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ABSTRACT

The Inc P-1 plasmid, R26 carries an inducible chloramphenicol resistance determinant, cml. This specifies low level chloramphenicol resistance (30 µg/ml) in Escherichia coli. The mechanism does not involve drug detoxification or ribosomal modification, but may involve a permeability barrier to drug uptake acting at the cytoplasmic membrane. Experiments described in this thesis show that cml also specifies resistance to some fluorinated derivatives of chloramphenicol.

The determinant was cloned (initially on a 7 Kb EcoRI fragment of R26 DNA) in the cloning vector pBR322. It was subsequently sub-cloned on a 1.8 Kb HindIII fragment, also in pBR322. Mutagenesis with transposon Tn5 and deletion mutagenesis showed that a minimum of 1.4 Kb was required to specify chloramphenicol resistance.

In pBR322, cml was constitutively expressed. However, on sub-cloning in pUB5572, a low copy number vector, expression became inducible. This indicated that the 1.8 Kb HindIII fragment carried the entire determinant, both regulatory and structural elements.

The mechanism of cml regulation was studied using both transcriptional and translational lac fusions. These showed that transcription of cml is constitutive and that the cml promoter is weak. The determinant was found to be regulated at the translational level. In addition, the translational fusion permitted the direction of expression of cml to be deduced. The DNA sequence of the 1.8 Kb HindIII fragment was determined. An open reading frame was identified which encoded a protein with a molecular weight of 33.8 Kd. This was preceded by a ribosome binding site and features which may be involved in post-transcriptional regulation of cml. The possible mechanism of regulation is similar to that proposed for the post-transcriptional control of inducible chloramphenicol acetyltransferase genes in gram-positive bacteria.

The product of cml was identified in the E. coli minicell system. This was found to be a chloramphenicol-inducible protein with an apparent molecular weight of 31 Kd. This agreed well with the gene product size predicted by DNA sequence analysis. A cell fractionation experiment was carried out to determine the cellular location of the product. The result indicated that the 31 Kd protein is associated with the cell envelope. This concurs with the model proposed for the cml-determined chloramphenicol resistance mechanism.

ANALYSIS OF THE CHLORAMPHENICOL
RESISTANCE DETERMINANT OF
PLASMID R26

by

CHARLES JAMES DORMAN

A dissertation submitted for the degree of
Doctor of Philosophy at the University of Dublin,
Trinity College.

THESIS
972

DECLARATION

I declare that this thesis represents my own unaided work,
except where acknowledged.

Charles J Dorman
Charles J. Dorman

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PUBLICATIONS

At the time of writing, material from Chapters 3, 4 and 5 has been published (see below) and material from Chapters 6 and 7 is being submitted for publication.

Dorman, C.J. and T.J. Foster. 1982. Non-enzymatic chloramphenicol resistance determinants specified by plasmids R26 and R55-1 in Escherichia coli K12 do not confer high-level resistance to fluorinated analogues. *Antimicrob. Agents Chemother.* 22: 912-914

Dorman, C.J. and T.J. Foster. 1985. Posttranscriptional regulation of the inducible non-enzymatic chloramphenicol resistance determinant of the IncP plasmid R26. *J.Bacteriol.* 161:

SUMMARY

The Inc P-1 plasmid, R26, carries an inducible chloramphenicol resistance determinant, cml. This specifies low level chloramphenicol resistance (30 µg/ml) in Escherichia coli. The mechanism does not involve drug detoxification or ribosomal modification, but may involve a permeability barrier to drug uptake acting at the cytoplasmic membrane. Experiments described in this thesis show that cml also specifies resistance to some fluorinated derivatives of chloramphenicol.

The determinant was cloned (initially on a 7 Kb EcoRI fragment of R26 DNA) in the cloning vector pBR322. It was subsequently sub-cloned on a 1.8 Kb HindIII fragment, also in pBR322. Mutagenesis with transposon Tn5 and deletion mutagenesis showed that a minimum of 1.4 Kb was required to specify chloramphenicol resistance.

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The mechanism of cml regulation was studied using both transcriptional and translational lac fusions. These showed that transcription of cml is constitutive and that the cml promoter is weak. The determinant was found to be regulated at the translational level. In addition, the translational fusion permitted the direction of expression of cml to be deduced. The DNA sequence of the 1.8 Kb HindIII fragment was determined. An open reading frame was identified which encoded a protein with a molecular weight of 33.8 Kd. This was preceded by a ribosome binding site and features which may be involved in post-transcriptional regulation of cml. The possible mechanism of regulation is similar to that proposed for the post-transcriptional control of inducible chloramphenicol acetyltransferase genes in gram positive bacteria.

The product of cml was identified in the E. coli minicell system. This was found to be a chloramphenicol-inducible protein with an apparent molecular weight of 31 Kd. This agreed well with the gene product size predicted by DNA sequence analysis. A cell fractionation experiment was carried out to determine the cellular location of the product. The result indicated that the 31 Kd protein is associated with the cell envelope. This concurs with the model proposed for the cml-determined chloramphenicol resistance mechanism.

CHAPTER 1

GENERAL INTRODUCTION

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GENERAL INTRODUCTION

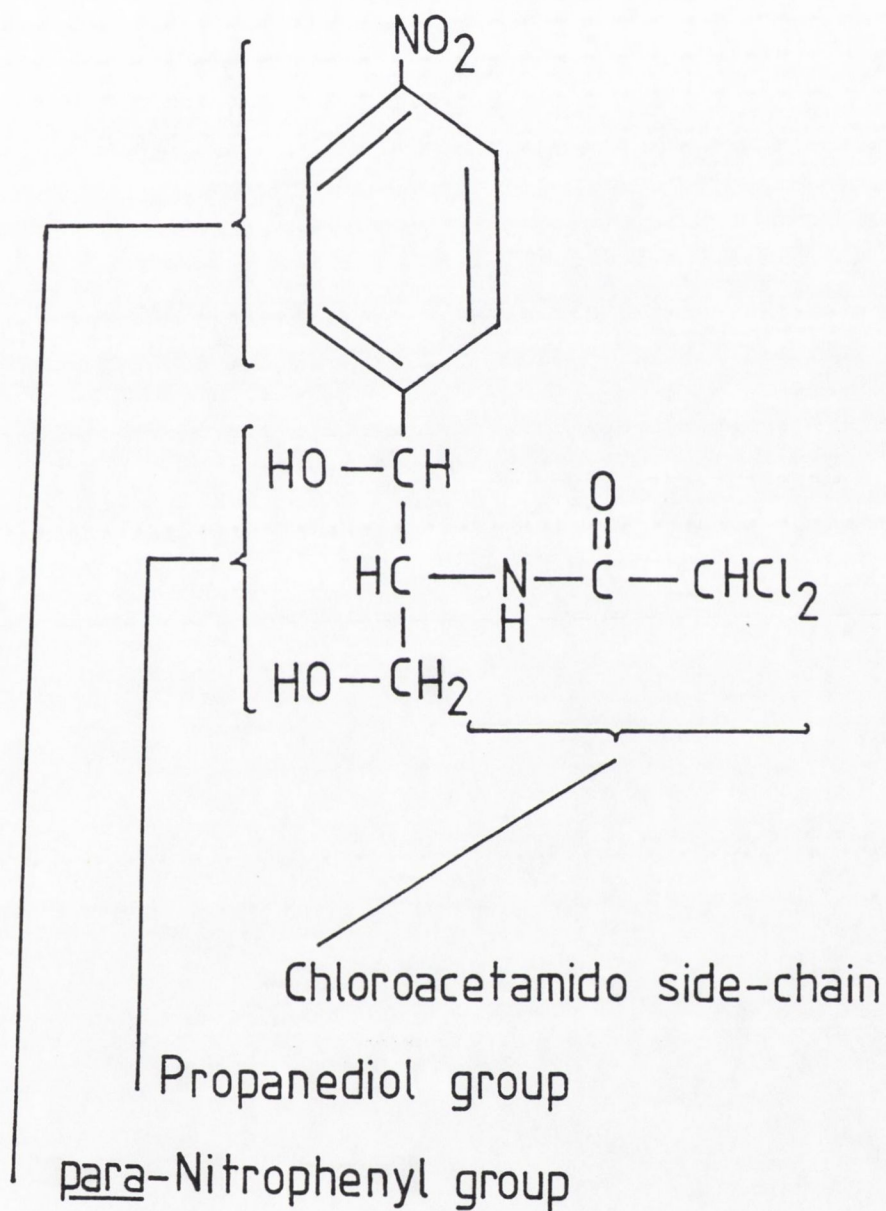
A. Chloramphenicol

Chloramphenicol is a bacteriostatic agent which inhibits growth of a wide range of gram-positive and gram-negative bacteria. It was the first broad-spectrum antibiotic to be used clinically. The compound, formerly called chloromycetin (D (-)-threo-2,2-dichloro-N-[β -hydroxy- α -(hydroxymethyl)-p-nitrophenyl] acetamide) was independently isolated by Burkholder, from an actinomycete from the soil in Venezuela (Erlich et al., 1947) and by Carter, Gottlieb and Anderson (1947) from a similar organism from a compost heap in Illinois, U.S.A.

By 1948 the crystalline form of the compound had been isolated by these groups, working in collaboration. Within a year its chemical structure had been established and a substance identical to the natural compound had been synthesised chemically and given the name chloramphenicol (Garrod and O'Grady, 1971).

Structurally, this is a simple molecule and so it can be produced easily by chemical synthesis, this method soon replacing fermentation as the primary means of production. The molecular structure of the antibiotic is shown in Fig. 1.1. For optimal activity, the aromatic ring should be substituted in the para position by an electronegative group (e.g. the nitro group), but with no preferred geometry or configuration. The propanediol group, in the D. threo configuration is essential for activity and the hydroxyls must not be substituted, although they may be replaced. Removal of the chloroacetamido side-chain results in almost complete loss of biological activity. It can, however, be substituted in a number of ways (Garrod and O'Grady, 1971).

FIGURE 1.1 Structural formula of chloramphenicol



B. Mode of action of chloramphenicol

Chloramphenicol inhibits protein synthesis in bacteria (Gale and Paine, 1951). It also inhibits protein synthesis in intact mitochondria (Wheeldon and Lehringer, 1966) and in mitochondrial extracts (Kroon, 1965). The same is true of chloroplasts and their extracts (Ellis, 1969; Margulies and Brubacker, 1970).

Chloramphenicol binds to bacterial ribosomes (Vazquez, 1964). It binds to the 50s subunit of the ribosome and this binding requires Mg^{2+} and K^{+} ions and is readily reversed by washing (Vazquez, 1964). At bacteriostatic concentrations, one molecule of chloramphenicol binds per 70s ribosome, although another molecule can bind at high drug concentrations (Wolfe and Hahn, 1965). This second molecule is thought to bind to a low-affinity site on the 30s ribosomal subunit (Grant et al., 1979). The bacteriostatic effect of chloramphenicol is due to the binding of the drug to the 50s subunit of the ribosome. Here, it interrupts the transpeptidase reaction of protein synthesis and so prevents polypeptide chain elongation (Gale et al., 1981).

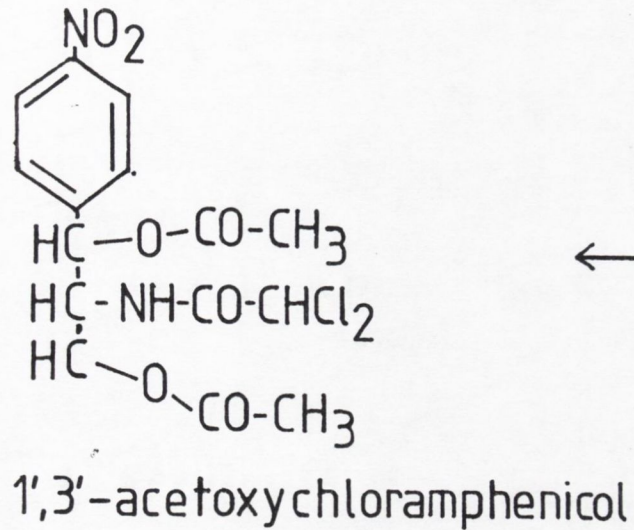
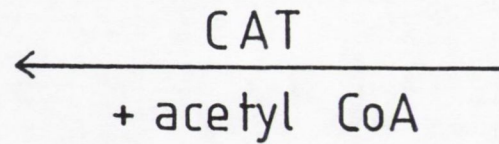
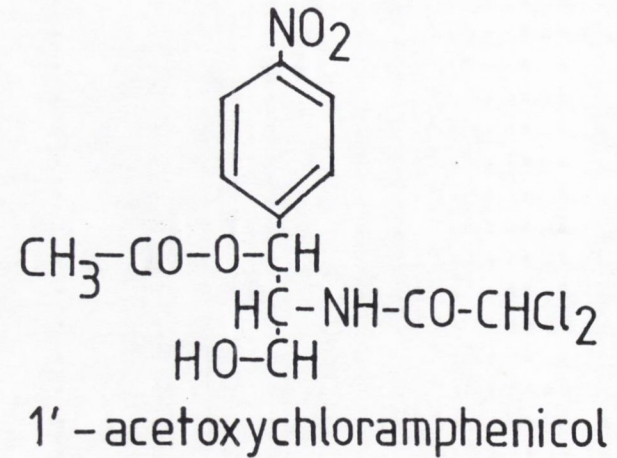
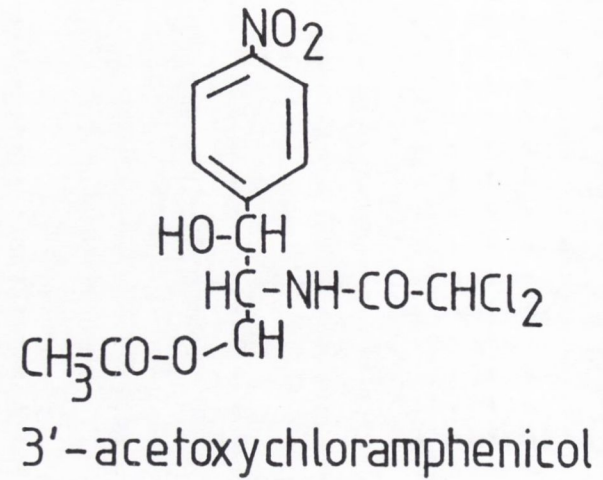
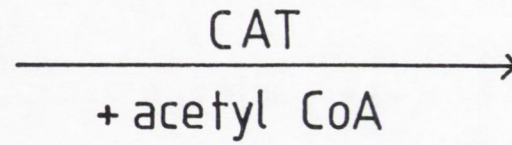
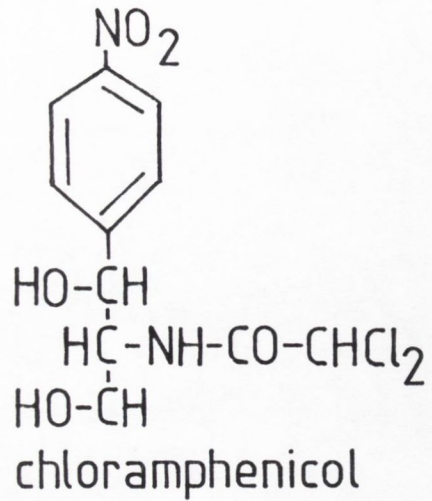
C. Bacterial resistance to chloramphenicol

I. Chloramphenicol acetyltransferase (CAT)

Chloramphenicol resistance (Cm^r) in bacteria is mediated primarily by CAT. This enzyme inactivates the drug by acetylation (Fig. 1.2). For a detailed review of the properties of CAT, see Shaw (1983). CATs have been discovered in a wide range of both gram-positive and gram-negative bacteria. Each functional enzyme is a tetramer of a subunit protein whose molecular weight is in the range 23,000 to 25,000 d. The various CAT variants have been classified according to their kinetics, electrophoretic mobility, inhibitor susceptibility and reaction to antisera (Shaw, 1983).

FIGURE 1.2 Acetylation of chloramphenicol catalysed by
chloramphenicol acetyltransferase (CAT)

In the presence of acetyl-CoA and CAT, chloramphenicol is acetylated at its 3'-OH group. The product, 3'-acetoxychloramphenicol, is inactive as an antibiotic. This molecule can undergo an intramolecular rearrangement to yield 1'-acetoxychloramphenicol, also inactive as an antibiotic. The 3'-OH group of this compound can again be acetylated by CAT to yield 1',3'-acetoxychloramphenicol, a product that is not functional as an antibiotic.



Genes specifying CAT in bacteria can be chromosomal or can be plasmid-located. In gram-negative bacteria, plasmid-encoded CATs are expressed constitutively (Fitton et al., 1978). Among the Enterobacteriaceae there are three types of plasmid-encoded CAT (Gaffney et al., 1978). The Type I CAT is widely distributed throughout this bacterial family. It is an integral part of transposon Tn9. In addition to catalysing the acetylation of Cm, it can bind fusidic acid strongly (Volker et al., 1982) and also rosaniline dyes (Procter and Rownd, 1982). The amino acid sequence of Type I CAT is known, as is the nucleotide sequence of its gene (Alton and Vapnek, 1979; Marcoli et al., 1980; Shaw et al., 1979). The amino acid sequences of the Type II and Type III CATs are known in part (Nitzan (Zaidenzaig) and Gozhansky, 1980; Zaidenzaig and Shaw, 1978). The nucleotide sequence of the Type III CAT gene is known (I.A. Murray, personal communication).

In Proteus mirabilis, a chromosomally-determined enzyme has been described which is related to Type I CAT (Zaidenzaig and Shaw, 1978). The gene encoding this enzyme has now been cloned and sequenced (I.G. Charles, personal communication). The plasmid-specified CAT of Haemophilus influenzae is related to CAT Type II (Roberts et al., 1982). The CAT enzyme from Bacteroides fragilis is also similar to CAT Type II (Bretz and Wilkinson, 1978). The Bacteroides ochraceus CAT is similar to CAT Type I (Shaw, 1983). Chromosomally-determined enzymes have been described in Agrobacterium spp. These enzymes are inducible (Haag et al., 1976; Zaidenzaig and Shaw, 1978). Chromosomally specified CATs have also been described in Flavobacterium sp. and Myxococcus sp. (Zaidenzaig and Shaw, 1978).

CAT enzymes from gram-positive bacteria have also been studied. Five variants have been described for staphylococcal CATs. Types A to D have been described by Davies and Smith (1978), Shaw (1971, 1983)

and Shaw et al., (1970). A fifth variant is that encoded by plasmid pC194 (Horinouchi and Weisblum, 1982a). Amino terminal sequences are available for the A, B and D proteins together with a more extended partial sequence for the Type C CAT (Fitton and Shaw, 1979).

Streptococcal CATs specified by the following species are related to the staphylococcal enzymes; Streptococcus agalactiae (Zaidenzaig and Shaw, 1978); S. faecalis (Courvalin et al., 1975); and S. pneumoniae (Dang-Van and Bouanchand, 1978). CAT activity has also been found in Clostridium perfringens (Zaidenzaig and Shaw, 1978), Bacillus pumilis (Keggins et al., 1978), and Streptomyces spp. (Shaw and Hopwood, 1976).

II Mechanism of acetylation of chloramphenicol by CAT

CAT inactivates Cm by catalysing the acetylation of the 3'-OH group of the drug. The source of the acetyl group is the cofactor acetyl-CoA, Fig. 1.2 (Shaw, 1967; Suzuki and Okamoto, 1967). An intramolecular rearrangement allows the acetyl group of 3'-acetoxy-chloramphenicol to be transferred to the 1'-OH position, yielding 1'-acetoxychloramphenicol. This molecule may then be enzymatically re-acetylated at the 3'-OH position to give the 1',3'-diacetoxy derivative of chloramphenicol (Fig. 1.2). All three acetoxy variants of Cm are inactive as antibiotics. They are unable to bind to bacterial ribosomes (Shaw and Unowsky, 1968). It is thought that this expenditure of a second molecule of acetyl-CoA to reacetylate the 3'-OH position of Cm may exert physiological pressure on cells carrying multiple copies of the CAT gene and so lead to the observed non-linear relationship between the number of enzyme molecules and the Cm^r level (Nordstrom et al., 1972).

III Catalytic properties of CAT

The catalytic properties of CAT have been reviewed by Shaw (1983). The staphylococcal C variant, the gram negative Type I and the ribosomal target for Cm (peptidyltransferase) all display an absolute specificity for the D-threo isomeric form of Cm. The amino group of carbon atom 2 of the propanediol chain must be substituted and the protons on carbon atoms 1 and 3 must be unsubstituted, although CAT is more tolerant than peptidyltransferase of C-2 amino group substitutions and para-substitutions of the phenyl group.

Type I and C variant CATs differ in their sensitivities to thiol inhibitors. The three types of enteric CAT are now known to have a cysteine residue near to the active site. This residue does not have a direct role in Cm inactivation but stabilises the active centre of the enzyme, hence the sensitivity of those CATs to thiol inhibitors. The current model for Cm-inactivation by CAT proposes the formation of a ternary complex in which Cm and acetyl-CoA are bound, together, at the active site. The active sites of CATs are highly conserved and are made up of seven amino acids. The second residue, a histidine, has long been known to be highly reactive and of central importance (Shaw, 1983). It has recently been shown to be directly involved in binding chloramphenicol (K. Kleanthous, personal communication).

IV Chloramphenicol resistance which does not involve CAT

(a) Chromosomally-determined non-CAT Cm^r

In the 1950s it was discovered that high-level Cm^r mutants of E. coli could be isolated by growing sensitive cells in progressively higher concentrations of Cm until the solubility limit for the drug, 2 mg/ml, was reached (Cavalli, 1952; Cavalli and Maccacaro, 1952). During the 1960s, mutations in the E. coli chromosome were reported

which conferred low-level Cm^r on the cells. The cells were resistant to between 1 and 5 $\mu\text{g}/\text{ml}$ of the antibiotic (Reeve, 1966). More than one locus was involved and in some cases cross-resistance to other drugs was discovered. One such mutation was designated cmlA. E. coli strains which carried it exhibited a four-fold increase in Cm^r level over the cmlA⁺ wild-type cells (Reeve, 1966; 1968). The mutations cmlB and lon specified resistance to tetracycline (Tc) as well as Cm (Reeve and Suttie, 1968). Strains which were cmlB showed a two-fold increase in Cm^r over the wild-type, and synergy was observed between cmlB and CAT when the latter was introduced on a plasmid (Foster, 1975). The locus cmlB has now been re-designed ompF, as it encodes the major outer membrane protein, OmpF (Lee et al., 1979; Reeve and Dougherty, 1968; Bachmann, 1983). It is thought that these mutations bring about a change in cell envelope permeability to Cm (Chopra and Eccles, 1978). George and Levy (1983a, b) have described amplifiable, high-level co-ordinate expression of Tc^r and Cm^r in E. coli. Cross-resistance to other, structurally unrelated drugs, was also noted. The strains involved did not contain plasmids and the Cm^r was not due to CAT. A locus, marA, was identified and shown to have a role in specifying an energy-dependent efflux mechanism for Tc. Whether this mechanism was involved in Cm^r is not known (George and Levy, 1983b). The mutations involved in Cm^r could not be co-transduced by phage P1 to sensitive cells, perhaps suggesting that the loci are widely spaced on the chromosome. The mechanism of amplification to high-level Cm^r (i.e. $>100 \mu\text{g}/\text{ml}$) was found to be polA and recA independent and it was proposed that amplification might involve gene duplication via illegitimate recombination (Anderson and Roth, 1977). Amplifiable chromosomally-specified co-resistance to Cm and Tc has also been reported in Staphylococcus epidermidis (Sugarman and Pesanti, 1980). There have also been reports of chromosomally-specified Cm^r in

Bacillus subtilis which does not involve CAT. One report gives details of a locus, cam-1 which encodes a modified ribosomal protein, thus giving rise to Cm^r via ribosomal modification (Osawa and Takata, 1973). The validity of this observation is now in doubt (W.V. Shaw, personal communication). A second report involves a locus, camZ, which specifies non-inducible Cm^r in B. subtilis strain 168. The mechanism of Cm^r is unknown, but does not involve CAT or altered ribosomal proteins, although camZ maps in the ribosomal protein region near dal. A change in cell permeability to Cm has been proposed as the possible mechanism (Anderson et al, 1984).

An enzymatic mechanism of Cm^r which does not involve CAT has been reported in anaerobic bacteria. Here, the drug is inactivated by reduction of the para-nitro group of chloramphenicol to yield an inactive amino derivative (Thadepalli et al, 1977). A similar enzyme has also been described by O'Brien and Morris (1971).

It is interesting to note that the chloramphenicol-producing organism, Streptomyces venezuelae, does not produce CAT, whereas other Streptomyces do produce this enzyme (Shaw and Hopwood, 1976). Here, Cm^r is thought to be due to a permeability barrier to drug re-entry following excretion from the cytoplasm, or perhaps even an active efflux mechanism capable of exporting Cm against a concentration gradient (Malik, 1972).

(b) Plasmid-determined non-CAT Cm^r

There have been numerous reports of plasmid-borne non-CAT Cm^r determinants, usually involving gram-negative bacteria, often from the genus Pseudomonas.

The plasmid KR102 of Pseudomonas aeruginosa carries a non-CAT Cm^r determinant which is expressed constitutively. It was described

by Kono and O'Hara (1976) and these authors advanced evidence for a permeability barrier to drug entry to the cell located at the outer membrane.

A non-CAT Cm^{r} determinant which may be carried within a transposon has been reported. Tn2001, the putative transposon, was discovered on an R plasmid of incompatibility group Inc P-2 in P. aeruginosa. The estimated size of the transposon was 2.1 Kb (Iyobe et al., 1981).

Four R plasmids from Escherichia coli have been reported which specify inducible non-CAT Cm^{r} . Resistance was found to be lost upon sphaeroplast formation, suggesting that the outer membrane has a role to play in Cm^{r} in this system (Nagai and Mitsuhashi, 1972).

Inc C plasmids from Serratia marcescans have been shown to encode a non-CAT Cm^{r} determinant. These plasmids include R935, R936, and R937 (Shapiro, 1977; Hedges et al., 1975).

Ingram and Hassan (1975) reported plasmids from P. aeruginosa which carried both a non-CAT and a CAT Cm^{r} determinant. These plasmids, R55 and R57b, were from incompatibility group C. R57b is distinct from R57b Inc N of Foster and Shaw, (1973). The non-CAT determinants of R55 and R57b were inducible and seemed to encode a Cm-specific permeability barrier. Segregants of R55 and R57b were isolated which no longer specified CAT. These plasmids confer Cm^{r} via the putative permeability barrier only. The plasmid derivatives were designated R55-1 and R57-1 respectively (Gaffney et al., 1978). R55-1 was used in some of the experiments described in this study (Chapter 3).

D. The multiple drug resistance R plasmid, R26

This plasmid was originally isolated from Pseudomonas aeruginosa by Stanisich et al. (1976). It specifies resistance to ampicillin, chloramphenicol, gentamicin, kanamycin, mercuric ions, neomycin,

streptomycin, sulphonamides, and tetracycline. It has an estimated molecular weight of 52 Md, which equates to about 75 Kb. R26 is self-transmissible over a broad host range. This self-transmissibility is due to the possession by the plasmid of transfer genes, tra. Features of the F and I tra systems have been reviewed recently and give some insight into how such systems function (Willetts and Wilkins, 1984). This system confers upon the plasmid the ability to transfer itself from one host to another via a conjugation mechanism, a process requiring direct cell-to-cell contact mediated by a plasmid-encoded pilus on the cell surface (Achtman and Skurray, 1977). R26 belongs to incompatibility group P-1 (Stanisich et al., 1976). Inc P plasmids encode short, blunt-ended pili (Bradley, 1974, 1976; Bradley and Cohen, 1977; Coetzee et al., 1979) which provide binding sites for male-specific bacteriophage PRR1, Pf₃, PR3, PR4, PRD1, and PR772 (Coetzee et al., 1979; Kehoe, 1979, Ph.D. thesis, Dublin University; Olsen and Shipley, 1973; Olsen et al., 1974; Olsen and Thomas, 1973; Stanisich, 1974). Plasmids of the Inc P-1 group do not express fertility inhibition (Sagai et al., 1977; Stanisich, 1974) and are thus designated fi⁻. An early system of plasmid classification assigned plasmids to one of two groups, fi⁺ or fi⁻, on the basis of their ability to inhibit the fertility of the F factor (Watanabe et al., 1964). This overly-simple method was superseded by incompatibility typing which relies on the inability of two plasmids from the same incompatibility group to coexist stably in the same cell (Datta, 1974). In simple terms, incompatibility can be looked upon as a manifestation of plasmid copy number control (Pritchard et al., 1969) or as a result of competition for a common segregation apparatus (Jacob et al., 1963; Novick and Hoppensteadt, 1978). For large plasmids, such as R26, copy number control is stringent [R26 exists in bacterial cells at one or two copies per chromosome (Stanisich et al., 1976)]. At such low copy numbers, these plasmids

require an efficient segregation mechanism if each daughter cell is to receive a copy at cell division. Thus, early theories regarded this requirement as the key to the incompatibility mechanism (Jacob et al., 1963). Since then much attention has been given to the role of plasmid copy number control mechanisms in determining incompatibility. Copy number control has been shown to be exerted in many plasmids by plasmid-encoded, diffusible molecules which bind to specific sites on the plasmid to modulate replication. In some systems these regulatory elements are RNA molecules, while in others they are proteins. In ColE1, an RNA of 108 nucleotides inhibits primer formation for the initiation of plasmid replication (Tomizawa et al., 1981); in Inc F II plasmids, such as R1, R6-5 and R100, incompatibility is exerted via a small RNA molecule (Kamio and Terawaki, 1983). In R6 K and F plasmids, a positively operating initiator protein has been found to interact with directly repeated nucleotide sequences in the replication region (Murotsu et al., 1981; Stalker et al., 1979). Early evidence for a plasmid-encoded, trans-acting diffusible molecule involved in incompatibility in F came from the observation that an F plasmid integrated into the host chromosome (and therefore no longer autonomously replicating) could exert an incompatibility effect on an autonomous F plasmid in the cytoplasm. Recently, an analogous phenomenon has been described for RP4, an Inc P-1 plasmid (Grinter, 1984).

The incB and incC regions of F contain the 196p directly repeated sequences which function as binding sites for the replication regulatory protein. In addition, a third incompatibility locus, incD, has been described. This is not involved in controlling plasmid replication but specifies functions required for the normal segregation of F into daughter cells at cell division (Hillenbrand et al., 1984). This finding shows that segregation can play a role in exerting incompatibility, in addition to copy number control.

The transfer region and replication region make up two of the three major components of R plasmids. The third component consists of the drug resistance genes. These are arranged differently in different R plasmids. In the Inc F II plasmid, R100, the resistance genes (with the exception of Tc^r) are clustered within an amplifiable region, the r-det (Perlman and Rownd, 1975; Fig. 1.3). In the Inc P-1 plasmid, RP4, the resistance genes are scattered randomly through the plasmid (Fig. 1.3). The physical arrangement of plasmid R26 is unknown.

E. Mechanism of chloramphenicol resistance specified by plasmid R26

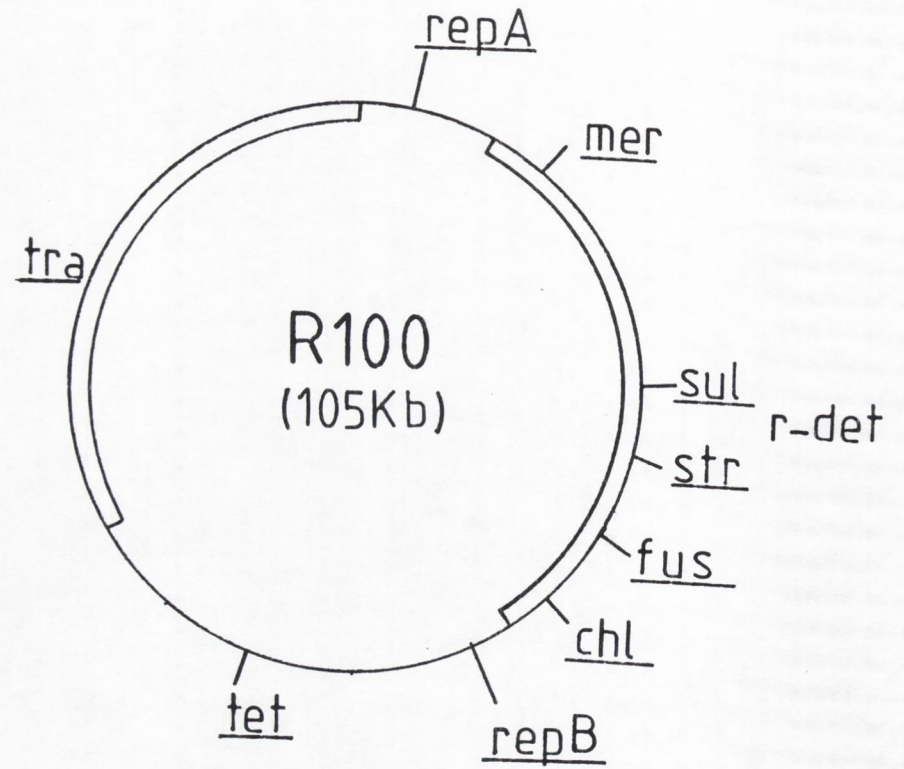
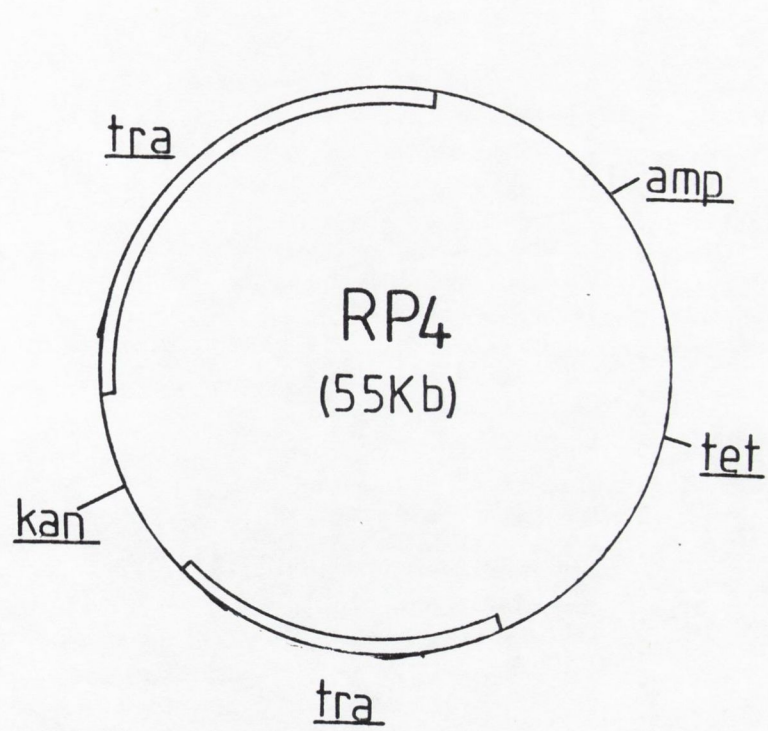
Experiments which attempted to elucidate the nature of the Cm^r mechanism specified by R26 were carried out by Gaffney et al., (1981). It was found that uninduced R26-carrying strains of E. coli K12 incorporated less (¹⁴C) leucine into cellular protein when challenged with high concentrations of Cm than was the case with induced strains providing evidence for enhanced gene expression following induction. It was also found that E. coli K12 strains harbouring R26 did not degrade the drug or modify it in any detectable way. This indicated that neither CAT nor any other enzymatic detoxification mechanism was involved in the resistance mechanism.

Ribosomes from sensitive and resistant cells were tested for Cm-sensitivity in an in vitro poly (U,G)-directed protein synthesising system. No detectable difference was recorded in the inhibition by Cm of (¹⁴C) valine incorporation into TCA-precipitable material with ribosomes from resistant or sensitive (i.e. plasmidless) cells. Results were unchanged whether induced or uninduced E. coli K12 J5-3 (R26) strains were used. This suggested that ribosome modification was not the mechanism of resistance.

Sphaeroplasts from R26-bearing strains were tested for Cm^r by monitoring the incorporation of (¹⁴C) leucine into cellular protein

FIGURE 1.3 Physical map of plasmids RP4 and R100

Both plasmids are self-transmissible, multiple drug resistance molecules. RP4 provides an example of an Inc P group plasmid, while R100 represents Inc F II. In RP4, the drug resistance determinants are scattered at random about the plasmid. One, the kanamycin resistance gene, lies between the two portions of the tra region. In R100, by contrast, all but one of the drug resistance determinants lie within the amplifiable r-det region. The outlying resistance determinant is the tetracycline resistance function. These plasmids are not drawn to scale.



in the presence of Cm. Sphaeroplasts were from both induced and uninduced cells and incorporation profiles were compared with both induced and uninduced intact cells. The sphaeroplasts and intact cells behaved similarly throughout, indicating that the resistance mechanism did not require the outer membrane or cell wall.

A constitutive derivative of R26 was generated by nitrosoguanadine mutagenesis (Gaffney et al., 1981) and used to construct double-plasmid strains with a CAT-specifying plasmid. Only marginal inactivation of the drug by CAT was detected, suggesting that Cm was being prevented from reaching the intracellular enzyme. These results implied that a permeability barrier to Cm entry was at work in these strains. The sphaeroplast experiment data indicated that this barrier was associated with the cytoplasmic membrane.

The Inc C plasmid, R55-1, described and studied in Chapter 3, was subjected to the same tests as R26 and similar results were obtained throughout. This suggested that the Cm^r mechanism specified by R55-1 was similar to that of R26.

F. Other resistance mechanisms which involve permeability barriers

Mechanisms for resistance to antibiotics and heavy metal ions, which involve permeability barriers, have been reported. These include resistance to tetracycline (Tc^r), Arsenate (As^r) and Cadmium (Cd^r).

Resistance to Tc is now known to depend on an energy-driven efflux mechanism (Foster, 1983). This was established by using spectrofluorimetry to demonstrate that resistant cells took up less Tc than sensitive cells and that any drug which had accumulated within the cytoplasm was actively excreted upon transfer of the Tc^r cells to a drug-free medium (Ball et al., 1980). Other workers demonstrated energy-dependent efflux using inverted plasma membrane vesicles

prepared from Tc^r bacteria. These were found actively to accumulate the antibiotic via a Tc-specific transport system (Hedstrom et al., 1982; McMurray et al., 1980). The resistance mechanisms described by these authors are plasmid-encoded. Chromosomally-located Tc^r determinants which involve drug efflux have also been described (George and Levy, 1983b). It is possible that ribosome protection may have a role to play in Tc^r, in addition to the efflux mechanism (Levy and McMurray, 1978; Levy et al., 1977). Tetracycline resistance has been reviewed recently by Foster (1983), and Chopra (1984).

Arsenate ions are toxic to bacterial cells (Summers and Silver, 1978). The metal ions are excreted from Staphylococcus aureus and Escherichia coli cells by an arsenate-specific, energy-dependent efflux system. This is plasmid-encoded and does not depend on chemiosmotic potential or a pH gradient across the membrane. Studies which led to its elucidation were conducted with resistant cells pre-loaded with radioactive $^{74}\text{AsO}_4^{3-}$ (Mobley and Rosen, 1982; Silver and Keach, 1982).

Cadmium ions are respiratory poisons in bacterial cells (Tynecka et al., 1981a; 1981b; Weiss et al., 1978). Cd^r is plasmid-encoded in S. aureus but has not been described in other bacteria (Foster, 1983). It is mediated via two different determinants. CadA specifies high-level resistance and involves a Cd²⁺-specific efflux mechanism (Perry and Silver, 1982; Smith and Novick, 1972; Tynecka et al., 1981). CadB determines low-level Cd^r via an uncharacterised mechanism which does not involve efflux (Perry and Silver, 1982). It has been shown by experiments using respiratory chain uncouplers and ionophores that cadA Cd^r cells expel the metal ions via a Cd²⁺/2H⁺ antiport system. This is chemiosmotically-driven, with two protons taken up for each Cd²⁺ ion expelled (Tynecka et al., 1981a; 1981b).

Some other, less well-understood, resistance determinants are thought to involve permeability barriers. Plasmid-determined inducible

fusidic acid resistance in S. aureus may involve decreased permeability. This hypothesis is based on the findings that ribosomes from resistant and sensitive organisms were equally sensitive to the drug in vitro, there was no detectable drug modification, and the ratio of phosphatidylglycerol to lysylphosphatidylglycerol differed between resistant and sensitive cells (Chopra, 1976).

Resistance to silver ions (Ag^{2+}) is thought to be located at the cell envelope in gram-negative bacteria. This function prevents resistant cells from extracting ionic silver from non-toxic AgNO_3 in the presence of halide ions. Sensitive cells do extract silver from AgCl_2 precipitate. The metal is a respiratory poison in its cationic form (Bragg and Rainnie, 1974; Silver, 1981). The determinant is expressed constitutively and confers a high level of resistance (Silver, 1981).

Decreased permeability has been proposed as the mechanism of plasmid-encoded resistance to acridine, acriflavine, ethidium bromide and proflavine. This proposal is based on the results of binding studies (Johnson and Dyke, 1969).

A binding or "sponge" mechanism has been proposed for Type I CAT-mediated fusidic acid resistance in gram-negative bacteria. Fusidic acid diffuses slowly into the gram-negative cells cytoplasm. There it can be bound by CAT within a hydrophobic domain within the enzyme. The drug is not modified in any way, but is sequestered by CAT before it can reach its target (Shaw, 1983). Type I CAT can also bind rosanilin dyes, such as crystal violet, in this way (Proctor and Rownd, 1982). A similar mechanism has been proposed for the poorly-understood cadB-determined Cd^{F} system already mentioned (Perry and Silver, 1982).

G. Aims of the project

The work documented in this thesis was undertaken to provide some insight into (i) the mode of regulation of the R26 Cm^r determinant (cml) and (ii) the mechanism of resistance which it specifies. The approach taken was a molecular genetic one, involving cloning, mapping, Southern blotting, and deletion and transposon mutagenesis to discover the extent of DNA involved in specifying Cm^r; the generation of both transcriptional and translational lac fusions to determine the level at which cml expression is controlled; the use of the E. coli minicell system to identify the gene product and the use of DNA sequencing techniques to reveal (i) the nucleotide sequence of the determinant, (ii) the predicted amino acid sequence of the gene product and (iii) information concerning the mechanism of regulation of cml.

CHAPTER 2

MATERIALS AND METHODS (GENERAL)

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D. Composition of the media	20
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MATERIALS AND METHODS (GENERAL)

A. Bacterial strains, plasmids and bacteriophages

The bacterial strains, plasmids and bacteriophages used in this study are described in the Materials and Methods sections of those chapters in which their use is documented.

B. Chemicals

These were of analytical grade (unless otherwise stated) and were purchased from Sigma (U.K.) or British Drug Houses (B.D.H., U.K.). Details of the chemicals employed are given in Chapters 3, 4, 5, 6 and 7.

C. Antibiotics

Ampicillin was a gift from Beecham (U.K.); chloramphenicol, gentamicin, kanamycin, neomycin, streptomycin, sulphamylamide and tetracycline were purchased from Sigma. Nalidixic acid was obtained from Winthrop (U.K.). Trimethoprim was purchased from Wellcome (U.K.) and mercuric chloride was from B.D.H. (U.K.).

D. Composition of the media

L broth: contained 10.0g tryptone (Oxoid) and 5.0g yeast extract (Difco) per litre of distilled water; pH 7.0-7.2.

L agar: as for L broth but with 1% (w/v) Oxoid No. 3 agar.

Nutrient broth: 5.0g lab-lemco (Oxoid), 10.0g peptone (Oxoid), 5.0g NaCl. This was made up to 1 litre with distilled water; pH 7.4.

Nutrient agar: as for nutrient broth but with 20.0g agar (B.D.H.)

M9 medium: KH_2PO_4 (3.0g), NaCl (0.5g), Na_2HPO_4 (6.0g), and NH_4Cl (1.0g) were added to distilled water and autoclaved. Then 1.0 ml of a 1M MgSO_4 solution and 10.0 ml of a 0.01M CaCl_2 solution were added, the final volume being adjusted to 1 litre with distilled sterile H_2O .

Minimal broth: This contained 10% (v/v) of M9 solution, 10% (v/v) of a 20% (v/v) glucose solution, 1% (v/v) of a 30% (w/v) vitamin B_1 solution, 10% (v/v) of a 20% (w/v) Casamino acids (Oxoid) solution and 0.1% (v/v) of a 30% (w/v) MgCl_2 solution. Distilled water was added to give required volume.

Minimal agar: As for minimal broth but with 2% Difco Bacto agar added in place of distilled water.

MacConkey's agar: 20.0g peptone (Oxoid), 5.0g sodium taurocholate (Oxoid), 5.0g NaCl, 10.0g lactose and 20.0g agar (B.D.H.) per litre distilled water; pH 7.4. 7.5 ml of 2.0% (w/v) neutral red in 50% (v/v) alcohol was added per litre before use. For short-term storage, MacConkey's agar was kept in the dark at room temperature.

