STUDIES ON STAPHYLOCOCCAL CHLORAMPHENICOL-RESISTANCE PLASMIDS.

S.E. SKINNER - 1984

Six staphylococcal chloramphenicol-resistance (Cm^R) plasmids were compared by incompatibility tests and restriction digest analysis: pCW8 was indistinguishable from pC194; pCW6 was very similar to pUB112. While interruption of the HindIII site of pCW6 failed to prevent the inducible synthesis of chloramphenicol acetyltransferase (CAT), the BstEII site was shown to lie in or near the Cm^R determinant. Four of the plasmids were compared by electronmicroscopy of heteroduplexes between derivatives of them. A 2 kilobase-pair (kb) region of homology which pCW6 and pCW7 shared with pC221 coincided with pCW41, a 2 kb derivative of pC221 carrying the functions for inducible synthesis of CAT and replication, maintenance and incompatibility in Staphylococcus aureus. While neither pC221 nor pCW6 showed any homology with pCW8, 0.28 kb of pCW7 and pCW8 hybridised. Detailed restriction maps were constructed for pC221 and pCW7 by Smith-Birnstiel mapping and double digests. These results are discussed in terms of the evolution of staphylococcal Cm^R plasmids.

The organisation of the Cm^R determinant of pC221 was studied by induction experiments using S.aureus carrying derivatives of pC221, including pCW41. Interruption of the HpaII site failed to prevent the inducible synthesis of CAT; however, interruption of the BstEII site abolished detectable CAT synthesis. The induction of CAT was prevented by rifampicin. A small increase in cat RNA was observed on induction. Like the 3-deoxy analogue of Cm, 3-fluorochloramphenicol could be used as an inducer of CAT. Levels of expresson of the cat gene of pC221 in Escherichia coli were low and induction was generally poor unless transcription from an adjacent pBR322 promoter was possible. Sequences in pC221 could act as promoters for the tetracycline-resistance determinant of pBR322. These results are discussed in relation to various models for the regulation of Gram-positive cat genes.

STUDIES ON STAPHYLOCOCCAL CHLORAMPHENICOL-RESISTANCE PLASMIDS

by

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PREFACE

Except where explicitly stated, this thesis is based on experiments conducted by the author in the Department of Biochemistry of the University of Leicester mainly during the period January 1978 to July 1983. None of this work has been submitted for another degree in this or any other University.

Sarah E. Skinner. 9 May 1984

Sarah E. Skinner

Some of this work has already been published:-

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CHAPTER 3 : COMPARISON OF STAPHYLOCOCCAL Cm^R PLASMIDS

CHAPTER 4 : HETERODUPLEXES OF Cm^R PLASMIDS

ABBREVIATIONS

b	:base(s)
bp	:base-pair(s)
kb	:kilobase-pairs(s)
S	:Svedberg unit (10^{-13} seconds)
DNA	:deoxyribonucleic acid
RNA	:ribonucleic acid
mRNA, rRNA, tRNA	:messenger, ribosomal, transfer RNA
DNase	:deoxyribonuclease
RNase	:ribonuclease
CCC	:covalently closed circular
OC	:open circular
SC	:supercoiled
ss	:single-stranded
ds	:double-stranded
cpm	:counts per minute
pfu	:plaque-forming units
rpm	:revolutions per minute
uv	:ultra-violet
CAT	:chloramphenicol acetyltransferase
BLA	:β-lactamase

cat	:gene encoding CAT
bla	:gene encoding BLA
tet	:gene specifying resistance to tetracycline
Cm	:chloramphenicol (D-*threo* unless otherwise stated); 1-*p*-nitrophenyl-2-dichloroacetamido-propan-1,3-diol
3-deoxyCm	:1-*p*-nitrophenyl-2-dichloroacetamido-propan-1-ol
3-fluoroCm	:1-*p*-nitrophenyl-2-dichloroacetamido-3-fluoro-propan-1-ol
Tc, Ap, Em, Sm	:tetracycline, ampicillin, erythromycin, streptomycin
Cm^R	:resistance or resistant to Cm
Tc^R	:resistance or resistant to Tc
A_x	:absorbance at x nm
A	:adenine, adenosine
ABM-	:aminobenzoxymethyl-
AcCoA	:acetyl Coenzyme A
APT-	:aminophenylthioether-
ATP etc.	:adenosine triphosphate etc.
BHI	:Brain-heart infusion
BME	:2-mercaptoethanol
BRL	:Bethesda Research Labs
BSA	:bovine serum albumin

C	:cytidine, cytosine
CoA	:Coenzyme A
dATP etc.	:deoxyadenosine triphosphate etc.
DBM-	:diazobenzoxymethyl-
DPT-	:diazophenylthioether-
DTNB	:5,5',dithiobis(2-nitrobenzoic acid)
DTT	:dithiothreitol
EDTA	:diaminoethane tetra-acetic acid (disodium salt)
EthBr	:ethidium bromide
G	:guanidine, guanosine
L	:Luria (broth) (see section 2.2.1, p. 21)
LCm10	:L medium containing 10 μg Cm/ml
MIC	:minimal inhibitory concentration
NBM-	:nitrobenzoxymethyl-
NC	:nitrocellulose
NEB	:New England Biolabs
PAGE	:polyacrylamide gel electrophoresis
PVP 360	:polyvinylpyrrolidine of average molecular weight 360,000
SDS	:sodium dodecyl sulphate

T	:thymine, thymidine
TAE	:see Chapter 2, section 2.6.1, p. 31
TBE	:see Chapter 2, section 2.6.1, p. 31
TE	:see section 2.8.6, p. 37
Tris	:tris(hydroxymethyl)aminomethane
TSB	:Trypticase soy broth
TSBTc10	:TSB containing 10 μg Tc/ml
U	:uracil, uridine or unit(s)

All temperatures are in ^{o}C.

1 bp has been assumed to be equivalent to 0.67 kD.

pC221/EcoRI indicates pC221 digested with EcoRI.

CHAPTER 1

GENERAL INTRODUCTION

1.1. Resistance to Chloramphenicol

1.1.1 Bacterial resistance to antibiotics.

The strategies which could in theory be adopted by a bacterium to avoid inhibition or killing by an antibiotic were discussed by Davis and Maas (1952). Most of their predictions have been fulfilled (Benveniste and Davies, 1973; Davies and Smith, 1978). To summarise, mechanisms of resistance to antibiotics have fallen into the following categories:

1) modification of the target site so that the binding of the antibiotic is severely reduced;
2) decreased entry or increased efflux of the antibiotic;
3) inactivation of the drug by modification or degradation;
4) replacement of a sensitive enzyme or use of a different pathway.

Mutations in chromosomal genes can cause resistance to antibiotics: for instance, one-step mutants possessing an altered ribosomal protein, S12, are resistant to streptomycin (Ozaki et al., 1969). Similarly, permeability mutants can be isolated: these may simultaneously acquire reduced sensitivity to antibiotics other than that used for the initial selection (Reeve, 1966; Foster, 1975).

It has become apparent, however, that a bacterium may also acquire resistance by receiving the genetic determinant for the resistance-mechanism from another bacterium by conjugal transfer of extrachromosomal DNA. During the rapid increase in antibiotic-resistant bacteria following the introduction of antibiotics into clinical use, transmissible resistance-factors

(R-factors) were rapidly disseminated by conjugation between and among different genera of Gram-negative enteric bacteria, and, as these R-factors could carry determinants for resistance to five or six antibiotics simultaneously they became a major problem in the treatment of infectious diseases (Watanabe, 1963).

A similar phenomenon was observed among Gram-positive bacteria e.g. staphylococci: in this case, transduction by phage seemed to be the most important means of transmission of resistance genes (Lacey, 1973 and 1975).

1.1.2. Chloramphenicol and resistance to it.

The structure of chloramphenicol (Cm) is shown in fig. 1.1. Of the four possible stereoisomers (C_1 and C_2 are chiral) only the D, threo configuration is synthesised by Streptomyces venezuelae (Rebstock et al., 1949) and only this form shows potent antibacterial activity (Maxwell and Nickel, 1954). Cm inhibits the peptidyltransferase activity of 70S ribosomes (reviewed by Gale et al., 1981). Analogues of Cm with electronegative groups at the para position of the aromatic ring retain antibacterial activity: the dichloroacetamido group on C_2 can also be altered to some extent, though not removed altogether. Little alteration can be tolerated in the propandiol structure, however: substitution of the protons of the hydroxyl groups abolishes antibiotic activity (Pongs, 1979; Gale et al., 1981; section 1.1.3). If the C_3 hydroxyl group is replaced by fluorine antibacterial activity is retained, however (Hahn, 1980; Syriopoulou et al., 1981). Nuclear magnetic resonance studies by Tritton (1979) also suggested that this part of the molecule

is most important in binding to the ribosome.

Cm^R mutants of yeast, whose mitochondrial ribosomes resemble those of prokaryotes, have been reported: these have single base-changes in their 21S rRNA (Dujon, 1980).

Some R-factors of Gram-negative species and Pseudomonas transposons confer Cm^R by a mechanism inducible by Cm which probably involves decreased permeability of the cell envelope to Cm. (Gaffney et al., 1978 and 1981; Iyobe et al., 1981). Chromosomal cmlB mutants of Escherichia coli were found to be deficient in a membrane protein, Ia (Chopra and Eccles, 1978).

