showing banding pattern produced after isolation of the following plasmids by the method of Kado and Liu, (1981). A, R387 (harbouring a type III cat gene). B, pSa (harbouring a type II cat gene). C, R994 (harbouring a type I cat gene). Track D was a HindIII digest of $\lambda$. In tracks A, B, and C, the lower of the 2 bands (running with the 23.0-kb size marker) corresponds to chromosomal DNA, while the upper band corresponds to the plasmid.

(b) Autograph of the DNA shown in part (a) after transfer to nitrocellulose, and hybridization to an $\alpha$ [$^{32}$P]-labelled 773-bp catI probe. Under the stringency conditions of the hybridization (refer to text), homology is only revealed in track (C) corresponding to the plasmid harbouring a type I cat gene.


(a)
size in kb
-23.0
-9.7
-6.4
A B C D

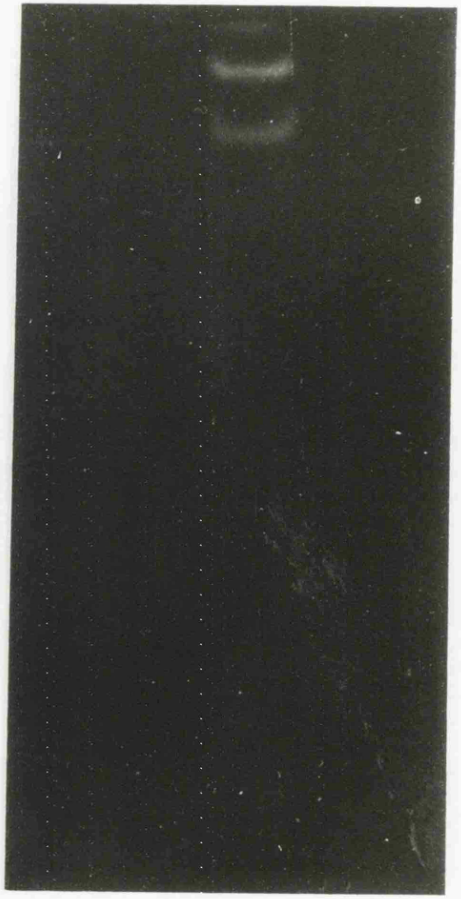
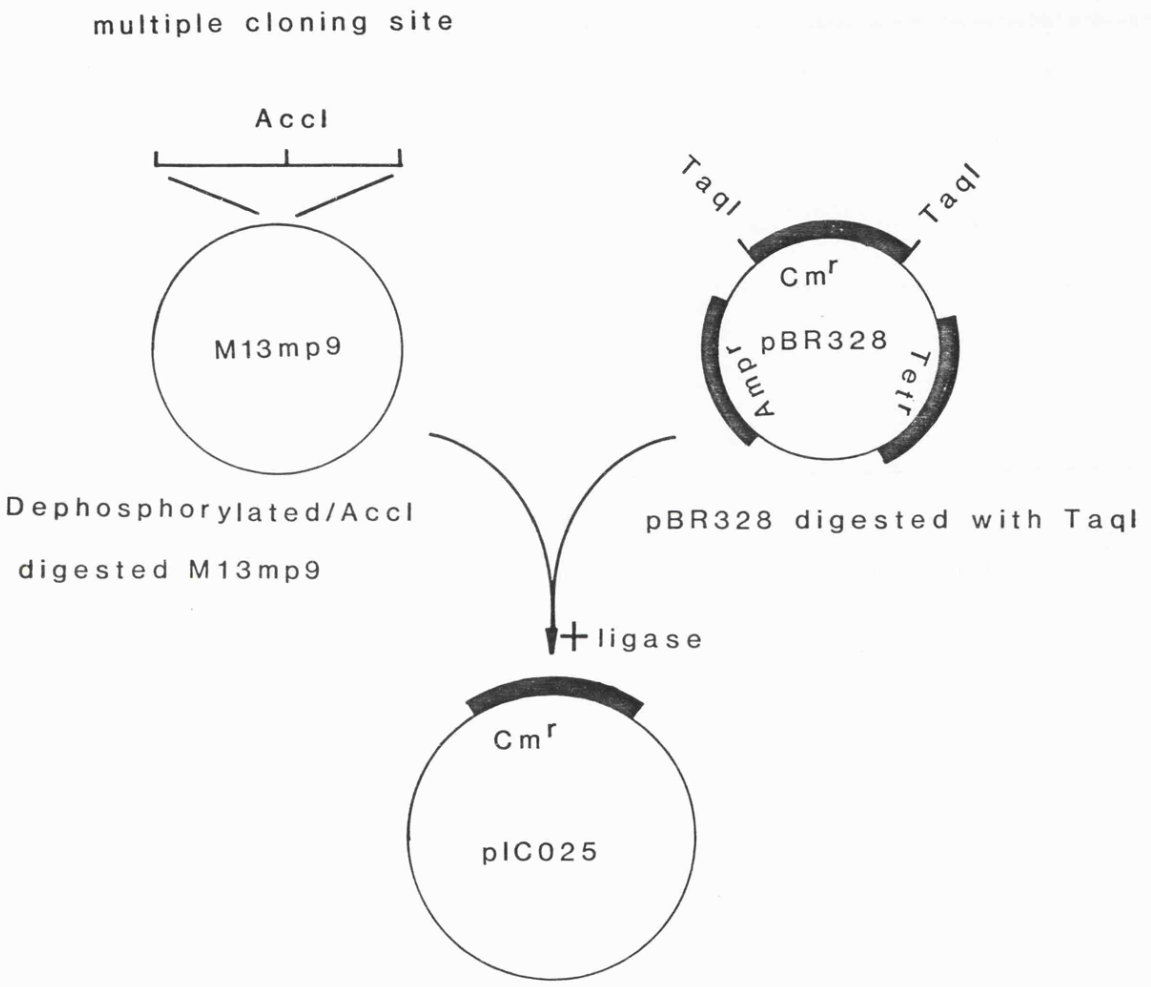

(b)
A B C D

FIG. 4.3

Construction of plasmid pICO25.


multiple cloning site
AccI
M13mp9
Dephosphorylated/AccI
digested M13mp9
TaqI
TaqI
Cm^r
pBR328
Amp^r
Tet^r
pBR328 digested with TaqI
+ ligase
Cm^r
pIC025

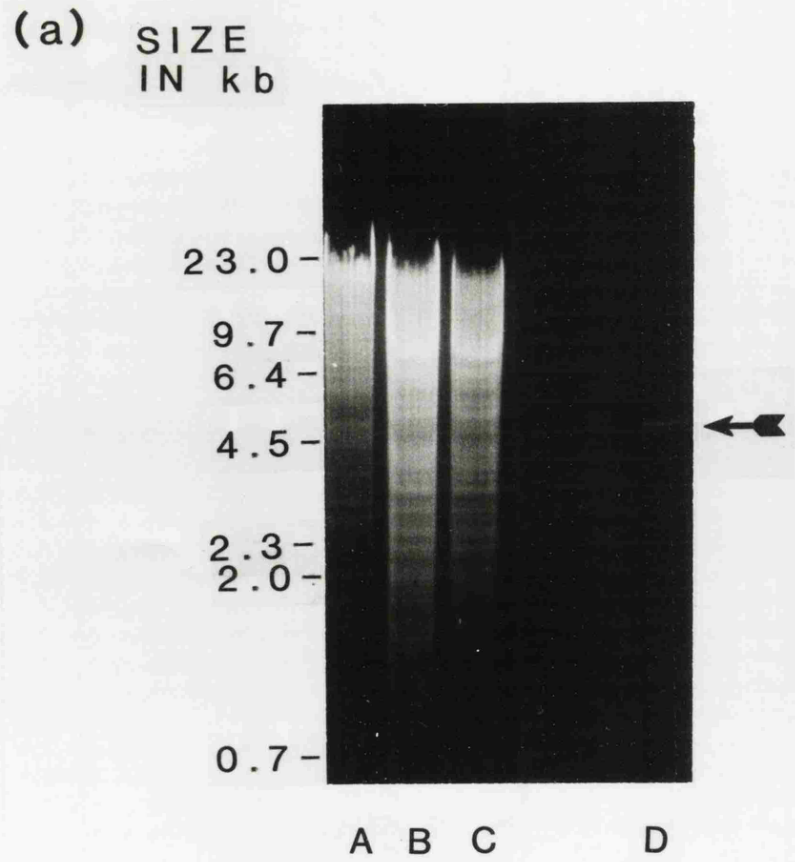
4.2.5 Hybridization of the type I cat to P.mirabilis chromosomal DNA

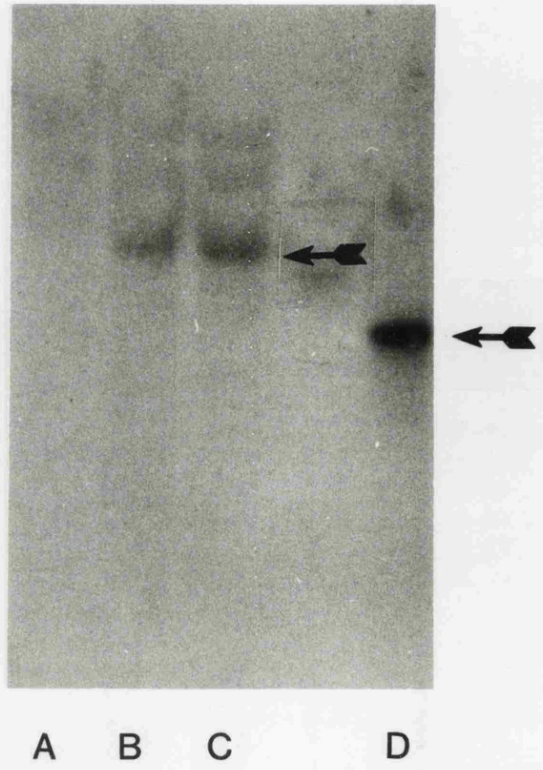
The type I cat gene isolated from pIC025 was used to probe total DNA isolated from P.mirabilis PM13. Approximately 3μg of total DNA prepared either from cells grown in the presence (50μg/ml) or the absence of chloramphenicol were digested separately with PstI, loaded onto a 0.7% (w/v) agarose gel, and electrophoresed at 150V for 2 hours. A positive hybridization control of pBR328 digested with EcoRI was included, as were size markers (a Hind III digest of λ DNA). After electrophoresis the gel was stained with ethidium bromide. Fig. 4.4(a) shows a photograph of the banding pattern produced after staining, while Fig. 4.4(b) shows the result of hybridization of the tracks to the type I cat probe after transfer to nitrocellulose paper (Methods, 2.3). Hybridization has occurred equally well to an 8.5-kb PstI fragment present in total DNA prepared from P.mirabilis PM13 cells grown in the presence and absence of chloramphenicol.

Fig. 4.4(a) Agarose (0.7%) gel electrophoresis of PstI digests of DNA isolated from (A) P.mirabilis PM2 (negative control), (B and C) P.mirabilis PM13 grown in the presence (50µg/ml) and absence of chloramphenicol respectively, and D, a PstI digest of plasmid pBR328 (positive control).

Fig. 4.4(b) Autoradiograph of the DNA in (a) after transfer to nitrocellulose by the method of Southern and hybridization with the cat I probe from pIC025.

Hybridization has occurred equally well (arrows) to P.mirabilis PM13 chromosomal DNA prepared from cells grown in the presence and absence of chloramphenicol.


(a)
SIZE
IN kb
23.0
9.7
6.4
4.5
2.3
2.0
0.7
A B C D


(b)
A B C D

### 4.2.6 Use of the type I cat 'cartridge' to detect the presence of a plasmid in P.mirabilis PM13

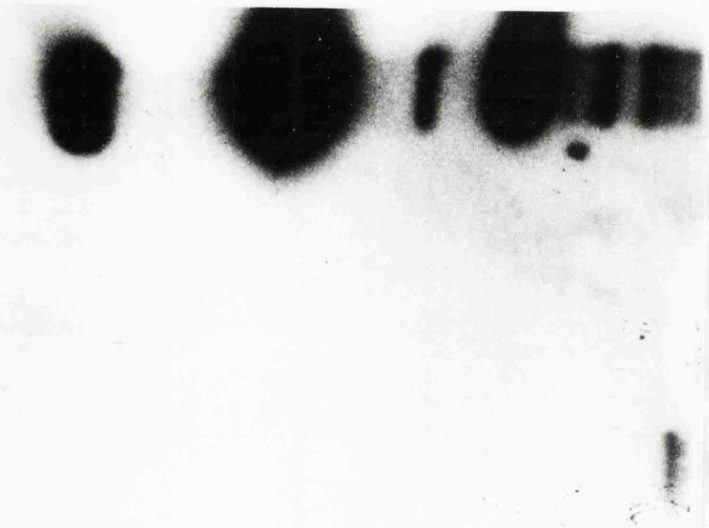
The method of chromosome isolation used in this study in fact yields total bacterial DNA, that is, a mixture or chromosomal DNA and plasmid DNA, if present. Since it had been demonstrated previously that the type I cat probe could identify a specific 8.5-kb PstI fragment in DNA prepared in this way, it was decided to use the probe to detect a plasmid harbouring a cat gene. Specific protocols, designed to isolate plasmid DNA have been developed (reviewed by Broda, 1979). The method used in this experiment was that of Kado and Liu (1981). Overnight cultures of P.mirabilis PM13 separately harbouring the plasmids pBR328, and R100 respectively were grown alongside P.mirabilis PM13 grown in the presence (50µg/ml) and absence of chloramphenicol. P.mirabilis PM2 was grown as a negative control, having been shown previously to be CAT$^-$ by the sensitive radiometric assay (see Chapter 1). The overnight cultures were then subjected to the plasmid isolation procedure, after which the DNA was recovered by ethanol precipitation and electrophoresed in 0.6% agarose. (Methods, 2.3). After electrophoresis, and subsequent transfer to nitrocellulose paper (see Methods, 2.3), the filter was probed with the [$^{32}$P]-labelled 773-bp cat 'cartridge'. Fig. 4.5 shows that hybridization has occurred to the two positive controls, P.mirabilis PM13 harbouring either pBR328 or R100. No positive hybridization signal is present in the tracks containing DNA prepared from P.mirabilis PM2, or P.mirabilis PM13 grown in the presence or absence of chloramphenicol. The plasmids pBR328 and R100 were chosen as

positive controls, as both are representative of the extremes of variability in size and copy number of plasmids. The rationale of the experiment is that if a large low copy number plasmid (R100) and a small high copy number (pBR328) plasmid can be identified, then any plasmid naturally carried by P.mirabilis PM13 is likely to be identifiable. The lack of any detectable hybridization signal in this experiment suggests that the cat gene of P.mirabilis PM13 is not plasmid borne. Furthermore the positive hybridization signal to an 8.5-kb PstI fragment in Southern blot experiments involving total bacterial DNA preparations suggest that the cat gene is chromosomal.

FIG. 4.5

Autoradiograph of plasmid DNA isolated from P.mirabilis PM13 and PM2 by the method of Kado and Liu (1981), after gel electrophoresis (0.6%) and transfer to nitrocellulose by the method of Southern (1975). Track (A) PM13 harbouring pBR328 (positive control), (B) PM2 (cat-free, and plasmid-free negative control), (C) PM13 grown in the absence of chloramphenicol, (D) PM13 grown in the presence of chloramphenicol (50µg/ml) and (E) PM13 harbouring R100 (positive control).

The filter was hybridized with an $\alpha[^{32}P]$-labelled type I cat gene isolated from pIC025. Hybridization has only occurred to plasmids isolated from the positive controls.


A
B
C
D
E
PM13 pBR328
PM2
PM13 Cm−
PM13 Cm+
PM13 R100

## 4.3 SUMMARY

The work described in this chapter demonstrated that the likely structural homology between the type I CAT and its counterpart in P.mirabilis PM13 can be detected at the DNA level. This homology prompted DNA:DNA hybridization studies which revealed (a) that the cat gene of P.mirabilis PM13 is located on an approximately 8.5-kb PstI fragment and (b) that this cat gene was found in DNA prepared from cells grown in the absence of chloramphenicol as well as its presence.

# CHAPTER 5

# CLONING OF THE *P.MIRABILIS* *CAT* GENE

# CHAPTER 5 : Cloning of the P.mirabilis cat gene

## 5.1 Introduction

The essence of in vitro gene cloning is the joining of a stretch of DNA of interest to a vector molecule which allows propagation (and amplification) of that DNA segment in a host cell. There are five requirements for a successful in vitro gene cloning experiment: (i) a method of generating DNA fragments; (ii) a vector to carry the fragments; (iii) a means of joining the vector and fragment together; (iv) a method of introducing hybrid molecules into a host cell, and finally; (v) a way of identifying the desired recombinant. The mechanisms by which fragments are generated and subsequently joined to vector molecules are equally applicable to all in vitro cloning strategies. However, the choice of vector and host depends upon the nature and origin of the fragment of DNA to be cloned. Host-vector systems have now been described for a variety of gram-positive and gram-negative bacteria (reviewed by Thompson, 1982). As P.mirabilis spp. is closely related to E.coli the same vectors can be employed, unfortunately, those P.mirabilis strains tested could not be transformed to high efficiency (see Chapter 8), so a self-cloning strategy was not viable. Initially it was decided to attempt to clone the P.mirabilis PM13 cat gene in an E.coli host as other workers (Beck and Coetzee, 1983; Eitner et al., 1982; Eitner et al., 1983) had reported success with this approach.

In parallel with the experiments described above, it was decided to employ an alternative procedure to clone the P.mirabilis

PM13 cat gene. This strategy involved screening a P.mirabilis PM13 gene-bank (made with chromosomal DNA prepared from cells grown in the absence of chloramphenicol) with an $\alpha[^{32}P]$-labelled cat probe. This experiment was primarily undertaken as a back-up strategy to attempt to clone the cat gene from the CAT$^-$ state by expression, as conceivably such an approach may have been unsuccessful.

## 5.2 RESULTS

### 5.2.1 Cloning of the P.mirabilis cat gene from cells in the CAT+ or CAT− state by expression in an E.coli host

The strategy used to clone the P.mirabilis PM13 cat gene(s) from either state relied on the homology observed between the P.mirabilis PM13 cat and the type I variant (Chapter 4). Southern hybridization of an $\alpha$[$^{32}$P]-labelled type I cat probe against PstI chromosomal digests of P.mirabilis PM13 DNA (prepared from cells grown in the presence or absence of chloramphenicol) yielded the same unique positive hybridization signal in both instances. This was interpreted as evidence to suggest that the entire structural gene for the P.mirabilis PM13 cat was located within the 8.5-kb fragment. The cloning strategy therefore involved the separate ligation of PstI digested chromosomal DNA (prepared from cells grown in the presence (50µg/ml) and absence of chloramphenicol) into the PstI digested dephosphorylated vector, pAT153 (Twigg and Sherratt, 1980). In a typical experiment, 1µg of vector DNA was ligated to 3µg of insert DNA, and the ligation-mix used to transform competent E.coli SK3430 (Methods, 2.3). After the heat-shock, 1ml of L-broth was added, after which the cells were incubated at 37°C for 1 hour to allow expression of the transformed DNA. The cells were then pelleted by centrifugation in a microfuge for 1 minute, resuspended in 200µl of L-broth, and spread on L-agar plates containing either chloramphenicol (10µg/ml) or tetracycline (12.5µg/ml). Controls included in the transformation experiment were: supercoiled vector DNA; cut and phosphatase treated vector; cut and phosphatased vector ligated to itself; and a 'blank' of no DNA.

Analysis of the control experiments allowed an interpretation of the likelihood of success for the cloning strategy by application of the Clarke & Carbon gene-bank equation (Clarke and Carbon, 1976). In the summary at the end of this chapter, the implications of this equation to the P.mirabilis enhancement phenomenon are discussed.

If analysis of the controls revealed that a representative gene-bank was likely to have been constructed the recombinants were allowed to grow at 37°C for 48 hours. Any chloramphenicol-resistant colonies were then picked from the plates and tested, by replica-plating, for resistance to ampicillin (50µg/ml), tetracycline (12.5µg/ml), and chloramphenicol (10µg/ml). Those colonies which were $Cm^r$, $Tc^r$, and $Ap^s$ were selected for further analysis. From the cloning experiments five $Cm^r$, $Tc^r$, and $Ap^s$ colonies were isolated from ligation mixes which included chromosomal DNA isolated from cells grown in the absence of chloramphenicol, while 31 such colonies were isolated from the parallel experiments wherein chromosomal DNA was prepared from cells grown in the presence of chloramphenicol. Restriction digest analysis carried out on mini-plasmid preparations (Methods, 2.3) of each of the positive colonies revealed, in all cases, a common insert band of approximately 8.5-kb, along with the band of 3.4kb corresponding to linearized vector (Fig. 5.1).

Several criteria have to be fulfilled in order to establish that the recombinants carrying the 8.5-kb inserts are bona fide, these include: (i) restransformation, to demonstrate that the desired trait

(chloramphenicol resistance) is carried by the recombinant plasmid; (ii) enzyme assays to establish that chloramphenicol resistance is mediated by CAT rather than alternative mechanisms, and finally, (iii) Southern blotting to show that the insert DNA originated from the chromosome of P.mirabilis PM13 rather than any other strain.

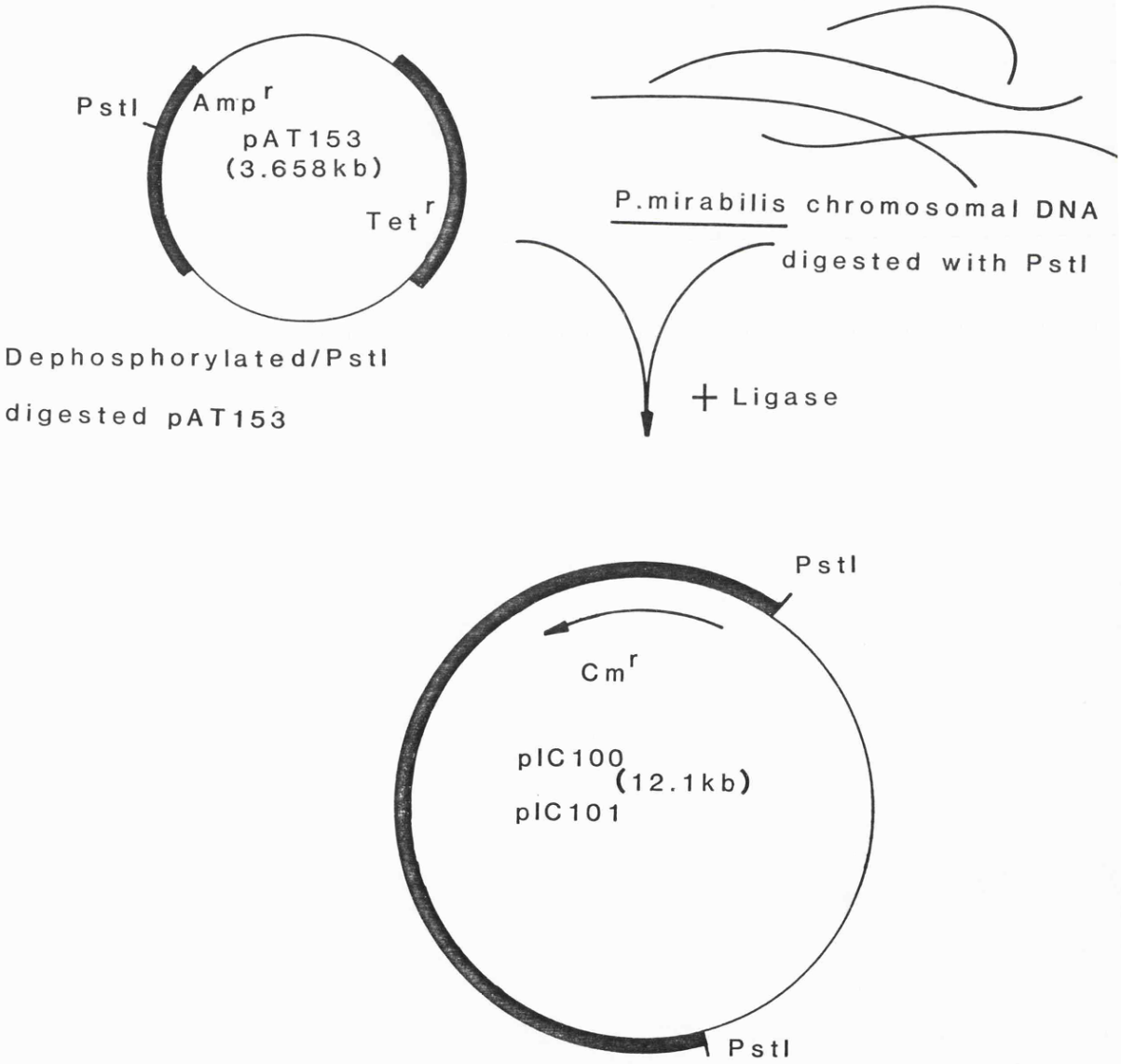
5.2.2 Retransformation

To establish that the plasmids carrying the 8.5-kb insert were indeed responsible for the observed chloramphenicol resistance, purified plasmid from four of the 8.5-kb insert carrying recombinants from each of the transformations were prepared. These plasmids were used to separately transform E.coli SK3430, and after the heat shock cells were spread onto Penassay-agar supplemented with chloramphenicol at a concentration of 10µg/ml. A random selection of colonies (100) corresponding to each class of transformant were patch-plated onto Penassay-agar, grown overnight at 37°C, and replica-plated onto Penassay-agar containing either ampicillin (50µg/ml), tetracycline (12.5µg/ml), or chloramphenicol (10µg/ml). The replicas were examined (after overnight growth at 37°C for colonies inoculated on Penassay-ampicillin agar and Penassay-tetracycline agar, and after 48 hours for those on Penassay-chloramphenicol). All colonies proved to be $Cm^r$, $Tc^r$, and $Ap^s$, thus demonstrating that the resistant determinants were each carried on the recombinant plasmids, and that chloramphenicol resistance was encoded by the 8.5-kb insert.

One recombinant plasmid carrying the 8.5-kb insert in the same orientation from each of the cloning experiments was selected for

FIG. 5.1 Plasmid construction

Construction of plasmids pIC100 and pIC101 , refer to text f details.


PstI
Amp r
pAT153
(3.658kb)
Tet r
Dephosphorylated/PstI
digested pAT153
P.mirabilis chromosomal DNA
digested with PstI
+ Ligase
PstI
Cm r
pIC100
(12.1kb)
pIC101
PstI

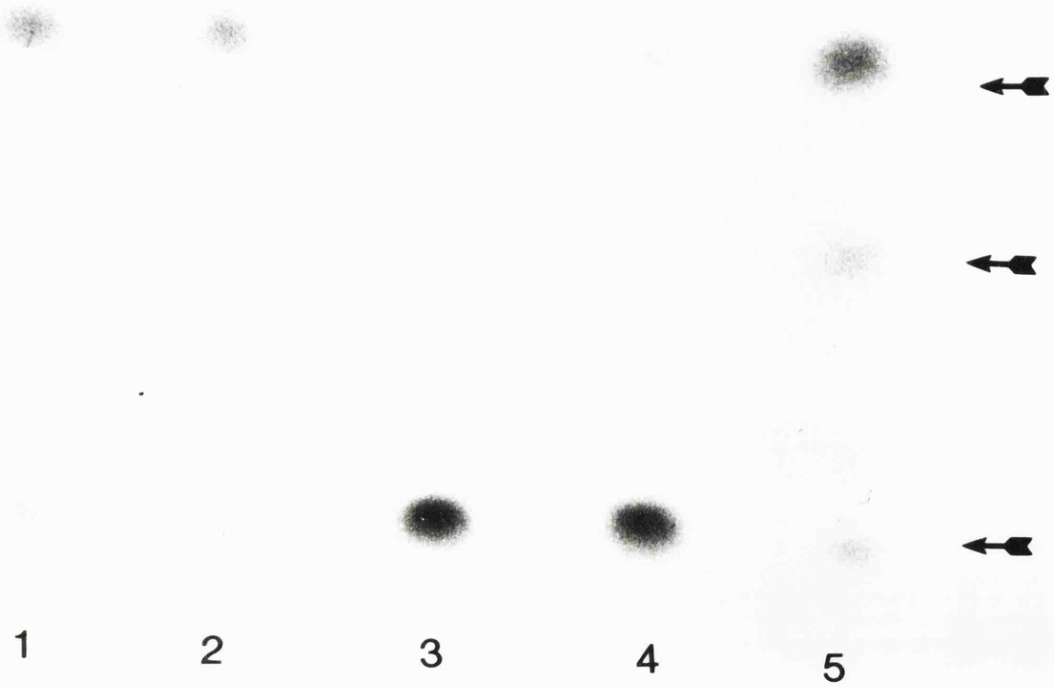
further study. The plasmid cloned from chromosomal DNA prepared from P.mirabilis PM13 grown in the presence of chloramphenicol ($CAT^+$ state) was designated pIC100, whereas that carrying insert DNA prepared from cells grown in the absence of chloramphenicol ($CAT^-$ state) was designated pIC101. Both recombinants carried the 8.5-kb insert in the same orientation in pAT153 (Fig. 5.1).

### 5.2.3 Assay of chloramphenicol acetyltransferase activity

To confirm that the chloramphenicol resistance expressed by E.coli SK3430 harbouring pIC100 or pIC101 was due to chloramphenicol acetyltransferase activity, and not one of the other resistance mechanisms (discussed in Chapter 1), the enzyme activity of crude cell extracts were measured. Initial attempts to measure CAT activity by the spectrophotometric assay were unsuccessful, and the more sensitive radiometric assay had to be employed. E.coli SK3430 harbouring pBR328, pIC100, pIC101, or no plasmid were grown separately in the presence of [$^{14}C$]-labelled chloramphenicol (Methods, 2.3) after which ethylacetate extracts of the growth medium were chromatographed on thin layer sheets. Fig. 5.2 shows an autoradiograph from such an experiment, showing that acetylated products (marked with arrows) only occur in tracks containing pBR328 (positive control), pIC100, and pIC101. This demonstrates that the 8.5-kb insert encodes a gene for chloramphenicol acetyltransferase.

FIG. 5.2 Measuring CAT activity in crude cell extracts

Radiometric demonstration of CAT activity in crude cell extracts from E.coli SK3430 harbouring pIC100 (lane 1), pIC101 (lane 2) or pBR328 (lane 5). Lanes 3 and 4 contain negative controls which correspond respectively to extracts from E.coli SK3430 (no plasmid) and Methylophilus methylotrophus, an obligate methylotroph which has been demonstrated to lack both CAT activity and a cat gene. (Beschle et al., 1984).


1
2
3
4
5

5.2.4 Southern blotting of the P.mirabilis PM13 chromosome

As a means of verifying that the 8.5-kb insert responsible for chloramphenicol resistance in pIC100 and pIC101 originated from the P.mirabilis PM13 chromosome, Southern hybridization experiments were carried out using the nick-translated 8.5-kb fragment, and the cat consensus 20-mer oligonucleotide as probes (Chapter 1). Chromosomal DNA prepared from P.mirabilis PM13 grown in the presence and absence of chloramphenicol were separately digested with PstI, and electrophoresed through a 0.7% (w/v) agarose gel. Negative controls of PstI digests of P.mirabilis PM2, Providencia stuarti P6 and Methylophilus methylotrophus (AS1) DNA were included, and the resulting fragments transferred to nitrocellulose by the method of Southern (Method, 2.3). The 8.5-kb hybridization probe was prepared by digesting plasmid pIC100 with PstI, and separating the insert away from the vector by electrophoresis through a 0.7% (w/v) agarose gel. The 8.5-kb insert band was cut from the gel, the DNA recovered by the optimized "freeze-squeeze" method, and subsequently nick-translated. The cat consensus oligonucleotide was end-labelled with [$^{32}$P] (Methods, 2.3). Fig. 5.3 shows photographs of autoradiographs produced by hybridizing the 8.5-kb probe (b), and the cat consensus oligonucleotide (c) to the nitrocellulose filter. Hybridization has occurred in both instances to an 8.5-kb fragment in P.mirabilis PM13 chromosomal DNA. This suggests that the 8.5-kb insert from plasmid pIC100 (and by inference pIC101) originated from P.mirabilis PM13. Perhaps not surprisingly the 8.5-kb PstI probe also hybridized to an equivalent locus in the chromosome of P.mirabilis PM2, however

this chromosomal locus did not hybridize to the cat consensus active site oligonucleotide probe, indicating that in this strain, no functional cat gene is present.

5.2.5 Restriction mapping of pIC100 and pIC101

The plasmids pIC100 and pIC101 were digested with a series of restriction enzymes, and electrophoresed through either 0.7% or 1.5% (w/v) agarose gels (Fig. 5.4). A restriction map for both plasmids was constructed, and was found to be indistinguishable for those enzymes tested (Fig. 5.5).

Fig. 5.3 Southern blotting of the P.mirabilis chromosome

(a) Photograph of an ethidium bromide stained agarose gel (0.7%) showing the banding pattern produced when chromosomal DNA samples from the following strains were digested with the restriction endonuclease PstI: (B) AS1, (C) Providence strain P6 NCTC 8113, (D) P.mirabilis PM2, (E) P.mirabilis PM13 (50), (F) P.mirabilis PM13 (Cm0). Figures in parentheses represent the concentration of chloramphenicol cells were grown in prior to lysis and DNA preparation. Track (A) contained phage λDNA digested with HindIII (as size markers). Tracks (G) and (H) were PstI digests of pIC100 and were included as hybridization and visualization positive controls respectively.

(b) Autoradiograph of the DNA in (a) after transfer to nitrocellulose by the method of Southern, and hybridization to the 8.5-kb nick-translated PstI insert fragment of pIC100. Hybridization has occurred to P.mirabilis PM13 chromosomal DNA (tracks E and F), and to the taxonomically related strain PM2, as well as the plasmid positive control (G). No hybridization was seen to the chromosomal DNA from AS1 or Providence P6.

(c) Autoradiograph of the DNA shown in (a) after transfer to nitrocellulose, and hybridization to a $\gamma[^{32}P]$-labelled consensus oligonucleotide directed at the highly conserved active site region of cat. Both tracks containing DNA isolated from PM13 yield a positive hybridization signal (E, and F) as did the positive hybridization control (G) arrowed.

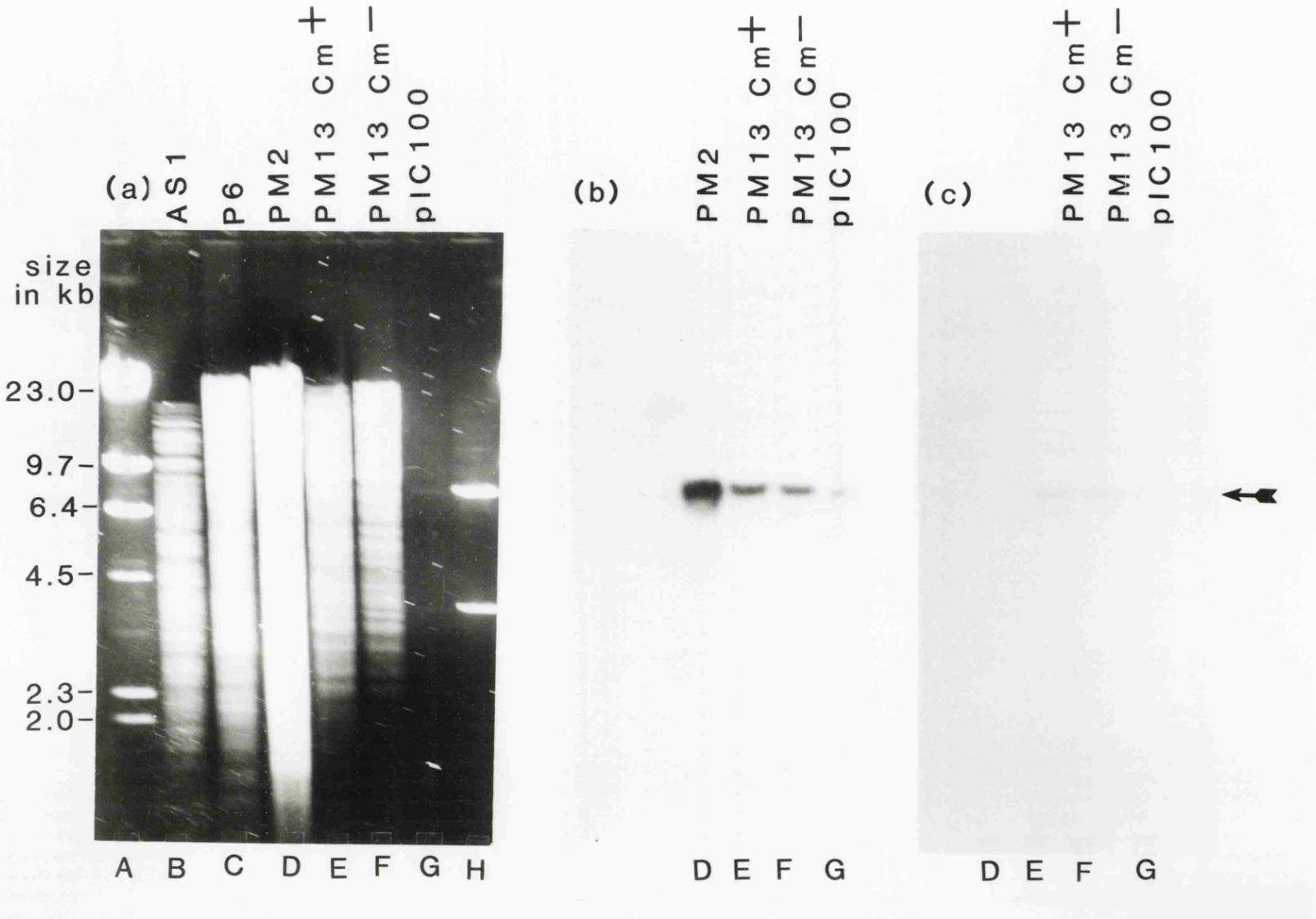
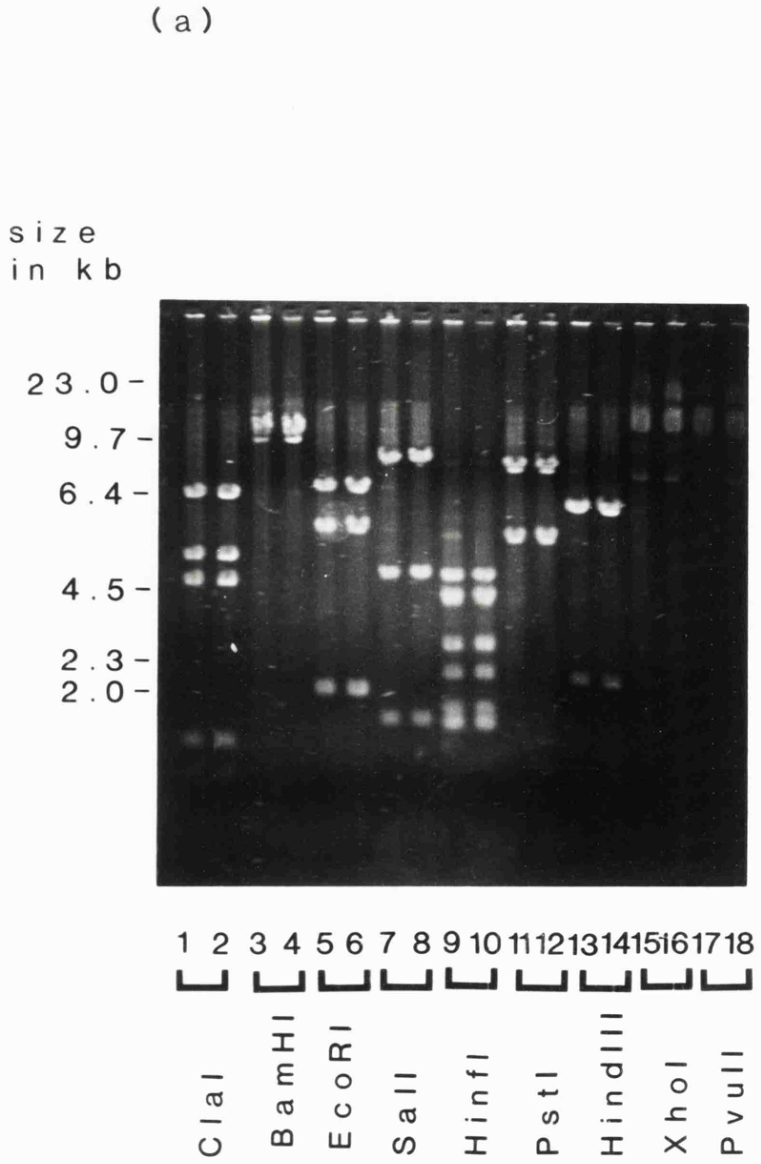

(a)
AS1
P6
PM2
PM13 Cm+
PM13 Cm−
pIC100
size in kb
23.0
9.7
6.4
4.5
2.3
2.0
A B C D E F G H
(b)
PM2
PM13 Cm+
PM13 Cm−
pIC100
D E F G
(c)
PM13 Cm+
PM13 Cm−
pIC100
D E F G

FIG.5.4 Restriction digests of plasmids pIC100 and pIC101

(a) Photograph of an ethidium bromide stained agarose gel (0.7%) showing the banding pattern produced when the plasmids pIC100 (tracks 1, 3, 5, 7, 9, 11, 13, 15 and 17) and pIC101 (tracks 2, 4, 6, 8, 10, 12, 14, 16 and 18) were digested with the restriction endonucleases ClaI, BamHI, EcoRI, SalI, HinfI, PstI, HindIII, XhoI and PvuII.