Lambda base agar: 10.0g tryptone (Oxoid), 2.5g NaCl, 1.0% (w/v) Difco Bacto agar per litre of distilled water; pH 7.4.

Lambda top agar: as for the base but with 0.5% (w/v) Difco Bacto agar.

Brain Heart Infusion Broth: 12.5g calf brain infusion solids, 5.0g beef heart infusion solids, 10.0g protease peptone (Oxoid L46), 2.0g dextrose; 5.0g NaCl, 2.5g Na_2HPO_4 in distilled water; pH 7.4

Dorset Egg medium: Two volumes of whole chicken eggs were mixed with one volume of beef infusion broth, filtered through gauze and dispensed into glass Bijou bottles. The medium was sterilised by heating to 75° for 20 min on two successive days.

E. Lambda phage buffer

This contained 0.01M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.4% (w/v) NaCl in 0.01M Tris-hydrochloride (pH 7.2).

F. General culture conditions

For most routine purposes, L broth or L agar was employed. Minimal medium, appropriately supplemented, was used to select auxotrophic markers or to detect resistance to sulphanilamide or trimethoprim. In general, cultures were incubated for 15h at 37° (broth cultures with shaking). Bacterial strains were stored at room temperature on Dorset Egg slopes. Detailed information about culture conditions for specific purposes is given in Chapters 3, 4, 5, 6 and 7.

All other procedures and materials used in this study are described in the relevant Results Chapters.

CHAPTER 3

STUDIES ON RESISTANCE TO FLUORINATED
DERIVATIVES OF CHLORAMPHENICOL SPECIFIED
BY THE NON-CAT CHLORAMPHENICOL RESISTANCE
DETERMINANTS OF PLASMIDS R26 AND R55-1

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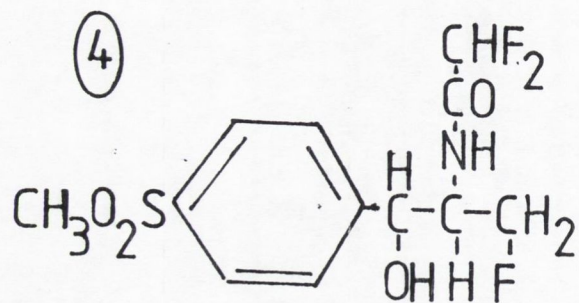
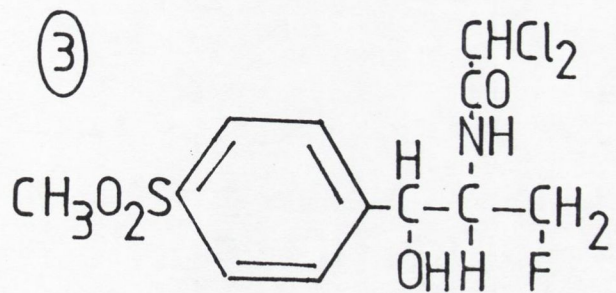
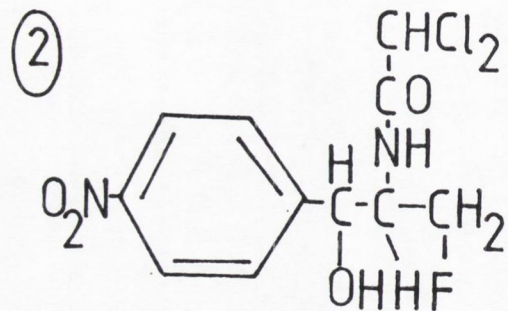
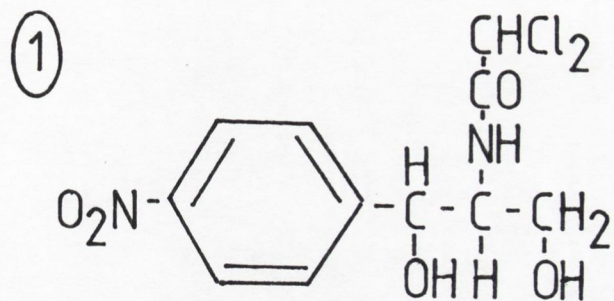
Studies on resistance to fluorinated derivatives of chloramphenicol specified by the non-CAT Cm^r determinants of plasmids R26 and R55-1

INTRODUCTION

Derivatives of chloramphenicol have been chemically synthesised with structures which enable them to evade acetylation by CAT (Nagabhushan et al., 1980). These drugs, designated SCH24893, SCH25298 and SCH25393, were developed by the Schering Corporation and their structures are illustrated in Figure 3.1. In each case, the 3'-OH group (the site of acetylation by CAT), has been fluorinated. These drugs are no longer a substrate for CAT and so are effective against chloramphenicol resistant bacteria which rely on this enzyme for resistance (Neu et al., 1980; Schafer et al., 1980; Syriopoulou et al., 1981). No investigation had been conducted to see whether or not these antibiotics were effective against bacterial strains which rely on non-CAT determinants to specify Cm^r. Here, experiments are reported which investigated the possibility that the non-CAT Cm^r determinants of plasmids R26 and R55-1 might confer resistance to the fluorinated analogues.

FIGURE 3.1 Structural formulae of chloramphenicol and its analogues

1. Chloramphenicol D(-)-threo-2-dichloroacetamido-1-p-nitrophenyl-1,3-propanediol .
2. SCH24893 D(-)-threo-2-dichloroacetamido-1-p-nitorphenyl-3-fluoropropan-1-ol .
3. SCH25298 D(-)-threo-2-dichloroacetamido-1-p-methylsulphonylphenyl-3-fluoropropan-1-ol .
4. SCH25393 D(-)-threo-2-difluoracetamido-1-p-methylsulphonyl-3-fluoropropan-1-ol .



MATERIALS AND METHODS

A. Bacterial strains and plasmids

The host strain used was Escherichia coli K12 J5-3 (pro met) [Clowes and Hayes, 1968]. The plasmid R26 has been described in Chapter 1. The plasmid R55-1 belongs to Inc C and is a spontaneous deletion mutant of R55, a plasmid specifying Cm^r via both CAT and a non-CAT determinant (Gaffney et al., 1981). R55-1 has lost the CAT determinant while retaining the non-CAT determinant. It has a molecular weight of about 135 Kb and also specifies resistance to sulphonamides. It is self-transmissible by conjugation.

B. Minimum inhibitory concentration (MIC) determinations

These were carried out by inoculating 10^3 mid-log phase cells onto Lemco nutrient agar plates containing antibiotics at the following concentrations: 0.5, 1, 2, 3, 4, 5, 7.5, 10, 20, 30, 40, 50, 75, 100, 125 and 150 µg/ml. The lowest concentration which inhibited growth was recorded as the MIC. The reported values were the means of four independent experiments. Uninduced and induced cultures were compared for both R26- and R55-1-containing strains. Cultures were induced by adding 1 µg/ml of the appropriate drug 60 min. prior to plating. Plates were then incubated overnight at 37°.

C. Growth and Challenge Tests

Cultures to be tested were grown in Lemco broth to an optical density of 0.1 ($\lambda = 657$ nm, Varian Techtron Spectrophotometer) in 250 ml flasks (Gallenkamp Orbital incubator, 37° 200 r.p.m.). Where appropriate, cultures were induced by adding 1 µg/ml of antibiotic 60 min prior to challenge. The challenging doses were as follows:

chloramphenicol: 15 $\mu\text{g}/\text{ml}$ (R26), 35 $\mu\text{g}/\text{ml}$ (R55-1); SCH24893: 5 $\mu\text{g}/\text{ml}$ (R26), 7.5 $\mu\text{g}/\text{ml}$ (R55-1); SCH25298: 5 $\mu\text{g}/\text{ml}$ (R26), 5 $\mu\text{g}/\text{ml}$ (R55-1); SCH25393: 5 $\mu\text{g}/\text{ml}$ (R26), 5 $\mu\text{g}/\text{ml}$ (R55-1). Graphs were then plotted of optical density against time.

Each compound was tested as an inducer of resistance to each of the four drugs. Thus, each drug was studied on the role of inducing agent and challenging agent. For the purpose of induction, 1 $\mu\text{g}/\text{ml}$ of the compound was used in each case. These experiments were done for both R26 and R55-1.

RESULTS

The host strain used in these experiments, E. coli K12 J5-3, showed a similar intrinsic resistance to Cm, SCH25298 and SCH25393 as measured in the MIC tests (Table 3.1). A higher intrinsic resistance was recorded to SCH24893. Growth and challenge tests were carried out to test the response of broth cultures of the plasmid-free host to the inducing concentrations of the four drugs and to record the growth pattern of the host under uninduced/challenged or induced/challenged conditions (Fig. 3.2). Data for all four drugs in the role of inducer are shown, although only data for SCH25298 as challenging agent are given. The results obtained with the other three antibiotics as challenging agents were similar. From the growth curves, it can be seen that 1 µg/ml of any of these drugs depressed the growth-rate of the plasmid-free host strain considerably and that no resistance whatsoever was recorded to the challenging concentration. As expected, prior incubation with a sub-toxic concentration of antibiotic made no difference to the growth rate when challenged, as compared with the uninduced control.

A marked difference in MIC value was recorded for J5-3 (R26) and J5-3 (R55-1) with respect to resistance to Cm (Table 3.1). In each case a two-fold increase in resistance was recorded for the induced culture over the uninduced control but the MIC value for J5-3 (R55-1) was about two-fold higher than that for J5-3 (R26).

These strains were tested in growth and challenge experiments in which each strain was induced in turn with each of the four antibiotics and then challenged with Cm (Fig. 3.3 and 3.7). It was found that resistance to Cm was inducible in each strain and that all four drugs were efficient inducers of resistance. Furthermore it was found that Cm^r was induced more rapidly in J5-3 (R55-1) than

TABLE 3.1 Minimum inhibitory concentration determinations

- a. The numbers are the means* of four independent experiments.
- b. Induction was with $1\mu\text{g/ml}$ of Cm or analogue for 60 min.
prior to plating.

*Note: no variation was seen between replicates.

(i) E. coli K12 J5-3 (plasmid-free)

Minimum inhibitory concentration ($\mu\text{g/ml}$)^a

Induction ^b Challenge	-	Cm	SCH24893	SCH25298	SCH25393
Cm	1	1	1	1	1
SCH24893	4	4	4	4	4
SCH25298	2	1	1	1	1
SCH25393	2	1	1	1	1

(ii) E. coli K12 J5-3 (R26)

Minimum inhibitory concentration ($\mu\text{g/ml}$)^a

Induction ^b Challenge	-	Cm	SCH24893	SCH25298	SCH25393
Cm	15	30	30	30	30
SCH24893	4	4	4	4	4
SCH25298	3	5	5	5	5
SCH25393	3	4	4	4	4

(iii) E. coli K12 J5-3 (R55-1)

Minimum inhibitory concentration ($\mu\text{g/ml}$)^a

Induction ^b Challenge	-	Cm	SCH24893	SCH25298	SCH25393
Cm	30	50	50	50	50
SCH24893	4	4	4	4	4
SCH25298	7.5	10	10	10	10
SCH25393	5	5	5	5	5

FIGURE 3.2 Growth and challenge test data for the plasmid-free host, *E. coli* K12 J5-3

In all panels, the challenging drug was SCH25298 (7.5 μ g/ml). Similar results were obtained with Cm (15 μ g/ml), SCH24893 (5 μ g/ml), SCH25298 (5 μ g/ml), and SCH25393 (5 μ g/ml). Panel 1, "induction" by Cm; Panel 2, "induction" by SCH24893; Panel 3, "induction" by SCH25298; Panel 4, "induction" by SCH25393. These data illustrate the effects of inducing concentrations of the four drugs on the plasmid-free host. Symbols: (●) uninduced, unchallenged; (o) induced, unchallenged; (□) induced, challenged; (■) uninduced, challenged.

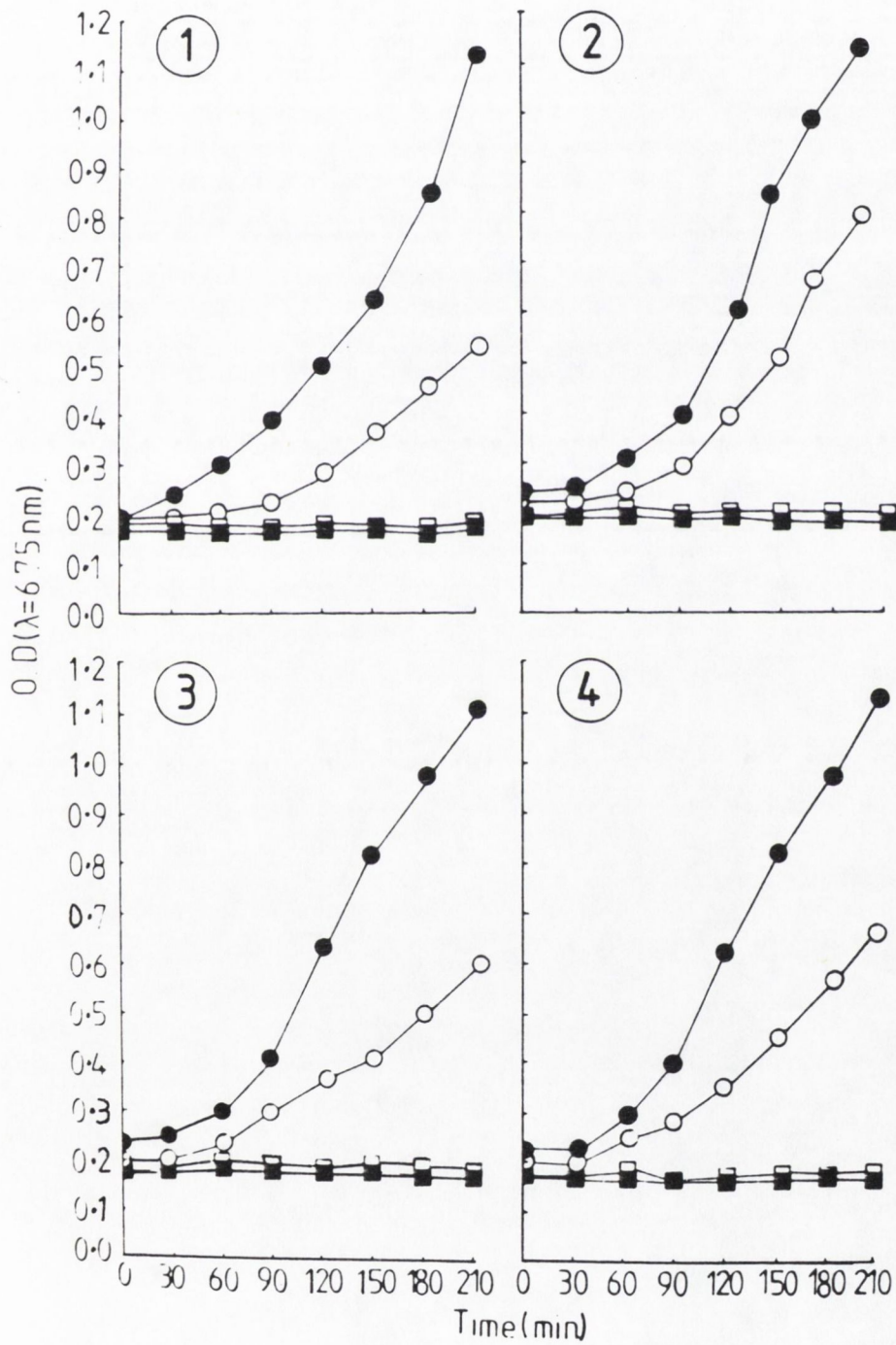


FIGURE 3.3 Growth and challenge test data for E. coli K12 J5-3
(R26) challenged with chloramphenicol (15 μ g/ml)

Panel 1, induction by Cm; Panel 2, induction by SCH24893; Panel
3, induction by SCH25298; Panel 4, induction by SCH25393.

Symbols: (●) uninduced, unchallenged; (◐) induced, unchallenged;
(◑) induced, challenged; (■) uninduced, challenged.

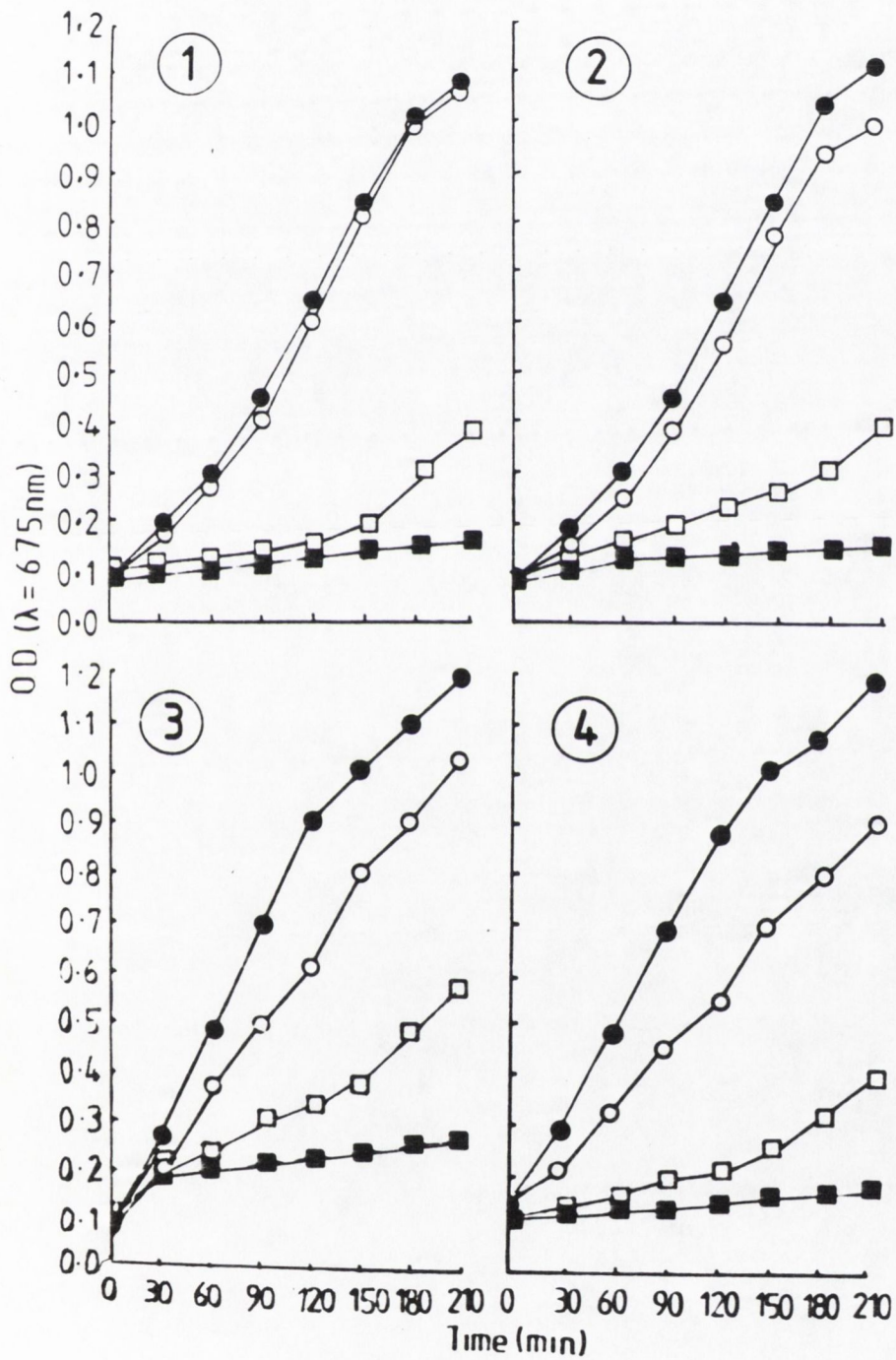


FIGURE 3.4 Growth and challenge test data for *E. coli* K12
J5-3 (R26) challenged with SCH24893 (5 µg/ml)

Panel 1, induction by Cm; Panel 2, induction by SCH24893;

Panel 3, induction by SCH25298; Panel 4, induction by SCH25393.

Symbols: (●) uninduced, unchallenged; (○) induced, unchallenged;

(□) induced, challenged; (■) uninduced, challenged.

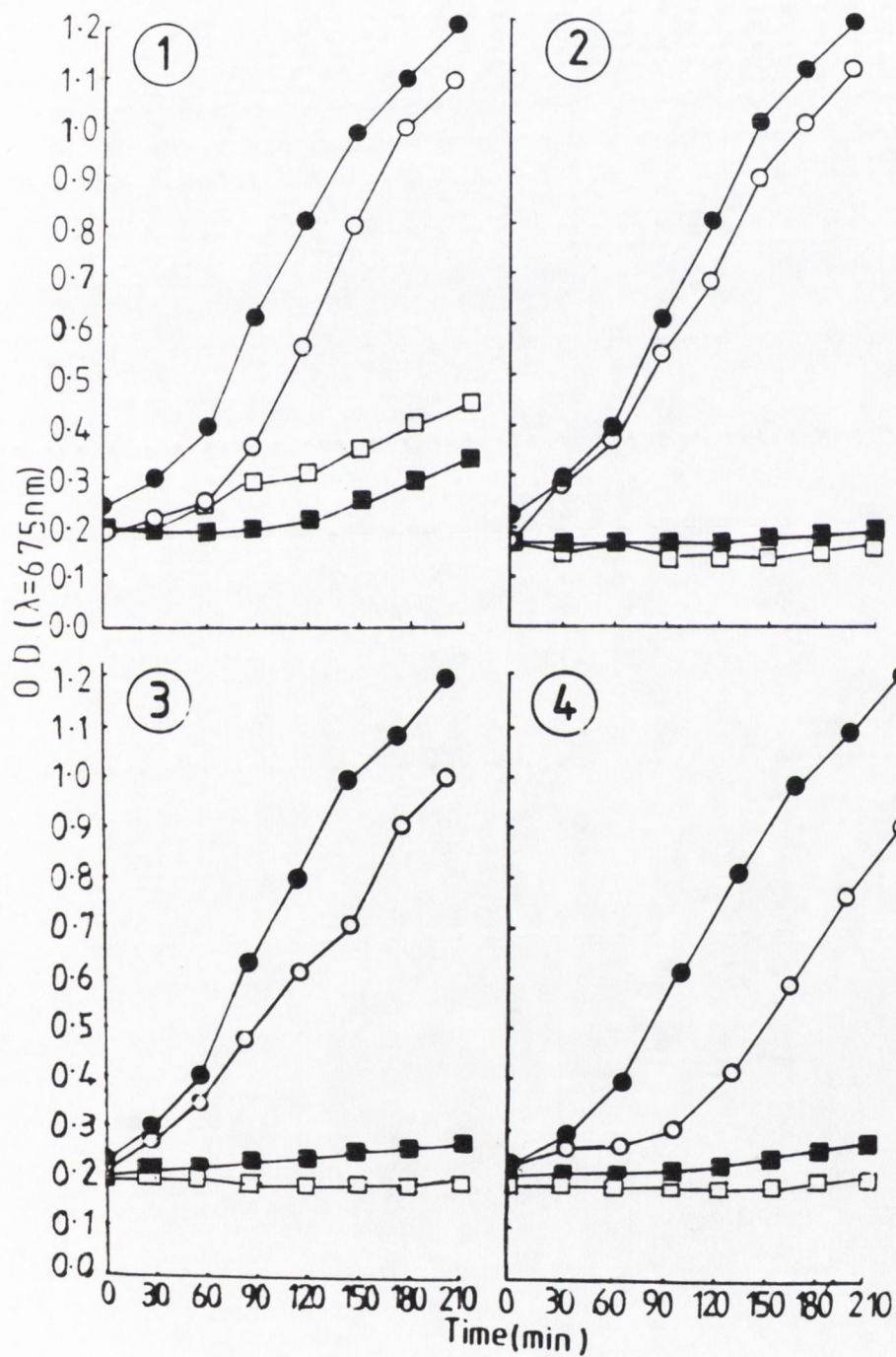


FIGURE 3.5 Growth and challenge test data for *E. coli* K12 J5-3
(R26) challenged with SCH25298 (5 µg/ml)

Panel 1, induction by Cm; Panel 2, induction by SCH24893; Panel
3, induction by SCH25298; Panel 4, induction by SCH25393.
Symbols: (●) uninduced, unchallenged; (○) induced, unchallenged;
(◻) induced, challenged; (■) uninduced, challenged.

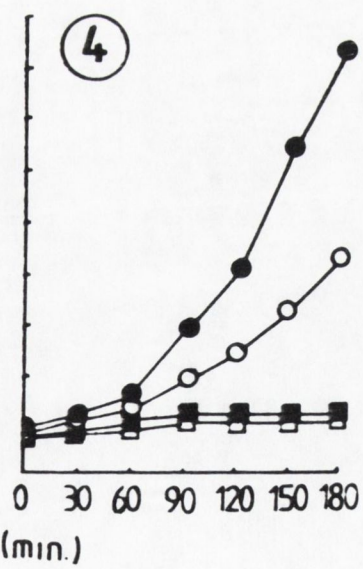
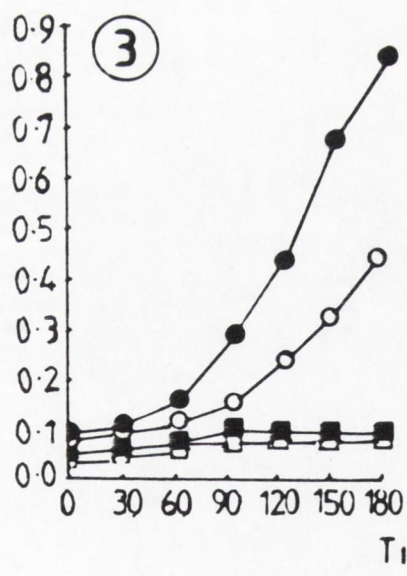
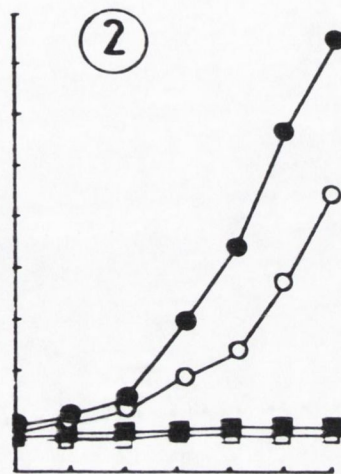
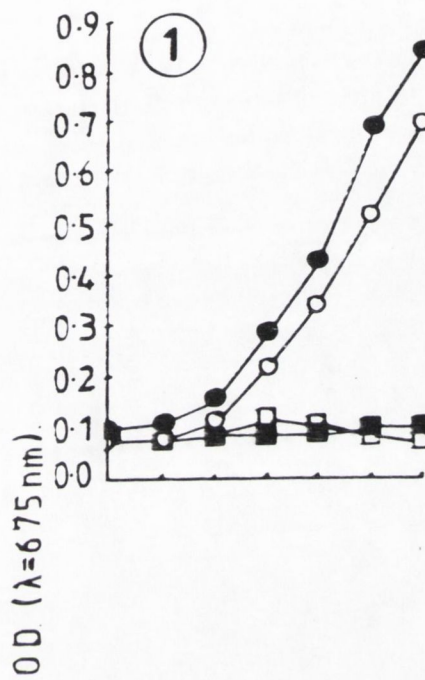


FIGURE 3.6 Growth and challenge test data for *E. coli* K12 J5-3
(R26) challenged with SCH25393 (5 µg/ml)

Panel 1, induction by Cm; Panel 2, induction by SCH24893; Panel 3, induction by SCH25298; Panel 4, induction by SCH25393.

Symbols: (●) uninduced, unchallenged; (○) induced, unchallenged; (◻) induced, challenged; (◼) uninduced, challenged.

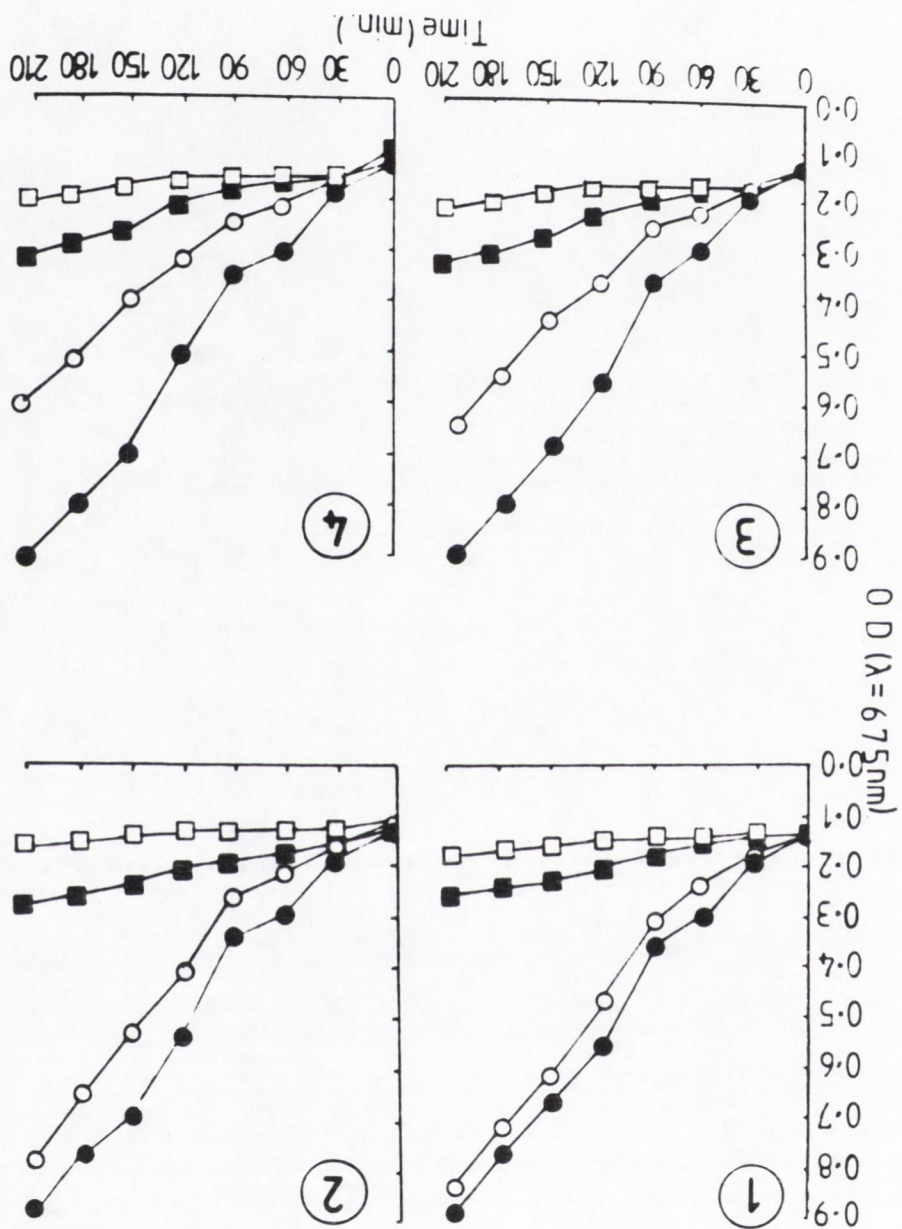
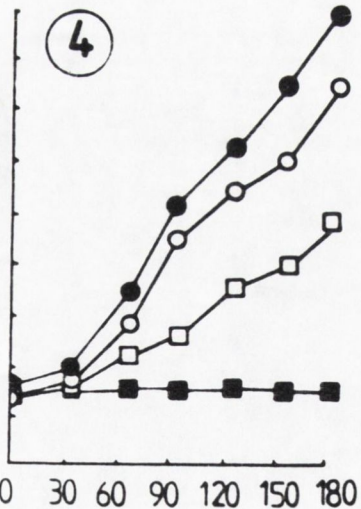
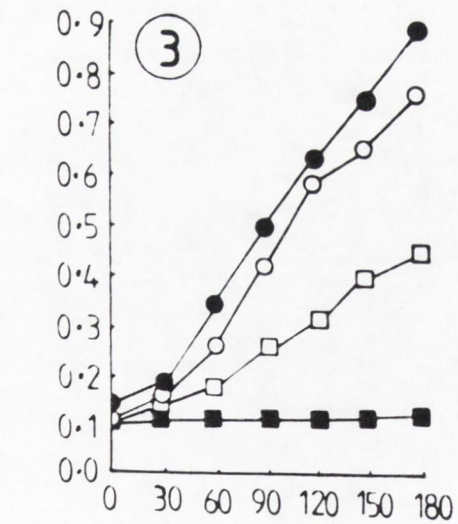
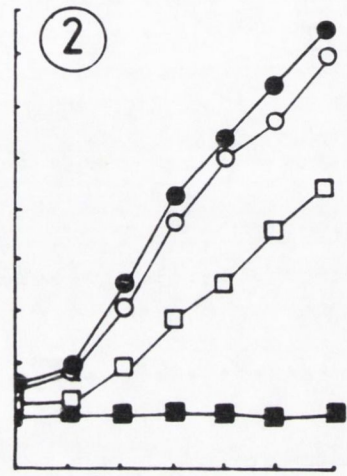
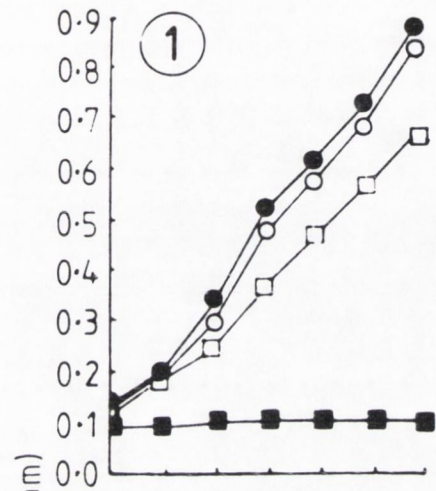


FIGURE 3.7 Growth and challenge test data for *E. coli* K12 J5-3
(R55-1) challenged with chloramphenicol (35 µg/ml)

Panel 1, induction by Cm; Panel 2, induction by SCH26893; Panel
3, induction by SCH25298; Panel 4, induction by SCH25393.

Symbols: (●) uninduced, unchallenged; (○) induced, unchallenged;
(◻) induced, challenged; (◼) uninduced, challenged.



Time (min.)

O.D. ($\lambda = 675 \text{ nm}$)

FIGURE 3.8 Growth and challenge test data for *E. coli* K12 J5-3
(R55-1) challenged with SCH24893 (8.5 μ g/ml)

Panel 1, induction by Cm; Panel 2, induction with SCH24893; Panel
3, induction with SCH25298; Panel 4, induction with SCH 25393.
Symbols: (●) uninduced, unchallenged; (○) induced, unchallenged;
(□) induced, challenged; (■) uninduced, challenged. .
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FIGURE 3.9 Growth and challenge test data for *E. coli* K12 J5-3
(R55-1) challenged with SCH25293 (5 µg/ml)

Panel 1, induction by Cm; Panel 2, induction by SCH24893; Panel 3, induction by SCH25298; Panel 4, induction by SCH25393.

Symbols: (●) uninduced, unchallenged; (○) induced, unchallenged;
(◻) induced, challenged; (◼) uninduced, challenged.

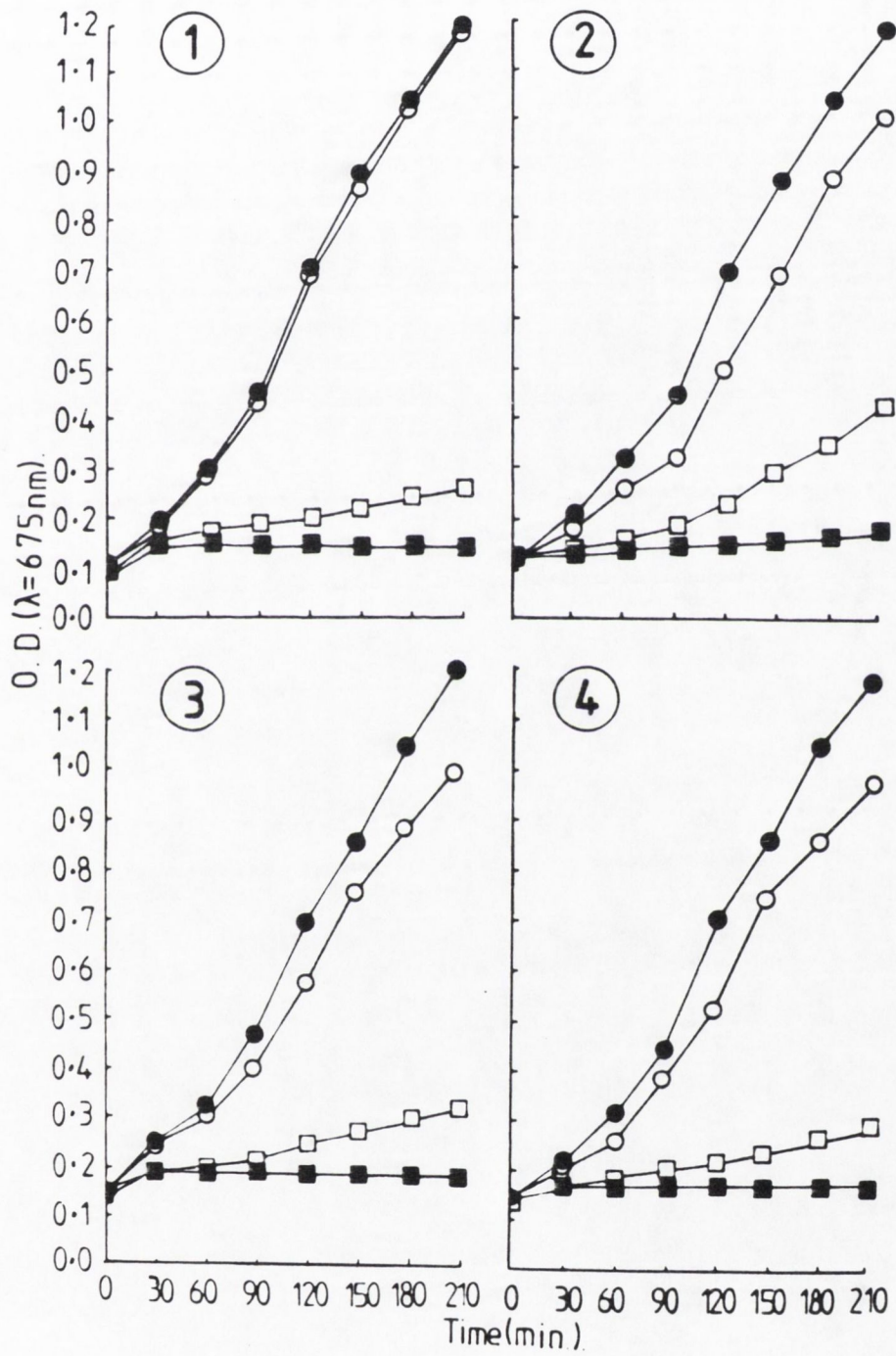
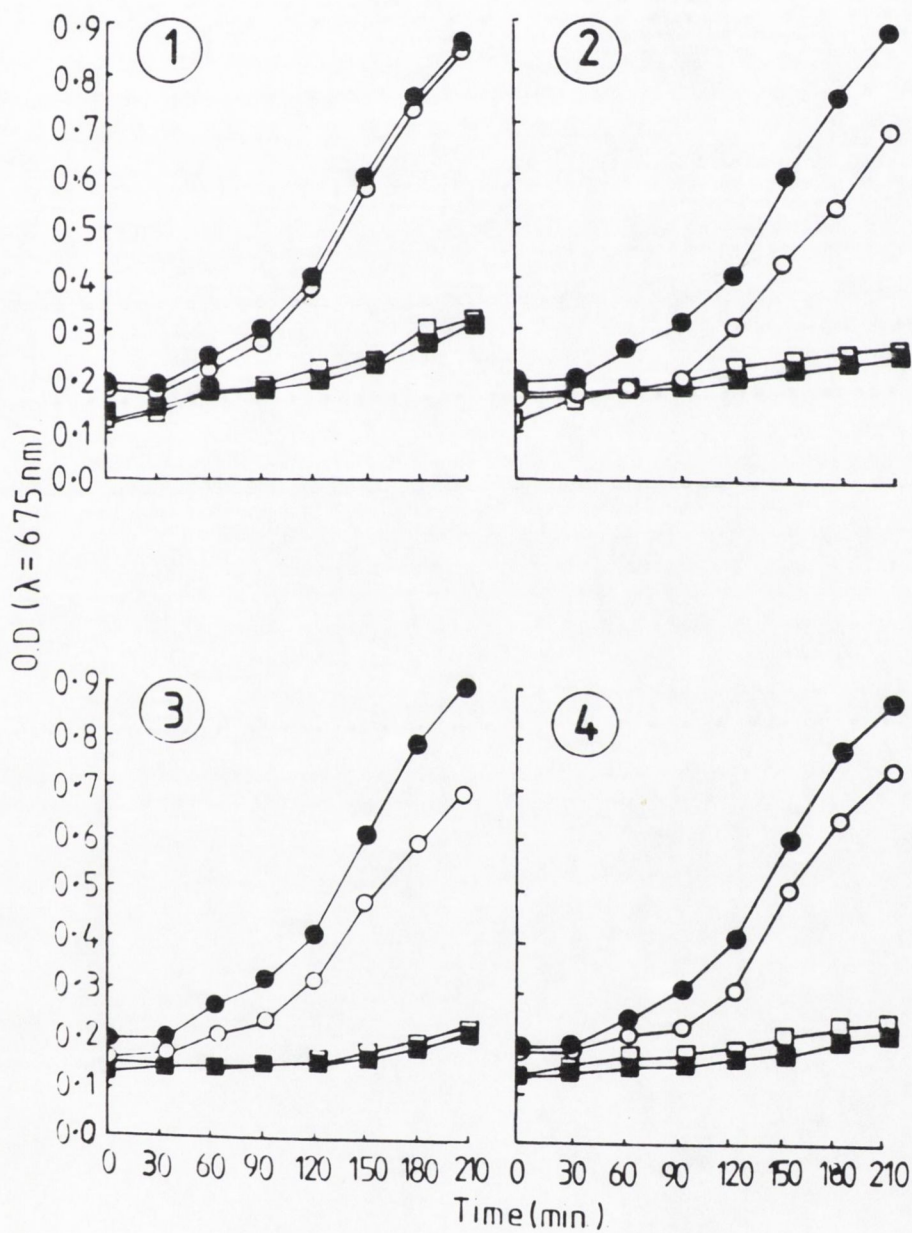


FIGURE 3.10 Growth and challenge test data for *E. coli* K12 J5-3
(R55-1) challenged with SCH25393 (5 μ g/ml)

Panel 1, induction by Cm; Panel 2, induction by SCH24893; Panel 3, induction by SCH25298; Panel 4, induction by SCH25393.

Symbols: (●) uninduced, unchallenged; (○) induced, unchallenged;
(◻) induced, challenged; (■) uninduced, challenged.



in J5-3 (R26), as indicated by the time taken for the induced/challenged growth curves to diverge from the uninduced/challenged controls. In all growth and challenge tests the effect of the inducing concentration of drug on the plasmid-bearing host was investigated. Each was found to have some inhibitory effect, Cm being less inhibitory than the other three (Figs. 3.3-3.10).

Neither R26 nor R55-1 conferred additional resistance to SCH24893 on E. coli K12 J5-3, plasmid-free and plasmid-containing cells having the same MIC values (Table 3.1). Resistance levels were not increased upon induction by any of the four antibiotics. This observation was supported by the results of growth and challenge tests (Figs. 3.4 and 3.8).

A different situation was found to obtain in the case of SCH25298. Here, induced resistance levels were higher than those of uninduced controls (Table 3.1). In the case of J5-3 (R26) the induced MIC value was 60% higher than the uninduced value (5 $\mu\text{g/ml}$ compared to 3 $\mu\text{g/ml}$) while in the case of J5-3 (R55-1) the induced value was 30% greater than the uninduced MIC (10 $\mu\text{g/ml}$ compared to 7.5 $\mu\text{g/ml}$). All four drugs proved to be equally effective as inducers of resistance in both strains (Table 3.1). Growth and challenge tests did not confirm the observation that low-level (i.e. 5 $\mu\text{g/ml}$) resistance to SCH25298 could be induced in J5-3 (R26) by the four drugs (Fig. 3.5). However, inducible resistance to SCH25298 was recorded in the case of J5-3 (R55-1) and the growth curves suggested that induction occurred with equal efficiency with all four antibiotics (Fig. 3.9).

MIC experiments showed a slight (25%) increase in resistance to SCH25393, the third fluorinated analogue, for induced cultures of J5-3 (R26) compared to the uninduced control. These experiments showed no evidence of inducible resistance to this drug in the case

of J5-3 (R55-1) [Table 3.1]. Growth and challenge data indicated that resistance to SCH25393 was not inducible in J5-3 (R26) [Fig. 3.6] or in J5-3 (R55-1) [Fig. 3.10]. This was true regardless of which drug was used as an inducer.