Some soil bacteria, e. g. Flavobacter spp., can degrade Cm (Süssmuth et al., 1979). Clostridium acetobutylicum can reduce the aryl nitro group, which effectively inactivates Cm (O'Brien and Morris, 1971). The mechanism of Cm^R mostly found in clinical isolates, however, and that conferred by the plasmids which are the subject of this work, is chloramphenicol acetyltransferase (CAT).

1.1.3. CAT as a mechanism of resistance to chloramphenicol.

Miyamura (1964) found that almost all of 110 clinical isolates of Cm^R Shigella, Escherichia and Staphylococcus, when incubated with Cm, inactivated most of it. Strains which acquired the Cm^R determinant during mixed culture were also able to inactivate Cm, and treatment with acriflavine caused simultaneous loss of the transferred Cm^R determinant and the ability to inactivate Cm. Okamoto and Suzuki (1965) showed that crude extracts of E.coli carrying an R-factor could inactivate Cm in vitro if supplied with acetate, ATP and Coenzyme A to generate

acetylCoA. Shaw (1967) and Suzuki and Okamoto (1967) showed that this was due to acetylation of Cm on the C_3 hydroxyl (see figs. 1.1 and 1.2). The 3-acetoxyCm is in (non-enzymic) equilibrium with 1-acetoxyCm, which can be further acetylated (by CAT) to give 1,3-diacetoxyCm. Acetylation of Cm was found to lead to a large decrease in inhibitory activity: the concentration required for 50% inhibition of the growth rate of E.coli K10 rose from 3 μM for Cm to 190 μM for 3-acetoxyCm and greater than 500 μM for 1,3-diacetoxyCm (cited by Shaw, 1967). In vitro, ribosomes from E.coli bound twenty-fold less 3-acetoxyCm than Cm when incubated with 3 μM of either (Shaw and Unowsky, 1968). Corresponding reductions in the inhibition of protein synthesis in vitro have been observed (Piffaretti et al., 1970; Piffaretti and Froment, 1978).

1.1.4. Survey of cat genes.

The cat genes of Gram-negative, enteric bacteria such as Klebsiella and Shigella are found on R-factors and have usually been studied after conjugative transfer to laboratory strains of E.coli. CAT is produced constitutively by strains carrying these plasmids and can be classified as one of three types (I, II or III) by a variety of physical and biochemical criteria (Foster and Shaw, 1973; Zaidenzaig and Shaw, 1976). The synthesis of type I CAT is subject to catabolite repression (see section 1.4.2). This type of CAT is encoded by the transposon Tn9 in which the cat gene is flanked by direct repeats of the insertion sequence IS1 (Alton and Vapnek, 1979). Perhaps because of this, type I CAT is the most prevalent among Gram-

negative enteric bacteria (Shaw, 1983).

The CAT of Proteus mirabilis is similar to, but not identical with, type I (Zaidenzaig et al., 1979): those of Haemophilus influenzae and parainfluenzae are similar to type II (Shaw et al., 1978; Roberts et al., 1982). The cat gene of Agrobacterium tumefaciens is atypical of those of Gram-negative bacteria in that its expression is inducible by Cm or its 3-deoxy analogue (Shaw, 1974) and encodes a CAT different from types I, II and III (Zaidenzaig et al., 1979).

Synthesis of CAT by Cm^R Gram-positive bacteria is almost always inducible by Cm or 3-deoxyCm (see section 1.3.1). The notable exceptions are Streptomyces spp. (Shaw and Hopwood, 1976). The cat gene of Streptomyces acrimycini is chromosomal (Wright and Hopwood, 1977). The cat gene of Bacillus pumilus NCIB 8600 was also apparently chromosomal, and inducible by Cm after molecular cloning onto a multi-copy plasmid in Bacillus subtilis (Williams et al., 1981). Streptococcal cat genes are also inducible (Miyamura et al., 1977) and either plasmid-borne (Horodniceanu et al., 1976; Dang Van et al., 1978; Courvalin et al., 1978) or chromosomal (Shoemaker et al., 1979). In the latter case they can still be transferred horizontally among pneumococci (Shoemaker et al., 1980). In clinical isolates of Staphylococcus aureus, cat genes have been found only as solitary resistance genes on generally small (<5 kb) plasmids, which will be described in section 1.3.2.

1.2 Replication, maintenance and incompatibility of plasmids.

1.2.1 Replication

Replication of plasmids has been studied most extensively in E.coli. Functions involved in the replication of the host chromosome are necessary for plasmid replication, the usual exception being the dnaA gene which codes for a factor required for the initiation of chromosomal replication. Replication proceeds uni- or bidirectionally from the origin, generating thetoid replication intermediates (Kornberg, 1980). Different groups of plasmids have different requirements for host functions: pSC101 requires the dnaA function for replication (Hasunuma and Sekiguchi, 1977; Felton and Wright, 1979) while ColE1 and similar plasmids require DNA polymerase I (Kingsbury and Helinski, 1973).

Similarly, in S.aureus certain host mutations are specific for the replication of plasmids of only one incompatibility (Inc) group (Novick, 1974; Iordanescu, 1983). Shivakumar and Dubnau (1978) showed that pUB110, a staphylococcal plasmid conferring neomycin-resistance (Inc13), required functional host genes dnaF, dnaG and dnaH but not polymerase I activity for replication in B.subtilis. Replication proceeded unidirectionally generating thetoid replication intermediates (Scheer-Abramowitz et al., 1981).

Most plasmids also require plasmid-encoded proteins for replication. Replication of R1 in vitro has been shown to require plasmid-directed protein synthesis (Diaz et al., 1981). Primase proteins can often suppress host mutations; for instance, R64drd11 primase substitutes for the dnaG protein in the

replication of phage G4 *in vitro* (Lanka *et al.*, 1979). In *S.aureus*, *trans*-acting replication functions have been reported for plasmids of Inc groups 1, 2, 3, and 8 (Wyman and Novick, 1974; Iordanescu, 1979; Iordanescu and Surdeanu, 1983). Replication of the Inc3 plasmid pT181 both *in vivo* and *in vitro* requires a functional RepC protein (Iordanescu, 1979; Khan *et al.*, 1981).

Conversely, the replication of ColE1 and similar plasmids in *E.coli* can continue for several hours after the addition of Cm has abolished chromosomal replication (Clewell, 1972): this is referred to as "amplification". Neither *in vivo* (Donoghue and Sharp, 1978; Kahn and Helinski, 1978) nor *in vitro* (Tomizawa *et al.*, 1975) was plasmid-directed protein-synthesis necessary for the replication of ColE1.

There is some evidence that replicating plasmids associate specifically with membranes in *B.subtilis* (Winston and Sueoka 1980a, b; Korn *et al.*, 1983) and Novick *et al.* (1980) observed significant curing of certain plasmids during the regeneration of the cell wall by protoplasts of *S.aureus*. Whether these observations are related to replication itself or some other maintenance function is as yet unclear.

1.2.2. Control of copy-number.

Jacob *et al.* (1963) and Pritchard and his colleagues (Pritchard, 1969; Pritchard *et al.*, 1969) discussed control of the replication of F-factors in their models of control of chromosomal replication. While Jacob *et al.* suggested that binding-sites on the membrane were necessary for replication and equipartition of replicas, Pritchard and his colleagues proposed that

dilution of an inhibitor, whose synthesis was linked to initiation of replication of the plasmid, was necessary to allow fresh initiation of replication to occur.

Nordström and co-workers (Nordström, 1971; Nordström et al., 1972) showed that the copy-number of an R-factor of E.coli could be altered by mutations on the plasmid. For the systems studied, control of copy-number was generally negative, that is, in accord with the predictions of Pritchard's model (Nordström, 1983). Cabello et al. (1976) showed that the copy-number of a pSC101/ ColE1 hybrid was characteristic of the replicon being used (normally ColE1) rather than the sum of the parental copy-numbers (which would be predicted by Jacob's model).

For ColE1, formation of the primer for initiation of DNA synthesis is regulated: binding of a small RNA (RNA I, 108 b) to the preprimer RNA prevents RNase H from processing the latter RNA into primer (Itoh and Tomizawa, 1980). For the IncFII plasmid, R1, synthesis of the replication protein, RepA, is controlled both by CopB (an 11 kD basic protein), which represses transcription of repA, and CopA (a 90 b RNA), which prevents efficient translation of the repA transcript (Light and Molin, 1983). Where plasmid-encoded proteins are necessary for replication their availability may not always be the limiting factor, however: Shafferman et al. (1982) showed that although π regulates its own expression, the copy number of R6K is not altered by changes in the concentration of π.

Sequences of about 20bp, repeated directly six or seven times, are implicated in the regulation of copy-number of some

plasmids, e.g. R6K (Kolter and Helinski, 1982). Trans-acting replication functions may act on direct repeats upstream and downstream of the promoter for the putative primer for initiation of replication of RK2 (Stalker et al., 1981; Thomas, 1981). Direct repeats are also seen at a cop locus of F-factor (Seelke et al., 1982.)

1.2.3. Distribution of plasmid copies at cell division.

Partition loci have been reported for several systems in E.coli. Hogan et al. (1982) found that mutations at the IncD/par locus affected the segregation of F-factor into mini-cells. Mutations in the parA and parB functions of ClODF13 (a colicinogenic plasmid) caused the accumulation of multimers if the plasmids were simultaneously high copy-number mutants. The cis-acting par site of pSC101 accomplished active distribution of plasmid DNA to daughter cells (Meacock and Cohen, 1980). Nordström et al. (1980) reported a par locus distinct from cop, mapping away from the origin and "basic replicon" functions.