(b) Photograph of an ethidium bromide stained agarose gel (1.5%) showing the banding pattern produced when pIC100 (tracks 2, 4, 6 and 8) and pIC101 (tracks 3, 5, 7 and 9) were digested with the enzymes ClaI, HinfI, TaqI, and Sau3A. Track 1 was a HinfI digest of plasmid pAT153 as size markers.

Both plasmids produced the same size restriction fragments when digested with the above enzymes. The significance of the restriction digest pattern is discussed in Chapters 6, 7 and 8.


(a)
size
in kb
23.0
9.7
6.4
4.5
2.3
2.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
ClaI
BamHI
EcoRI
SalI
HinfI
PstI
HindIII
XhoI
PvuII

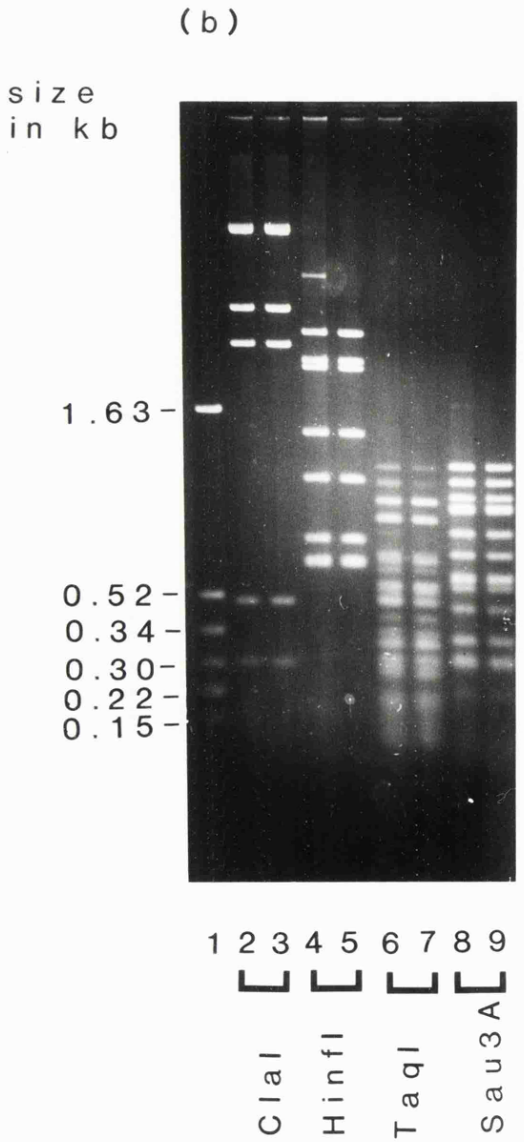
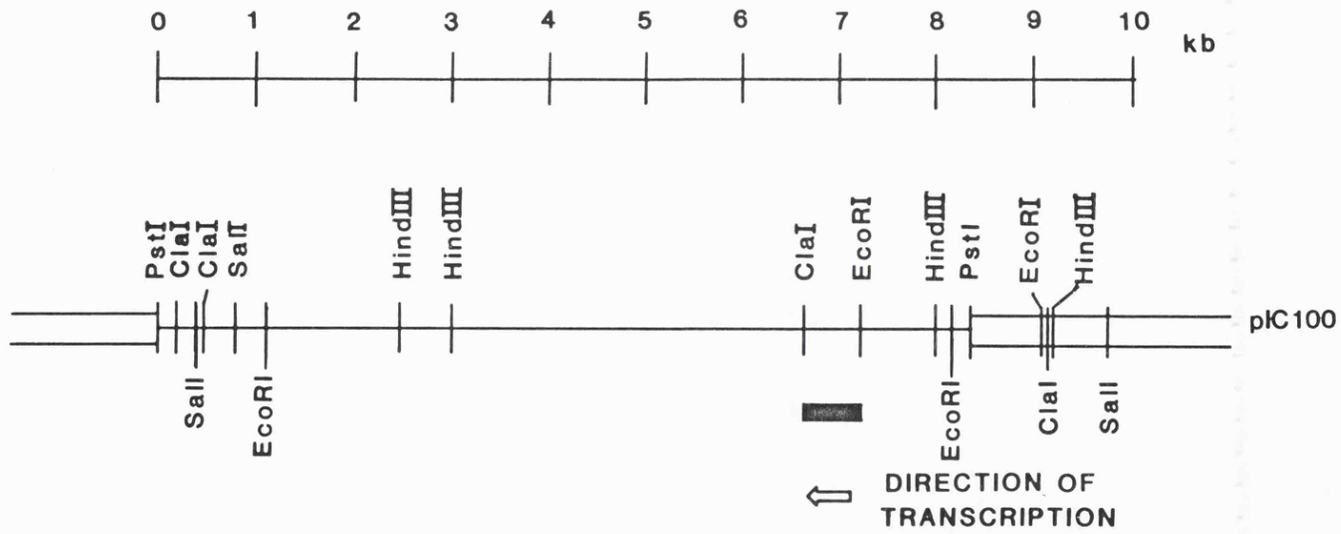

(b)
size
in kb
1.63
0.52
0.34
0.30
0.22
0.15
1 2 3 4 5 6 7 8 9
ClaI
HinfI
TaqI
Sau3A

FIG. 5.5 Restriction map of pIC100 (and pIC101)

The map was compiled using restriction endonucleases ClaI, EcoRI, HindIII, PstI and SalI. The black box bounded by sites for EcoRI and ClaI represents the smallest restriction fragment detected by hybridization to the consensus active site oligonucleotide (Charles et al., 1985).

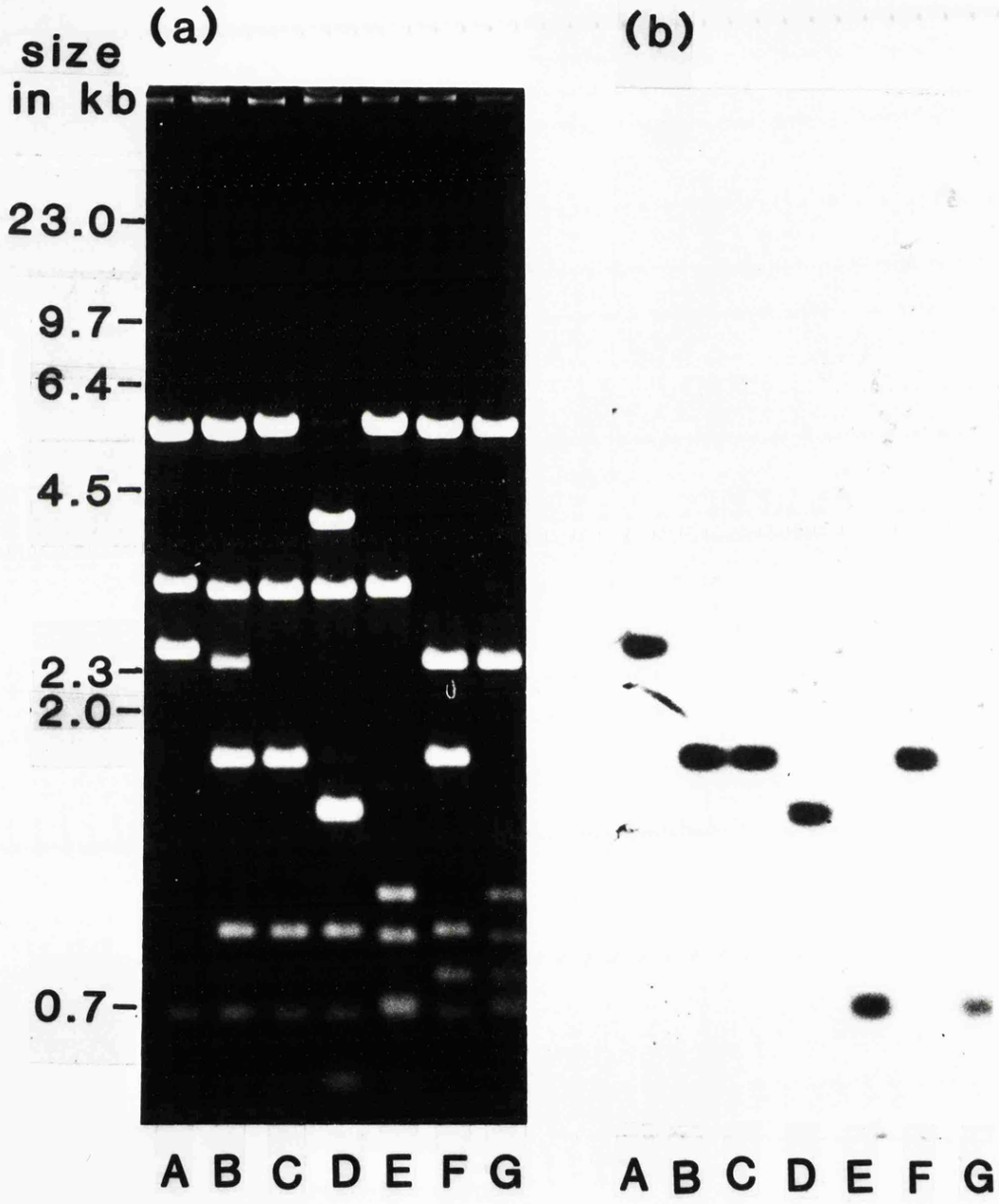

0
1
2
3
4
5
6
7
8
9
10
kb
PstI
ClaI
ClaI
SalI
HindIII
HindIII
ClaI
EcoRI
HindIII
PstI
EcoRI
HindIII
pIC100
SalI
EcoRI
EcoRI
ClaI
SalI
DIRECTION OF
TRANSCRIPTION

5.2.6 Localization of the *cat* gene within the 8.5-kb insert

The sequence around the active site regions of several different *cat* genes from gram-negative and gram-positive bacteria is highly conserved (Shaw, 1983). A 20-base synthetic oligonucleotide, complementary to the coding strand of the active site region of the gene for a type I CAT has been used (Beschle *et al.*, 1984) to identify novel, as well as known *cat* elements by DNA:DNA hybridization. This synthetic oligonucleotide 5'-CCATCACAGACGGCAT GATG-3' was used to locate the *cat* gene within the 8.5-kb of cloned insert of pIC100. Hybridization of the $\gamma[^{32}P]$-labelled oligonucleotide yielded results which were interpreted as evidence for the localization of the *cat* gene on a 500-bp fragment located near one end of the insert (Fig. 5.6).

FIG. 5.6 Localisation of the *cat* gene in pIC100

Agarose gel (0.7%) showing endonuclease digest pattern of pIC100 after ethidium bromide staining. (a) Lanes after digestion with the following enzymes: A, *Cla*I; B, *Cla*I and *Pst*I; C, *Cla*I, *Pst*I, and *Pvu*II; D, *Cla*I, *Pst*I, and *Hin*dIII; E, *Cla*I, *Pst*I, *Eco*RI and *Sal*I. (b) Autoradiograph of the DNA shown in part (a) after transfer to nitrocellulose and hybridization with a $\gamma$-[$^{32}$P]-labelled consensus *cat* active site oligonucleotide.


(a)
(b)
size
in kb
23.0
9.7
6.4
4.5
2.3
2.0
0.7
A B C D E F G
A B C D E F G

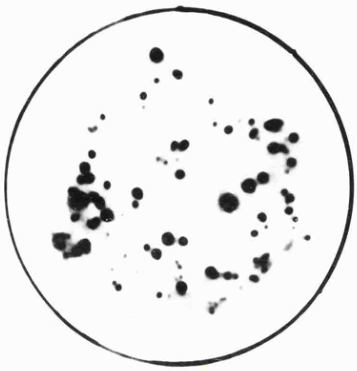
### 5.2.7 Cloning the *P.mirabilis* PM13 *cat* gene by use of a hybridization probe

Chromosomal DNA isolated from cells of *P.mirabilis* PM13 grown in the absence of chloramphenicol was digested with *Pst*I and ligated into plasmid pAT153 as described earlier in this chapter. Competent cells of *E.coli* SK3430 were transformed with the ligation mix, (Methods, 2.3), spread onto L-agar plates supplemented with tetracycline at a concentration of 20μg/ml, and were allowed to grow overnight at 37°C. The resulting colonies were transferred to nitrocellulose, and hybridized to an $\alpha[^{32}P]$-labelled probe comprising the entire 773-bp *cat* insert of pIC025. Five separate colonies showing positive hybridization signals (Fig. 5.7) were picked into M9 medium (Methods, 2.3), diluted, and replated onto tetracycline-agar plates (20μg/ml) so as to give approximately 100 colonies per plate. After overnight growth the colonies were once again transferred to nitrocellulose and subjected to another round of hybridization to the $\alpha[^{32}P]$-labelled 773-bp *cat* insert from pIC025 (see Chapter 4). An individual colony showing a positive hybridization signal from each of the five separate isolates was picked, and grown overnight on an L-agar plate supplemented with tetracycline at a concentration of 20μg/ml. Individual colonies from each plate were then grown overnight in L-broth and subjected to the mini-plasmid lysis procedure described in Methods (2.3). Fig. 5.8 shows the result of digesting each of the resulting five plasmids (named pIC200 to pIC204) with *Pst*I. In each case, an 8.5-kb band was identified, corresponding in size to the *Pst*I inserts in pIC100 and pIC101.

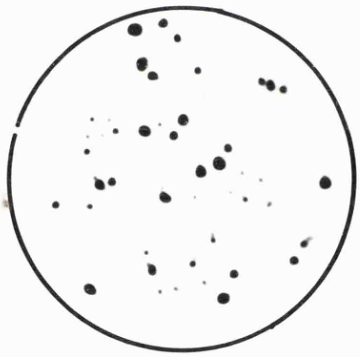
Fig 5.7 Screening a *P.mirabilis* PM13 chromosomal DNA library

The library, constructed in the vector pAT153, was screened for the presence of the *cat* gene. In the first round of screening five separate colonies returning positive hybridization signals to the $\alpha[^{32}P]$-labelled 8.5-kb *PstI* *cat* probe were picked. In each case the presence of the *cat* gene was verified by re-screening (second round, numbered 1 to 5). The resulting plasmids were named pIC200, pIC201, pIC202, pIC203, and pIC204 respectively.

FIRST ROUND

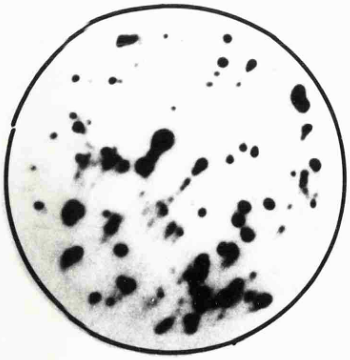


SECOND ROUND

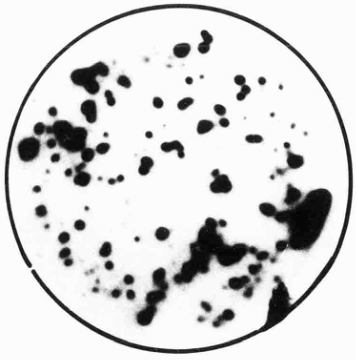


1

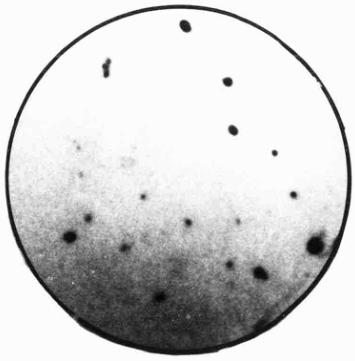
2



3



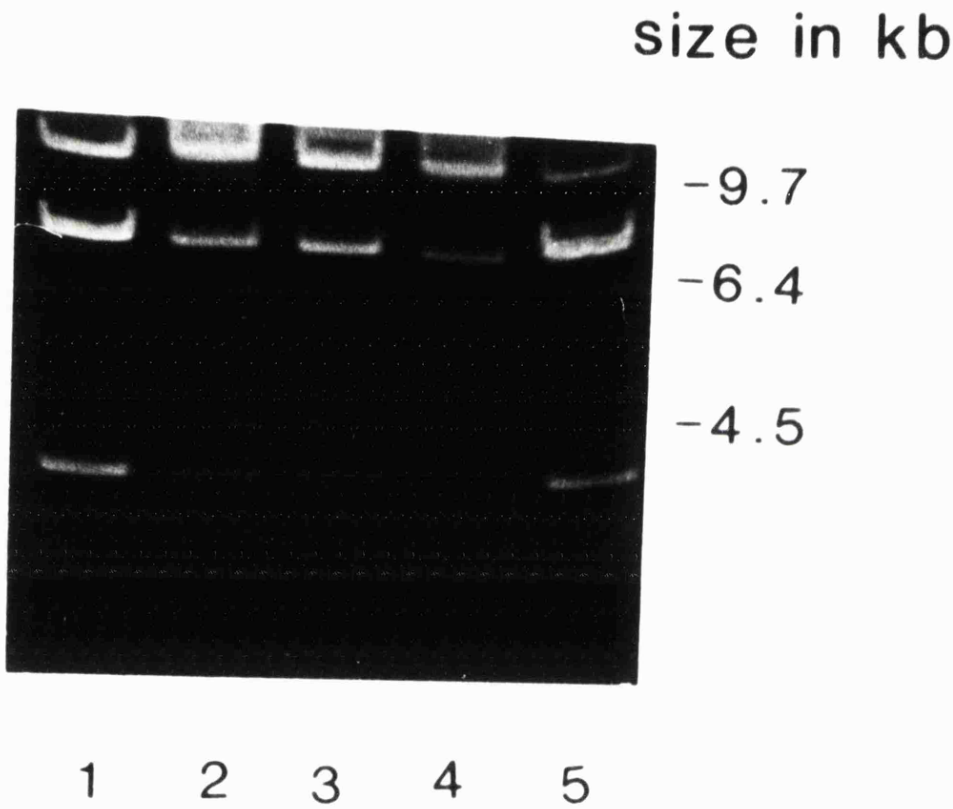
4



5

Fig. 5.8 Analysis of *cat*-containing recombinants by electrophoresis

Photograph of an ethidium bromide stained agarose gel (0.7%) showing (tracks 1 to 5) five recombinants arising from the screening experiment (see Fig. 5.7) digested with *Pst*I. The band at the top of the gel represents a partial digest product, and is probably linearized plasmid. The middle band is the 8.5-kb *cat* containing insert (found in pIC100 and pIC101), while the lower band represents the vector pAT153.


size in kb
-9.7
-6.4
-4.5
1
2
3
4
5

### 5.2.8 Do the plasmids pIC200 to pIC204 confer resistance to chloramphenicol

In order to test whether or not the five plasmids pIC200, pIC201, pIC202, pIC203, and pIC204 conferred resistance to chloramphenicol, replica-plating experiments were carried out. Strains of E.coli SK3430 harbouring the above plasmids were replica-plated (along with pIC101 as a positive control, and the host strain alone as a negative control) from L-agar plates onto L-agar supplemented with tetracycline (20µg/ml) thence onto chloramphenicol (10µg/ml) and finally chloramphenicol (50µg/ml). The strains harbouring the plasmids pIC200, pIC201, pIC202, pIC203, and pIC204 showed a similar resistance pattern to the strain harbouring pIC101 in that growth was supported on L-agar plates supplemented with chloramphenicol at a concentration of 10µg/ml, but not on any higher concentrations of the antibiotic. This CAT expression data suggests that the cat genes harboured by pIC200, pIC201, pIC202, pIC203, and pIC204 are indistinguishable from those harboured by pIC100 and pIC101.

## 5.3 Summary

The cat gene(s) of P.mirabilis PM13 have been cloned as 8.5-kb PstI fragments from chromosomal DNA isolated from P.mirabilis PM13 grown both in the presence (pIC100) or the absence (pIC101) of chloramphenicol. Identical restriction maps were produced from both plasmids when digested with the enzymes ClaI, EcoRI, HindIII, PstI and SalI.

The cat gene was also cloned by a strategy involving hybridization of a P.mirabilis PM13 gene-bank to an $\alpha$[$^{32}$P]-labelled type I cat probe. The five recombinant plasmids isolated (pIC200 to pIC204) were all demonstrated to contain an 8.5-kb PstI insert, and to produce low CAT levels, and thus were indistinguishable from pIC100 and pIC101.

The expression of the P.mirabilis PM13 cat gene in an E.coli host was very poor and CAT could be detected only with the sensitive radiometric assay. This initially raised the query as to whether or not the entire coding and control regions for the gene had been cloned. However, restriction mapping in conjunction with oligonucleotide-directed active-site probing located the active-site approximately 1.5-kb away from PstI site at one end of the fragment (this was later confirmed by sequencing (Chapter 7)). Based on the knowledge of the size of other cat genes, this was judged to be sufficient DNA to encode the rest of the structural gene and still leave enough room for transcriptional initiation and possible translational control elements. The absence of high-level expression

of the P.mirabilis cat in E.coli might be related to the general problem of the anomalous expression which has been observed reciprocally between genes in E.coli and P.mirabilis (Baumberg et al., 1980; Baumberg and Dennison, 1975).

Because the expression of the P.mirabilis PM13 cat gene harboured by pIC100 and pIC101 in E.coli SK3430 (IC100 and IC101 respectively) was very low, attempts were made to increase cat gene expression by growing IC100 and IC101 on increasingly high levels of chloramphenicol. That is, the strategy involved a recapitulation of the procedure used to detect the enhancement phenomenon originally observed for chloramphenicol resistance in wild-type P.mirabilis PM13. Such efforts to produce high level chloramphenicol resistance colonies failed, a result which suggested that although the cat gene has been cloned successfully from P.mirabilis PM13 into E.coli, the enhancement phenomenon has not.

The equation of Clarke and Carbon (1976) describes the number of recombinant clones required to contain an entire genome in any cloning strategy. The probability (P) that a unique sequence is contained within a set of N recombinant transformants is given by

$$P = 1 - (1 - f)^N$$

where f is the average restriction fragment size as a fraction of total genome size. This can be rearranged to give

$$N = \frac{\ln (1 - P)}{\ln (1 - f)}$$

In the cloning experiment described for the P.mirabilis PM13 cat gene, (for DNA prepared from cells grown in the presence and absence of chloramphenicol) if an average insert of approximately 4.3-kb is assumed, and the P.mirabilis PM13 genome is taken to be the same size as that of E.coli, then

$$N = \frac{\ln (1-0.99)}{\ln [1-(4.3/4.3 \times 10^3)]} = 4.6 \times 10^3$$

Therefore, on average, $4.6 \times 10^3$ recombinants must be screened in order to have a 99% chance of cloning the cat gene. Had there been any large-scale amplification of the P.mirabilis PM13 cat gene, (consistent with the massive increase in CAT levels seen in chloramphenicol-resistant cell populations), then such a phenomenon would be reflected in the ease with which the gene might be expected to be cloned. Experimentally, five positive clones (i.e. chloramphenicol resistant), were isolated from DNA prepared from cells grown in the absence of chloramphenicol, while 31 positive clones were isolated from DNA prepared from cells grown in the presence of chloramphenicol. This represents a six-fold increase in frequency of cloning of the cat gene from DNA prepared with cells grown in the presence of chloramphenicol compared with those grown in the absence of antibiotic. It is unknown whether or not this six-fold increase has any significance as far as the enhancement phenomenon is concerned. The higher number of positive clones (chloramphenicol resistant), recovered from chloramphenicol resistant P.mirabilis PM13 DNA is, however, more likely to represent an unrelated increase in the efficiency of ligation and/or transformation in those experiments.

The question of gene amplification in P.mirabilis PM13 as a possible mechanism for the enhancement phenomenon is the subject of experiments in Chapter 6.

# CHAPTER 6

# CHROMOSOMAL ORGANIZATION AND EXPRESSION OF THE *P.MIRABILIS* PM13 *CAT* GENE

# CHAPTER 6 : CHROMOSOMAL ORGANIZATION AND EXPRESSION OF THE P.MIRABILIS PM13 CAT GENE

## 6.1 INTRODUCTION

The experiments carried out in Chapters 3 and 4 clarified certain features about the enhancement of chloramphenicol resistance in P.mirabilis PM13. The phenomenon appears to involve mutation, in that pre-existing mutant cells (which occur spontaneously at a frequency of between $10^{-4}$ and $10^{-5}$ per cell per generation), are selected by exposure to chloramphenicol and are maintained in the population for so long as they are under such selection. Back-mutation (estimated as occurring at a frequency of $10^{-2}$ per cell per generation), plus counter-selection against resistant cells in the absence of chloramphenicol were judged to account for the loss of resistant cells from a population propagated in antibiotic-free medium.

Spontaneous point mutations which inactivate gene function generally occur in bacteria at a rate of between $10^{-6}$ and $10^{-7}$ events per locus per generation. Back-mutations to restore gene function, may be more specific and occur typically at a 10-fold lower rate (Drake, 1970). Whereas point-mutations were believed formerly to be the principal means of change of individual genes, it has become clear recently that gene expression in prokaryotes and eukaryotes may be modulated by other types of change in DNA, ones which may occur at higher frequency than point mutations. These include:

(i) gene amplification;

(ii) specific and reversible DNA inversions;

(iii) DNA insertions and deletions via transposition events;

(iv) gene conversion.

Any of these above mechanisms could, in theory, account for the enhancement of chloramphenicol resistance in P.mirabilis PM13. Since the cat gene had been cloned (Chapter 5) from DNA prepared from cells grown in the presence and the absence of chloramphenicol, it was decided to use them as probes in Southern-blot and heteroduplex experiments:

(a) to search for changes in the chromosomal organization of DNA under such conditions and, in particular, for evidence of gene amplification and/or DNA re-arrangements, and

(b) to examine the organization of the 8.5-kb cloned fragment of P.mirabilis DNA in both pIC100 and pIC101 by heteroduplex analysis.

The possible control mechanisms noted above all operate at the transcriptional level, either through increasing gene expression via gene dosage (amplification) or by movement of a hitherto silent copy of the gene to the control of an effective promoter. In practice post-transcriptional control mechanisms can also control gene expression (see Chapter 1 for references). To examine whether or not such mechanisms played any part in the enhancement of chloramphenicol resistance in P.mirabilis PM13, "Northern" DNA:RNA hybridizations were performed with RNA isolated from cultures of P.mirabilis PM13 grown in the presence and the absence of chloramphenicol.

## 6.2 RESULTS

### 6.2.1 Gene amplification

Gene amplification is a widespread phenomenon in prokaryotes (Anderson and Roth, 1977), and the frequency of amplification within the genome often depends on the presence of a system for homologous recombination. P.mirabilis sp. can amplify cat genes carried on plasmids such as NR1 when grown in high concentrations of chloramphenicol (Rownd and Mickel, 1971), and chromosomal gene amplification has accounted for increased chloramphenicol resistance mediated by CAT in E.coli (Meyer and Iida, 1979) and also in B.subtilis (Young, 1984).

A process of reversible gene amplification can be imagined to account for the appearance and the disappearance of chloramphenicol resistance in P.mirabilis PM13. To test this hypothesis, the P.mirabilis PM13 genome was examined by Southern blotting for evidence of selective amplification of the cat gene.

Chromosomal DNA was prepared (Methods, 2.3) from P.mirabilis PM13 cells grown in 0, 50, 100, 200 or 500µg of chloramphenicol per ml. Each DNA sample (3µg) was digested with PstI and subjected to electrophoresis through a 0.7% (w/v) agarose gel at 150V for 2 hours. A positively hybridizing control of pIC100 digested with PstI was loaded at a concentration corresponding to that calculated for a single copy of the gene. Phage λ DNA digested with HindIII was loaded as size markers. When the gel had run, it was stained with ethidium bromide, photographed, and transferred to nitrocellulose

paper (Methods, 2.3). An $\alpha[^{32}P]$-labelled probe, comprising the entire P.mirabilis PM13 cat gene on a PstI-ClaI fragment from pIC100 was used to detect the presence of chromosomal cat genes (Methods, 2.3). Efforts were made to ensure that an equal amount of chromosomal DNA was loaded in each of the tracks. The autoradiograph in Fig. 6.1 shows that there is no significant difference in hybridization signal intensity in the restriction fragments arising from DNA prepared from cells which had been grown in the presence of chloramphenicol as compared with those which had not. The higher fraction of high molecular weight DNA-fragments in lanes C, D and E (marked with arrows) indicates that samples in these tracks had not cut to completion. In order to further characterize these hybridization data, the autoradiograph gel tracks were scanned at 550nm using an SP1800 spectrophotometer fitted with density tracing facility. The resulting trace is shown in Fig. 6.2. Integration, and summation of the major and minor peaks, and comparison with the notional value of unity for the pIC100 control produces the values of 2.5:1, 2.4:1, 2.6:1, 3.0:1 and 3.1:1 for the tracks C through G respectively. The results support the contention that there is no amplification of cat when P.mirabilis PM13 is grown in the presence of chloramphenicol.

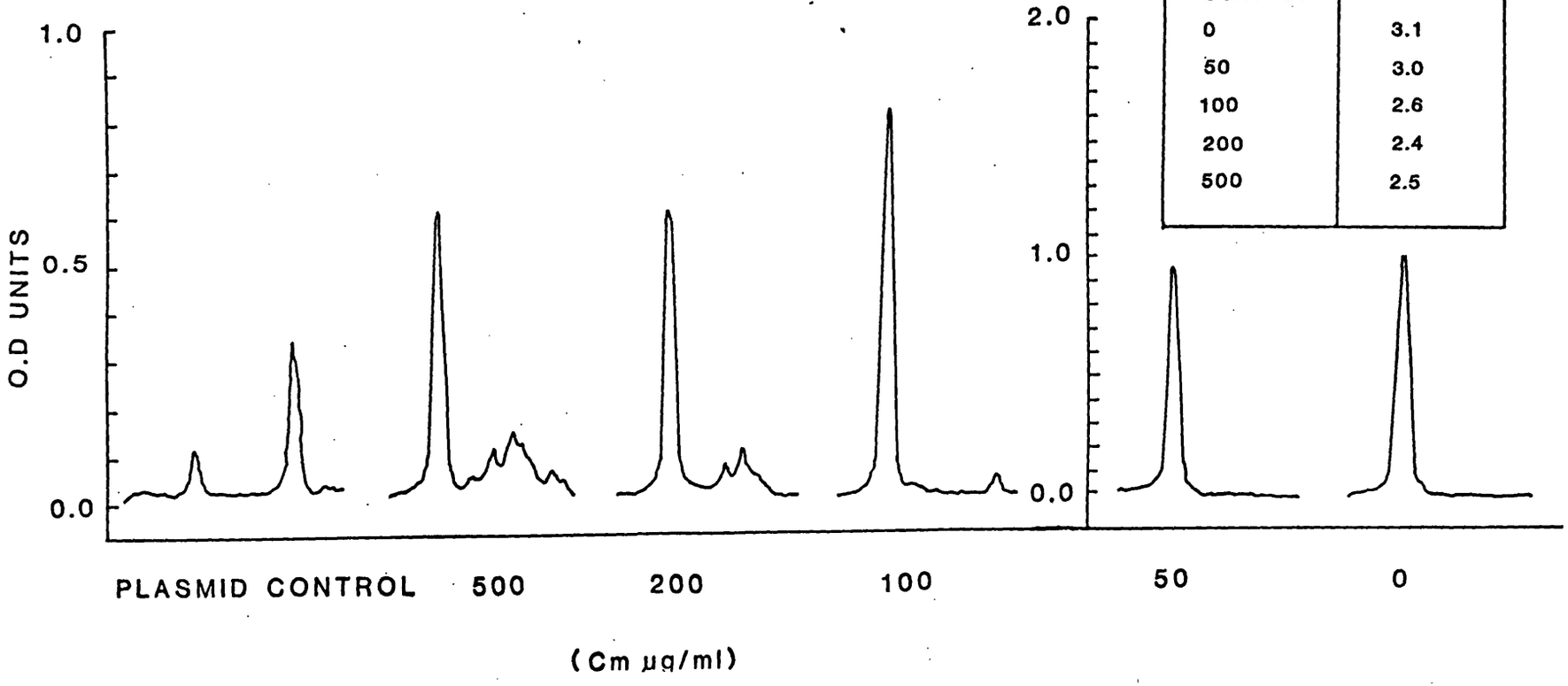
FIG. 6.1

Agarose (0.7%) gel electrophoresis (a) of PstI digests of (A) pIC100 at high concentration for visualization with ethidium bromide and (B) pIC100 at a concentration estimated to correspond to approximately one copy of cat per chromosome for comparison with subsequent (C through G) loadings of chromosomal DNA. Lanes C through F were loaded with PstI digests of chromosomal DNA from P.mirabilis PM13 cells grown in the presence of chloramphenicol at (C) 500, (D) 200, (E) 100, and (F) 50μg/ml. Lane G was a PstI digest of chromosomal DNA from strain PM13 grown in the absence of chloramphenicol. (b) Autoradiograph of the DNA in (a) after transfer to nitrocellulose by the method of Southern (Methods, 2.3) and hybridization with a $\alpha[^{32}P]$-labelled cat probe comprising the 1.7-kb PstI-ClaI fragment of pIC100 (Methods, 2.3 and Fig 5.5). Efforts were made to load equal amounts of chromosomal DNA in tracks C through G. The higher fraction of high-molecular-weight DNA fragments in lanes C, D and E and the weakly positive discrete hybridization signals (marked with arrows) suggest that the corresponding PstI digests may have been incomplete. Scanning of the autoradiograph with integration and summation of the major and minor peaks yielded the intensities of hybridization for comparison with the notional value of unity for the pIC100 control. The relative intensities for lanes C, D, E, F, and G in relation to that of lane B were 2.5, 2.4, 2.6, 3.0 and 3.1 respectively (Fig. 6.2).

(a)
size
in kb
23.0
9.7
6.4
4.5
2.3
2.0
0.7
A B C D E F G
pIc100
PM13 Cm500
PM13 Cm200
PM13 Cm100
PM13 Cm50
PM13 Cm0
(b)
A B C D E F G
pIC100
PM13 Cm500
PM13 Cm200
PM13 Cm100
PM13 Cm50
PM13 Cm0

FIG. 6.2

Fixed wavelength scan of the autoradiograph shown in Fig. 6.1. Peaks represent the absorbance of the positive hybridization bands, numbers under the peaks describe the concentration of chloramphenicol cells were grown in prior to DNA extraction.




| GROWTH CONDITIONS (µg/ml Cm) | RATIO OF BAND INTENSITIES |
|---|---|
| PLASMID CONTROL | 1 |
| 0 | 3.1 |
| 50 | 3.0 |
| 100 | 2.6 |
| 200 | 2.4 |
| 500 | 2.5 |

6.2.2 DNA inversions and other rearrangements

Some metastable phenotypes in bacteria can be attributed to specific DNA rearrangements. For example, phase variation in S.typhimurium mediated by hin (Silverman and Simon, 1980), G-loop inversion in phage Mu mediated by gin (Kamp and Kahmann, 1981; Plasterk et al., 1983), C-loop inversion in P1 mediated by cin (Iida et al., 1982), and P-region inversion in certain strains of E.coli, mediated by pin (Plasterk et al., 1983) form a family of highly conserved invertible systems. The predicted amino acid sequences of the enzymes encoded by pin, gin, hin, and cin show homology with each other, and with that of TnpR, the resolvase of transposon Tn3, and all show high frequency inversions (Simon et al., 1980; Plasterk, and Van de Putte, 1984).

A number of other rearrangements have been demonstrated to affect gene expression directly. Several transposons have been shown to carry promoters that are able to direct transcription of adjacent genes following a transposition event (Iida et al., 1983; Kleckner 1983). Thus, the insertion of a transposon within or adjacent to a cryptic gene can result in transcription initiation by a latent promoter carried on the transposable element.