DISCUSSION

The E. coli K12 strains J5-3 (R26) and J5-3 (R55-1) were tested for resistance to the fluorinated derivatives of chloramphenicol, SCH24893, SCH25298 and SCH25393. The resistance patterns, as determined by MIC tests and growth and challenge tests, were compared with those for Cm^R as specified by these two strains. The induced Cm^R MIC values were different for J5-3 (R26; 30 μ g/ml) and J5-3 (R55-1; 50 μ g/ml) suggesting that the Cm^R determinant in the latter strain was more effective at protecting its host from chloramphenicol inhibition. Growth and challenge tests gave a qualitative impression of the responses of these strains to chloramphenicol challenge. Induced strains of J5-3 (R55-1) entered log phase much sooner than did induced strains of J5-3 (R26) after challenge with Cm, suggesting that the Cm^R determinant of R55- responds more rapidly to induction.

Both MIC experiments and growth and challenge tests showed that SCH24893 was the least inhibitory of the four drugs to E. coli K12 J5-3. However, possession of a Cm^R R plasmid gave no increase in resistance to this drug, suggesting that it can evade the Cm^R mechanism in these strains. SCH25298 and SCH25393 were more effective than SCH24893 against the plasmid-free host. Perhaps these drugs bind more effectively to the ribosomes in this strain. The MIC data for these antibiotics were very similar to those for chloramphenicol itself (Table 3.1). This may be because their ribosome-binding efficiency matches that of Cm closely. However, in the plasmid-containing strains, these drugs inhibited growth more effectively than Cm, as shown by the growth curves from growth and challenge tests. Moreover, the MIC values for these compounds were several

fold lower than those for Cm itself. Thus SCH25298 and SCH25393 are more effective at evading the non-CAT Cm^r specified by R26 and R55-1 than is Cm. SCH25393 was effective against the plasmid-bearing strains to the same degree as SCH24893, as reflected by similar MIC values for the two compounds and the fact that increased resistance to them could not be induced.

SCH25298 differed from the other analogues in that an increase in resistance was recorded upon induction. Of the three fluorinated analogues, this was the least effective against the plasmid-containing strains.

Possibly, the 3'-OH group of Cm has a role to play in the non-CAT Cm^r specified by plasmids R26 and R55-1. When this group is replaced by a fluorine atom the resulting compound is no longer as effectively prevented from causing inhibition of translation by the Cm^r determinants of these R-factors.

In contrast, fluorination did not impair the ability of these inhibitors to induce resistance to Cm. With both J5-3 (R26) and J5-3 (R55-1) 1µg/ml of each analogue induced resistance to Cm with the same efficiency as 1µg/ml of Cm itself, as determined by MIC experiments. Growth and challenge tests confirmed this. Therefore, the mechanism of induction by the drugs does not depend upon the retention of the 3'-OH group. Nor does it depend upon the presence of the para-nitro group nor the two chlorine atoms on the dichloro-acetamido side chain. The former group is replaced in both SCH25298 and SCH25293 while the chlorine atoms are replaced in SCH25393 (Fig. 3.1). The induction phenomenon may be due to either a structural or a functional facet of the drugs. The structural aspect could involve some part of the Cm molecule other than those which were

altered in the synthesis of the analogues. The functional facet could involve the ability of these drugs to inhibit translation through binding to the ribosome. Alternatively, induction may be due to the interaction of the antibiotics with some cellular constituent other than the ribosome.

No other investigation of the responses of inducible non-CAT Cm^r determinants to fluorinated Cm analogues has been carried out to date. Syriopoulou et al., (1981) reported strains of Haemophilus influenzae which were Cm^r but had extremely low levels of intracellular CAT indicating the presence of an alternative, non-CAT, Cm^r mechanism. These strains were resistant to the analogues. Whether or not those Cm^r determinants were inducible was not reported and the relationship (if any) between the H. influenzae determinant(s) and those of J5-3 (R26) and J5-3 (R55-1) is not known.

CHAPTER 4

MOLECULAR CLONING AND PHYSICAL MAPPING
OF THE CHLORAMPHENICOL RESISTANCE DETERMINANT
OF PLASMID R26

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Molecular Cloning and Physical Mapping of the Chloramphenicol
Resistance Determinant of Plasmid R26

INTRODUCTION

This Chapter describes the molecular cloning of the Cm^r determinant of plasmid R26. This was performed in order to facilitate a detailed genetic analysis of the determinant. The cloning and subsequent sub-cloning experiments were performed using techniques which are now commonplace in molecular biology laboratories. The first gene cloning experiments were reported in 1972 (Jackson et al., 1972) and these depended on the availability of three techniques which are fundamental to such experiments. These are (A) a system which permits the precise cutting and joining together of DNA from different sources; (B) a method for monitoring this cutting and joining and (C) a system for introducing recombinant DNA into a host organism. These three fundamental techniques will be briefly reviewed here.

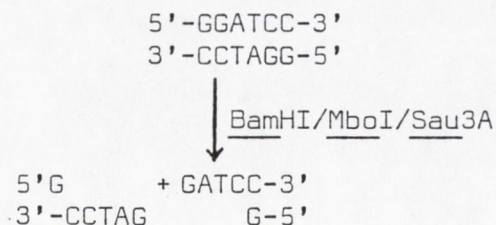
A. Cleaving DNA molecules

Modern DNA technology is absolutely dependent upon our ability to cleave DNA molecules at specific sites with enzymes called restriction endonucleases. Early work with bacteriophage lambda predicted the existence of these enzymes (Dussoix and Arber, 1962; Lederberg and Meselson, 1964). The first restriction endonuclease (or restriction enzyme) to be isolated and studied in detail was obtained from E. coli K by Meselson and Yuan (1968). It was designated a Type I endonuclease and had a number of features which made it unsuitable for routine use in DNA manipulation: (i) it required magnesium cations, ATP and s-adenosyl-methionine, (ii) DNA was cleaved on one strand only, (iii) the cleavage occurred at

between one and five kilobases from the recognition site for the enzyme (Bickle *et al.*, 1978; Rosamund *et al.*, 1979).

The prototype of the Type II restriction enzymes was isolated from Haemophilus influenzae in 1970 (Kelly and Smith, 1970; Smith and Wilcox, 1970). Type II enzymes recognise a particular sequence within a duplex DNA molecule and cut both strands within that sequence. The cleavage products are discrete fragments of DNA which give characteristic banding patterns in gel electrophoresis (Old and Primrose, 1981).

The DNA sequences recognised by restriction enzymes vary greatly. They may contain as few as four nucleotides but may be much longer; they may or may not have rotational symmetry; the enzyme may cut within the sequence or immediately next to it; the fragments resulting from digestion may be flush-ended (e.g. Hinc II), have 3'-cohesive ends [e.g. EcoRI], or 5' cohesive ends (e.g. PstI). Several enzymes may recognise the same site (e.g. BamHI, MboI and Sau3A will all cut within the sequence 5'-GGATCC-3'). MboI and Sau3A are termed isoschizomers. In the example given, BamHI recognises the hexanucleotide 5'-GGATCC-3' but MboI and Sau3A only require the sequence 5'-GATC-3' in order to cut. However, all three enzymes will generate the same 'ends' from cutting the sequence 5'-GGATCC-3':



In any given piece of DNA, the sequence GATC has a higher statistical probability of occurring than has the hexanucleotide GGATCC. Thus MboI and Sau3A will generate more fragments from any given DNA molecule than will BamHI.

B. Joining DNA molecules

In gene manipulation experiments, DNA molecules are usually joined together by T4 DNA ligase. First, cohesive ends are generated by restriction enzyme cleavage. For example, a vector plasmid such as pBR322 is linearised (i.e. cut once) by BamHI. The cohesive ends generated by this enzyme have been illustrated in the previous section. The DNA to be cloned can then be cut by, e.g., Sau3A to generate complementary cohesive ends. These cohesive ends can anneal with those of the BamHI-cleaved vector, i.e. hydrogen bonding can occur between the complementary base-pairs of the overhanging ends. Bacteriophage T4 DNA ligase is an enzyme which can then seal the single-stranded nicks between the adjacent nucleotides in the duplex DNA molecules, yielding a new 'recombinant' plasmid (Olivera et al., 1968; Gumpert and Lehman, 1971). The nick to be sealed must expose a 5'-phosphate and a 3'-hydroxyl group. The enzyme complex binds to this nick and a covalent bond is formed in the phosphodiester backbone of the DNA molecule (Old and Primrose, 1981).

Agarose gel electrophoresis provides a method for monitoring the cutting and joining of DNA molecules. Aaji and Borst (1972) showed that the migration rates of DNA molecules in agarose gels were proportional to the inverse of their molecular weights. By plotting the logarithms of molecular weight against migration, accurate measurements of the sizes of DNA molecules can be performed. Radiolabelling is unnecessary; instead the DNA is visualised with ethidium bromide, an intercalating dye which fluoresces in ultraviolet light.

C. Transformation of *Escherichia coli*

Mandel and Higa (1970) showed that *E. coli* cells which had been treated with CaCl_2 could take up bacteriophage lambda DNA. In 1972 Cohen et al., (1972) showed that CaCl_2 -treated *E. coli* cells could take up plasmid DNA. Systematic studies have since been done to optimise transformation conditions for plasmid DNA uptake by *E. coli* (Humphreys et al., 1979).

D. Restriction enzyme analysis

A 'physical' map of the cloned DNA fragment can be formed by ordering different restriction endonuclease recognition sites with respect to one another. Known recognition sites within the vector can be used as reference points so that the locations of recognition sites within the cloned DNA fragment may be determined by single and multiple digests with different enzymes. The physical map is essential for further molecular genetic analysis of the cloned gene(s).

E. In vitro deletion mutagenesis

The physical map can be used to plan in vitro deletion mutagenesis experiments. Here, specific fragments of DNA are removed from the recombinant plasmid by cutting with endonucleases and religating. Two tasks are performed in this way: (i) DNA which is not essential for the specification of the phenotype of interest (e.g. chloramphenicol resistance) can be removed and (ii) DNA essential for the expression of the gene(s) of interest can be identified. This second type of experiment is known as deletion mutagenesis. The technique of transposon mutagenesis with Tn5 was also used here to map the cloned genes.

F. Transposon Tn5

Tn₅ belongs to a class of transposable genetic elements, called transposons, whose properties have been widely reviewed (Berg, 1983a; Berg and Berg, 1981; Campbell, 1981; Campbell *et al.*, 1979; Goodenough, 1983; Kleckner, 1981; Shapiro, 1983). Tn₅ consists of a 5.7 Kb DNA sequence made up of two 1.5 Kb terminal inverted repeats flanking a 2.7 Kb unique central region. This was determined by mapping restriction endonuclease cleavage sites (Fergensen *et al.*, 1979), by DNA sequence analysis (Auerswald *et al.*, 1980; Beck *et al.*, 1982) and electron microscope heteroduplex analysis (Berg, 1983a).

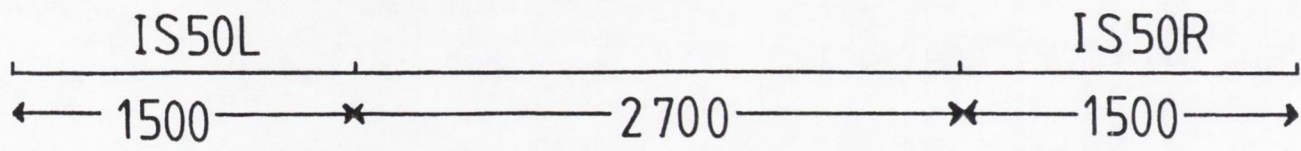
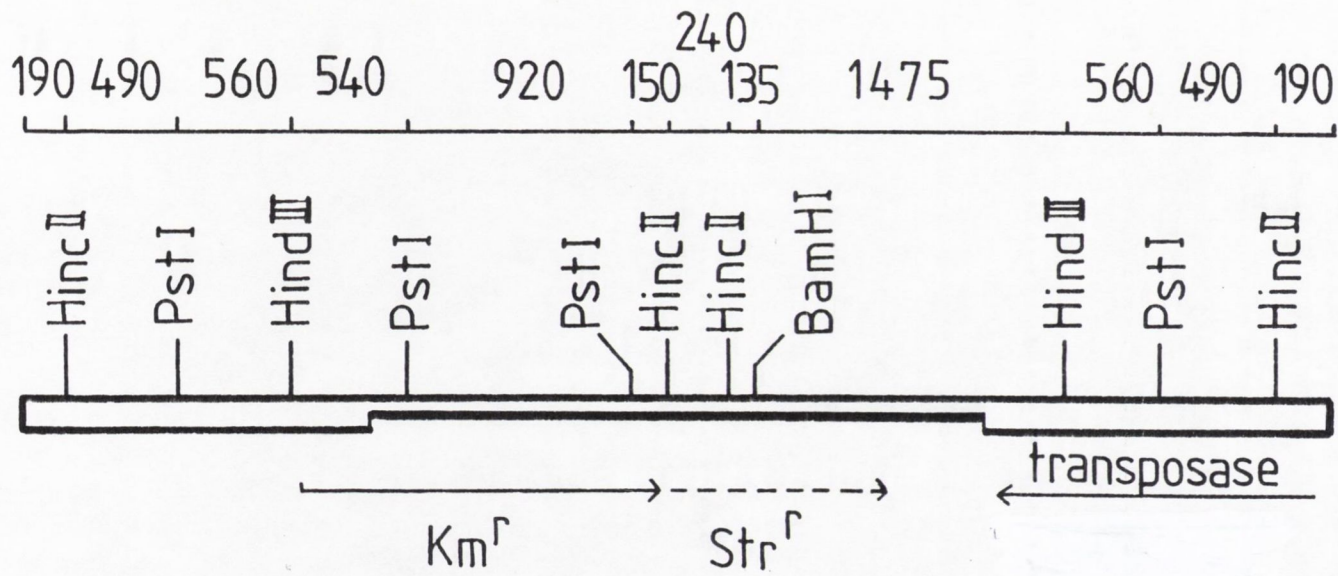
The inverted repeats are termed IS_{50L} (left) and IS_{50R} (right), their orientations being defined by the direction of transcription of a Km^r gene within the unique 2.7 Kb sequence (Fergensen *et al.*, 1979). The structure of the transposon is illustrated in Fig. 4.1.

IS_{50L} and IS_{50R} are not perfect repeats of one another; they differ by a single base-pair non-homology (Rothstein and Reznikoff, 1981). It has been shown that IS_{50R} is an autonomous transposable element, an insertion sequence. It carries the necessary genetic information to promote its own translocation from one bacterial replicon to another (Berg *et al.*, 1982b). The single base-pair change in IS_{50L} produced an ochre mutation (a translational stop signal) in an open reading frame now known to encode the transposase function (Rothstein *et al.*, 1980; Rothstein and Reznikoff, 1979). This IS_{50L} can no longer produce an active transposase protein but has acquired a functional promoter which permits transcription of the Km^r gene of Tn₅ (Fig. 4.1; Rothstein and Reznikoff, 1979). The recently-discovered streptomycin resistance gene of Tn₅, which functions in non-enteric bacteria, is also transcribed from this promoter (Fig. 4.1;

Fig. 4.1 Physical map of transposon Tn5

Tn₅ sequences are represented by the open bar. The narrow portion of this represents the unique sequences between the inverted repeats, these being denoted by the wider portions of the bar. The respective lengths of these components of Tn₅ are given at the base of the figure. The locations of recognition sites of the restriction endonucleases HincII, HindIII and PstI are shown, together with the distances between them (at top of figure). All distances are in base pairs. The locations and directions of expression of the genes for Km^r and Str^r are given, together with that for transposase.

This figure was based on those of Rothstein and Reznikoff (1980) and Berg (1983a).



De Vox et al., 1984; Mazodier et al., 1982; Mazodier et al., 1983; O'Neill et al., 1984; Putnoky et al., 1983; Selverey and Iyer, 1984). **1S50L** will produce an active transposase in an ochre-suppressing host (Rothstein and Reznikoff, 1981). The transposase gene has a second, in phase, translation initiation signal 40 codons downstream from its own initiation site. This internal start signal has been shown to initiate a second, smaller protein which co-terminates with transposase and has a function in transposition regulation. The ochre mutation in **1S50L** abolishes the function for this element in non-ochre-suppressing hosts (Rothstein and Reznikoff, 1981; Rothstein et al., 1980).

Transposase has been shown to be a largely cis-acting function which requires the ends of an **1S50** element to bind to in order to promote transposition (Berg et al., 1982c). The ends of **1S50** are nine base-pair inverted repeats and Tn5 carries four copies of this sequence (Auerswald et al., 1980). Transposase is a DNA binding protein and is thought to bind to the outside 17 bp of **1S50L** and **1S50R** to promote transposition (Sasakawa et al., 1983). The smaller protein, which is co-translated with transposase, represses transposition, possibly by complexing with the transposase protein itself (Berg, 1983). It does not cause repression by inhibiting either transcription or translation of transposase (Johnson et al., 1982; Isberg et al., 1982). This protein has also been abolished in **1S50L** by the ochre mutation.

Tn5 does not depend upon the host cell's recA gene for transposition (Berg, 1983), although gyrA and polA products are needed for transposition at maximum efficiency (Isberg and Syvanen, 1982; Syvanen et al., 1982). It does not have an absolute requirement for any particular insertion site in E. coli, transposing to different

places in the genome in a near-random fashion (Shaw and Berg, 1979). However, there have been reports that insertion occurs at certain sites at a higher frequency than elsewhere (Berg et al., 1980b; Miller et al., 1980; Bossi and Ciampe, 1981). The properties of these preferred sites or hotspots are not clearly understood (Berg, 1983).

Two general models have been advanced to describe transposition. One involves conservative transposition (Berg, 1977; Berg, 1983b); the other involves replicative transposition (Arthur and Sherratt, 1979; Berg, 1983b; Galas and Chandler, 1981; Harshey and Bukhari, 1981; Shapiro, 1979).

The properties of Tn₅ which have been briefly reviewed above confer upon that element characteristics which make it a useful tool in the genetic manipulation of gram-negative bacteria. The uses of Tn₅ in this way have recently been reviewed (Berg, 1983b; de Bruijn and Lupski, 1984).

Tn₅ may be used to study operon organisation (Ní Bhriain et al., 1983; Merrick et al., 1978); as a mobile region of homology for use in chromosome mobilisation (Berg and Curtiss, 1967; Hooybaas et al., 1982; Kleckner et al., 1977; Pischl and Farrand, 1983). Tn₅ can be used to provide duplications, i.e. produce partially diploid strains, for complementation analysis (Avery and Kaiser, 1983). Tn₅ has been directly exploited as a tool for gene cloning (Guarente et al., 1980); and it can be used as a portable set of restriction enzyme recognition sites for use in in vitro deletion experiments (Coleman et al., 1983). It can be used to generate deletions with fixed end-points in vivo (Berg et al., 1980a) and as an agent for mapping silent regions of bacterial genomes (Fouts and Barbour, 1982). A number of workers have exploited the fact that the Km^r gene of Tn₅ specifies resistance to an aminoglycoside antibiotic called G418, which is toxic

to eukaryotic cells. The Km^R determinant from the transposon has been introduced as a selectable marker into a number of cloning vectors used in plants and animals (Colberre-Garapin et al., 1982; McKinnon et al., 1982; Southern and Berg, 1982).

In this study, Tn5 was used to interrupt the coding sequence of a specific gene by insertion. The methodology used to achieve this effect is described in the Materials and Methods section.

MATERIALS AND METHODS

A. Bacterial strains and plasmids

E. coli K12 strain J5-3 was described in Chapter 3. Strain C600 (thr leu lac supE) was obtained from N. Kleckner (Harvard University). Strain XAc Su⁻ [Δ(pro-lac) XIII ara argE rpoB gyrA thi] was from J. Beckwith via N. Kleckner. The plasmid R26 was described in Chapter 1. The other plasmids used in the work described here are listed in Table 4.1.

B. Bacteriological media, chemicals and antibiotics

The compositions of L broth, L agar and M9 minimal medium have been described in Chapter 2. Antibiotics were incorporated into agar as follows: ampicillin, 50 µg/ml; chlormaphenicol, 20 µg/ml; kanamycin, 20 µg/ml; sulphathiozole, 20 µg/ml; tetracycline, 10 µg/ml; trimethoprin, 30 µg/ml. Chemicals were purchased from Sigma or were the best available from B.D.H.

C. Enzymes and enzyme buffers

The restriction endonucleases and ligase used were purchased from New England Biolabs or Boehringer and were used according to the manufacturers' instructions.

D. DNA isolation techniques

I. Large-scale cleared-lysate procedure.

A 500 ml L broth culture of the strain harbouring the desired plasmid was incubated for 15h with shaking in a 1L baffled flask at 37°. Antibiotic selection for the desired plasmid was used where appropriate. Cells were harvested in 250 ml centrifuge bottles by centrifuging for 10 min at 16,000 x g in a GSA Sorvall rotor. The

supernatant was discarded and the pellet thoroughly drained. It was then resuspended in 3 ml of a solution containing 25% sucrose in 0.05M Tris-HCl (pH 8.0), and transferred to a 30 ml Sorvall centrifuge tube. To this was added 1ml of a lysozyme solution (25 mg lysozyme per ml of 0.25M Tris-HCl (pH 8.0)). This mixture was allowed to stand at 4° for 15 min. Then 7 ml of a triton lysing mix was added (2% Triton-X100 in 0.05M Tris-HCl and 0.625M EDTA [pH 8.0]). This was left at 4° for a further 15 min. It was then centrifuged at 48,000 x g in an SS-34 Sorvall rotor for 30 min at 4°. 9.5 ml of supernatant was decanted into an acid-washed glass universal bottle. 1g of CsCl was added per ml of cleared lysate, together with 0.5 ml of ethidium bromide. When the CsCl had dissolved, the mixture was centrifuged at 8,000 x g in a MSE bench centrifuge. This caused any proteinaceous debris remaining in the lysate to collect at the surface. The liquid was transferred to heat-sealable ultracentrifuge tubes (Beckman) and centrifuged at 125,000 x g for 40 h at 15° in a 70.1.Ti fixed angle rotor. Chromosomal (upper) and plasmid (lower) DNA bands were then visualised using ultra violet light. The plasmid band was extracted with a syringe and transferred to an acid-washed glass universal bottle. The ethidium bromide was extracted from the recovered fraction by adding 1 volume of isopropanol saturated with CsCl. The upper (isopropanol) layer was then removed with a pasteur pipette and the procedure repeated until the lower layer was colourless. The plasmid fraction was then dialysed against TES buffer (10 mM Tris-HCl, 5 mM NaCl, 1mM EDTA, pH 7.6) for 1h to remove the CsCl. The plasmid fraction was then extracted with phenol, one volume of phenol, saturated with 1M Tris-HCl (pH 7.5), was added and the mixture shaken vigorously for 30 sec. The aqueous layer was

recovered using a pasteur pipette and transferred to a 30 ml Corex tube (DuPont). 10% volume of 20% sodium acetate and 2 volumes of absolute ethanol were added and the mixture stored at -20° for 15h to precipitate the plasmid DNA. DNA was recovered by spinning at $48,000 \times g$ for 30 min in a SS-34 Sorvall rotor at 4° . The DNA pellet was washed in 90% ethanol to remove residual salt and then dried under vacuum. The pellet was dissolved in TES and stored at -20° until required. This method was adapted from that of Clewell and Helinski, (1970).

II. Small-scale cleared lysate procedure

20 ml cultures of the desired plasmid-containing strains were grown in L broth for 15h at 37° with shaking. Selective drugs were used where appropriate. The cells were harvested in 30 ml Sorvall tubes ($16,000 \times g$ for 10 min in SS-34 rotor). The supernatant was discarded and the pellet drained, resuspended in 0.25 ml of 25% sucrose in 10 mM Tris-HCl (pH 8.0) and transferred to a 1.5 ml microfuge tube. To this was added 0.1 ml of a lysozyme solution (25 mg/ml in 0.25M Tris-HCl, pH 8.0) and 0.1 ml of EDTA solution (0.25M, pH 8.0). The mixture was kept at 4° for 15 min and then 0.5 ml of a Triton lysing mix was added (2% Triton-X100 in 0.05M Tris-HCl and 0.625 M EDTA, pH 8.0). This was allowed to stand at 4° for a further 15 min before centrifuging for 15 min in an Eppendorf microfuge. The cleared lysate was transferred to a fresh 1.5 ml tube and twice extracted with an equal volume of Tris-saturated phenol. The aqueous layer was transferred to a fresh 1.5 ml tube and a 10% volume of 20% sodium acetate and two volumes of absolute ethanol were added. This mixture was stored at -20° for 15h (or -70° for 30 min) to precipitate the plasmid DNA. The plasmid

DNA was pelleted by centrifuging for 15 min in an Eppendorf microfuge. The pellet was washed with 90% ethanol to remove residual salt and dried under vacuum. It was then dissolved in 100 μ l of TES buffer and stored at -20° until required. This DNA was sufficiently pure for use in transformation experiments. If the DNA was to be cleaved by restriction enzymes an additional step was usually included to remove any remaining phenol. Sephadex G50 was equilibrated in TES as follows: 1g of G50 was dissolved per 15 ml of TES and the solution boiled for 2 min. This was allowed to cool and then stand at 4° for 15h. A large Gilson Pipetman tip was plugged with silanised glass wool. To this was added about 1 ml of the G50 slurry. The minicolumn formed in this way was mounted in a 2 ml plastic tube (Sarstedt) and spun at 8,000 x g for 1 min to remove excess TES buffer. This fluid was discarded and the DNA sample loaded onto the column. The column was centrifuged at 8,000 x g for 1 min and 100 μ l of TES buffer was added to the column to wash through the remaining DNA. This was centrifuged for a further 1 min at 8,000 x g. The DNA was present in the TES buffer in the Sarstedt tube, contaminating phenol remained in the G50 column. The column was discarded and the DNA was stored at -20° until required.

III. Rapid plasmid screening technique

For routine estimation of plasmid molecular weights, the method of Kado and Liu (1981) was employed. 2 ml cultures of strains harbouring the desired plasmids were grown in L broth for 15h at 37° with shaking. Cells were harvested in 1.5 ml microfuge tubes by centrifuging in an Eppendorf microfuge. The supernatant was discarded and the pellet resuspended in 100 μ l of E buffer (2 mM EDTA, 40 mM Tris-HCl, pH 7.9). To this was added 200 μ l of lysing mix (3% SDS

and 50 mM Tris-HCl, pH 12.6). The mixture was vortexed briefly and incubated at 60° for 20 min. Two volumes of a phenol-chloroform (50:50) mix were added and the mixture centrifuged for 15 min in a microfuge. The aqueous layer was recovered using a micropipette and mixed with 10 µl of final sample *solution* (0.25% bromophenol blue, 0.25% xylene cyanol, 15% Ficoll type 400 in H₂O) to give a final volume of 40 µl. This was then electrophoresed using an agarose gel (see below).

E. Electrophoresis of DNA

I. Agarose gel electrophoresis

DNA molecules were electrophoresed routinely in gels containing 0.7%, 0.8% or 1.0% (w/v) agarose (Miles labs). Concentrations were chosen depending on the expected Mr of the molecules to be electrophoresed. With molecules smaller than 700 bp, polyacrylamide gels were employed (see below). The gels were made up in and run in TBE buffer (89 mM Tris-HCl, 89 mM Boric acid, 2 mM EDTA, pH 8.0). Vertical slab gels were used routinely and electrophoresis was performed at 100V (constant voltage).

II. Polyacrylamide gel electrophoresis

5% (w/v) polyacrylamide gels were used to electrophorese small DNA fragments (i.e. less than 700 bp). A 30% acrylamide stock was prepared containing 1% (w/v) bisacrylamide and 29% (w/v) acrylamide. 20 ml of this was added to 15 ml of x10 concentrated TBE and 80 ml of distilled water, giving a final volume of 116 ml. The acrylamide was polymerised with 4 ml of 10% (w/v) ammonium persulphate (fresh) and 100 µl of TEMED (NNN'N'-tetramethylethylenediamine). Vertical slab gels were used throughout and electrophoresis was performed using x1 TBE in the gel rig reservoirs at 100V (constant voltage).

F. Gel photography

Gels were stained for 20 min in a 5 $\mu\text{g}/\text{ml}$ solution of ethidium bromide in distilled water. They were then destained in distilled water for 1h. DNA bands were visualised using a long wavelength ultraviolet light source (Chromato-Vue Transilluminator C-62) and photographed with Kodak Tri-X Pan 35 mm film through a red photographic filter. The film was developed with Kodak Universal Developer ($\frac{1}{8}$ v/v in distilled water) and fixed using Kodafix ($\frac{1}{4}$ v/v in distilled water). Printing was on Kodak F4 photographic paper using the same chemicals as for the film.

G. Extraction of DNA molecules from gels

I. From agarose gels

Gels were stained in the usual way following electrophoresis. The desired band was cut from the gel using a scalpel and frozen at -70° for 10 min in a 30 ml Corex tube. As thawing commenced, the agarose was macerated with a tissue macerator in the presence of an extraction buffer (0.5M NaCl, 10 mM Tris-HCl, 1mM EDTA, pH 7.8). The agarose was removed by filtration through silanised glass wool and the DNA precipitated, in the presence of a carrier polynucleotide (yeast tRNA), using the method described previously.

II. From polyacrylamide gels

The desired band was cut from a stained polyacrylamide gel and transferred to a Gilson tip (large-size) which had been plugged with glass wool and heat-sealed at the tip. The acrylamide was macerated with a glass rod in the presence of TES buffer. The macerate was incubated in the tube for 15h at 37° . The seal at the tip was broken

and the aqueous phase centrifuged out via the glass wool. It was then extracted with phenol-chloroform (50:50), then with chloroform and the DNA precipitated as previously described.

H. Nick translation reactions

DNA molecules to be nick translated were recovered from preparative gels by the extraction procedures already described. The nick translation reaction mixture contained 2 μg of DNA dissolved in 5 to 10 μl of TES buffer, 5 μl of $\times 10$ nick translation buffer (500 $\mu\text{g}/\text{ml}$ BSA, 1mM dithionthreitol, 0.1M MgSO_4 , 0.5M Tris-HCl, pH 7.2), 1 μl each of 1 mM dATP, 1 mM dGTP and 1 mM TTP (each in TES buffer), 10 μl of (α - ^{32}P) dCTP (100 pM, at 1000 Ci/mM), 0.5 μl of DNaseI (0.1 $\mu\text{g}/\text{ml}$), 5 units of DNA polymerase I (as defined by Richardson et al., 1964) and distilled, sterile water to a final volume of 50 μl . The reaction was incubated at 15 $^\circ$ for 2h. The polynucleotide molecules were ethanol precipitated in the presence of yeast tRNA and the unincorporated (^{32}P - α) dCTP was discarded with the ethanol. The radiolabelled probe DNA was dried under vacuum and dissolved in TES buffer. It was then stored at -20 $^\circ$ until required.

I. Southern blotting

The DNA to be probed was electrophoresed in an agarose gel. This was stained, destained and photographed. The gel was then soaked for 15 min in 0.25M HCl to partially hydrolyse the DNA by depurination. (This helps in transferring large DNA fragments to nitrocellulose filters). The DNA was then denatured by soaking in 1.5M NaCl and 0.5M NaOH for 60 min, with shaking. It was then neutralised by soaking in several volumes of 1.5M NaCl, 1M Tris-HCl (pH 8.0) for

60 min, with shaking. These incubations were performed at room temperature. The gel was then placed in a Southern transfer apparatus. This was as described by Maniatis et al., (1982). DNA transfer was allowed to proceed for 15h. It was mediated by 20 x SSC (17.53% NaCl (w/v), 8.82% sodium citrate (w/v), pH 7.0, in distilled water). The nitrocellulose filter was then removed from the gel and soaked in 6 x SSC for 5 min at room temperature. It was dried on Whatman 3 mm paper and baked at 80° for 2h.

J. Hybridisation

The baked filter was immersed in 6 x SSC for 5 min at room temperature. It was transferred to a plastic bag, to which was added prewarmed (68°) prehybridisation fluids at 0.2 ml per cm² of filter. Prehybridisation fluid contained 6 x SSC, 0.5% SDS, 5 x Denhardt's solution (a x50 stock solution contains 1% (w/v) ficoll (Sigma 400-DL), 1% (w/v) polyvinylpyrrolidone (Sigma PVP-LO), 1% (w/v) BSA, in distilled water), 100 µg/ml of heat-denatured salmon sperm DNA (from a 10 mg/ml stock solution of Sigma (type III sodium salt) salmon sperm DNA, in distilled water)]. The DNA was sheared by passing the solution through a syringe and denatured by boiling for 10 min. It was stored at -20° until required). When all air had been expelled from the bag, it was heat-sealed and submerged in water at 68° for 4h. The prehybridisation fluid was then removed and replaced with hybridisation fluid (68°) at 50 µl per cm² of filter. Hybridisation fluid contained 6 x SSC, 0.5% SDS, 0.01M EDTA, 5 x Denhardt's solution, 100 µg/ml heat-denatured salmon sperm DNA, 0.1 µg of ³²P-labelled probed DNA (heat-denatured). Hybridisation was allowed to proceed for 15h at 68°. The filter was then removed from the bag

and immersed in 2 x SSC, 0.5% (w/v) SDS in distilled water for 5 min at room temperature. It was then transferred to 2 x SSC, 0.1% (w/v) SDS in distilled water for 15 min at room temperature. Finally, the filter was submerged in 0.1 x SSC, 0.5% (w/v) SDS in distilled water for 2h at 68^o. The solution was changed and incubation continued for a further 30 min. The filter was then dried, wrapped in Cling Film and autoradiographed at -70^o using Kodak X-omat X-ray film. Autoradiographs were developed using Kodak DX-80 X-ray developer ($\frac{1}{5}$ in distilled water) and fixed using Kodak FX-40 X-ray fixer ($\frac{1}{5}$ in distilled water).

K. Transformation experiments

A 2 ml starter culture of the recipient strain was grown in L broth for 15h at 37^o with shaking. This was used to inoculate a volume of L broth (1 ml per individual transformation) and this was incubated at 37^o with shaking until the culture O.D. revealed 0.4 ($\lambda = 500 \text{ nm}$). The culture fluid was transferred to sterile 1.5 ml microfuge tubes (1ml per tube) and the cells pelleted by centrifuging for 10 min in an Eppendorf microfuge. The cells were resuspended in 500 μl of sterile 30 mM CaCl_2 and allowed to stand at 4^o for 20 min. They were again pelleted and then resuspended in 50 μl of 30 mM CaCl_2 . To each tube was added 1 μg of DNA (dissolved in 1 to 5 ml of TES buffer) and the mixture kept at 4^o for 60 min. The cells were heat shocked by immersing the tubes in water at 42^o for 2 min. The cells were transferred to tubes containing 2 ml L broth and incubated at 37^o with shaking for 2h. These cultures were then plated onto a selective medium (0.1 ml of culture per plate) and incubated for 15h at 37^o to allow transformant colonies to develop.

L. Cloning the chloramphenicol resistance determinant of plasmid R26 in vectors pBR322 and pUB5572

20 μ g of R26 DNA, prepared using the large scale cleared lysate technique, was cleaved with EcoRI and ligated with EcoRI-linearised pBR322. A Cm^{r} transformant was isolated which carried a 7 Kb insert in the EcoRI site of pBR322. This plasmid was called pDU1244 (Fig. 4.2; 4.3). The orientation of this insertion was reversed by cleaving pDU1244 with EcoRI and religating to yield pDU1245 (Fig. 4.3). Strains carrying these recombinant plasmids also expressed resistance to sulphonamides (Table 4.1). The Cm^{r} determinant was sub-cloned from pDU1244 as follows: pDU1244 was digested with HindIII and the products religated. A new plasmid was recovered which consisted of a large (4600 bp) HindIII fragment (made up from the large HindIII-EcoRI fragment of pBR322 and a 240 bp EcoRI-HindIII fragment of R26) and a smaller (1.9 kb) HindIII fragment of pDU1244. This plasmid was termed pDU1246 (Fig. 4.2; 4.3). The orientation of the 1.9 kb HindIII fragment was reversed by digesting pDU1246 with HindIII and religating. The plasmid formed in this way was called pDU1247 (Fig. 4.3). Both pDU1246 and pDU1247 specified Cm^{r} (Table 4.1).

Data obtained from experiments with these plasmids (detailed below) suggested that the copy number of the cloned 7 Kb and 1.8 Kb Cm^{r} fragments should be reduced. This was accomplished by cloning the fragments into the EcoRI and the HindIII sites respectively of the R388-derived cloning vector pUB5572 (Fig. 4.4). The pUB5572 derivative with the 7 Kb Cm^{r} insert was termed pDU1248 while that with the 1.8 Kb Cm^{r} insert was designated as pDU1249 (Table 4.1).

Fig. 4.2 Representative gels showing the cloning, physical mapping and subsequent subcloning of *cml*

4.2.1 Screening of pBR322 derivative plasmids specifying *Cm^r*

Lanes: A, pBR322 (uncut); B, pBR322 (EcoRI); C, pDU1244 (EcoRI); D, pDU1244 (uncut); E, H and J, other recombinant derivatives of pBR322 which specify *Cm^r* (cleaved by EcoRI); F, I and K, uncut samples of plasmids in E, H and J, respectively; G, λ DNA cleaved by BamHI and EcoRI to provide molecular weight marker. In lane C, note the 7 Kb band which carries *cml*. The lower band corresponds to pBR322 (EcoRI). Both bands are common to E, H and J.

4.2.2 Mapping the 7 Kb EcoRI fragment of pDU1244

Lanes: A, pDU1244 (PstI); B, pDU1244 (PstI + BamHI); C, pNK80 (AccI + HindIII, to provide molecular weight markers); D, pDU1244 (PstI + HindIII); E, pDU1244 (PstI + HindIII); F, pDU1244 (PstI + BglII); G, pNK80 (AccI + HindIII); H, pDU1244 (PstI + EcoRI).

4.2.3 Mapping the 7 Kb EcoRI fragment of pDU1244 (continued from 4.2.2)

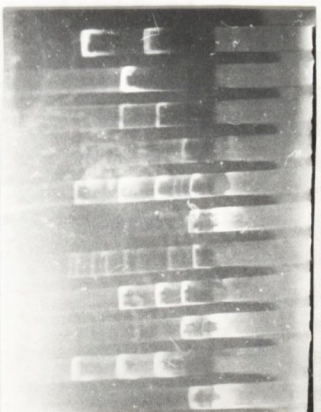
Lanes: A, pDU1244 (HincII); B, pDU1244 (HindIII + EcoRI); C, pDU1244 (HindIII + BglII); D, pNK80 (AccI + HindIII); E, pDU1244 (HindIII + HincII); F, pDU1244 (HindIII); G, pDU1244 (BamHI + EcoRI); H, pDU1244 (BamHI + BglII); I, pNK80 (AccI + HindIII); J, pDU1244 (BamHI + HincII); K, pDU1244 (BamHI + HindIII); L, pDU1244 (BamHI).

4.2.4 Subcloning *cml* from the 7 Kb EcoRI fragment of pDU1244

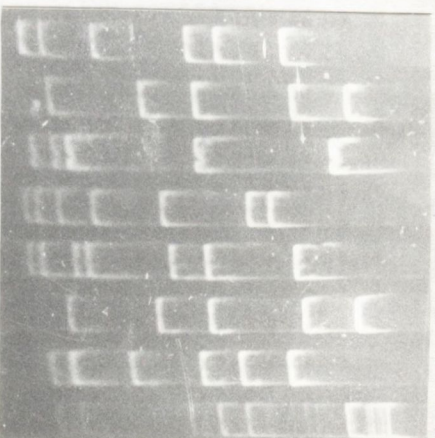
Lanes: A, pDU1244 (HindIII); B, C and D, derivatives of pDU1244 which specify *Cm^r*, D is the simplest of these, having only two HindIII fragments (1.8 and 4.6 Kb). This plasmid was designated pDU1246 (see text).

The molecular weights of the pNK80 fragments were communicated by T. Foster.

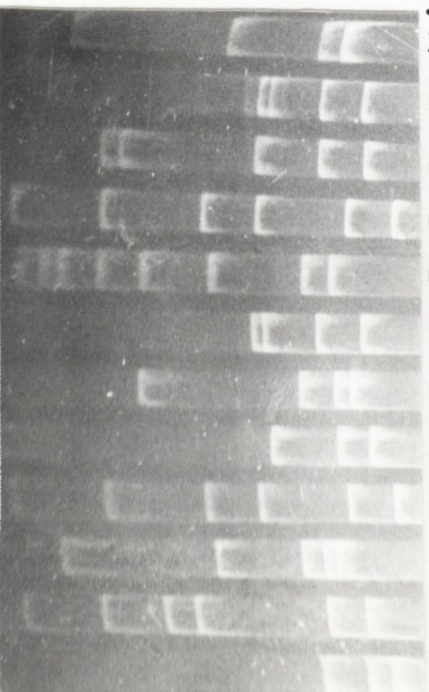
1. A B C D E F G H I J K



2. H G F E D C B A



3. A B C D E F G H I J K L



4. A B C D



Figure 4.3 Structure of pBR322 derivative plasmids specifying resistance to chloramphenicol

The structures of plasmids pDU1244, pDU1245, pDU1246 and pDU1247 are illustrated. In each case, pBR322 sequences are denoted by the broad part of the horizontal bars, while R26 DNA is represented by the narrow portions. Drug resistance genes are represented by the solid bands within the bars. Restriction endonuclease recognition sites are indicated. The origins of the various plasmids are indicated by the arrows. The symbol Tc' indicates a Tc^r determinant which has had its promoter sequence deleted. See Figure 4.5 for a more detailed restriction map of the cloned sequences.