A likely cellular location for partitioning sites would appear to be the cell membrane. The observation of Novick et al. (1980) that certain plasmids were cured during the regeneration of cell walls by staphylococcal protoplasts (referred to in 1.2.1) might be related to this.

1.2.4 Incompatibility of plasmids.

When two different plasmids cannot co-exist stably in the same host cell in the absence of continued selection pressure for both they are said to be incompatible (Novick et al., 1976). Incompatibility is expressed by plasmids sharing the same

replication control or maintenance systems. For systems whose incompatibility has been studied in detail often several Inc loci have been identified: for example, incompatibility among F replicons is determined by IncB, associated with a secondary origin of replication, and IncC which maps with mutations in a copy-number control locus and a site determining the sensitivity of replication of F to acridine orange (reviewed by Lane, 1981). Direct repeats appear to be associated with the IncC function (Tolun and Helinski, 1981). IncD, which determines incompatibility with other plasmids of the IncFI group, is associated with par, which affects segregation (Hogan et al., 1982, see 1.2.3). For pSC101, however, mutations in par do not appear to affect incompatibility (Meacock and Cohen, 1980).

Cowan and Scott (1981) showed that the segregation of homologous P1 plasmids (IncY; low copy-number) was due to the lack of replication of either, then separation at cell division, while incompatibility among heterologous plasmids of IncY was due to the preferential replication of one plasmid then equal distribution of replicas to the daughter cells.

For ColE1 single base-changes at seven bases in the region coding for RNA I and the pre-primer (see 1.2.2) can affect both the inhibitory activity of RNA I on primer formation and the sensitivity of primer formation to RNA I action. This leads to changes in copy-number and incompatibility (Tomizawa and Itoh, 1981; Lacatena and Cesareni, 1981 and 1983).

1.3 Antibiotic-resistance plasmids

1.3.1 Evolution of antibiotic-resistance plasmids.

Evolution of plasmids could occur not only at the level of base-changes in the DNA sequence (micro-evolution) but also by translocation of segments of DNA (macro-evolution) (Cohen, 1976). The segmental composition of large plasmids has been attributed to multiple translocations of DNA segments and recombinations at hotspots (Kopecko *et al.*, 1976). In addition to the contributions made by transposon-borne antibiotic-resistance determinants, transposable DNA elements (reviewed by Starlinger, 1980) can have positive as well as negative effects on gene expression in *cis*, and imprecise excision can cause novel juxtapositions of DNA sequences. Deletion events can involve repeated sequences of six or seven base-pairs upwards (Albertini *et al.*, 1982; Jones *et al.*, 1982).

The segmental nature of large plasmids has been observed in numerous hybridisation studies on R-factors of Gram-negative bacteria. For example, it has been shown by electronmicroscopy and Southern blots (Southern, 1975) that IncFI plasmids (F-factor and similar plasmids) are homologous for the region containing the genes for autonomous replication, though not the vegetative origin (Palchaudhuri and Maas, 1977) while they share homologies in the transfer function region with IncFII plasmids (e.g. R6-5) (Sharp *et al.*, 1973; Timmis *et al.*, 1981). Similarly, the IncW plasmids share a 13 MD region containing both transfer and replication functions while the antibiotic-resistance regions differ (Gorai *et al.*, 1979; Ward and Grinsted, 1982). Thus, these

plasmids could be considered as backbones bearing common replication and/or transfer functions in and out of which antibiotic-resistance determinants move. Hughes and Datta (1983) observed that, while isolates of Enterobacteriaceae stored before most antibiotics came into use contained conjugative and non-conjugative plasmids, the incidence of antibiotic-resistance was low. Presumably as antibiotic therapy became more widespread loss of resistance-determinants from the plasmid backbone became more disadvantageous.

Antibiotic-resistance genes appear to be shared by different plasmids within and between genera, for instance, the cat genes of Gram-negative R-factors (Gaffney et al., 1978, Shaw, 1983: section 1.1.4). Among Gram-positive cocci Em^R determinants are sufficiently similar to hybridise in Southern blot experiments (Weisblum et al., 1979). It is possible that some plasmid-borne antibiotic-resistance determinants originated in the producing organisms while others might be spontaneously mutated chromosomal genes which had become translocated onto a plasmid.

Plasmid evolution has been followed during many epidemiological studies: for example, in Gram-negative enteric bacteria, transposition between co-existing plasmids in Serratia marcescens (Rubens et al., 1979), the spread and gradual change of Cm^R plasmids in pathogenic E.coli infecting Danish piglets (Jørgenson et al., 1980), and sequential acquisitions and deletions of transposons in Inc7-M plasmids conferring resistance to gentamicin (Labigne-Roussell et al., 1982). Thus, it has been possible (and realistic) to propose schemes for the evolutionary

relationships of plasmids (e.g. for the "R5" plasmids originating in Shigella sonnei, (Haas and Davies, 1980).

This has also been done for the penicillinase-encoding plasmids of S.aureus Inc groups 1 and 2. Shalita et al. (1980) were able to divide almost all the plasmids into four families, on the basis of restriction endonuclease digests and electronmicroscopy of heteroduplexes, and to postulate their evolution through insertion and deletion events. Iordanescu et al. (1978) have proposed that modular evolution also occurs for the smaller staphylococcal plasmids, from the results of hybridisation and incompatibility studies. Polak and Novick (1982) found that Tc^R plasmids from soil bacilli shared little homology with a staphylococcal Tc^R plasmid: instead, they shared homology and were incompatible in B.subtilis with a staphylococcal neomycin-resistance plasmid, pUB110 (Incl3), suggesting that their replication and maintenance functions came from a common source.

1.3.2 Staphylococcal Cm^R plasmids.

Plasmids have been found in many species of Staphylococcus; in S.epidermidis (e.g. by Baldwin et al., 1969; Minshew and Rosenblum, 1973; Rosendorf and Kayser, 1974), S.simulans (Keller et al., 1983), and S.xylosus (Götz et al., 1983); but have mainly been isolated from and studied in S.aureus, where they can be divided into at least thirteen incompatibility groups (Novick and Richmond, 1965; Ruby and Novick, 1975; Iordanescu et al., 1978; Iordanescu and Surdeanu, 1980). As isolated from S.aureus they tend either to be large (about 20 MD) and to carry resistance-determinants to penicillin, metal ions

and sometimes Em, or to be small (about 3 MD) and to carry solitary resistance-markers. The Cm^R plasmids are in the latter group.

Evidence for the plasmid-linkage of Cm^R determinants in S.aureus came from the "curing" studies of Chabbert et al. (1964). Novick's studies (1976) on the relaxation complexes of small staphylococcal plasmids included four Cm^R plasmids (pC221, pUB112, pC223, pC194). Ehrlich (1977) successfully transformed B.subtilis with these and other small staphylococcal plasmids, opening the way for their use in the development of vehicles for molecular cloning in B.subtilis (e.g. Ehrlich, 1978; Gryczan and Dubnau, 1978; Keggins et al., 1978). Iordanescu et al. (1978) found that the four Cm^R plasmids were mutually compatible and that each of them exhibited a different degree of homology with pC221, and also with staphylococcal Sm^R and Tc^R plasmids. Wilson and Baldwin (1978) used a different set of Cm^R plasmids; in addition to pC221 they used pCW6, pCW7 and pCW8 (isolated in different parts of the U.S.A.) and constructed preliminary restriction maps for them.

In 1974, Chang and Cohen showed that a staphylococcal penicillinase gene could be expressed in E.coli. Some staphylococcal plasmids (including pC194 and pUB112, but not pC221) have been shown to replicate in E.coli (Goze and Ehrlich, 1980). Cm^R plasmids (pC221, pUB112, pC223 and pC194) replicated autonomously in yeast, although pUB110, a neomycin-resistance plasmid, did not (Goursot et al., 1982). The complete nucleotide sequence of pC194 has been determined by Horinouchi and Weisblum (1982).

1.4 Regulation of Gram-positive *cat* genes

1.4.1 Regulation of bacterial gene expression.

Regulation of gene expression can, in principle, take place at either the transcriptional or translational level (leaving aside processing mechanisms). In their operon model Jacob and Monod (1961) suggested that regulation of the lactose-utilisation system occurred at the level of transcription of genetic information into an unstable messenger RNA. Since then extensive research has confirmed that initiation of transcription of the three structural genes of the *lac* operon (which are regulated co-ordinately) is inhibited by the tight binding ($K_d = 10^{-13}$ M under the conditions used by Riggs *et al.*, 1970b) of a repressor protein to an operator site on the DNA near the promoter for the *lac* operon (Gilbert and Müller-Hill, 1966 and 1967; de Crombugghe *et al.*, 1971). If an inducer, e. g. allolactose, binds to the repressor its affinity for the operator decreases (the dissociation rate increases: Riggs *et al.*, 1970a) and transcription can proceed.