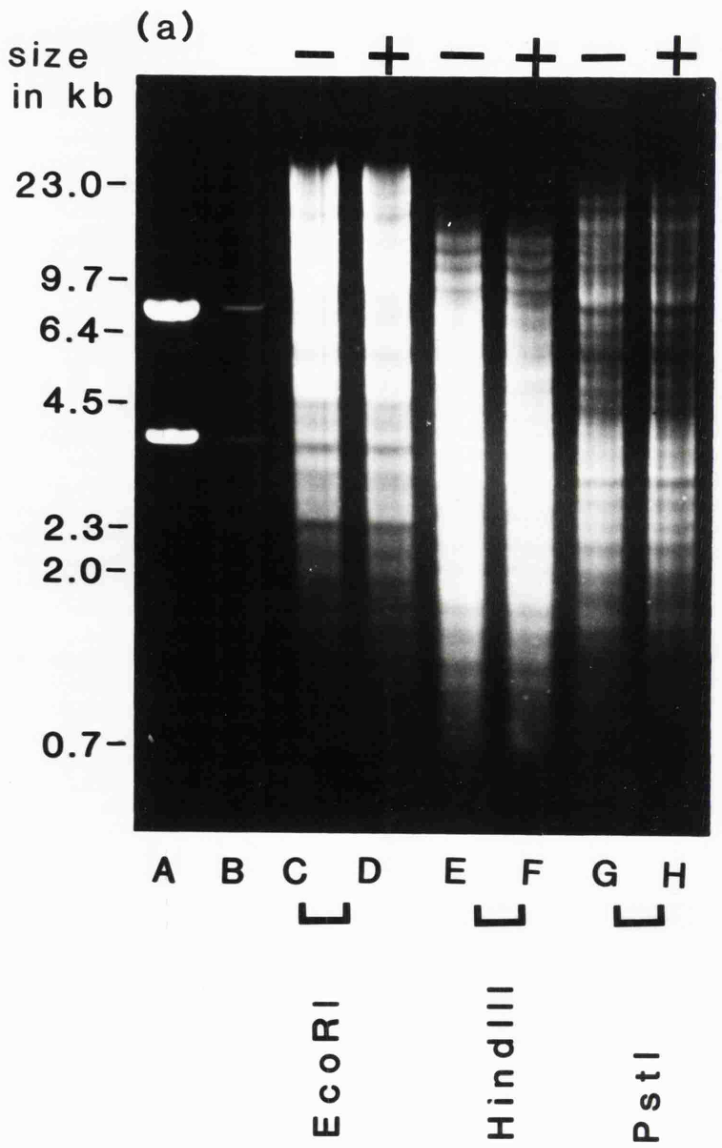
In addition to such familiar rearrangements which regulate gene activity, a variety of more complex, and less well understood systems have been described. In Neisseria gonorrhoeae, for example, antigenic variation brought about by switching "on" or "off" opacity proteins (Op) is believed to be due to recombination

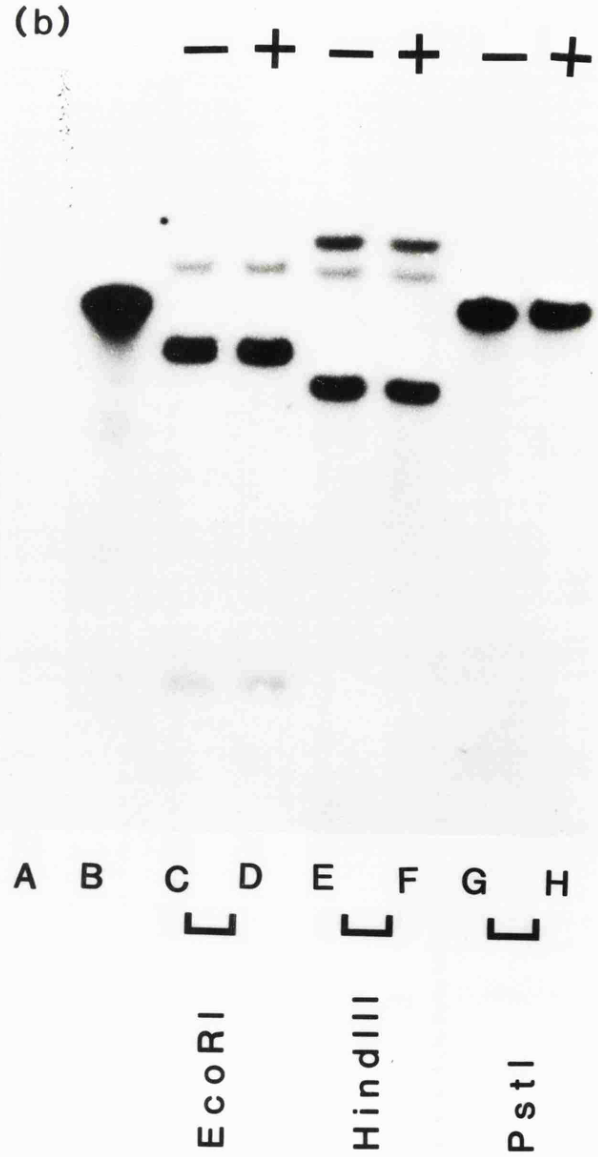
and/or gene conversion at the Op expression site. Also in N.gonorrhoeae, pilin phase variation has been correlated with rearrangements in chromosomal structure, but the precise mechanism is not known.

Because of the superficial similarity between reversible gene expression systems, and the phenomenon of the appearance and disappearance of chloramphenicol resistance in populations of P.mirabilis PM13, it was decided to use Southern blotting to detect a possible change in orientation of cat DNA within the chromosome. P.mirabilis PM13 chromosomal DNA was prepared from late exponential phase cultures grown in Penassay-broth containing either no antibiotic ($CAT^-$) or chloramphenicol (500µg per ml, $CAT^+$). Both DNA preparations were digested separately with PstI, HindIII and EcoR1, subjected to agarose (0.7% w/v) gel electrophoresis, transferred to nitrocellulose sheets, and probed with the 8.5-kb $\alpha[^{32}P]$-labelled PstI insert of pIC100. Fig. 6.3 shows that the pattern of hybridization produced for each pair of digests ($CAT^-$ and $CAT^+$) is indistinguishable, indicating that a DNA rearrangement cannot be detected within this 8.5-kb cat fragment. In the event that a rearrangement of the sort described above is responsible for the appearance and disappearance of chloramphenicol resistance in populations of P.mirabilis PM13, it must be occurring at a locus outside the cat gene and outside the flanking 8.5-kb of insert DNA.

FIG. 6.3

Use of the cloned fragment containing the cat of P.mirabilis PM13 to probe chromosomal DNA from cells of strain PM13 grown in the presence (+) or absence (-) of chloramphenicol (500μg/ml) as indicated. (a) Agarose (0.7%) gel electrophoresis showing digests of chromosomal DNA with EcoRI (C and D), HindIII (E and F), and PstI (G and H). (b) Autoradiograph of the DNA shown in (a) after transfer to nitrocellulose and hybridization with a [$^{32}$P]-labelled probe comprising the PstI fragment from pIC100 (see Fig. 5.5 and Methods, 2.3). Lanes A and B contain PstI digests of pIC100. Only the lower concentration (B) was used for the hybridization control in (b) wherein an intense signal at approximately 8.5-kb may be seen to correspond with that in lanes G and H for the PstI digest of chromosomal DNA.


(a)
size
in kb
23.0
9.7
6.4
4.5
2.3
2.0
0.7
− + − + − +
A B C D E F G H
EcoRI
HindIII
PstI


(b)
− + − + − +
A B C D E F G H
EcoRI
HindIII
PstI

### 6.2.3 Heteroduplex formation between pIC100 and pIC101

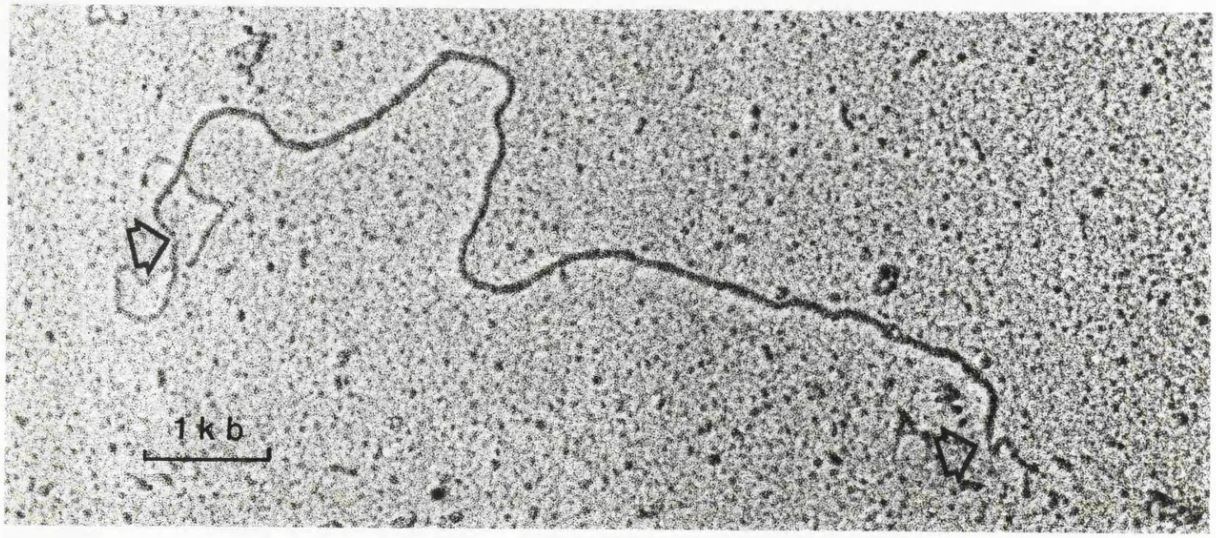
In parallel with the DNA:DNA hybridizations designed to detect rearrangements within the chromosome of *P.mirabilis* PM13 (encompassed by the 8.5-kb *cat* containing fragment), heteroduplex experiments between the plasmids pIC100 and pIC101 were carried out. Plasmid pIC100 (generated from *P.mirabilis* PM13 chromosomal DNA isolated from cells grown in the presence of chloramphenicol) was digested with *Pst*I, while pIC101 (generated from DNA isolated from cells grown in the absence of chloramphenicol) was digested with *Bam*H1. *Bam*H1 linearizes the plasmid pIC101, whereas *Pst*I resects the 8.5-kb cloned insert of pIC100 from the vector, (pAT153). Heteroduplexes can thus be differentiated from self-annealed plasmids since the former must have single stranded ends. The electron micrographs in fig. 6.4 show two heteroduplexes between plasmids pIC100 and pIC101. No "bubbles", corresponding to regions of non-homology are apparent along the length of the annealed DNA, indicating that there are no gross rearrangements within the 8.5-kb *cat* insert of pIC100 as compared with that of pIC101.

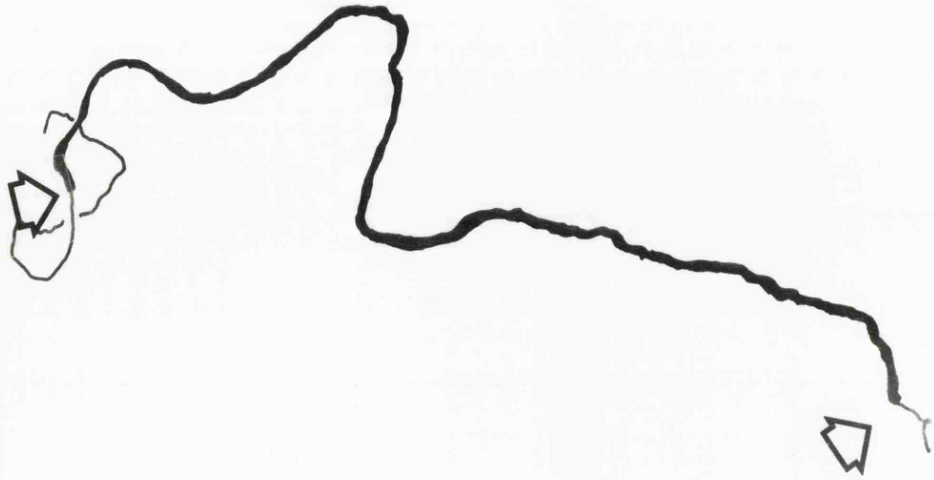
FIG. 6.4

Heteroduplex formation between plasmids pIC100 and pIC101.

The electron micrograph in (a) and (b) show two separate isolates of heteroduplexes formed between plasmids pIC100 (digested with PstI), and pIC101 (digested with BamH1). Heteroduplexes can be discriminated from homoduplexes as the former have single stranded ends (marked by arrows). Fig. (c) represents a line drawing of the hetroduplex in (b).

1kb


1kb



6.2.4 Quantification of cat in RNA by DNA:RNA hybridization

Post-transcriptional regulation operating at the mRNA level has been evoked as a possible means of control of gene expression. Such mechanisms may involve (a) "transcriptional attenuation" of the sort described for the trp operon (reviewed by Yanofsky, 1981), (b) "translational attenuation", as is the case for inducible erythomycin resistance in the Staphylococcal plasmid pE194 (reviewed by Weisblum, 1984), or (c) post transcriptional RNA processing (reviewed by Gegenheimer and Apirion, 1981).

To test whether or not the P.mirabilis PM13 cat gene is regulated by the formation of a stable cat mRNA species, which itself is controlled by the adoption of a secondary structure favourable to expression (see Chapter 1), "Northern" dot-blot experiments were carried out.

RNA was isolated (Methods, 2.3) from cells of P.mirabilis PM13 grown in the presence (500µg/ml) and absence of chloramphenicol, and from the strain P.mirabilis PM2 (which is free of a detectable cat gene on the basis of (a) Southern hybridization data (Chapter 4) and (b) the sensitive radiometric assay for CAT (Chapter 1)).

The RNA samples prepared from P.mirabilis PM13, and P.mirabilis PM2 were spotted in a dilution series (from 5µg to 0.75pg) onto nitrocellulose paper, baked, and hybridized to an $\alpha[^{32}P]$-labelled 1.7-kb PstI-ClaI fragment of pIC100 containing the cat gene (Methods, 2.3). After washing, filter-bound counts

were determined by autoradiography (Fig. 6.5) and by scintillation counting. The graph in Fig. 6.6 shows that hybridization to the cat probe has only occurred with RNA extracted from P.mirabilis PM13 grown in the presence of chloramphenicol. RNA extracted from cultures of P.mirabilis PM13 grown in drug-free conditions produce approximately the same (background) signal level as RNA extracted from P.mirabilis PM2 which lacks a cat gene by hybridization criteria. Although the cat gene has been demonstrated to be present in P.mirabilis PM13 regardless of the presence of chloramphenicol, its product is only observed in cell populations exposed to (and allowed to grow in) the presence of the antibiotic. Such a result strongly suggests that the cat gene of P.mirabilis PM13 is under transcriptional control, occurring at the level of initiation of transcription, rather than any post-transcriptional mechanism involving the secondary structure of the mRNA. It is also unlikely that mRNA processing is involved as a mechanism of the control of P.mirabilis PM13 cat gene expression. In those systems examined wherein the processing of mRNA alters the message stability (Panayotatos and Truong, 1985), the half life of the mRNA is altered, at most, by an order of magnitude (Gegenheimer and Apirion, 1981). Were the expression of cat to be under such control, then intermediate cat-specific mRNA levels might be expected, rather than the basal levels seen in Fig. 6.6.

FIG. 6.5

RNA 'dot-blots' (DNA:RNA Hybridizations)

(a) Autoradiograph of RNA prepared from cultures of P.mirabilis PM13 grown in the presence and absence of chloramphenicol, and P.mirabilis PM2, after hybridization to a 1.7-kb PstI-ClaI cat probe. The dot of RNA at the top of the autoradiograph represents 20μg of total RNA, and the dilution series decreased the RNA content in each sample by 50%. The RNA for this 'dot-blot' was purified away from contaminating DNA by centrifugation through a 5.7M CsCl 'plug' (Methods, 2.3)

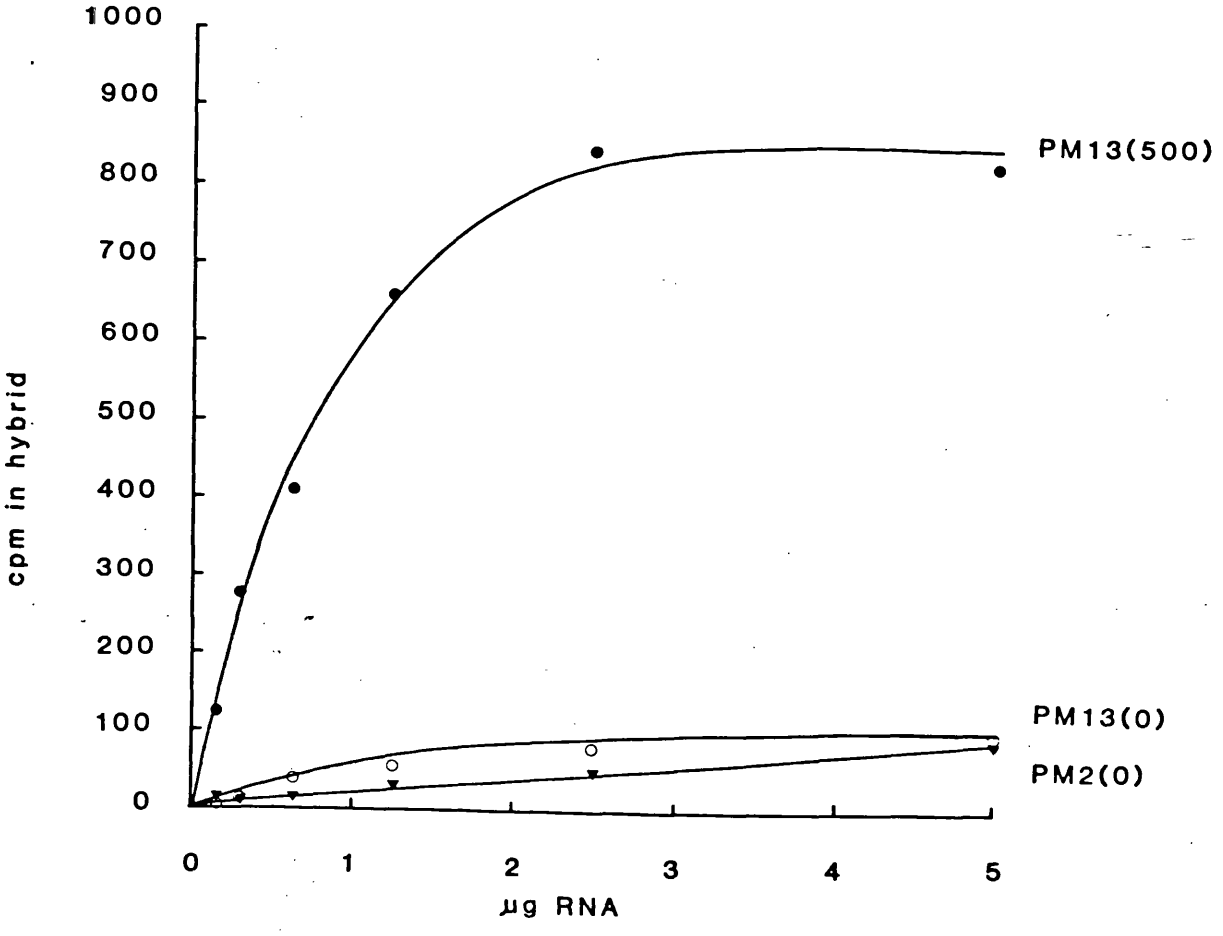
The autoradiograph in (b) is the result of an identical hybridization experiment as described for (a) above, however in this case, RNA was purified away from DNA by incubation with 'RNase-free DNase' (BRL).

In both cases hybridization has only occurred to RNA prepared from cultures of P.mirabilis PM13 grown in the presence of chloramphenicol.

(a)
PM2
PM13(Cm 500)
PM13(Cm 0 )
1 2 3
(b)
PM13(Cm 500)
PM13(Cm 0)
PM2
1 2 3

FIG. 6.6

Titration of cat mRNA from P.mirabilis strains PM13 and PM2 grown in different concentrations of chloramphenicol. Figures in parenthesis (500 or 0) denote the concentration of chloramphenicol in μg/ml. Determination of mRNA was by hybridization of total RNA to a 1.7-kb ClaI-PstI fragment of pIC100 containing cat. A titration series of RNA was dot blotted onto a nitrocellulose filter, incubated with the nick-translated probe, washed, and specific filter bound radioactivity determined by scintillation counting.


1000
900
800
700
600
500
400
300
200
100
0
cpm in hybrid
PM13(500)
PM13(0)
PM2(0)
0
1
2
3
4
5
µg RNA

## 6.3 SUMMARY

The P.mirabilis PM13 chromosome has been examined by Southern hybridization for evidence of cat gene amplification, with the result that there is no significant difference between the copy number of the cat gene in cells from populations grown in the presence of chloramphenicol as compared with those not exposed to the antibiotic. Such a result is consistent with the results of cloning experiments (see Chapter 5) wherein the frequency of isolation of the cat genes from DNA prepared from cells grown in the presence of chloramphenicol was similar to that for cells grown in antibiotic-free medium.

Southern blotting experiments also revealed no evidence of DNA rearrangements (i.e. DNA inversions, or insertions and deletions) in the region of the P.mirabilis PM13 chromosome spanned by the 8.5-kb of cloned insert in pIC100. Such a result does not, however, rule out a rearrangement occurring at a distant locus - as is the case in Salmonella phase variation (see Chapter 1 for references).

Heteroduplex studies of the two recombinant plasmids constructed with DNA prepared from cells grown in the presence ($CAT^+$) or absence ($CAT^-$) of chloramphenicol (pIC100, and pIC101 respectively) reveal identity along their length, at least within the limits of resolution of the technique (differences as small as 100-bp may be detected, P. Mc.Turk personal communication). This apparent identity of pIC100 and pIC101 is in agreement with restriction map data (Chapter 5), and is reflected in the

organization of the gene within the chromosome.

Finally, RNA 'dot-blot' experiments have demonstrated that the control of cat gene expression is likely to occur at the level of initiation of transcription.

# CHAPTER 7

# DNA SEQUENCING

# CHAPTER 7 : DNA SEQUENCING

## 7.1 INTRODUCTION

There are two general methods for the sequencing of DNA, the chemical method of Maxam and Gilbert (1979), which relies on the selective chemical cleavage of bonds in single stranded DNA, and the enzymic methods developed by Sanger and co-workers. The first of the enzymic methods was the so-called "plus and minus" technique (Sanger and Coulson, 1975), which was used to sequence the 5386-bp genome of the DNA phage ØX174. This technique was superceded by the dideoxy chain termination method (Sanger et al., 1977) which relies on the random incorporation of 2', 3' dideoxynucleotides into a growing DNA chain and the consequent premature termination of chain growth with such events. With suitably primed template, the 'Klenow' fragment of E.coli DNA polymerase I, will, in the presence of all four deoxynucleoside triphosphates, extend DNA synthesis in the 5' to 3' direction. If the four dideoxy analogues (ddATP, ddTTP, ddGTP, ddCTP) are included in separate sets of reactions, chain termination will result at the point at which a dideoxynucleoside is incorporated. The ratio of deoxy to dideoxy nucleotide is carefully controlled, which results in a nested set of fragments where chain termination has occurred at each dideoxy nucleoside. Such fragments can then be separated by electrophoresis through denaturing gels, which may be formed with an ionic gradient to increase resolution (Biggin et al., 1983). When the DNA is made radioactive, for example by inclusion of [$^{35}$S]- ATP in each reaction mix (Biggin et al., 1983), the resulting bands can be detected by autoradiography (Fig. 7.1). The obvious difficulty with

this method is that a single stranded template must be obtained. Historically this was overcome by sequencing single stranded phage. ØX174 has a double stranded DNA replicative form, (RF DNA), which can be isolated from the cell as a supercoiled plasmid or as infective phage comprising single stranded DNA. Small restriction fragments of the RF DNA could be isolated, heat denatured, and allowed to anneal to a suitable single stranded DNA, yielding primed single stranded template. For success with this strategy, not only must single stranded primer DNA be available, but also a detailed restriction map must be constructed so as to generate small fragments as primers. Such considerations have been greatly simplified by the construction of M13 phage vectors (reviewed by Messing, 1983).

FIG. 7.1

Autoradiograph of a sequencing gel. 4 clones are shown loaded left to right A T C G. The lower region of track 4 illustrates the high A+T content of the P.mirabilis cat gene.

ATCG ATCG ATCG ATCG

1 2 3 4

Bacteriophage M13 Vectors

M13 is a male specific bacteriophage which absorbs to the sex pili produced by E.coli cells carrying (and expressing) an F plasmid. (Reviewed by Denhardt et al., 1978). After the single stranded (+ strand) has infected a cell, the complementary strand is synthesized to form the double stranded replicative form. The latter is then used as a template to produce more '+ strand' phage which are packaged and extruded from the cell. The infection of host cells with M13 is not lethal but results in a 2-3 fold reduction in growth rate, an effect which is observed as a turbid plaque on a lawn of indicator cells. The life-cycle of M13 has several features which make it desirable as a cloning/sequencing vehicle: (i) The double stranded form can be isolated as a plasmid, and used to clone specific restriction fragments; (ii) the RF DNA recombinants thus generated can be used to transform E.coli cells, which subsequently give rise to infective phage particles, such phage can be propagated and processed to yield single stranded DNA as a sequencing template; and (iii) there is no defined limit on the size of fragment which may be cloned, molecules with inserts of additional 'foreign' DNA simply yield longer phage filaments.

Messing and co-workers have developed a series of cloning/sequencing vectors based on M13 (Messing, 1983; Yanisch-Perron et al., 1985). The phages in the M13mp-series have a built in screen to allow visual identification of recombinant plaques. The vectors contain the first 145 amino acids of $\beta$-galactosidase, which can be translated to yield the so-called $\alpha$-peptide. This $\alpha$-peptide

complements the lacΔZM15 deletion carried by an Flac plasmid in the host strain JM103. This Flac plasmid has been further modified and carries a traD36 mutation as a safety feature, allowing the cells to make F pili, but removing their ability to transfer DNA by conjugation (Messing et al., 1981). Infection of cells harbouring the Flac plasmid with an M13mp-series phage results in α-complementation, and the production of active β-galactosidase. On plates containing the lac operon inducer IPTG, and the chromogenic substrate X-gal, blue plaques will be produced (see Methods, 2.3). The M13mp-series of vectors have had an array of cloning sites introduced into codon six of the β-galactosidase α-segment. Cloning into this site abolishes α-complementation, and produces the blue to white phenotypic change which can easily be detected by visual inspection of the plates. As a consequence of the cloning sites being closely spaced, a single synthetic primer can be used to prime the sequencing of all cloned fragments. A variety of single stranded phage vectors are now available (reviewed by Thompson, 1982).

Sequencing Strategies

The major problem encountered in any M13 sequencing strategy is the generation of subclones which will enable the entire fragment of interest to be completely sequenced on both strands. Two broad families of techniques have been utilised to generate subclones: (a) the random, or "shotgun" approach, and (b) the non-random, or "sequential" approach. Both families encompass a variety of enzymic and/or mechanical operations to generate subclones, and the selection of any particular method depends upon the fragment of DNA to be sequenced.

Techniques based on the random approach rely upon the DNA to be sequenced to be fragmented by the action of restriction enzymes (Messing, 1981), DNase (Anderson, 1981) or sonication (Deininger, 1983). The subfragments thus generated are arranged in sequential order by finding overlaps between the sequences, this procedure can be computer-aided (Staden, 1982). Data accumulation, although rapid at first, tends to slow down as the same regions are repeatedly sequenced.

The simplest of the sequential techniques currently in use starts with cloned double stranded DNA which is cleaved at a unique site, and then shortened by a time-course with Bal31 digestion (Poncz et al., 1982) or exonuclease III, followed by either S1 nuclease or exonuclease VII digestion to generate blunt ends. (Guo et al., 1983). With these methods both insert and vector sequences are deleted, thus the shortened insert must be recloned prior to sequencing.

Improvements on this method can be used when specific sites are available in the M13 polylinker (this is consequently facilitated by using the vectors M13mp18 and/or M13mp19, which currently possess the largest number of sites). The technique requires the presence of two restriction endonuclease sites that are not present in the fragment to be sequenced, but which do occur in the polylinker region of M13. One of the enzymes (the one nearer the primer binding site) must leave a 3'-overhang (e.g. PstI, SstI), a fragment cut in this way is resistant to attack from exonuclease III. The other restriction enzyme must cut between this site and the insert, and leave a 5'-overhang, which is sensitive to attack from exonuclease III. The insert fragment is then sequentially digested with exonuclease III over a time-course, followed by digestion with S1 nuclease, or exonuclease VII (Henikoff, 1984; Yanisch-Peron *et al.*, 1985). The blunt ends thus generated are ligated, bringing the primer binding site (which was protected from exonuclease III by the 3'-overhang) up against sequentially deleted fragments.

The above methods require double stranded DNA as starting material, and are limited in their utility by the requirements for specific restriction sites. A rapid procedure for generating sequential deletions in a cloned insert, without the prior knowledge of internal restriction sites, has been described (Dale *et al.*, 1985). The technique employs single stranded M13 DNA as a template to anneal either one of two specific oligonucleotides (the choice of which oligonucleotide is employed depends on which of the pairs of

M13 vectors were used, i.e. one oligonucleotide (RD29) can be annealed to mp8, 10, or 18, while the other (RD20) can be annealed to mp9, 11, or 19). The DNA is then cut with a restriction endonuclease, the recognition site of which had been generated by the annealed oligonucleotide. The single stranded insert DNA can then be digested using the specific 3'→5' exonuclease activity of T4 DNA polymerase. The digestion can be carried out over a time-course, the reaction stopped, and a short poly A or poly G 'tail' added to the 3' ends of the digested insert. This homopolymeric tail generates a complementary end for either oligonucleotide RD29 or RD20, which can be subsequently reannealed over the ends to generate a circular molecule. Ligase is added, and the total ligation mix used to transform JM103. White plaques can be picked, and selected according to size on agarose gels prior to sequencing.

## 7.2 RESULTS

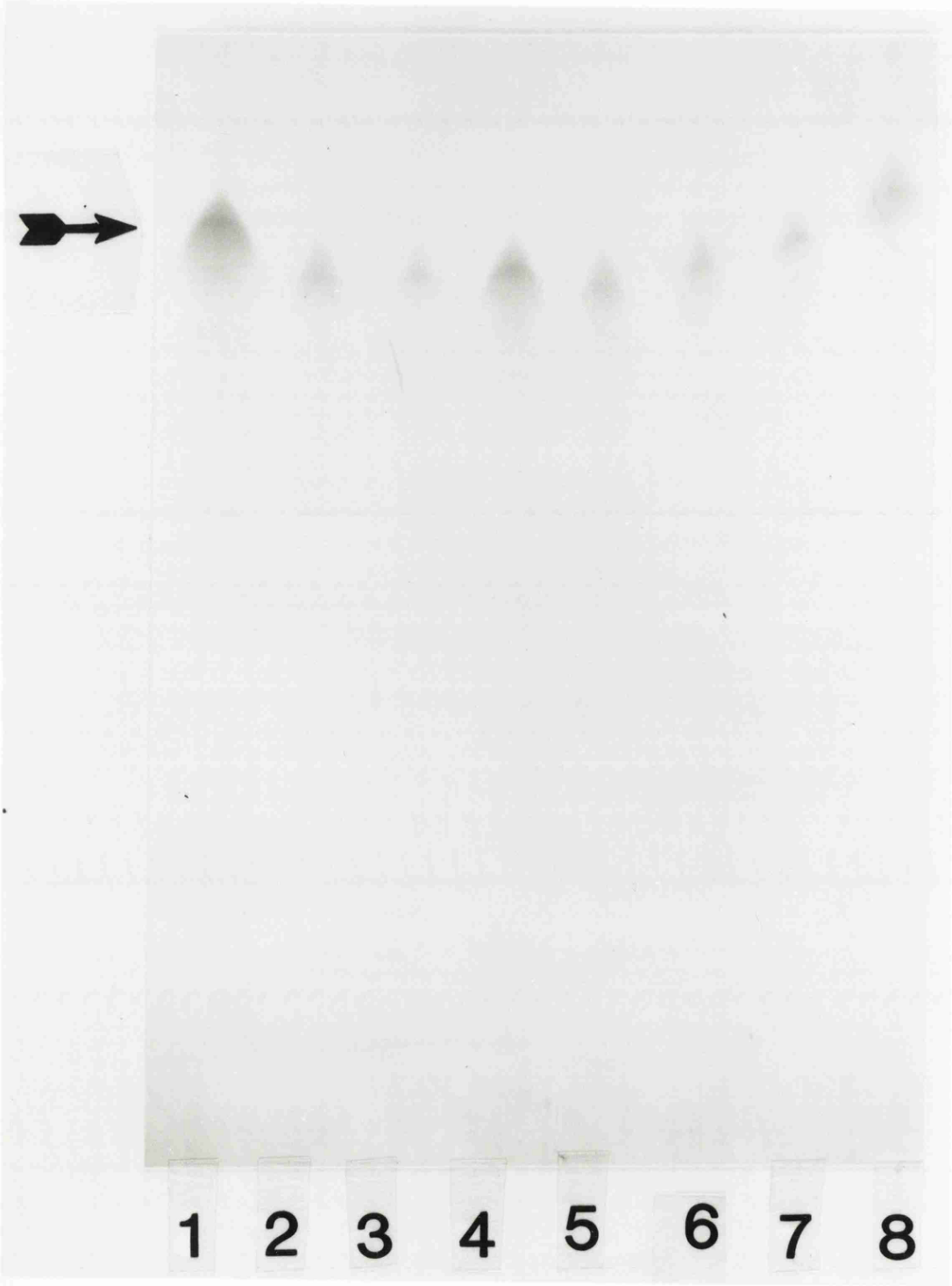
### 7.2.1 Sequencing the P.mirabilis PM13 cat

The method used to sequence the P.mirabilis PM13 cat gene was essentially that of the random ("shot-gun") approach. The problem of generating complete sequence was greatly simplified, however, by the use of synthetic oligonucleotides as specific primers. These were used to generate the overlaps in regions where no favourable restriction sites were present. Oligonucleotides were synthesized by J. Keyte on paper discs (Mathes et al., 1984) using the modification of Brenner and Shaw, 1985. Up to 80 oligonucleotides could be produced in a single round of synthesis, and the crude full-length oligonucleotides were purified away from lower molecular weight components by electrophoresis through a 20% polyacrylamide gel (Methods, 2.3). Fig. 7.2 shows a photograph of the crude oligonucleotides after UV. shadowing of a polyacrylamide gel.

Previous experiments (Chapter 5) had shown that a cat consensus oligonucleotide hybridized to a 1.7-kb PstI - ClaI fragment at one end of the PstI fragment of pIC100. This was the presumed site of the cat gene, of P.mirabilis PM13. The sequencing strategy involved the cloning of subfragments into M13mp8 and M13mp9. Plasmid pIC100 was double digested with HindIII and EcoRI, and the resulting fragments ligated with similarly (EcoRI, HindIII) digested M13mp8 and M13mp9. The resulting ligation mixes were used to transform E.coli JM103. Recombinants, identified as white plaques, were spotted onto a lawn of E.coli JM103, grown overnight, and transferred to nitrocellulose paper (Methods, 2.3).

FIG. 7.2

Photograph of 17-mer oligonucleotides after electrophoresis through a denaturing 20% polyacrylamide gel. Tracks 1 to 8 represent different oligonucleotides which were used as specific sequencing primers. The arrow indicates the position of the full-length 17-mer oligonucleotides, which were cut from the gel, and further purified by G-25 spun-column chromatography (Methods, 2.3).


1
2
3
4
5
6
7
8

Fragments containing the cat sequence were identified by hybridization to the ClaI - PstI fragment which was made radioactive by nick-translation (Methods, 2.3). In a similar fashion, pIC100 was digested with Sau3A, PstI, and ClaI and the fragments produced separately ligated into M13mp9 digested with BamH1, PstI, and AccI respectively. After transformation the cat containing recombinants were similarly identified by hybridization (Methods, 2.3). Once the sequence data had been compiled using the information generated using these recombinants, specific synthetic oligonucleotides were made, and purified (Methods, 2.3) for use as internal primers (Fig. 7.3). The complete sequence of the P.mirabilis PM13 cat gene is shown in Fig. 7.4 Computer programs were employed to examine the sequence, to attempt to answer some of the questions of P.mirabilis cat gene regulation raised in earlier chapters.

Because of the high degree of similarity between the cat gene(s) of P.mirabilis cloned in pIC100 and pIC101 (i.e. cloned from P.mirabilis PM13 chromosomal DNA extracted from cells grown in the presence and absence of chloramphenicol) it was decided to initially only sequence the cat gene from pIC100. This decision is discussed in more detail in Chapter 8.