PBR322
R26
EcoRI, ligate

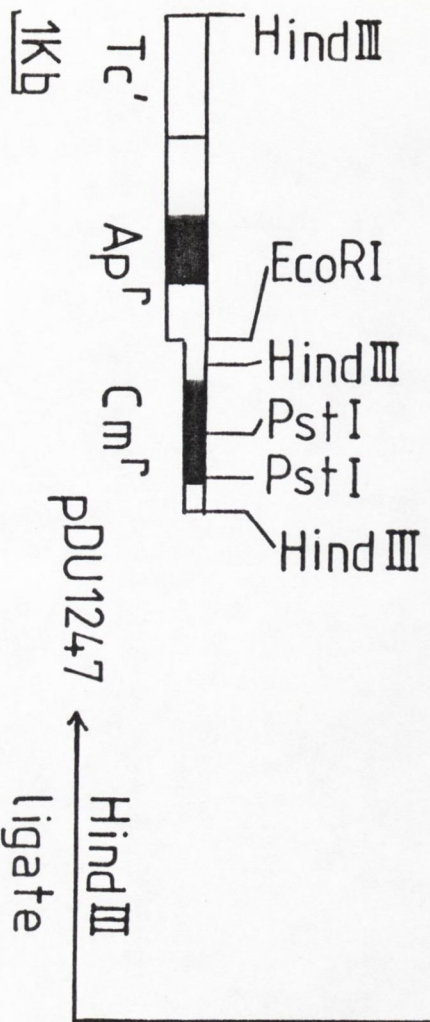
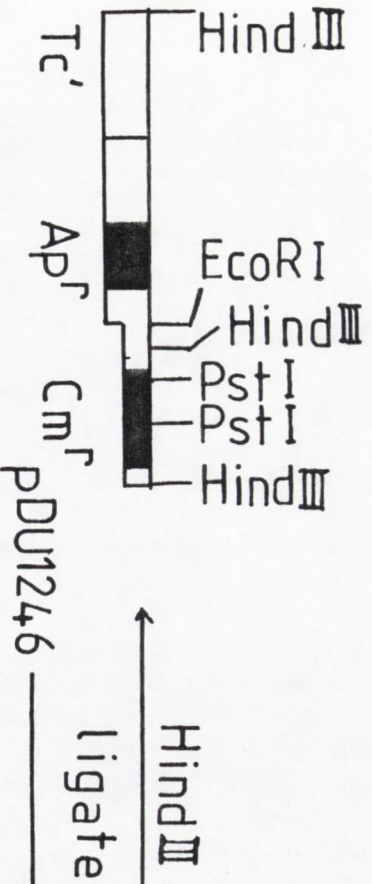
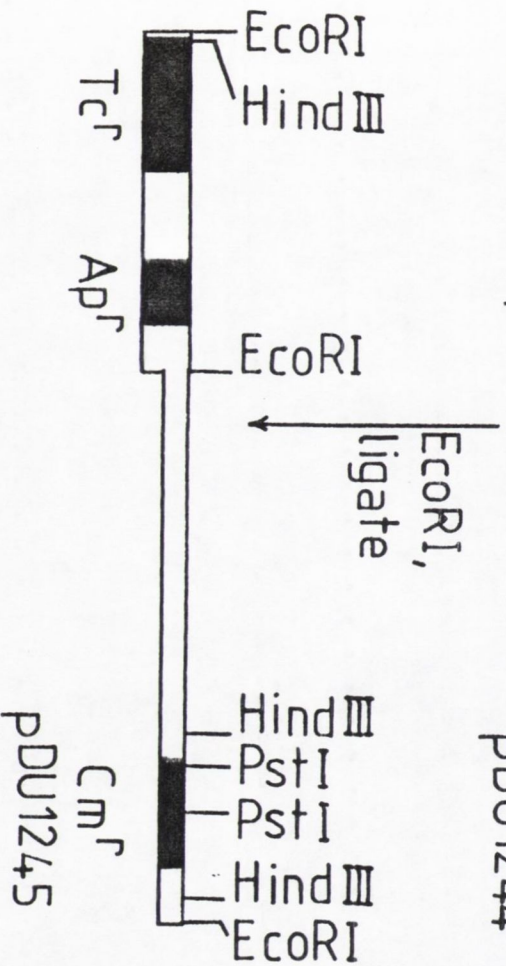
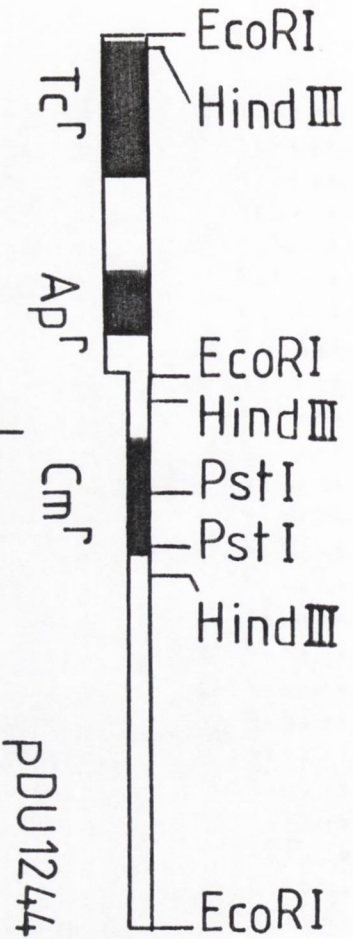
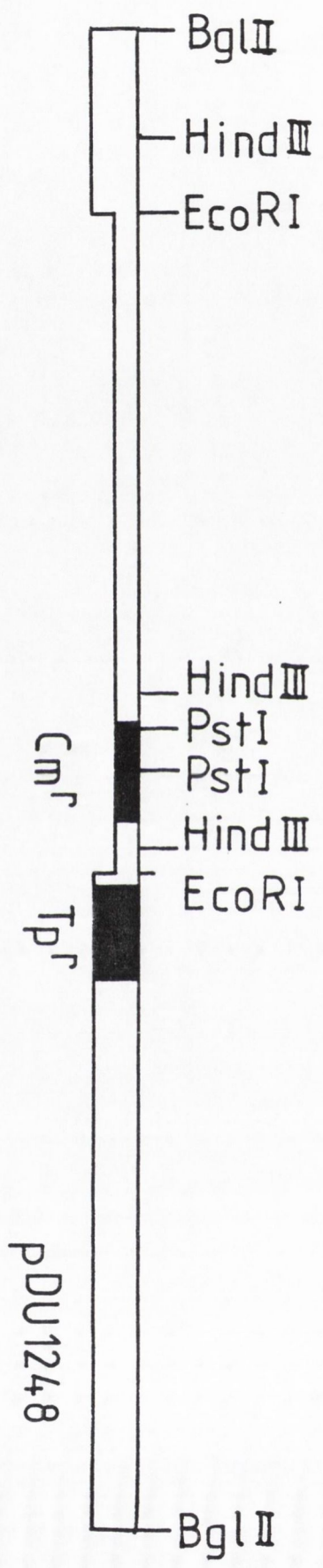
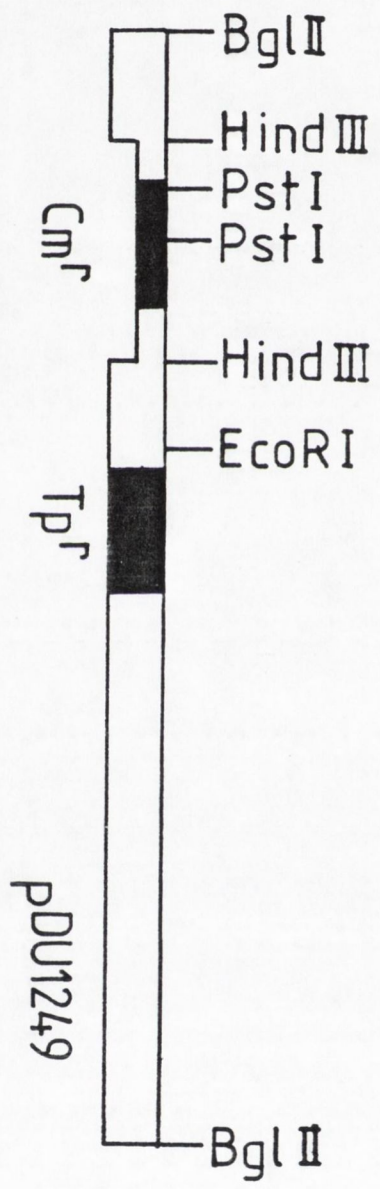


Fig. 4.4 Structures of pUB5572 derivative plasmids which specify resistance to chloramphenicol

The structures of plasmids pDU1248 and pDU1249 are shown. In each case, the broad part of the horizontal bar represents pUB5572 sequences. The cloned R26 sequences are denoted by the narrow portions of the bar. Drug resistance genes are represented by the solid bands within the bars. The locations of restriction endonuclease recognition sites are indicated.

1Kb



M. Physical mapping of the cloned DNA fragments

A physical map was constructed of the 7 Kb EcoRI and 1.8 Kb HindIII fragments in pBR322. The locations of recognition sites for restriction endonucleases within the cloned fragments were determined with respect to the known positions of sites within pBR322, the vector plasmid. These determinations were performed using single and double digests with a number of restriction enzymes. The sizes of the digest products were measured on agarose and polyacrylamide gels (Fig. 4.2). The maps obtained are shown in Fig. 4.5.

N. Isolation of deletions in the cloned cml region

Deletions in the Cm^R determinant were constructed in vitro by removing the DNA located between restriction endonuclease recognition sites. Plasmids pDU1246 (pBR322 Cm^R) and pDU1247 (pBR322 Cm^R) were digested with BamHI. The DNA was ligated in each case to yield a plasmid consisting of only the larger BamHI fragment. $Ap^R Cm^S$ transformants were selected and the desired derivative plasmids obtained. These were called pDU1334 and pDU1335, respectively. In these plasmids the DNA to either side of the unique BamHI site within the 1.8 Kb Cm^R HindIII fragment is alternatively removed (Fig. 4.6 and Table 4.1). Similarly, pDU1335 was constructed by partial digestion of pDU1244 with PstI, ligation and transformation of C600 with selection for Ap^R . pDU1335 is essentially pDU1246 without the small PstI-HindIII sequence of the 1.8 Kb Cm^R fragment (Fig. 4.6; Table 4.1).

Fig. 4.5 Physical map of the 7 Kb EcoRI fragment from R26
which carries cm1

The 7 Kb EcoRI fragment is represented by the broad horizontal bar. Recognition sites for the following restriction endonucleases are shown: BamHI, BglIII, EcoRI, HincII, HindIII, and PstI. The HindIII fragment to the left of the 7 Kb fragment, indicated by the bracket, is the 1.8 Kb HindIII sequence subsequently shown to carry cm1. A more detailed physical map of this sequence is shown in Fig. 4.6.

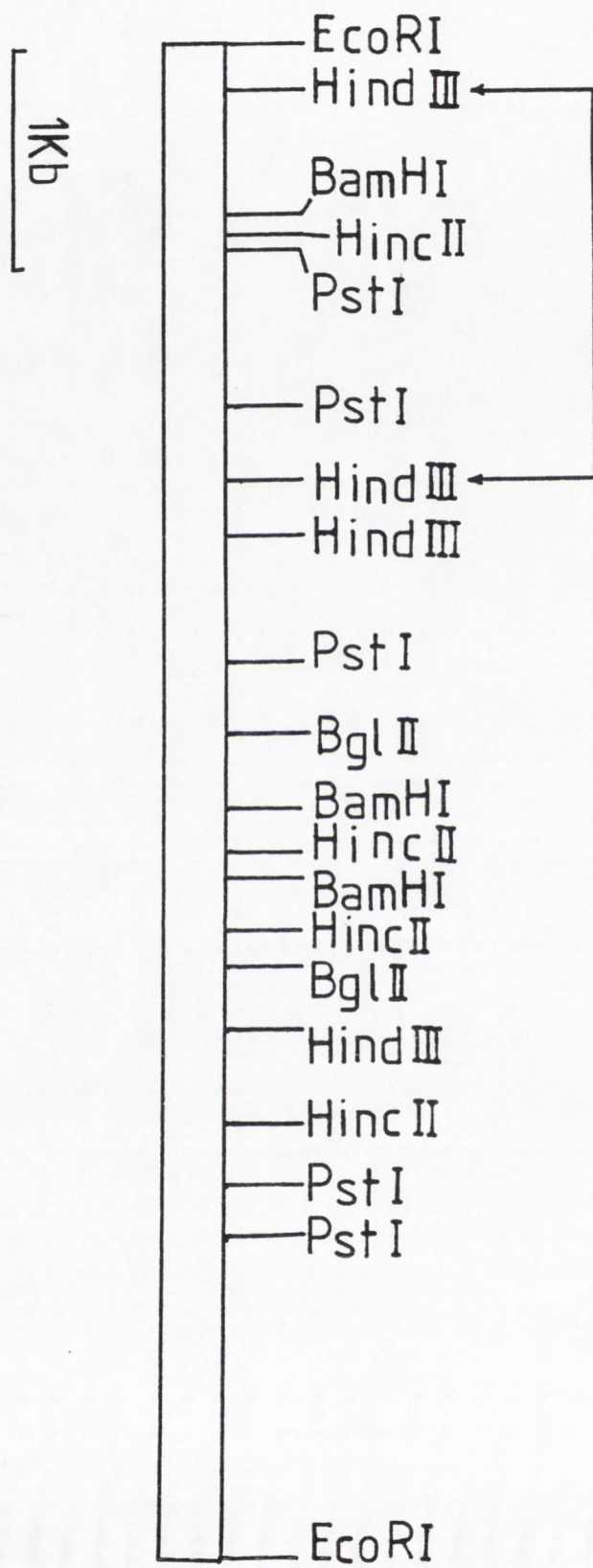
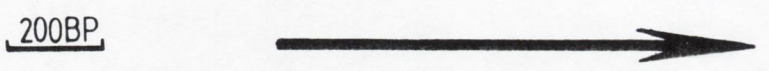
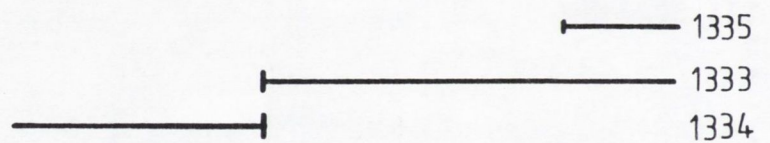
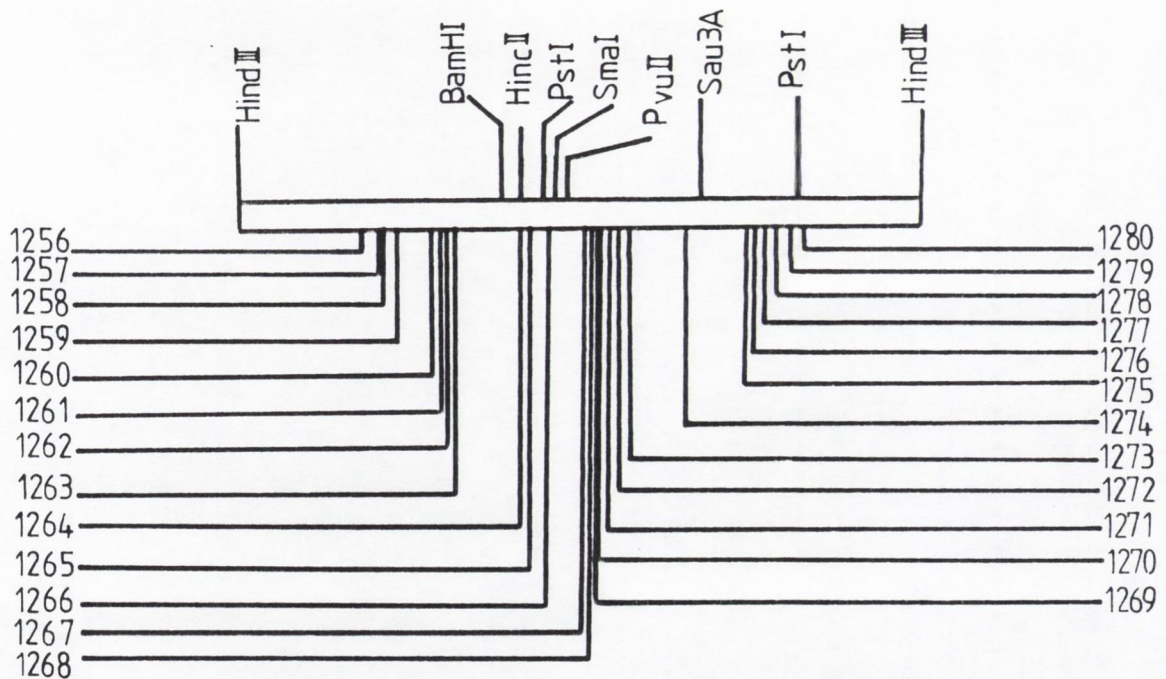


Fig. 4.6 Physical map of the 1.8 Kb HindIII fragment of pDU1246 which carries cml: structures of deletion and Tn5 insertion mutants

The upper horizontal bar represents the 1.8 Kb Cm^{r} HindIII fragment of pDU1246. The locations of restriction endonuclease recognition sites are shown above the bar. DNA sequences deleted from this fragment to form plasmids pDU1333-35 are shown at the base of the figure. The sites of Tn5 insertion in the cml::Tn5 mutant plasmids pDU1256-80 are shown by the right-angles below the horizontal bar. The arrow at the base of the figure shows the extent of the minimum of DNA required for Cm^{r} as determined by these mutation experiments. The arrow head shows the direction of expression by cml (see Chapter 5).



O. Mutagenesis with transposon Tn5

I. Preparation of a $\lambda::Tn5$ stock

The indicator strain used throughout was C600. The bacteriophage Tn5-vector was $\lambda 467$ (rex::Tn5 Oam 29 Pam 80 cI 837) from N. Kleckner, Harvard University. Mutations in this strain of λ prevent lysogeny and lysis can only be achieved with an amber suppressing host, such as C600. In an Su^- host, such as XAc, Km^r is indicative of transposition of Tn5 from $\lambda 467$ to a host replicon (de Bruijn, 1984). Cells to be infected with $\lambda 467$ were grown in LBM (L broth supplemented with 0.2% maltose). 0.2 ml of a mid-exponential phase C600 culture was pipetted into sterile culture tubes. To each was added 3 ml of molten λ top agar, precooled to 60°. This was spread onto λ base agar plates and allowed to set. A loopful (0.01 ml) of a λ phage suspension (in λ buffer) was streaked across the surface of each plate. The plates were incubated at 37° for 15h to allow single plaques to form. Plaques were recovered using sterile pasteur pipettes and transferred to 0.2 ml of an LBM culture of C600 in a culture tube (3 plaques per 0.2 ml culture). These were allowed to stand for 15 min at room temperature. To each tube was added 7.5 ml of molten λ top agar, cooled to 60°, and this was dispensed onto the surface of each of a pair of λ base plates. These were incubated at 37°, base-downwards, until plaques formed. This took 4 to 5h. Plates on which confluent lysis had occurred were chosen and the top layers emulsified in 1 ml of λ buffer. The fluid was transferred to a sterile universal bottle, 0.5 ml of chloroform added, and the mixture vortexed vigorously. The suspensions were then centrifuged at 16,000 x g for 15 min in a MSE bench centrifuge. The supernatants were decanted into sterile universal storage bottles. To each was added 3 drops of chloroform and they were stored at 4° until required.

II Titration of $\lambda::Tn5$ phage stocks

An exponential culture of C600 was prepared in LBM. The phage stock was diluted to 10^{-2} , 10^{-4} , 10^{-6} and 10^{-7} in buffer and 0.1 ml of the 10^{-6} and 10^{-7} dilutions were transferred to sterile culture tubes. To each tube was added 0.2 ml of indicator culture (i.e. C600). The mixtures were allowed to stand for 30 min at room temperature. 3 ml of molten λ top agar was then poured into each tube and this was overlaid onto λ base plates. These plates were incubated base-upwards at 37° for 15h. Plaques were then counted and the titre calculated as plaque forming units (pfu) per ml. In the experiments described below, titres of 10^{11} and 10^{12} pfu/ml were obtained.

III. Selection of transposon insertions following infection with $\lambda::Tn5$

The chloramphenicol resistance determinant of plasmid pDU1246 (see Table 4.1 and below) was mutagenised by $Tn5$ insertion. 5 ml cultures of E. coli K12 XAc Su^{-} harbouring pDU1246 were grown to mid-log phase in L broth plus maltose. To each tube was added 1 ml of the stock suspension of $\lambda::Tn5$ ($CHCl_3$ -free). The mixture was allowed to stand at 37° for 60 min and the 0.1 ml aliquots were spread on L agar plates containing 20 μ g/ml Km to select for $Tn5$ transpositions. Pools of confluent colonies were made from the plates in 1.0 ml L broth and diluted into 20 ml of L broth. After incubation at 37° for 15h plasmid DNA was isolated using the small scale cleared lysate method and used to transform C600. Km^R transformants had $Tn5$ inserted in pDU1246 and those which were Cm^S were assumed to have $Tn5$ inserted within the Cm^R determinants. One

mutant from each transposition pool was retained for further analysis.

IV. Mapping pDU1246 cml::Tn5 insertions

Physical maps of Tn5 and of the 1.8 Kb Cm^r HindIII fragment of pDU1246 are given in Figures 4.1 and 4.6 respectively. Each contains a unique BamHI recognition site. Analysis of the deletion plasmids pDU1333 (pDU1247 Cm^r) and pDU1334 (pDU1246 Cm^r) revealed that the BamHI site in the 1.8 Kb fragment lay within cml (Fig. 4.6 and Table 4.1). Tn5 insertions in cml were initially localised with respect to this BamHI site.

Tn5 possesses HincII sites within 190 bp of the outer ends of its inverted repeats (Fig. 4.1). cml has a unique HincII site (Fig. 4.6). By measuring the sizes of Tn5-pDU1246 HincII junction fragments by gel electrophoresis, the distance of Tn5 from the unique cml HincII site was computed for each insertion. This position was then cross-checked by cutting with another enzyme, such as PstI. Examples of gels from these experiments are provided (Fig. 4.7). The accumulated data from these measurements allowed an estimate to be made of the minimum extent of the DNA sequence required to specify Cm^r.

P. Minimum inhibitory concentration determinations and growth and challenge tests

These were performed as described in Chapter 3.

Figure 4.7 Representative gels used in mapping the sites of Tn5 insertions in pDU1246 cml::Tn5 plasmids

4.7.1 Localisation of Tn5 insertion sites by PstI digestion

Tn5 contains three internal PstI fragments (920 bp, 1135 bp, 2460 bp, Fig. 4.1). These are indicated to the left of the figure. pDU1246 contains three PstI fragments (750 bp, 1000 bp, 4500 bp). The locations of the 1000 bp and 4500 bp fragments are shown at the left of the figure. The 750 bp fragment is absent from the pDU1246 cml::Tn5 plasmid tracks, indicating that this fragment is the site of insertion of these particular Tn5 insertions (Fig. 4.6). Bands other than those indicated by the markers at the left hand margin must be Tn5-cml junction fragments. Lanes: C and J, pNK80 (AccI + HindIII, molecular weight markers - right side of figure; A, pDU1279 (PstI); B, pDU1277 (PstI); D, pDU1270 (PstI); E, pDU1274 (PstI); F, pDU1275 (PstI); G, pDU1272 (PstI); H, pDU1269 (PstI); I, pDU1276 (PstI); K, pDU1267 (PstI); L, pDU1268 (PstI).

4.7.2 Localisation of Tn5 insertion sites by HincII digestion

Tn5 contains three internal HincII fragments (240 bp, 2400 bp and 2600 bp, Fig. 4.1). These are indicated to the left of the figure. pDU1246 has one internal HincII fragment not involved in cml (3255 bp). The other two fragments (1450 bp and 1700 bp) are involved in cml and so one or other is absent from each of the pDU1246 cml::Tn5 sample tracks. Lanes: D and J, pBR322 (HaeIII, to provide molecular weight markers - right-side of the figure); A, pDU1268 (HincII); B, pDU1267 (HincII); C, pDU1258 (HincII); E, pDU1256 (HincII); F, pDU1276 (HincII); G, pDU1273 (HincII); H, pDU1269 (HincII); I, pDU1259 (HincII); K, pDU1272 (HincII); K, pDU1272 (HincII); L, pDU1275 (HincII); M, pDU1274 (HincII). Bands other than the internal HincII fragments of Tn5 or pDU1246 described above are Tn5-cml junction fragments. pBR322 (HaeIII) fragment sizes are from Old and Primrose (1981).

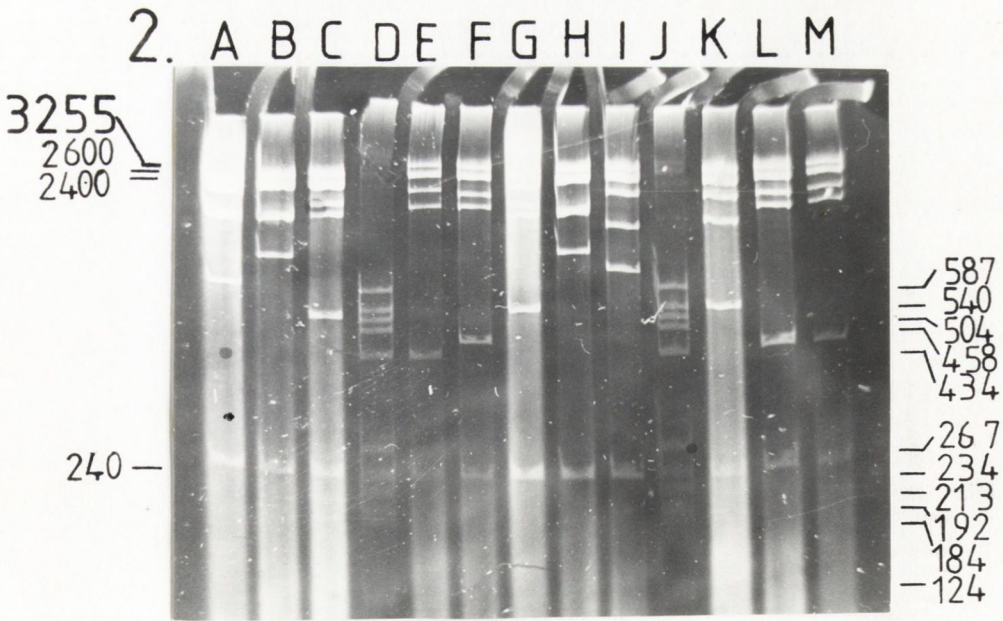
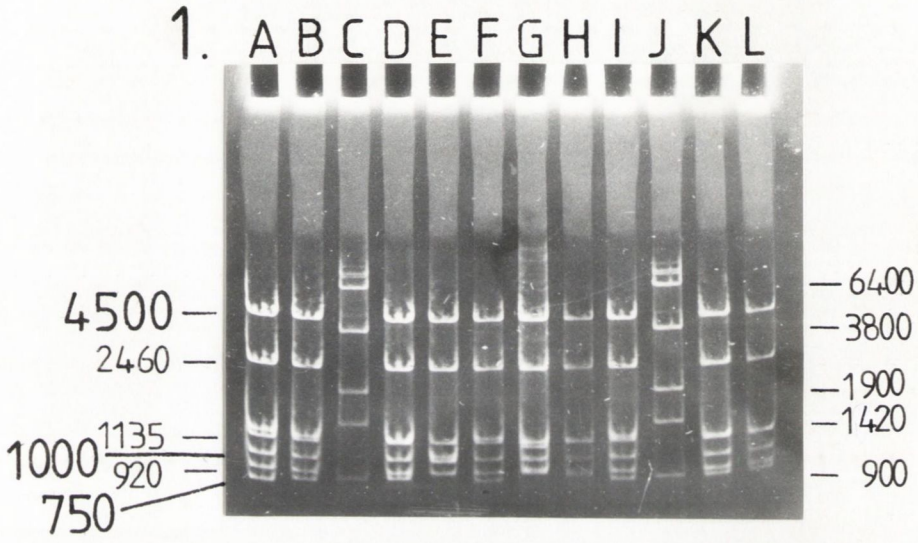


Table 4.1 Plasmids

<u>Plasmid</u>	<u>Phenotypic markers</u>	<u>Comments</u>
pBR322	Ap ^r Tc ^r	High copy number cloning vector (<u>ca.</u> 40 copies per chromosome) (Bolivar <u>et al.</u> , 1977a; 1977b)
pUB5572	Tp ^r	Derived from R388 (Inc W). It is a low copy number plasmid (<u>ca.</u> 8 copies per chromosome [Chopra <u>et al.</u> , 1981])
pDU1244	Ap ^r Cm ^r Su ^r Tc ^r	pBR322 with 7 Kb <u>EcoRI</u> fragment cloned from R26
pDU1245	Ap ^r Cm ^r Su ^r Tc ^r	pDU1244 with <u>EcoRI</u> fragment in reverse orientation
pDU1246	Ap ^r Cm ^r	Essentially pBR322 with 1.8 Kb <u>HindIII</u> fragment of R26 DNA from pDU1244
pDU1247	Ap ^r Cm ^r	pDU1246 with 1.8 Kb fragment reverse orientation
pDU1248	Cm ^r Tp ^r	pUB5572 with 7 Kb <u>EcoRI</u> fragment from pDU1244
pDU1249	Cm ^r Tp ^r	pUB5572 with 1.8 Kb <u>HindIII</u> fragment from pDU1246
pDU1256- pDU1280	Am ^r Km ^r	pDU1246 <u>cm1::Tn5</u> mutants
pDU1333	Ap ^r	Cm ^s deletion derivative of pDU1247
pDU1334	Ap ^r	Cm ^s deletion derivative of pDU1246
pDU1335	Ap	Cm ^s deletion derivative of pDU1244

RESULTS

A. The chloramphenicol resistance determinant of plasmid R26 is carried on a 7 Kb EcoRI fragment

The Cm^{r} determinant of plasmid R26 was cloned on a 7 Kb EcoRI fragment. This fragment was inserted in both possible orientations into the EcoRI site of pBR322 to yield pDU1244 and pDU1245 (Fig. 4.3). The screening gel from the initial cloning experiment is shown in Fig. 4.2. Lane B shows pBR322 linearised by EcoRI. In lane C is pDU1244 cleaved by EcoRI. The lower band corresponds to the linear pBR322 band in lane B. The EcoRI digest patterns of other recombinant plasmids from the cloning experiment are shown in lanes E, H and J. All share the 7 Kb band of pDU1244, although each has a number of additional bands. All of these plasmids specified Cm^{r} in C600. It was therefore concluded that the Cm^{r} determinant was carried on the 7 Kb EcoRI fragment. Once the locations of some restriction endonuclease recognition sites within this fragment had been determined (see next section), its orientation was reversed by digesting pDU1244 with EcoRI and religating. This yielded pDU1245. Both recombinant plasmids specified Su^{r} in addition to Cm^{r} (Table 4.1).

B. Physical mapping of the 7 Kb EcoRI fragment

Using single and multiple digests, the locations within the 7 Kb fragment of the following restriction endonuclease recognition sites were determined, using the known positions of sites within the pBR322 component of pDU1244 as reference points: BamHI (3 sites), BglIII (2 sites), HincII (4 sites), HindIII (4 sites), and PstI (5 sites), (Fig. 4.2). The physical map obtained from this experiment is shown in Fig. 4.5. This was used to plan subcloning experiments.

C. Subcloning the chloramphenicol resistance determinant from plasmid pDU1244

The pBR322 Cm^r plasmid, pDU1244 was digested with HindIII and religated. A transformant was detected which was resistant to Ap and Cm but not Tc. It contained a plasmid, pDU1246, which consisted of two HindIII fragments (Fig. 4.2). The larger fragment was composed of the 4334 bp HindIII-EcoRI fragment of pBR322 together with a 240 bp EcoRI-HindIII fragment of R26 DNA. The smaller fragment was a 1.8 Kb sequence from the R26 component of pDU1244 (Fig. 4.3; 4.5). The plasmid pDU1246 was Tc^s because the 29 bp EcoRI-HindIII fragment of pBR322 has been deleted. This sequence includes the promoter for the tetA gene (Sutcliffe, 1979). The orientation of the 1.8 Kb HindIII fragment was reversed by cleaving pDU1246 with HindIII and religating. This yielded pDU1247 (Fig. 4.3).

D. The chloramphenicol resistance determinant is expressed constitutively in pBR322

The results of minimum inhibitory concentration (MIC) determinations and growth and challenge tests showed that Cm^r was constitutively expressed by the pBR322 derivatives pDU1244, pDU1245, pDU1246 and pDU1247 (Table 4.2 and Fig. 4.8). It was also found that the MIC conferred by these plasmids, 30 µg/ml, was equivalent to that of induced R26-containing hosts (Table 4.2). This showed that increasing gene dosage did not bring about an increase in protection against Cm (the copy number of the pBR322 derivatives was some forty-fold higher than that of R26).

The constitutive expression of the Cm^r determinant (cm1) could have been due to one or more of the following causes (i) constitutive

Table 4.2 Minimum inhibitory concentration determinations

Table 4.2 Minimum inhibitory concentration
determinations

<u>Plasmid</u> ^a	<u>Minimum inhibitory concentration</u> ($\mu\text{g/ml}$ of chloramphenicol)	
	<u>uninduced</u>	<u>induced</u> ^b
none	2	2
R26	15	30
pDU1244	30	30
pDU1245	30	30
pDU1246	30	30
pDU1247	30	30
pDU1248	15	30
pDU1249	15	30
pDU1256-1279	2	2
pDU1280	20	20
pDU1333	2	2
pDU1334	2	2
pDU1335	5	5

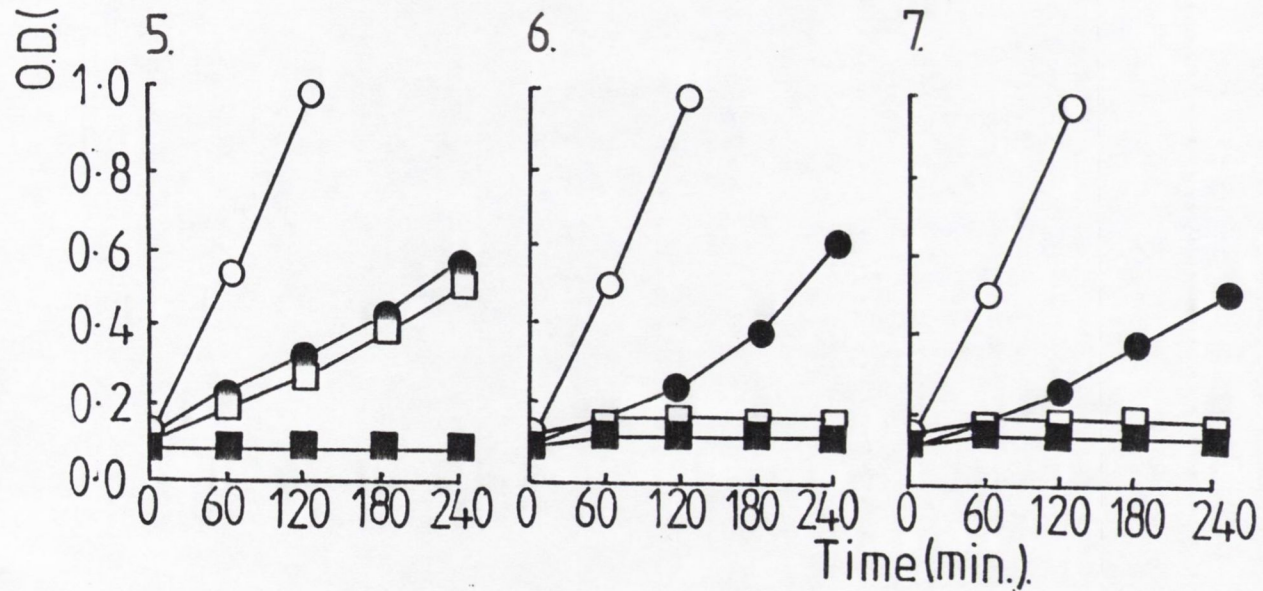
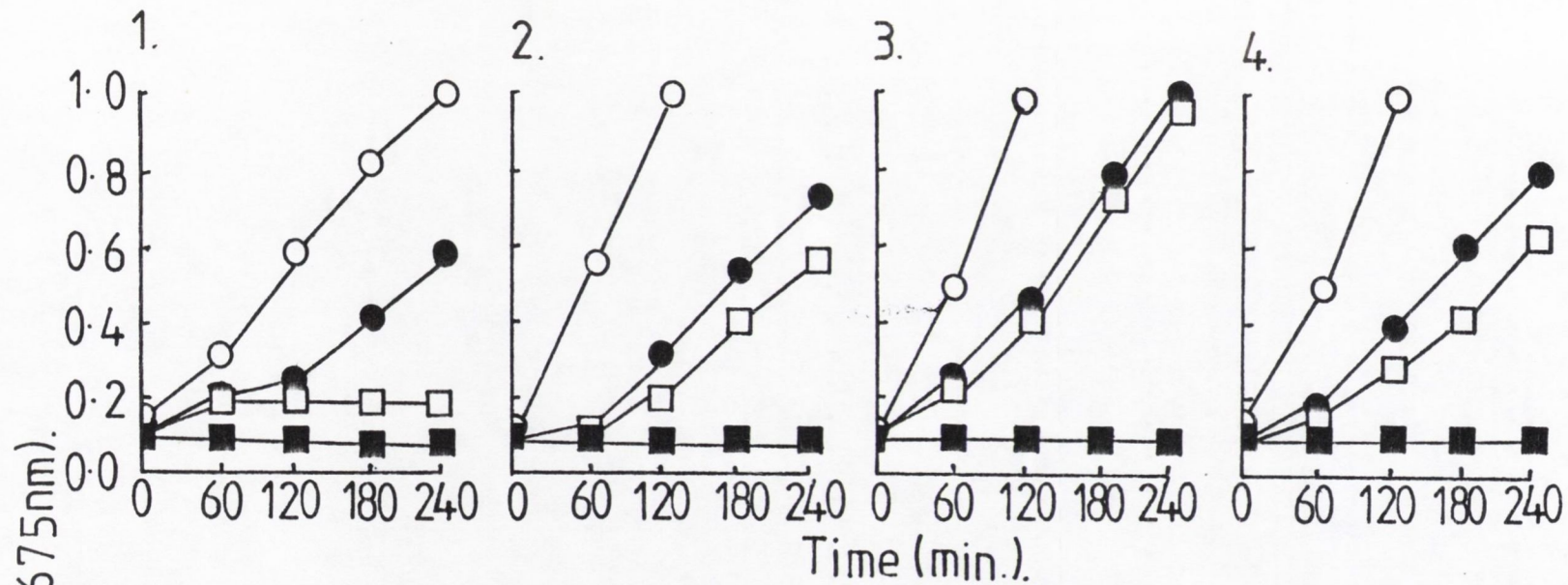
a. The host used throughout was E. coli K12 strain J5-3.

b. Induction was with 1 $\mu\text{g/ml}$ chloramphenicol .
added to exponentially growing cells 60 min
prior to plating

Fig. 4.8 Data from growth and challenge experiments with
E. coli strains harbouring chloramphenicol
resistance plasmids

Each panel shows the experimental data in the form of growth curves expressed as optical density per unit time. In each case, the following symbols were used: (o) uninduced, unchallenged; (●) induced, challenged; (□) uninduced, challenged; (■) plasmid-free control, challenged. Induction was with 1 µg/ml of Cm for 1 hr prior to challenge. The challenging dose was 15 µg/ml of Cm added at time zero.

Panel 1, J5-3 (R26); Panel 2, J5-3 (pDU1244); Panel 3, J5-3 (pDU1245); Panel 4, J5-3 (pDU1246); Panel 5, J5-3 (pDU1247); Panel 6, J5-3 (pDU1248); Panel 7, J5-3 (pDU1249).



expression could be a function of a gene dosage effect (the copy number of cml had been increased forty-fold in pBR322 as compared with R26); (ii) the cloning could have disconnected the structural part of the determinant from its regulatory function, possibly a repressor gene, thus producing a constitutive mutant; (iii) cml could have come under the influence of strong pBR322 promoters, with read through from these giving enhanced expression of cml.

It was decided to deal with the first possibility by reducing the copy number of cml by transferring the 7 Kb and 1.8 Kb fragments to pUB5572, an R388-derivative cloning vector with a low copy number (ca. 8 per chromosome) and specifying resistance to trimethoprim (tp^r).

E. The cloned chloramphenicol resistance determinant is expressed inducibly in the low copy number state

The 7 Kb EcoRI and 1.8 Kb HindIII Cm^r fragments were cloned into the appropriate sites of the R388-derivative pUB5572. This is a low copy number vector (ca. 8 copies per chromosome) which specifies resistance to trimethoprim (Table 4.1). The structures of the EcoRI-generated recombinant plasmid (pDU1248) and the HindIII-generated recombinant plasmid (pDU1249) are shown in Fig. 4.4. The results of MIC determination and growth and challenge tests showed that cml was expressed inducibly in both plasmids (Table 4.2 and Fig. 4.8). These results showed that the Cm^r determinant had probably been cloned in its entirety and that both structural and regulatory functions were carried within the 1.8 Kb HindIII fragment.

F. The 1.8 Kb HindIII fragment originated in plasmid R26 and a chloramphenicol resistance determinant similar to cml exists on an Inc C plasmid from Serratia marcescans

A Southern blot hybridisation experiment was conducted to confirm that R26 was indeed the source of the 1.8 Kb Cm^r fragment. This piece of DNA was excised from an agarose gel and nick translated using (α -³²P) CTP. It was then used to probe for homology with R26, R55-1 (the Inc C plasmid described in Chapter 3) and pSM-1. The plasmid pSM-1 was isolated from Serratia marcescans and was a member of Inc C. It conferred low-level, inducible Cm^r on its E. coli host (H. Griffin, personal communication). The plasmid pDU1246, cleaved by HindIII was included as a positive control. DNA from R26, R55-1 and pSM-1 was isolated by the method of Kado and Liu (1981) and electrophoresed on an agarose gel with pDU1246 (Fig. 4.9). The positive control track (lane A) was clearly overloaded. E. coli chromosomal DNA can be seen in lanes B, C and D (the R plasmid tracks). An unidentified plasmid, of lower molecular weight than pSM-1, can also be seen in the track for this sample (lane D). A scale was provided (left of figure) to permit correlation of the agarose gel data with those from the autoradiograph. The probe had hybridised with R26 and pSM-1, but not R55-1, the E. coli chromosome or the low molecular weight plasmid from the pSM-1 sample. No hybridisation occurred in the region of the gel occupied by pBR322 (4 cm mark). The probe did hybridise to the 1.8 Kb HindIII fragment of pDU1246 (5.25 cm mark). Additional bands in lane A of Fig. 4.9.2 were due to (i) partial digestion of pDU1246 with HindIII and (ii) contamination of the HindIII enzyme solution by HincII. Evidence for HincII contamination was obtained from a comparison of digest products from

Figure 4.9 Data from Southern hybridisation experiment

4.9.1 Agarose gel of "target" DNA molecules

Lane A, pDU1246 (HindIII). The upper band is the vector fragment, the lower is the 1.8 Kb cml fragment. This fragment was the probe used in the hybridisation experiment; lane b, R26 (upper band), chromosomal DNA (lower); lane C, R55-1 (upper band), chromosomal DNA (lower); lane D, from top to bottom, pSM-1, an unknown plasmid, chromosomal DNA. A scale, graduated in centimetres, is given to the left of the figure to allow correlation with the autoradiograph (Fig. 4.9.2).

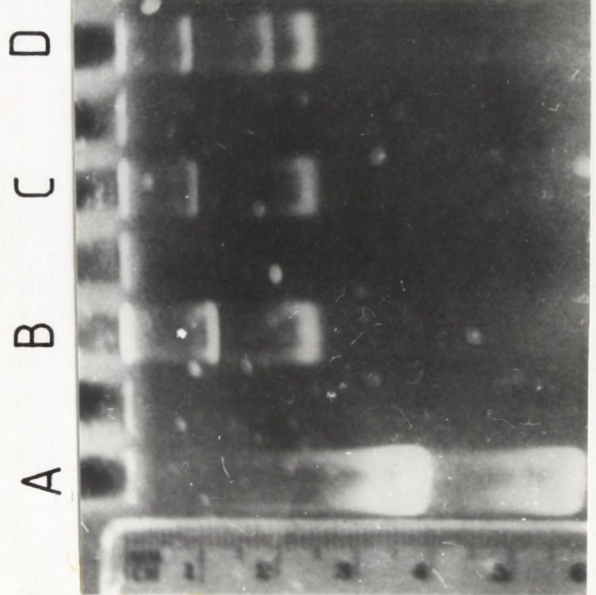
4.9.2 Autoradiograph of filter following hybridisation with
³²P-labelled probe

Lane A, pDU1246 (see text); lane B, R26 sample track, only R26 has bound the probe; lane C, R55-1 sample track, probe has not bound; lane D, pSM-1 sample track, probe has bound to pSM-1, but not to the unknown plasmid or the chromosomal DNA.

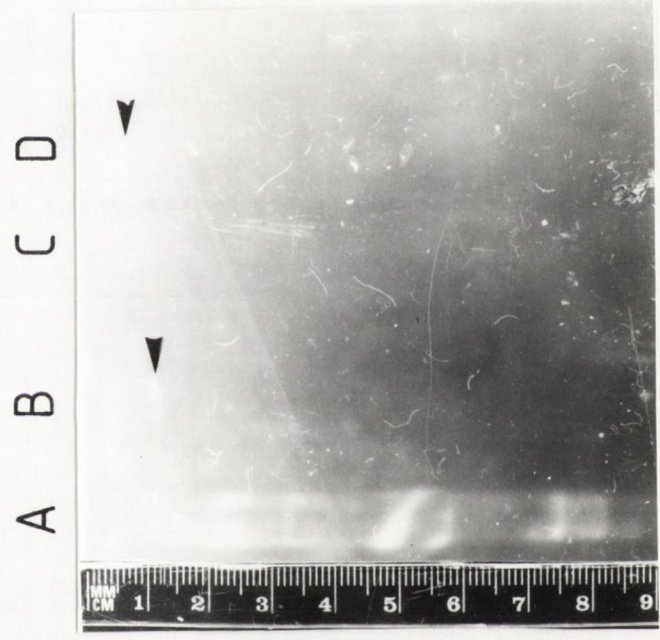
4.9.3 Comparison of digest products of pDU1246 cleaved by
HincII, HindIII or both

Lane A, pDU1246 (HindIII); lane B, pDU1246 (HincII); Lane C, pDU1246 (HincII + HindIII). Note four low molecular weight bands in lane B corresponding to those in lane C. See text for discussion of results.

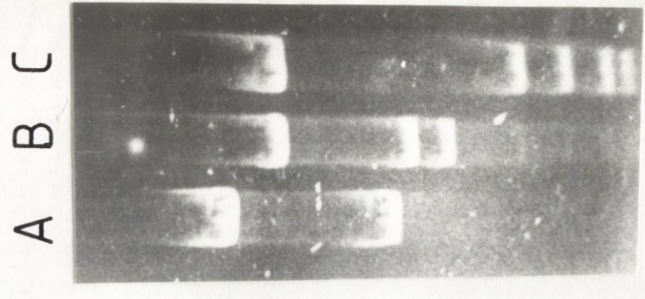
1.



2.



3.



pDU1246 cleaved by HindIII, HincII and HindIII plus HincII (Fig. 4.9.3). The low molecular weight bands in Fig. 4.9.3 (lane C) are equivalent to those in Fig. 4.9.2 (lane A), at and below the 7.5 cm mark. Indeed, the corresponding bands can be detected in Fig. 4.9.3, (lane B), showing that HincII was contaminated by HindIII (both enzymes are produced by Haemophilus influenzae).