The initiation of transcription can also be stimulated by the binding of activator proteins. For example, the *araC* protein in its activator form stimulates the initiation of transcription from the P_{BAD} and P_{FG} promoters in the arabinose-utilisation operon of *E.coli* (Lee *et al.*, 1981; Kosiba and Schlief, 1982). Again, when cyclic AMP levels are high in response to glucose starvation, catabolite activator protein (CAP) stimulates initiation of transcription of operons for the utilisation of various sugars, e. g. galactose (Nissley *et al.*, 1971), as well as

the gene for type I CAT (Harwood and Smith, 1971; de Crombugghe et al., 1973; Le Grice and Matzura, 1980). Both of these regulatory proteins can also act negatively; araC protein at its own promoter (Lee et al., 1981), and CAP at the P_2 promoter of the gal operon (Musso et al., 1977). Apparently, both compete directly with RNA polymerase for binding to the promoters.

Termination of transcription can also be important in the regulation of gene expression. It was noticed that the expression of the tryptophan-biosynthesis (trp) operon in E.coli was controlled not only by a repressor of transcriptional initiation (Zubay et al., 1972) but also by attenuation; that is, termination of transcription before the start of the structural genes (Morse and Morse, 1976; Bertrand et al., 1976). Attenuation also occurs in biosynthetic operons for other amino-acids in E.coli, and in S.marcescens and Salmonella typhimurium (Kolter and Yanofsky, 1982). The tight coupling of transcription and translation which occurs in prokaryotes is thought to be obligatory for optimal regulation by attenuation (Platt, 1981).

The structure of the 5'-terminal portion of trp RNA is shown schematically in fig. 1.3. When tryptophan is scarce ribosomes translating the leader RNA stall at the repeated codons for tryptophan due to a shortage of tryptophanyl-tRNA (Bertrand and Yanofsky, 1976). As sequence 1 is then no longer able to base-pair with sequence 2, the latter can now base-pair with sequence 3, pre-empting the formation of the terminator structure (3:4) (Farnham and Platt, 1980; Stroynowski et al., 1983). Transcription can now proceed through the structural genes, generating

full-length *trp* RNA.

The structure of the 3'-terminus of RNA can also be important; for instance, the mRNA of the *int* gene of bacteriophage λ is only a substrate for RNase III if transcription has proceeded through the terminator, t_I, under the influence of the N protein of λ and the products of the host genes, *nusA* and *nusB* (Luk *et al.*, 1982). Whether or not transcription is sensitive to anti-termination depends on whether it initiated at P_L and proceeded through the *nutL* site (Salstrom and Szybalski, 1978; Drahos and Szybalski, 1981) or at P_I which is downstream of this. Cleavage of the anti-terminated transcript by RNase III removes the terminator structure from the mRNA, which is then susceptible to degradation. This suggests that terminator structures may play a general role in the stabilising of mRNA (Rosenberg and Schmeissner, 1982).

The secondary structure of the mRNA is also important in the regulation of the Em^R genes of Gram-positive bacteria. Gryczan *et al.* (1980) and Horinouchi and Weisblum (1980) proposed similar models for the regulation of the Em^R gene of staphylococcal plasmid, pE194. Briefly, a stable mRNA (Shivakumar *et al.*, 1980) is transcribed and is in equilibrium between several stable secondary conformations (fig. 1.4). In the absence of Em the preferred conformation is that in which the ribosomal binding-site (Shine and Dalgarno, 1974, 1975) for the structural gene is buried in a ds RNA stem. Stalling of ribosomes inhibited by Em in the leader RNA favours the alternative conformation in which the Shine-Dalgarno sequence is available for the initiation

of translation of the Em^R methylase. As the different secondary structures of the mRNA and the role of ribosomes are reminiscent of those involved in transcriptional termination (see above and fig. 1.3), this mechanism has been termed "translational attenuation" by Horinouchi and Weisblum (1981). Interestingly, the DNA sequence of the Tc^R gene of a staphylococcal plasmid, pT181, suggests that a similar mechanism may regulate expression of Tc^R in S.aureus (Khan and Novick, 1983).

1.4.2 The induction of CAT.

Dunsmoor and his colleagues (1964) observed that a culture of Cm^R S.aureus could be "pre-adapted" to grow in the presence of Cm by pre-incubation of the culture with Cm. The presence of significant levels of CAT activity in extracts of Cm^R S.aureus was also shown to depend on pre-incubation with Cm (Suzuki et al., 1966) or certain close structural analogues (Shaw and Brodsky, 1968b; Shaw and Winshell, 1969). The specific activity of CAT, which is present at low levels in uninduced cultures, increased by as much as one hundred-fold on induction by Cm; however, as the concentration of Cm was lowered by acetylation, and the acetylated products were inactive as inducers, the kinetics of induction by Cm were complicated (Shaw and Brodsky, 1968b).

For more detailed studies of the kinetics of induction it was necessary to use the 3-deoxy analogue of Cm (fig. 1.1). As this is not a substrate for CAT (Shaw and Brodsky, 1968a) its concentration remains constant during the course of an induction experiment and the increase in the rate of CAT synthesis is sustained. Winshell and Shaw (1969) found that maximal induction

occurred when 5 to 10 μM 3-deoxyCm was used; 5 μM 3-deoxyCm inhibited growth by only 5%. A linear rate of CAT synthesis was observed within five minutes of adding the inducer. Removal of inducer (by washing the cells) caused a rapid decrease in the rate of synthesis of CAT. The addition of puromycin (50 μg/ml) prevented further synthesis of CAT in an induced culture, demonstrating that protein synthesis was required for induction. Genetic analysis of the regulatory mechanism was held back, however, by the failure to obtain mutants which produced CAT constitutively; this was attributed to the relatively high copy-number of the staphylococcal Cm^R plasmid (Shaw, 1974).

Fig. 1.1 Chloramphenicol

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

1·1

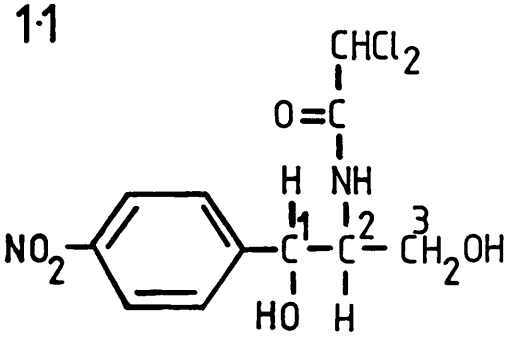


Fig. 1.2 The reactions catalysed by CAT

The equilibrium between 3-acetoxyCm and 1-acetoxyCm is non-enzymic. After Shaw (1983).

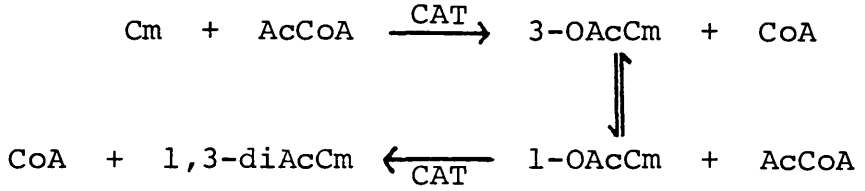


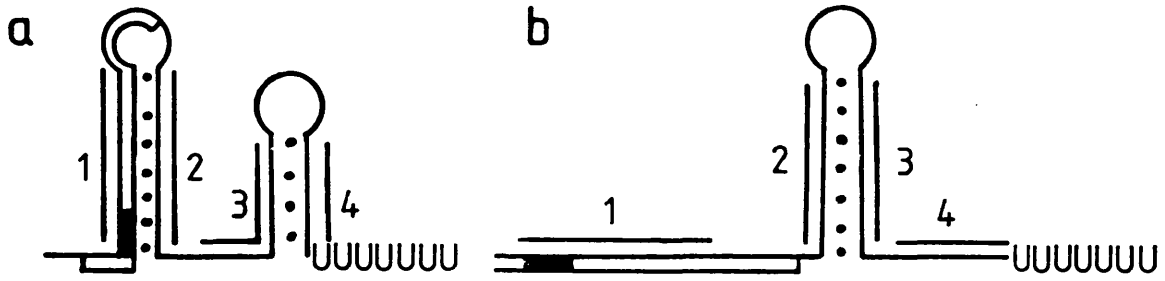
Fig. 1.3 Secondary structures of trp RNA

The boxed area indicates the coding-region for the leader-peptide, the shaded region the two codons for tryptophan. In the secondary structure shown on the left (a), sequences 3 and 4 have paired to form a transcriptional termination signal (after Kolter and Yanofsky, 1982).

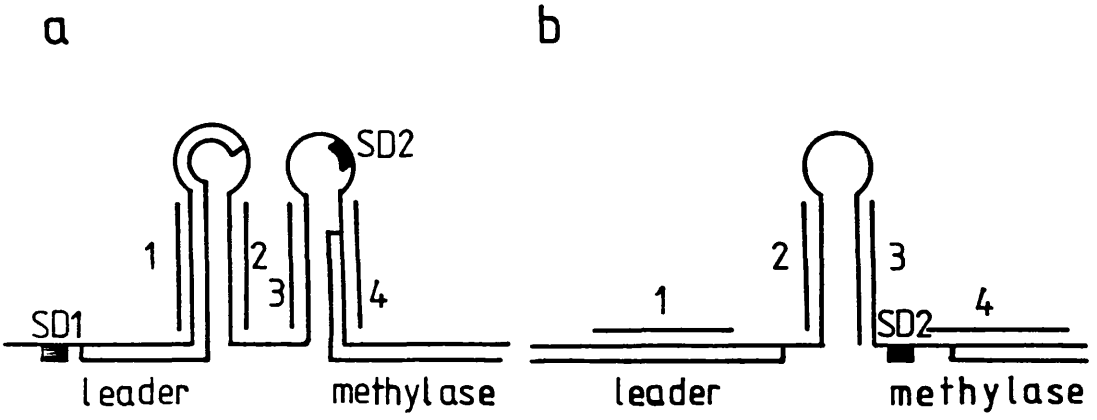
Fig. 1.4 Secondary structures for Em^R gene RNA

The boxed areas indicate the coding-regions for the leader-peptide and the Em^R methylase. SD-1 and SD-2 are the Shine-Dalgarno sequences for translation of the coding-regions. In the RNA conformation shown on the left (a), the ribosome binding site for the Em^R methylase would be inaccessible (after Horinouchi and Weisblum, 1981).