Fig. 7.3 Sequencing Strategy. The heavy dark lines represent pAT153 vector DNA, and the light lines represent inserted DNA. Arrows represent DNA sequences determined either from restriction endonuclease sites, or from specific oligonucleotides used as internal primers (listed below).

| | OLIGONUCLEOTIDE | CO-ORDINATES |
|---|---|---|
| a | 3'-GTACTTATTGTTTATA-5' | 3' to sequence |
| b | 3'-GTCCAAACTCACCTTG-5' | 1561-1576 |
| c | 3'-CGCATATTATAAACGG-5' | 1399-1414 |
| d | 3'-GTAGTACGGCAGACACTACC-5' | cat active site oligonucleotide |
| e | 3'-GTTCACGTAGTACGGC-5' | 1447-1462 |
| f | 3'-CCCCTGCTTTTCTTGT-5' | 8226-942 |
| g | 3'-AGGTTTAAATTTAATT-5' | 843-859 |
| h | 3'-TTACTGAGACAATAAA-5' | 696-711 |
| i | 3'-TTTACATTAATACTAC-5' | 526-541 |
| j | 3'-GTAGTATTAATGTAAA-5' | 392-417 |
| k | 3'-ACCGTATACTTGAAAA-5' | 392-417 |
| l | 3'-GTGCTATTTTCGGACC-5' | 228-243 |
| m | 3'-GGTCCGAAAATAGCAC-5' | 228-243 |

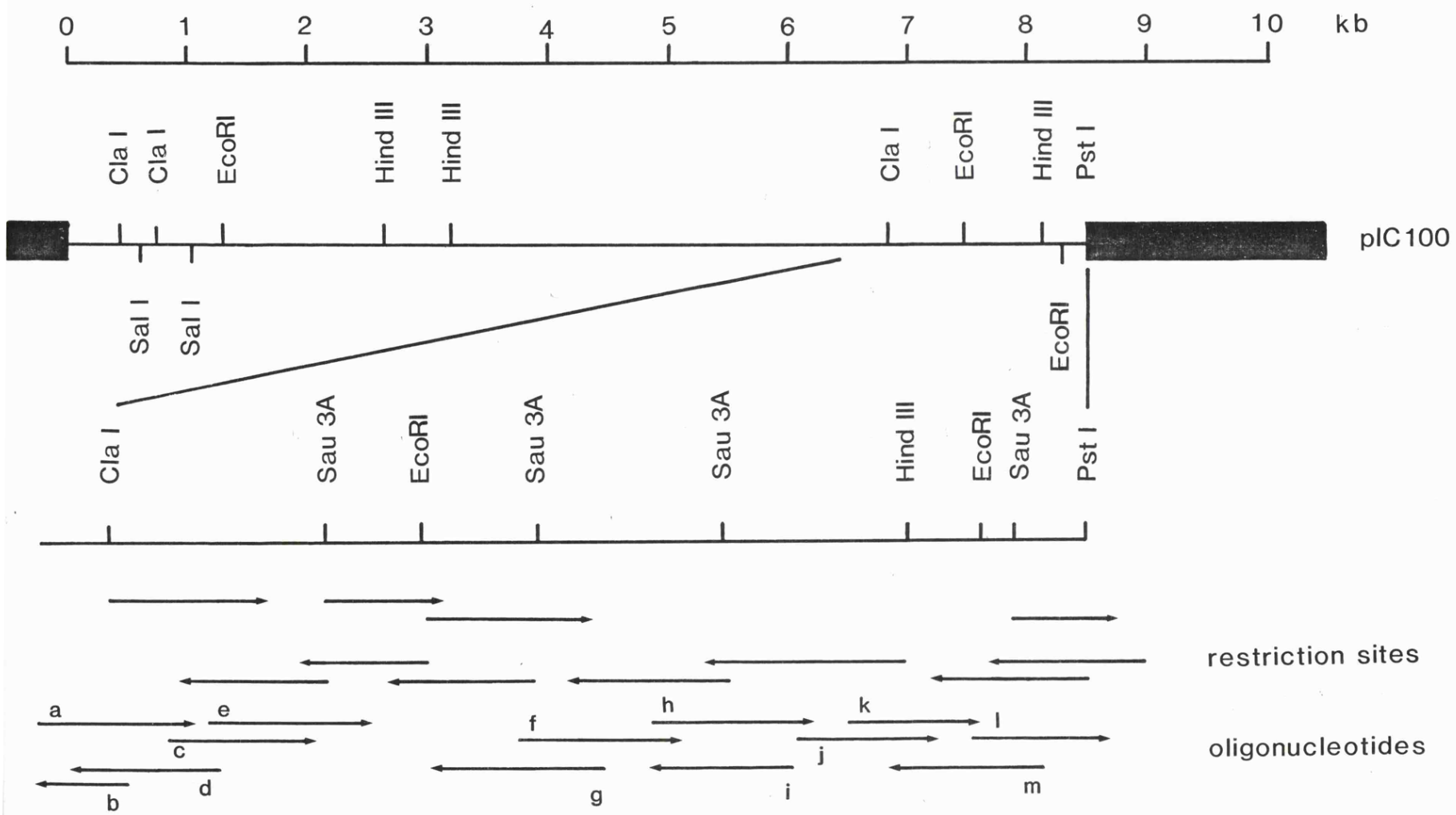

0
1
2
3
4
5
6
7
8
9
10
kb
Cla I
Cla I
EcoRI
Hind III
Hind III
Cla I
EcoRI
Hind III
Pst I
pIC100
Sal I
Sal I
EcoRI
Cla I
Sau 3A
EcoRI
Sau 3A
Sau 3A
Hind III
EcoRI
Sau 3A
Pst I
restriction sites
a
e
h
k
l
f
c
j
oligonucleotides
b
d
g
i
m

Fig. 7.4 Nucleotide sequence of the P.mirabilis PM13 cat gene displayed as a 1,760 base-pair fragment, along with the predicted amino acid sequence of CAT.

Another open reading frame of 132 base pairs and its predicted amino acid sequence is shown 5' to the start of the cat gene. The underlined nucleotide clusters -35 and -10 are the putative RNA polymerase recognition sites. SD indicates the likely ribosome-binding site. Opposing arrows indicate regions with imperfect dyad axis of symmetry. Another dyad (omitted for clarity), spans nucleotides 817 through 862. The inverted complementary repeat sequence from nucleotides 1645 through 1665 is followed immediately by the five T residues; this structure is characteristic for a rho-independent termination site.

```
        10        20        30        40        50        60        70        80        90       100       110       120
CTGCAGGAATATTTAAACCTTCTTGAACATATTTAGTCACACAGGTCTCTGTTTTAGTCTGTGTACGACCGGTAATGAAATAAACAGTATCACCGCGTTTTAAGTGCATCTGAACAAGAT
       130       140       150       160       170       180       190       200       210       220       230       240
CGATACCGACTTGTTTAGGCATACTGAATTTATCCCATTCGTTGTTCATTTTTTCCAGAATTCTGGGTTTTTCAGATAACTGAAATCGTTTGGAGATATTCAGTTTACACGATAAAAGCC
       250       260       270       280       290       300       310       320       330       340       350       360
TGGGCTTGAGAACCAGAACGTGTCATCAATATCAAAACCTACCGCCATTGGTGCTTGGCCTTCTAAGCTTTTTTCAATTTCTGCAACAGAAACCCATTTAATTGGTTGCTGATGTGCAAG
       370       380       390       400       410       420       430       440       450       460       470       480
TTCAGTAACAGTAACCCTGGCTAACAACTTCTGGCATATGAACTTTTGCCATTGCTGCGCCATTTAAACTTAACGCCAACGCAATTGCACTAAGAGTAAGTGTGACTTTACGCATTCTCT
       490       500       510       520       530       540       550       560       570       580       590       600
TTCCTTTAATTTTTATATTAATCACAGAAGGTGATAATAATAAACATCATAATTACATTTAAATGAGATATAGCGCACGTTATTTTATTTAGGCTGGTACTCTTTTGTTCAGTTTACTTT
                                                                                                                  -35
       610       620       630       640       650       660       670       680       690       700       710       720
CATTTTTTGATCTATCTACATTTGAAAAAAGAAAGCACTATCTACGAAAGCAGATAGCGCCGTTATTATCTAAGTCGTTCCCAAATGAACACATCAATGACTCTGTTATTTTTGAAACAT
                                                                                                MetThrLeuLeuPheLeuLysHis
            -10                                                                   -35                       -10
       730       740       750       760       770       780       790       800       810       820       830       840
TTTTTGGTAAAAAATAAACATTTTAAAATTAGCCAAGAAAAACCTATTACCTATCATAGGTTATGGGTTTATCTACAGATAGAGGTTCGAAATTGTTTTAATAATTTATAAGTAATATAC
PheLeuValLysAsnLysHisPheLysIleSerGlnGluLysProIleThrTyrHisArgLeuTrpValTyrLeuGlnIleGluValArgAsnCysPheAsnAsnLeu***
                                SD
       850       860       870       880       890       900       910       920       930       940       950       960
AATTAATTTAAATTTGGATTAGAAAATAGGAAAGCTAATATGGACACAAAGCGCGTGGGTATATTGGTTGTTGATCTATCCCAATGGGGACGAAAAGAACACTTTGAAGCATTTCAGTCT
                                    MetAspThrLysArgValGlyIleLeuValValAspLeuSerGlnTrpGlyArgLysGluHisPheGluAlaPheGlnSer

       970       980       990      1000      1010      1020      1030      1040      1050      1060      1070      1080
TTTGCTCAATGCACCTTTAGCCAGACCGTTCAACTGGATATTACTTCATTATTAAAAACCGTAAAGCAAAATGGGTACAAATTCTATCCGACATTTATATACATCATTAGCCTATTGGTA
PheAlaGlnCysThrPheSerGlnThrValGlnLeuAspIleThrSerLeuLeuLysThrValLysGlnAsnGlyTyrLysPheTyrProThrPheIleTyrIleIleSerLeuLeuVal

      1090      1100      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
AATAAACATGCAGAATTCCGCATGGCAATGAAAGACGGGGAATTGGTGATATGGGATAGTGTTAACCCTGGGTACAATATCTTTCATGAACAGACCGAAACATTTTCATCTTTATGGAGT
AsnLysHisAlaGluPheArgMetAlaMetLysAspGlyGluLeuValIleTrpAspSerValAsnProGlyTyrAsnIlePheHisGluGlnThrGluThrPheSerSerLeuTrpSer

      1210      1220      1230      1240      1250      1260      1270      1280      1290      1300      1310      1320
TACTACCACAAAGATATCAATCGTTTTTTGAAAACTTATTCAGAAGATATAGCACAATACGGCGACGATCTAGCCTATTTTCCAAAAGAATTTATCGAAAATATGTTTTTTGTGTCAGCA
TyrTyrHisLysAspIleAsnArgPheLeuLysThrTyrSerGluAspIleAlaGlnTyrGlyAspAspLeuAlaTyrPheProLysGluPheIleGluAsnMetPhePheValSerAla

      1330      1340      1350      1360      1370      1380      1390      1400      1410      1420      1430      1440
AATCCTTGGGTAAGTTTCACCAGTTTTAACTTGAATATGGCGAATATAAACAATTTCTTTGCCCCTGTATTTACAATAGGTAAATATTATACGCAAGGAGATAAAGTTCTGATGCCACTA
AsnProTrpValSerPheThrSerPheAsnLeuAsnMetAlaAsnIleAsnAsnPhePheAlaProValPheThrIleGlyLysTyrTyrThrGlnGlyAspLysValLeuMetProLeu

      1450      1460      1470      1480      1490      1500      1510      1520      1530      1540      1550      1560
GCAATTCAAGTGCATCATGCCGTATGTGATGGCTTCCATGTAGGCAGATTACTCAATGAAATACAGCAATACTGCGATGAGGGATGTAAATAATCAATGGGACTTAAAGCACATGTTTTT
AlaIleGlnValHisHisAlaValCysAspGlyPheHisValGlyArgLeuLeuAsnGluIleGlnGlnTyrCysAspGluGlyCysLys***

      1570      1580      1590      1600      1610      1620      1630      1640      1650      1660      1670      1680
GTTCCACTCAAACCTGTTCGCTATCCTCTGTTTCGCACTTTTATTGGATTATTTTAACATCGATATCGATATAAAATTGATGAGGGGCAATTAGTATTTCCCTTTTTATTGAGGGGTCAC
      1690      1700      1710      1720      1730      1740      1750      1760
GCTATAAAATTTGTACACTGATTTGTACTAATACGGTGATAAAATACTAATGCCATTACACTCATGAATAACAAATATAA
```

### 7.2.2 Homology with the type I (Tn 9) cat

The DNA sequence of the P.mirabilis cat, and the type I (Tn 9) variant were aligned, and the homology between them is presented in Fig. 7.5. This aligment shows the high degree of homology between the structural gene sequences, 73 and 76% at the nucelotide and amino acid levels respectively (Fig. 7.6).

### 7.2.3 Codon usage in P.mirabilis cat

Codon usage is believed to be mainly dependent on the relative amounts of iso-accepting tRNA molecules present in the cell (Ikemura, 1981). The codon usage for the cat of P.mirabilis is similar to that of the type I cat (Fig. 7.7), and is consistent with the codon usage of E.coli genes which are not highly expressed (Grantham et al., 1981). How significant the E.coli codon bias is to the expression of a gene from P.mirabilis is not yet known. The G + C content of P.mirabilis is 39 $\pm$ 1.4% whereas that of E.coli is 51% (Coetzee, 1972), and a different codon usage may therefore be expected. Infrequently used codons (Konigsberg and Godson, 1983) do occur more often in the P.mirabilis cat gene than in the type I variant, and this may contribute to some degree to the poor expression of the P.mirabilis cat in E.coli (Chapter 5).

FIG. 7.5

Graphic representation of nucleotide similarity between type I cat and P.mirabilis cat. Each point on the graph represents the percentage of nucleotide homology present in a window of 49 nucleotides. The two parallel lines across the graph encompasses the level of background homology that might be expected by chance. The arrow below the graph represents the coding region of both cat genes. The numbering corresponds to the sequence shown in Fig. 7.4.

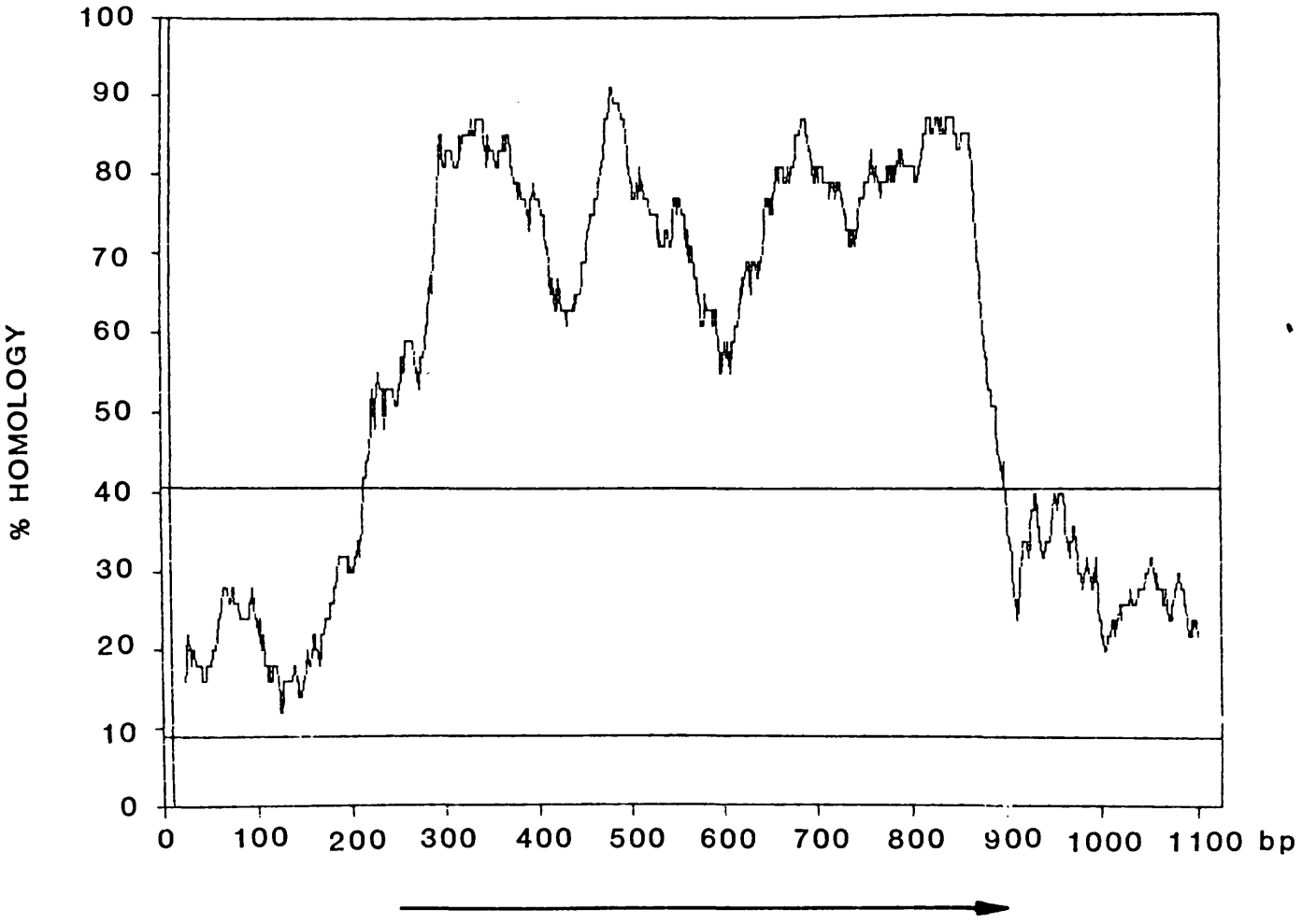

% HOMOLOGY
100
90
80
70
60
50
40
30
20
10
0
0
100
200
300
400
500
600
700
800
900
1000
1100 bp

FIG. 7.6 Amino acid comparison between the type I chloramphenicol acetyltransferase peptide, encoded by Tn9 with the chloramphenicol acetyltransferase of P.mirabilis PM13. The 76% identity (underlined residues) was deduced by inspection.

```
            1   5   10   15   20   25   30   35   40   45   50   55   60   65   70   75   80
I           MEKKITGYTTVDISQWHRKEHFEAFQSVAQCTYNQTVQLDITAFLKTVKKNKHKFYPAFIHILARLMNAHPEFRMAMKDG
Proteus     MDTKRVGILVVDLSQWGRKEHFEAFQSFAQCTFSQTVQLDITSLLKTVKQNGYKFYPTFIYIISLLVNKHAEFRMAMKDG

              85   90   95  100  105  110  115  120  125  130  135  140  145  150  155  160
I           ELVIWDSVHPCYTVFHEQTETFSSLWSEYHDDFRQFLHIYSQDVACYGDNLAYFPKGFIENMFFVSANPWVSFTSFDLNV
Proteus     ELVIWDSVNPGYNIFHEQTETFSSLWSYYHKDINRFLKTYSEDIAQYGDDLAYFPKEFIENMFFVSANPWVSFTSFNLNM

              165  170  175  180  185  190  195  200  205  210  215  220
I           ANMDNFFAPVFTMGKYYTQGDKVLMPLAIQVHHAVCDGFHVGRMLNELQQYCDEWQGGA
Proteus     ANINNFFAPVFTIGKYYTQGDKVLMPLAIQVHHAVCDGFHVGRLLNEIQQYCDEGCK
```

FIG. 7.7 Codon usage of E.coli type I (Tn9) cat compared with the P.mirabilis PM13 cat. An asterisk marks those codons which are said to be rare in E.coli genes (Konigsberg and Godson, 1983).

Codon usage for P.mirabilis cat and E.coli type I cat

| Amino Acid | Codon | Codons in gene P.mirabilis | Codons in gene E.coli | Amino Acid | Codon | Codons in gene P.mirabilis | Codons in gene E.coli |
|---|---|---|---|---|---|---|---|
| Phe | TTT | 15 | 10 | TERM | TAA | 1 | 1 |
| | TTC | 5 | 10 | | TAG | 0 | 0 |
| Leu | TTA | 4 | 3 | His | CAT | 5 | 7 |
| | TTG | 5 | 0 | | CAC | 2 | 5 |
| | CTT | 0 | 2 | | | | |
| | CTC | 1 | 1 | *Gln | CAA | 8 | 6 |
| | CTA | 4 | 1 | | CAG | 4 | 7 |
| | CTG | 2 | 6 | | | | |
| | | | | *Asn | AAT | 10 | 6 |
| Ile | ATT | 3 | 5 | | AAC | 3 | 4 |
| | ATC | 4 | 1 | | | | |
| * | ATA | 7 | 3 | Lys | AAA | 11 | 7 |
| | | | | | AAG | 2 | 5 |
| Met | ATG | 6 | 9 | | | | |
| | | | | Asp | GAT | 9 | 8 |
| Val | GTT | 5 | 7 | | GAC | 3 | 4 |
| | GTC | 0 | 3 | | | | |
| | GTA | 6 | 1 | Glu | GAA | 10 | 6 |
| | GTG | 4 | 5 | | GAG | 1 | 6 |
| Ser | TCT | 2 | 0 | Cys | TGT | 2 | 4 |
| | TCC | 1 | 1 | | TGC | 2 | 1 |
| | TCA | 4 | 3 | | | | |
| * | TCG | 0 | 2 | TERM | TGA | 0 | 0 |
| Pro | CCT | 3 | 2 | Trp | TGG | 4 | 5 |
| | CCC | 0 | 2 | | | | |
| | CCA | 2 | 0 | Arg | CGT | 1 | 2 |
| | CCG | 1 | 3 | | CGC | 2 | 1 |
| | | | | | CGA | 1 | 0 |
| Thr | ACT | 2 | 2 | | CGG | 0 | 1 |
| | ACC | 5 | 8 | | | | |
| | ACA | 4 | 0 | Ser | AGT | 4 | 4 |
| * | ACG | 1 | 3 | | AGC | 2 | 0 |
| Ala | GGT | 1 | 2 | Arg | AGA | 1 | 1 |
| | GCC | 3 | 8 | | AGG | 0 | 0 |
| | GCA | 6 | 2 | | | | |
| | GCG | 1 | 3 | Gly | GGT | 2 | 2 |
| | | | | | GGC | 3 | 5 |
| Tyr | TAT | 5 | 7 | | GGA | 3 | 1 |
| | TAC | 7 | 4 | | GGG | 3 | 2 |

7.2.4 Putative regulatory sequences in the P.mirabilis PM13 cat

The striking sequence homology between the cat of P.mirabilis and the Tn9 cat ended abruptly in the 5' and 3' flanking regions (Fig. 7.5). Unlike the type I cat, which is under cyclic AMP-mediated catabolite control, the cat of P.mirabilis contains no apparent comparable binding site for the cyclic AMP receptor protein (based on the absence of the consensus sequence (Ebright et al., 1984)). Such an observation is not surprising, since catabolite repression of cat was not observed in P.mirabilis (Smith et al., 1972). Neither is there any evidence of potential secondary structure in the mRNA involving the folding of the ribosome binding site (Shine, and Dalgarno, 1974) as is found in the gram-positive cat genes (Byeon and Weisblum, 1984; Duvall et al., 1983 and Shaw et al., 1985), and which is believed to play a part in the phenomenon of induction mediated by chloramphenicol and certain analogues. The upstream flanking region of the PM13 cat is very A + T rich, and contains several notable features 5' to the start of cat at nucleotide 880. These include the following: (i) an open reading frame from nucleotides 697 through 828 with a predicted polypeptide chain of 44 amino acids. (ii) Several possible RNA polymerase binding sites for cat. Of these, the two most likely (predicted by the algorithm described by Harr et al., 1983) extend from nucleotides 712 to 741 (promoter 1) comprising the presumptive -35 sequence (5'-TTGAAA-3') and the -10 (Pribnow box), (Pribnow, 1975) sequence (5'-TAAACA-3'), and from nucleotides 813 through 844 (promoter 2), comprising a -35 sequence (5'-TTGTTT-3') or the overlapping candidate (5'-TTTAAT-3') and the -10 (Pribnow box) sequence

(5'-TACAAT-3'). (iii) Two inverted complementary repeat sequences, one from nucleotides 756 through 795 lying between the two best-fit RNA polymerase binding sites (Rosenberg and Court, 1979), and the other from nucleotides 817 through 862, covering the -35 and -10 sequences of putative promoter 2. (iv) Sequence homology (Fig. 7.8) with the 5' flanking regions of the flagellin genes H1 and H2 of Salmonella typhimurium. These features are summarized in Fig. 7.9.

Examination of the 3' flanking region of the P.mirabilis cat reveals an inverted complementary repeat region which resembles cannonical rho-independent termination sequences. (Rosenberg and Court, 1979), a feature which is conspicuously absent from the corresponding region of the Tn9 cat.

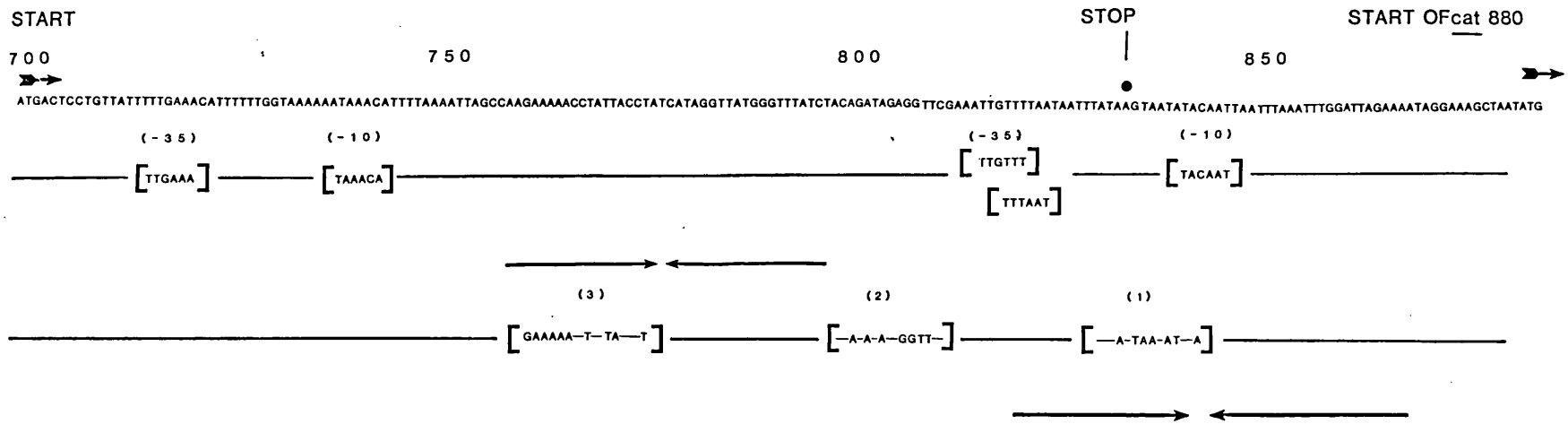
FIG 7.8

Comparison of conserved sequences present upstream from the H1 gene (a) from S.typhimurium and (b) the cat gene from P.mirabilis PM13. Alignments, detected by visual inspection, are underlined and the number of nucleotides separating each block is indicated in parenthesis. The triplet ATG in each case corresponds to the start codon for the H1 protein or chloramphenicol acetyltransferase in (a) and (b) respectively.

GAAAAATTTTCTAAAGTT...(14)...CGATACAAGGGTTAC...(22)...CCCAATAACATCAA...(25)...ATG a

GAAAAACCTATTACCTAT...(21)...ACAGATAGAGGTTCG...(18)...ATAAGTAATATACA...(37)...ATG b

FIG. 7.9 Putative regulatory sequences at the 5' flanking region of the P.mirabilis cat gene. The -10 and -35 regions (located by the algorithm of Harr et al., 1983) are shown along with the predicted open reading frame, and the dyad axis of symmetry (detected using a program written by D. Burt, University of Leicester). The alignment of the 3 boxes sharing homology with the 5' flanking region of the genes H1 and H2 (involved in phase variation in S.typhimurium) was by visual inspection.


START
STOP
START OFcat 880
700
750
800
850
ATGACTCCTGTTATTTTTGAAACATTTTTTGGTAAAAAATAAACATTTTAAAATTAGCCAAGAAAAACCTATTACCTATCATAGGTTATGGGTTTATCTACAGATAGAGGTTCGAAATTGTTTTAATAATTTATAAGTAATATACAATTAATTTAAATTTGGATTAGAAAATAGGAAAGCTAATATG
(-35)
(-10)
(-35)
(-10)
TTGAAA
TAAACA
TTGTTT
TTTAAT
TACAAT
(3)
(2)
(1)
GAAAAA—T—TA—T
—A-A-A—GGTT—
—A-TAA-AT—A

## 7.3 SUMMARY

The nucleotide sequence of the P.mirabilis cat gene, including the 5' and 3' flanking regions has been determined. The coding sequence can be aligned with the Tn9 cat (type I). The two genes have similarities of 76% at the amino acid level and 73% at the nucleotide level, suggesting that the P.mirabilis cat and the Tn9 type I variant are likely to have diverged from a common ancestor.

The 5' and 3' sequences flanking the cat of P.mirabilis, and the corresponding segments of the type I cat are dissimilar, which may account for the poor expression of the P.mirabilis cat gene in E.coli (Chapter 5).

# CHAPTER 8

# SUBCLONING AND EXPRESSION OF THE *P.MIRABILIS* PM13 *CAT* GENE

# CHAPTER 8 : SUBCLONING AND EXPRESSION OF THE P.MIRABILIS PM13 CAT GENE

The experiments described in this chapter were carried out jointly with Fiona Scott whose project on the P.mirabilis cat system was submitted (July 1985) for the intercalated degree (B.Sc.) in Medical Sciences. Ms. Scott performed the CAT assays, and I performed the in vitro genetic manipulations.

## 8.1 INTRODUCTION

The experiments in chapter 5 demonstrated that expression of the P.mirabilis PM13 cat gene (carried by pIC100 and pIC101) was poor in E.coli SK3430, and that CAT activity was detected only by use of the sensitive radiometric assay. The work in this chapter was undertaken to investigate the above observation by (i) subcloning the P.mirabilis PM13 cat gene into a high copy number vector, and (ii) to examine the expression of the cat gene following its reintroduction into the parental strain, P.mirabilis PM13, as well as other related hosts.

It was hoped that such a strategy might distinguish between the presence of a cis-acting function associated with the P.mirabilis cat gene in pIC100 or pIC101 and required for the enhancement of chloramphenicol resistance, and the possibility that a host-specific component acting in trans might be responsible for modulating gene expression.

## 8.2 RESULTS

### 8.2.1 Expression of the P.mirabilis PM13 cat gene in a cat⁻ strain of P.mirabilis

In Chapter 1 the P.mirabilis strain PM2 was described. This isolate had previously been shown to be devoid of detectable CAT by the sensitive radiometric assay, and cat⁻ by Southern hybridization experiments (Chapter 4). In order to examine the expression of the cat of P.mirabilis PM13 in another representative of Proteus. spp transformation experiments were carried out to introduce the plasmids pIC100 and pIC101 separately into P.mirabilis PM2. Initially the transformation procedure described by Kushner, (1979) was used in an attempt to transform P.mirabilis PM2 (with either pIC100 or pIC101), but no chloramphenicol-resistant colonies were recovered. However, the use of a protocol originally described for the transformation of Pseudomonas. spp (Methods 2.3), yielded chloramphenicol-resistant colonies at a frequency of between 5 and 20 per microgram of added supercoiled DNA. Separate transformants of P.mirabilis PM2 harbouring either pIC100 or pIC101 were grown overnight on Penassay-agar plates supplemented with chloramphenicol at a concentration of 10µg/ml, and then successively replica-plated onto Penassay-agar plates containing 50, 100, 200 and 500µg of chloramphenicol per ml. No growth was observed on the agar plates containing 500µg of chloramphenicol per ml, but P.mirabilis PM2 harbouring either pIC100 or pIC101 were capable of growth on 200µg/ml, suggesting that the cat gene carried by each plasmid was expressed at a similar level in strain PM2. To verify this observation CAT assays (Methods, 2.3) were performed

on cell-free extracts prepared from cultures of P.mirabilis PM2 harbouring pIC100 or pIC101, and grown either in the presence or absence of chloramphenicol. Table 8.1 summarizes the results from which it may be concluded that (i) the cat genes specified by the plasmids pIC100 and pIC101 are expressed at the same efficiency in P.mirabilis PM2, and (ii) measured CAT activity is independent of the presence of chloramphenicol in the growth medium. The cat gene dose was assumed to be identical for pIC100 and pIC101 since plasmid mini preps (Methods, 2.3) showed indistinguishable levels of plasmid DNA (data not shown).

Table 8.1.

Comparison of the expression of pIC100 and pIC101 in *P.mirabilis* PM2 and *E.coli* SK3430.

| strain | plasmid | Specific Activity of CAT (nmoles/min/mg) (a) | (b) |
|---|---|---|---|
| PM2 | - | 0 | 0 |
| PM2 | pIC100 | 85 | 87 |
| PM2 | pIC101 | 88 | 85 |
| SK3430 | - | 0 | 0 |
| SK3430 | pIC100 | n.d. | n.d. |
| SK3430 | pIC101 | n.d. | n.d. |

(a) cells grown in the absence of chloramphenicol.

(b) cells grown in the presence of 10μg/ml chloramphenicol prior to lysis and measurement of the specific activity of cat (see Methods, 2.3). n.d. indicates that CAT activity was not detected by the spectrophotometric assay in these crude cell extracts.

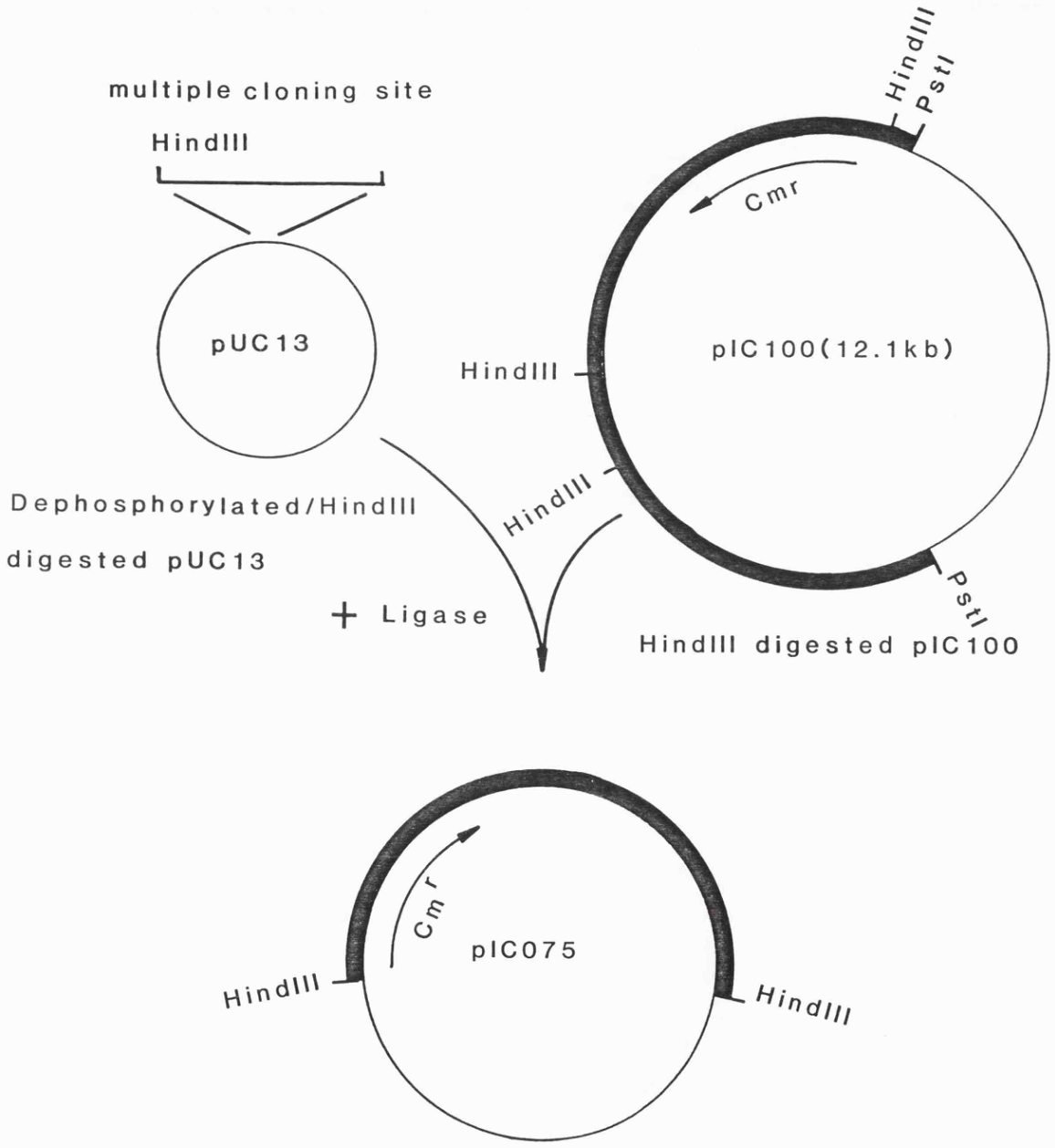
8.2.2 Subcloning the P.mirabilis PM13 cat gene

The introduction of the plasmids pIC100 and pIC101 into the strain P.mirabilis PM2 demonstrated that the cat genes harboured by either plasmid were expressed at a similar level, suggesting that both genes had functionally identical promoters. Hence, the initial subcloning experiments were only performed with the cat gene carried by pIC100.

P.mirabilis PM13 is phenotypically resistant to tetracycline and chloramphenicol and consequently, before the cat gene could be introduced into the parental strain it was necessary to subclone it into a vector carrying another selectable resistance marker. The plasmid pUC13 (Vieira and Messing, 1982) was chosen for this purpose as it carries an ampicillin resistance determinant. The vector was digested with HindIII [a site likely to lie outside cat and flanking control sequences] and ligated to HindIII digested pIC100. The ligation mix was then used to transform E.coli JM83, and the resulting recombinants, identified as white colonies, were replica plated onto L-agar plates supplemented with chloramphenicol at a concentration of 10µg/ml. Those colonies resistant to both ampicillin (50µg/ml) and chloramphenicol (10µg/ml) were subjected to the plasmid lysis procedure described in Methods (2.3). One such cat harbouring plasmid (pIC075) was identified by restriction enzyme analysis, and was chosen for subsequent transformation experiments.

FIG. 8.1

Construction of pIC1075, refer to text for explanation.


multiple cloning site
HindIII
pUC13
Dephosphorylated/HindIII
digested pUC13
+ Ligase
HindIII
PstI
Cmr
pIC100(12.1kb)
HindIII
HindIII
PstI
HindIII digested pIC100
Cmr
pIC075
HindIII
HindIII

### 8.2.3 Transformation of P.mirabilis PM2, P.mirabilis PM13 and S.typhimurium LT2 with pIC075.

The strains P.mirabilis PM2, P.mirabilis PM13, and S.typhimurium LT2 were separately transformed (Methods 2.3) with either plasmid pBR328 (Soberon et al., 1981) or pIC075, and maintained on agar plates supplemented with either chloramphenicol (50µg/ml) or ampicillin (50µg/ml) (Table 8.2).

The transformants were grown overnight in Penassay-broth supplemented with the appropriate antibiotic, subcultured into fresh antibiotic-medium, and harvested when apparent cell density corresponded to $A_{650}$ = 0.8. CAT levels were assayed on crude cell extracts as described in Methods (2.3).

The transformation experiments were designed to answer two questions: (i) How well might the cat gene of P.mirabilis be expressed in the parental strain (P.mirabilis PM13) as compared with related strains?, and (ii) how did its expression alter when cells harbouring the plasmids were grown in the presence of chloramphenicol?

Table 8.2

| Bacterial strain | | Plasmid | Level of Antibiotic in medium (µg/ml) |
|---|---|---|---|
| P.mirabilis | Pm13 | - | - |
| | *Pm13 | - | Cm50 |
| | *IC3328 | pBR328 | Cm50 |
| | IC3328 | pBR328 | Amp50 |
| | *IC3075 | pIC075 | Cm50 |
| | IC3075 | pIC075 | Amp50 |
| | Pm2 | - | - |
| | ICI328 | pBR328 | Cm50 |
| | ICI328 | pBR328 | Amp50 |
| | ICI075 | pIC075 | Cm50 |
| | ICI075 | pIC075 | Amp50 |
| S. typhimurium | LT2 | - | - |
| | IC5328 | pBR328 | Cm50 |
| | IC5328 | pBR328 | Amp50 |
| | IC5075 | pIC075 | Cm50 |
| | IC5075 | pIC075 | Amp50 |

* indicates that the P.mirabilis PM13 colony had been isolated after growth on Penassay-agar supplemented with chloramphenicol at a concentration of 50µg/ml (i.e. had been subjected to the enhancement procedure).