G. 1.4 Kb of the 1.8 Kb HindIII fragment are required to specify chloramphenicol resistance

Analysis of the deletion mutant plasmids pDU1333 and pDU1334 indicated that the unique BamHI site within the 1.8 Kb HindIII fragment was located in cml (Fig. 4.6 and Table 4.2). The MIC specified by pDU1335 (Fig. 4.6) suggested that the deleted Pst, - HindIII sequence lay at one end of cml (Table 4.2). Mutagenesis with transposon Tn5 revealed that the minimum amount of DNA required to specify Cm^R was 1.4 Kb. The locations of the Tn5 insertions in 25 independent mutants (pDU1256-1280) are shown in Fig. 4.6. Plasmids pDU1256-1279 were fully Cm^S but pDU1280 still expressed Cm^R albeit at a lower level than pDU1246 (Table 4.2). The Tn5 insertion in pDU1280 lies to the right of the PstI site at which the deletion in pDU1335 was made (Fig. 4.6). This confirmed the suggestion that this PstI site lay near to one end of cml promoter or of the carboxy terminus of the gene until the direction of expression of cml was known. The next Chapter deals with experiments designed to elucidate this.

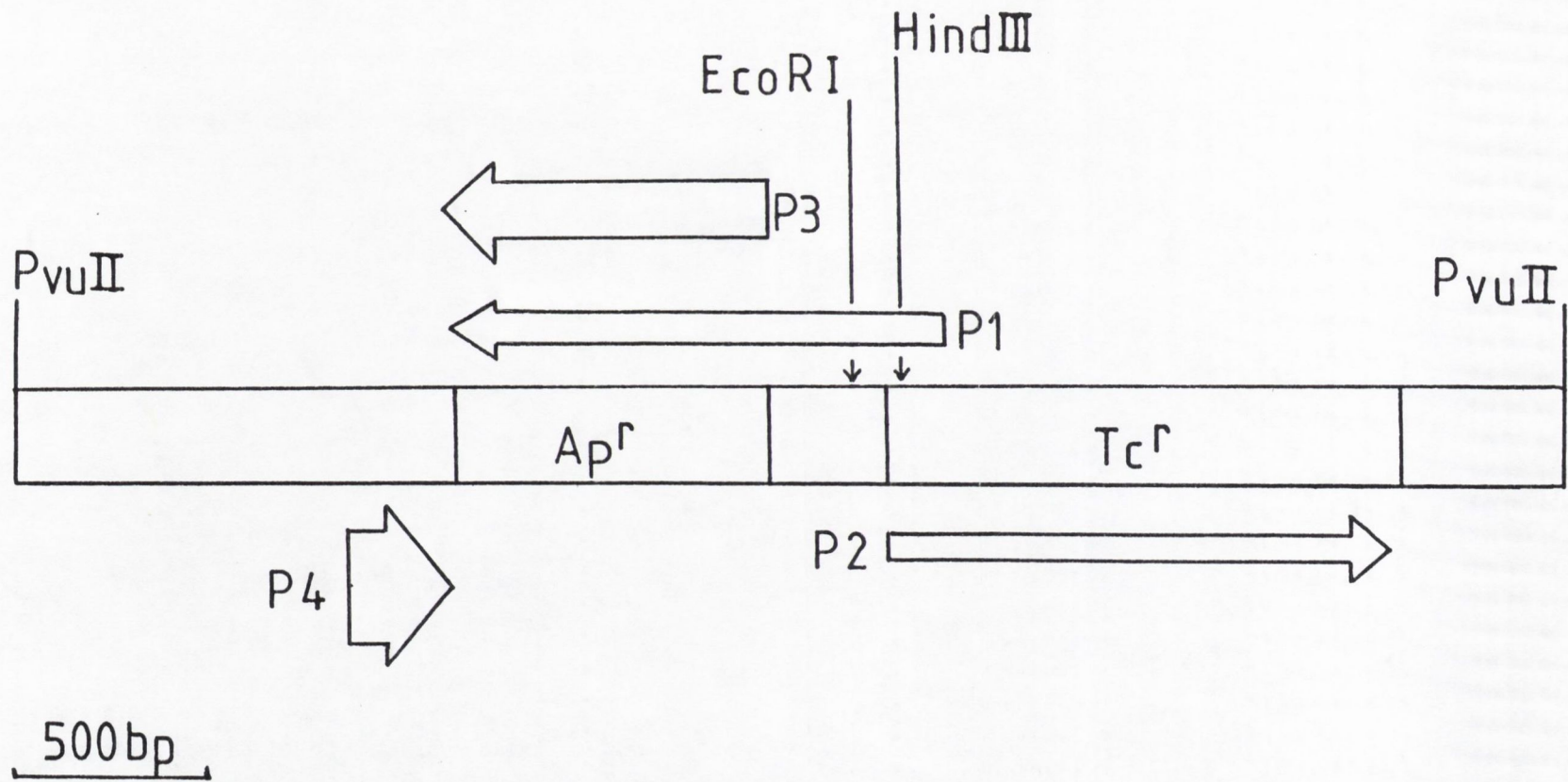
DISCUSSION

This Chapter describes the cloning and preliminary genetic analysis of cml, the Cm^r determinant of plasmid R26. The determinant was cloned on a 7 Kb EcoRI fragment in pBR322. Cm^r was expressed with the cloned DNA in both possible orientations. The 7 Kb fragment was physically mapped using various restriction endonucleases as a prelude to subcloning cml. It was discovered that cml was contained within a 1.8 Kb HindIII fragment which lay close to one end of the 7 Kb sequence. This was cloned in both orientations in pBR322 and the phenotypes conferred on E. coli by the recombinant plasmids studied. The results showed that cml was being expressed constitutively from pBR322. Three reasons were advanced to explain this, any or all of which could have been correct. First, constitutive expression could be a function of gene dosage; second, it could be due to a failure to clone a regulatory function along with the structural gene(s) (this would most probably be a repressor gene); third, constitutive expression could be due to read-through of cml from a powerful pBR322 promoter.

Of the three possible explanations, the third is the least likely. The situation with regard to promoters within pBR322 has been studied in detail by Stuber and Bujard (1981). Their findings are illustrated in Fig. 4.10. Genes inserted at the EcoRI or HindIII sites of pBR322 will come under the influence of only one vector promoter, P1. Constitutive expression of cml was observed when the DNA fragments carrying it were inserted in either orientation. The strong promoter, P4, reads against P1, but since it must read through the Ap^r gene (against the direction of transcription) it is unlikely to have a significant influence on genes inserted at the EcoRI or HindIII sites.

Fig. 4.10 The promoters of the cloning vector plasmid, pBR322

pBR322 sequences are represented by the broad horizontal bar. The plasmid is shown linearised at its unique PvuII site. The positions of the Ap^R and Tc^R genes are indicated. The locations of the EcoRI and HindIII recognition sites, used in constructing the pDU1244-47 Cm^R plasmid series, are indicated. The four major promoters of this vector are represented by arrows. The blunt end of each arrow represents the location of the promoter sequence and the arrow head shows the direction of expression. The extent of the influence of each promoter is shown by the length of the arrows. The depth of the arrows is indicative of the relative strengths of the promoters. The figure is based on that of Stuber and Bujard (1981) and their promoter designations (i.e. P_1 , P_2 , P_3 and P_4) have been retained.



The first and second explanations were investigated simultaneously by the pUB5572 cloning experiments. It was found that cml, whether carried on the 7 Kb EcoRI or 1.8 Kb HindIII fragment, was expressed inducibly in this vector. This shows that cml still possesses its regulatory functions and so the second explanation is incorrect. This means that constitutive expression is primarily a function of gene dosage.

This finding is intuitively appealing in the light of the permeability barrier model proposed by Gaffney *et al.* (1981) to explain the mechanism of Cm^R specified by R26. A permeability barrier at the cytoplasmic membrane could involve a limited number of membrane sites for the cml product. These sites are adequately supplied by induced cml in the single copy state. Increasing the gene dosage could raise the uninduced MIC to the induced level by saturating the available membrane sites with many additional copies of the gene product. At this point the upper limit has been reached and further copies of the gene product are redundant, having no membrane sites to occupy. The overall effect is that the determinant appears to be constitutively expressed.

These results show that the entire Cm^R determinant resides with the 1.8 Kb HindIII fragment. Deletion mutagenesis and transposon Tn5 mutagenesis showed that of this 1.8 Kb, 1.4 Kb was the minimum amount of DNA required to specify Cm^R . Furthermore, data obtained from experiments with two mutant plasmids, (the deletion mutant plasmid pDU1335 and the Tn5 insertion mutant plasmid, pDU1280) allowed one of the boundaries of cml to be approximately determined. Whether this is the promoter or the carboxy terminus of the gene cannot be determined until the direction of expression of cml is known. This is dealt with in the next Chapter.

In Chapter 3, the Cm^r determinants of plasmids R26 and R55-1 were compared with regard to their ability to confer resistance to chloramphenicol and a number of chloramphenicol analogues. In this Chapter it was shown that these determinants are not closely related at the DNA sequence level, as determined by Southern blot hybridisation. It was shown, however, that cml is closely related to an inducible, low-level Cm^r determinant on an Inc C plasmid from a clinical isolate of Serratia marcescans. It is now known that this plasmid also resides in clinical isolates of Proteus spp. and E. coli. In Proteus the plasmid exists in two forms, one Tc^r and one Tc^s (H. Griffin and D.C. Coleman, personal communication). A possible evolutionary origin for cml is discussed in Chapter 8.

CHAPTER 5

THE REGULATION OF EXPRESSION OF THE
CHLORAMPHENICOL RESISTANCE DETERMINANT
OF PLASMID R26

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The Regulation of Expression of the Chloramphenicol Resistance
Determinant of Plasmid R26

INTRODUCTION

This Chapter deals with experiments which were performed to investigate the control of expression of cml (the Cm^r determinant of R26). Before turning to these, various models for procaryotic gene regulation will first be described.

A. The regulation of bacterial gene expression

I. Positive and negative control of transcription

(a) The lactose operon

The prototype system for the study of bacterial gene expression has been the lactose operon (for review, see Miller and Reznikoff, 1978). Work carried out on this system in the early 1960s gave rise to the concepts of "structural" and "regulatory" genes (Jacob and Monod, 1961). In the lactose operon there are three structural genes, transcribed in the order lacZYA (Beckwith, 1978). lacZ encodes the alpha subunit of β -galactosidase, the enzyme which catalyses the cleavage of lactose to glucose and galactose; lacY encodes a permease to admit the carbohydrate to the cytoplasm and lacA encodes a transacetylase to acetylate the sugar. The three genes are transcribed as a continuous message, that is a polycistronic mRNA species, from a promoter (or binding site for RNA polymerase) which lies upstream from lacZ (Beckwith, 1978). The lacI gene lies upstream from this promoter, and is transcribed from a promoter of its own (Miller, 1978). This gene encodes the regulatory protein, a repressor, which in the absence of β galactoside substrate will bind to a stretch of DNA, called the operator, which

lies between the lacZYA promoter and lacZ. When the repressor is bound to the DNA at that site, transcription of lacZYA is precluded and the operon is said to be "repressed", a phenomenon first described by Monod and Cohen-Bazire (1953) for the tryptophan synthetic pathway genes. Under such conditions, only about five molecules of β -galactosidase will be found per cell (Lewin, 1973). When lactose or an equivalent β galactoside is present in the cell, the sugar binds to the lacI gene product, altering the conformation of this protein tetramer such that binding to the lac operator cannot occur. RNA polymerase is then able to bind to the lacZYA promoter and transcription of the polycistronic message begins. In about 200 seconds, the level of β -galactosidase in the cell increases to around 5,000 molecules (Lewin, 1973; Miller and Reznikoff, 1978). This phenomenon is known as "induction"; the lactose molecule is the inducer and the lac operon is said to have been induced. It provides the bacterium with a genetic switch to bring genes into use only when they are required, thus saving metabolic energy.

Other early work on gene regulation involved studies of bacteriophage genes, particularly of coliphages. Bacteriophage lambda was studied in great detail from the point of view of the control of expression of its genes (Hendrix et al., 1983), as were the T-even phage, T4, and the T-odd phages T-3 and T-7 (reviewed by Lewin, 1977). During the 1960s these systems supported the idea of negative control of bacterial genes mediated by repressor molecules. Since that time it has been realised that the processes involved in regulation are much more complex (Englesberg and Wilcox, 1974).

(b) Catabolite repression and the *lac* operon

There are now a number of well-studied systems which illustrate the various facets of gene regulation (Glass, 1983; Lewin, 1983). The lactose operon (Fig. 5.1A) remains an excellent example of negative control of a polycistronic genetic system. This negative control is now known to co-exist with another transcriptional control mechanism, in this case a positive effector. This enhancement of transcription is brought about by the level of cyclic AMP in the cell and the effect is directed at the promoter, not the operator, (Pastan and Perlman, 1968). Glucose represses the level of cAMP in cells, possibly by promoting its excretion into the incubation medium. In the absence of glucose, cAMP binds to an apoinducer protein known alternatively as CAP (catabolite activator protein) or CRP (cyclic AMP receptor protein) and this complex may then bind to a DNA sequence known as a CAP-binding site, in the vicinity of the promoter for *lacZYA* (Fig. 5.1). This binding enhances the rate of transcription of the operon. When glucose is present, this enhancement is precluded due to the reduction in the intracellular concentration of cAMP and so glucose is metabolised by the cell in preference to galactoside sugars. This is more energetically favourable as less metabolic energy is required to utilise glucose as a carbon source than lactose or other galactosides (Lewin, 1974; 1983). This control of transcription, exerted by glucose, is called catabolite repression. It occurs in many other genetic systems.

(c) The arabinose operon

This system consists of five structural genes, three of which are cotranscribed as a polycistronic message (*araBAD*) and two others

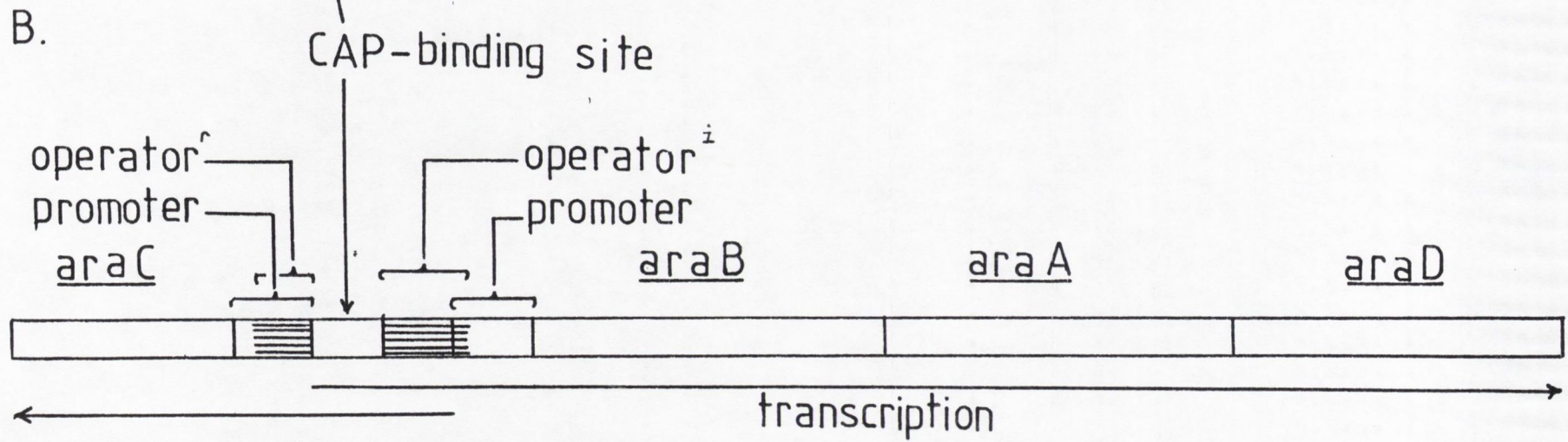
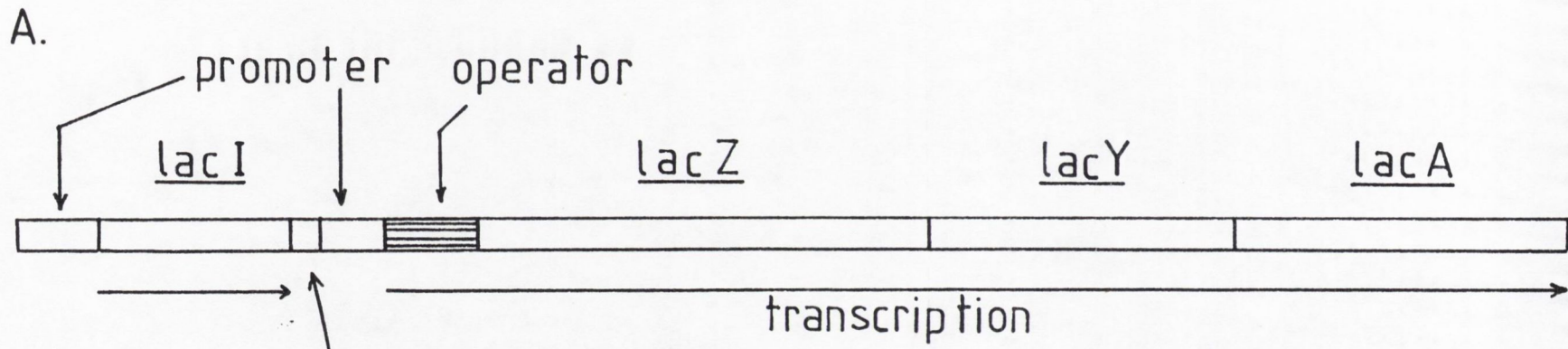
Figure 5.1 The lactose and arabinose operons of *Escherichia coli*

A. The lactose operon

The DNA sequence which includes the genes and regulatory regions of the operon is represented by the broad bar. The lac repressor gene, lacI, is the most leftward of the four lac genes, and is transcribed independently. The lacZ, lacY and lacA genes are co-transcribed. The directions of transcription of the cistrons are shown by the horizontal arrows. The locations of the promoters, the operator and the CAP-binding site are indicated.

B. The arabinose operon

The DNA sequence encoding the genes and regulatory regions of the operon is represented by the broad bar. The ara regulatory gene, araC, is the most leftward of the four ara genes. araB, araA, and araD, are co-transcribed. The promoters for araC and araBAD are opposed to one another. The directions of transcription of these genes are indicated by horizontal arrows. The operator sequences are denoted by shaded areas and these overlap with the diverging promoters. Operator^r is the binding site for the araC product when the operon is repressed, and operatorⁱ is used when the system is induced. The CAP binding site is located between the promoters.



araE and araF which are unlinked (Lee et al., 1981; Lewin, 1983; Ogden et al., 1980). There is a regulatory gene, araC, which subjects all five structural genes to both positive and negative control, these genes being further controlled positively by cAMP (Englesberg and Wilcox, 1974). This system may be termed a regulon, in that a number of unlinked genes or gene clusters share a common control function. araC is linked to araBAD but is transcribed in the opposite orientation (Wilcox et al., 1974). The expression of araC is repressed by its own product and stimulated by cAMP (Lee and Carbon, 1977). Thus the araC product has three functions: (1) Negative control of araC; (2) negative control of araBAD; (3) positive control of araBAD. As a multifunctional control factor, the amino acid sequence of the araC protein has been highly conserved in different bacterial species (Stoner and Schleif, 1982). The main features of the ara system are shown in Fig. 5.1B. In the absence of arabinose, the araC product binds to an operator site to prevent transcription of the ara genes. Arabinose binds to this protein and prevents its functioning as a repressor, just as in classical negative control. However, this binding has an additional effect in that the repressor protein is converted to a positive effector (Englesberg et al., 1965). The positive effector no longer binds to the operator but to an alternative site, called the initiator region, at which it actively enhances transcription (Fig. 5.1B). Those control regions lie in the 147 bp gap between araC and araBAD, thus allowing simultaneous control of these systems. When the araC product is acting as a repressor, no other protein can bind in this region. When it is converted to a positive effector and is bound to its initiator site, the cAMP-CRP complex can bind to its site and RNA polymerase can

bind to the promoters for araC and araBAD (Fig. 5.1B). The transcription of a regulatory gene from the same control region as is involved in regulation of the structural genes allows coordinate regulation of the entire system. Such an arrangement is now known to obtain in a number of other operons.

(d) Other examples of positive and negative control of transcription

Some other systems which can be grouped under this heading include: the galactose operon (Busby et al., 1982); the arginine system (Beny et al., 1982), the biotin operon (Kotval et al., 1982); the maltose regulon (Debarbouille et al., 1982); the deo operon (Valentin-Hansen et al., 1982); the hexuronate operon (Hugouvieux-Cotte-Pattat and Robert-Baudouy, 1982). Each of these systems represents a variation on the theme of positive and negative transcriptional control.

II. Transcriptional Attenuation

(a) The Tryptophan operon

This system has yielded a great deal of insight into another mode of transcriptional regulation of bacterial genes. The trp operon consists of five structural genes, trpEDCBA, whose products are required to synthesise tryptophan (Imamoto and Yanofsky, 1967). These genes are under repressor control, the regulatory protein being encoded by the unlinked trpR gene (Zubay et al., 1972). The operator site for this protein lies between the trp promoter and trpE (Rose and Yanofsky, 1974). There is a weak internal promoter between trpD and trpC, allowing basal expression of the trpCBA genes, even under repressed-operon conditions (Bauerle and Margolin, 1967; Morse and Yanofsky, 1968).

The trp operon is responsive to the level of tryptophan in the cell. The amino acid functions as a co-repressor to repress transcription from the main promoter. In addition to this control, a more subtle form of regulatory circuit is at work. The trpE gene is preceded by a 162bp translated leader. The regulatory function of this part of the message is shown in Fig. 5.2. Regulation depends upon the formation or non-formation of a transcription terminator structure (Rosenberg and Court, 1979). Suitably placed ribosomes, on the message, can preclude the base-pairing required for this terminator to form. The positioning of the ribosome in the anti-termination mode is determined by two sequential Trp codons (Lee and Yanofsky, 1977). When tryptophan is in short supply, the ribosomes stall at these codons and anti-termination is achieved. Transcription of trpEDCBA can now proceed (Yanofsky, 1981). Transcriptional attenuation has now been shown to control several biosynthetic operons. These include the his operon (Barner, 1978a; Dinocera et al., 1978); the thr and ilv operon (Gardner, 1979, 1982; Lawthur and Hatfield, 1980; Nargang et al., 1980); the leu operon (Gemmill et al., 1979; Keller and Calvo, 1979); the phe operon (Zurawski et al., 1978).

A regulatory system, which is closely analogous to those of these biosynthetic operons, controls the E. coli phenylalanine-tRNA synthetase operon (Fayat et al., 1983; Springer et al., 1983). This is not true of all tRNA synthetases. Alanyl-tRNA synthetase is autogenously regulated at the transcriptional level (Putney and Schimmel, 1981).

III. Autogenous Control of Transcription

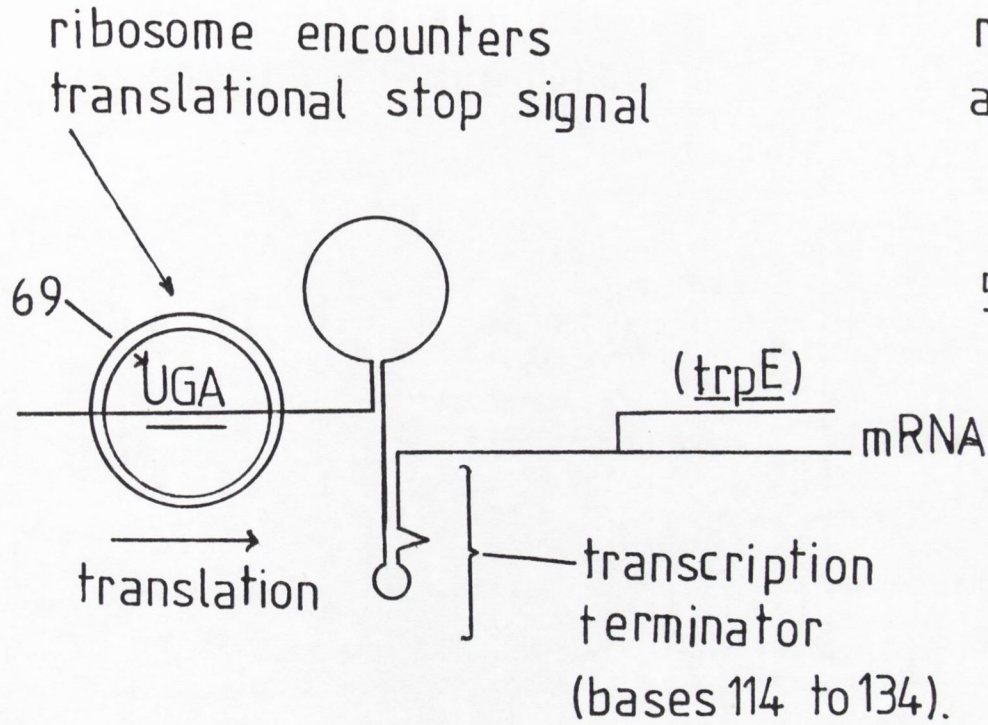
Genes which are regulated in this way encode bifunctional

Figure 5.2 Tryptophan operon sequences involved in transcriptional attenuation

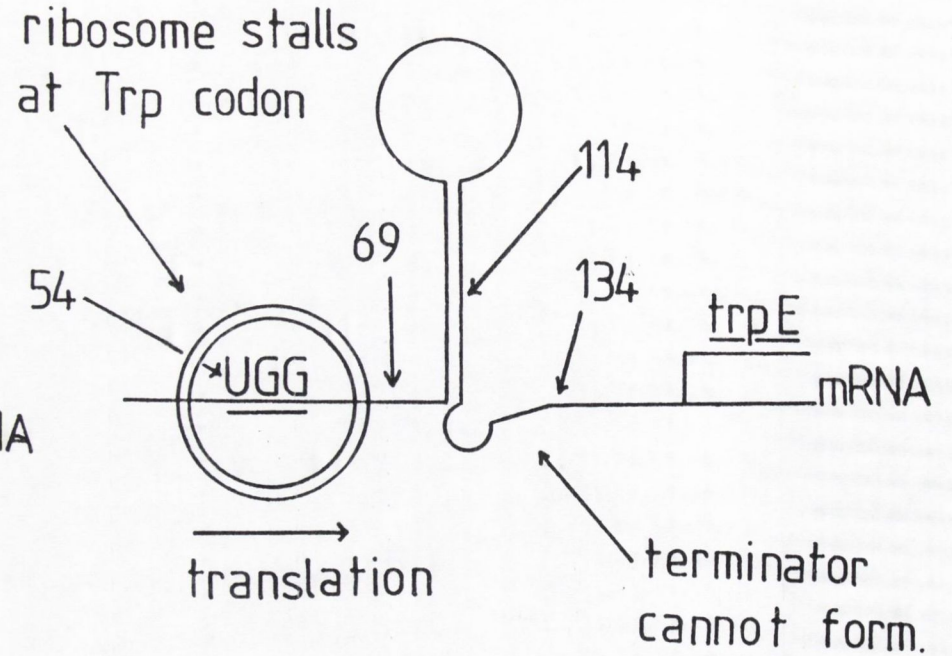
Possible conformations for the trp operon leader mRNA are shown. Regions of base-paired ribonucleotides are indicated by parallel lines. The 5' end of the trpE gene is denoted by an open bar. Ribosomes are represented by large concentric circles. The direction of translation is shown by a horizontal arrow.

A. The mRNA conformation which leads to transcriptional termination is shown. The ribosome is shown at the translational stop codon of the trp leader open reading-frame. Translation of this leader is only possible in the presence of tryptophan and permits formation of the transcriptional terminator stem-and-loop structure. This causes dissociation of mRNA and DNA template before trpE can be transcribed. For this reason trpE is shown in parentheses.

B. The anti-terminator conformation is illustrated here. The ribosome is shown stalled at the first of two sequential Trp codons in the leader peptide open reading-frame. This stalling occurs when tryptophan is in low concentrations in the cell. It permits the formation of a stem-and-loop structure which is an alternative to the transcription terminator shown in A. Without the terminator structure, the remainder of the trp operon can be transcribed.



A. TERMINATION.



B. ANTI TERMINATION.

products. The products can perform a role as regulatory elements in addition to their primary function. The alanyl-tRNA synthetase gene has already been listed as being autogenously controlled, as has the araC gene. The trpR gene is autogenously regulated at the level of transcription (Kelley and Yanofsky, 1982). So is the tyrR gene, which encodes the regulatory function for the tyrosine operon (Camakaris and Pittard, 1982). Autogenous regulation has been described for phage genes, such as the arc gene of P22, which is required for lysogeny (Youderian et al., 1982). Repressor synthesis in lambda phage is also controlled by transcriptional autoregulation (Pastrana, 1982).

IV. Translational Control Circuits

These have been described for ribosomal protein genes (Nomura et al., 1980; Yates et al., 1980). These genes are organised into operons, within which one gene product functions as a regulatory protein as well as a ribosomal component. Such dual-purpose proteins include L4 (Yates and Nomura, 1980; Sengel et al., 1980), L10 (Brot et al., 1980; Fukuda, 1980; Yates et al., 1981), S4 (Jinks-Robertson and Nomura, 1982), S7 (Dean et al., 1981a) and S8 (Dean et al., 1981b). The mechanism involves binding of the regulatory proteins directly to the mRNA at the translational initiation site, thus preventing gene expression. The proteins normally bind to rRNA in ribosomes but when the level of protein production exceeds requirements they can perform this alternative RNA-binding function. The rate of production of rRNA is controlled transcriptionally, possibly via attenuation (Kingston and Chamberlin, 1981; Tsugawa, 1982).

Translational autoregulation controls expression of gene 32 in bacteriophage T4. The product, gp32 is a protein which binds to single-stranded DNA. When this protein is overproduced, it can bind to an AT-rich region which straddles the ribosome-binding site of gene 32 mRNA and prevent translation initiation (Kirsch and Allet, 1982). Single-stranded polynucleotide binding proteins regulate translation in phage M13 also. Here the gene V product regulates the expression of gene II and gene X by binding to their mRNA products (Model et al., 1982; Yen and Webster, 1982).

Translational control can be exerted through mRNA modification in bacteria. Gene 1.2 mRNA of phage T7 must be processed by RNase III before it can become functional. Processing removes a base-paired region which sequesters the ribosome binding site in unmodified mRNA (Saito and Richardson, 1981). Sites distal to genes can exert post-transcriptional control. The lambda int gene is stabilised by a base-paired region in its mRNA. If this fails to form, the mRNA is rapidly degraded. This mechanism underlies a transcriptional control circuit involving lambda-encoded positive effectors (Guaneros et al., 1982). In phage MS2, frame-shifting during translation is essential if overlapping genes are to be expressed. Here expression of the lysis gene depends on initiation of translation of the coat protein gene (Kasteleiss et al., 1982).

The foregoing list of examples, which is by no means exhaustive, illustrates the types of control circuits employed by bacteria and their bacteriophages to regulate expression of their genes. Antibiotic resistance genes are also often subject to regulation (for the same reasons as are other genes, namely to avoid wasteful synthesis of products not required by the cell in order to survive in its present environment). The following is a list

of regulated genes involved in expression of antibiotic resistance in bacteria.

B. Regulated antimicrobial resistance determinants

Plasmid-determined resistance to tetracycline is usually inducible, both in gram-positive and gram-negative bacteria. The type B tet determinant of transposon Tn10 is controlled at the level of transcription. Two genes are involved, a structural gene, tetA, and a repressor gene, tetR. These are divergently transcribed and the tetR gene product binds to an operator region between the genes to repress transcription of tetA and tetR (Beck et al., 1982; Hillen and Schollmeir, 1983; Wray et al., 1981; Wray and Reznikoff, 1983; Yang et al., 1976). [Among gram-positive bacteria, the tet determinant of plasmid pT181 may be regulated via an attenuation mechanism. This observation is based on DNA sequence data alone (Khan and Novick, 1983)].

Divergent transcription of structural and regulatory tet genes in gram-negative bacteria resembles the situation in the arabinose operon. Other systems which have features in common with ara in terms of gene regulation are the mercuric ion resistance operons of plasmid R100 and transposon Tn501 (Foster, 1983). Here, the regulatory gene product (encoded by merR) negatively regulates its own expression and also acts as a positive and negative regulator of the mer operon structural genes (Foster, 1983; Ni Bhriain et al., 1983).

Catabolite repression plays a role in the regulation of resistance determinants, including tet, mer, and gram-negative cat genes. It may also have a role to play in the regulation of some aminoglycoside resistance determinants (Davies and Smith, 1978).

Transcription of genes in Staphylococcus aureus which specify penicillinases type A, B or C is inducible (Dyke, 1979; Richmond, 1965a; Richmond, 1965b). The repressor is the product of the penI gene (Richmond, 1965b). This is thought to bind to an operator site (a 26 bp inverted repeat) which overlaps the structural gene promoter (McLaughlin et al., 1981). Evidence has also been advanced for a role for a chromosomal gene, acting as an antirepressor, which complexes with the plasmid-determined repressor in the presence of inducer (Imsande, 1973; 1978).

The transcription of ampC, a chromosomal gene specifying β -lactamase in E. coli, is regulated by an attenuation mechanism which is growth-rate related (Jaurin et al., 1981). Regulation depends upon the increase in ribosome numbers within the cell which occurs with increased growth rate (Maaloe, 1979). At high growth rates, the higher ribosome concentration means that ribosomes are more likely to bind to the amp leader and so stall at a translational stop codon in the message. In this way they prevent the formation of a transcriptional terminator, analogous to that found in some amino acid synthetic pathway operons (Jaurin et al., 1981). The result is anti-termination and therefore increased expression of ampC.

Attenuation of a different sort plays a role in the regulation of expression of MLS (macrolide-lincosamide-spectogramin B) resistance encoded by plasmid pE194. In this case it is translation rather than transcription which is being attenuated (Halin et al., 1982; Horinouchi and Weisblum, 1980; Horinouchi and Weisblum, 1981; Shivakimar et al., 1980; Gryczan et al., 1980).

The gene for MLS resistance carried on pE194 is ermC (Horinouchi and Weisblum, 1980; 1982b) whose product is a 29K methylase

which methylates 23S ribosomal RNA, making the ribosome resistant to MLS drugs (Lai et al., 1973; Shivakumar and Dubnau, 1981). This open reading-frame is preceded in the message by a regulatory region which includes another, short, open reading-frame which is prefaced by an efficient ribosome-binding site (Halin et al., 1982; Horinouchi and Weisblum, 1980; Grycran et al., 1980; McLaughlin, 1981; Shine and Dalgarno, 1974). This region is capable of forming secondary structures by base-pairing of complementary sequences (Fig. 5.3). In uninduced cells the ribosome binding site (RBS2) of the methylase reading frame is sequestered in a hairpin-loop structure, ribosomes cannot bind and the message is not translated. Induction is achieved by altering the secondary structure of the transcript such that this sequence becomes available for ribosome binding (Gryczan et al., 1980; Hahn et al., 1982; Horinouchi and Weisblum, 1980). In the presence of the drug, a ribosome stalls while translating the short leader peptide. This precludes the formation of the secondary structure which normally masks RBS2 and so the ermC gene can be expressed. The message takes up an alternative secondary structure, leaving RBS2 free for ribosome binding (Fig. 5.3). Other factors at work include a phenomenon described by a kinetic trapping model (Halin et al., 1982) which proposes that RBS2 is initially available as that part of the sequence is translated thus permitting a ribosome to bind and give a basal level of methylase prior to sequestering of the Shine-Dalgarno sequence; it is also proposed that the nature of the amino acids to be included in the leader peptide has a controlling influence on where the ribosome stalls in its translation.

The incorporation of amino acids with bulky hydrophobic side chains in the carboxy terminus favours ribosome stalling at that part

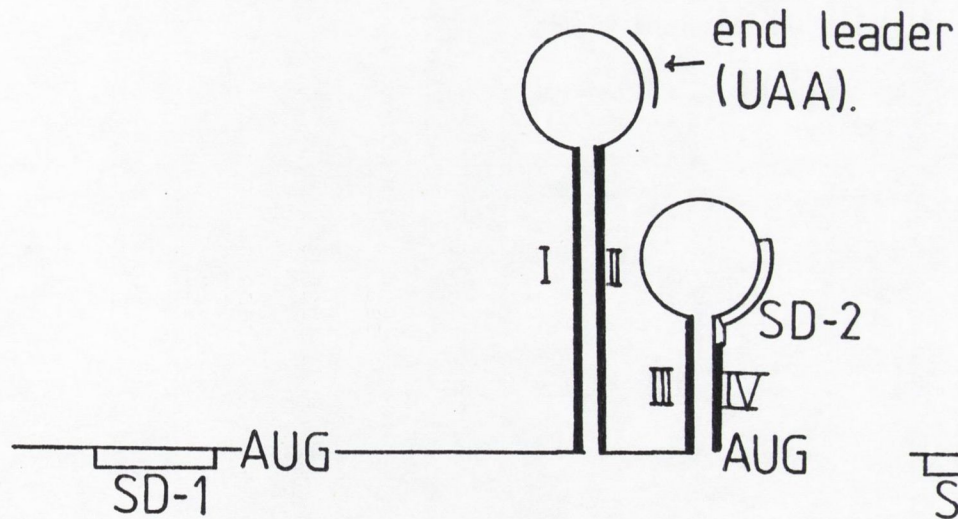
Figure 5.3 Alternative conformations of mRNA sequences involved in posttranscriptional control of erythromycin resistance (Em^r) in gram-positive bacteria

Possible conformations of the regulatory region of Em^r mRNA are shown. Regions of base-paired ribonucleotides are denoted by parallel lines. SD-1 is the Shine-Dalgarno sequence preceding the Em^r leader peptide open reading frame. The translation initiation codon for the leader peptide is shown, as is the terminator codon (UAA). SD-2 is the Shine-Dalgarno sequence preceding the methylase gene of the Em^r determinant. The AUG start codon for this gene is shown. Regions of mRNA which take part in base-pairing are numbered with Roman Numerals. The ribosome is represented by the hatched circle and an erythromycin molecule is shown bound to it.

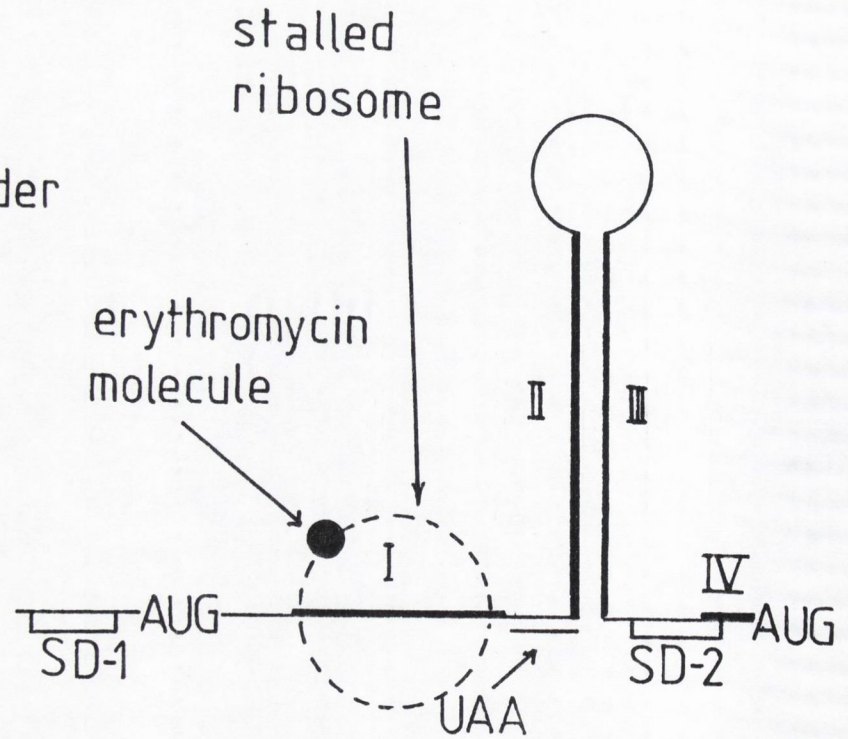
A. This shows the situation in the uninduced state. Region I pairs the region II and region III pairs with region IV. The Shine-Dalgarno site for the methylase gene (SD-2) is inaccessible to ribosomes and the gene is not translated. Ribosomes can, however, bind at SD-1 and translate the leader peptide.

B. In the induced state, a ribosome in the act of translating the leader peptide binds a molecule of erythromycin. The ribosome then stalls while translating region I. This prevents the I-II paired structure from forming. An alternative structure is formed when regions II and III pair. Region IV remains unpaired and so SD-2 is available for ribosome binding, permitting translation of the methylase gene.

A. UNINDUCED.



B. INDUCED.



of the message (Mao and Robinshaw, 1971; 1972). Attenuation of translation also features in the regulation of MLS resistance in Streptococcus sanguis (Horinouchi et al., 1983) and Bacillus licheniformis (Gryczan et al., 1984).

Among gram-positive bacteria, the regulation of CAT synthesis has been studied in the most detail at the molecular level in S. aureus (Ambulos et al., 1984; Byeon and Weisblum, 1984; Horinouchi and Weisblum, 1982a) and Bacillus pumilus (Duval et al., 1983; Williams et al., 1981a, 1981b).

As with their work on MLS resistance encoded by pE194, Horinouchi and Weisblum relied heavily on DNA sequence data to propose a mechanism of regulation of CAT expression in pC194, a small S. aureus plasmid. The open reading frame encoding CAT was preceded by an untranslated leader message in which the ribosome binding site for the cat gene could be sequestered in a stable secondary structure formed by pairing complementary bases (Horinouchi and Weisblum, 1982). Only one secondary structure was predicted, unlike the MLS resistance situation, in which alternative structures could be demonstrated (Horinouchi and Weisblum, 1980; 1981; 1982b). For this reason, the same type of translational attenuation model could not be proposed for pC194 CAT. Ambulos et al., (1984) have shown the secondary structure in the cat message to be important in regulating expression of the gene. Byeon and Weisblum (1984) put forward a regulation model based upon the RNA sequence of procaryotic 23S rRNA. A detailed description of this model is given in Chapter 7, where the DNA sequence of cml is considered. Here, the main features of the model are briefly outlined. In 23S rRNA a sequence may be found which has the potential of binding to that part of the cat leader which is thought to pair with the

cat ribosome binding site (Noller et al., 1981). In the absence of Cm, the model proposes that this 23S rRNA sequence is itself sequestered in a stem-and-loop structure. When Cm is present, the conformation of the ribosome is altered through drug binding such that the 23S rRNA sequence now becomes exposed and can take part in binding to cat mRNA. The secondary structure which masks the cat ribosome binding site cannot now form, so the cat message can be translated by unmodified (i.e. Cm-free) ribosomes. The situation with regard to the control of CAT gene expression in B. pumilus is broadly similar (Duval et al., 1983; Harwood et al., 1983; Williams et al., 1981a; 1981b).

Having discussed the strategies employed by bacteria to regulate gene expression, and then seen how these apply to genes for antibiotic and heavy metal ion resistance, it is now possible to discuss experiments aimed at determining the mode of regulation of the inducible chloramphenicol resistance determinant of plasmid R26. These experiments involved the in vitro construction of hybrid genes by fusing the cml coding sequence to that of the lacZ gene in E. coli lactose operon. lacZ is the structural gene for the α -subunit of β -galactosidase. This enzyme can be readily assayed and so provides a powerful tool for studying the control of gene expression.

C. lac fusion technology

Techniques exist which permit fusions to be made at the transcriptional level or translational level, in vivo or in vitro. In vitro methods involve the joining of restriction fragments using specially constructed recombinant plasmids as the source of the lac genes (Casadaban et al., 1980). To generate in vivo

fusions, a derivative of bacteriophage Mu is used which carries an ampicillin resistance determinant and the lacZ and lacY genes. The Mu derivative employed to obtain transcriptional fusions is termed MudI (Casadaban and Cohen, 1979), while that used to generate translational fusions is MudII (Casadaban and Chou, 1984).

The in vivo bacteriophage system is used for genes which occur at single copies within the cell. Insertion of the fusion phage into the target is random. Thus a variety of fusions can be generated in the gene of interest (Hall and Silhavy, 1981).

Since the in vitro method depends upon the ligation of DNA fragments generated by restriction endonuclease digestion, the locations of the junctions between the fused sequences are predictable.

The construction of transcriptional or "operon" fusions requires a lacZ gene which does not have a transcription start signal of its own. It has its own ribosome binding site and translational start codon. The lacZ gene will only be transcribed if an exogenous promoter is supplied, thus the β -galactosidase gene acts as a promoter "probe". The relative strengths of exogenous promoters can be measured by assaying the amount of β -galactosidase produced (Miller, 1972).