1·3


a
1
2
3
4
UUUUUUU
b
1
2
3
4
UUUUUUU

1·4


a
SD2
1
2
3
4
SD1
leader
methylase
b
1
2
3
4
SD2
leader
methylase

CHAPTER 2

MATERIALS AND GENERAL METHODS

2.1 Chemicals

Most reagents were purchased from BDH or Fisons Ltd. and, unless otherwise stated, were of analytical grade purity. Agarose was from Miles Labs, except for low melting-point agarose which was obtained from Bethesda Research Labs (BRL). Tris (Trizma) base, DTNB, lysostaphin, lysozyme, ethidium bromide, amino-acids, vitamin B1 and antibiotics were purchased from Sigma: spectinomycin was from Upjohn. Coenzyme A (lithium salt) was obtained from Boehringer Corporation Ltd. Brain-heart infusion (BHI) was from BBL or Oxoid; trypticase soy broth (TSB) was from BBL; Bacto-tryptone, yeast extract and Bacto-agar were from Difco. 3-fluoroCm was a gift from Dr. G. H. Miller, Schering-Plough Corporation, Bloomfield, New Jersey. 3-deoxyCm, unless otherwise stated, was of the D,*threo* configuration and prepared by Prof. W. V. Shaw or Dr. M. Webb according to the procedure of Rebstock *et al*. (1951): where stated, a preparation containing both D, and L,*threo*-3-deoxyCm (prepared by Dr. J. E. Fitton) was used.

2.2 Preparation of media

2.2.1 Nutrient media.

TSB was the routine nutrient medium for S.aureus. Luria (L) broth (the nutrient medium used for E.coli) contained: Bacto-tryptone, (10 g/l); yeast extract, (5 g/l); sodium chloride, (10 g/l). The pH was adjusted by the addition of 1.5 ml of 1 M sodium hydroxide per litre. For solid media, agar was added to 1.5 to 2.0% (w/v) before autoclaving. Media were sterilised at 15 lbs/in^2 for at least 15 min. For selective plates, sterile antibiotic solutions (2.2.3) were added after cooling the sterilised nutrient agar to 55^o.

2.2.2 M56 plates.

For one litre of M56 agar, 61.1 ml of 0.5 M disodium hydrogen phosphate, 19.3 ml of 1 M potassium dihydrogen phosphate, 0.5 ml of 10% (w/v) ammonium sulphate and 0.5 ml of 0.05% (w/v) ferrous sulphate were mixed, made up to 500 ml with distilled water, and sterilised by autoclaving. 500 ml of 4% (w/v) agar were also sterilised. After cooling the salt solution to 50^o the following additions were made aseptically: 1 ml of 10% (w/v) magnesium sulphate heptahydrate, 0.5 ml of 1% (w/v) calcium nitrate and 4 ml of 50% (w/v) D-glucose (all autoclaved separately), and, as required, L-amino-acids (to 50 μg/ml final concentration) and vitamin B1 (5 μg/ml). Amino-acid and vitamin B1 solutions had been sterilised by passage through a 0.45 μm pore-size cellulose nitrate filter (Sartorius). The sterilised agar was then mixed in thoroughly and the plates poured.

2.2.3 Antibiotics.

Solutions of antibiotics were sterilised by passage through a 0.45 μm pore-size cellulose nitrate filter (Sartorius). Tetracycline (up to 12.5 mg/ml) was dissolved in ethanol and the solution stored, protected from the light, at +4^{o} or -20^{o}. Chloramphenicol solutions were 5 mg/ml in 30% (v/v) aqueous ethanol or 10 mg/ml in absolute ethanol and stored at +4^{o}. Rifampicin was dissolved in 0.1 M hydrochloric acid before use. Ampicillin and spectinomycin were dissolved in water and used the same day (stored at 4^{o} until required).

2.3 Bacterial strains

2.3.1 Sources of strains.

Table 2.1 lists the strains of S.aureus and E.coli used in this work. WS 2001-2069 were obtained from C. R. Wilson; WS 2204 and 2206-9 were from R. P. Novick; WS 2301 was from A. R. Hawkins, GM48(pBR322) from A. C. Boyd. Propagating strain 55 was from the Central Public Health Labs, Colindale, London. Plasmids are described in the relevant chapters.

2.3.2 Storage of S.aureus.

For short-term storage S.aureus strains were transferred every one or two months on TSB plates containing appropriate selective antibiotics. For longer storage, selective TSB agar stabs and slopes, frozen (-70°) TSB cultures, or glycerol/phosphate stocks were made. The last were prepared by resuspending cultures grown overnight in selective TSB in 1 ml of 100 mM sodium phosphate buffer, (pH 7.0), and mixing in 1 ml of glycerol (both sterilised by autoclaving): 1 ml portions were stored at -20°.

2.3.3 Storage of E.coli.

Master plates were transferred at monthly intervals. Cultures were stored in selective L agar stabs, or at -70° after the addition of 10% (v/v) sterile glycerol and 5% (v/v) dimethyl sulphoxide to an overnight culture in L broth.

2.4 Measurement of CAT in cultures

2.4.1 Induction and measurement of CAT in cultures of S.aureus.

(Modified from Winshell and Shaw, 1969)

TSB (50 to 125 ml) was inoculated with 1 to 4 ml of an overnight culture and shaken at 37°: growth was followed by measurement of A_{600}. 3-deoxyCm or 3-fluoroCm was added at the concentrations indicated in individual experiments (usually 5 to 10 μM 3-deoxyCm) and incubation at 37° continued. The concentrations of the stock solutions were estimated from the A_{277}, assuming a molar extinction co-efficient of 9,700 $M^{-1}cm^{-1}$ (Rebstock et al., 1951.) When D,L,threo-3-deoxyCm was used the concentration was approximately doubled (Shaw and Winshell, 1969). Control (uninduced) cultures received a similar volume of ethanol.

Culture samples were harvested by centrifugation (10 min. at 9,000 rpm, 6 x 250 ml rotor or 15,000 rpm in the 8 x 50 ml or 6 x 100 ml rotor at 5° in the MSE 18, or 10 min. at 2,000 rpm in the MSE Minor at room temperature) and washed in 5 to 10 ml of Tris-HCl (50 mM, pH 7.8, or 10 mM, pH 8.0) The washing step was sometimes omitted when the sample size was 10 ml or less. In some experiments the washed pellets were held at -70° before proceeding to the lysis step.

The pellets were resuspended with 0.5 ml of 50 mM Tris-HCl, pH 7.8, containing 0.145 M sodium chloride. Lysostaphin (25 or 50 μg) was added to each sample and the suspensions incubated at 37° for 30 to 60 min., 20 or 50 μg DNase (100 or CL, Sigma) being added towards the end of the incubation to reduce viscosity. Cell debris was removed by 5 to 10 min. centrifugation in a

microfuge (approx. 11,000 x g). Supernatants were stored at -20° until assayed for CAT activity (2.4.3.) and protein concentration (2.4.2.).

2.4.2 Protein determinations.

The protein concentrations of cell extracts were determined essentially as described by Lowry et al. (1951). Copper sulphate solution (0.5 ml of 1% (w/v) $CuSO_4.5H_20$) was mixed with 0.5 ml of 2% (w/v) potassium-D-tartrate and made up to 50 ml with 2% (w/v) sodium carbonate in 0.1 M sodium hydroxide. This reagent (1 ml) was added to 0.2 ml of protein solution (diluted if necessary) and mixed thoroughly. After ten minutes at room temperature 0.1 ml Folin reagent (Folin and Ciocalteau's Reagent, BDH, diluted 1:2 with water immediately before use) was added and mixed thoroughly by vortexing. The A_{660} was read after 30 min. at room temperature and the protein concentration determined from a standard curve constructed using 0 to 400 μg/ml BSA (Sigma, fraction V). The standards also contained the same concentrations of Tris buffer and sodium chloride as the sample tubes.

2.4.3 CAT assays.

CAT activity was measured by the spectrophotometric assay of Shaw and Brodsky (1968b) using a Pye-Unicam SP1800 spectrophotometer with chart recorder. Assays were performed at 37°: 10 μl of cell extract (diluted with 50 mM Tris-HCl, pH 7.8, if necessary) were added to a 1 ml cuvette containing 0.96 ml of 1mM DTNB in 50 mM Tris-HCl, pH 7.8, and 0.01 ml of 10 mM AcCoA, pre-equilibrated at 37°. After 5 minutes at 37° to allow any background rate to decrease to zero or become quasi-linear, the

rate of increase in A_{412} was recorded. Chloramphenicol (20 μl of a 5 mM aqueous solution) was added and the rate of increase of A_{412} again recorded. CAT activity was taken to be proportional to the Cm-dependent rate of increase in A_{412}; i.e., the first rate ("background") subtracted from the second rate ("total").