Table 8.3 summarizes the results of the transformation experiments; several conclusions can be drawn from the data:

(i) The most obvious feature is that isolates of P.mirabilis PM13, P.mirabilis PM2, and S. typhimurium LT2 harbouring pIC075 all express CAT at a sufficiently high level so as to be measured by the spectrophotometric assay, a result which is in sharp contrast to the results with E.coli harbouring pIC075, wherein CAT activity was detected only by the radiometric assay.

(ii) The CAT activity measured for cultures harbouring the P.mirabilis PM13 cat gene is not as high as that encountered in those cultures harbouring the type I cat (carried by pBR328).

(iii) A comparison of the CAT levels detected in cultures of P.mirabilis PM13 harbouring pBR328 reveals an approximate 2-fold increase when cultures are grown in the presence of chloramphenicol compared with those grown in the presence of ampicillin. This may be due to plasmid amplification (Clewell, 1972). When a similar comparison is made between isolates harbouring pIC075, there is a much larger increase in CAT activity of either 23-fold (column a) or 7-fold (column b). This large increase in CAT levels only occurs in P.mirabilis PM13 harbouring pIC075, and suggests that a host specific element is modulating cat gene expression.

Table 8.3

| Strain | | Plasmid | Antibiotic in media (µg/ml) | Specific Activity µmole/min/mg (a) | (b) |
|---|---|---|---|---|---|
| P.mirabilis | PM13 | - | - | 0.04 | 0.05 |
| | PM13 | - | Cm50 | 0.48 | 0.26 |
| | IC3075 | pIC075 | Cm50 | 1.58 | 0.67 |
| | IC3075 | pIC075 | Amp50 | 0.07 | 0.10 |
| | IC3328 | pBR328 | Cm50 | 15.01 | 9.26 |
| | IC3328 | pBR328 | Amp50 | 6.73 | 3.99 |
| P.mirabilis | PM2 | - | - | 0.0 | 0.0 |
| | IC1075 | pIC075 | Cm50 | 1.35 | 0.72 |
| | IC1075 | pIC075 | Amp50 | 0.68 | 0.63 |
| | IC1328 | pBR328 | Cm50 | 3.70 | 2.13 |
| | IC1328 | pBR328 | Amp50 | 3.16 | 2.11 |
| S.typhimurium | LT2 | - | - | 0.0 | 0.0 |
| | IC5075 | pIC075 | Cm50 | 0.37 | 0.94 |
| | IC5075 | pIC075 | Amp50 | 0.34 | 0.43 |
| | IC5328 | pBR328 | Cm50 | 11.54 | 8.77 |
| | IC5328 | pBR328 | Amp50 | 11.41 | 9.56 |

## 8.3 SUMMARY

The P.mirabilis PM13 cat gene was cloned into pUC13 (to generate pIC075) and reintroduced into the parental strain (P.mirabilis PM13). The vector pUC13 is a multicopy plasmid, and the cat containing derivative (pIC075) may be expected to exist at a high copy number in each cell. The expected increase in gene dose was reflected by an increase in CAT levels when transformants were grown in the presence of chloramphenicol. Surprisingly, this high cat copy number did not result in increased CAT levels when plasmid harbouring cells were grown in the presence of ampicillin.

This chloramphenicol-dependent difference in CAT levels was not observed in other related bacterial strains (P.mirabilis PM2, and S.typhimurium LT2) harbouring pIC075, and strongly suggests that a P.mirabilis PM13 host-specific factor is responsible for modulating cat gene expression.

# CHAPTER 9

# DISCUSSION

# CHAPTER 9 : DISCUSSION

## 9.1.1 Homology with the type 1 (Tn9) variant

The cat determinant found in P.mirabilis PM13 has not been detected in another closely related strain (P.mirabilis PM2), and thus constitutes a 'variable trait', typical of those which are expressed by some strains of a given species but not by others. Such traits may be contrasted with 'constant' ones which are present as part of the standard genetic complement of the chromosome and are usually of taxonomic importance. Novick and Murphy (1985) studied the phenomenon of resistance to macrolide-lincosamide-streptogramin B antibiotics ($MLS^r$) in Staphylococcus aureus as a model for the concept of variable genetic traits. They suggested that these traits were associated with variable and mobile genetic elements, and thus were spread horizontally through the species. The observation that the cat gene of P.mirabilis PM13 shows a high degree of homology to that of the transposon Tn9-associated type I variant suggests a possible origin for the chromosomal location of the P.mirabilis cat gene: via transposition from an ancestral Tn9-like element.

The initial suggestion of likely sequence homology (as revealed by immunological cross-reactivity, see Chapter 4) between the type I CAT and the P.mirabilis CAT was confirmed by DNA sequencing. An earlier report (Zaidenzaig et al., 1979) that both variants might share a common N-terminus for the first 12 amino acids has, however, not been confirmed (see Fig. 7.6). The two CAT variants

do show 73% residue identity at the DNA level, and 76% when the amino acid sequences are compared. Such a high degree of homology is a strong argument for the hypothesis that both genes have evolved from a common ancestor. Indeed an alignment of all the CAT variants sequenced to date (Fig. 9.1) confirms the view that they represent a family of closely related enzymes arising by divergence from an ancestral protein.

The homology between the type I cat and the cat of P.mirabilis PM13 breaks down in the 5' and 3' flanking regions. No sequence homology corresponding to the insertion sequence IS1 (which flanks the type I cat in Tn9) was detected in the P.mirabilis cat sequence. This observation has been confirmed by Southern blotting experiments wherein a $\gamma$[$^{32}$P]-labelled IS1 DNA fragment was used to probe chromosomal DNA from a variety of bacteria (Nyman et al., 1981). No positive hybridization signal was identified with DNA isolated from P.mirabilis, although greater than 40 copies could be identified in certain enteric strains such as Shigella dysenteriae.

Fig. 9.1 Comparison of the amino acid sequences of chloramphenicol acetyltransferase variants

The type I sequence is from three sources; the nucleotide sequences of (i) Alton and Vapnek, 1979; (ii) Marcoli *et al.*, 1980; and (iii) the protein sequence of Shaw *et al.*, 1979. The remaining amino acid sequences were all derived from the nucleotide sequence. The two *Staphyloccus aureus* plasmids (pC221 and pC194) carrying the genes for the type C and D *cats* were sequenced by Shaw *et al.*, 1985, and Horinouchi and Weisblum, 1982, respectively. The *Bacillus pumilus cat* was sequenced by Harwood *et al.*, 1983, while the type III *cat* was sequenced by Murray *et al.*, 1985 (Manuscript in preparation). Hatched areas represent amino acids conserved in a minimum of five out of six listed sequences.

```
          1   5   10  15  20  25  30  35  40  45  50  55  60  65  70  75  80
I         MEKKITGYTTVDISQWHRKEHFEAFQSVAQCTYNQTVQLDITAFLKTVKKNKHKFYPAFIHILARLMNAHPEFRMAMK D
Proteus   MDTKRVGILVVDLSQWGRKEHFEAFQSFAQCTFSQTVQLDITSLLKTVKQNGYKFYPTFIYIISLLVNKHAEFRMAMK D
III            MNYTKFDVKNWVRREHFEFYRHRLPCGFSLTSKIDITTLKKSLDDSAYKFYPVMIYLIAQAVNQFDELRMAIK D
pC221          MTFNIIKLENWDRKEYFEHY FNQQTTYSITKEIDITLFKDMIKKKGYEIYPSLIYAIMEVVNKNKVFRTGINSE
pC194          MNFNKIDLDNWKRKEIFNHY LNQQTTFSITTEIDISVLYRNIKQEGYLFYPAFIFLVTRVINSNTAFRTGYNSD
Bacillus       M FKQID ENYLRKEHFHHYMTLTRCSYSLVINLDITKLHAILKEKKLKVYPVQIYLLARAVQKIPEFRMDQVND

             85  90  95  100 105 110 115 120 125 130 135 140 145 150 155 160
I         GELVIWDSVHPCYTVFHEQTETFSSLWSEYHDDFRQFLHIYSQDVACYGDNLAYFPKGFI ENMFFVSANPWVSFTSFDL
Proteus   GELVIWDSVNPGYNIFHEQTETFSSLWSYYHKDINRFLKTYSEDIAQYGDDLAYFPKEFI ENMFFVSANPWVSFTSFNL
III       DELIVWDSVDPQFTVFHQETETFSALSCPYSSDIDQFMVNYLSVMERYKSDTKLFPQGVTPENHLNISALPWVNFDSFNL
pC221     NKLGYWDKLNPLYTVFNKQTEKFTNIWTESDNNFTSFYNNYKNDLLEYKDKEEMFPKKPIPENTIPISMIPWIDFSSFNL
pC194     GDLGYWDKLEPLYTIFDGVSKTFSGIWTPVKNDFKEFYDLYLSDVDKYNGSGKLFPKTPIPENAFSLSIIPWTSFTGFNL
Bacillus   ELGYWEILHPSYTILNKDTKTFSSIWTPFDENFAQFYKSCVADIETFSKSSNLFPKPHMPENMFNISSLPWIDFTSFNL

            165 170 175 180 185 190 195 200 205 210 215 220 225 230 235 240
I         NVANMDNFFAPVFTMGKYYTQGDKVLMPLAIQVHHAVCDGFHVGRMLNELQQYCDEWQGGA
Proteus   NMANINNFFAPVFTIGKYYTQGDKVLMPLAIQVHHAVCDGFHVGRLLNEIQQYCDEGCK
III       NVANFTDYFAPIITMAKYQQEGDRLLLPLSVQVHHAVCDGFHVARFINRLQELCNSKLK
pC221     NIGNNSNFLLPIITIGKFYSENNKIYIPVALQLHHAVCDGYHASLFMNEFQDIIHKVDDWI
pC194     NINNNSNYLLPIITAGKFINKGNSIYLPLSLQVHHSVCDGYHAGLFMNSIQDLSDRPNDWLL
Bacillus  NVSTDEAYLLPIFTIGKFKVEEGKIILPVAIQVHHAVCDGYHAGQYVEYLRWLIEHCDEWLNDSLHIT
```

9.1.2 Control of P.mirabilis PM13 cat gene expression

In the general introduction (Chapter 1) a variety of mechanisms were described by which bacteria regulate gene expression. These were characterized according to the level at which regulation occurred, i.e. transcriptional control, or post-transcriptional control. In this section the phenomenon of the appearance of chloramphenicol-resistant cclonies from a predominantly sensitive population at a frequency of betwen $10^{-4}$ to $10^{-5}$ per cell per generation (enhancement), is discussed in relation to each of the various models.

First, and most important, an analysis of the data from the Luria-Delbruck fluctuation test (Chapter 3) indicated that chloramphenicol-resistant cells of P.mirabilis PM13 arose spontaneously from a predominantly sensitive population as a result of mutation, rather than by a mechanism involving induction. Furthermore, the contour of the plot of the decrease in the number of resistant cells (efficiency of plating) as a function of time, when grown in the absence of antibiotic, was shown to be inappropriate for a model wherein a putative intracellular inducer might be diluted by cell division. A mechanism involving back-mutation, and counter-selection of resistant cells was proposed to explain the data.

Support for mutation rather than induction as the explanation for the enhacement phenomenon came from two other sources: (i) the data from 'Northern dot-blot' experiments (Chapter 6), demonstrated

that expression of the P.mirabilis cat gene was under transcriptional rather than post-transcriptional control (an observation not tenable with the current models of gram-positive cat induction); and (ii) examination of the DNA sequence 5' to the start of cat at 880 (see Fig. 7.4) revealed no sequences analogous to those implicated in gram-positive cat induction. These points are discussed in more detail below.

(i) 'Nothern dot-blots' reveal transcriptional control

The induction of antibiotic resistance determinants by the inclusion of the appropriate drug in the growth medium has been observed to operate for a number of genes, e.g: the erythromycin resistance gene harboured by plasmid pE194 is induced by erythromycin (reviewed by Weisblum, 1984); the cat gene of Agrobacterium tumefaciens is induced by chloramphenicol, as are the gram-positive cat genes carried by pCW41 and pC194 (reviewed by Shaw, 1983) and the chromosomal cat-86 gene of B.pumilus (Harwood et al., 1983).

Those examples of induction (described above) which have been studied at the molecular level have revealed some conserved features. The first observation is that the frequency of transcription of the resistance gene is not increased by the presence of the appropriate inducer, thus control of gene expression occurs post-transcriptionally. 'Northern dot-blot' experiments (Chapter 6) using an homologous [$^{32}$P]-labelled cat probe against total RNA prepared from P.mirabilis PM13 cells grown in the presence and

absence of chloramphenicol detected a substantial increase in the amount of cat specific mRNA when antibiotic was included in the growth medium. Such a result supports the view that regulation of the P.mirabilis PM13 cat gene occurs at the transcriptional level, and as such is not formally analogous to the gram-positive induction mechanisms described above.

(ii) Sequence motifs 5' to the start of the P.mirabilis cat gene

Examination of the sequence 5' to the start of the inducible antibiotic resistance genes described above reveals dyad axes of symmetry which can form putative hairpin-loop structures in mRNA. In the case of the $Em^r$ determinant, four inverted repeats are present (which can form two hairpin loop structures), one of which sequesters the Shine-Dalgarno sequence (SD-sequence) in the double-stranded stem (see Chapter 1). In this configuration, ribosomes are unable to bind to the mRNA and consequently cannot translate the message. In the presence of the antibiotic, ribosome 'stalling' is believed to explain the exposure of the SD-sequence thus allowing translation to proceed. Dyad axes of symmetry (which can potentially sequester the SD-sequence in a double stranded form) have also been found 5' to the start of the cat genes of pCW41, pC194 and B. pumilus. The $\Delta G^o$ for the three structures at 25°C are: pCW41, -19.1 kcal.mol.; pC194, -28.1 kcal.mol.; and B. pumilus, -26.4 kcal.mol., suggesting that stem-loop formation is energetically favourable. Models for cat induction in all three cases have been proposed (but not confirmed), and assume that the action of chloramphenicol in some way (possibly by interaction with

ribosomes) alters the configuration of the stem-loop so as to free the SD-sequence and thus allowing ribosomes to bind, and thence translate the mRNA. Examination of the sequence 5' to the start of the cat gene of P.mirabilis PM13 reveals two regions that could potentially form stem-loop structures. These are shown (as DNA, rather than RNA structures) in Fig 9.2 with their calculated $\Delta G^{o}$ values in kcal/mol. Comparison of these potential structures with the stem-loop structures discussed above for the gram-positive cat genes suggest that they are not so energetically favourable and are thus less likely to exist in vivo. Furthermore, neither of the putative stem-loop structures 5' to the start of P.mirabilis cat gene can sequester the Shine-Dalgarno sequence in secondary structure. It seems unlikely, therefore, that either putative stem-loop has any significance in the control of the cat gene expression of P.mirabilis PM13 via an induction mechanism as proposed for the gram-positive genes.

Unlike the stem-loop structures involved in transcription termination, such as those involved in attenuation (reviewed by Kolter and Yanofsky, 1982), neither of the putative stem-loops 5' to the cat gene of P.mirabilis PM13 are G+C rich, nor are they followed by a U-rich tract. These observations suggest that they are unlikely to function as attenuators in regulating cat gene expression.

The two dyad axes 5' to the start of the cat gene of P.mirabilis PM13 are, however, suggestive of sequence targets for certain

trans-acting control elements (Reznikoff and Abelson, 1978). Such binding sites often possess an imperfect axis of symmetry, thus the lac-operator comprises an imperfect inverted repeat spanning 35-nucleotides, while the cAMP-CAP binding site spans a region of 14-nucleotides with imperfect symmetry.

Another putative stem-loop structure was identified 3' to the cat structural gene (Fig. 9.2), and in sequence resembles the consensus for a rho-independent terminator. As this sequence exists 3' to the structural gene it is unlikely to be involved in any mechanism of the control of initiation of cat gene expression, however, it may be important in transcriptional termination and message stability.

### 9.1.3 A mutation to chloramphenicol resistance

The high frequency and reversible nature of the mutation responsible for the expression of chloramphenicol resistance in P.mirabilis PM13 suggests a genetic-switch mechanism may be operating (reviewed by Plasterk and Van de Putte, 1984). Such mechanisms have been described for a variety of genes (see Chapter 1) wherein an invertible DNA segment alternates between two orientations, consequently altering the expression of different genes or operons. These site-specific inversions can occur over a frequency range of between $10^{-6}$ to $10^{-2}$ per cell per generation, often with a preference for one orientation over the other (Silverman and Simon, 1983). The experiments described in Chapter 6 were designed to detect the occurrence of such a rearrangement either

FIG. 9.2

Potential secondary structure 5' to the start of the *cat* gene

The structures are drawn as they could potentially form in the DNA. The - $G^o$ values are shown underneath in k calories.

(a) (i) $\Delta G^o = -9.4$Kcal/mol

(ii) $\Delta G^o = -7.2$Kcal/mol

FIG. 9.3

Potential secondary structure 3' to the *cat* gene

The structure is drawn as it could potentially form in the DNA, and is characteristic of a rho-independent terminator.

$\Delta G^o = -6.0$Kcal/mol.

FIG.9.2

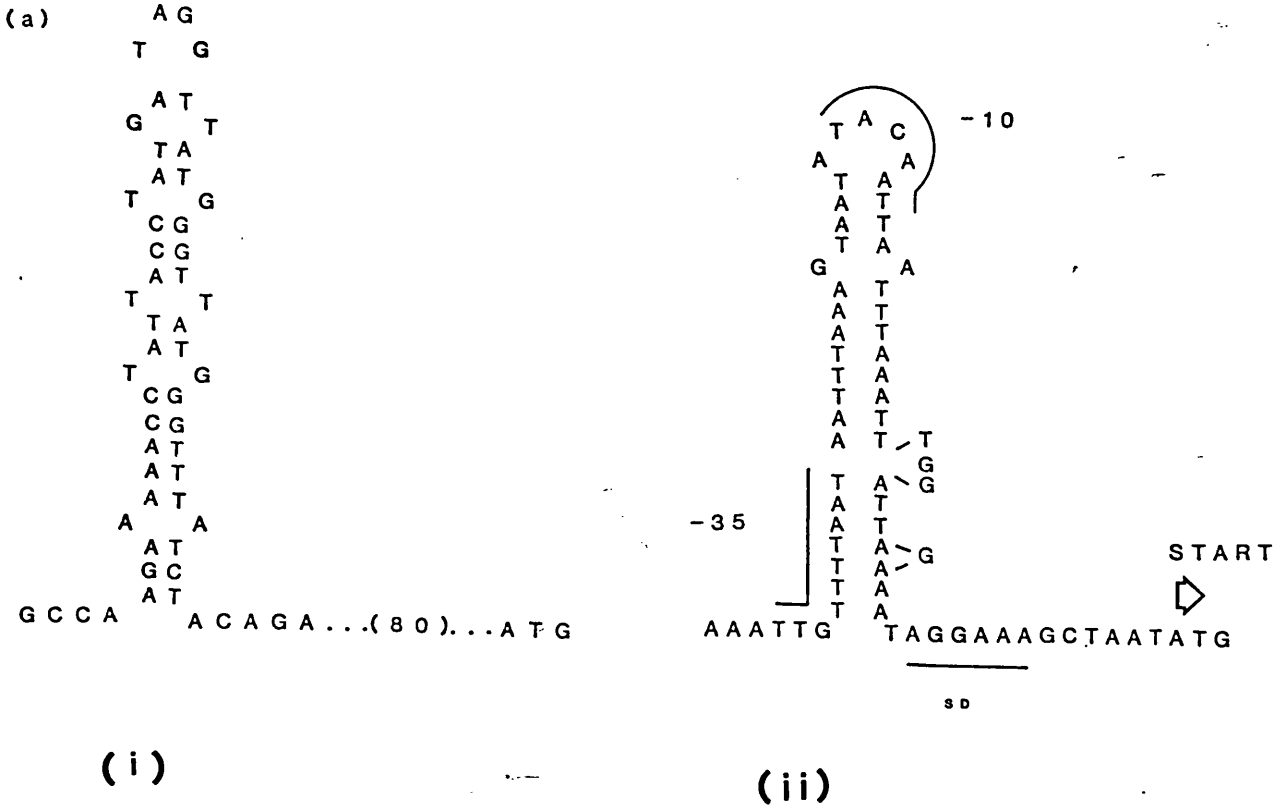
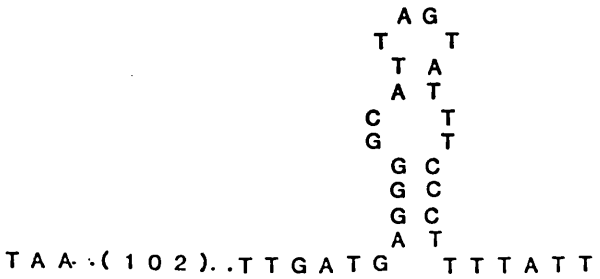

(a)
GCCA
ACAGA...(80)...ATG
-10
-35
START
AAATTG
TAGGAAAGCTAATATG
SD
(i)
(ii)

FIG.9.3


TAA..(102)..TTGATG
TTTATT

(i) within the genome of P.mirabilis PM13 when grown in the presence as compared with the absence of chloramphenicol, or (ii) between the two cloned cat genes harboured by pIC100 and pIC101. Southern blotting, heteroduplex analysis, and restriction mapping has failed, however, to identify any difference between the two cat genes.

This observation was supported by the data on the expression of the cat gene of P.mirabilis PM13 in different enteric hosts (Chapter 8). The most interesting finding was that the difference in specific activity of CAT measured in crude extracts of P.mirabilis PM13 harbouring pIC075 mirrored the behaviour of cat expression for the plasmid-free host. This was a surprising find as pIC075 was constructed from pIC100 (i.e. from DNA isolated from cultures of P.mirabilis PM13 grown in the presence of chloramphenicol). Thus, if any structural rearrangement had occurred within pIC075 to increase cat gene expression, re-introducing it into P.mirabilis PM13 should have immediately produced a high specific activity of CAT. This was not observed. A high specific activity of CAT was only obtained when plasmids were introduced into isolates of P.mirabilis PM13 which had been grown in the presence of chloramphenicol suggesting that: (i) a trans-acting host-specific factor is likely to be responsible for regulating cat gene expression; and (ii) the putative host-specific factor is itself subject to 'ON/OFF' control of the type encountered in genetic switch mechanisms. As a consequence of this, it follows that the cat genes present in cells expressing the CAT+ or CAT− phenotype will be the same; alternate

expression being controlled by the presence or absence of a trans-acting factor.

A precedence for the regulatory action of a trans-acting element, which is itself controlled by a genetic-switch element is given by the example of phase variation in S. typhimurium (see Chapter 1). Interestingly, a comparison of the DNA sequence 5' to the start of the flagellin genes H1 and H2 (alternately expressed in phase variation) reveals blocks of homology with the 5' region of the cat gene of P.mirabilis (Fig. 7). Whether or not this homology has any significance for the mechanism of the regulation of cat in P.mirabilis PM13, (i.e. is the cat gene switched 'ON' and 'OFF' in a similar fashion to the alternate expression of H1 and H2) can only be speculation at present.

The conclusions on the nature of cat gene expression in P.mirabilis PM13 are summarized below:

(i) chloramphenicol-resistant colonies of P.mirabilis PM13 occur as a result of spontaneous mutation at a frequency of between $10^{-4}$ to $10^{-5}$ per cell per generation;

(ii) these resistant cells are lost from cultures (grown in the absence of chloramphenicol) at a frequency of approximately $10^{-2}$ per cell per generation as a result of back-mutation and counter-selection;

(iii) the frequency of the CAT$^-$ to CAT$^+$ phenotypic change (and *vice versa*) occurs within the same frequency limits as many 'genetic-switch' elements;

(iv) the mutation controlling the alternate expression of *cat* is host specific, suggesting the involvement of a *trans*-acting element.

The work undertaken during the course of these studies has contributed towards the understanding of the mechanism whereby *P.mirabilis* PM13 can become resistant to chloramphenicol. Further work, however, is required to elucidate the specific mechanism of control. The first aim of any future work should be to attempt to isolate the proposed *trans*-acting control element. Experiments to elucidate the mechanism of action of this element could then be devised. Work along these lines has already been initiated in the laboratory (F. Scott, B.Sc. Thesis, 1985, University of Leicester), but so far has not led to any further understanding of the mechanism.

# APPENDIX A

# CHLORAMPHENICOL RESISTANCE IN *FLAVOBACTERIUM* sp. CB60

# APPENDIX A : Chloramphenicol Resistance in *Flavobacterium* sp. CB60

This work was carried out as a joint project with Hans Georg Beschle during his visit to the Department of Biochemistry on a short term fellowship awarded by the European Molecular Biology Organization. Dr. Beschle provided the microbiological expertise. The bulk of the *in vitro* recombinant DNA work was mine. An account of the study has been published (Beschle *et al.*, 1984).

## A.1 INTRODUCTION

*Flavobacterium* sp. CB60 is a gram-negative isolate which has been tentatively identified as *Flavobacterium devorans*, (Sussmuth *et al.*, 1979). As the *Flavobacterium devorans* type species, ATCC 10829, has recently been identified as a strain of *Pseudomonas pacimobilis* (Yabuuchi *et al.*, 1979) the present taxonomic position of *Flavobacterium* sp. CB60 is uncertain.

Resistance to chloramphenicol in *Flavobacterium* sp. CB60 is mediated by the constitutive synthesis of CAT, and the acetylated products are further degraded by co-metabolism (Sussmuth *et al.*, 1979; Beschle *et al.*, 1982). Since a previous report implicated R-plasmids as vectors of resistance to ampicillin and erythromycin in clinical isolates of *Flavobacterium odoratum* (Kono *et al.*, 1980), it was appropriate to characterize the chloramphenicol resistance determinant of *Flavobacterium* sp. CB60.

## A.2 RESULTS

### A.2.1 Presence of a 15.6-kb plasmid in *Flavobacterium* sp. CB60

Since the genetic determinant, *cat*, is associated in most gram-negative bacteria with R-plasmids (reviewed by Shaw, 1983) it was decided to screen *Flavobacterium* sp. CB60 for the presence of a plasmid. Three very closely related *Flavobacterium* strains CB60 ($Cm^r$), CB61 ($Cm^s$), and CB67 ($Cm^s$) were subjected to the plasmid lysis procedure of Birnboim and Doly, (1979). The DNA was subjected to agarose gel (0.7%) electrophoresis, and stained with ethidium bromide (Methods, 2.3). Only strain CB60 was observed to harbour a plasmid (pCB60), the molecular weight of which was estimated to be 15.6-kb by addition of the apparent sizes of fragments produced by single and double digests with the enzymes *Acc*I, *Eco*RI, *Pvu*II, and *Sal*I (Fig. A.1).

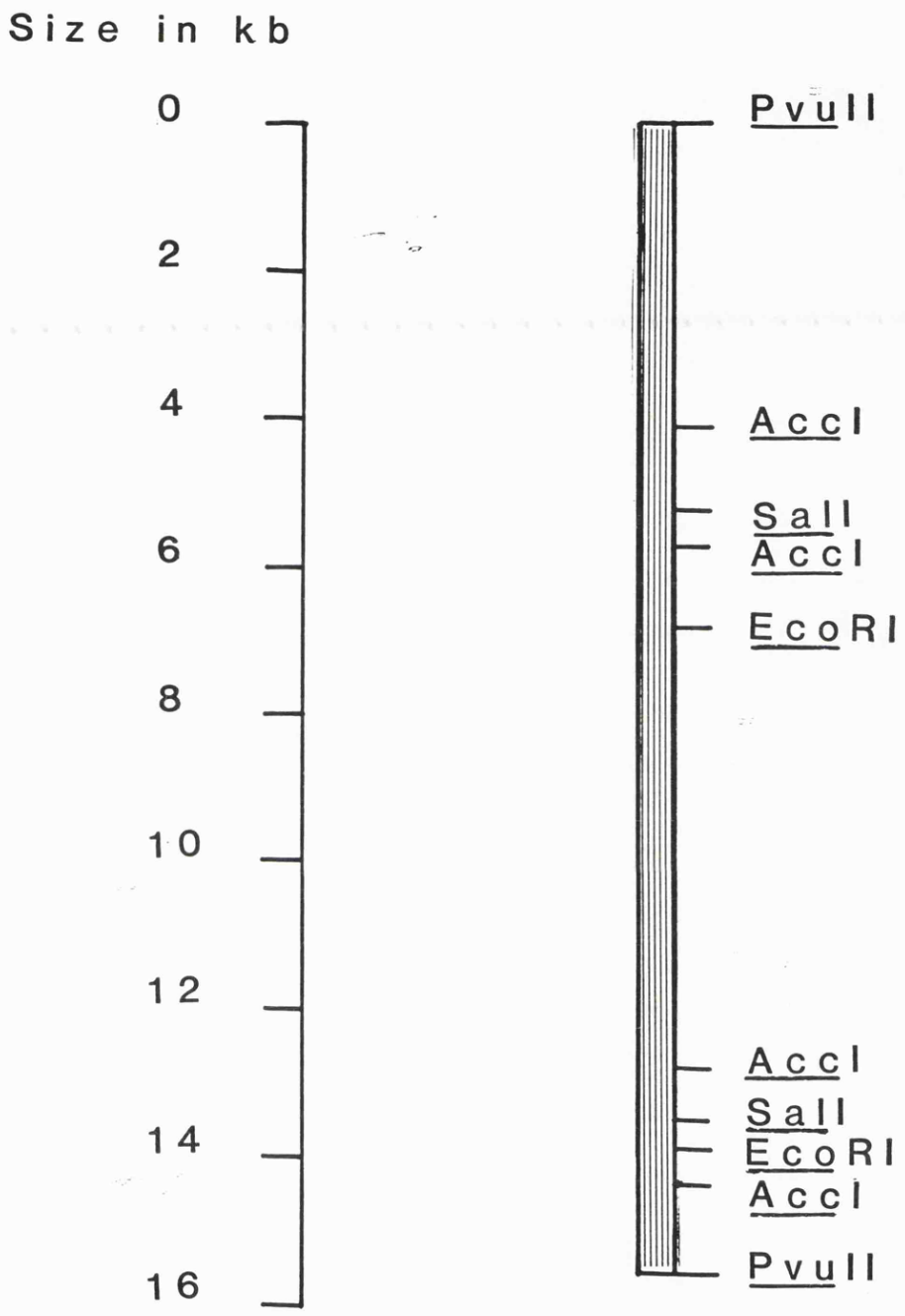
The possibility that the $Cm^r$ determinant was encoded by pCB60 was investigated by two strategies:

(a) by attempting to transfer it to different bacterial strains (*Flavobacterium* strains CB61 and CB67, *E.coli*, and *Pseudomonas* sp.) and

(b) Southern hybridization against a *cat* active site oligonucleotide.

FIG A.1 Restriction Map

Restriction map of plasmid pCB60 generated by the enzymes AccI, EcoRI, PvuII and SalI.




restriction map of pCB60

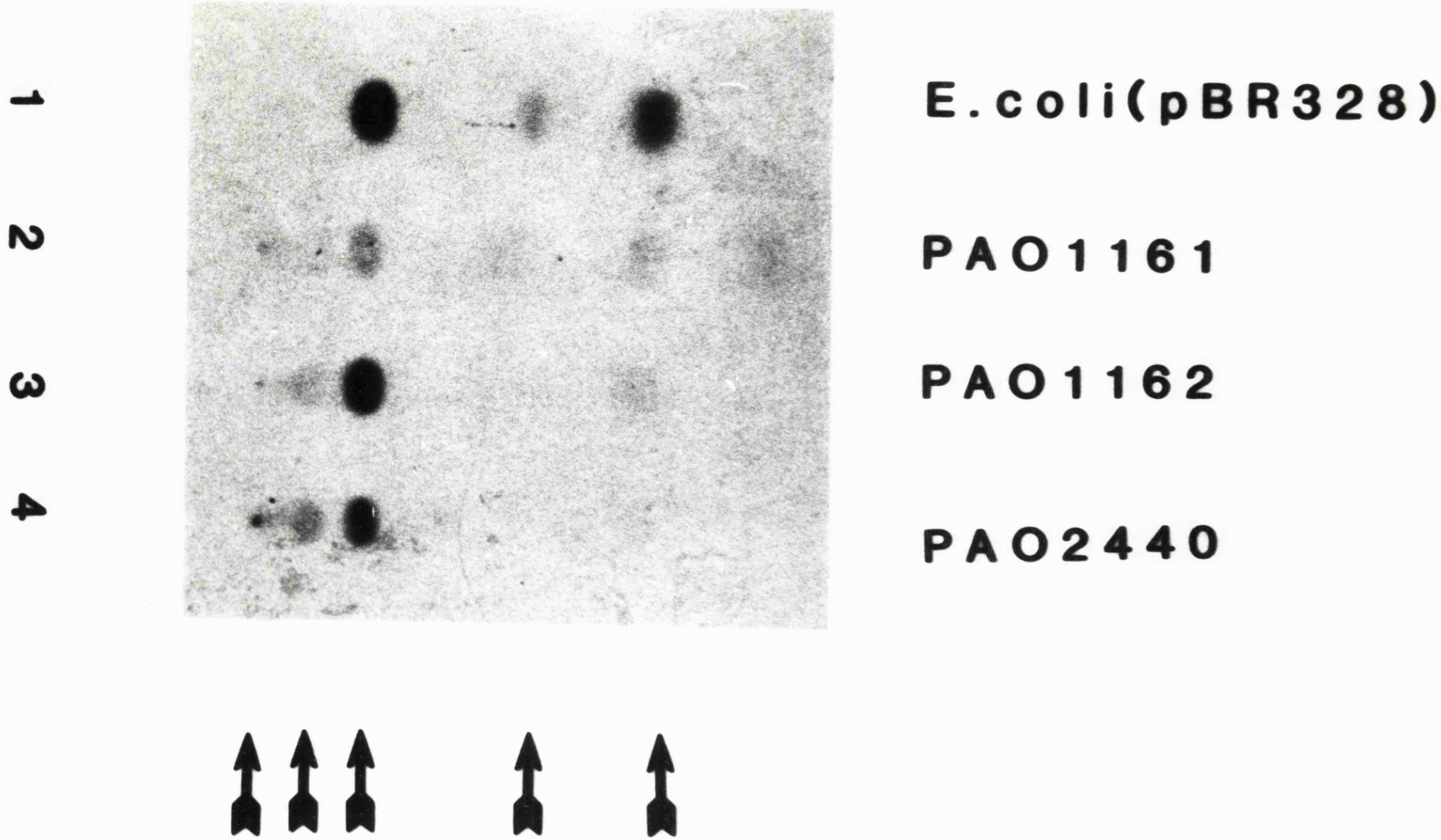
A.2.2 Transfer Studies

The transformation of the $Cm^s$ *Flavobacterium* strains CB61 and CB67 with pCB60 using protocols described for the transformation of *E.coli* and *Pseudomonas* (Methods, 2.3) failed to yield any $Cm^r$ *Flavobacteria*. The *Pseudomonas* strains PA01161, PA01162, and PA02440 subsequently proved unsuitable as hosts for any further transformation experiments because of their intrinsic resistance to chloramphenicol. This chloramphenicol resistance phenotype only became apparent after the strains PA01161, PA01162 and PA02440 had been grown on L-agar plates containing chloramphenicol (10µg/ml) for 5 days at 37°C. Growth of resistant colonies on progressively higher levels of chloramphenicol (50, 100, 200, 500µg/ml) resulted in strains which could ultimately grow on 1mg of chloramphenicol per ml. Mechanisms of chloramphenicol resistance in *Pseudomonas* spp. often rely on the relative impermeability of the cell envelope to the drug, and this barrier may be enhanced by the presence of a plasmid, the products of which lead to further exclusion of chloramphenicol from the cell interior (Kono and O'Hara, 1976). This barrier has been reported to act in concert with CAT to yield even higher levels of resistance (Gaffney *et al.*, 1981), and this was observed to be the case for the amplifiable chloramphenicol resistance detected in strains PA01161, PA01162 and PA02440. Attempts to detect CAT activity by the spectrophotometric assay were unsuccessful, but the more sensitive radiometric assay carried out with cell-free extracts of $Cm^r$ isolates of strains PA01161, PA01162, and PA02440 successfully demonstrated CAT activity (Fig. A.2). These results suggest that both an exclusion mechanism, and CAT are responsible for chloramphenicol resistance in these strains.

Attempts were made to transform E.coli SK3430 with pCB60, but no $Cm^r$ (10μg/ml) colonies were detected. As it was not known whether the plasmid pCB60 could replicate in an E.coli host it was decided to clone the plasmid into an E.coli vector, pUC13, (Vieira and Messing, 1982). The plasmid pUC13 was digested with the restriction endonuclease EcoRI, and 5'-phosphate groups removed by inclusion of calf intestinal phosphatase in the digest-mix (Methods, 2.3). Plasmid pCB60 was partially digested with EcoRI to produce predominantly linear fragments. After electrophoresis, the band corresponding to linearized pCB60 was cut from the gel, the DNA extracted, and ligated into the linearized pUC13 vector. After transformation of competent E.coli JM83, recombinants (identified as white colonies on plates containing X-GAL) were subjected to the plasmid mini-prep protocol (Methods, 2.3) and 4 plasmids, representing pCB60 linearized at either EcoRI site, and inserted into pUC13 in both orientations selected. None of these plasmids were capable of supporting growth on Cm when subsequently transformed into E.coli strain SK3430 and plated onto L-agar containing chloramphenicol at a concentration of 10μg/ml.

FIG. A.2 CAT activity in crude cell extracts

Radiometric demonstration of CAT activity in crude cell extracts from E.coli SK3430 harbouring plasmid pBR328 (lane 1 - positive control), PA01161 (lane 2), PA01162 (lane 3) and PA02440 (lane 4). The assay involves ascending chromatography on silica gel in chloroform-methanol (85:15 vol/vol) of the products of acetylation of [$^{14}$C]-chloramphenicol by CAT in the presence of coenzyme A. From top to bottom, the arrows represent: 1,3-diacetyl-chloramphenicol; 3-acetyl-chloramphenicol; 1-acetyl-chloramphenicol; unmodified chloramphenicol; and the bottom arrow indicates the initial loading spot.


1
2
3
4
E.coli(pBR328)
PAO1161
PAO1162
PAO2440

A.2.3 A synthetic oligonucleotide to identify cat genes

A synthetic oligonucleotide, complementary to the active site region of the gene for a type I cat (Chapter 5) was used in an attempt to identify the location of the Flavobacterium sp. CB60 cat gene by DNA:DNA hybridization.