The generation of translational or "gene" fusions depends upon the observation that the amino-terminal end of the β -galactosidase protein is not needed for enzymatic activity (Muller-Hill and Kania, 1974). Up to 27 amino acid codons of lacZ can be removed from the amino terminus and the remainder joined to a DNA segment that encodes the amino terminus and the regulatory signals of another gene, yielding a hybrid protein that retains a

β -galactosidase component that is enzymatically active and has the same specific activity as the intact enzyme (Casadaban et al., 1980; Sarthy et al., 1979; Welply et al., 1980). There is virtually no limit to the amount of DNA which can be fused to lacZ to give a "gene" fusion (Chou et al., 1979a; 1979b; Cohen et al., 1979; Muller-Hill and Kania, 1974).

As with the "operon" fusion, the lacZ derivative used in making "gene" fusions must be supplied with an exogenous promoter, but it must also be given a ribosome binding site and in-phase translational start codon. This makes the system a useful one for determining unambiguously the direction of transcription/translation of a gene (Casadaban et al., 1980; Casadaban and Chou, 1984).

MATERIALS AND METHODS

A. Bacterial strains and plasmids

E. coli K12 strains C600 and XAc Su⁻ have been described in Chapter 4. The characteristics of the plasmids discussed in this Chapter are given in Table 5.1.

B. Bacteriological media, chemicals and antibiotics

MacConkey agar (oxid) was used to grow bacteria containing putative cml-lac fusions. The nutrient broth and agar used were as described in Chapter 2. Antibiotics (ampicillin, chloramphenicol, kanamycin, tetracycline and trimethoprin) were used as described in Chapter 4. ONPG (o-nitrophenyl- β -D-galactoside), used in β -galactosidase assays, were purchased from Sigma.

C. Construction of transcriptional (operon) fusions

A promoter-less lacZ-lacY operon is carried within a 7.7 Kb BamHI fragment of pMC903 (Casadaban et al., 1980). This plasmid was digested with BamHI and the 7.7 Kb fragment excised from an agarose gel using the freeze-thaw method described in Chapter 4. The pBR322 cml⁺ chimeric plasmid, pDU1246, contains two BamHI sites, one of which is within cml (Fig. 5.5 and Chapter 4). It was partially digested with BamHI and the digest products mixed with the 7.7 Kb lac BamHI fragment from pMC903 and ligated. Transformants of C600 which were Ap^r Cm^s were selected. Plasmid DNA was isolated using the small scale cleared lysate method (Chapter 4). A plasmid was obtained which consisted of pDU1246 with the 7.7 Kb fragment from pMC903 inserted in the BamHI site within cml (Fig. 5.4.1). This recombinant plasmid was designated

Table 5.1 Plasmids

Note: Lac^+ indicates sufficient expression of β -galactosidase to form red or pink colonies on MacConkey agar. (Lac^+) indicates insufficient expression of β -galactosidase to form pink colonies on MacConkey agar.

<u>Plasmid</u>	<u>Phenotypic markers</u>	<u>Comments</u>	<u>Reference</u>
pMC903	Ap ^r Km ^r	Has <u>Bam</u> HI fragment with promoter-less <u>lac</u> operon	Casadaban et al., 1980
pMC931	Ap ^r Km ^r	Has <u>Bam</u> HI fragment with <u>lacZ</u> derivative suitable for constructing gene fusions	Casadaban et al., 1980
pDU1252	Ap ^r	pBR322 with a 1.1 Kb deletion in the <u>tc^r</u> region	This study
pDU1253	Ap ^r Cm ^r	pDU1252 with 1.8 Kb <u>Hind</u> III fragment from pDU1246	This study
pDU1290	Ap ^r Lac ⁺	7 Kb <u>Bam</u> HI fragment from pMC903 in <u>Bam</u> HI site of pDU1246	This study
pDU1291	Ap ^r Lac ⁺	As for pDU1290, but with insert in reverse orientation	This study
pDU1292	Tp ^r (Lac ⁺)	<u>cml-lac</u> construction from pDU1290 in <u>Hind</u> III site of pUB5572	This study
pDU1293	Tp ^r (Lac ⁺)	<u>cml-lac</u> construction from pDU1291 in <u>Hind</u> III site of pUB5572	This study
pDU1294	Ap ^r (Lac ⁺)	ϕ (<u>cml'-lacZ⁺</u>) formed from 6.8 Kb <u>Bam</u> HI fragment of pMC931 in pDU1253	This study
pDU1295	Ap ^r	6.8 Kb <u>Bam</u> HI fragment of pMC931 in pDU1253 in reverse orientation	This study

Figure 5.4 Representative agarose gels from lac fusion construction experiments

1. Construction of pDU1290, a transcriptional fusion plasmid derived from pDU1246

Lane A, bacteriophage λ cleaved by BamHI and EcoRI to provide molecular weight markers; lane B, pDU1290, cleaved by BamHI and HindIII, note 7.7 Kb lac BamHI fragment; lane C, pDU1246 cleaved by BamHI and HindIII.

2. Construction of pDU1252, a 3.2 Kb derivative of pBR322, and construction of pDU1253, a Cm^r derivative of pDU1252

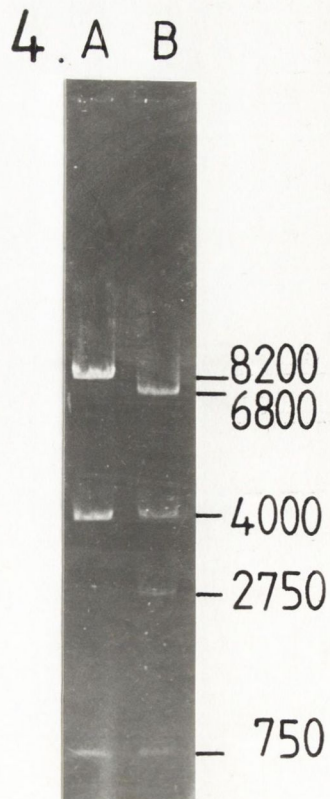
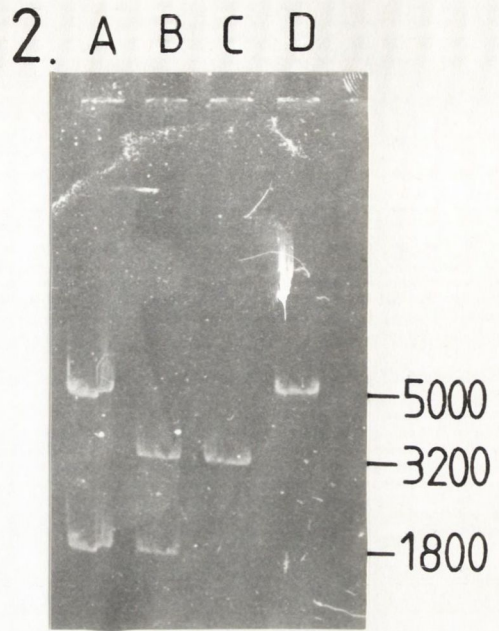
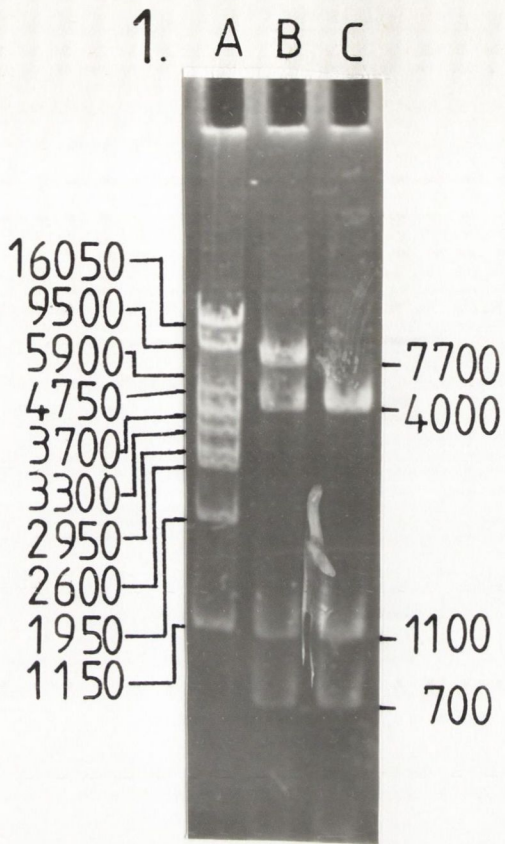
Lane A, pDU1246 cleaved by HindIII, note 1.8 Kb Cm^r HindIII fragment; lane B, pDU1253 cleaved by HindIII, note the 1.8 Kb Cm^r HindIII fragment and the 3.2 Kb pDU1252 component; lane C, pDU1252, linearised with HindIII to give a 3.2 Kb band (this plasmid contains a unique HindIII site); lane D, pDU1253 linearised with BamHI (this plasmid has a unique BamHI site; that within the 1.8 Kb Cm^r HindIII fragment).

3. Insertion of the 6.8 Kb BamHI lac fragment of pMC931 into the BamHI site of pDU1253: construction of a translational fusion

Lane A, pDU1294, cleaved by BamHI (the 5000bp band is pDU1253); lane B, pMC931 cleaved by BamHI (the 3700 band contains the Ap^r and Km^r genes of this plasmid); lane C, pDU1295 cleaved by BamHI (the 5000 bp band is pDU1253).

4. Analysis of orientation of 6.8 Kb insert: isolation of pDU1294 and pDU1295

Lane A, pDU1294 cleaved by BglII and PstI; lane B, pDU1295, cleaved by BglII and PstI. See text for rationale.



pDU1290 (Fig. 5.5). It was digested with BamHI and religated to reverse the orientation of the lac insert, yielding pDU1291 (Fig. 5.5). Both pDU1290 and pDU1291 were transformed into XAc Su⁻ and the strains assayed for β -galactosidase activity.

D. Copy number reductions with the transcriptional cml-lac fusions

For reasons discussed below, it seemed prudent to reduce the copy numbers of the cml-lac components of plasmids pDU1290 and pDU1291. This was achieved by cloning the 9.6 Kb HindIII fragment from each plasmid (consisting of the cml and lac sequences) into the HindIII site of the low copy number R388-derivative cloning vector pUB5572. The new plasmids were designated pDU1292 and pDU1293 respectively (Fig. 5.5). These plasmids were used to transform XAc Su⁻ and the new strains were assayed for β -galactosidase activity.

E. Construction of a translational (gene) cml-lac fusion

The plasmid pMC931 carries a promoter-less lacZ-lacY operon in which lacZ also lacks a ribosome binding site and translation initiation codon. The lac sequences are carried in a 6.8 Kb BamHI fragment (Casadaban et al., 1980). It was desired to generate a cml-lac fusion by cloning this 6.8 Kb fragment into the BamHI site of cml. The properties of the hybrid polypeptide which this would create could not be predicted. If these were deleterious to the host cell, plasmids containing the desired construction might have been selected against, making them less likely to be detected. For this reason it was decided to construct a pBR322 cml⁺ recombinant plasmid with a single BamHI site (that

within cml) to improve the likelihood of obtaining the desired chimera in the fusion experiment.

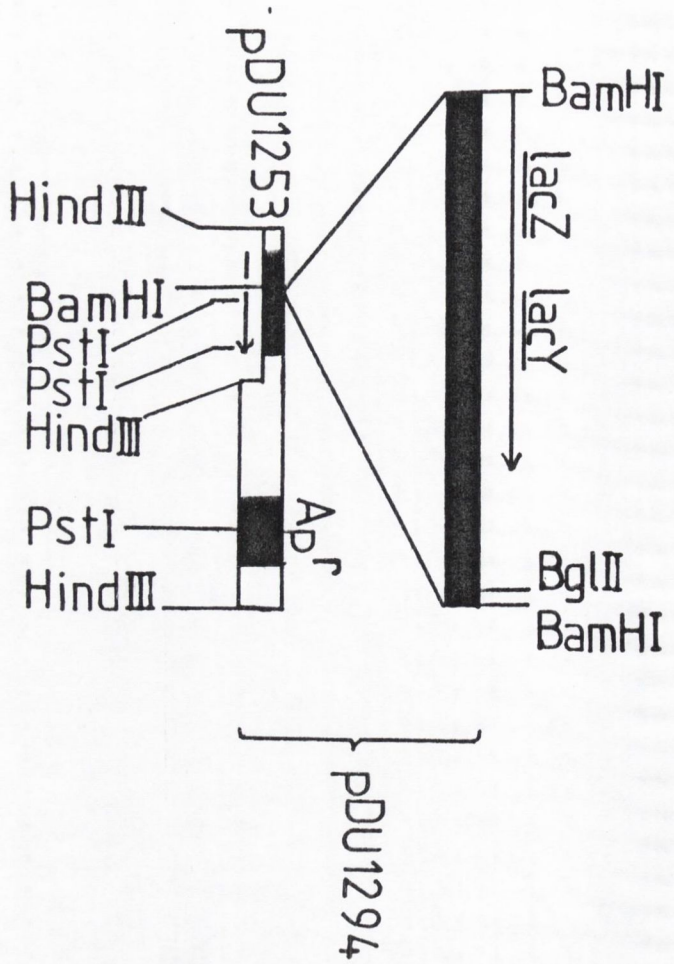
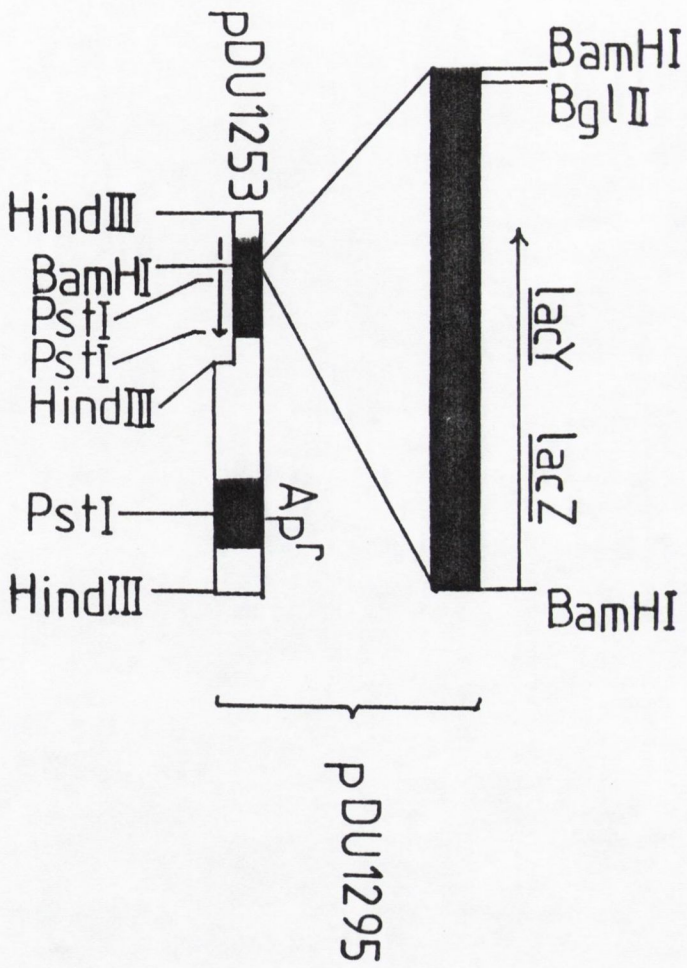
The BamHI site was removed from pBR322 by digesting with BamHI and transforming C600 with the linear DNA, selecting for Ap^r Tc^s transformants. Deletion and ligation occurred in vivo to yield colonies with the desired phenotype. One was retained for further study. This contained a plasmid, pDU1252, with a 1.1 Kb deletion in the Tc^r region. This plasmid retained its HindIII site and all functions required for plasmid maintenance.

Retention of the HindIII site is demonstrated in Fig. 5.4.2 (lane C) in which pDU1252 is shown linearised by HindIII, yielding a 3.2 Kb fragment (about 1.1 Kb smaller than pBR322). The 1.8 Kb Cm^r HindIII fragment from pDU1246 was cloned into the HindIII site of pDU1252 to yield pDU1253 (Fig. 5.4.2, lanes B and D; Fig. 5.6). This recombinant plasmid had a unique BamHI site, that within cml. The 6.8 Kb lac BamHI fragment from pMC931 was cloned into this BamHI site in both orientations to yield pDU1294 and pDU1295. The presence of the 6.8 Kb lac fragment in pDU1294 and pDU1295 is shown in Fig. 5.4.3, lane A and C. The orientations of the lac inserts in these plasmids were deduced from double digests with PstI and BglIII (Fig. 5.4.4). Both pDU1294 and pDU1295 contain the 750 bp PstI site of cml together with a 2.75 Kb PstI junction fragment made up of pBR322 and R26 DNA (Fig. 5.6). In pDU1294, there are BglIII-PstI fragments of 8.2 Kb and 200 bp (this latter band has run off the gel in Fig. 5.4.4). In pDU1295, the BglIII-PstI fragment sizes are 6.8 Kb and 1.5 Kb, showing that the lac inserts in these plasmids are in opposite orientations (Fig. 5.6). pDU1294 and pDU1295 were used to transform XAc and the strains were assayed for β -galactosidase activity.

Figure 5.6 Structures of translational fusion plasmids

The upper solid bar in each diagram represents the 6.8 Kb BamHI fragment from pMC931. The direction of transcription of the lac operon is shown by the arrow. The broad part of pDU1253 represents pBR322 DNA, while the narrow part represents R26 sequences. The direction of transcription of the Cm^r determinant is indicated by the lower arrow. The position of the ampicillin resistance (Ap^r) gene is shown.

1KB



F. Transformation protocol

The method described in Chapter 4 was used for transforming E. coli K12 strains C600 and XAc Su⁻ (Δlac).

G. β-galactosidase assays

The method used was essentially that of Miller (1972). 2 ml starter cultures of XAc Su⁻ (Δlac), with or without plasmids (with drugs where appropriate) were used to inoculate 10 ml L broth cultures in 250 ml flasks. 0.2 ml of starter culture was added to each flask and these were incubated with shaking at 37° for 2 to 3h. Where necessary, cultures were induced with 1 μg/ml of Cm 60 min. prior to assay. When the optical density ($\lambda = 600 \text{ nm}$) had reached 0.4, the cultures were put on ice. 3 ml samples were withdrawn for O.D._{600 nm} measurements (Varian Techtron spectrophotometer). Assays were then set up as follows: 0.2 ml of culture was added to 1.8 ml Z buffer in a clean test tube. Z buffer contained: 0.06M Na₂HPO₄ · 7H₂O, 0.04M NaH₂PO₄ · H₂O, 0.01M KCl, 0.001M MgSO₄ · 7H₂O, 0.05M 2-mercaptoethanol. To each tube was added 2 drops CHCl₂ and SDS (0.1% w/v in distilled H₂O). The tubes were incubated at 30° for 5 min in a water bath. Then 0.4 ml of a 4 mg/ml solution of ONPG (O-nitrophenyl-β-D-galactoside) was added to successive tubes at 15 sec intervals. The reaction was stopped by adding 1 ml of 1.0M Na₂CO₃. This raised the pH to 11, inactivating β-galactosidase. The ONPG solution was colourless but β-galactosidase catalysed its conversion to galactose and o-nitrophenol, a yellow compound. The concentration of this compound was measured at $\lambda = 420 \text{ nm}$. Since cell debris caused light scattering at this wavelength, the optical density at

$\lambda = 550$ nm was also measured. Here, o-nitrophenol did not absorb while the cell debris still caused light scattering. Thus the debris light scattering component could be computed and corrected for. The blank used was Z buffer + ONPG + Na_2CO_3 , without cells. Enzyme activity was calculated thus:

$$\text{UNITS} = \frac{1000 \times \text{O.D.}_{420} - (1.75 \times \text{O.D.}_{550})}{t \times V \times \text{O.D.}_{600}}$$

where t = time (min.) and V = volume of cells (ml).

RESULTS

A. β -galactosidase activity is expressed constitutively by high copy number cml-lac operon fusion plasmids

Plasmids pDU1290 and pDU1291 are cml-lac fusion derivatives of the pBR322 cml⁺ plasmid pDU1246 having the 7.7 Kb lac fragment from pMC903 inserted in either orientation in the BamHI site within cml (Fig. 5.3). Strains of XAc Su⁻ (Δ lac) harbouring these plasmids were assayed for β -galactosidase activity. In both cases, induced and uninduced cultures were compared. It was found that both XAc (pDU1290) and XAc (pDU1291) expressed the lacZ gene constitutively (Table 5.2). This indicated the presence of a promoter to either side of the BamHI site, reading toward each other. In the case of pDU1290, the promoter was quite strong, giving 1200 units of β -galactosidase activity in both uninduced and induced samples. The orientation of lacZ-lacY in this plasmid aligns these genes with the P1 promoter of pBR322 (Stuber and Bujard, 1981 and Fig. 4.10). The promoter which controls lac transcription in pDU1291 is not as strong, giving less than half as much β -galactosidase activity as that in pDU1290. Since both plasmids are fully Cm^S, constitutive expression cannot be for the reasons proposed for the parental pBR322 cml⁺ plasmid, pDU1246, i.e. that the cml product saturates membrane sites at high copy number and so gives maximum resistance, even in the absence of induction (Chapter 4).

The direction of expression of cml was not known at this stage of the project. If the cml promoter was aligned with promoter P1 of pBR322, then the influence of this strong vector promoter could mask the effects of induction on the cml promoter

Table 5.2 β -galactosidase activity of putative transcriptional
cml-lac fusions

<u>Plasmids</u>	<u>β-galactosidase activity (units)^a</u>	
	uninduced	induced ^b
pDU1291	500	470
pDU1290	1200	1200
pDU1293	13	13
pDU1292	20	20
pDU1293 + R26	18	18
pDU1292 + R26	22	25
pDU1292 + pBR322	128	128
pDU1292 + pDU1246	120	120
pDU1292 + pDU1256	90	90
pDU1292 + pDU1257	80	80
pSU1292 + pDU1258	80	80
pDU1292 + pDU1259	85	85
pDU1292 + pDU1261	80	80
pDU1292 + pDU1262	75	75
pDU1292 + pDU1263	75	75
pDU1292 + pDU1265	80	80
pDU1292 + pDU1272	90	90
pDU1292 + pDU1274	80	80
pDU1292 + pDU1275	70	70
pDU1292 + pDU1276	75	75
pDU1292 + pDU1277	85	85
pDU1292 + pDU1278	80	80
pDU1292 + pDU1279	115	115
pDU1292 + pDU1280	130	130

a. The numbers were obtained from at least three experiments.

b. Induction was with 1 μ g/ml chloramphenicol for 60 min prior to assay.

in pDU1290. In pDU1291, there is no vector promoter to override the cml promoter. If lacZY and cml are aligned in pDU1291, then constitutive expression could be due to some unexplained effect of high copy number on transcription of the operon fusion. This could also be true of pDU1290, together with the effect of P1 promoter readthrough. Alternatively, cml could be transcribed constitutively in any event, with induction being effected at another level in its expression (e.g. translation). It was decided to reduce the copy number of the operon fusion by cloning the 9.6 Kb HindIII lac-cml fragments from pDU1290 and pDU1291 into pUB5572, a low copy number cloning vector.

B. β -galactosidase activity is expressed constitutively by low copy number cml-lac operon fusion plasmids

The pUB5572 derivative generated by cloning the 9.6 Kb cml-lac fragment of pDU1290 into this vector was termed pDU1292. That obtained by cloning the cml-lac sequence from pDU1291 into pUB5572 was called pDU1293 (Fig. 5.5). Once again, the cml-lac sequences expressed β -galactosidase constitutively (Table 5.2). This was also the case when a wild-type cml determinant was introduced into the same cell on R26 (Table 5.2), confirming the earlier observation that the entire regulatory mechanism for cml was contained within the 1.8 Kb HindIII Cm^r fragment and that there was no requirement for additional regulatory factors to be supplied in trans (Chapter 4). When the high copy number pBR322 cml⁺ plasmid, pDU1246, was introduced into the same cell as pDU1292, expression of lacZ increased about six fold (Table 5.2). The same occurred with pDU1293 (data not shown). Subsequently, a similar effect was shown to occur when various pDU1246 cml::Tn5

Cm^S mutant plasmids were coresident with pDU1292 and finally pBR322 itself was shown to exert this effect (Table 5.2). Therefore this increased expression of lacZ was probably due to a copy number effect exerted by pBR322 and its derivatives on pUB5572-derived plasmids. Such an effect has not been described previously for these replicons.

C. β -galactosidase activity is expressed inducibly by a cml-lac translational fusion: elucidation of the direction of expression of cml

A 3.2 Kb derivative of pBR322 was constructed by deleting the BamHI site of that vector together with about 1.1 Kb of DNA in the Tc^r region. This plasmid, pDU1252 was then cleaved by HindIII and the 1.8 Kb Cm^r HindIII fragment from pDU1246 was cloned into it (Fig. 5.6). The resulting chimera, pDU1253, contained a unique BamHI site, that within cml. Into this was cloned the 6.8 Kb lac BamHI fragment of pMC931, in both orientations to give pDU1294 and pDU1295 (Fig. 5.6). The lacZ gene carried within this BamHI fragment did not have an indigenous promoter, ribosome binding site or translation initiation codon. In order to be expressed, these had to be supplied exogenously. In the case of pDU1295, no β -galactosidase activity was detected in assays for this enzyme. This was true of both uninduced and induced cultures (Table 5.3). Furthermore, no activity was recorded when pDU1295 was accompanied in the host cell by pDU1249 (pUB5572 cml⁺), a low copy number recombinant plasmid specifying inducible Cm^r (Table 5.3). However it was discovered that cells containing pDU1294 expressed β -galactosidase, albeit at a low level (Table 5.3). Moreover, the level of expression increased

Table 5.3 β -galactosidase activity of putative translational
cml-lac fusions

<u>Plasmids</u>	<u>β-galactosidase activity (Units)^a</u>		
	Uninduced	Induced	
pDU1295	0	0	
pDU1295 + pDU1249 <u>cml</u> ⁺	0	0	
pDU1294 φ <u>cml-lacZ</u>	4	20	(x5) ^c
pDU1294 φ <u>cml'-lacZ</u> + pDU1249 <u>cml</u> ⁺	1.5	4.5	(x3)
pDU1294 φ <u>cml'-lacZ</u> + pUB5572	2	10	(x5)

- a. The numbers were obtained from three independent experiments.
- b. Induction was with 1 μg/ml chloramphenicol for 60 min prior to assay.
- c. The numbers in parentheses are the induction ratios, the ratio of β-galactosidase in induced/uninduced samples.

five-fold upon induction with Cm (Table 5.3). These results show that the promoter governing expression of lacZY in pDU1290 and pDU1292 is the cml promoter, and so cml is expressed in the same direction as lacZY in these plasmids, as in pDU1294. It was found that when pDU1294 was accompanied by the pUB5572 cml⁺ plasmid, pDU1249, the gene fusion was still inducible but that the overall level of expression had declined (Table 5.3). This could have been due to the cml product of pDU1249 preventing entry of the inducer (Cm) to the cell together with a diminishing effect exerted by pDU1249 on the copy number of pDU1294 (pUB5572- and pBR322-derivatives, respectively). It has already been shown that pBR322 may exert an enhancing effect on the copy number of co-resident pUB5572 derivatives (Table 5.2). Further evidence for this copy number effect was provided by the observation that pUB5572 could itself cause a reduction in the overall level of expression of β -galactosidase by pDU1294 when both plasmids resided in the same cell. However, β -galactosidase expression was still inducible by Cm (Table 5.3).

Since the cml-lac fusion in plasmid pDU1294 was made at the level of translation and since this fusion brought expression of the lacZ gene under the control of Cm, cml must be controlled at the level of translation.

DISCUSSION

The results of experiments described in this Chapter permit several conclusions to be drawn concerning the regulation of expression of cml. It can now be seen that cml is posttranscriptionally regulated and that the mechanism required for this control is wholly contained within the cloned 1.8 Kb Cm^r HindIII fragment. This was deduced from data obtained with the cml-lac translational fusion plasmid, pDU1294. Analysis of the structure of this plasmid also revealed the direction of expression of cml. The level of β -galactosidase activity obtained with this plasmid was low, even in the induced state. This could have been due to the fusion being out of frame and the observed activity being the result of translational slippage or restarts. Analysis of the DNA sequence of cml (Chapter 7) revealed that the fusion was in-frame. Thus cml is poorly expressed at the translational level. It is also poorly transcribed. Knowledge of the direction of expression of the determinant demonstrates that lacZ was under the control of the cml promoter in pDU1292 [pUB5572 ϕ (cml-lac)]. Here, β -galactosidase was expressed at a low level (Table 5.2), indicating that the cml promoter is a weak one. The constitutive expression of lacZ seen with pDU1292 was real, meaning that since cml is posttranscriptionally regulated, transcription occurs at a low, constant rate whether the determinant is induced or not. The high level constitutive expression seen when the transcriptional fusion was carried on pBR322 (in pDU1290) was due to the constitutive transcription system of cml being at an elevated copy number and coming under the influence of a strong pBR322 promoter (the P1 promoter of Stuber and Bujard, 1981).

CHAPTER 6

THE GENE PRODUCT OF THE R26
CHLORAMPHENICOL RESISTANCE DETERMINANT

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INTRODUCTION

This Chapter describes experiments which identified the gene product of the cloned R26 Cm^r determinant, using the E. coli minicell system.

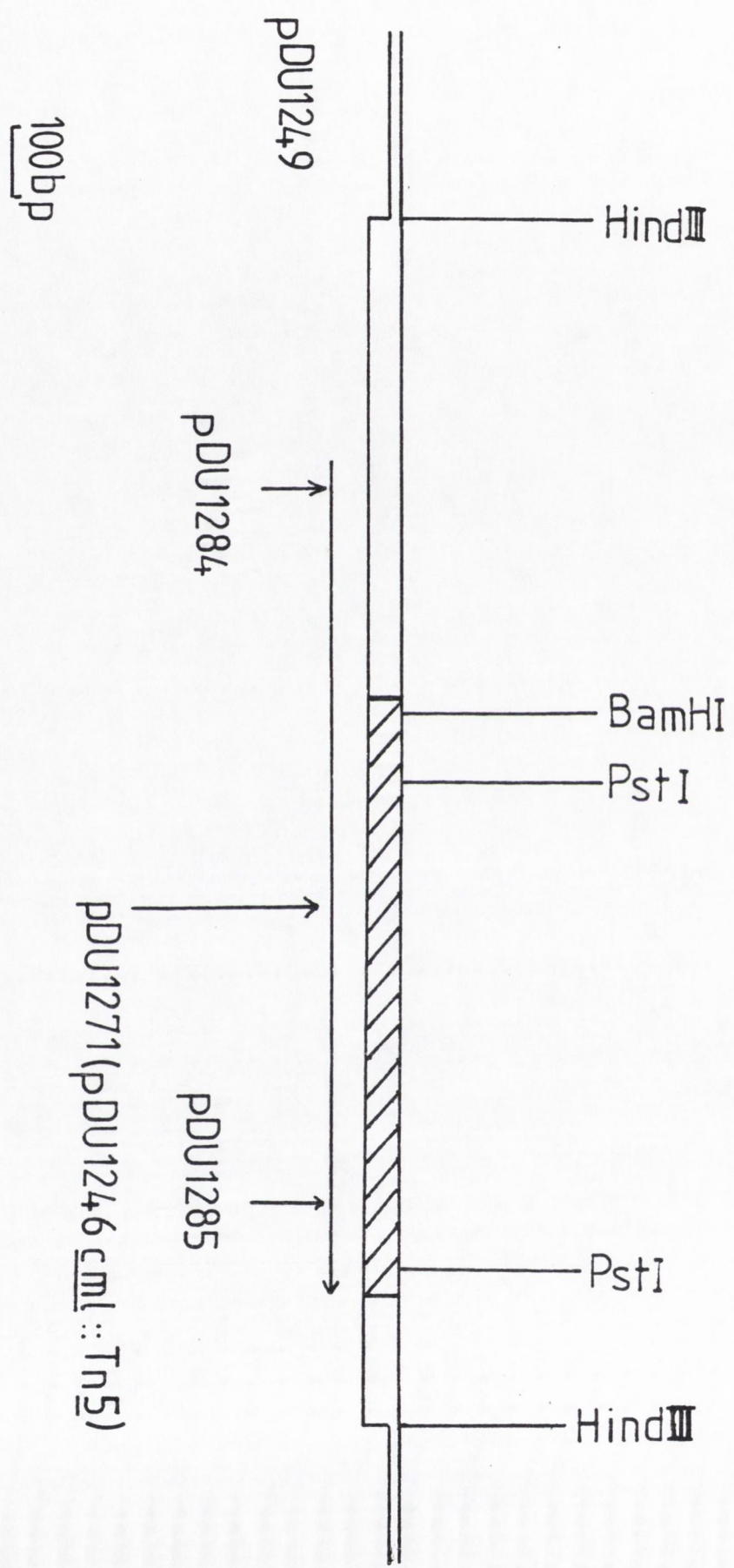
Through the processes of transcription and translation, a gene is ultimately expressed in the form of a protein. Techniques exist which allow a protein of interest to be synthesised in a bacterial host in the absence of synthesis of most of the other proteins of the cell. For a study such as this, the properties of the protein which can be investigated are its size and its location within the cell. Knowledge of its size permits a deduction to be made concerning the amount of DNA required to encode it. Knowing where the protein resides in the cell allows some conclusions to be drawn about its possible function.

Two techniques are available for in vivo analysis of gene products from cloned DNA sequences in E. coli. The first to find widespread use was the minicell system. This employs a mutant of E. coli K12 which is transformed with the recombinant plasmid encoding the protein(s) of interest. Mutations in the E. coli strain, DS410, affect its ability to divide normally (see Materials and Methods section, below). Instead of producing two equal daughter cells at cell division, in many cases two "cells" of unequal size are produced. These are analogous to the mother and bud produced in budding. The smaller, or minicell, cannot accommodate a copy of the chromosome although a plasmid can be taken in. With multi-copy cloning vehicles, there is a high

probability that most minicells will receive at least one plasmid molecule. In addition to the DNA molecule, the minicell will receive all of the factors necessary for gene expression. By mechanically separating the minicells from the mother cells and then supplying them with a radiolabelled derivative of an amino acid, a centre for in vivo synthesis of proteins specified by the plasmid has been established. The radioactive proteins can then be extracted from the minicells and analysed by electrophoresis (Adler et al., 1967; Crooks et al., 1983; Dougan et al., 1979; Frazer and Curtiss, 1975; Hogan et al., 1982; Levy, 1971; Levy, 1974; Levy and Norman, 1970; Meagher et al., 1977; Rogerson and Stone, 1974; Roozen et al., 1971). This system was the one utilised in this study. The second method is termed the maxicell system since the bacterial cells are of normal size. The cells carry the plasmid encoding the protein of interest and are irradiated with ultraviolet light. The success of this method depends upon the differential susceptibility of the bacterial chromosome and the plasmid in E. coli recA uvrA cells. Being much smaller, the latter molecule should survive the UV treatment while the chromosome is destroyed. The end result is a full-sized bacterial cell in which the only viable replicons are plasmids. When supplied with a radiolabelled amino acid, these cells synthesise proteins specified by the plasmids and these may then be detected as in the minicell system (Sancar et al., 1979).

Figure 6.1 Structures of cml::Tn5 mutant plasmids used in minicell experiments

The broad bar represents R26 DNA. The hatched area within this denotes the open reading frame of cml. The narrow, open bar indicates pUB5572 sequences adjacent to the cloned 1.8 Kb Cm^r HindIII fragment. The horizontal arrow shows the direction of transcription, together with the putative start and end points, as deduced from Tn5 mutagenesis (Chapter 4). The sites of insertion of Tn5 in pDU1284 and pDU1285 are illustrated by vertical arrows. Similarly, the site of insertion of Tn5 within the 1.8 Kb Cm^r HindIII fragment of pDU1246 in the Tn5 mutant plasmid pDU1271 is shown. Recognition sites for BamHI, HindIII and PstI are indicated.



MATERIALS AND METHODS

A. Bacterial strains and plasmids

Escherichia coli K12 strain DS410 (minA, minB rpsL) was the minicell producing strain (Dougan and Sherratt, 1977). E. coli K12 strain C600 has been described in Chapter 4. It was used as a transformation recipient for newly-constructed plasmids prior to transfer to DS410. It was also employed as the host for long-term storage of plasmids.

Plasmids pDU1244, pDU1246, pDU1249, pUB5572 and pBR322 have been described previously (Table 4.1). Plasmid pDU1254 was pBR322 with a small deletion in the bla gene. This was constructed by cleaving pBR322 with PstI and transforming C600 with the linear DNA and selecting for Ap^S Tc^R colonies. pDU1254 specifies a 15K truncated derivative of β -lactamase (see below). pDU1255 was constructed by cloning the 1.8 Kb Cm^R HindIII fragment of pDU1246 into pDU1254 (Ap^S Tc^R) to give a derivative which was Cm^R Ap^S Tc^R. The plasmids pDU1284 and pDU1285 were Cm^S derivatives of pDU1249 generated by Tn5 mutagenesis (see Chapter 4 for methodology). The structures of these plasmids are shown in Fig. 6.1.

B. Chemicals and antibiotics

These were as previously described in Chapter 2. 3-deoxy-chloramphenicol was a gift from W.V. Shaw. It is a gratuitous inducer of gram-positive CAT genes, not being a substrate for CAT (Shaw, 1983). It has also been shown to induce the R26 Cm^R determinant (D.F. Gaffney, Ph.D. Thesis, University of Dublin).

C. Preparation and labelling of *E. coli* minicells

DS410 was transformed as described in Chapter 4 with plasmids to be studied. However, 100 mM CaCl_2 was used in place of 30 mM CaCl_2 . Minicells were prepared from shaken cultures grown for 15h at 37° . When induction of Cm^r with Cm or 3'-deoxy Cm was carried out, this was for 30 min prior to labelling minicells or throughout the 15h growth period. Whole cells were pelleted in 250 ml Sorvall centrifuge bottles using a GSA rotor at $1500 \times g$ for 5 min. Minicells and any contaminating whole cells were then pelleted in fresh 250 ml bottles at $16,000 \times g$ for 10 min. The pellet was then resuspended in 2 ml BSG (0.85% NaCl, 0.03% KH_2PO_4 , 0.06% Na_2HPO_4 and $100 \mu\text{g ml}^{-1}$ gelatine) at 4° . All subsequent steps in the isolation were performed at 4° . This suspension was layered onto a 5-20% BSG sucrose gradient (sterile BSG + 20% sucrose, frozen to -40° and then allowed to thaw for 15h at 4°) in 30 ml Corex tubes (DuPont). The gradients were spun at $1700 \times g$ for 20 min at 4° in a Sorvall HS-4 swing-out rotor. The minicells banded near the top of the gradient and were removed with a pasteur pipette, pelleted at $12000 \times g$ for 10 min in a SS-34 Sorvall rotor at 4° , resuspended in 1 ml sterile BSG and then loaded onto a 5-20% BSG sucrose gradient in a 15 ml Corex tube. This gradient was spun at $1700 \times g$ for 20 min in a Sorvall HS-4 swing-out rotor and the procedure was repeated. The pelleted minicells were resuspended in labelling medium (4.1% methionine assay medium (DIFCO), 8% X10 M9 salts, 0.2% glucose, 0.2% MgSO_4 , boiled for 3 min). They were pre-warmed to 37° for 10 min, and then induced (where appropriate) with $1 \mu\text{g/ml}$ of Cm or 3-deoxy Cm for 30 min and then labelled for a further 30 min with

with 50 μCi of (^{35}S) methionine per ml (600 Ci/mmol), obtained from Amersham. Labelled minicells were then either fractionated or prepared immediately for electrophoresis. Cells to be electrophoresed were pelleted and resuspended in Final Sample Buffer (10% glycerol, 5% mercaptoethanol, 3% SDS, 0.0625 M Tris pH 6.8 and 0.01% bromophenol blue). They were boiled for 3 min and then electrophoresed or stored at -70° until required.

D. Minicell fractionation

Minicells to be fractionated were lysed as follows. Labelled minicells, from 2L of culture were chilled on ice and resuspended in 2 ml of ice-cold 200 mM Tris-hydrochloride (pH 8.0) and maintained at 4° throughout the procedure. 2 ml of 20% (wt/vol) sucrose in 200 mM Tris-hydrochloride (pH 8.0) and 50 μl of 100 mM EDTA (pH 8.0) were added, followed immediately by lysozyme to a final concentration of 40 $\mu\text{g/ml}$. An equal volume of sterile water (ice-cold, distilled) was added and the mixture was allowed to stand on ice for 30 min to permit sphaeroplasts to form. These were checked optically, using a phase-contrast microscope. Sphaeroplasts were lysed in a French pressure cell (Aminco) at 14,000 psi (97×10^6 Pa). Lysis was observed by phase contrast microscopy. Minicell envelopes were separated from cytoplasmic components by centrifugation at $80,000 \times g$ for 2h in a Beckman L8-55 preparative ultracentrifuge using a 70.1 Ti fixed angle rotor. The envelopes were washed once in 10 mM Tris-hydrochloride (pH 7.5) and then resuspended in final sample buffer. The cytoplasmic fraction was allowed to stand for 15h at 4° in the presence of 20% TCA ($\frac{1}{4}$ volume). Precipitated protein was then

pelleted at 17,000 x g for 30 min and resuspended in final sample buffer. Several drops of 1M NaOH were added to neutralise the acid, bromophenol blue being the indicator. These procedures were based on those of Whitholt et al., 1976 and of Dougan et al., 1983.

E. Electrophoresis of proteins

The procedure used was based on that of Laemmli (1970). 15% acrylamide gels were used, with 4.5% stacking gels. The acrylamide solution contained (wt/vol) 29% acrylamide and 1% bisacrylamide in distilled water. This solution was filtered before use through Whatman No. 3 filter paper. The gel buffer consisted of 1.5M Tris-hydrochloride and 0.4% SDS (wt/vol) pH 8.8. To cast a 15% gel, 1 volume of gel buffer was mixed with 2 volumes of the acrylamide/bisacrylamide solution. To this was added 10 μ l of TEMED (NNN'N'-tetramethylethylenediamine) and 200 μ l of a 10% Ammonium persulphate solution (freshly prepared each time). The stacking gel buffer consisted of 0.5 M Tris-hydrochloride and 0.4% SDS, pH 6.8 in distilled water. To cast a 4.5% stacking gel, 1 volume of this buffer were mixed with $\frac{3}{5}$ volume of acrylamide/bisacrylamide solution. To this was added 10 μ l of TEMED (NNN'N'-tetramethylethylenediamine). The running buffer for electrophoresis was 0.3% Tris-hydrochloride, 1.44% Glycine and 0.1% SDS (wt/vol), made up in distilled water. Samples were run through the stacking gel¹ at a fixed current of 10 mAmperes and then through the gel at a constant current of 20 mAmperes. Gels were stained for 15h in 0.25% Coomassie Brilliant Blue R, 45.4% methanol and 9.2% acetic acid in distilled water. Destaining was with 45.5% laboratory-grade spirit, 9.2% acetic acid in distilled water.* Gels were fixed/stored under 5% glacial

* For as long as required

acetic acid in distilled water. They were dried down and then autoradiographed against Kodak X-omat X-ray film at -70° . Films were developed using Kodak DX-80 X-ray developer (20% vol/vol in distilled water) and fixed with Kodak FX-40 X-ray fixer (20% vol/vol in distilled water).

RESULTS

A. A chloramphenicol-inducible polypeptide was not detected in minicells containing pBR322 Cm^r derivative plasmids

Cultures of DS410 harbouring pDU1246 (pBR322 with 1.8 Kb Cm^r fragment in HindIII site) or pDU1244 (pBR322 with 7 Kb Cm^r fragment in EcoRI site), both uninduced and induced, were tested for the presence of a Cm-inducible protein (Fig. 6.2.1). The protein profiles of DS410 (pDU1246) uninduced (lane A) and induced (lane B) were identical. The same was true of DS410 (pDU1244) uninduced (lane C) and induced (lane D). No additional protein bands were seen with induced cultures in either case. Both plasmids specified β -lactamase, this being seen in its unprocessed (31K) and processed (28K) forms (Cornish et al., 1982). A 25K protein, probably host-encoded, was seen in all experiments. In Chapter 4 it was reported that pDU1244 specifies Su^r, in addition to Cm^r and Ap^r. Su^r is mediated by a 40 Kd dihydropteroate synthetase enzyme in gram-negative bacteria (Svedberg and Skold, 1980). A 40 Kd polypeptide band was seen in the pDU1244 tracks (lanes C and D).