One unit of CAT activity was defined as the activity which produced 1 μmol of CoA in 1 min., under standard conditions (above). CoA, released on acetylation of Cm, reacts with DTNB to form a mixed disulphide and the displaced thionitrobenzoate ion absorbs light at 412 nm. The molar extinction co-efficient was assumed to be 13,600 $M^{-1}cm^{-1}$ (Kredich and Tompkins, 1966).

2.4.4 Preparation of acetylCoA.

(Modified from Simon and Shemin, 1953).

CoA (100 mg) was dissolved in 7 ml of water, 1 ml of 1 M potassium bicarbonate was added, and the solution was chilled on ice. Acetic anhydride (16 μl) was added dropwise while vortexing the solution. After 10 min. on ice the concentration of free CoA remaining was assayed: 10 μl of reaction mixture were added to 1 ml of 1 mM DTNB in 50 mM Tris-HCl, pH 7.8. A change in A_{412} of less than 0.02 indicated that the amount of free CoA still present was negligible; if the change was 0.02 or greater, 5 or 10 μl of acetic anhydride were added as before (and the change in A_{412} on mixing with DTNB solution checked again). The concentration of AcCoA was determined by the addition of 10 μl to a 1 ml cuvette containing 1 mM DTNB in 50 mM Tris-HCl, pH 7.8, 500 μM Cm and sufficient pure CAT (usually type III* prepared by A. J. Corney or K. Kleanthous) to catalyse the

complete reaction in 2 min. The AcCoA concentration was deduced from the maximum change in A_{412}, and was normally about 12mM. This was diluted to 10 mM and stored in aliquots at -20^{o} until required.

2.5 Preparation of purified plasmid DNA

2.5.1 Preparation of a cleared lysate from S.aureus.

(After Stiffler et al., 1974).

One half of a ml of an overnight culture was used to inoculate 0.5 l of TSB (in a 2 l flask) containing an appropriate antibiotic (Cm, 20 μg/ml or Tc, 10 μg/ml). The flask was incubated with shaking at 37° for 16 to 18 hr. After being harvested by centrifugation (10 min. at 9,000 rpm in the 6 x 250 ml rotor, MSE 18, 5°) the cells were washed in 100 ml of 10 mM Tris-HCl, pH 8.0, and resuspended with 18 ml of 2.5 M sodium chloride, 0.05 M EDTA, pH 7.1. Lysostaphin (0.5 mg) was added and the suspension incubated with occasional gentle swirling at 37° for 10 to 15 min.: 27 ml of 1% (w/v) Brij 58, 1% (w/v) sodium deoxycholate (both from Sigma), in 0.04 M EDTA, pH 7.5, were then added, and, after gentle swirling, the preparation was left standing at room temperature for 20 min. Cell debris and most of the chromosomal DNA were removed by centrifugation (39,000 x g, 45 min., 5°).

2.5.2 Preparation of a cleared lysate from E.coli.

(Modified from Clewell and Helinski, 1970).

Five ml of an overnight culture were used to inoculate 0.5 l of L broth in a 2 l flask and shaken at 37° until the A_{600} reached 0.6. Spectinomycin solution (1 ml of 100 mg/ml in water) was added and incubation continued for at least 12 hr. Cm (200 μg/ml final concentration) was used instead of spectinomycin for Cm^S strains. The cells, harvested by centrifugation (10 min. at 10,000 rpm, Beckman JA-10 rotor, 5°) were resuspended in 20 ml

of 0.2 M Tris-HCl, pH 8.0, containing 25% (w/v) sucrose, and 30 mg of lysozyme were added. The suspension was incubated on ice for 5 min. with occasional gentle swirling before 6 ml of 0.2 M EDTA, pH 8.0 were added. After a further 20 min. incubation on ice, 10 ml of Brij reagent (1% (w/v) Brij 48, 0.4% (w/v) sodium deoxycholate, in 0.0625 M EDTA, 50 mM Tris-HCl, pH 8.0) were added dropwise at 20°. After 20 min. incubation on ice, cell debris and most of the chromosomal DNA were removed by centrifugation (60 min. at 39,000 x g, 5°). The supernatant was extracted once with an equal volume of phenol: chloroform: iso-amyl alcohol (25:24:1) and the phases separated by centrifugation (10 min. at 10,000 rpm, 6 x 250 ml rotor, MSE 18, 20°). After adjusting the sodium chloride concentration of the aqueous layers to 0.3 M, the nucleic acids were precipitated with 2 vols of 95% (v/v) ethanol and cooling for at least 4 hr. in a -70° freezer. The precipitate was recovered by centrifugation (30 min. at 10,000 rpm, 6 x 250 ml rotor, MSE 18, 0°) and resuspended in water before being adjusted to 50 mM Tris-HCl, 10 mM EDTA, pH 8.0.

2.5.3 Dye-buoyant equilibrium density-gradient centrifugation. (Radloff et al., 1967).

Ethidium bromide was added (to 300 μg/ml) to the lysates from procedure 2.5.1 or 2.5.2. Caesium chloride (Fisons AR or that reclaimed by Wykeham Field Services) was added to give a refractive index (at 20°) of 1.3920. The lysates were centrifuged either in a fixed-angle rotor, (Beckman Ti50 or Ti75, or MSE 10 x 10 Ti) at 36,000 to 40,000 rpm and 20° for at least

40 hr., or in a vertical rotor. If a vertical rotor was to be used for S.aureus lysates they were first centrifuged at 10,000 rpm (6 x 100 ml rotor, MSE 18) for 30 min. at 20° to allow the removal of a pellicle of membrane, lipids and proteins. The refractive index was checked, and re-adjusted if necessary. This extra centrifugation step was unnecessary for E.coli lysates. Lysates were then centrifuged in Quickseal tubes in the Beckman VTi50 rotor for 8 to 16 hr. (20°, 40,000 rpm): better resolution was obtained after 16 hr. than after 8 hr.

The lower (CCC plasmid) band was collected under long-wave uv illumination. If re-banding was necessary, the caesium chloride concentration was corrected (as above) before recentrifugation. The purified plasmid was extracted repeatedly with 0.2 vol iso-propyl alcohol (propan-2-ol) to remove ethidium bromide, until both layers were colourless, then dialysed against sterile TE (2.8.6) at 5° to remove caesium chloride and other low molecular-weight solutes (four or five changes of 1 l for at least three hours each) using dialysis tubing prepared as described (2.8.4).

It was found necessary to concentrate the S.aureus plasmid DNA by pervaporation or iso-butyl alcohol extractions and ethanol precipitation (2.8.3). The nucleic acid concentration was measured (2.8.1): The A_{260}/A_{280} ratio was usually not less than 1.8. Yields were 50 to 250 μg plasmid per litre of S.aureus culture, 200 to 1,000 μg per litre of E.coli. Purity (lack of chromosomal contamination and RNA content) was assessed by gel electrophoresis of samples.

2.6 DNA techniques

2.6.1 Agarose gel electrophoresis.

Vertical slab gels (14 cm wide x 10 cm high x 3 mm thick) were supported by plugs of 10% (w/v) Cyanogum 41 (gelling agent; Sigma or BDH) in electrophoresis buffer. Agarose concentrations varied from 0.7 to 2.4% (w/v) as appropriate, in electrophoresis buffer (TAE or TBE). 10 x TAE was 0.4 M Trizma base, 0.2 M sodium acetate, 0.02 M EDTA, pH adjusted to 8.2 with glacial acetic acid. 10 x TBE was 0.89 M Trizma base, 0.89 M boric acid, 0.02 M EDTA, pH 8.3 without further adjustment. TAE gels were usually run for 3 hr. at 60 V or overnight at 15 V: TBE gels were run at about 100 V for 2 hr. or 20 V overnight. (For exact conditions see individual experiments). For preparative purposes gels of similar dimensions were cast, except that wider wells were sometimes used to accommodate the larger amounts of DNA loaded. Low melting-point agarose was sometimes used for preparative gels.

Analytical gels were stained by immersion in 1 μg/ml ethidium bromide in electrophoresis buffer for at least 30 min. (20^{o}) and destained by soaking in water for 10 min. Gels were viewed using a short-wave uv transilluminator and photographed on Polaroid 55 or 57 film through an orange or yellow filter. Preparative gels were stained in a similar manner, protected from the light, and bands were excised under long-wave uv light.

2.6.2 Size-markers for linear DNA fragments.

Sizes for pBR322 digests were from Sutcliffe (1978); those for λ digests were from the BRL Catalogue and Reference

Guide* (from the nucleotide sequence of Sanger et al., 1982). Unknown sizes were obtained from plots of log(size) against mobility (Meyers et al., 1976).

Some of the pBR322 DNA was prepared by A. D. Bennett: PM2 DNA was prepared by Dr. C. R. Wilson. Some of the λ DNA was a gift from Prof. W. J. Brammar and Mrs. A. Smith, the remainder being a gift from Dr. A. R. Hawkins.