Plasmids pBR322 (negative control containing no cat gene), pCB60, pBR328 (type I cat), pJK111 (type III cat) and pSES9 (type c cat) were cut with the restriction endonucleases TaqI, PstI, Sau3A, and Sau3A respectively, electrophoresed through a 1.0% agarose gel, and transferred to nitrocellulose paper by the method of Southern, 1975. The filter was hybridized with the $\gamma[^{32}P]$-labelled active-site specific probe as previously described (Methods, 2.3). Autoradiography revealed that hybridization occurred only to those plasmids carrying the three well characterized genes specifying CAT (Fig. A.3). Both pCB60 and pBR322 failed to show any hybridization. This result is in agreement with the expression experiments, and are compatible with the view that pCB60 does not carry a typical cat gene . The alternative hypothesis, namely that Flavobacterium sp. CB60 harbours cat on its chromosome (or that the cat gene does not hybridize to the active site oligonucleotide) was explored in a similar fashion. Endonuclease PstI was used to digest chromosomal DNA prepared from strains CB60, CB61, and CB67. The digested DNA was subjected to electrophoresis, and was transferred to nitrocellulose by the method of Southern. Hybridization of the resulting nitrocellulose-bound DNA to the $\gamma[^{32}P]$-labelled cat active site probe only produced a positive signal with chromosomal DNA prepared from strain CB60 (Fig. A.4).

This result, combined with the failure of the plasmid pCB60 to give a hybridization signal strongly suggests that: (a) the cat gene in Flavobacterium sp. CB60 is chromosomally located; (b) there is a substantial degree of homology at the active site between the CAT of strain CB60 and CAT variants studied previously; and (c) the finding of pCB60 in this $Cm^r$ strain (CB60) was either fortuitous, or reflects a secondary role which it may play in specifying gene products necessary for the subsequent metabolism of acetylated chloramphenicol (Beschle et al., 1982).

FIG. A.3 A synthetic oligonucleotide to identify *cat* genes

(a) 1.0% agarose gel showing banding pattern produced after ethidium bromide staining of: A, pBR322 digested with *Taq*I (negative control); B, pBR328 digested with *Taq*I (type I *cat*); C, pCB60 digested with *Pst*I; D, pSES9 digested with *Sau*3A (type C *cat*); E, pJK111 digested with *Sau*3A (type III *cat*). Dots indicate the known position of *cat* genes in each case.

(b) Autoradiograph of the DNA shown in (a) after transfer to nitrocellulose by the method of Southern and hybridization with the $\gamma[^{32}P]$-labelled oligonucleotide. Hybridization has only occurred with the bands known to contain *cat* genes.

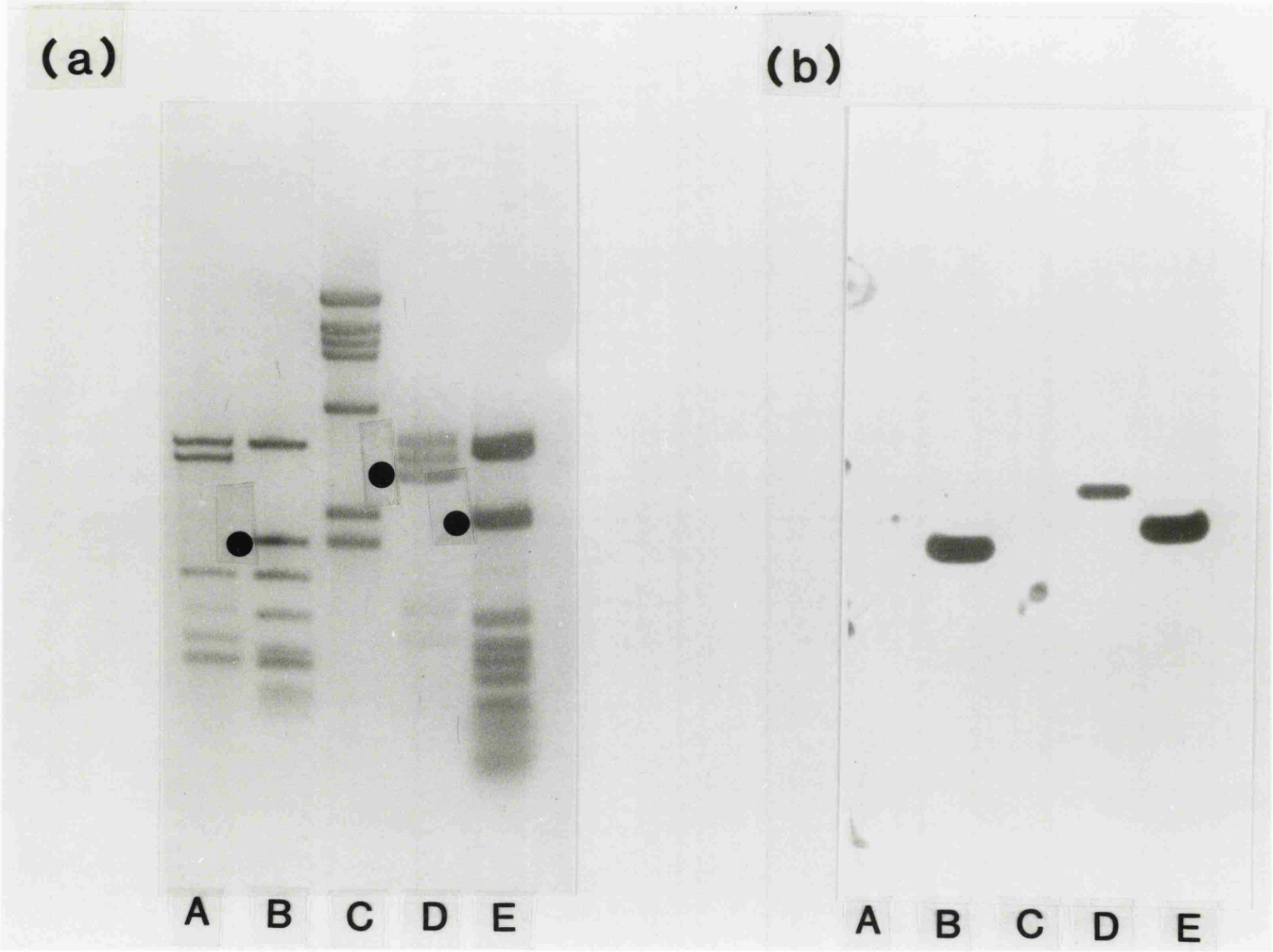
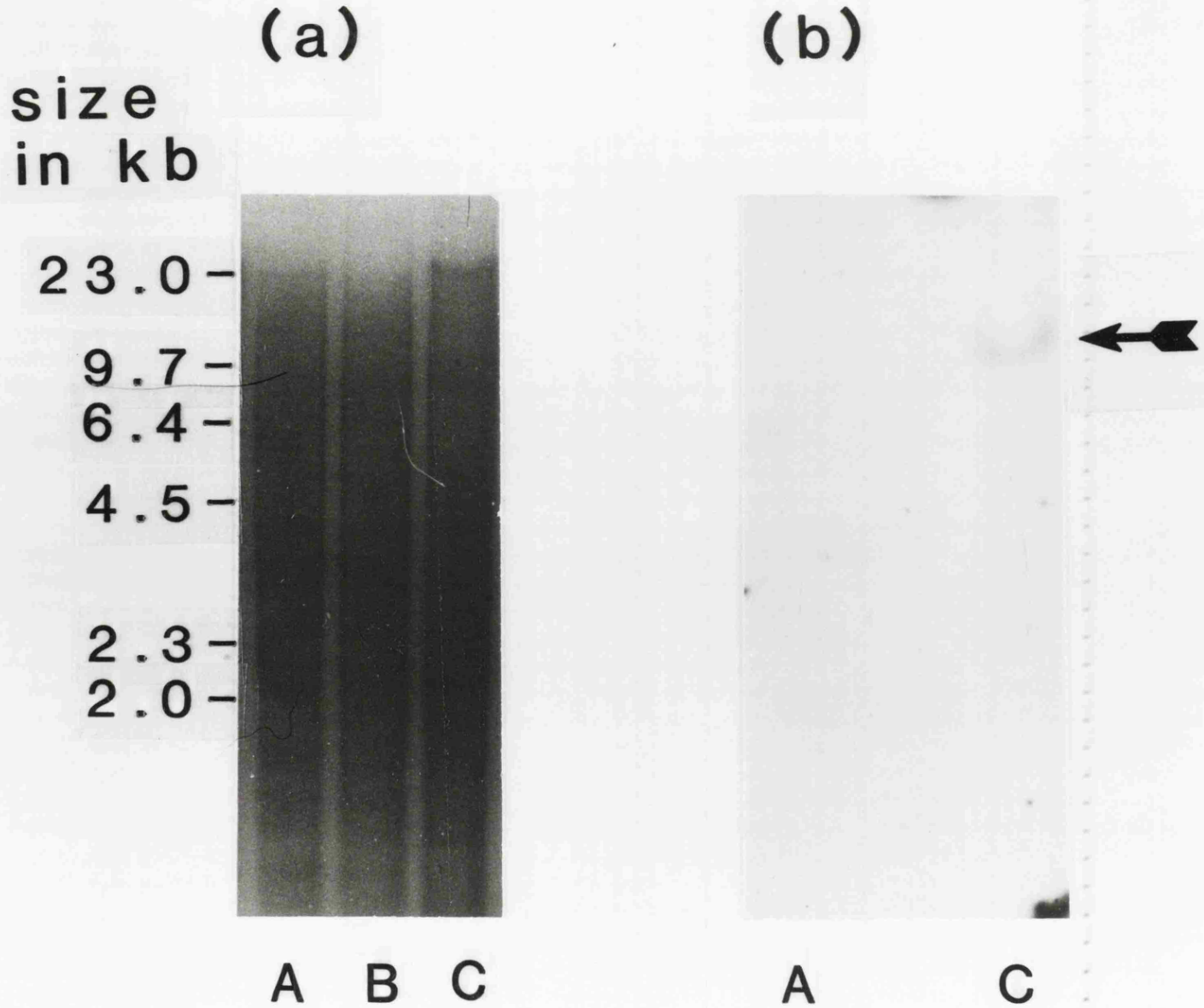

(a)
A B C D E
(b)
A B C D E

FIG. A.4 Hybridization of a *cat*-specific oligonucleotide to *Flavobacterium* DNA

(a) 0.7% agarose gel showing the banding pattern produced after ethidium bromide staining of: A, CB67 chromosomal DNA digested with *PstI*; B, CB61 chromosomal DNA digested with *PstI*; and C, CB60 chromosomal DNA digested with *PstI*.

(b) Autoradiograph of the DNA shown in (a) after transfer to nitrocellulose by the method of Southern, and hybridization to the $\gamma[^{32}P]$-labelled *cat* active site oligonucleotide. The arrow indicates that hybridization has only occurred to DNA prepared from CB60.


(a)
(b)
size
in kb
23.0
9.7
6.4
4.5
2.3
2.0
A B C
A C

A.2.4 Cloning of the Flavobacterium sp. CB60 cat gene

Initial cloning experiments were carried out to isolate the Flavobacterium sp. CB60 cat gene by virtue of its expression in an heterologous host. A potential host/vector system for such a cloning strategy would have been the Pseudomonas spp. protocol (Bagdasarian et al., 1981). However, the strains described (PA01161, PA01162, and PA02440) proved to be amplifiable to high level chloramphenicol resistance, and were thus unsuitable. An E.coli host/vector system was consequently used. Chromosomal DNA isolated from Flavobacterium sp. CB60 was digested with the restriction endonuclease Sau3A (under varying conditions of enzyme concentration and time) which produced fragment sizes in the range from 5 to 15-kb. These fragments were further purified away from higher and lower molecular weight components by centrifugation through a 10-40% sucrose gradient (Methods, 2.3). Once the insert DNA was purified it was ligated with BamHI digested pAT153 which had been treated with calf intestinal phosphatase to remove the 5'-phosphate groups. After transformation of competent E.coli SK3430, recombinants were selected on L-agar plates supplemented with the appropriate antibiotics, and the control plates examined (see Chapter 5) in order to calculate the probability of having generated a representative gene-bank (Clarke and Carbon, 1976). Despite examining enough recombinants to have had a 99.9% probability of finding the Flavobacterium sp. CB60 cat gene, no chloramphenicol resistant colonies were seen. A possible explanation for this is that the cat gene was not expressed in E.coli through an inability of the hosts machinery to recognize CB60 control sequences

for transcription and translation. In view of the lack of success with this approach it was decided to attempt to clone the Flavobacterium sp. CB60 gene by a strategy based on screening for the gene rather than selecting for it. This screening procedure could be achieved by using the cat consensus active-site oligonucleotide as a radio-active probe to locate the cat gene among sequences cloned into an appropriate vector. In practice, the λ-replacement vector λDB286 (Brammar, 1982) was chosen for this purpose. This vector, a derivative of λL47.1 (Loenen and Brammar, 1981) contains the "non-essential" regions of the phage (including red and gam) bounded by specifically engineered pairs of restriction sites. Thus digesting the vector DNA (100μg) with BamHI separated the left and right arms away from the non-essential central region. These arms were then purified by centrifugation through a 10 to 40% sucrose gradient (Fig. A.5). Flavobacterium sp. CB60 chromosomal DNA was digested with Sau3A under conditions which had been optimized to give DNA fragments in the size range of 10-20-kb. These fragments were also purified by centrifugation through a 10-40% sucrose gradient.

Once the vector arms and insert DNA had been purified, they were heated at 70°C for 10 minutes, then held at 42°C for 1 hour (to allow arms and insert to anneal). Samples were ligated together at high concentration (250μg/ml) with a molar ratio of vector to insert of 3:1. After overnight ligation at 10°C the samples were again heated at 70°C for 5 minutes, and packaged using extracts derived from the E.coli strains BHB2688, and BHB2690 (Methods, 2.3). The

resulting phage particles were serially diluted in $\lambda$-phage buffer and plated onto an E.coli P2 lysogen (Q359) and E.coli 5K (the P2 lysogen only permits growth of red$^-$ gam$^-$ phage, i.e. recombinants). The library was thus shown to contain approximately $1 \times 10^5$ recombinant phage particles.

A.2.5 Identification of the Flavobacterium sp. CB60 cat

Representative samples of the $\lambda$DB286 library were plated onto lawns of E.coli WJB910, (a recBC host which minimizes recombination between cloned sequences) grown overnight, and the resulting plaques transferred to nitrocellulose using the method of Benton and Davis, 1977. After hybridization to the $\gamma[^{32}P]$-labelled cat active site oligonucleotide-probe (Methods, 2.3) areas of lysis which showed a putative positive signal were picked, and replated onto lawns of E.coli WJB910 for re-screening. Probing nitrocellulose filters made against these phage should have resulted in an increased signal intensity as more "positive" (i.e. cat-containing phage) should be present. In practice, however, this did not occur, indeed, positively hybridizing plaques obtained from one round of screening were discovered not to be reproducibly positive on subsequent screening. This was attributed to the high background of radio-activity on the filter resulting as a consequence of the low-stringency hybridization and wash experimental conditions.

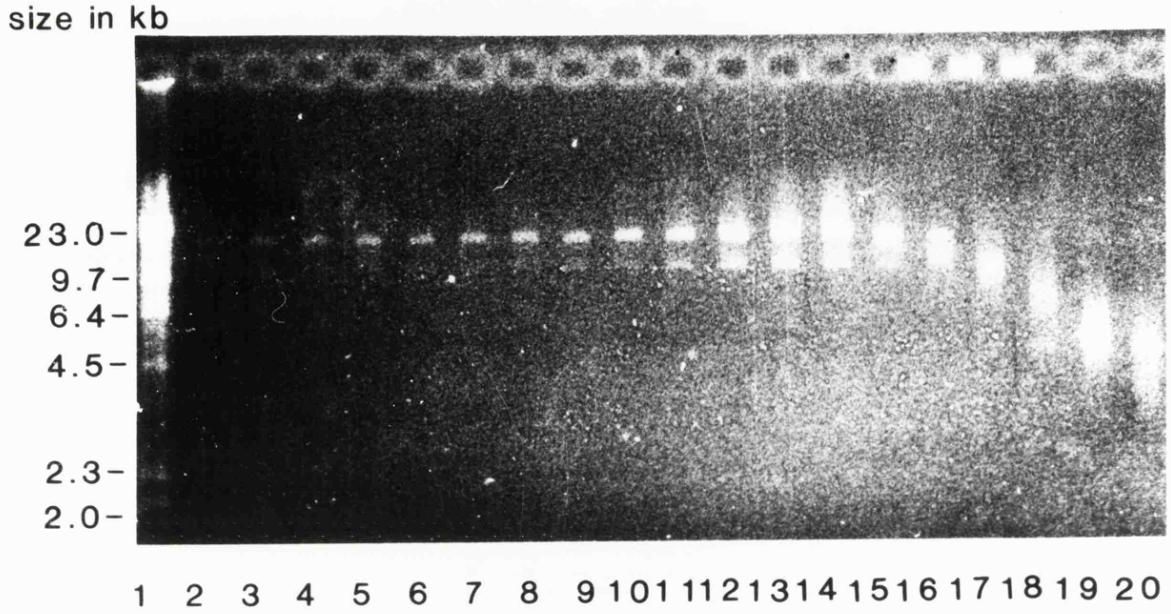
FIG. A.5

Centrifugation of DNA samples through a sucrose gradient

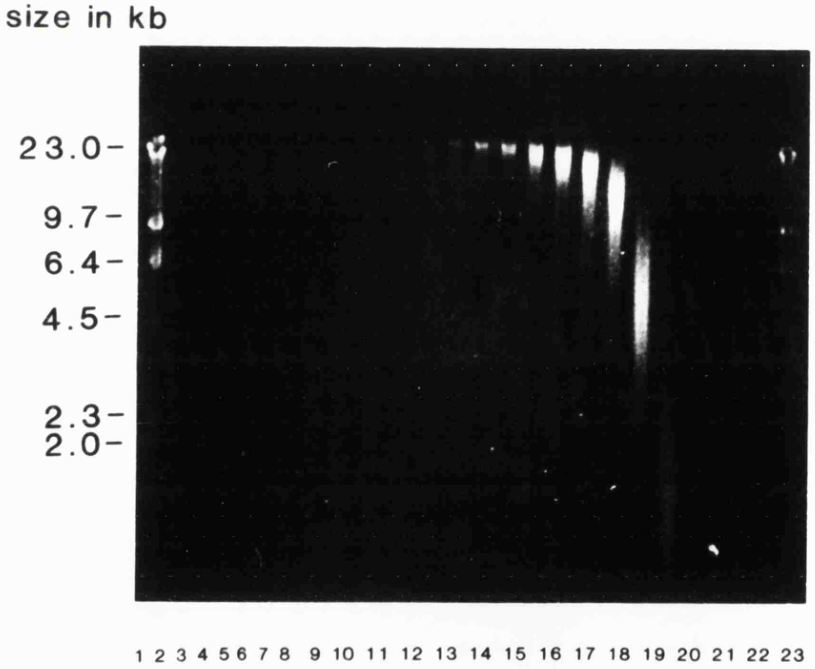
(a) 0.6% agarose gel showing the purification of λ -arms away from the central non-essential region. The tracks 2 to 20 represent fractions collected from a 10-40% sucrose gradient. Tracks 2-15 show the left and right arms with no contaminating central fragment (tracks 19 and 20). The fractions containing arms were pooled and used in subsequent cloning experiments.

(b) 0.6% agarose gel showing the purification of Sau3A digested Flavobacterium sp. CB60 chromosomal DNA after centrifugation through a 10-40% sucrose gradient. Track 17 shows DNA in the size range of 10-20-kb which was used in the cloning strategy.

(a)


size in kb
23.0-
9.7-
6.4-
4.5-
2.3-
2.0-
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

(b)


size in kb
23.0-
9.7-
6.4-
4.5-
2.3-
2.0-
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23

## A.3 SUMMARY

The results presented in this appendix have demonstrated the presence of a 15.6-kb plasmid (pCB60) in Flavobacterium sp. CB60. This plasmid does not appear to play a role in chloramphenicol acetylation. Furthermore, DNA:DNA hybridization experiments using a synthetic oligonucleotide probe (the sequence of which was based upon known sequences corresponding to the highly conserved active site region of several CAT variants) revealed a positively hybridizing restriction fragment in the CB60 chromosome, but failed to do so with pCB60 plasmid DNA. This strongly suggests that the cat gene of Flavobacterium sp. CB60 is chromosomally located.

Attempts to clone the Flavobacterium sp. CB60 cat gene by transformation of Pseudomonas spp. hosts (Bagdasarian et al., 1981) failed, due to the surprising discovery of amplifiable high-level chloramphenicol resistance in these strains. Resistance was mediated by a combination of CAT, and a barrier mechanism excluding Cm from the cell.

Attempts to clone the cat gene by virtue of its expression in E.coli SK3430 were also unsuccessful, as were attempts to screen for the presence of the gene by DNA:DNA hybridization.

## A.4 FUTURE WORK

It has been demonstrated that the cat consensus oligonucleotide hybridized to a 14.5-kb PstI fragment in the Flavobacterium sp. CB60 chromosome. Future cloning experiments could utilize this observation by cutting a limited range of "positive" restriction fragments out of an agarose gel. Reliable methods (Methods, 2.3) now exist for recovering DNA from such fragments, and the resulting cat-containing DNA cculd be ligated with an appropriate vector. Screening for the presence of the cat gene would then be considerably simplified, as far fewer recombinants would need to be examined. This strategy has been successfully employed (Kinnaird and Fincham, 1981), in the cloning of the N.crassa gdhA gene.

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# SHORT COMMUNICATION

## Use of a Synthetic Oligonucleotide to Identify a Chromosomal Gene for Chloramphenicol Acetyltransferase in a Plasmid-bearing Flavobacterium

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A micro-organism previously designated *Flavobacterium* sp. CB60 is resistant to chloramphenicol as a consequence of antibiotic acetylation by the enzyme chloramphenicol acetyltransferase and subsequent degradation of the acetylated product by co-metabolism. Although a 15·6 kb plasmid (pCB60) was demonstrated in this *Flavobacterium* strain, it did not appear to play a role in chloramphenicol acetylation. DNA hybridization was used to identify a fragment of DNA presumptively carrying the *cat* gene. The sequence of the synthetic probe was based upon known nucleotide sequences corresponding to the highly-conserved active site region of several chloramphenicol acetyltransferase variants. The structural gene for the enzyme in strain CB60 appeared to be chromosomal since the radioactive probe hybridized with a unique restriction fragment from chromosomal DNA but failed to do so with the DNA of plasmid pCB60.

### INTRODUCTION

Chloramphenicol resistance ($Cm^r$) has been observed in many genera of bacteria, wherein resistance to chloramphenicol of more than 25 μg $ml^{-1}$ is most commonly plasmid-borne and the result of antibiotic acetylation by the enzyme chloramphenicol acetyltransferase (CAT: EC 2.3.1.28) (Shaw, 1967; Suzuki & Okamoto, 1967). The genetic determinant (*cat*) is associated in Gram-negative bacteria with R-plasmids which can be assigned to a number of distinct incompatibility groups (Gaffney *et al.*, 1978). *Flavobacterium* sp. CB60, a Gram-negative isolate which is highly resistant to chloramphenicol, acetylates the antibiotic by means of the constitutive synthesis of CAT and further degrades the acetylated product in co-metabolism (Sussmuth *et al.*, 1979; Beschle *et al.*, 1982). Since a previous report implicated R-plasmids as vectors of resistance to ampicillin and erythromycin in clinical isolates of *Flavobacterium odoratum* (Kono *et al.*, 1980), it seemed appropriate to characterize the $Cm^r$ determinant of *Flavobacterium* sp. CB60.

### METHODS

*Bacteria.* The isolation and properties of *Flavobacterium* sp. CB60 have been described previously (Sussmuth *et al.*, 1979). *Flavobacterium* strains CB61 and CB67 are yellow pigmented flavobacteria which are chloramphenicol sensitive ($Cm^s$) and were kindly provided as controls by R. Sussmuth (Institute of Microbiology, University of Hohenheim, Stuttgart, FRG). *Methylophilus methylotrophus* (*mmeA*) was the gift of C. Boyd (Dept. of Genetics, University of Glasgow, UK) and *Escherichia coli* strains JM83 (*ara* Δ*lac-pro strA thi* φ80 *dlacZ*ΔM15) and BW162 (*thr leu*) were obtained from G. Sharpe (ICI, Runcorn, UK). *Pseudomonas aeruginosa* PAO 1162 (*leu-38*) and PAO 1161 (*leu-38*) are both phenotypically recombination deficient ($Rec^-$) and modification proficient ($Mod^+$) as is *Pseudomonas putida* mt-2 KT 2440. These strains have been described by Bagdasarian *et al.* (1981) and were kindly provided by D. Jeenes (University College of North Wales, Bangor, UK).

*Abbreviation*: CAT, chloramphenicol acetyltransferase.

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*Plasmids.* pSW 1.1 ($Su^r$ $Tc^r$ $Mob^+$) and pUC 13 ($Ap^r$) were from G. Sharpe (ICI, Runcorn, UK). The use of pSW 1.1 was through the courtesy of W. Schuch (ICI, Runcorn, UK). Plasmid pJK111 ($cat^{III}$) encodes the type III enteric variant of CAT specified by plasmid R387; pSES9 carries the $cat^c$ gene of staphylococcal plasmid pC221 (Shaw, 1983).

*Media and growth conditions.* Flavobacteria were grown according to Sussmuth *et al.* (1979). *E. coli* strains were grown on Luria medium or minimal medium with appropriate supplements and drugs as described by Maniatis *et al.* (1982). *M. methylotrophus* was cultivated in methanol-minimal medium (Windass *et al.*, 1980). *Pseudomonas* strains were grown as described by Timmis *et al.* (1978).

*Plasmid preparations.* Plasmids were prepared by the method of Birnboim & Doly (1979) using the following modifications in large scale preparations. After potassium acetate precipitation, the supernatants were shaken with aqueous phenol, precipitated with isopropanol and centrifuged to equilibrium in a caesium chloride buoyant density gradient containing ethidium bromide.

*Chromosomal DNA preparations.* Flavobacterial DNA was purified by the method of Chow *et al.* (1977) with the addition of a final caesium chloride buoyant density centrifugation step.

*Transformation. E. coli* strains were transformed by the method of Kushner (1978). The *Pseudomonas* transformation system was that of Bagdasarian & Timmis (1981).

*Bacterial conjugations.* Strains were conjugated by plate-mating as described by Barth *et al.* (1981).

*DNA digestions and agarose gel electrophoresis.* DNA samples were digested with commercial restriction endonucleases, using the manufacturers' recommended buffers, and analysed in 0·7% (w/v) agarose in Tris/acetate buffer (pH 8·1) as previously described by Maniatis *et al.* (1982). Molecular weights of DNA fragments were estimated from the migration of bacteriophage $\lambda$ DNA fragments of known size.

*Hybridizations.* DNA fragments were transferred to nitrocellulose filters by the method of Southern (1975). Samples of the synthetic oligodeoxyribonucleotide 5′–CCATCACAGACGGCATGATG–3′ (synthesized by Celltech, Slough, by the method of Patel *et al.*, 1982) were labelled using [$\gamma$-$^{32}$P]ATP and polynucleotide kinase by the method of Maxam & Gilbert (1980). Other DNA samples were nick-translated as described by Rigby *et al.* (1977). Filters were prehybridized for 4 h at 30 °C and probed with [$^{32}$P]oligonucleotide (sp. act. 5·5 × $10^7$ d.p.m. $\mu g^{-1}$) for approximately 18 h. Filters were routinely washed in 2 × SSC containing 0·5% SDS at 30 °C or at 37 °C for higher stringency in selected cases (1 × SSC is 0·15 M-NaCl/0·015 M-trisodium citrate, pH 7).

*Ligation procedures and enzyme assays.* DNA fragments were ligated to vectors using DNA ligase following treatment of linearized vector DNA with alkaline phosphatase followed by phenol extraction and ethanol precipitation. CAT was measured in crude extracts by the spectrophotometric and radioactive methods described by Shaw (1975).

## RESULTS AND DISCUSSION

*Presence of a 15·6 kb plasmid in Flavobacterium sp. CB60.* Three very closely related *Flavobacterium* strains, CB60 ($Cm^r$), CB61 ($Cm^s$) and CB67 ($Cm^s$), were screened for the presence of plasmids after lysis and electrophoresis in agarose. Only strain CB60 was observed to contain a plasmid, the molecular weight of which was estimated to be approximately 15·6 kb by addition of the apparent sizes of fragments produced by digestion with individual restriction endonucleases. A map of the plasmid (designated pCB60) derived from the results of single and double digestions with restriction endonucleases *Pvu*II, *Eco*RI, *Sal*I and *Acc*I is shown in Fig. 1. The possibility that the $Cm^r$ determinant was encoded by pCB60 was investigated both by attempting to transfer it to *Flavobacterium* strains CB61 and CB67, *E. coli*, *Pseudomonas* spp. and *M. methylotrophus*, and by probing with an active site synthetic oligonucleotide.

*Transfer studies.* The transformation with pCB60 of $Cm^s$ *Flavobacterium* strains (CB61 and CB67), using conventional protocols for the transformation of *E. coli* and *Pseudomonas*, failed to yield $Cm^r$ flavobacteria. All the *Pseudomonas* strains proved unsuitable because of their intrinsic resistance to chloramphenicol and low-level CAT activity. An effort was made to transform *E. coli* but no $Cm^r$ (10 $\mu g\ ml^{-1}$) colonies were detected. Because of the absence of a transformation system for *M. methylotrophus* special conjugation vectors containing pCB60, pUC13, and the broad host range vector pWS1.1 were prepared by ligating the products of restriction endonuclease partial digests of each. Although such vectors were shown to replicate in *E. coli*, no consistent evidence of their transfer to *M. methylotrophus* was obtained (data not shown).

*A synthetic oligonucleotide to identify CAT genes.* The sequence around the active site of several different CAT genes from Gram negative and Gram-positive bacteria is highly conserved

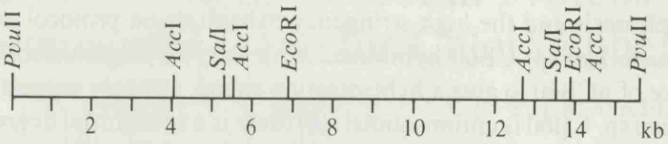


Fig. 1. Restriction map of plasmid pCB60 using restriction endonucleases *AccI*, *Eco*RI, *Pvu*II and *Sal*I.

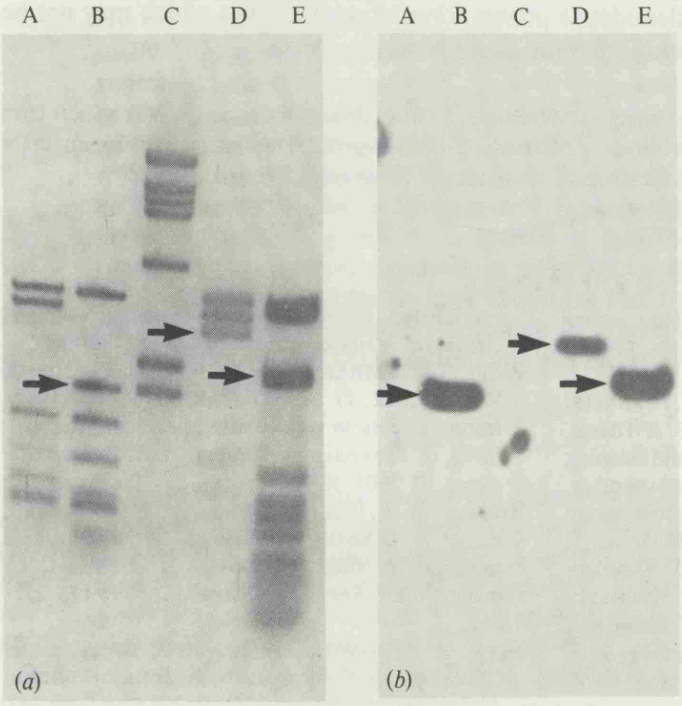


Fig. 2

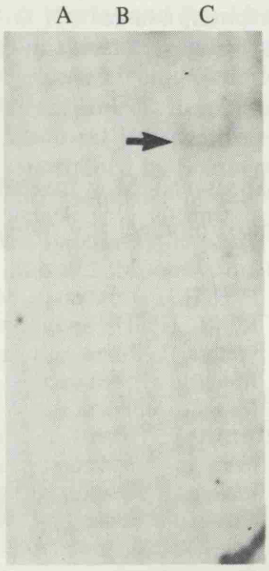


Fig. 3

Fig. 2. (*a*) 1·0% agarose gel showing banding pattern after ethidium bromide staining of: A, pBR322 digested with *Taq*I (negative control); B, pBR328 digested with *Taq*I ($cat^{I}$); C, pCB60 digested with *Pst*I; D, pSES9 digested with *Sau*3 ($cat^{c}$); E, pJK111 digested with *Sau*3A ($cat^{III}$). Arrows indicate the known position of the *cat* sequence in each case. (*b*) Autoradiograph of the DNA shown in (*a*) after transfer to nitrocellulose, by the method of Southern, and hybridization with [5′-$^{32}$P]oligonucleotide (see Methods). Hybridization only occurred with the bands known to contain the *cat* genes (arrowed).

Fig. 3. Autoradiograph from a Southern transfer experiment in which chromosomal DNA from CB67 (A), CB61 (B), and CB60 (C) was subjected to digestion by endonuclease *Pst*I, electrophoresis in 0·7% agarose and hybridization to a [$^{32}$P]oligonucleotide probe as in Fig. 2(*b*). The discrete radioactive band for DNA from CB60, indicated by the arrow, was not observed for CB67 and CB61. The agarose gel itself revealed no detectable banding pattern before or after endonuclease digestion (not shown).

(Shaw, 1983). A 20-base synthetic oligonucleotide, complementary to the coding strand of the active site region of the gene for a type I CAT ($cat^{I}$, carried for example, by Tn*9*, pBR325, and pACYC184), was used in an attempt to identify novel as well as known *cat* elements by DNA:DNA hybridization. Initially, pBR322 (negative control), pCB60, pBR328 ($cat^{I}$), pJK111 ($cat^{III}$) and pSES9 ($cat^{c}$) were cut with appropriate restriction endonucleases, subjected to electrophoresis in 0·7% (w/v) agarose, transferred to nitrocellulose paper, and probed with [$^{32}$P]oligonucleotide under high and low stringency conditions as described in Methods.

Autoradiography revealed that hybridization occurred only with plasmids carrying the three well-characterized genes specifying CAT. Both pCB60 and pBR322 failed to show any hybridization (see Fig. 2*a*, *b*), a result compatible with the view that pCB60 does not carry a typical gene for CAT. The alternative hypothesis that *Flavobacterium* sp. CB60 harbours *cat* on its chromosome was explored in similar fashion. Endonuclease *Pst*I was used to digest pCB60 as well as total DNA from *Flavobacterium* strains CB60, CB61, and CB67. The digested DNA was

subjected to electrophoresis and the high stringency hybridization protocol as described; only the DNA of *Flavobacterium* sp. CB60 hybridized with the [$^{32}$P]oligonucleotide (Fig. 3). This result, and the failure of pCB60 to give a hybridization signal, strongly suggests that: (*a*) the *cat* gene in *Flavobacterium* sp. CB60 is chromosomal; (*b*) there is a substantial degree of homology at the active site between the CAT of strain CB60 and CAT variants studied previously; (*c*) the finding of pCB60 in this Cm$^r$ strain was either fortuitous or reflects a secondary role which it may play in specifying gene products necessary for the subsequent metabolism of acetylated chloramphenicol (Beschle *et al.*, 1982). The studies reported here also emphasize the likely general utility of synthetic oligonucleotides as probes for prokaryotic genes which may not be expressed in bacterial hosts commonly used for cloning studies.

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# Resistance to Chloramphenicol in *Proteus mirabilis* by Expression of a Chromosomal Gene for Chloramphenicol Acetyltransferase

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***Proteus mirabilis* PM13 is a well-characterized chloramphenicol-sensitive isolate which spontaneously gives rise to resistant colonies on solid media containing chloramphenicol (50 μg ml$^{-1}$) at a plating efficiency of $10^{-4}$ to $10^{-5}$. Such chloramphenicol-resistant colonies exhibit a novel phenotype with respect to chloramphenicol resistance. When a single colony grown on chloramphenicol agar is transferred to liquid medium and grown in the absence of antibiotic for 150 generations, a population of predominantly sensitive cells arises. This mutation-reversion phenomenon has been observed in other *Proteus* species and *Providencia* strains, wherein resistance has been shown to be mediated in each case by the enzyme chloramphenicol acetyltransferase. The *cat* gene responsible for the phenomenon is chromosomal and can be cloned from *P. mirabilis* PM13 with DNA prepared from cells grown in the absence or the presence of chloramphenicol. Recombinant plasmids which confer resistance to chloramphenicol carry an 8.5-kilobase *Pst*I fragment irrespective of the source of host DNA. The location of the *cat* gene within the *Pst*I fragment was determined by Southern blotting with a *cat* consensus oligonucleotide corresponding to the expected amino acid sequence of the active site region of chloramphenicol acetyltransferase, and the direction of transcription was deduced from homology with the type I *cat* variant.**

The mechanisms by which microorganisms express resistance to antibiotics is of clinical and theoretical importance. This report describes a novel phenomenon whereby a population of chloramphenicol-sensitive cells of *Proteus mirabilis* gives rise to resistant cells at a very high frequency ($10^{-4}$ to $10^{-5}$ per cell per generation).

Genes specifying the enzyme chloramphenicol acetyltransferase (CAT) occur in many genera of bacteria, wherein they confer resistance to chloramphenicol. The regulation of *cat* gene expression depends in the main on factors which correlate with the taxon of the bacteria in which they reside. The *cat* genes of gram-negative bacteria are commonly found on large R-plasmids and produce CAT constitutively. The most commonly observed variant in this category is the type I polypeptide encoded by Tn*9* and naturally occurring plasmids such as NR1 (also called R100 or R222) and R6 or the in vitro plasmid constructs such as pACYC184 and pBR328 (reviewed by Shaw [35]). The nucleotide sequence of the type I *cat* has been determined (1), and the gene has been demonstrated to be subject to catabolite repression mediated by cyclic AMP and the catabolite gene activator protein at the level of transcription (14, 22). Circumstantial evidence suggesting transcriptional activation by the cyclic AMP-catabolite gene activator protein system has not been observed for the type II and type III genes found on non-F plasmids in enteric bacteria (35).

The synthesis of CAT in gram-positive bacteria is induced by chloramphenicol and selected analogs (43), and the regulation appears to be one of posttranscriptional control, which may be regulated by the secondary structure of the CAT mRNA (9, 15).