When the protein profiles of DS410 (pDU1246) and DS410 (pDU1244) were compared with that of DS410 (pBR322), no additional polypeptide bands were seen in the Cm^r samples (Fig. 6.2.2; lane A). Results from Chapter 4 showed that Cm^r was constitutively expressed by E. coli strains containing pDU1246 or pDU1244, so the similarities of uninduced and induced protein profiles for DS410 strains harbouring these plasmids was perhaps not surprising. The protein(s) involved in specifying Cm^r might be expected to be present in both uninduced and induced cultures. The fact that the DS410 (pBR322) profile

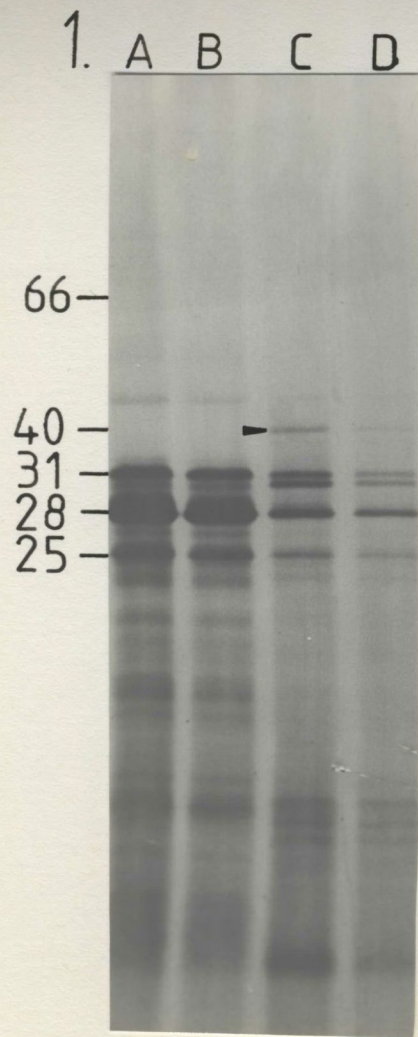
Figure 6.2 Autoradiographs of ^{35}S -methionine-labelled polypeptides
fractionated by SDS-PAGE

6.2.1 Protein profiles of minicells containing pBR322 Cm^r
derivatives

lane A, pDU1246; lane B, pDU1246, induced (Cm, 30 min); lane C, pDU1244; lane D, pDU1244, induced (Cm, 30 min). 66: shows the location of bovine serum albumin (66 Kd) on coomassie blue-stained gel (not shown).

6.2.2 Identification of a chloramphenicol inducible polypeptide

Lane A, pBR322; lane B, pDU1246- lane C, pDU1244; lane D, pDU1249; lane E, pDU1248; lane F, pDU1249; lane G, pDU1249, induced (Cm, 15h); lane H, pUB5572; lane I, pDU1248, induced (Cm, 15h); lane J, pDU1248; lane K, pDU1248, induced (Cm, 30 min); lane L, pDU1248, induced (Cm, 15h); lane M, pUB5572; lane N, pDU1249, induced (Cm, 15h); lane O, pDU1249, induced (Cm, 30 min). All molecular weights in kilodaltons. Solid arrowheads: 40 Kd Su^r protein; open arrowheads 31 Kd Cm^r protein.



matched that for the Cm^r plasmids (with the exception of the putative Su^r protein of pDU1244) is more difficult to explain. Either (i) the Cm^r protein was not labelled by this experimental technique or (ii) it was co-migrating with host- or vector-encoded polypeptides. Experiments described below showed that the latter was the case.

B. A chloramphenicol-inducible protein is encoded by plasmids pDU1248 and pDU1249

In Chapter 4 it was shown that when the 7 Kb EcoRI Cm^r fragment of pDU1244 or the 1.8 Kb HindIII Cm^r fragment of pDU1248 was cloned into pUB5572, the resulting recombinant plasmids specified inducible Cm^r. These plasmids, pDU1248 and pDU1249 respectively, were tested for the production of a Cm-inducible protein in the minicell system. When the polypeptide profiles of DS410 (pDU1248) and DS410 (pDU1249) were compared, it was found that both contained the 25K protein mentioned previously, together with a protein of 28K (Fig. 6.2.2, lanes D and E). Both of these proteins were also seen in DS410 (pUB5572) (lane M). The 40K, putative Su^r protein, was seen in DS410 (pDU1248) only (lane E).

When DS410 (pDU1249) and DS410 (pDU1248) were induced for 15h with 1 µg/ml of Cm, a 31K, heavily-labelled protein was detected (lanes G and I respectively). This protein was not seen in the uninduced controls (lanes F and J). Induction with Cm for 30 min was not sufficient to give induction (pDU1248, lanes K and L- pDU1249, lanes N and O). The minimum period required for induction was not determined.

C. The chloramphenicol-inducible protein co-migrates with β -lactamase

A direct comparison was made of the protein profiles of DS410 harbouring pDU1246 (pBR322 Cm^{r}) and DS410 harbouring pDU1249 (pUB5572 Cm^{r}) (Fig. 6.3.1, lanes A and B respectively). The pDU1249-containing strain had been induced for 15h with Cm. The heavily-labelled Cm-inducible protein was shown to migrate adjacently to the unprocessed form of β -lactamase (Fig. 6.3.1; lanes B and A respectively). This could explain the failure to detect the Cm^{r} -specific protein with pBR322-derived Cm^{r} plasmids.

D. The chloramphenicol-inducible polypeptide is specified by pBR322 Cm^{r} recombinant plasmids

The protein profiles of DS410 containing pDU12 (pBR322 Δ PstI, an Ap^{S} derivative of pBR322), pDU1255 (pDU1254 with the 1.8 Kb Cm^{r} fragment in the HindIII site), or pDU1249 (pBR5572 with 1.8 Kb Cm^{r} fragment in the HindIII site) were compared (Fig. 6.3.2). Lanes D and E show the pattern for DS410 (pDU1249) uninduced and induced, respectively. The induced sample shows the heavily-labelled, 31 Kd, Cm-inducible protein band. A faintly labelled band of corresponding M^{r} can be seen in the uninduced sample track (lane D), possibly representing the background level of the protein in uninduced cells. The uninduced (lane B) and induced (15h, lane C) profiles for DS410 (pDU1255) are illustrated. Both contain the 31 Kd protein, and at approximately the same concentration. The band is absent from the DS410 (pDU1254) track (lane A). The 20K polypeptide observed in lanes A, B and C is probably the truncated β -lactamase specified by pDU1254 and pDU1255. The calculation of

Fig. 6.3 Autoradiographs of ^{35}S -methionine-labelled polypeptides
fractionated by SDS-PAGE

Fig. 6.3.1 Co-migration of the chloramphenicol-inducible protein
with β -lactamase

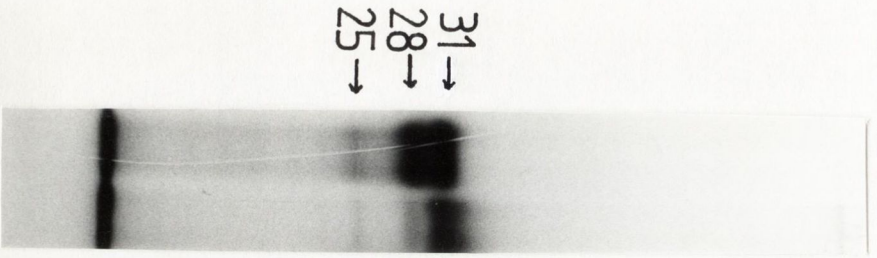
Lane A, pDU1246; lane B, pDU1249, induced (Cm, 15h).

Fig. 6.3.2 The chloramphenicol-inducible protein is specified
constitutively by pBR322 Cm^{r} derivative plasmids

Lane A, pDU1254; lane B, pDU1255; lane C, pDU1255, induced
(Cm, 15h); lane D, pDU1249; lane E, pDU1249 induced (Cm, 15h).

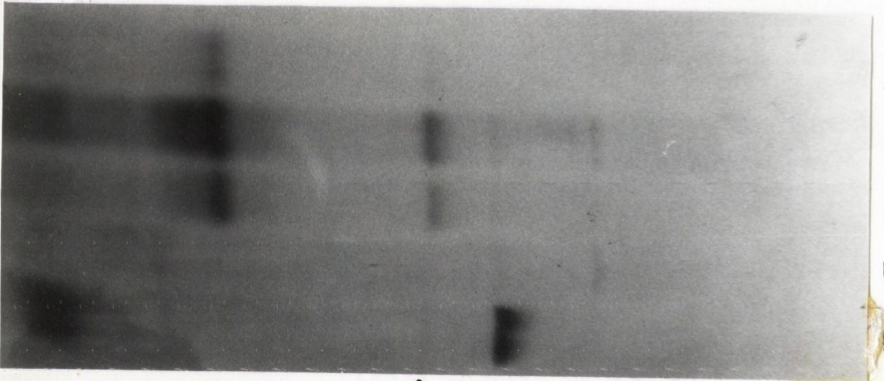
All molecular weights are in kilodaltons. The 20K protein in
6.3.2, lanes A, B and C is probably a truncated form of β -lactamase
(see text).

1. A B



← 31
← 28
← 25

2. A B C D E



← 31
← 25
← 20

the expressed M^r of this protein was based on the assumption that the Ap^r coding region had been interrupted at the PstI site, a distance of 540 bp from the beginning of the open reading frame (Sutcliffe, 1979). Evidence that the truncated reading frame was not significantly shorter than this distance was gained from agarose gel electrophoresis of pDU1254 and pBR322, both linearised with HindIII. No detectable difference in M^r was observed (data not shown). This indicated that the deletion in the Ap^r region of pDU1254 was a small one. Assuming an average molecular weight of 110d per amino acid, a protein encoded by this truncated reading frame would have a molecular weight of about 20 Kd.

The results obtained with these plasmids suggest that the 31 Kd, Cm-inducible protein specified by pDU1248 and pDU1249 is also specified by the pBR322 Cm^r -derivatives. These findings correlate with the phenotypic data on Cm^r expression as specified by these plasmids (Chapter 4).

E. The chloramphenicol resistance protein is inducible by 3-deoxychloramphenicol

Cultures of DS410 (pDU1249) were induced for 15h with Cm or 3-deoxychloramphenicol (3-deoxy Cm) and their protein profiles compared with that of the uninduced control (Fig. 6.4.1). The 31 Kd protein was detected in both the Cm-induced (lane B) and 3-deoxy Cm-induced (lane C) but not the uninduced (lane A) samples. As before, proteins of 25 Kd and 28 Kd were present in all lanes. The 10 Kd protein is possibly the dihydrofolate reductase enzyme encoded by the Tp^r gene of pDU1249 (Fling and Elvell, 1980).

Figure 6.4 Autoradiographs of ³⁵S-methionine labelled polypeptides
fractionated by SDS-PAGE

6.4.1 Polypeptides specified by cml::Tn5 mutants

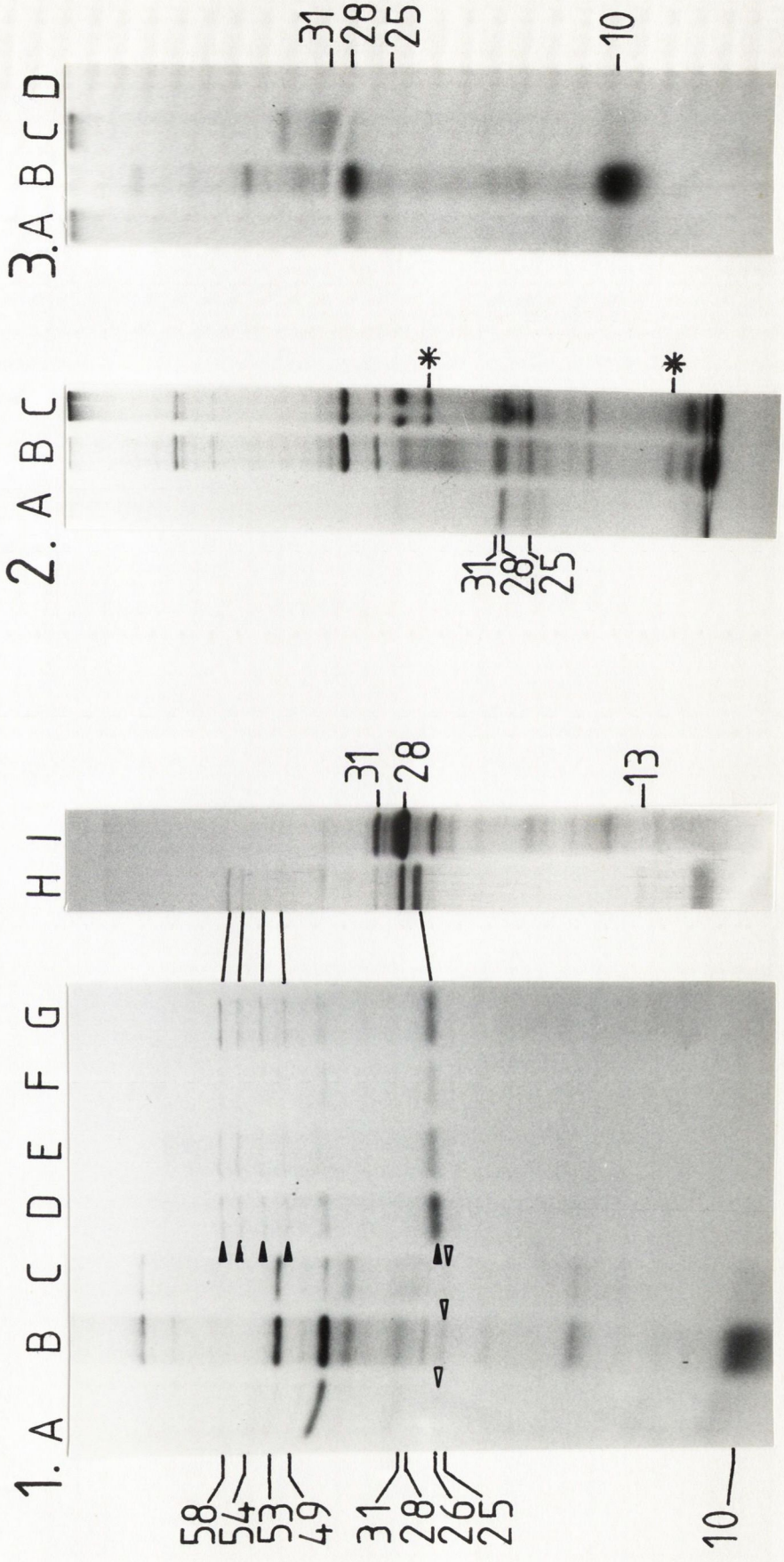
Lane A, pDU1249; lane B, pDU1249 induced (Cm, 15h); lane C, pDU1249 induced (3-deoxy Cm, 15h); lane D, pDU1284; lane E, pDU1284, induced (3-deoxy Cm, 15h); lane F, pDU1285; lane G, pDU1285, induced (3-deoxy Cm, 15h); lane H, pDU1271; lane I, pDU1246. Solid arrow heads: Tn5-encoded proteins; open arrowheads: 25K protein (see text).

6.4.2 Results of heat-treatment experiment

Lane A, pDU1249; lane B, pDU1249, induced (Cm, 15h), 60°; lane C, pDU1249, induced (Cm, 15h) 100°. Asterisks: proteins whose migration rate is temperature variable.

6.4.3 Results of fractionation experiment

Lane A, pDU1249; lane B, pDU1249, induced (Cm, 15h); lane C, pDU1249, envelope fraction, induced (Cm, 15h); lane D, pDU1249, cytoplasmic fraction, induced (Cm, 15h). All molecular weights are in kilodaltons.



F. Proteins encoded by transposon Tn5 insertion mutants

The plasmid-encoded protein profiles of DS410 harbouring pDU1284 (pDU1249 cml::Tn5) and DS410 harbouring pDU1285 (pDU1249 cml::Tn5), both uninduced and induced (with 3-deoxy Cm for 15h), were examined. The structures of pDU1284 and pDU1285 are illustrated in Fig. 6.1. Five Tn5-encoded polypeptides were detected (Fig. 6.4.1). These were the 26 Kd neomycin phosphotransferase type II (NPTII) enzyme and four larger proteins ranging in molecular weight from 49 Kd to 58 Kd (Rothstein and Reznikoff, 1981). No 3-deoxy Cm-inducible polypeptides were detected. This suggests that production of the 31 Kd Cm^r protein had been prevented due to insertion of Tn5 into the Cm^r determinant.

Knowledge of the direction of transcription of cml was gained from the work described in Chapter 5. In the light of this information, the cml Tn5 mutation in pDU1280 (pDU1246 cml::Tn5, Chapter 4) must affect the carboxy terminus of the 31 Kd protein. This information can be used to estimate the position of the translational initiation site of cml. Assuming an average molecular weight of 110d per amino acid, a protein of 31 Kd would require approximately 900 bp of DNA to encode it. If the site of insertion Tn5 within pDU1280 is about 20 bp to the right of the PstI site (Fig. 4), then the open reading frame for cml must commence about 50 bp to the left of the BamHI site. In Fig. 6.1, the Tn5 insertion in pDU1284 is shown to be 350 bp to the left of the BamHI site, presumably between the cml promoter and the start of the open reading frame for the 31 Kd protein. In this case, no part of the reading frame would be transcribed and so no 3-deoxy Cm-inducible truncated protein would be seen.

With pDU1285, the Tn5 insertion is about 100 bp to the left of the promoter-distal PstI site. An insertion here could theoretically result in a 26K truncated protein. This would be expected to co-migrate with the NPTII encoded by Tn5 and so not be detected.

The protein profile of DS410 harbouring pDU1271 (pDU1246 cml::Tn5, Chapter 4) was also studied. Here, the Tn5 insertion was about 500 bp from the promoter-distal PstI site (Fig. 6.1). An insertion at that point could result in a truncated protein of about 13 Kd. This was calculated using the assumptions about amino acid molecular weight as before. Such a protein was detected in the minicell system (Fig. 6.4.1, lane H). No induction was required as pBR322 Cm^r-derivatives express the determinant constitutively (Chapter 4). As with the pDU1249 cml::Tn5 plasmids, the five Tn5 encoded proteins were detected, as were the β -lactamase bands (28 Kd and 31 Kd). pDU1271 is a pDU1246 derivative. The plasmid-encoded protein profile of DS410 (pDU1246) is included for comparison (lane I).

G. The rate of migration of the chloramphenicol-inducible polypeptide is not affected by boiling in sodium dodecylsulphate

The migration rates of some membrane-associated proteins in polyacrylamide gels vary depending on whether or not they have been boiled in SDS prior to electrophoresis (Beher et al., 1980). Proteins prepared from an induced culture of DS410 containing pDU1249 (pUB5572 Cm^r) were either heated to 60^o for 3 min in SDS or boiled for 3 min in SDS. The samples were then electrophoresed with an uninduced control (Fig. 6.4.2). The 31 Kd protein is absent from

the uninduced control (lane A) but is present in the induced samples (lanes B and C). Moreover, it migrated in a similar fashion in both the 60⁰ sample (lane B) and the boiled sample (lane C). Other proteins, marked by asterisks, migrated differently under these conditions.

H. The chloramphenicol-inducible protein may be associated with the cell envelope

The results of a fractionation experiment with DS410 (pDU1249) are illustrated in Fig. 6.4.3. The uninduced pattern for whole-cells is shown in lane A, while the induced pattern is in lane B. The cell envelope fraction was electrophoresed in lane C and the cytoplasmic fraction in lane D. The 31 Kd Cm-inducible polypeptide is present in the induced control (lane B) but not the uninduced control (lane A). It is also present in the cell envelope fraction (lane C). No label whatsoever was detected in the cytoplasmic fraction (lane D) indicating failure to adequately recover labelled polypeptides from the supernatant following minicell fractionation.

DISCUSSION

The results of minicell experiments described in this Chapter show that R26 mediates chloramphenicol resistance via a 31 Kd polypeptide. This protein is inducible by both Cm and 3-deoxy Cm, as is the Cm^r phenotype. Results from earlier Chapters showed that the phenotype was not expressed immediately following induction. The data on induction of the 31 Kd protein in minicells carrying the low copy number pUB5572 Cm^r plasmids show that it too is slowly induced, lending weight to the hypothesis that it is the product of cml. Further evidence was obtained from the Tn5 mutant plasmids pDU1271, pDU1284 and pDU1285. All three were incapable of specifying Cm^r, each lacked the 31 Kd protein and pDU1271 encoded a possible truncated derivative of it. The direction of expression of cml has been determined in Chapter 5 and from this information it was deduced that the Tn5 mutation in pDU1280 (Chapter 4) defined the carboxy terminus of the cml open reading frame. It was then possible to estimate the amount of DNA required to encode a 31 Kd protein, and so localise the start of the reading frame (Fig. 6.1). The size of the possible truncated protein specified by pDU1271 supports the evidence for the location of the start of the reading frame. There is sufficient DNA between the proposed translational initiation site and the Tn5 insertion site in pDU1272 to encode a polypeptide of about 13K, as seen with this plasmid (Fig. 6.1; Fig. 6.4.1, lane H).

The apparently constitutive expression of the 31 Kd protein in pDU1255 (pBR322 bla cml) correlates well with the constitutive expression of Cm^r by pBR322 Cm^r derivatives pDU1244, pDU1245,

pDU1246 and pDU1247 (Chapter 4). This finding also helps to identify the cml product as the 31 Kd protein.

The fractionation experiment with DS410 carrying pDU1249 (pUB5572 cml) provided a clue as to the cellular location of the 31 Kd protein. The outcome of such a crude experiment cannot be wholly reliable but the suggestion of an envelope location for the cml product is in keeping with the model proposed for the resistance mechanism by Gaffney et al., (1981).

CHAPTER 7

DNA SEQUENCE ANALYSIS OF THE R26
CHLORAMPHENICOL RESISTANCE DETERMINANT

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DNA sequence analysis of the R26 chloramphenicol resistance determinant

INTRODUCTION

A. DNA sequencing methods

Knowledge of the nucleotide sequence of any piece of DNA under study is very valuable for the following reasons. The extent of open reading frames within genes can be deduced, the precise points at which translation initiates and terminates can be determined and the location of signals promoting transcription and of other sequences with possible involvement in gene regulation may be discovered. In addition, the primary structure of any proteins encoded by the sequenced region can be deduced from the DNA sequence and something can be learned of the properties such proteins might have. Hydrophobic and hydrophilic regions may be discerned within the structure, giving clues as to the possible sites within the cell which the protein could reside in. Functional domains may be discovered within the protein, pointing to the role the molecule plays in the cell. For these reasons the DNA sequence of the Cm^r determinant of R26 was obtained. This Chapter deals with the experiments which led to its elucidation and the conclusions drawn from the results. Before turning to these, a brief review will be made of DNA sequencing techniques.

At the time of writing, the method of DNA sequence par excellence is that of Sanger, employing dideoxy chain terminator analogues of nucleoside triphosphates in conjunction with the M13 filamentous phage cloning vehicles of Messing. This technology has only been developed in the last five years and is an

essential element of modern molecular biology. Methods of DNA sequencing have, however, been available for nearly twenty-five years. Nucleotide sequences of the bacteriophage ϕ X174 were determined in the early 1960s using a depurination method published in 1960 (Burton and Petersen, 1960; Hall and Sinsheimer, 1963; Sanger et al., 1977). In the early 1970s degradation methods using exonuclease IV were in use (Ling, 1971; Sadowski and Bakyt, 1972). Around this time a method involving the analysis of the nascent mRNA produced by RNA polymerase from a given DNA template was available (Takanami, 1980), and it was used to solve the sequence of the lac operator (Gilbert and Maxam, 1973) and of the ϕ d phage promoters (Takanami et al., 1976). The method depended upon being able to sequence the RNA product. Techniques for doing this have been reviewed by Diamond and Dudock, 1983.

In 1973, Sanger et al. published details of a sequencing method which involved the use of a synthetic primer, DNA polymerase and ^{32}P -labelled triphosphates. A ribosubstitution method was used to aid the sequencing (Sanger et al., 1973; Barnes, 1978b). The sequence determination depended on fractionation of the polymerised products by two dimensional homochromatography. This method led to the development of the 'plus and minus' method of DNA sequencing (Sanger and Coulson, 1975). Here, synthetic primers, or, more often, restriction fragments, were used to prime the polymerisation of a radiolabelled complement to a single-stranded template. The system was applied to ϕ X174, sequencing both the viral (+) and complementary (-) strands (Sanger et al., 1977; Brown and Smith, 1977; Smith et al., 1977). This system was eventually to lead to the dideoxy chain terminator method used today. Around the same

time, an independent and totally different method for DNA sequencing was being developed. This was the chemical cleavage method of Maxam and Gilbert, 1977; 1980. Here, the DNA to be sequenced is first end-labelled (Richardson, 1965; Chaconas and Van de Sande, 1980), then partially cleaved at each of the four bases in four reactions. The products are ordered by size on a slab gel and the sequence is read from an autoradiogram by noting which base-specific reagent cleaved at each successive nucleotide along the strand. The technology involved in electrophoresing the products was worked out by Sanger and Coulson (1978). With the chemical cleavage method, either single- or double-stranded DNA can be sequenced. The DNA must first be cut by a restriction endonuclease. The technique can reveal the sequence for a distance of about 250 bases from this cut site. From the same cut, the corresponding 500 bases can be sequenced on the complementary strand. The distance after which the sequence can be read is a function of the gel electrophoresis technology. An early, and important, use of this technique involved the determination of the entire DNA sequence of the cloning vector, pBR322 (Sutcliffe, 1979; Peden, 1983).

Other approaches to DNA sequencing have involved making radiolabelled RNA from a DNA template using RNA polymerase and α -³²P-labelled ribotriphosphates (Blackburn, 1975; 1976). The labelled mRNA is then sequenced by established methods (Diamond and Dudock, 1983). Wu and others have employed exonuclease III to sequence both short DNA fragments, i.e. up to 20 bases (Tu and Wu, 1980) and more extensive DNA segments (Guo and Wu, 1983).

It has already been noted that the chain terminator technique is the most widely used method of DNA sequencing today. Like many

important technological advances, it was only made possible in its present form by drawing together knowledge from a number of different fields. This narrative has already traced the development of sequencing techniques by Sanger's group based on priming of DNA synthesis on single-stranded DNA templates from either synthetic oligonucleotides or restriction fragments using DNA polymerase I to drive the reaction. The chain terminator technique is a refinement of this method. It makes use of the 2',3'-dideoxy analogues of the deoxynucleoside triphosphates and their incorporation by DNA polymerase I onto the 3'-hydroxyl of an extending transcript. Once incorporated, the 3' end is no longer a substrate for further chain growth. This is because the dideoxy analogue lacks the 3'-hydroxyl necessary for further elongation of the chain, this being replaced by a hydrogen atom (Atkinson et al., 1969).

The chain terminator method also depends on DNA synthesis by the Klenow subfragment of DNA polymerase I. This lacks the 5'-3' exonuclease activity of the intact enzyme (Klenow et al., 1971). It will synthesise a complementary copy of the single-stranded target sequence, primed from a directly adjacent annealed strand of a primer molecule. The synthesis is carried out in the presence of the four deoxyribonucleoside triphosphates, one or more of which is α -³²P labelled (or, more latterly, α -³⁵S-labelled) and in turn in the presence of each dideoxy nucleoside triphosphate in separate incubations. In each reaction, there is a base-specific incorporation of a terminating analogue onto the 3' ends of the extending transcripts throughout the sequence. Parallel fractionation by gel electrophoresis of the size ranges of terminated labelled transcripts from each reaction, each with the common 5' end of the primer, allows a sequence to be deduced

(Maat and Smith, 1978; Sanger et al., 1977, Smith, 1980).

B. Cloning in M13 phage vectors

The use of single-stranded DNA cloning vectors is an important feature of the chain terminator sequencing system. The F-specific, filamentous coliphage, M13, is usually the vector of choice. This phage belongs to a group which includes f1 and fd and which do not lyse host cells. They are capable of packaging greater than unit-length viral DNA (Salivar et al., 1967). In fact, viral DNA up to six times the length of M13 DNA has been packaged (Messing et al., 1981).

In order to appreciate the usefulness of M13 in recombinant DNA work, it is necessary to understand its lifecycle. This may be summarised as follows. The rod-shaped virus penetrates the F pilus and is stripped of its major coat protein in the cell membrane. The viral single-stranded DNA (ss DNA) is converted to a double-stranded, circular form (RF, or replicative form, DNA) without the synthesis of any viral product. The virion DNA is always the same strand (the (+) strand). The complementary strand, the (-) strand, is the sense strand, and contains all of the coding information.

Once the RF is established, a new (+) strand is synthesised using the (-) strand as a template. This new (+) strand is then converted to RF through the synthesis of a new, complementary, (-) strand. This process continues until there are between 100 and 200 RF molecules in the cell. By then, the gene V product has accumulated sufficiently to interfere with the formation of new RF molecules. It binds to (+) strands, thus ensuring that they remain single-stranded. The protein-ss-DNA complex moves to the

periplasm, where the gene V product is displaced and the ss DNA is packaged. The mature virus particle is then released without lysing the host cell (Messing, 1983). Up to 1000 phage particles may be released into the medium per cell per generation (Old and Primrose, 1981). In addition to entry via the F-pilus, both RF and ss DNA forms can be introduced to cells rendered competent by CaCl_2 treatment. This process is termed transfection (Messing et al., 1977).

In the RF form, M13 can be handled like a plasmid in terms of DNA manipulations. Much work has been invested in improving the phage as a cloning vehicle. M13 does not have non-essential genes (unlike λ phage) into which foreign DNA may be cloned. The M13 cloning vectors developed to date exploit a 507 bp intergenic region between positions 5498 and 6005 of the DNA sequence.

The E. coli lac regulatory region and the sequence encoding the α -peptide of β -galactosidase have been inserted into this region to yield M13 mp1 (Messing et al., 1977). Since that vector was constructed, in-frame poly-linkers containing restriction sites for many commonly used enzymes have been inserted into the α -peptide region. These vectors still specify a functional α -peptide, capable of complementing mutations in this gene in the bacterial host (Messing et al., 1981; Rothstein et al., 1979). The cloning vectors used in this study, M13 mp8 and M13 mp9, carry the same polylinker, inserted in opposite orientations (Messing and Vieira, 1982; Fig. 7.1).

The vectors are used in conjunction with E. coli K12 strain JM103. This host is lac⁻ pro⁻ and can be complemented to lac by the phage. In addition, it carries a tra⁻ F factor. The F factor

is required to encode the pilus needed for M13 uptake. When the lac mutation in JM103 is complemented by M13, small amounts of β -galactosidase are produced. This production is induced by IPTG (isopropyl- β -D-thiogalactopyranoside), a gratuitous inducer of the enzyme, and may be detected by X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside), a chromogenic substrate. When complementation occurs, a blue colour results. Should a piece of foreign DNA be inserted into the polylinker within the lac α -fragment, complementation will not occur within JM103 and the phage will yield colourless rather than blue plaques (Messing et al., 1981). The M13/JM103 vector/host system is now an integral part of the sequencing system based on chain-termination (Sanger et al., 1980).

C. Analysis of DNA sequences

Once the DNA sequence has been established, it can then be scanned for interesting features whose presence has been suggested by previous genetic studies. At this point, the assistance of a computer may be enlisted to expedite matters. A comprehensive listing of available computer aids for DNA research may be found in *Nucleic Acids Research*, Volume 12, number 1, parts 1 and 2, published in January, 1984.

The features in the sequence which are of importance will now be detailed in turn. Open reading frames consist of runs of codons beginning with translational initiation signals and terminating with translational stop codons. The initiation signal is a start codon in mRNA, usually AUG, or less frequently, GUG which is prefaced by a ribosome binding site (Shine and Dalgarno, 1974). This is a short sequence with some complementarity to the 3' end of 16s rRNA. The base sequence at this part of the rRNA

is HO-A-U-U-C-C-U-C-C-A-C-U-A-G-5'. The ribosome binding site usually consists of all or part of the sequence AGGAGG. The f-Met codon, AUG or GUG, is normally within 5-9 bases of the binding site (Gold et al., 1981; Kozak, 1983). The open reading frame closes with a translational stop signal, one of UAA, UAG or UGA. These are thought to function by binding to the last three bases at the 3'-OH end of 16s rRNA (Shine and Dalgarno, 1974; Stormo et al., 1982).

Between the f-Met initiation codon and the translational stop signal stretches the string of sense codons which specify the primary structure of the gene product. In addition to determining the nature of the protein, the codons exert an influence on the translation mechanism itself. Rules have now been drawn up based on the type of codon sequence which mRNA presents to the ribosome. These describe the strategy for codon usage specified by the sequence.

Strategies of codon usage employed in E. coli have been reviewed by Gouy and Gautier (1982); Grantham et al., (1981); Grosjean and Fiers (1982); Ikemura (1981). The conclusions of these authors may be summarised as follows. Codon usage is markedly different in efficiently-expressed genes compared with genes coding for rare proteins, such as repressors. Two different aspects of selective codon usage are distinguished. Degenerate codons ending in U or C and recognised by the same tRNA are selected on the basis of codon-anticodon initiation energy: this should be neither too strong nor too weak. Secondly, a number of modulating codons are recognised by minor tRNAs or by a weakly interacting iso-accepting tRNA. These codons are clearly avoided in efficiently expressed genes.

In addition, certain sequences of codons may be favoured because they limit the probability of missense or frameshift errors.

In E. coli, NNC and NNU are read by their corresponding tRNAs having an anticodon GNN or QNN (INN for major arginine tRNA). Unmodified adenosine is never found in the "wobble" position of anticodons (Gauss and Sprinyl, 1981). NNU codons form a weak complex with GNN, QNN and INN anticodons (Freier and Tinoco, 1975). During translation, having NN as U and A, *optimises* the interaction with GNN, QNN (or INN) anticodons if a C is in the "wobble" position. For example, in the genome of the MS2 RNA phage, there is a systematic preference for the NNC codons over NNU for phenylalanine (Fiers et al., 1976; Atkins et al., 1979; Beremaud and Blumenthal, 1979).

If the NNs were C and/or G, a preference would exist for U as a third "letter" in the code "word" to avoid too sticky a codon-anticodon interaction. In this way, the codon-anticodon interaction is neither too weak nor too strong in highly expressed genes.

With weakly expressed genes, the opposite situation obtains. Here, the codon usage is apparently random and specific codons are never avoided, e.g. the trp repressor (Rose and Yanofsky, 1974; Singleton et al., 1980), araC (Miyada et al., 1980); Tn₃ repressor (Heffron et al., 1979). Among those codons not avoided are the so-called modulating codons. These have cognate tRNAs which are minor species in E. coli and are avoided in highly-expressed genes. They include AUA for isoleucine; CGG/AGA/AGG/CGA for arginine; CUA for leucine; GGA/GGG for glycine. In the rare cases that these appear in well-expressed genes, they may have a role in fine-tuning the rate of translation.

Consensus sequences for transcriptional start signals in E. coli have been published (Hawley and McClure, 1983; Maniatis et al., 1975; Schaller et al., 1975; Siebenlist et al., 1980; Rosenberg and Court, 1979; Pribnow, 1975a,b; 1979). This signal is a binding site for RNA polymerase and in its simplest form it may be written: T T G A C A 17 bases-TATAAT. The polymerase enzyme binds to this sequence and transcription begins, usually at a purine residue, at a position designated 1. Immediately upstream is position -1, there being no "position zero" in the sequence. The sequence TATAAT is located at position -10 and is called the Pribnow Box, after its discoverer or simply the "minus ten" region. This sequence is preceded by a 17 base hiatus before the next major consensus sequence. The piece of DNA, with the sequence TTGACA, is 35 bases upstream from the transcriptional start point. Thus it is referred to as the "minus thirty-five" region.

It should be stressed that this is an idealised sequence and that the published sequences of promoters usually differ from it in a number of respects. Differences may concern changes in base composition or in the spacing between -35 and -10 regions, or both. Such differences affect promoter strength (see Hawley and McClure, 1983; and below). In general, the more a promoter sequence diverges from the consensus, the weaker it is.

Transcription termination signals can be found at the ends of open reading frames. These are sequences which, once transcribed into mRNA, are likely to destabilise the mRNA-DNA complex, allowing the message to dissociate from the template and so end transcription. A palindrome normally occurs and this is often followed by a run of U residues. The hairpin structure probably causes RNA polymerase

to slow down or pause in transcription and the U-rich region provides a site for mRNA-DNA dissociation. This dissociation is brought about because the rU-dA RNA-DNA hybrid is a particularly weak base-paired structure. Bacterial terminators have been divided into two groups based on their apparent dependence on or independence of rho (σ) factor. This is a 55 Kd protein, probably active as a tetramer, which is thought to bind to mRNA and then pursue polymerase through the transcription process. Should polymerase stall, rho will reach it and form a complex which dissociates from the DNA. Terminator structures which possess hairpins followed by runs of U residues are classed as rho-independent. These hairpins also have GC-rich regions in their stem structures which enhance stability (Tinoco et al., 1973). Rho-dependent terminators lack the U-rich region after stem-loop structure and the stem structure itself does not contain the stability enhancing GC-rich regions. The transcription termination features discussed here were reviewed by Rosenberg and Court (1979).

Secondary structure in the mRNA can also have an effect on the initiation of translation. Systems in which such features play a part in regulating gene expression have been described in the Introduction to Chapter 5. Secondary structures which sequester the ribosome-binding site within a base-paired region block initiation of translation (Hall et al., 1982). This finding was confirmed recently by Schottel et al., (1984). These authors point out that while translational frequency declines when the ribosome binding site is sequestered, it is not affected when the AUG initiation codon is sequestered but the ribosome binding site remains free. The presence of such structures may be predicted from the DNA sequence and their stability can be determined using the rules of Tinoco et al., (1973).

MATERIALS AND METHODS

A. Bacterial strains, plasmids and phage

Escherichia coli K12 strain JM103 (Δ [lac pro], thi strA, supE, cndA, sbcB, hsdR⁻, F'traD36, proAB, lacI^a, 2 Δ M15) was used to propagate phages M13 mp8 and M13 mp9 (Messing et al., 1981). The structures of the coliphage M13 variants mp8 and mp9 have been described by Messing and Vieira (1982). The plasmid pDU1246 was used as the source of the Cm^r determinant for the M13 cloning and sequencing experiments. This plasmid has been previously described (Chapter 4).

B. Preparation of M13 RF DNA

A blue plaque was chosen from a transformation experiment in which M13 mp8 or mp9 was transfected into E. coli K12 JM103. The plaque was picked into 5 ml of L broth using a sterile pasteur pipette. Enough host cells were carried over to co-inoculate the broth. The culture was grown for 15h at 37° with shaking and the cells pelleted in 1.5 ml microfuge tubes. The supernatants were transferred to fresh tubes and to one tube 100 μ l of 10% Polyethylene glycol (10% wt/vol); 2.5 M NaCl was added and allowed to stand for 30 min. The tube was spun for 5 min in an Eppendorf microfuge. A white pellet was indicative of phage. The supernatant from the other tubes was then used in the RF prep. 1L of JM103 cells were grown to OD₅₅₀ = 0.5 in L Broth, at 37°, with shaking. Optical density measurements were performed with a Varian Techtron spectrophotometer. At OD₅₅₀ = 0.5, the phage suspension was added to the JM103 culture. This was grown for a further 15h, allowing the phage to come to the double-stranded DNA phase of its life cycle (see Introduction, this

Chapter). Chloramphenicol was added to a final concentration of 250 µg/ml to amplify the RF. The culture was grown for a further 15h and the cells were pelleted at 16,000 x g in a Sorvall GSA rotor. The CsCl-ethidium bromide plasmid DNA protocol described in Chapter 4 (Materials and Methods) was then followed to obtain purified RF DNA.

C. Transformation of *E. coli* K12 JM103

A colony from a minimal agar plate was used to inoculate an L-broth culture (2 ml per transformation experiment). The cells were grown to an OD₆₀₀ of 0.4. They were pelleted in sterile 1.5 ml microfuge tubes, resuspended in 500 µl of 30 mM CaCl₂ (4°) and left on ice for 20 min. The cells were again pelleted and then resuspended in 50 µl of 30 mM CaCl₂ (4°). M13 or M13 recombinant phage DNA was added and the solutions were allowed to stand on ice for 60 min. Luria base plates were prepared (1% Bactotryptone, 0.5% Bacto yeast extract, 1% NaCl, 1 pellet NaOH, 1.5% Difco agar) and dried. The cells were heat shocked at 42° for 2 min in a water bath. 30 µl of a 25 mg/ml solution of IPTG (in water) and 30 µl of an X-GAL solution (25 mg/ml in dimethyl-formamide) were added. To this was added 200 µl of a stationary phase culture of JM103. 3 ml of Luria overlay agar (as for Luria base but with 0.7% agar) was added (55°) and the mixture poured onto the dried base plates. Plates were incubated for 15h at 37°, inverted. Colourless plaques were then chosen for further work.

D. Preparation of templates for sequencing

Colourless plaques from transformation experiments were picked into sterile lambda storage buffer in 1.5 ml microfuge tubes (0.58% NaCl, 0.2% MgSO₄ 7H₂O, 1M Tris-hydrochloride, pH 7.5, 2% gelatin).

100 μ l of this phage stock was inoculated into a fresh 2 ml L broth culture of JM103. Cultures were then incubated for 15h with shaking, 37°. Then, 1.5 ml was transferred to a microfuge tube and spun for 5 min in an Eppendorf microfuge. 800 μ l of the supernatant was transferred to a fresh microfuge tube. To this was added 200 μ l of 2.5M NaCl, 10% PEG 6000. The tubes were mixed by inverting and allowed to stand at room temperature for 30 min. The tubes were microfuged for 10 min and the supernatant poured off. ALL of the remaining supernatant was removed with a heat-drawn glass capillary tube fitted to a micropipette. The pellet was resuspended in 100 μ l of 1.1M NaOAc, pH 7.0. To this was added 100 μ l of Tris-saturated phenol:chloroform:isoamylalcohol 50:50:1. The tubes were vortexed for 10 sec and allowed to stand for 5 min before microfuging for 60 sec. The aqueous layer was removed to a fresh tube and to this was added 60 μ l of chloroform:isoamylalcohol 50:1. This was vortexed and spun for 1 min. The aqueous layer was removed to a third tube. To this was added 250 μ l ethanol and the single-stranded DNA was allowed to precipitate for 15h at -20°. The DNA was pelleted, washed with 90% ethanol (1 ml) and dried under vacuum. The pellet was then resuspended in 15 μ l of sterile buffer (10 mM Tris-hydrochloride, 0.1 mM EDTA, pH 7.5). This provided enough material for three separate sequencing runs.

E. Cloning fragments of the Cm^r gene into M13

The methods of DNA manipulation, use of restriction enzymes, ligase, etc. have been described previously (Materials and Methods, Chapter 4).

F. Sequencing the Cm^r determinant

This was carried out by the method of Sanger et al., 1977.

The sequencing strategy is summarised in Fig. 7.1. α -³⁵S-ATP was purchased from Amersham Corporation; Klenow fragment of DNA polymerase I was purchased from BRL; a 15-base primer was synthesised by Celltech (U.K.).

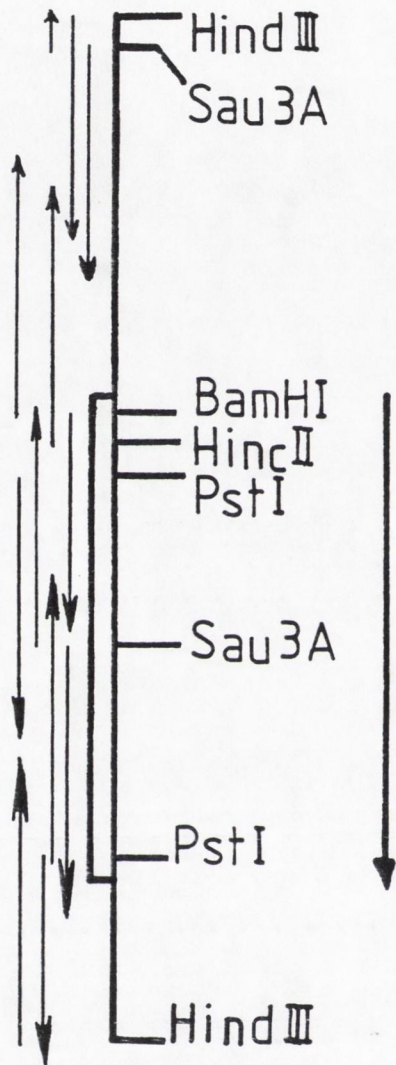
This primer is complementary to the 15 bases to the right of the polylinker in M13 derivatives mp8 and mp9 as these are drawn in Fig. 7.2. All other nucleotides, both deoxy and dideoxy, were purchased from Sigma.