2.6.3 Restriction digests.

These were generally performed according to the makers' instructions (Boehringer-Mannheim, Bethesda Research Labs or New England Biolabs). Some of the AluI and BstEII used were gifts from Dr. R. B. Meagher. The restriction buffers are described in table 2.2. TaqI digests were performed at 60° or 65°, BstEII digests at 37° (RBM's preparation) or 60°, all others at 37°. (Reactions at 60° or 65° were under Fisons' water-white paraffin). When necessary, reactions were stopped by heating thermolabile enzymes to 65° for 10 min., phenol extraction (2.8.2), or mixing in stop mix (to give final concentrations of 10% (v/v) glycerol, 1% (w/v) SDS, 0.005% (w/v) bromophenol blue) as appropriate to the particular experiment.

When digestion by more than one enzyme was required the digests were performed either simultaneously, if the enzymes required sufficiently similar conditions, e.g. MboI and HindIII, or sequentially, the first enzyme being inactivated (by heat or phenol) before changing the pH and/or salt content of the buffer.

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* Bethesda Research Labs, Inc., P. O. Box 6009, Gaithersburg, MD 20877.

Sometimes this could be achieved by adding sodium or potassium chloride: more often the DNA was precipitated with ethanol (2.8.3), resuspended in TE (2.8.6), and a suitable stock buffer added. Usually a sample from the first digest was subjected to gel electrophoresis to check for complete cleavage before proceeding to the second digest.

2.6.4 Ligations.

After the restriction digests had been stopped by heat-inactivation or phenol extraction the DNAs were precipitated with ethanol and resuspended in TE before the addition of Tris-HCl, pH 7.5 (to 50 mM), magnesium chloride (to 10 mM), DTT (to 1 mM) and ATP (to 1 mM). Usually 0.5 to 1.0 U of T4 DNA ligase (Boehringer-Mannheim or Bethesda Research Labs) was added to a total reaction volume of 10 to 50 μl and incubated at 10° overnight. A sample of the ligation mixture was subjected to gel electrophoresis to check that ligation had occurred.

2.7 E.coli techniques

2.7.1 Transformation of E.coli

(Modified from Mandel and Higa, 1970)

An exponential culture of E.coli ($A_{600} \simeq 0.18$) was washed with an equal volume of 50 mM calcium chloride and resuspended in 0.2 vol of the same solution. Portions (0.2 ml) of this suspension were incubated on ice for 45 min., then about 0.2 μg DNA per tube (in TE or ligation buffer) was added and incubation on ice continued for a further 45 min. A heat-shock, 1 min. at 47° (glass tubes), or 1.5 min. at 47° or 1 min. at 55° (plastic tubes), was given before spreading the transformation mixtures on L plates containing selective antibiotics (after dilution in L broth if necessary). Negative controls (omitting the DNA) were performed in each experiment, as were positive controls (usually 0.2 μg of CCC pBR322 DNA).

After incubation at 37° for 1 to 4 days a number of putative transformants (usually six representative colonies) were streaked for single-colony isolation on L plates containing the selective antibiotic: after incubation single colonies were picked with sterile toothpicks and used to make patches on different selective plates in succession (always ending with a non-selective L plate to check that sufficient growth had been transferred on the toothpick). Ap^R was scored after 18 hr. incubation at 37°, Cm^R and Tc^R after 40 hr., and the nutritional markers after several days, as the control WS 2301 patches often took this long to grow. WS 2302 was also patched as a control.

2.7.2 Mini-lysates of E.coli.

(Modified from Birnboim and Doly, 1979; Maniatis et al., 1982).

About 1 mm^3 of growth from a selective L plate (inoculated from a single colony and incubated 18 hr. at 37°) was resuspended in an Eppendorf tube (1.5 ml capacity) containing 100 µl of 25 mM Tris-HCl, 10 mM EDTA, pH 8.0, 50 mM glucose and 2 mg/ml lysozyme. After incubation at room temperature for 5 min., 200 µl of 1% (w/v) SDS in 0.2 M sodium hydroxide were added and the tube was chilled on ice for 5 min. After mixing in 150 µl of potassium acetate (pH 4.8 with glacial acetic acid, 3 M in K^+) by gentle inversion, incubation on ice continued for a further 30 min. After this time centrifugation in a microfuge (5 min.; 11,000 x g) removed a coarse, white precipitate containing most of the dodecyl sulphate ions, chromosomal DNA and proteins. The plasmid DNA and RNA in the supernatant were precipitated by the addition of 2.5 vols ethanol and chilling briefly (5 to 10 min.) in dry ice/industrial ethanol, and recovered by centrifugation (as above). The pellet was dissolved in 100 mM sodium acetate in 50 mM Tris-HCl, pH 8.0, and reprecipitated with 2 vols of ethanol and chilling (as before). This was repeated once. The final pellet was dissolved in 40 µl of water: the material obtained was suitable for transformation of E.coli or for gel electrophoresis.

For restriction digests residual RNA was removed by incubation with 4 µg of "DNase-free" RNase (2.8.5) at 37° for 30 min. before or during the incubation with restriction enzymes. Normally 2 to 10 U of restriction enzyme were incubated with 5 to 40 µl of mini-lysate for 5 hr. or overnight as convenient.

2.8 Other procedures and solutions

2.8.1 Estimation of nucleic acid concentrations.

Usually the concentrations of purified nucleic acids were determined by measuring the A_{260} of suitably diluted samples and assuming that an A_{260} of 20 was given by a 1 mg/ml solution of nucleic acid: however, when insufficient material was available for this method (e.g. after purification of a restriction fragment by preparative gel electrophoresis) or when DNA preparations contained residual RNA, concentrations were estimated by comparison of band intensity of a sample with that of a known amount of DNA after gel electrophoresis.

2.8.2 Phenol extractions.

Phenol to be used with purified DNA was redistilled,and stored under water or buffer, protected from light, at $+4^{o}$ or -20^{o} until required. Normally, one volume of phenol (saturated with 10 mM Tris-HCl, pH 8.0) was vortexed for 30 s with the DNA solution in an Eppendorf tube and the phases were separated by centrifugation for 3 min. at 11,000 x g (microfuge). Residual phenol was removed from the aqueous layer by extraction with 2 to 3 vols of diethyl ether and ethanol precipitation. For plasmid purification from E.coli (2.5.2) or RNA extraction from S.aureus (6.2.2) Fisons' AR (detached crystals) phenol was used without further purification after saturation with 10 mM Tris-HCl, pH 8.0.

2.8.3 Ethanol precipitation of nucleic acids.

Unless otherwise stated this was performed by the addition of 0.1 vol each of 3 M sodium acetate and 0.1 M magnesium acetate

followed by 2.5 vols of absolute ethanol, mixing by inversion (at least seven times) and chilling, either for at least 4 hr. in a -70° freezer or by immersion in a dry ice/industrial ethanol bath for at least 5 min. The nucleic acids were recovered by centrifugation and, after rinsing the pellets with ethanol, were dried briefly (5 min.) in vacuo and resuspended in TE buffer (2.8.6).

2.8.4 Preparation of dialysis tubing.

(Modified from McPhie, 1971).

For all DNA work dialysis tubing (Visking, $8/_{32}$" or $18/_{32}$") was soaked for 2 hr. in two changes of 50% (v/v) aqueous ethanol then thoroughly rinsed in distilled water. After autoclaving for 15 min. at 15 lbs/in^2 in 10 x TE buffer, the tubing was rinsed in sterile distilled water and stored (under the same) at 4° until required.

2.8.5 "DNase-free" RNase.

RNase (Sigma; 2 mg/ml in water) was incubated in a boiling-water bath for 10 min. then chilled on ice and stored at -20° in aliquots until required.

2.8.6 TE buffers.

100 x TE was 1 M Tris-HCl, 0.1 M EDTA, pH 7.5. This was diluted one hundred-fold and sterilised by autoclaving before use. TE(7.6) was 10 mM Tris-HCl, 1 mM EDTA, pH 7.6.

2.8.7 Preparation of solutions.

Where possible, solutions for use in the extraction of nucleic acids or incubation with them were autoclaved before use, or if not feasible, made up in sterile distilled water.

Similarly, equipment in contact with nucleic acid solutions in such procedures was sterilised before use whenever possible. Deionised, double-distilled water ("Milli-Q") was used for making up solutions, except that "house" deionised, distilled water was used for culture media, and occasionally for electrophoresis buffers for analytical gels and the first two changes of dialysis buffer. The pH of solutions was adjusted at ambient temperature (about 22^{o}).

Table 2.1 Bacterial strains used in this work.

a) Host strains

Strain no.	Organism	Alias	Comments/References
WS 2001	S.aureus	RN450, 8325-4	8325 cured of prophages, Novick (1967)
WS 2064	"	PS 55	propagating strain for phage 55
WS 2065	"	RN981	8325-4 recA1 his7 Wyman et al. (1974)
WS 2204	"	RN27	8325 (80∝)
WS 2301	E.coli K12	3430	aroD$^-$ leu$^-$ thi$^-$ r$^-$ m$^+$
GM48	"		dam3 dcm6 gal$^-$ ara$^-$ lac$^-$ thr$^-$ leu$^-$ thi$^-$? tonA tsx ; Marinus (1973)

Table 2.1, continued.

b) WS 2001 as host strain

Strain no.	Plasmid	Resistance phenotype	Comments/References
WS 2003	pCW3	Tc^R	
WS 2006	pCW6	Cm^R	
WS 2007	pCW7	Cm^R	Wilson and Baldwin (1978)
WS 2008	pCW8	Cm^R	
WS 2009	pC221	Cm^R	
WS 2015	pCW15	$Cm^R Tc^R$	
WS 2016	pCW16	$Cm^R Tc^R$	
WS 2041	pCW41	Cm^R	Wilson *et al*. (1981)
WS 2046	pCW46	Cm^R	
WS 2048	pCW48	$Cm^R Tc^R$	
WS 2054	pCW54	Tc^R	C.R. Wilson (unpublished)
WS 2055	pCW55	Tc^R	
WS 2059	pCW59	$Cm^R Tc^R$	Wilson *et al*. (1981)
WS 2066	pCW66	Tc^R	
WS 2118	pSES18	$Cm^R Tc^R$	
WS 2119	pSES19	$Cm^R Tc^R$	Chapter 3, this work
WS 2120	pSES20	Tc^R	
WS 2206	pC221	Cm^R	RN1305
WS 2207	pUB112	Cm^R	RN2438 Novick (1976)
WS 2209	pC194	Cm^R	SA231

Table 2.1, continued.