Any study of chloramphenicol resistance and CAT synthesis in *P. mirabilis* is bound to be complicated by two considerations. The first is the well-documented phenomenon of selective amplification of genes encoding resistance determinants carried by certain R plasmids in *P. mirabilis*. Rownd and Mickel (33) described this phenomenon as transitioning and have shown that the increase in CAT synthesis due to gene amplification is reversible in that growth of transitioned cells in drug-free medium results in loss of resistance determinants with concomitant decrease in resistance. The second consideration has been the knowledge that strains of *P. mirabilis* lacking detectable plasmids may nevertheless harbor *cat* determinants which (i) are expressed poorly, (ii) specify a polypeptide similar to that of the type I CAT (45), and (iii) have been described as mutable to high-level expression (37).

The present studies were undertaken to gain a better understanding of the structure and function of the putative chromosomal *cat* gene of *P. mirabilis*, its relationship to analogous genes carried on plasmids in gram-negative and gram-positive bacteria, and the mechanism underlying the high-level expression of CAT in selected strains of *P. mirabilis*.

## MATERIALS AND METHODS

**Bacteria, plasmids, and phage.** The bacteria, plasmids, and phage used in this report are listed in Tables 1 and 2. All bacteria were grown in Penassay nutrient broth (Difco Laboratories) unless otherwise specifically stated.

**Chromosomal DNA preparations.** *P. mirabilis* genomic DNA was purified by the method of Chow et al. (11), with an additional final cesium chloride buoyant density centrifugation step.

**Plasmid preparations.** Plasmids were prepared by the rapid method of Birnboim and Doly (7) with the protocol described by Maniatis et al. (25).

**Conjugation and transformation of *E. coli* and *P. mirabilis*.** Strains were transformed by a modified version (19) of the

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TABLE 1. Bacterial strains and plasmids

| Strain or plasmid | Genotype or comment | Reference or source |
|---|---|---|
| Plasmids | | |
| pAT153 | *tet bla*, cloning vehicle | (41) |
| pIC100 | pAT153 with *P. mirabilis cat* in *Pst*I site | This study |
| pIC101 | pAT153 with *P. mirabilis cat* in *Pst*I site | This study |
| M13mp9 | Cloning vehicle | (42) |
| pIC025 | M13mp9 with pBR328 *cat* in *Acc*I site | This study |
| *P. mirabilis* | | |
| PM13 | Wild type, $tet^+$ $cat^+$ | (13) |
| PM2 | Wild type, $tet^+$ $cat^-$ | NCTC 3177 and this study |
| IC100 | PM2 containing pIC100 | This study |
| IC101 | PM2 containing pIC101 | This study |
| *E. coli* | | |
| Sk3430 | *thi aroD6 leuC* | S. Kushner via A. R. Hawkins |
| IC500 | Sk3430 containing pIC100 | This study |
| IC501 | Sk3430 containing pIC101 | This study |
| JM103 | Δ(*lac pro*) *thi rpsL supE endA sbcB hsdR* F'*traD36 proA lacI* Δ*lacZM15* | (27) |
| IC025 | JM103 containing pIC025 | This study |

protocol of Kushner (21). Plate matings were carried out as described by Barth et al. (2).

**Southern blotting and hybridization.** Restriction endonuclease fragments were separated by gel electrophoresis and transferred to nitrocellulose filters by the method of Southern (39). The consensus active site *cat* 20-mer oligonucleotide (5'-CCATCACAGACGGCATGATG-3') was labeled with [$\gamma$-$^{32}$P]ATP and polynucleotide kinase by the method of Maxam and Gilbert (26). Hybridization was carried out as previously described (6). Other DNA samples were nick translated (31) and hybridized under the conditions outlined by Maniatis et al. (25).

**Purification of DNA fragments.** DNA fragments generated by endonuclease digestion were purified by separation through low-melting-point agarose and extracted by the freeze-squeeze method of Tautz and Renz (40).

**Construction of recombinant plasmids.** Plasmids were cleaved with restriction endonucleases under incubation conditions recommended by the vendors. 5'-Phosphate groups were removed by inclusion of calf intestinal phosphatase (0.5 U $\mu g^{-1}$). Digested plasmid DNA was incubated with T4 phage DNA ligase and a threefold molar excess of insert DNA in a buffer containing 50 mM Tris (pH 7.6), 10 mM $MgCl_2$, 1 mM ATP, and 100 μg of bovine serum albumin per ml in a volume of 10 to 30 μl (8). Other reactions containing one-half and twice the amount of insert DNA were set up to optimize ligation conditions, and a linearized plasmid control without insert DNA was included to check the effectiveness of the phosphatase treatment.

**Enzyme purification.** The affinity chromatography purification of CAT from *P. mirabilis* cells grown in the presence of chloramphenicol has been described (45). CAT was purified from cultures of chloramphenicol-sensitive *P. mirabilis* by the same method, but with a 10-fold larger cell mass.

**Enzyme assays.** CAT was measured by spectrophotometric and radiometric methods (34). Protein concentration was determined by the method of Lowry et al. (23). Induction experiments were carried out as described previously (43) with a range of inducer concentrations from 0.1 to 10 μM of either 3-deoxychloramphenicol or the 3-fluoro analog of chloramphenicol.

**Expression of chloramphenicol resistance.** Cultures were grown in Penassay broth at 37°C to the midexponential phase, and approximately $10^6$ cells were spread on Penassay agar plates containing 50 μg of chloramphenicol per ml and incubated at 37°C for 48 h. The resulting colonies containing resistant cells were transferred to plates containing 100 μg of chloramphenicol per ml and thence stepwise to 200 and 500 μg of chloramphenicol per ml.

The fall-off of chloramphenicol resistance in *P. mirabilis* cultures grown in the absence of selective pressure (as determined by the drop in efficiency of plating on agar containing 50 μg of chloramphenicol per ml and by the concomitant and parallel decrease in CAT levels) was assessed by direct plating experiments and CAT enzyme assays. Studies of the underlying basis of the resistance

TABLE 2. Strains of *Proteus*, *Morganella*, and *Providencia* tested for presence of chloramphenicol resistance and CAT synthesis

| Species | NCTC no. | Selectable CAT synthesis[a] |
|---|---|---|
| *Proteus mirabilis* | 10374 | + |
| | 9559 | + |
| | 6396 | + |
| | 6197 | − |
| | 3177 | − |
| | 60 | − |
| *Proteus vulgaris* | 10376 | − |
| | 10015 | − |
| | 4175 | − |
| *Morganella morganii* | 10375 | + |
| | 10041 | − |
| | 7381 | + |
| | 5845 | + |
| | 2818 | + |
| | 2815 | + |
| | 1707 | − |
| | 235 | − |
| | 232 | + |
| *Providencia rettgeri* | 10377 | C |
| | 8893 | + |
| | 7481 | + |
| | 7480 | − |
| | 7479 | − |
| | 7477 | − |
| | 7476 | + |
| | 7475 | + |
| *Providencia* species | 10318 | C |
| | 10286 | − |
| | 8113 | − |
| | 8056 | − |
| | 6345 | − |
| | 2481 | − |

[a] + and − indicate the presence and absence, respectively, of selectable chloramphenicol resistance mediated by CAT. Strains marked (+) have the same phenotype as *P. mirabilis* PM13. C indicates constitutive CAT production. These strains may harbor R plasmids conferring resistance to multiple antibiotics (see Results).

phenotype and estimates of the spontaneous mutation rate were carried out by means of the Luria-Delbruck fluctuation test (24). A cell suspension prepared from a single colony of *P. mirabilis* PM13 was used to inoculate 10 1-ml tubes of Penassay broth as well as a single 10-ml sample. After growth of all cultures to the midexponential phase of growth (approximately $10^7$ cells per ml) the large culture was divided into 10 aliquots, and an equal volume (0.1 ml) of each of the 20 samples was plated on Penassay agar plates containing chloramphenicol (50 μg $ml^{-1}$). After growth at 37°C for 24 h, the number of colonies on each plate was counted, and the results with samples from independent cultures were compared with the number of resistant cells detected in the larger culture volume (see Results).

**Definitions.** In this paper we refer to enhancement as the phenomenon whereby there is an increase in the level of CAT produced in *P. mirabilis* cells that have been grown in the presence of chloramphenicol as compared with cells that have not. No mechanism is implied by this term. The phenotype of cells of *P. mirabilis* which have been grown in the presence of chloramphenicol is referred to as $CAT^+$, whereas that of *P. mirabilis* cultured in the absence of chloramphenicol is described as $CAT^-$. The latter do contain CAT activity that can be detected by the radiometric method and purified by affinity chromatography, but it is dramatically lower than that in $CAT^+$ cells (see Results).

**Sources of materials.** All restriction endonucleases, DNA polymerase I, T4 DNA kinase, and low-melting-point agarose were purchased from BRL Ltd.; T4 DNA ligase and calf-intestinal phosphatase were purchased from Boehringer Mannheim Biochemicals; Fujimex RX X-ray film was from Hannimex UK Ltd.; sheets of nitrocellulose were from Sartorius; agarose was from Miles Laboratories, Inc.; Penassay broth and solid media were from Difco Laboratories; salmon sperm DNA, polyvinyl pyrollidone, bovine serum albumin, Ficoll 400, and all antibiotics were from Sigma Chemical Co. All other reagents were of analytical grade.

## RESULTS

**Characterization of the CAT in *P. mirabilis* PM13.** The CAT from strain PM13 was purified to homogeneity both from cells exhibiting the resistant ($CAT^+$) state and the sensitive ($CAT^-$) state, and various properties and kinetic parameters were compared. They were eluted in identical fashion from highly substituted chloramphenicol base-agarose at 1.0 M NaCl in 5 mM chloramphenicol (45) and gave lines of identity with one another by two-dimensional gel diffusion in agar against a goat antiserum to the type I (*Escherichia coli*) variant of CAT (45). Both preparations were observed to migrate at the same rate in polyacrylamide gel electrophoresis under nondenaturing conditions (18). The apparent $K_m$ values for chloramphenicol were 16 and 14 μM for enzyme from $CAT^-$ and $CAT^+$ cells, and the values were 73 ± 5 and 82 ± 6 μM for acetyl coenzyme A, respectively. Although the CAT purified from cells grown in the absence of chloramphenicol is present at low levels, it is indistinguishable from the CAT purified from cells grown in the presence of chloramphenicol (50 μg $ml^{-1}$). For cells grown in 0, 50, 100, 200, and 500 μg of chloramphenicol, the CAT specific activity was <1, 124, 150, 134, and 118 nmol $min^{-1}$ $mg^{-1}$. (Extracts from cells grown in the absence of chloramphenicol contained CAT which could be detected by the radiometric assay [34] and purified by affinity chromatography [45], but which was below the sensitivity of the spectrophotometric assay [1 $nmol^{-1}$ $min^{-1}$ $mg^{-1}$] used in these experiments.) The results support the view that the resistance phenotype is likely to be due to an increase in the amount of enzyme produced rather than to any alteration in the properties of the protein or the synthesis of a second CAT variant.

**Enhancement of CAT synthesis in *P. mirabilis* and origin of the resistance phenotype through mutation.** Experiments carried out under conditions which induce CAT synthesis in *Staphylococcus aureus* (43) and *Bacillus pumilus* (15) produced no effect on the CAT levels in *P. mirabilis* PM13. The incubation of resting cell suspensions with chloramphenicol, 3-deoxychloramphenicol, or 3-fluorochloramphenicol was not accompanied by significant increases in the specific activity of CAT (data not shown). Such results, although negative, strongly suggest that induction by chloramphenicol or its analogs is unlikely to play a role in the enhancement of chloramphenicol resistance observed in *Proteus* spp.

The application of the Luria-Delbruck fluctuation test to the efficiency of plating of chloramphenicol-resistant cells revealed a non-Gaussian variation among samples taken from independent cultures as compared with equal samples from a single large culture. Since a mutation to chloramphenicol resistance can occur in any generation before exposure to the selective agent, the large variance observed favors the hypothesis of a mutational event rather than induction of CAT by exposure of cells to the antibiotic. In a typical experiment (see Materials and Methods) comparing 10 small cultures with a single 10-fold larger culture volume the results were as follows. Six of the small cultures yielded no chloramphenicol-resistant cells of *P. mirabilis* PM13 in samples containing approximately $10^7$ cells per ml, whereas the other four cultures yielded 5, 12, 61, and 267 cells resistant to chloramphenicol from comparable populations. By way of contrast, 10 equivalent samples from the larger culture each yielded from 167 to 233 resistant cells (mean 207 ± 14).

Single step mutants resistant to 50 μg of chloramphenicol per ml after growth for 48 h (see Materials and Methods) were transferred sequentially to plates containing higher concentrations of chloramphenicol (100, 200, and 500 μg $ml^{-1}$). Individual colonies were taken from each plate and grown for transfer to liquid media containing the same concentration of chloramphenicol used in the selection plate, and cells were harvested at the midexponential phase of growth. The specific activity of CAT in crude cell extracts was below the limits of detection by the routine spectrophotometric assay in extracts prepared from bacteria grown in the absence of chloramphenicol (Table 3), whereas the specific activity of CAT from cells grown in the presence of chloramphenicol was high and essentially independent of the concentration of antibiotic in the growth medium. The mutation to the $CAT^+$ state, (i.e., high efficiency of plating on chloramphenicol agar) seems to be of an all-or-nothing nature in that all cells which will grow on 50 μg $ml^{-1}$ will also grow on 500 μg $ml^{-1}$. Once selected, $CAT^+$ cells are maintained in the population so long as there is selective pressure favoring their persistence. Figure 1 epitomizes the results obtained with *P. mirabilis* PM13 (as well as other strains of *Proteus* spp. and related genera) when $CAT^+$ cells are grown in the absence of the antibiotic. The exponential decline with time of the efficiency of plating of cells on 50 μg of chloramphenicol per ml and the parallel fall in the specific activity of CAT for the population as a whole constitute the phenomenon which is analyzed in greater detail below (see Discussion).

*P. mirabilis* PM13 was tested for enhanced levels of resistance to other antibiotics after selection on 50 μg of

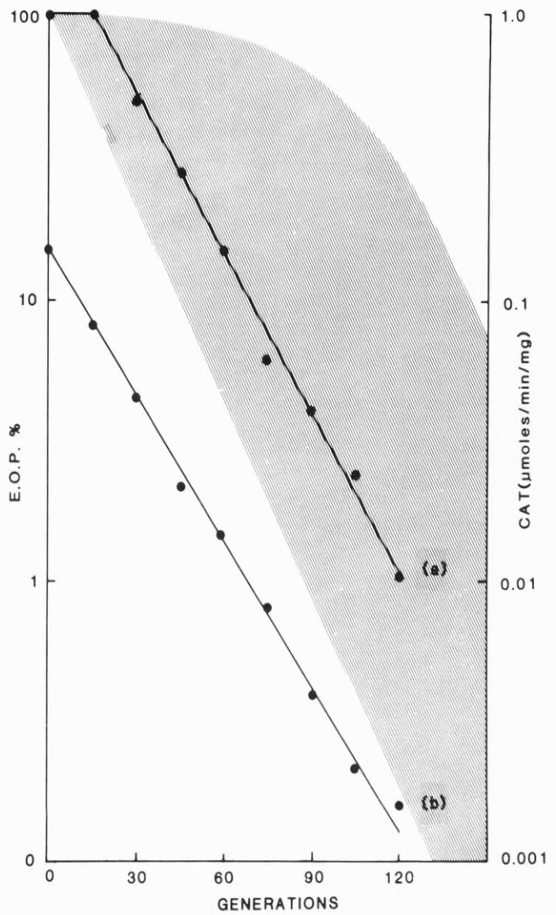


FIG. 1. Disappearance of chloramphenicol-resistant cells of *P. mirabilis* PM13 from a uniformly resistant population as a function of time. Cells were collected by centrifugation after growth in Penassay broth containing chloramphenicol (50 μg $ml^{-1}$) and, after washing, were suspended in antibiotic-free medium. Samples taken at appropriate intervals were divided and taken for simultaneous plating on Penassay agar containing 50 μg of chloramphenicol per ml and on plates lacking the antibiotic and for lysis before measurement of the specific activity of CAT. The experimental points giving line (a) represent the efficiency of plating (E.O.P.) of cells on chloramphenicol, and line (b) depicts the parallel and exponential fall in CAT for the population. Growth in the absence of chloramphenicol was followed for 120 generations by means of repeated dilutions ($10^{-2}$) of late-exponential-phase cells into fresh medium. The shaded area represents an envelope containing a range of theoretical curves for the fall in efficiency of plating as a function of growth, such plots being generated by solutions to the equation (see Discussion) which relates the efficiency of plating to parameters for both selection and mutation. For the case wherein the disappearance of resistant cells from the population is solely due to selection against their persistence, the initial rate of reduction in the efficiency of plating should be low. For the other extreme, wherein the rate of back-mutation to sensitivity is the sole determinant of disappearance of resistant cells, an immediate and exponential fall in the efficiency of plating is predicted. The position of the experimental points between the two extremes described by the boundaries of the shaded area suggests that both back-mutation to sensitivity and selection against resistant cells are likely to play a role.

chloramphenicol per ml but remained uniformly sensitive to ampicillin, kanamycin, nalidixic acid, spectinomycin, and streptomycin (data not shown).

**How widespread is the phenomenon?** Additional representatives of *Proteus* spp., *Morganella* spp., and *Providencia* spp. were screened for the occurrence of the enhancement phenomenon described above, i.e., the appearance of chloramphenicol-resistant cells arising from a predominantly chloramphenicol-sensitive population at high frequency (approximately $10^{-5}$ per cell per generation). Thirteen of 32 strains from the National Collection of Type Cultures (United Kingdom) showed the phenomenon (Table 2), whereas only two produced CAT at high levels without prior exposure to chloramphenicol. The latter were not studied further because they displayed resistance to multiple antibiotics, suggesting the presence of plasmid-mediated resistance (data not shown).

**Absence of a plasmid in *P. mirabilis* PM13.** *P. mirabilis* PM13 was screened for the presence of plasmids by two methods (7, 20), neither of which yielded evidence of plasmids when the DNA from cell lysates was subjected to agarose gel electrophoresis. Positive controls in such experiments included *E. coli* and *P. mirabilis* strains harboring well-characterized plasmids (data not shown). Attempts to transfer chloramphenicol resistance from strain PM13 to suitable *E. coli* recipients by conjugation were also unsuccessful. Subsequent experiments (see below) were consistent with the view that the *cat* gene is a chromosomal one in strain PM13.

**Cloning of the *P. mirabilis cat* gene from the $CAT^+$ and $CAT^-$ states.** Preliminary studies revealed that the CAT from *P. mirabilis* was likely to be similar to the type I variant in structural and kinetic properties (45). Also, the *P. mirabilis* CAT bound the dye crystal violet (I. G. Charles, unpublished experiments), a property demonstrated by the type I enzyme (30) but by none of the other CAT variants found in gram-negative bacteria (A. D. Bennett, Ph.D. thesis, University of Leicester, 1984). The likely high degree of homology of the CAT of *P. mirabilis* with its type I counterpart was exploited in the strategy used to clone the *cat* gene of *P. mirabilis*.

Plasmid pBR328 (38) contains a type I *cat* gene on a 773-base-pair *Taq*I fragment. To facilitate the isolation of this fragment for hybridization experiments, the entire 773-base-pair fragment was cloned into phage M13 mp9 (42) as outlined in Materials and Methods. One recombinant (pIC025) was isolated as the replicative form, and the entire structural gene for *cat* was isolated by a double digest with *Pst*I and *Bam*HI.

Identification and isolation of the DNA fragments of the *P. mirabilis* chromosome carrying the *cat* gene was achieved by first using the type I *cat* gene (isolated from pIC025) to probe various restriction endonuclease digests of chromosomal DNA from *P. mirabilis*. A *Pst*I digest yielded a single band on hybridization (data not shown), indicating that this fragment was likely to contain the entire *P. mirabilis cat* gene. *Pst*I-digested chromosomal DNA samples (prepared from *P. mirabilis* in both the $CAT^+$ and $CAT^-$ states) were separately ligated with pAT153 which had been cut by *Pst*I and treated with alkaline phosphatase. After transformation, the CAT-containing cells, which were likely to harbor recombinant plasmids (selected on 10 μg of chloramphenicol per ml), were then screened (7) for insert DNA with positive results in each case for an insert of approximately 8.5 kilobases (kb). Plasmids from one each of the resulting transformants (designated pIC100 for the recombinant produced from DNA in the $CAT^+$ state and pIC101 for the recombinant produced from DNA in the $CAT^-$ state) were used for large-scale plasmid preparations.

**Identification and localization of the *cat* genes in plasmids pIC100 and pIC101.** The synthetic 20-mer *cat* active site consensus probe was used to locate the *cat* gene within the

8.5-kb *Pst*I fragment of plasmids pIC100 and pIC101. Hybridization of the $^{32}$P-labeled probe to separated restriction fragments yielded results (Fig. 2) which were interpreted as evidence for the localization of the *cat* gene on a 500-base-pair fragment located near one end of the insert (Fig. 3). The direction of transcription of the gene was inferred from this hybridization data in relation to the restriction map of the type I *cat* gene.

**Chromosomal organization of the *P. mirabilis cat* gene.** *P. mirabilis* can amplify *cat* genes carried on plasmids such as NR1, and the phenomenon of chromosomal *cat* gene amplification has accounted for increased chloramphenicol resistance in *E. coli* and *B. subtilis* (28, 44). The *P. mirabilis* genome was examined by Southern blotting for selective gene amplification. A *Cla*I-to-*Pst*I fragment of pIC100, encompassing the entire *cat* gene, was isolated after electrophoresis through low-melting-point agarose and labeled with [$^{32}$P]dATP by nick translation. *P. mirabilis* chromosomal DNA samples prepared from late-exponential-phase cultures grown in 0, 50, 100, 200, and 500 μg of chloramphenicol per ml were each digested with *Pst*I and, after electrophoresis, were probed with the labeled fragment. No significant difference in hybridization signal intensity was detected in the restriction fragments arising from DNA prepared from cells that have been grown in chloramphenicol as compared which those that had not (Fig. 4). Southern blotting was also used to detect a possible change in orientation of *cat* DNA within the chromosome of the CAT$^+$ state compared with the CAT$^-$ state, at least within the 8.5 kb of DNA spanning

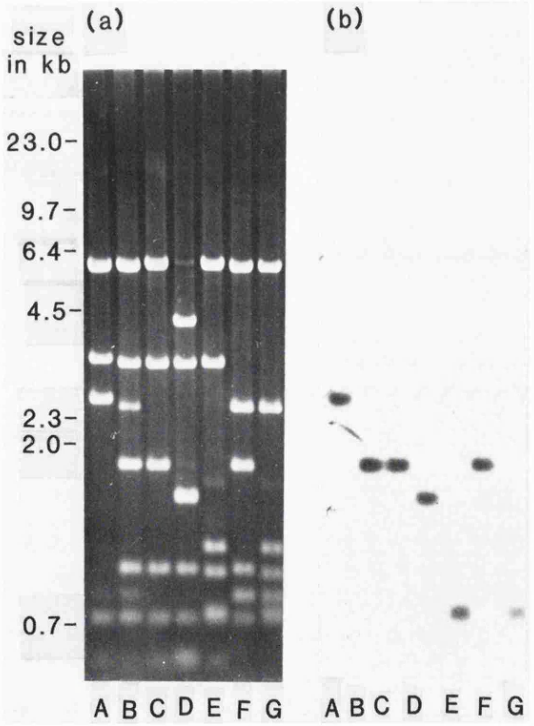


FIG. 2. Agarose gel (0.7%) showing endonuclease digest pattern of pIC100 after ethidium bromide staining. (a) Lanes after digestion with the following enzymes: A, *Cla*I; B, *Cla*I and *Pst*I; C, *Cla*I, *Pst*I, and *Pvu*II; D, *Cla*I, *Pst*I, and *Hin*dIII; E, *Cla*I, *Pst*I, and *Eco*RI; F, *Cla*I, *Pst*I, and *Sal*I; G, *Cla*I, *Pst*I, *Eco*RI, and *Sal*I. (b) Autoradiograph of the DNA shown in part (a) after transfer to nitrocellulose and hybridization with a γ-$^{32}$P-labeled consensus *cat* active site oligonucleotide (See Fig. 3 and Materials and Methods).

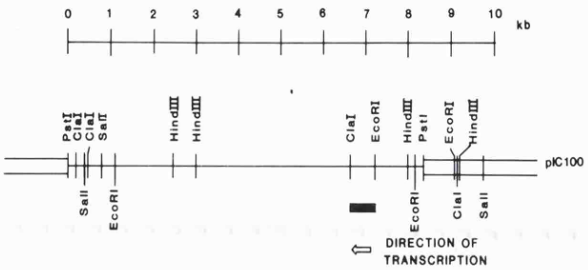


FIG. 3. Restriction map of pIC100 (and pIC101) with restriction endonucleases *Cla*I, *Eco*RI, *Hin*dIII, *Pst*I, and *Sal*I. The black box bounded by sites for *Eco*RI and *Cla*I represents the smallest restriction fragment detected by hybridization to the consensus active site oligonucleotide (see Fig. 2). The arrow indicates the direction of transcription as inferred from the location of the *Eco*RI site in the gene for the type I *cat* (1) and from subsequent nucleotide sequence analysis (10).

the cloned fragment. *P. mirabilis* chromosomal DNA was prepared from late-exponential-phase cultures grown in either no antibiotic or 500 μg of chloramphenicol per ml. Both preparations were separately digested with *Pst*I, *Hin*dIII, and *Eco*RI, subjected to agarose (0.7%) gel electrophoresis, and probed with the entire 8.5-kb $^{32}$P-labeled insert of

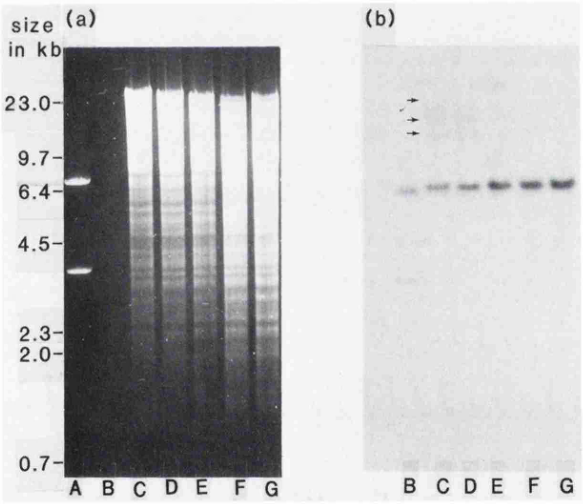


FIG. 4. Agarose (0.7%) gel electrophoresis (a) of *Pst*I digests of (A) pIC100 at high concentration for visualization with ethidium bromide and (B) pIC100 at a concentration estimated to correspond to approximately one copy of *cat* per chromosome for comparison with subsequent (C through G) loadings of chromosomal DNA. Lanes C through F were loaded with *Pst*I digests of chromosomal DNA from *P. mirabilis* PM13 cells grown in the presence of chloramphenicol at (C) 500, (D) 200, (E) 100, and (F) 50 μg ml$^{-1}$. Lane G was a *Pst*I digest of chromosomal DNA from strain PM13 grown in the absence of chloramphenicol. (b) Autoradiograph of the DNA in (a) after transfer to nitrocellulose by the method of Southern (39) and hybridization with a $^{32}$P-labeled *cat* probe comprising the 1.7-kb *Pst*I-*Cla*I fragment of pIC100 (see Materials and Methods and Fig. 3). Efforts were made to load equal amounts of chromosomal DNA in tracks C through G. The higher fraction of high-molecular-weight DNA fragments in lanes C, D, and E and the weakly positive discrete hybridization signals (marked with arrows) suggest that the corresponding *Pst*I digests may have been incomplete. Scanning of the autoradiograph with integration and summation of the major and minor peaks yielded the intensities of hybridization for comparison with the notional value of unity for the pIC100 control. The relative intensities for lanes C, D, E, F, and G in relation to that of lane B were 2.5, 2.4, 2.6, 3.0, and 3.1, respectively.

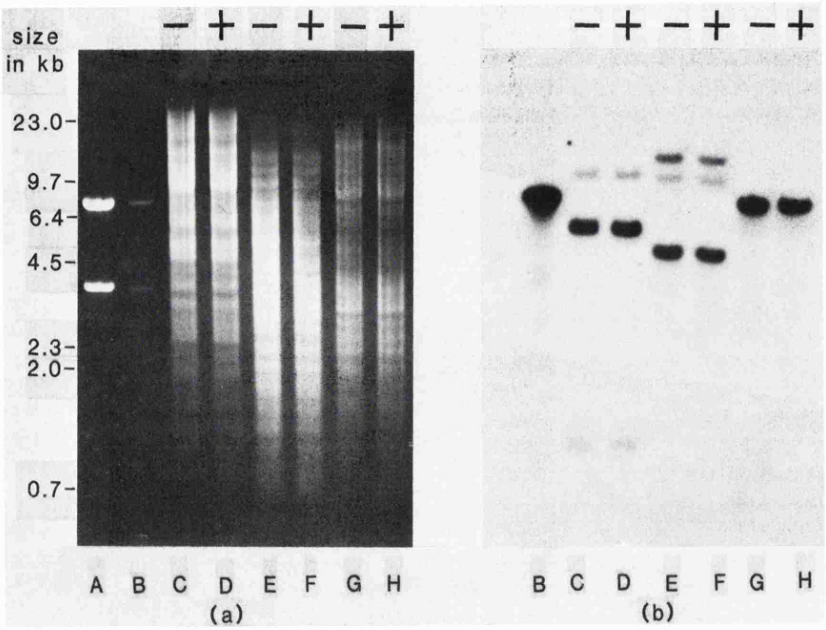


FIG. 5. Use of the cloned fragment containing the *cat* of *P. mirabilis* to probe chromosomal DNA from cells of strain PM13 grown in the presence (+) or absence (−) of chloramphenicol (500 μg $ml^{-1}$) as indicated. (a) Agarose (0.7%) gel electrophoresis showing digests of chromosomal DNA with *Eco*RI (C and D), *Hin*dIII (E and F), and *Pst*I (G and H). (b) Autoradiograph of the DNA shown in (a) after transfer to nitrocellulose and hybridization with a $^{32}$P-labeled probe comprising the *Pst*I fragment from pIC100 (see Fig. 3 and Materials and Methods). Lanes A and B contain *Pst*I digests of pIC100. Only the lower concentration (B) was used for the hybridization control in (b), wherein an intense signal at approximately 8.5 kb may be seen to correspond with that in lanes G and H for the *Pst*I digest of chromosomal DNA.

pIC100. Figure 5 shows that the pattern of hybridization produced for each pair of digests is indistinguishable, indicating that no detectable rearrangement has occurred within this 8.5-kb *cat*-containing fragment.

**Expression of the *P. mirabilis cat* gene in *E. coli* and in a *cat*⁻ strain of *P. mirabilis*.** Plasmids pIC100 and pIC101 were both transferred into *E. coli* and into *P. mirabilis* PM2, the latter strain having previously been demonstrated to be negative for CAT by a sensitive radiometric assay (34) and to lack homologous *cat* DNA by Southern blot experiments (data not shown). The resulting transformants were then tested for enhanced expression of *cat* as observed in strain PM13 (Table 3). Expression of the *P. mirabilis cat* gene was sufficiently poor in *E. coli* that the specific activity of the enzyme could not be measured by the spectrophotometric method and required use of the radiometric technique (Fig. 6). The minimum inhibitory concentration for chloramphenicol with *E. coli* Sk3430 carrying pIC100 or pIC101 is only 10 μg $ml^{-1}$, whereas the corresponding concentration for strain PM2 with either plasmid is 100 μg $ml^{-1}$ as compared with 10 μg $ml^{-1}$ without pIC100 or pIC101. The constitutive expression of the *cat* gene from strain PM13 in the genetic background of different *P. mirabilis* strains is addressed in the Discussion.

TABLE 3. Synthesis of CAT from the cloned *cat* gene of *P. mirabilis* PM13 in chloramphenicol-sensitive (*cat*⁻) bacteria

| Bacterium | Plasmid | Sp act of CAT (nmol $min^{-1}$ $mg^{-1}$) | |
|---|---|---|---|
| | | No chloramphenicol | Chloramphenicol[a] |
| *P. mirabilis* PM2 | None | 0 | |
| *P. mirabilis* PM2 | pIC100 | 85 | 87 |
| *P. mirabilis* PM2 | pIC101 | 88 | 85 |
| *E. coli* Sk3430 | None | 0 | |
| *E. coli* Sk3430 | pIC100 | <1 | |
| *E. coli* Sk3430 | pIC101 | <1 | |

[a] CAT specific activity by the spectrophotometric method in extracts of cells grown in the absence or presence of chloramphenicol (10 μg $ml^{-1}$). The zero value for *P. mirabilis* PM2 and for *E. coli* Sk3430 indicates that no CAT was detected by the more sensitive radiometric method. Although the presence of pIC100 or pIC101 in *E. coli* promoted CAT synthesis as detected by the latter method (Fig. 6), it was below the level of detection by spectrophotometry. Plasmid-free *P. mirabilis* PM2 and all three strains of *E. coli* failed to grow in 10 μg of chloramphenicol per ml.

## DISCUSSION

The results presented here show that a number of strains of the *Proteus-Providencia* group are capable of expressing high levels of chloramphenicol resistance under appropriate conditions.

The mechanism of the phenomenon appears to involve the selection of mutants in the population. Preexisting mutant cells (which occur spontaneously at a frequency of approximately $10^{-5}$ per cell per generation) are selected by growth on chloramphenicol and are maintained in the population for as long as they are exposed to chloramphenicol selection. Cultures of *P. mirabilis* in which 100% of the cells are phenotypically resistant to chloramphenicol undergo a population reversion to the chloramphenicol-sensitive state during growth in the absence of the antibiotic, the rate of this process being of the order of $10^{-2}$ per cell per generation. The rate of decrease in the efficiency of plating of cells on antibiotic agar (Fig.1) is too low to correspond with a model wherein a putative intracellular inducer is progressively diluted by cell division. An alternative explanation, the progressive reduction in the proportion of resistant cells in the population, may be seen to operate by mutation or selection or some combination of both factors. Backmutation to the sensitive phenotype at a very high rate would yield a concomitant reduction in the efficiency of plating of the population on antibiotic-containing media. Such a model

predicts that in any interval $\delta t$, a proportion of cells $\mu\delta t$ (where $\mu$ is the back mutation rate per generation) would revert to sensitivity. If $q$ is the frequency of resistant cells in a population after $t$ generations, then the integrated expression describing the phenomenon is

$$q_t = q_0 e^{-\mu t} \quad (1)$$

where $q_0$ is the initial frequency of resistant cells in the population and $q_t$ is their frequency after $t$ generations. A plot of $\ln(q_t)$ or ln(efficiency of plating) against time should yield a straight line, its slope being equal to the back-mutation rate.

A second alternative is selection against resistant cells in the absence of the antibiotic. If $s$ is the selective coefficient against resistant cells, then a proportion of the cells ($s\delta t$) is selectively eliminated in $\delta t$ generations, then the change in $q$ in $\delta t$ generations is

$$q = -s\delta t\, q(1 - q) \quad (2)$$

and, when integrated, yields the equation for the proportion of resistant cells remaining after $t$ generations

$$\delta q_t = \frac{q_0}{(1 - q_0)\, e^{st} + q_0} \quad (3)$$

Equation 3 describes a linear plot of ln(efficiency of plating) against time after an initial slow reduction in the efficiency of plating, the duration of such a lag being dependent upon how close $q_0$ is to 1. The plot becomes linear when nearly half of the cells in the population are sensitive, after which ln(efficiency of plating) decreases with a slope of $-s$. The compound expression corresponding to equations 1 and 3 in the case wherein both selection and back mutation are involved is

$$qt = \frac{q_0(\mu + s)}{[\mu + s(1 - q_0)]e^{(\mu+s)t} + sq_0} \quad (4)$$

The latter reduces to produce equations 1 and 3 as special cases when $s$ and $\mu$, respectively, are zero.

Irrespective of the relative contributions of mutation and selection, as $t$ becomes large, the slope of ln(efficiency of plating) against $t$ becomes $-(\mu + s)$. The amount of selection, however, determines the duration of the lag before the slope becomes linear.

The shaded region in Fig. 1 describes the graphical limits of predictions from equation 4 when $(\mu + s)$ is equal to the experimentally derived slope of $-0.044$. A comparison of the possible range of curves calculated from theory for different values of $\mu$ and $s$ with that actually determined experimentally suggests that mutation and selection are likely to be operating (calculations not shown). In addition to such direct evidence bearing on the phenomenon, there are reasons for believing that several conventional explanations are unlikely.

Although there is a superficial resemblance between the induction phenomenon observed for *cat* expression in gram-positive bacteria and the phenomenon observed with *P. mirabilis*, they differ in important respects. First, nondividing *P. mirabilis* cells cannot be induced by chloramphenicol or congeners known to induce *cat* in staphylococci (e.g., the 3-deoxy and 3-fluoro analogs). Second, the Luria-Delbruck fluctuation test produced marked variation in the frequency with which resistant colonies appeared from cells taken from independent cultures, indicative of a mutation event rather than a response to environmental change. Third, the decay

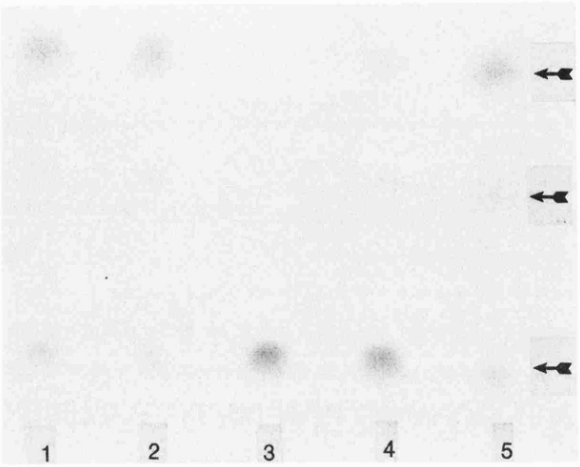


FIG. 6. Radiometric demonstration of CAT activity in crude cell extracts from *E. coli* Sk3430 harboring pIC100 (lane 1), pIC101 (lane 2), or pBR328 (lane 5). Lanes 3 and 4 contain negative controls which correspond, respectively, to extracts from *E. coli* Sk3430 without a plasmid and *Methylophilus methylotrophus*, an obligate methylotroph which has been shown previously to lack both CAT activity and a *cat* gene detectable by hybridization (6). The assay involves ascending chromatography on silica gel in chloroform-methanol (85:15, vol/vol) of the products of acetylation of [$^{14}$C]chloramphenicol by CAT in the presence of acetyl coenzyme A. The assay is quantitative only when CAT is limiting and both substrates are present in excess (34). In this instance the preponderance of 1,3-diacetylchloramphenicol (top arrow) suggests the presence of a large excess of CAT for each of the positive extracts. The middle arrow indicates the relative mobility of 3-acetylchloramphenicol, and the bottom arrow indicates the position of unmodified chloramphenicol.

in the efficiency of plating with time is far too slow to represent the dilution by cell division of a conventional inducer or gene product present only at time zero.