G. Electrophoresis of sequencing products

Gradient acrylamide gels were used throughout. 5mm float-glass gel plates were used in conjunction with large-size BRL gel-rigs. Spacers were 0.4 mm thick, as were the "shark-tooth" combs (BRL). Gradient gels had two components, a 0.5X and a 2.5X acrylamide mix. The 0.5X mix contained 43% ultrapure urea, 5% (vol/vol) X10 TBE, and 15% (vol/vol) of a 40% acrylamide Bis solution (38% acrylamide, 2% Bisacrylamide, deionised by Amerlite monobed resin and filtered through sintered glass). The 2.5X mix contained 43% ultrapure urea, 25% (vol/vol) of X10 TBE, 15% (vol/vol) of a 40% acrylamide Bis solution (as for 0.5X mix), 5% sucrose, 50 mg/L bromophenol blue. These solutions were stored in the dark at 4°. Gel plates were scrupulously cleaned with hexane and acetone: the inner surface of the large plate was silanised. Gels were poured with 15 ml of 2.5X mix and 60 ml of 0.5X mix, these being polymerised with 90 μ l of 10% APS, 5 μ l of TEMED and 360 μ l of 10% APS, 15 μ l of TEMED respectively. Gradients were generated by taking up first 8 ml of 0.5X mix and then 12 ml of 2.5X mix in the same 20 ml pipette. The gradient was established by introducing air bubbles and the gel then poured. The remaining 0.5X solution was then added. Gels were

Figure 7.1 Sequencing strategy employed in determining the DNA sequence of the Cm^r determinant

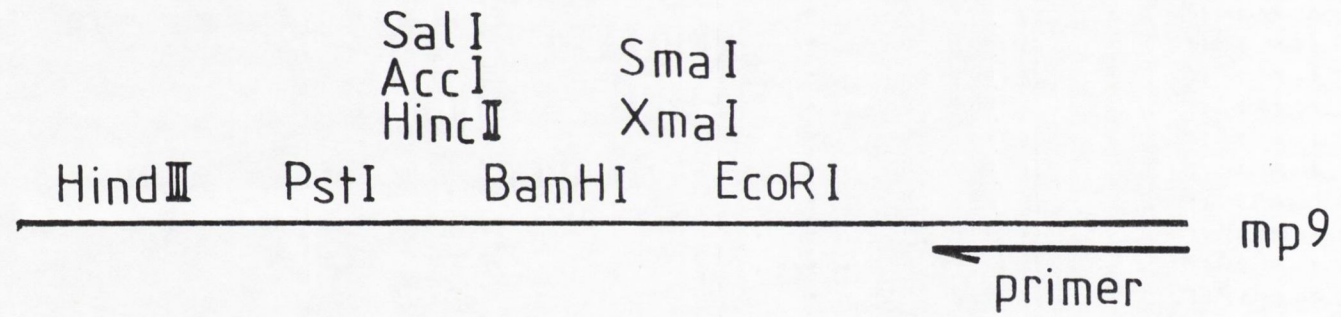
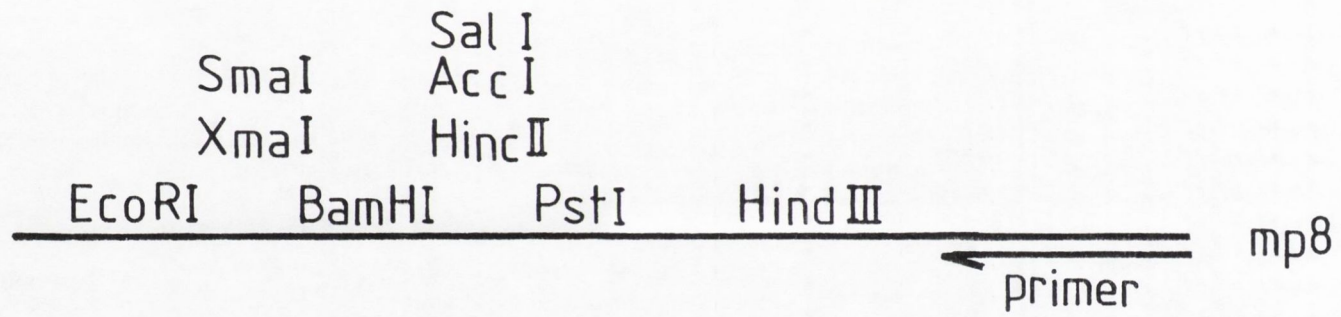
The horizontal line represents the 1.8 Kb Cm^r HindIII fragment. The locations of restriction endonuclease recognition sites are shown. The open box below the line represents the sequences required to specify the Cm-inducible polypeptide, based on genetic evidence and the results of minicell experiments. The heavy arrow above the line shows the direction of transcription of cml. The arrows below the line show the direction and extent of DNA sequence determinations. Sequencing was performed exclusively with restriction endonuclease-generated DNA fragments.



100bp

Figure 7.2 Structures of the multiple cloning site polylinkers
of vectors m13 mp8 and M13 mp9

The upper line represents the polylinker of M13 mp8 while the lower line shows that of M13 mp9. The DNA is in single-stranded form. The locations of the various restriction endonuclease recognition sites are shown. The binding site for the primer molecule is indicated by the arrow, the arrowhead shows the orientation of the priming reaction.



run with 0.5X TBE in the upper reservoir and 1X TBE in the lower. Gels were pre-run for 15 min in order to warm them as a precaution against uneven current distribution. A formamide dye mix was used as final sample buffer for samples being electrophoresed. This contained (in 100 ml de-ionised formamide) 0.1g Xylene cyanol FF, 0.1g bromophenol blue and 2 ml 0.5M EDTA. Gels were run at 1600 volts (constant voltage). Gels were fixed in 10% acetic acid, 10% methanol in distilled water for 20 min. They were then dried down onto Whatman 3HH paper at 80° under vacuum using a large gel drier (Biorad). Gels were autoradiographed at -70° using Fuji RX film. These procedures were based on those of Sanger and Coulson (1979). A representative autoradiograph is shown in Fig. 7.3.

H. Computer programmes for DNA sequence analysis

The programmes used in this study were written in BASIC programming language and ran on a Cyber 73 mainframe computer. The programmes were written by Dr Christopher Boyd, Department of Genetics, Glasgow University, Glasgow G11 5JS, U.K. and included AXXALIP, a programme for aligning, and translating DNA sequences; AXXFRAM, a programme for seeking out open reading frames within sequences; XEDIT, a programme for editing and updating sequences; AXXTIDY, a programme for packaging sequence information in a form for use by the computer; a programme called SUBMIT, which searched for restriction enzyme recognition sites; a programme called AXXRPT which searched for repeated sequences within the sequence file.

I. Probing with an oligonucleotide specific for chloramphenicol acetyltransferase genes

A synthetic oligonucleotide with the sequence

G.T.A. G.T.A. C.G.G. C.A.G. A.C.A. C.T.A. C.C.-5' was labelled at the 5' end using bacteriophage T4 kinase to transfer the gamma-phosphate from ATP to the DNA molecule. 4 µg of DNA were kinased in 10 µl of X10 kinase buffer, 150 µCi [γ -³²P] ATP, 20 units T4 polynucleotide kinase and water to a total volume of 50 µl. The reaction was allowed to proceed for 30 min at 37° and then quenched by adding 50 µl of 0.6M sodium acetate. The mixture was extracted once with phenol:chloroform, 50:50, and the DNA precipitated at -20° for 15h with ethanol. The pelleted DNA was redissolved in TE buffer (Maniatis, 1982) and centrifuged through Sephadex G-50 columns to separate labelled oligonucleotide molecules from free [γ -³²P] ATP molecules. The 10X kinase buffer contained: 0.5M Tris-hydrochloride (pH 7.6), 0.1M MgCl₂, 50 mM dithiothreitol, 1 mM spermidine and 1 mM EDTA. T4 kinase was purchased from BRL. These procedures were based on those of Maniatis et al., (1982) and Richardson, (1971).

Hybridisation was carried out against single-stranded DNA in M13 fixed to nitrocellulose filters for 15h at 37°. Washing was with 2 x SSC and 0.1% SDS at room temperature. These conditions exert low stringency (Southern, 1975). Target DNAs were: non-coding strand of cml (Fig. 6.3A); non-coding strand from Proteus mirabilis CAT (positive control); M13 DNA containing the cml "regulatory region" coding strand (negative control).

RESULTS

A. DNA sequence analysis

The DNA sequence of the Cm^r determinant of plasmid R26 was obtained by the method of Sanger et al (1977) using the sequencing strategy outlined in Fig. 7.1. The sequence data for both strands, including the locations of restriction endonuclease recognition sites, are given in Fig. 7.4. This information is correlated from independent sequencing experiments using the computer programmes AXXTIDY, XEDIT and SUBMIT. A total open reading-frame analysis was carried out using the programme AXFRAM and the results are shown in Fig. 7.5.

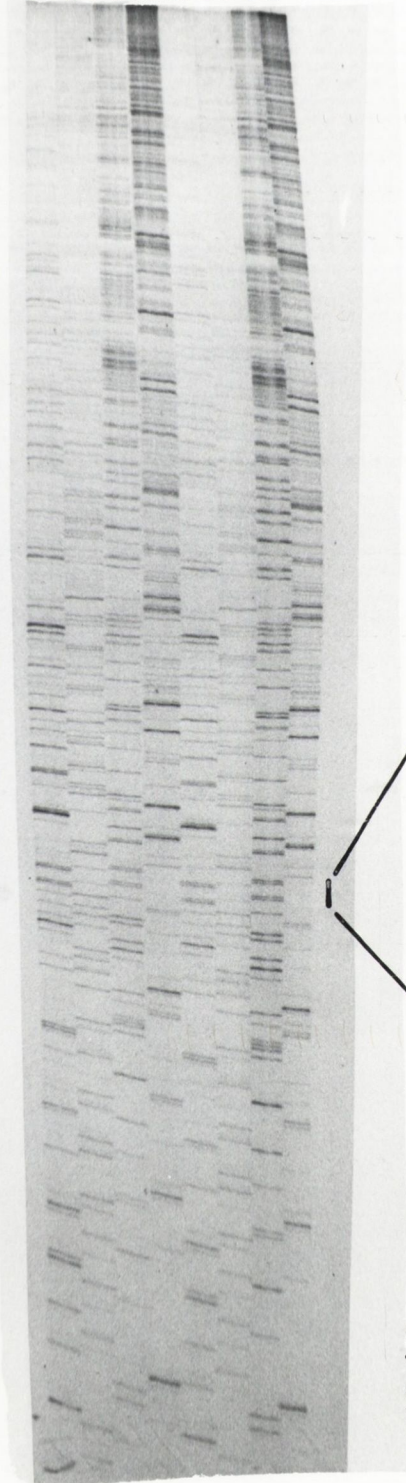
A major open reading-frame with the same orientation as cm1 can be discerned, initiating at position 583 of the sequence. The initiation codon is GUG, and the reading-frame terminates at position 1633 with a UGA codon. If this cistron was expressed, its product would be a protein of 350 amino acids, with a molecular weight of 39.9 Kd (this value being the sum of the molecular weights of the component amino acids).

The initiation codon is preceded by a possible Shine-Dalgarno sequence, GAGG, with a spacing of 15 nucleotides, or more unlikely, GUGG, with a spacing of 12 nucleotides. The spacing for the more favourable ribosome binding site is probably too great to permit initiation of translation. An alternative initiation codon lies within the same reading frame at position 727 (Fig. 7.5). As with the larger reading-frame, this open reading-frame is transcribed in the same direction as cm1 (Chapter 5). It occupies the stretch of DNA shown to be essential for Cm^r by genetic evidence (Chapter 4). The initiation codon here is AUG and this is prefaced by a good ribosome-binding site sequence (GAGG) which is separated from the AUG codon

Figure 7.3 An autoradiograph of a DNA sequencing gel

The loading pattern for the products of the A, C, G and T reactions is shown. Each reaction mix was halved and both portions loaded onto the gel in the manner shown. In this way no single sample is more than two lanes away from any other sample, increasing the accuracy with which gels may be read. This gel shows the sequence of a Sau3A-Pst1 clone inserted in M13 mp9. Electrophoresis was continued for a further 3h after the tracking dye had run off the bottom of the gel, revealing sequence information normally retained in the unreadable upper part of the gel.

GTACGTAC



A-1161
A
T
G
G
G
A
A
T
G
G
G-1172

Sequence
sample

Figure 7.4 DNA sequence of the 1.8 Kd HindIII fragment of
plasmid pDU1246 (Cm^r)

The most leftward HindIII site is not shown, the sequence beginning at the most leftward Sau3A site. The sequence of both strands is shown with coordinate numbers given above the sequence. The locations of restriction endonuclease recognition sites are indicated by small asterisks, with the names of the enzymes listed above the sequence.

Figure 7.5 A total reading-frame analysis of the sequenced
DNA fragment

The DNA sequence is as in Fig. 7.4, but with the coordinates given between the DNA strands. Predicted amino acid sequences for all six reading-frames are given, with arrows to indicate the orientation of translation. Translational stop codons are indicated by triple asterisk symbols.

Note: a copy of the original computer print-out is contained in a pocket at the rear of this volume.

by six nucleotides. These features make this a good candidate for an authentic translation initiation site. A protein expressed from this stretch of DNA would contain 302 amino acids, giving it a molecular weight of 33,845d. This makes this protein a good candidate for the Cm-inducible polypeptide of apparent molecular weight 31 Kd seen in minicells (Chapter 6). The predicted amino acid sequence of this protein is given in Fig. 7.6 and the codon-usage table is in Fig. 7.7. The protein contains many hydrophobic residues, often in sequence, which may be in keeping with a membrane location within the cell. The various features of the DNA sequence which may play a role in regulating the production of this protein and/or in determining its nature will now be considered in turn.

I. Control of Transcription

(i) Initiation. Genetic evidence discussed in Chapter 4 indicated that the DNA to the left of the BamHI site within cml was essential for Cm^r for a distance of 400 bp (Fig. 4.7). The DNA sequence of this region is shown in Fig. 7.6, the BamHI site being located at position 747. A possible transcription promoter can be discerned between positions 301 and 332. The "minus thirty-five" region has the sequence TTGCCT (consensus: TTGACA) and the "minus ten" has the sequence TCCTTT (consensus: TATAAT). The spacing between these hexanucleotides is twenty base-pairs. The possible transcription start (+1) is at position 343, an adenosine residue. Since the cml promoter was not genetically characterised, it is not possible to identify it unambiguously merely from a study of the DNA sequence. The potential promoter described here has certain features in common with that of hisJ, a genetically characterised promoter sequence (Higgins and Ames, 1982). The "minus thirty-five" regions are

Figure 7.6 DNA sequence and predicted amino acid sequence of the major open reading-frame within the 1.8 Kb HindIII fragment

The DNA sequence given is that of the non-coding strand of the Cm^r determinant. The sequence preceding position 300 is not shown. The predicted amino acid sequence for the 33.8 Kd protein is given below the DNA sequence string. The stop codon is marked by a treble-asterisk symbol. A possible Shine-Dalgarno sequence at position 717 is doubly underlined. Inverted repeats within the DNA sequence are marked by converging arrows. Potential promoter sequences in the 300 to 400 region of the sequence are singly, doubly or trebly underlined. Each pair or underlined hexanucleotides represents a "-35" or "-10" promoter sequence (see text for details).

310 320 330 340 350 360
7 : TTGCCTCTTACGGGAGCGCCGCCAAATCCCTTGTTCAGGAGATAGGTTCCGGTAGCCCT
370 380 390 400 410 420
7 : CAAAACCTTAGTTCGCTACTCTTGGCCGCACTTGGTATCACGGTTCGATATGCAACATC
430 440 450 460 470 480
7 : TGCATGCATTAATCCAEATCCCTTAGCCAAAATCGCGCTGGTACGACAGCAGCACACTCA
490 500 510 520 530 540
7 : GACGCTGACAACGTAATGTCATGATGTGCCGGTACAGCTCTATTTGGACCGCTATCGGA
550 560 570 580 590 600
7 : CCGTCAGCGCGCCCGCTTCTACTGGAGTGCCCTGCTACGTGTGTGCGTCAATGGGGC
610 620 630 640 650 660
7 : TCGCTCTTACGTCATCGGCTGAAGTCTTCTGGGGCTTCGGATCTTCAGGCTTGTGGTG
670 680 690 700 710 720
: CCTCGGCGTGCCTTGTTCACCATTTGCAACAGTACGTGACATTTAGCAGGTCGCGGAGG
730 740 750 760 770 780
: AAAGTAATGTCAATTTACGGCATACTCGGATCCATGCTGGCCATGGTCCCGCGGTAGGCC
MetSerPheThrAlaTyrSerAspProCysTrpProTrpSerArgGlyArgPro
790 800 810 820 830 840
: ATTGCTCGGAGCGCTCGTCGACATGTGGCTTGGGTGGCGGGCTATCTTTCGCTTCTAGG
IleAlaArgSerAlaArgArgHisValAlaTrpValAlaGlyTyrLeuCysValSerArg
850 860 870 880 890 900
: TTTGGGCATGATCGCTGCATCGCAGGCGTGGCAGCTTCTGGCCTGAAACCCGGGTGCAA
PheGlyHisAspArgCysIleCysArgArgGlySerPheTrpProGluThrArgValGln
910 920 930 940 950 960
: CGAGTTGCGGGCTTGCAATGGTCGACGTGCTACTCCCGTAAAGTGCCGTAACCTCTGG
ArgValAlaGlyLeuGlnTrpSerGlnLeuLeuLeuProValLysCysLeuAsnPheTrp
970 980 990 1000 1010 1020
: TTGTACACGTTGTGTACGCCGCTGGAATGGGTAGCTTCTCGCTCTTTCTCCATTCGG
LeuTyrThrLeuCysTyrAlaAlaGlyMetGlySerPhePheValPhePheSerIleAla
1030 1040 1050 1060 1070 1080
: CCCGGACTAATGATGGCCAGGCCAAGGTGTGTCTCAGCTTGGCTCAGCCTGCTGTTCGC
ProGlyLeuMetMetGlyArgProArgCysValSerAlaTrpLeuGlnProAlaValArg
1090 1100 1110 1120 1130 1140
: CACAGTCAATTTGCCATGGTGTTCACGGCTCGTCTTATGGGGAGTGTGATACCAAGTGG
HisSerAlaIleAlaMetValPheThrAlaArgPheMetGlySerValIleProLysTrp
1150 1160 1170 1180 1190 1200
: GGCAGCCCAAGTGTCTTGCSAATGGGAATGGGATGCCGTGATAGCTGGAGCAGTATTGCTT
GlySerProSerValLeuArgMetGlyMetGlyCysLeuIleAlaGlyAlaValLeuLeu
1210 1220 1230 1240 1250 1260
: GCCATCACCAGAAATATGGGCTTTGCAACCGTGTAGGCTTTATTTGCTCCAATGTGGCTAGT
AlaIleThrGluIleTrpAlaLeuHisArgValArgLeuTyrCysSerAsnValAlaSer
1270 1280 1290 1300 1310 1320
: GGTATGGTGTGCGCACAGCGGTATCTGTGCCAATGGCGCTCTTCGAGGATTCGACCATG
GlyIleGlyValAlaThrAlaValSerValProMetAlaLeuPheGluAspSerThrMet
1330 1340 1350 1360 1370 1380
: TTGCTGGAACGGTCACGCGAGTCTACTTCTGCTTGGGCGTACTGCTAGGAAGCATCGG
LeuLeuGluArgSerArgGlnSerThrSerAlaTrpAlaCysThrAlaArgLysHisArg
1390 1400 1410 1420 1430 1440
: AACGTGTGATCATTTTCGCTGTGCCGCGCAACGGCTTGGGCTTGTGCTGTAACGTGTGG
AsnValAspHisPheAlaValAlaAlaGlnArgLeuGlyProCysArgValThrValTrp
1450 1460 1470 1480 1490 1500
: ACCCTTGCAACAGTCGTCTCGGCTGTCTTGTGTTTCCCAGTGAAGGGCTCTCGCGGC
ThrLeuAlaThrValValLeuGlyLeuSerCysValSerArgValLysGlySerArgGly
1510 1520 1530 1540 1550 1560
: CAGGGGGAGCATGATGCTGGTTCGCGCTACAAATGTCGGAAAGTACATCAATCCCAATCG
GlnGlyGluHisAspAlaGlyArgAlaThrAsnValGlyLysTyrIleLysSerGlnSer
1570 1580 1590 1600 1610 1620
: TTGAGAGAATGTGGCAAGCTATCGCCCAACAAATGCTGCAGCCGACCCAAAACGTACCGG
LeuArgGluCysGlyLysLeuSerProAsnLysCysCysSerArgProLysThrTyrAla
1630 1640 1650 1660 1670 1680
: TTTCCGCTCGGCTGAGCTTCATTGGCGTGTAGATGCCTAAGCACATAATTTGCTACAGC
PheArgLeuGly***
1690 1700 1710 1720 1730 1740
: AACTATCAGGTCAGTCTGCTTTTATATTTTAAAGCGTGCATAATAAGCCCTACACA
1750 1760 1770 1780 1790 1800
: AATGGGAGATATATCATGAAGGCTGGCTTTTGTCTTATCGCAATAGTTGGCAAGTA
1810 1820 1830 1840 1850 1860
: ATGCGACATCGCAATTAATCTAGCGGGGCTTACTAAGCTT

Figure 7.7 Codon usage table for the 33.8 polypeptide

A standard codon usage table is presented, using conventional nomenclature. Modulating codons are denoted by an asterisk after the amino acid epithet.

	U	C	A	G	
U	Phe 6	Ser 6	Tyr 2	Cys 6	U
	Phe 7	Ser 5	Tyr 5	Cys 9	C
	Leu 0	Ser 3	Ter 0	Ter 1	A
	Leu 8	Ser 5	Ter 0	Trp 11	G
C	Leu 6	Pro 3	His 5	Arg 5	U
	Leu 4	Pro 6	His 2	Arg 5	C
	Leu* 3	Pro 5	Gln 4	Arg* 5	A
	Leu 5	Pro 0	Gln 4	Arg* 7	G
A	Ile 4	Thr 3	Asn 2	Ser 4	U
	Ile 3	Thr 4	Asn 3	Ser 5	C
	Ile* 3	Thr 3	Lys 3	Arg* 1	A
	Met 10	Thr 4	Lys 6	Arg* 7	G
G	Val 9	Ala 15	Asp 5	Gly 6	U
	Val 6	Ala 5	Asp 0	Gly 9	C
	Val 3	Ala 3	Glu 4	Gly* 6	A
	Val 9	Ala 9	Glu 2	Gly* 4	G

identical. Both have greater than optimal spacing between "minus ten" and "minus thirty-five" regions, 18 bp for hisJ and 20 bp for cml, the optimum being 17 bp (Hawley and McClure, 1983). There are a number of mismatches in the "minus ten" regions of both promoters as compared with the consensus (hisJ, CACGAT; cml, TCCTTT; consensus, TATAAT). Work with pDU1292, a cml-lac operon fusion plasmid, indicated that the cml promoter was weak (Chapter 5). The features of the possible cml promoter discussed here would be characteristic of a weak promoter (Hawley and McClure, 1983). Another potential promoter exists between positions 348 and 373. The "minus thirty-five" sequence, TTTCCG, has three mismatches with the consensus and the "minus ten", TTAGTT, has four. The spacing is 14 bp. This would also be expected to be a weak promoter. A third candidate for the cml promoter sequence lies between positions 367 and 398. The "minus thirty-five" region, TTTAGT, has three mismatches with the consensus, while the "minus ten", TTGTGT, has four. The spacing is 20 bp. Again, this would be a weak promoter. The fact that the most leftward-mapping Tn5-insertion giving a Cm^S phenotype would be located near position 347 implies that the 367 to 398 sequence is not likely to be the true promoter (Fig. 4.7)

(ii) Termination. The open reading frame illustrated in Figure 7.6 is followed by the sequence UUUUAUUUUUUU, beginning at position 1703. This sequence may play a role in transcription termination (see Chapter Introduction) but it is not preceded by a stem-and-loop structure characteristic of a rho-dependent terminator (Rosenberg and Court, 1979). However, two sets of inverted repeats in the post reading-frame region may function as rho-dependent terminators (Fig. 7.6).

II. Control of translation

(i) Codon usage. The codon usage table for the 33.8 Kd protein predicts that this is a poorly translated cistron (Fig. 7.7). Codon usage is random, which is characteristic of poorly translated genes, as is the use of modulating codons (see Chapter Introduction). These findings correlate with the observations reported in Chapters 3, 4 and 6 concerning the time taken to achieve a fully induced level of cml expressions as determined by expression of the Cm^r phenotype or by the production of the Cm-inducible polypeptide in minicells.

(ii) Initiation of translation. The AUG codon which initiates the 33.8 Kd protein reading-frame is preceded by a good potential ribosome-binding site, making it a likely site for translational initiation. This AUG triplet is preceded by a purine residue and followed by a pyrimidine. The reverse arrangement gives more efficient translation initiation (Eckhardt and Luhehmann, 1981; Kozak, 1983). Furthermore, the AUG forms part of an inverted repeat sequence capable of forming a stem-and-loop structure (Fig. 7.6). In addition, the ribosome-binding site can be sequestered within a potential stem-and-loop structure (Fig. 7.6). A possible role for this structure in regulating the expression of cml will be discussed below.

Translation of an open reading-frame initiating at position 585 (GUG) and terminating at 698 (UGA) could modulate initiation of translation of cml. However, the nearest ribosome binding site for this reading frame is 17 residues away, a distance too great to make expression likely (Fig. 7.5).

Two inverted repeats within the reading frame were predicted by the AXRPT computer programme. These lie between coordinates 1498 and 1525 and between 1602 and 1631 (Fig. 7.6). Both are near to the carboxy-terminus of the reading frame and their role in regulating expression of cml (if any) is unknown.

B. The cml-lac gene fusion is in frame

The DNA sequence which encodes the 33.8 Kd protein includes the unique BamHI site within cml. In Chapter 5, this site was used to construct a cml-lac translational or gene fusion plasmid, pDU1294. Analysis of the sequence (Fig. 7.1) shows that this fusion was in frame. The DNA sequence across the junction at which the fusion was made together with the predicted amino-acid sequence of the hybrid Cm^{r} -lac polypeptide is shown in Fig. 7.8. Sequence data for lacZ and β -galactosidase comes from Casadaban *et al.*, (1980).

C. Probing with an oligonucleotide specific for the chloramphenicol acetyltransferase active site

A radiolabelled, CAT-specific oligonucleotide probe was used in a hybridisation experiment to seek homology between cml and cat at the DNA level. The positive control was the non-coding strand of Proteus mirabilis cat, cloned in M13. Negative controls were TE buffer, and the "coding" strand of the small (700 bp) BamHI-HindIII fragment of cml, i.e. that carrying all but the first eight codons of the 33.8 Kd protein. Both cml fragments were in M13 and in the single-stranded state.

The outcome of the experiment is shown in Fig. 7.9. The non-coding strand of P. mirabilis cat provided strong homology for

Figure 7.8 DNA sequence at the *cml-lac* junction within plasmid pDU1294 and predicted amino acid sequence of the amino terminus of the hybrid polypeptide

The DNA sequence of both strands is given. The location of the BamHI recognition site used in the construction of the fusion is shown, together with the nature of the cohesive ends involved. The predicted amino acid sequence of the amino terminal end of the fusion product is given below the DNA sequence, with the respective contributions from the Cm^r protein and β -galactosidase indicated. The arrowhead shows the direction of translation.

cmI

lacZ

BamHI

ATGTCATTTACGGCATACTCGGATCCCGTCGTTTAA
TACAGTAAATGCCGTATGAGCCTAGGGCAGCAAAT

fMet Ser Phe Thr Ala Tyr Ser Asp Pro Val Val Leu

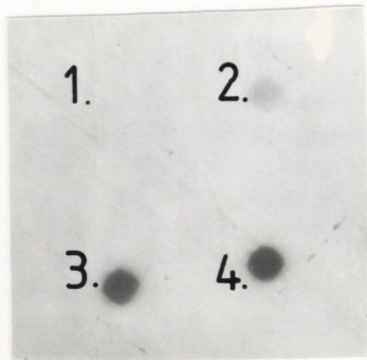
cm^r

β-GALACTOSIDASE

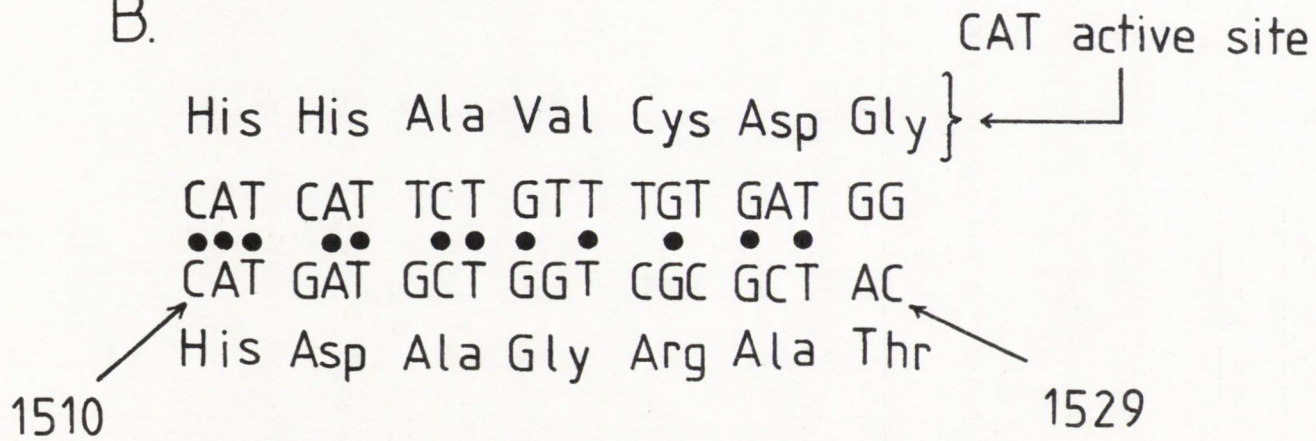
Figure 7.9 Results of hybridisation experiment with a radio-labelled probe specific for chloramphenicol acetyltransferase genes

- A. Autoradiograph of the hybridisation filter. Sample 1: DNA-free TE buffer; Sample 2: the coding strand of the 700 bp BamHI-HindIII Cm^r fragment from pDU1246; Sample 3: The non-coding strand of the 1100 bp BamHI-HindIII Cm^r fragment from pDU1246; Sample 4: the non-coding strand from Proteus mirabilis CAT which includes the complement of the CAT active-site-encoding sequence.
- B. The DNA sequence of the complement of the CAT active-site-encoding sequence, together with the amino acid sequence of the active site is given in the upper part of the diagram. A possible site of hybridisation for the CAT-specific probe within cmI is given in the lower part, together with the sequence coordinates and the predicted amino acid sequence of the 33.8 Kd protein encoded by that region of the DNA. The discs between the DNA strands show homologous bases.

A.



B.



the probe, as expected. Weak homology was detected between the "coding" strand of the 700 bp BamHI-HindIII fragment of cml, one of the negative controls. No binding occurred in the area of the filter occupied by TE buffer alone, as expected. However, strong homology was detected between the probe and the non-coding strand of cml, indicating some degree of sequence homology between it and cat. The experiment was carried out under conditions of low stringency, so considerable complementarity need not be required for binding to occur (see Materials and Methods section). A search of the cml sequence revealed a region at position 1510 at which binding may have occurred. The DNA sequence of this region, together with the predicted amino acid sequence of that part of the 33.8 Kd protein encoded by it is presented in Fig. 7.9.

DISCUSSION

The open reading-frame for the 33.8 Kd protein identified in the DNA sequence correlates with previous data on the extent of *the* Cm^r-essential region of the 1.8 Kb HindIII Cm^r fragment and on the direction of transcription of cml. The Cm-inducible polypeptide identified in Chapter 6 had an M_r of 31K, based on its rate of migration in SDS polyacrylamide gels. Work on other proteins, for example, the pBR322-encoded Tet protein, has shown that the actual molecular weights of polypeptides may differ somewhat from the values predicted from electrophoretic studies (Pedan, 1983). Thus, the 33.8 Kd protein predicted by the DNA sequence is a good candidate for the Cm-inducible protein.

Furthermore, the reading-frame for this protein is preceded by an untranslated leader sequence which may play a role in regulating expression of cml. This mechanism is based on the model proposed by Byeon and Weisblum (1984) to explain the regulation of gram-positive CAT genes (Fig. 7.10 and Chapter Introduction).

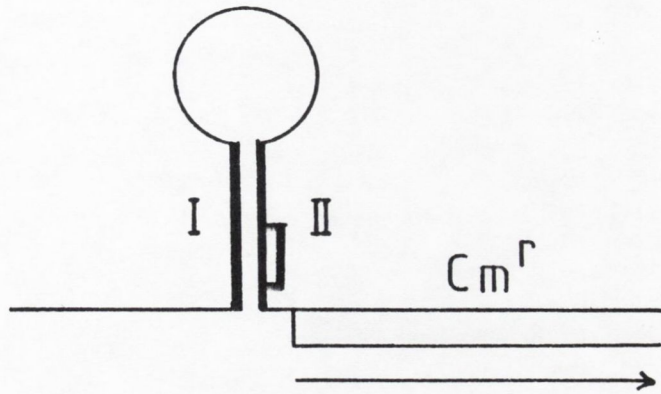
Their model requires that the ribosome-binding site for the Cm^r gene be sequestered in a secondary structure which the Cm^r mRNA adopts upon transcription (Fig. 7.10). This conformation prevents translation of cat in the absence of Cm. When Cm is added in sub-inhibitory concentrations, a conformational change occurs in the ribosomes which bind the drug, permitting binding of rRNA to the sequence CCUCCU within a region of mRNA normally involved in the stem-loop structure. Thus base pairing cannot now occur and unmodified ribosomes are free to bind to the cat ribosome-binding site to initiate translation (Fig. 7.10).

Figure 7.10 A model to explain the mechanism of induction of gram-positive cat genes

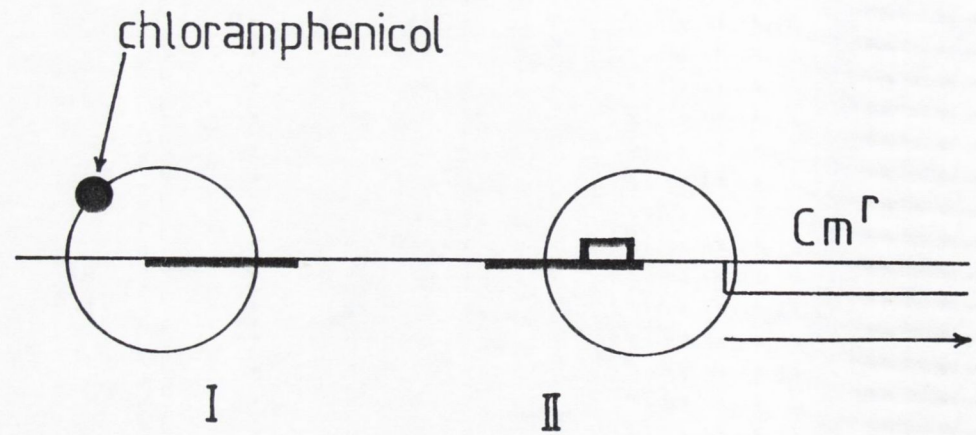
Possible conformations for the 5' end of cat mRNA are shown. Regions I and II of the mRNA are capable of base-pairing with each other. The cat ribosome-binding site is denoted by a box. The open bar represents the reading-frame for CAT. Ribosomes are represented by large circles. The arrow indicates the direction of translation.

- A. In the uninduced state, the cat ribosome-binding site is sequestered in the stem-and-loop structure formed by base-pairing of regions I and II. Translation of cat is thus precluded.
- B. In the induced state, a Cm-modified ribosome binds to a site in region I (see text), preventing base-pairing with II. An unmodified ribosome can bind to the free ribosome-binding site and initiate translation of cat.

A. UNINDUCED



B. INDUCED



The sequences involved in forming the stem-and-loop structure in cat mRNAs in B. pumilis cat and S. aureus (pC194) cat are shown in Fig. 7.11. The corresponding region of cml is also shown. The structures formed upon base pairing are illustrated in Fig. 7.12. In each case, the ribosome-binding site is unavailable for ribosome binding. Also shown is the region of E. coli 23s mRNA which may be involved in the induction event. The sequence in the modified ribosome which may bind to Cm^r mRNA is indicated. In cml, this would have fewer complementary bases to pair with and this may explain the poor rate of induction with the R26 system. The lac fusion data from the cml-lac gene fusion plasmid pDU1294 suggested that expression was poor, even in the induced state. The sequence data described in this Chapter shows that the poor expression of the hybrid protein was not due to an out-of-phase fusion. A combination of poor induction and poor translational initiation for the reasons discussed above may explain this.

Recent data on the role of mRNA secondary structure in controlling translation indicates that structures which sequester the AUG codon but not the Shine-Dalgarno sequence are unimportant in inhibiting initiation. Thus the potential stem-and-loop which includes the AUG in its stem but the ribosome-binding site in the unpaired loop has probably no role to play in controlling cml expression (Schottel et al., 1984).

The discovery of limited homology between the CAT active site and the cml gene product may be indicative of a common property with regard to Cm^r. Alternatively, it may be due purely to serendipity. The homology could reflect an ability to bind Cm, without being able to modify it. Binding the drug is essential whether a sponge-type mechanism or an efflux mechanism of resistance is envisaged.

Figure 7.11 Comparison of the putative regulatory region of R26
cml mRNA with the regulatory regions of *Bacillus*
pumilus and *Staphylococcus aureus* (pC194) CAT mRNAs

Initiation codons are boxed and ribosome-binding sites are underlined, as are the alternative binding sites for Cm-modified ribosomes. Data for the *B. pumilus* sequence comes from Duvall et al. (1984), while that for pC194 is from Byeon and Weisblum (1984).

B.pumilus CAT

CCUCCUGAAUACAGUAAAUCACAUUCAGGAGGAGAUAAAUUG

pCI94 CAT

CCUCCUAAAUUCACUUUAGAUAAAUUUAGGAGGCAUAUCAAUG

R26 cml

CCUCGGCGUGCCUUGUUCCACAUUUGCAACAGUACGUGACAUUACGCAGGUCGCGAGGAAAGUAAUG

Figure 7.12 RNA secondary structures involved in the control of expression of inducible chloramphenicol resistance

In each case, ribosome-binding site sequences or sequences analogous to ribosome binding sites are marked by vertical lines.

The free energy values for these structures are as follows:

B. pumilus CAT ($\Delta G = 25$ Kcal/mol); pC194 CAT ($\Delta G = 21$ Kcal/mol);

R26 cm1 ($\Delta G = -10$ Kcal/mol); 23s rRNA structure (-12 Kcal/mol).

Calculations were performed using the method of Tinoco et al.,

(1973). It is the ribosome-binding site-like structure of 23s

rRNA which is thought to be involved in disrupting the stem-and-

loop structures of the Cm^r determinants during induction (see text).

```

UAAA
G   U
A   C
C   A
A   C
  UA
  AU
  AU
  GC
  UA
  CG|
  CG|
  UA|
  CG|
  CG|
  UA
  CG
  UA
  AU

```

B. pumilus CAT

```

UUAGA
U   U
C   A
A   A
C   A
  UA
  UA
  AU
  AU
  UA
  CG|
  CG|
  UA|
  CG|
  CG|
  GC

```

pCI94 CAT

```

      AC
      A A
      CG
      GU
      UA
      U C
C C   ACAU   GUG   AC   AU
U U   UGUUA   CGC   AUU
      CG
      CG
      GU
      UI
      GC
      CG
      GI
      GC
      CG|
      UA|
      CG|
      CG|

```

R26 cml

```

AGAGU
A   A
A   A
U   C
  CG|
  CG|
  UA|
  CG|
  CG|
  UA|
  CG

```

23s rRNA

CHAPTER 8

GENERAL DISCUSSION

General Discussion

This thesis describes the molecular genetic analysis of the chloramphenicol resistance determinant of plasmid R26. The determinant, cml, was cloned, mapped and sequenced. It was subjected to deletion and to transposon mutagenesis. The cml gene product was identified and studied using the E. coli minicell system. The control of cml expression was studied by lac fusion experiments. In addition, the effects of fluorinated derivatives of Cm on E. coli cells harbouring R26 were investigated and the data compared with that for E. coli cells containing R55-1, a plasmid which also specified non-CAT Cm^r.

The Cm^r determinant was shown to be carried on a 1.8 Kd HindIII fragment, of which 1.4 Kb was essential for Cm^r. This DNA fragment was shown to specify a Cm-inducible protein of apparent molecular weight 31 Kd. DNA sequence analysis showed that the true value was 33.8 Kd. The direction of expression of cml was established by lac gene fusion experiments and cml was shown to be regulated posttranscriptionally. Analysis of the DNA sequence of the putative regulatory region of cml revealed that the mechanism of regulation proposed by Byeon and Weisblum (1984) for gram-positive cat genes may apply in this case. This model is intuitively appealing because in it the cellular target for Cm, the ribosome, provides a control circuit to activate resistance to the drug, so that resistance is only expressed in the presence of Cm.

Byeon and Weisblum (1984) showed that the fluorinated Cm derivative, SCH25298, was an effective inducer of pC194 CAT. This drug, together with other fluorinated Cm derivatives, was an efficient inducer of cml. In the case of pC194 cat, it was shown that a drug-

protein interaction was not required for induction. This was also established for cml, since the cml-lac gene fusion of pDU1294, which is fully Cm^S, is inducible by Cm (Chapter 5).

The similarities in induction mechanisms of cml and gram-positive cat genes are not reflected in large amounts of DNA sequence homology. Neither the regulatory regions nor the coding regions for the resistance proteins display any marked similarities. Taken together with the fact that the resistance mechanisms are also quite different (Gaffney et al., 1981), the common properties of the induction mechanisms are more likely to be the fruit of convergent evolution rather than a common evolutionary ancestry.

The resistance level specified by cml (30 µg/ml) is low. The induced/uninduced MIC ratio is only 2 and both induction and the rate of expression of cml are quite inefficient. These factors are indicative of a Cm^R determinant tailored to an environment in which Cm is in low concentration. R26, the plasmid from which cml was isolated, is a broad host-range plasmid from Inc P-1. Many of the gram-negative hosts which this plasmid might reside in, including Pseudomonas aeruginosa (the organism from which it was isolated) have a soil habitat. The actinomycete which synthesises chloramphenicol, Streptomyces venezuelae, has a soil habitat. It does not specify CAT (Shaw and Hopwood, 1976) but seems to depend upon an inducible permeability barrier to protect itself from the drug (Malik, 1972; Vining, 1979). Many authors currently propose that the ultimate source of antibiotic resistance determinants lies in the drug producing organisms (e.g. Foster, 1983; Gale et al., 1981). Thus S. venezuelae could be the source of permeability-type Cm^R determinants such as that of R26. The fact that cml and the Cm^R

determinant of R55-1 were not shown to be related by Southern hybridisation does not rule out a common ancestry for them. A precedent exists here in that the Tc^R determinants of plasmid RP1 and transposon Tn1721 (TetA), transposon Tn10 (TetB) and plasmid pSC101 (TetC) were not found to be related by this technique. However, DNA sequencing revealed considerable homology among these Tc^R determinants (Foster, 1983). Divergence over evolutionary time could lead to fine-tuning of these determinants to better fit them to their environmental niches. The same may have resulted in the divergence of cml and the R55-1 Cm^R determinant.

The model for the cml Cm^R mechanism was originally proposed as being permeability barrier to drug uptake (Gaffney *et al.*, 1981). Several pieces of indirect evidence from this thesis support this model. It was found that increasing the copy number of cml within the cell by forty times (as happened when the determinant was cloned in pBR322) did not lead to an increase in Cm^R above the fully-induced R26 value. This implies that there is an upper limit to the resistance level which this system can confer. The fact that resistance was constitutively expressed by pBR322 Cm^R plasmids implies that this limit is imposed by the host. In a membrane-located permeability barrier, there may be a limited number of membrane sites for the cml product. These sites can become saturated by R26-encoded product in the induced state, meaning that increasing the gene dosage level will not result in increased resistance. Gaffney *et al.*, (1981) showed that when R26 was accompanied by intracellular CAT specified by a second plasmid, no acetylation of Cm occurred, presumably because Cm did not reach the cytoplasm. In this work it was shown that when the cml-lac gene fusion plasmid pDU1294, was accompanied by pDU1249

(Cm^r) the ratio of induced/uninduced β -galactosidase activity declined from 5 to 3, supporting the permeability barrier model. It is also supported by the finding that the level of ³⁵S-labelled Cm-inducible polypeptide specified by pDU1255 (pBR322 Δ bla cml⁺) was the same in the induced and uninduced states. This suggested that the constitutively expressed cml prevented Cm from entering the cell to induce further production of the protein. Finally, the rather tenuous result of the crude minicell fractionation experiment described in Chapter 6 indicated an envelope location for the cml product, a finding in keeping with the resistance mechanism model.

This study has shed light on a somewhat enigmatic drug resistance determinant. The DNA sequence of the gene and its regulatory region is now known, as is the inferred amino acid sequence of the gene product. The level at which the gene is regulated has been elucidated and a model for this regulation has been assembled. A cellular location for the gene product has been proposed, supporting the original model for the resistance mechanism. Future work might involve the use of site-directed mutagenesis to generate mutations in the regulatory functions of the gene, or the generation of mutations which interfere with the localisation of the protein within the cell or its ability to confer resistance to Cm. In addition, protein-chloramphenicol binding studies would give new information on the resistance mechanism. The work described in this thesis has given an example of the power of modern molecular biological techniques to solve problems in biology.

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