An apparent precedent for the proposed reversible mutation event accounting for increased resistance in *Proteus* spp. is the so called transitioning phenomenon (reviewed by Rownd [32]). The level of chloramphenicol resistance in such a case is mediated by changes in *cat* gene dosage via a variation in plasmid copy number, the extent of tandem duplication of plasmid-borne genes, or by a combination of both mechanisms. Two observations argue against transitioning as the explanation for the reversible enhancement of *cat* expression in *P. mirabilis*. First, in the case of plasmid transitioning all cells appear to express *cat* constitutively and exhibit a high efficiency of plating on media containing chloramphenicol before an increase in gene dosage occurs. Second, and notwithstanding the limitations of negative experiments, all efforts to detect a plasmid in strain PM13 have been unsuccessful. Furthermore, the possibility of *cat* amplification by tandem duplication within the genome of *P. mirabilis* was investigated by Southern hybridization with negative results. No significant difference was observed in the extent of hybridization between the labeled *P. mirabilis cat* probe and its counterpart in restriction endonuclease digests of total DNA prepared from a population of resistant cells as compared with DNA from cells grown in the absence of chloramphenicol.

Recently it has become clear that gene expression in procaryotes may be modulated by specific and reversible DNA inversions, the best known example being phase variation in *Salmonella* spp. (36). One of the consequences of such inversions is that the restriction patterns of the DNA

from the alternate states are different. Southern blotting procedures designed to detect rearrangements within *cat* or its flanking sequences in *P. mirabilis* PM13 failed to identify any differences when DNA prepared from cells in the $CAT^+$ state was compared with DNA from $CAT^-$ cells. There is circumstantial evidence, on the other hand, that the site of action of the mutation may be outside of the *cat* gene and 5′ control regions. The efficiency of plating experiments show that for *P. mirabilis* in the $CAT^+$ state virtually 100% of the cells are expressing the *cat* gene at a high rate; thus, the highly transcribed gene must be represented at least once per genome. Furthermore, the Clarke and Carbon equation (12) predicts that not more than $10^4$ recombinants need be screened to isolate such a gene in a cloning experiment. In the present study the *cat* gene was cloned from *P. mirabilis* DNA isolated both $CAT^+$ cells (pIC100) and from $CAT^-$ cells (pIC101). The frequency of isolation of such clones was independent of the origin of the cells and was within the limits of the Clarke-Carbon prediction, and the *P. mirabilis* DNA inserts from $CAT^+$ and $CAT^-$ cells were observed to be identical by restriction endonuclease digestion. Taken together, the results favor the view that the site of the mutation which underlies the enhancement of chloramphenicol resistance and *cat* expression (and their reversion) is likely to be outside the *cat* gene and its flanking sequences as represented in pIC100 and pIC101. Expression studies employing these plasmids in backgrounds which lack *cat* (*E. coli* and *P. mirabilis* PM2) are consistent with the notion that the control of *cat* expression in *P. mirabilis* PM13 may reside outside the cloned segment. Whereas the absence of high-level expression of the *P. mirabilis cat* in *E. coli* might be related to the general problem of the anomalous expression which has been observed reciprocally between genes in *E. coli* and *P. mirabilis* (3, 4), there are examples of *P. mirabilis* genes being expressed at normal levels in *E. coli* backgrounds (5, 16, 17). Furthermore, it is more difficult to account for low-level expression of *cat* in PM2, since the apparent single copy of the chromosomal *cat* gene in strain PM13 yields resistance to chloramphenicol concentrations as high as 500 $\mu g\ ml^{-1}$. The data available are compatible with the hypothesis that a host specific element, present in PM13 but not in PM2, is responsible for promoting the high-level *cat* expression which is characteristic of the $CAT^+$ state.

Taken together, the results favor a mechanism wherein a reversible mutation event occurs at high frequency. Such mutations are characteristic of transposition or genetic switch mechanisms (29). Although the data for the *P. mirabilis cat* system do not support a rearrangement of a DNA segment within an 8.5-kb region which includes the 5′ control region, such a switching event could occur at a more distant locus as is the case in phase variation in *S. typhimurium*. The important predictions of such a model for the CAT system in *P. mirabilis* would be (i) the absence of a difference between the structural gene for *cat* in the $CAT^+$ and $CAT^-$ states, (ii) evidence of transcriptional control via a *trans*-acting element (protein or RNA); and (iii) the direct demonstration (by mutation or its isolation and characterization) of a control locus which is external to the *P. mirabilis* DNA in pIC100 and pIC101, and (iv) that the activity of the putative switch region shows positional dependence.

## ACKNOWLEDGMENTS

The CAT phenomenon in *P. mirabilis* has been a puzzle for more than a decade. W.V.S. thanks J. N. Coetzee, N. Datta, T. J. Foster, and R. W. Hedges for their advice and for their collaboration in preliminary (unpublished) studies which helped to shape our view of the problem. We are grateful to Dorothy Jones for access to her collection of bacterial strains and for guidance in taxonomic questions.

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# Nucleotide Sequence Analysis of the *cat* Gene of *Proteus mirabilis*: Comparison with the Type I (Tn*9*) *cat* Gene

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**In *Proteus mirabilis* PM13 chloramphenicol resistance is mediated by the *cat* gene, a single copy of which is present in both resistant and sensitive isolates and which reverts at a high frequency. RNA measurements show an about 8.5-fold increase in *cat*-specific mRNA in cells expressing the resistance phenotype as compared with those which are sensitive to chloramphenicol. DNA sequence analysis has revealed a high degree of homology between the *P. mirabilis cat* gene and the type I *cat* variant (Tn*9*), 76% at the amino acid level and 73% when nucleotides in the coding sequence are compared. Sequence homology between the strain PM13 *cat* variant and Tn*9* *cat* was not apparent however in the 5′ and 3′ flanking regions. Segments of near identity were seen when the upstream sequence of the *cat* of *P. mirabilis* was compared with the 5′ regions of the *Salmonella typhimurium* flagellin genes H1 and H2, which are alternately expressed by a flip-flop control mechanism involving an invertible promoter and a *trans*-acting product.**

A novel type of mutation to chloramphenicol resistance occurs in many strains of the *Proteus-Providencia-Morganella* group which do not appear to harbor plasmids. *Proteus mirabilis* PM13 has been the subject of detailed studies of the phenomenon (8).

Spontaneous chloramphenicol-resistant ($CAT^+$) mutants were isolated at a frequency of $10^{-4}$ to $10^{-5}$ per cell per generation by selection on complex agar medium containing chloramphenicol (8). The appearance of the resistance phenotype is associated with 1,000-fold-increased expression of a chloramphenicol acetyltransferase gene (*cat*), and there is no coincident increase in the level of resistance to any other antibiotic. During subsequent growth in the absence of chloramphenicol, the phenotype reverts within 150 generations to one of chloramphenicol sensitivity and low-level *cat* expression. The gene responsible for resistance (*cat*) has been cloned and shown by Southern blot hybridization to be present at near unit copy in cells of strain PM13, regardless of whether the DNA source was chloramphenicol resistant or not (8). Neither *cat* amplification nor gene rearrangement (within the 8.5 kilobases of cloned DNA) have been detected; it was proposed that (i) the enhancement of resistance may be a consequence of a *trans*-acting effector operating at the level of transcription, and (ii) the putative effector is itself controlled by a genetic rearrangement at a locus removed from the site of *cat*.

We present data here which are compatible with a model involving transcriptional control of the *cat* gene to produce the enhanced chloramphenicol resistance phenotype. Nucleotide sequence comparison of the *P. mirabilis cat* with the type I (Tn*9*) *cat* variant has shown a high degree of sequence conservation between both structural genes, but little or no evidence of homology in the flanking regions. A model is proposed for the regulation of *P. mirabilis cat* based on previous expression studies (8) and an unanticipated sequence homology with the 5′ flanking regions of the *Salmonella typhimurium* phase variation genes H1 and H2.

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## MATERIALS AND METHODS

**Bacterial and phage strains.** *P. mirabilis* PM13 (10) and PM2 (NCTC 3177) were the kind gifts of J. Coetzee (University of Pretoria) and D. Jones (University of Leicester), respectively. *Escherichia coli* JM103 [Δ(*lac-pro*) *thi rpsL supE endA sbcB hsdR*, F′ *traD36 proA lacI*$^q$ Δ*lacZM15*] and phage M13mp8 and M13mp9 have been described previously (20), and the double-stranded replicative form of the phage was kindly supplied by A. R. Hawkins (University of Leicester).

**Enzymes and DNA manipulations.** Restriction endonuclease digestions and ligations were carried out in the manufacturers' recommended buffers. Recombinant phage were analyzed by endonuclease digestion of the double-stranded form after extraction by the method of Birnboim and Doly (5). Transformation of bacterial cells was performed as described by Kushner (17).

**RNA-DNA hybridizations.** RNA was prepared by the phenol extraction protocol of Miller (21). Contaminating DNA in the preparation was removed by incubation with RNase-free DNase. The conditions for the RNA dot-blot hybridization procedure and the high-stringency washing were as described by Maniatis et al. (18).

**DNA sequence determination and analysis.** DNA sequencing was performed by the method of Sanger et al. (27). M13 manipulations were performed as described by Messing and Vieira (20), and the gradient polyacrylamide urea gel electrophoresis system and [α-$^{35}$S]dATP labeling were as described by Biggin et al. (4). The DNA sequence of the *cat* of *P. mirabilis* PM13 was compared with that of Tn*9* *cat* by a computer scan with a window of 49 nucleotides to search for homology.

**Oligonucleotides and purification.** Oligonucleotides synthesized on paper disks by the method of Mathes et al. (19) as modified by Brenner and Shaw (6), were purified by electrophoresis through a 20% polyacrylamide–50% urea gel. Bands corresponding to full-length oligonucleotides were visualized by UV shadowing and were cut from the gel, extracted with 100 μl of water for between 2 and 12 h, and desalted by spun-column chromatography (18). The concentrations of

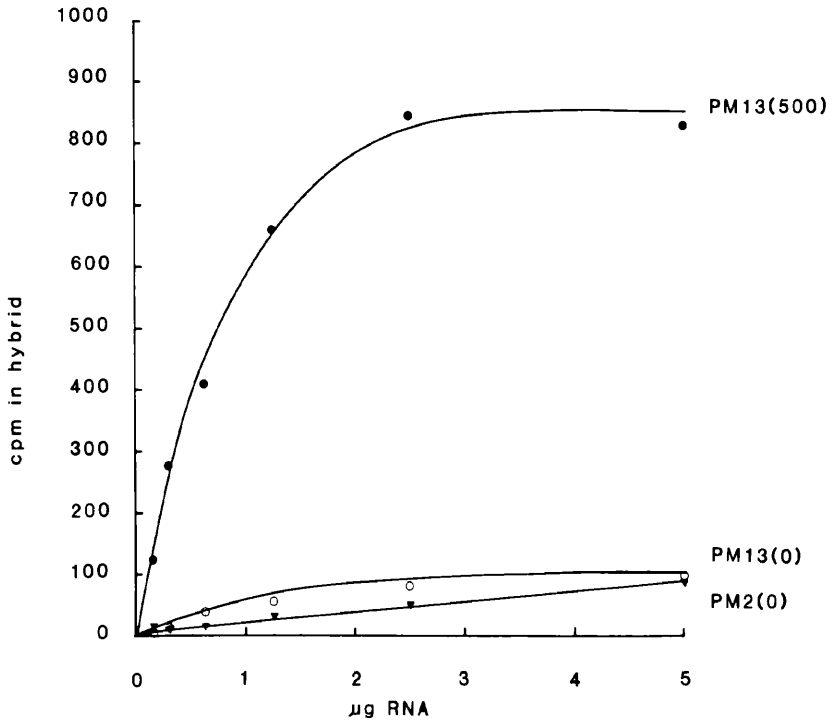


FIG. 1. Titration of *cat* mRNA from *P. mirabilis* PM13 and PM2 grown in different concentrations of chloramphenicol. Figures in parenthesis (500 or 0) denote the concentration during growth of chloramphenicol in micrograms per milliliter. Determination of mRNA was by hybridization of total RNA to a 1.7-kilobase *Cla*I-*Pst*I fragment of pIC100 containing *cat*. Contaminating DNA was removed by digestion with RNase-free DNase, and the absorbance at 260 nm of each of the three preparations was measured. A dilution series corresponding to a range from 5 to 0.05 µg of RNA was spotted onto nitrocellulose filters, baked at 80°C for 2 h, and hybridized with a nick-translated (24), α-$^{32}$P-labeled, 1.7-kilobase *Pst*I-*Cla*I fragment comprising the entire *cat* gene (8). Unbound probe was removed by washing, and filter-bound counts were determined by scintillation counting.

the oligonucleotides were calculated from the absorbance at 260 nm and adjusted to 5 ng/µl with water.

**Sources of materials.** All restriction endonucleases, DNA polymerase I large fragment (Klenow), T4 DNA kinase, and RNase-free DNase were purchased from BRL Ltd. Other sources were as previously described (8).

## RESULTS

**RNA dot-blot analysis.** RNA was isolated from cells of *P. mirabilis* PM13 grown in the presence (500 µg ml$^{-1}$) and absence of chloramphenicol, and from *P. mirabilis* strain PM2, which is free of a detectable *cat* gene on the basis of hybridization studies (data not shown). The graph in Fig. 1 shows that hybridization to the probe has only occurred with RNA extracted from PM13 grown in the presence of chloramphenicol. Cells of PM13 which were grown in drug-free conditions produce approximately the same background signal level as PM2. Although the *cat* gene has been shown to be present in the cells in the CAT$^-$ state as well as those with the CAT$^+$ phenotype, it is only transcribed in CAT$^+$ cells.

**Phage construction and sequencing strategy.** Previous experiments have shown that a *cat* consensus oligonucleotide (3) hybridizes to a 1.7-kilobase *Pst*I-*Cla*I fragment at one end of the 8.5-kilobase *Pst*I fragment in pIC100 (8), the presumed site of the *cat* gene of *P. mirabilis*; thus, the sequencing strategy involved the cloning of subfragments into M13mp8 and M13mp9. Plasmid pIC100 was double digested with *Hin*dIII and *Eco*RI, and the resulting fragments were ligated with similarly (*Eco*RI-*Hin*dIII)-digested M13mp8 and M13mp9. The resulting ligation mixes were used to transform *E. coli* JM103. Recombinants (identified as white plaques) were spotted onto a lawn of strain JM103, grown overnight, and transferred to nitrocellulose paper. Fragments containing *cat* sequence were identified by hybridization with the α-$^{32}$P-labeled 1.7-kilobase *Pst*I-*Cla*I probe (2). In a similar fashion, pIC100 was digested with *Sau*3A, *Pst*I, and *Cla*I and ligated into M13mp9 cut with *Bam*HI, *Pst*I, and *Acc*I, respectively. The *cat*-containing recombinants were similarly identified by hybridization. White plaques giving a positive hybridization signal were grown overnight in strain JM103, and the extracted phage DNA was subjected to the sequencing protocol. Extended sequences were generated by using oligonucleotides as internal primers (Fig. 2).

**Translation reading frame of *cat*.** Examination of all possible reading frames in the *Pst*I-*Cla*I sequence identified two reading frames of over 100 base pairs (Fig. 3). The 132-base-pair reading frame has the potential for encoding a protein containing 44 amino acids, whereas the 651-base-pair open reading frame could encode a protein of 217 amino acids. This longer open reading frame is clearly identifiable by comparison with the type I *cat* sequence (1) as the *cat* gene with a predicted molecular weight of 25,284 daltons.

**Amino acid and nucleotide similarities of *P. mirabilis cat* and type I (Tn*9*) *cat*.** The DNA sequences of the *P. mirabilis cat* and the type I variant were aligned, and the homology between them is presented in Fig. 4. This alignment shows the high degree of homology between the two structural gene sequences, 73 and 76% at the nucleotide and amino acid levels, respectively (Fig. 5).

**Codon usage in *P. mirabilis cat*.** Codon usage is believed to be mainly dependent on the relative amounts of *iso*-accepting tRNA molecules present in the cell (15). The

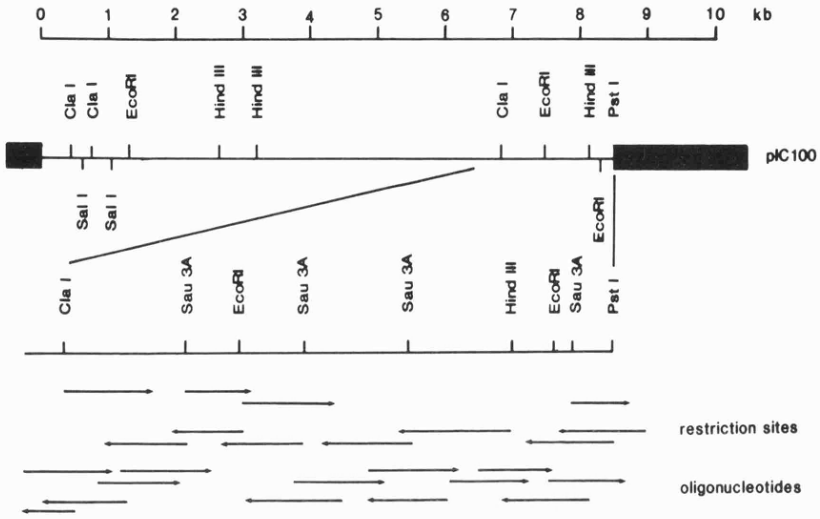


FIG. 2. Sequencing strategy. The heavy dark lines represent pAT153 vector DNA, and the light lines represent inserted DNA. Arrows represent the DNA sequences determined either from restriction endonuclease sites or from specific oligonucleotides used as internal primers.

codon usage for the *cat* of *P. mirabilis* is similar to that of type I *cat* identified with Tn*9* and frequently associated with R plasmids in enteric bacteria (data not shown) and is consistent with the codon usage of *E. coli* genes which are not highly expressed (13). How significant the *E. coli* codon bias is to the expression of a gene from *P. mirabilis* is not yet known. The G+C content of *P. mirabilis* is 39.3 ± 1.4% (9), whereas that of *E. coli* is 51%, and a different codon usage may therefore be expected. Infrequently used codons (16) do, however, occur more often in the *P. mirabilis cat* gene than in the type I variant, and this may partly explain why the expression of the *P. mirabilis cat* in *E. coli* is so poor (25).

**Putative regulatory sequences in *P. mirabilis* PM13 *cat*.** The striking sequence homology at the nucleotide level between the *cat* of *P. mirabilis* and the Tn*9 cat* ended abruptly in the 5′ and 3′ flanking regions (Fig. 4). Unlike the type I Tn*9 cat*, which is under cyclic AMP-mediated catabolite control, the *cat* of *P. mirabilis* contains no apparent comparable binding site for the cyclic AMP receptor protein (analysis not shown), a conclusion based on the absence of the consensus sequence (12). Such an observation is not surprising, since catabolite repression of *cat* has not been observed in *P. mirabilis* (30). Neither is there any suggestion of potential secondary structure in the mRNA around the ribosome-binding site (Shine-Dalgarno sequence) as is found in the gram-positive *cat* genes (7, 11) and which is believed to play a part in the phenomenon of induction mediated by chloramphenicol and certain of its analogs. The upstream flanking region to the PM13 *cat* is very A+T rich and contains several notable features 5′ to the start of *cat* at nucleotide 880. These include the following.

(i) An open reading frame from nucleotides 697 through 828 with a predicted polypeptide chain of 44 amino acids.

(ii) Several possible RNA polymerase binding sites for *cat*. Of these the two most likely by computer search (14) extend from nucleotides 712 to 741 (promoter 1), comprising the presumptive −35 sequence (5′-TTGAAA-3′) and the −10 (Pribnow box) (23) sequence (5′-TAAACA-3′) and from nucleotides 813 through 844 (promoter 2), comprising a −35 sequence (5′-TTGTTT-3′ or the overlapping candidate 5′-TTTAAT-3′) and the −10 (Pribnow box) sequence (5′-TACAAT-3′).

(iii) An inverted complementary repeat sequence from nucleotides 756 through 795 lying between the two best-fit RNA polymerase-binding sites (26).

(iv) Sequence homology (Fig. 6) with the 5′ flanking regions of the genes involved in phase variation in *S. typhimurium* (32).

Examination of the 3′ flanking region of *P. mirabilis cat* reveals an inverted complementary repeat region which resembles cannonical rho-independent termination sequences (26), a feature which is conspicuously absent from the corresponding region of the Tn*9 cat* gene.

## DISCUSSION

We have shown that chloramphenicol resistance in *P. mirabilis* is determined by a *cat* determinant which is present as a single-copy gene in both resistant and sensitive isolates of strain PM13 (8). RNA dot-blot analysis has determined that *cat*-specific mRNA is found only in cells in the $CAT^+$ state and never in cells in the $CAT^-$ state. Chloramphenicol resistance is achieved by active transcription of the single-copy gene which is not expressed in the sensitive ($CAT^-$) state, rather than by a mechanism which is likely to involve the sequestration of ribosome binding sites (7, 11) or altered RNA stability (22).

The nucleotide sequence of the *P. mirabilis cat* gene, including the 5′ and 3′ flanking regions, has been determined (Fig. 3). The coding sequence can be aligned with the Tn*9* type I *cat*. The two genes have similarities of 76% at the amino acid level and 73% at the nucleotide level, suggesting that *P. mirabilis cat* and the Tn*9* type I variant are likely to have diverged from a common ancestor. Since the type I *cat* is flanked by direct repeats of IS*1* sequences on the transposon Tn*9*, it is tempting to speculate that the evolutionary route for the chromosomal *cat* of *P. mirabilis* has involved an early transposition event from an ancestral transposon with subsequent loss of transposition functions to yield the present extent of divergence.

The 5′ and 3′ sequences flanking the *cat* of *P. mirabilis* and the corresponding segments of the type I *cat* are dissimilar, which may account for the poor expression of *P. mirabilis cat* in *E. coli* (8). Upstream from the start of the *P. mirabilis* PM13 *cat* gene is an open reading frame (nucleotides 697 through 828), which is suggestive of so-called leader sequences found in inducible operons. In the present case, however, the gene transcript might not be expected to

```
        10        20        30        40        50        60        70        80        90       100       110       120
CTGCAGGAATATTTAAACCTTCTTGAACATATTTAGTCACACAGGTCTCTGTTTTAGTCTGTGTACGACCGGTAATGAAATAAACAGTATCACCGCGTTTTAAGTGCATCTGAACAAGAT

       130       140       150       160       170       180       190       200       210       220       230       240
CGATACCGACTTGTTTAGGCATACTGAATTTATCCCATTCGTTGTTCATTTTTTCCAGAATTCTGGGTTTTTCAGATAACTGAAATCGTTTGGAGATATTCAGTTTACACGATAAAAGCC

       250       260       270       280       290       300       310       320       330       340       350       360
TGGGCTTGAGAACCAGAACGTGTCATCAATATCAAAACCTACCGCCATTGGTGCTTGGCCTTCTAAGCTTTTTTCAATTTCTGCAACAGAAACCCATTTAATTGGTTGCTGATGTGCAAG

       370       380       390       400       410       420       430       440       450       460       470       480
TTCAGTAACAGTAACCCTGGCTAACAACTTCTGGCATATGAACTTTTGCCATTGCTGCGCCATTTAAACTTAACGCCAACGCAATTGCACTAAGAGTAAGTGTGACTTTACGCATTCTCT

       490       500       510       520       530       540       550       560       570       580       590       600
TTCCTTTAATTTTTATATTAATCACAGAAGGTGATAATAATAAACATCATAATTACATTTAAATGAGATATAGCGCACGTTATTTTATTTAGGCTGGTACTCTTTTGTTCAGTTTACTTT
                                                                                                                 -35
       610       620       630       640       650       660       670       680       690       700       710       720
CATTTTTTGATCTATCTACATTTGAAAAAAGAAAGCACTATCTACGAAAGCAGATAGCGCCGTTATTATCTAAGTCGTTCCCAAATGAACACATCAATGACTCTGTTATTTTTGAAACAT
                                                                                                MetThrLeuLeuPheLeuLysHis
                 -10                                                                  -35                          -10
       730       740       750       760       770       780       790       800       810       820       830       840
TTTTTGGTAAAAAATAAACATTTTAAAATTAGCCAAGAAAAACCTATTACCTATCATAGGTTATGGGTTTATCTACAGATAGAGGTTCGAAATTGTTTTAATAATTTATAAGTAATATAC
PheLeuValLysAsnLysHisPheLysIleSerGlnGluLysProIleThrTyrHisArgLeuTrpValTyrLeuGlnIleGluValArgAsnCysPheAsnAsnLeu***
                                 SD
       850       860       870       880       890       900       910       920       930       940       950       960
AATTAATTTAAATTTGGATTAGAAAATAGGAAAGCTAATATGGACACAAAGCGCGTGGGTATATTGGTTGTTGATCTATCCCAATGGGGACGAAAAGAACACTTTGAAGCATTTCAGTCT
                                                   MetAspThrLysArgValGlyIleLeuValValAspLeuSerGlnTrpGlyArgLysGluHisPheGluAlaPheGlnSer

       970       980       990      1000      1010      1020      1030      1040      1050      1060      1070      1080
TTTGCTCAATGCACCTTTAGCCAGACCGTTCAACTGGATATTACTTCATTATTAAAAACCGTAAAGCAAAATGGGTACAAATTCTATCCGACATTTATATACATCATTAGCCTATTGGTA
PheAlaGlnCysThrPheSerGlnThrValGlnLeuAspIleThrSerLeuLeuLysThrValLysGlnAsnGlyTyrLysPheTyrProThrPheIleTyrIleIleSerLeuLeuVal

      1090      1100      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
AATAAACATGCAGAATTCCGCATGGCAATGAAAGACGGGGAATTGGTGATATGGGATAGTGTTAACCCTGGGTACAATATCTTTCATGAACAGACCGAAACATTTTCATCTTTATGGAGT
AsnLysHisAlaGluPheArgMetAlaMetLysAspGlyGluLeuValIleTrpAspSerValAsnProGlyTyrAsnIlePheHisGluGlnThrGluThrPheSerSerLeuTrpSer

      1210      1220      1230      1240      1250      1260      1270      1280      1290      1300      1310      1320
TACTACCACAAAGATATCAATCGTTTTTTGAAAACTTATTCAGAAGATATAGCACAATACGGCGACGATCTAGCCTATTTTCCAAAAGAATTTATCGAAAATATGTTTTTTGTGTCAGCA
TyrTyrHisLysAspIleAsnArgPheLeuLysThrTyrSerGluAspIleAlaGlnTyrGlyAspAspLeuAlaTyrPheProLysGluPheIleGluAsnMetPhePheValSerAla

      1330      1340      1350      1360      1370      1380      1390      1400      1410      1420      1430      1440
AATCCTTGGGTAAGTTTCACCAGTTTTAACTTGAATATGGCGAATATAAACAATTTCTTTGCCCCTGTATTTACAATAGGTAAATATTATACGCAAGGAGATAAAGTTCTGATGCCACTA
AsnProTrpValSerPheThrSerPheAsnLeuAsnMetAlaAsnIleAsnAsnPhePheAlaProValPheThrIleGlyLysTyrTyrThrGlnGlyAspLysValLeuMetProLeu

      1450      1460      1470      1480      1490      1500      1510      1520      1530      1540      1550      1560
GCAATTCAAGTGCATCATGCCGTATGTGATGGCTTCCATGTAGGCAGATTACTCAATGAAATACAGCAATACTGCGATGAGGGATGTAAATAATCAATGGGACTTAAAGCACATGTTTTT
AlaIleGlnValHisHisAlaValCysAspGlyPheHisValGlyArgLeuLeuAsnGluIleGlnGlnTyrCysAspGluGlyCysLys***

      1570      1580      1590      1600      1610      1620      1630      1640      1650      1660      1670      1680
GTTCCACTCAAACCTGTTCGCTATCCTCTGTTTCGCACTTTTATTGGATTATTTTAACATCGATATCGATATAAAATTGATGAGGGGCAATTAGTATTTCCCTTTTTATTGAGGGGTCAC

      1690      1700      1710      1720      1730      1740      1750      1760
GCTATAAAATTTGTACACTGATTTGTACTAATACGGTGATAAAATACTAATGCCATTACACTCATGAATAACAAATATAA
```

FIG. 3. Nucleotide sequence of the *P. mirabilis cat* gene and flanking regions displayed as a 1,760-base-pair fragment is shown along with the predicted amino acid sequence of CAT. Another open reading frame of 132 base pairs and its predicted amino acid sequence is shown 5′ to the start of the *cat* gene. The underlined nucleotide clusters −35 and −10 are the putative RNA polymerase recognition sites, two strong candidates being identified by a computer search (14). SD indicates the likely ribosome-binding site (28). Opposing arrows indicate regions with imperfect dyad axis of symmetry. The inverted complementary sequence from nucleotides 1645 through 1660 is followed immediately by five T residues; this structure is characteristic for rho-independent termination.

sequester the *cat* ribosome binding site (28) in stem-loop secondary structure, since (i) the putative mRNA is too short, and (ii) the open reading frame is not associated with a convincing ribosome-binding site. We propose, therefore, that this open reading frame is incidental to any mechanism of control of *cat* gene expression.

Of the several factors which may affect gene expression in heterologous backgrounds, those of special importance include the recognition by host RNA polymerase of signals for the initiation of transcription, 30S ribosomal RNA complements on the mRNA (31), and generic patterns of codon usage. Of particular interest is the lack of high-level expres-

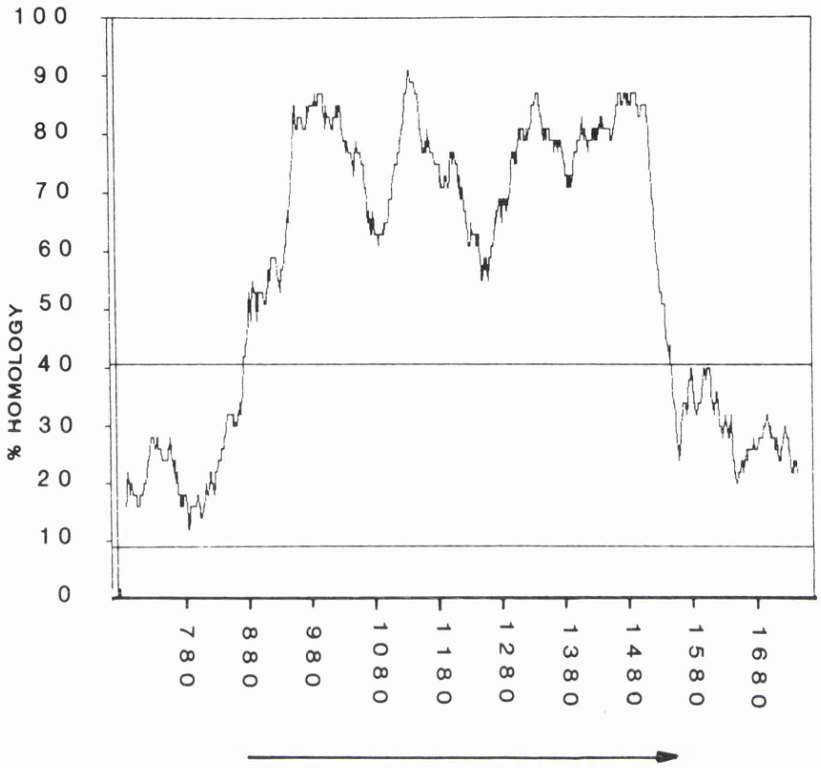


FIG. 4. Graphic representation of nucleotide similarity between type I *cat* (1) and *P. mirabilis cat*. Each point on the graph represents the percentage of nucleotide identity present in a window of 49 nucleotides. The two parallel lines across the graph encompasses the level of background homology that might be expected by chance. The arrow below the graph represents the coding region of both *cat* genes. The numbering corresponds to the sequence shown in Fig. 3.

sion of the PM13 *cat* gene in *P. mirabilis* PM2 (8), a background wherein arguments based on polymerase recognition and codon usage are not applicable.

A more convincing model hypothesis for the poor expression of the PM13 *cat* in strain PM2 is that of a requirement for a host-specific *trans*-acting effector, which is essential for expression of *cat* in *P. mirabilis* PM13, but which is absent in strain PM2. Two possibilities may be considered for such a control system: positive or negative effects on transcription by the putative regulator. Inverted complementary repeats in DNA may serve as binding sites for *trans*-acting regulatory proteins. One such inverted repeat was observed from nucleotides 756 through 795 lying between the two best-fit promoters. Should promoter 1 be preferred, the binding of a *trans*-acting protein would freeze transcription, whereas if promoter 2 is the preferred recognition site for RNA polymerase, the binding of the putative regulatory protein at the inverted complementary repeat might be expected to enhance the efficiency with which the polymerase initiates *cat* transcription. A second inverted complementary repeat (data not shown) spanning nucleotides 817 through 862 and including the −35 and −10 sequences of putative promoter 2 should also be noted.

To accord with the observed frequency of occurrence and disappearance of chloramphenicol resistance, the regulator itself would need to be under on-off control, switched on at a rate of $10^{-5}$ per cell per generation and off at a rate of $10^{-2}$ per cell per generation. Such a model is not unlike that

```
          1   5   10  15  20  25  30  35  40  45  50  55  60  65  70  75  80
I         MEKKITGYTTVDISQWHRKEHFEAFQSVAQCTYNQTVQLDITAFLKTVKKNKHKFYPAFIHILARLMNAHPEFRMAMKDG
Proteus   MDTKRVGILVVDLSQWGRKEHFEAFQSFAQCTFSQTVQLDITSLLKTVKQNGYKFYPTFIYIISLLVNKHAEFRMAMKDG

             85  90  95  100 105 110 115 120 125 130 135 140 145 150 155 160
I         ELVIWDSVHPCYTVFHEQTETFSSLWSEYHDDFRQFLHIYSQDVACYGDNLAYFPKGFIENMFFVSANPWVSFTSFDLNV
Proteus   ELVIWDSVNPGYNIFHEQTETFSSLWSYYHKDINRFLKTYSEDIAQYGDDLAYFPKEFIENMFFVSANPWVSFTSFNLNM

            165 170 175 180 185 190 195 200 205 210 215 220
I         ANMDNFFAPVFTMGKYYTQGDKVLMPLAIQVHHAVCDGFHVGRMLNELQQYCDEWQGGA
Proteus   ANINNFFAPVFTIGKYYTQGDKVLMPLAIQVHHAVCDGFHVGRLLNEIQQYCDEGCK
```

FIG. 5. Amino acid comparison between the type I chloramphenicol acetyltransferase polypeptide encoded by Tn9 with the chloramphenicol acetyltransferase of *P. mirabilis* PM13. The 76% identity (underlined residues) was deduced by inspection.

GAAAAATTTTCTAAAGTT...(14)...CGATACAAGGGTTAC...(22)...CCCAATAACATCAA...(25)...ATG a

GAAAAACCTATTACCTAT...(21)...ACAGATAGAGGTTCG...(18)...ATAAGTAATATACA...(37)...ATG b

FIG. 6. Comparison of conserved sequences present upstream from the H1 gene (a) from *S. typhimurium* (29) and the *cat* gene (b) from *P. mirabilis* PM13. A possible consensus sequence which was detected by visual inspection is underlined, and the number of nucleotides separating each block is indicated in parenthesis. The triplet ATG in each case corresponds to the start codon for the H1 protein or chloramphenicol acetyltransferase in (a) and (b), respectively. From left (5′) to right (3′), the coordinates for the aligned segments of *cat* in (b) are residues 757 to 774, 795 to 809, and 828 to 841 (Fig. 3).

proposed for phase variation in *S. typhimurium* (29). The alternate expression of the flagellin genes H1 and H2 is under the control of an invertible DNA region such that in one orientation or on state, H2 is transcribed along with a gene, *rhl*, a *trans*-acting repressor of transcription of H1. When the invertible region flips to the off state, H2 and *rhl* are no longer expressed, thus freeing transcription of H1. Such a flip-flop mechanism has been observed to operate at a frequency of $10^{-5}$ to $10^{-3}$ per cell per generation and often expresses a preference for one orientation (29).

A visual comparison of the 5′ flanking sequence of the *cat* of *P. mirabilis* aligned to correspond with regions upstream to the genes H1 and H2 revealed regions of partial identity (Fig. 6). Taking this together with all the data on PM13, it is tempting to propose a model according to which a *cat* gene (for example via transposition from an ancestral Tn*9*-like element) has come under control of a promoter which is itself regulated by a *trans*-acting element. No data are available as yet to guide a choice as to whether the *cat* gene of *P. mirabilis* is under positive or negative control.

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## ABSTRACT

Proteus mirabilis and CAT by Ian George Charles

Proteus mirabilis PM13 is a well characterized chloramphenicol-sensitive isolate which spontaneously gives rise to resistant colonies on solid media containing chloramphenicol (50µg/ml) at a plating efficiency of between $10^{-4}$ and $10^{-5}$ per cell per generation. When a chloramphenicol resistant colony is grown in liquid medium in the absence of the antibiotic for 150 generations a population of predominantly sensitive cells arises. The cat gene responsible for the phenomenon is chromosomal, and has been cloned from P.mirabilis PM13 with DNA prepared from cells grown in the absence or the presence of chloramphenicol. Recombinant plasmids which confer resistance to chloramphenicol carry an 8.5-kb PstI fragment irrespective of the source of host DNA. The location of the cat gene within the PstI fragment was determined by Southern blotting with a cat consensus 'active-site' oligonucleotide (5'-CCATCACAGACGGCATGATG-3') corresponding to the expected amino acid sequence of the active site region of chloramphenicol acetyltransferase. DNA sequence analysis has revealed a high degree of homology between the P.mirabilis cat gene and the type I cat variant (Tn9), 76% at the amino acid level and 73% when nucleotides in the coding sequence are compared. The mechanism for the appearance and disappearance of chloramphenicol resistance in P.mirabilis appears to be associated with a host-specific trans-acting element which controls cat gene expression. A precedent for such a control network is given by phase variation in Salmonella typhimurium, where an invertible DNA segment controls the transcription of a trans-acting regulatory element. A comparison of the 5' regions of the S.typhimurium flagellin genes H1 and H2 (which are alternately expressed by a flip-flop control mechanism) with the 5' region of P.mirabilis cat show blocks of homology. Whether or not this homology is significant in the regulation of cat gene expression has not been determined.