Strain no.	Plasmid(s)	Resistance phenotype	Comments/References
c) WS 2204 as host strain			
WS 2208	pC223	Cm^R	RN154; Novick (1976)
d) WS 2065 as host strain			Chapter 3, this work
WS 2070	pC221	Cm^R	pC221 from WS 2009
WS 2071	pCW6	Cm^R	
WS 2072	pCW7	Cm^R	
WS 2074	pCW41	Cm^R	
WS 2075	pCW54	Tc^R	
WS 2076	pCW66	Tc^R	
WS 2077	pCW7 + pCW54	$Cm^R Tc^R$	
WS 2080	pCW66 + pCW6	$Cm^R Tc^R$	
WS 2081	pC221	Cm^R	pC221 from WS 2206
WS 2082	pUB112	Cm^R	
WS 2083	pC223	Cm^R	
WS 2084	pC194	Cm^R	
WS 2085	pSES20	Tc^R	

Table 2.1(d), continued.

Strain no.	Plasmids	Resistance phenotype	Comments/References
WS 2086	pC221 + pCW54	$Cm^R Tc^R$	pC221 from WS 2206
WS 2087	pC221 + pCW54		pC221 from WS 2009
WS 2088	pCW41 + pCW54		
WS 2089	pCW6 + pCW54		
WS 2090	pCW7 + pCW66		WS 2072 transformed with pCW66
WS 2091	pCW7 + pCW66		WS 2076 transformed with pCW7
WS 2092	pCW66 + pUB112		
WS 2093	pCW66 + pC223		
WS 2094	pCW66 + pC194		
WS 2095	pSES20 + pCW6		
WS 2096	pSES20 + pC221		
WS 2097	pSES20 + pUB112		
WS 2098	pSES20 + pC223		
WS 2099	pSES20 + pC194		

Table 2.1, continued.

e) WS 2301 as host strain

Strain No.	Plasmid	Resistance phenotype	Comments/References
WS 2302	pBR322	$Tc^R Ap^R$	Chapter 7 (this work)
WS 2303	pSES3	$Cm^R Ap^R$	" 7
WS 2304	pSES4	$Cm^R Ap^R$	" 4
WS 2305	pSES5	$Cm^R Ap^R$	" 4
WS 2306	pSES6	$Cm^R Ap^R$	" 4
WS 2307	pSES7	$Cm^R Ap^R$	" 4
WS 2308	pSES8	$Cm^R Ap^R$	" 4
WS 2309	pSES9	$Cm^R Ap^R$	" 7
WS 2310	pSES10	$Cm^R Tc^R Ap^R$	" 7
WS 2311	pSES11	$Cm^R Tc^R Ap^R$	" 7
WS 2313	pSES13	$Cm^R Ap^R$	" 7
WS 2314	pSES14	$Cm^R Ap^R$	" 4
WS 2315	pSES15	$Cm^R Ap^R$	" 7
WS 2316	pSES16	$Cm^R Tc^R Ap^R$	" 7
WS 2317	pSES4	$Cm^R Ap^R$	" 4
WS 2318	pSES6	$Cm^R Ap^R$	" 4
WS 2319	pSES7	$Cm^R Ap^R$	" 4
WS 2320	pSES8	$Cm^R Ap^R$	" 4

Notes to table 2.2

* "core buffer"provided by Bethesda Research Labs (BRL)

Stock buffers were made up at 2X, 5X or 10X final concentrations: final concentrations are shown.

Gelatin (Sigma) was autoclaved in solution before use, generally at 200 μg/ml (final concentration); for MboI the final concentration used was 100 μg/ml.

Bovine serum albumin (BSA) was supplied by BRL and used at 100 μg/ml

Conditions used are those of Wilson and Baldwin (1978) or Maniatis et al. (1982), or following manufacturers' instructions.

Table 2.2 Restriction digest buffers.

Restriction enzyme	Tris-HCl mM	pH	NaCl mM	$MgCl_2$ mM	Other Additions
AluI (RBM)	6	7.5	0	6	6 mM BME gelatin
AluI	6	7.6	50	6	6 mM BME
BamHI	20	7.5	0	7	2 mM BME
BglII	10	7.4	0	10	6 mM KCl 1 mM DTT gelatin
BstEII (RBM)	as for AluI(RBM)				
BstEII	6	7.5	150	6	BSA
ClaI	10	8.0	0	10	
EcoRI	100	7.5	50	5	
HaeIII	6	7.4	6	6	6 mM BME
HindIII	20	7.4	60	7	
	20	8.0	60	7	1 mM DTT
HinfI	6	7.4	50	6	6 mM BME BSA
HpaII	as for BglII				
MboI	10	7.4	75	10	1 mM DTT gelatin
AccI PstI Sau3A	10	7.5	50	10	1 mM DTT
TaqI	6	7.4	6	6	6 mM BME gelatin
*	50	8.0	50	10	

CHAPTER 3

COMPARISON OF STAPHYLOCOCCAL Cm^R PLASMIDS

3.1 Introduction

Previous research on staphylococcal Cm^R plasmids focused on seven plasmids (see General Introduction, section 1.3.2 for references), either comparing pC221, pUB112, pC223 and pC194, or pC221, pCW6, pCW7 and pCW8. Wilson and Baldwin (1978) noted that pCW8 was similar to pC194 in size and the possession of a single HindIII site (Ehrlich, 1978; Löfdahl et al., 1978); their restriction map of pCW8 resembles that of pC194 constructed by Gryczan et al. (1978). No direct comparisons had been made between pUB112, pC223 and pC194, on the one hand, and pCW6, pCW7 and pCW8, on the other. It was therefore decided to investigate how these plasmids were related by the criteria of restriction analysis and incompatibility tests.

Shaw and his colleagues made pC221 the target of their studies on the induction of CAT synthesis (see General Introduction, section 1.4.2 for references). Sands and Shaw (1973) found that CAT synthesis was inducible in all of the fifty isolates of Cm^R staphylococci examined, and Iordanescu et al. (1978) had noted that Cm^R was inducible in S.aureus carrying pC221, pUB112, pC223 and pC194. For completeness, the expression of the cat genes of pCW6, pCW7 and pCW8 was also examined.

The construction by C. R. Wilson (described in Wilson et al., 1981) of $Cm^S Tc^R$ derivatives of pC221 (see also Chapter 6) and pCW7 had indicated that the BstEII site on both of these plasmids lay in or near the cat gene or its controlling elements, since interruption of this site abolished detectable CAT activity in S.aureus strains carrying the altered plasmids. As pCW6 also

contained a unique BstEII site (Wilson and Baldwin, 1978) a similar experiment was performed with this plasmid.

3.2 Methods

Tables 3.1 and 3.2 list the origins of plasmids referred to in this chapter. General methods such as the preparation of media, induction and measurement of CAT in S.aureus, preparation of purified plasmid DNA from S.aureus, and DNA techniques have been described in Chapter 2.

3.2.1 Incompatibility tests.

I am grateful to Dr. C. R. Wilson for purifying the pCW54 and pCW66 DNAs and some of the pCW6, pCW7 and pCW41 DNAs used in the construction of strains described in this section.

To avoid potential homologous recombination between closely related plasmids, double plasmid strains were constructed in WS 2065, a rec⁻ derivative of WS 2001 (Wyman et al., 1974). Transformations were carried out using the Ø55 technique described in section 3.2.5.

WS 2065 was transformed with the first plasmid (selecting for Cm^R or Tc^R as appropriate) and mini-lysates were prepared from putative transformants to screen for the presence of the plasmid (see section 3.2.2), before proceeding to transform with the second plasmid (selecting for both plasmids). Mini-lysates of the Cm^RTc^R transformants were prepared to check that both plasmids were indeed present. Double plasmid strains were streaked for single colonies on TSBCmTc plates (10 μg/ml of each drug). Colonies from these plates were used for mini-lysate analysis (3.2.2) and streaked for single colonies on TSB plates containing only one of the two antibiotics. Each of about fifty single colonies from these plates was patched onto a TSBTc and a

TSBCm plate. After 20 hr. at 37°, any patch which had grown uniformly or which contained three or more clearly visible colonies was scored as positive. (This leads to over-estimation of the resistance of the population). The values reported are generally the average of the results from two such experiments.

As controls for the stability of the individual plasmids, rec⁻ strains containing only one type of plasmid were streaked out on antibiotic-free TSB plates. Single colonies were then scored for Cm^R or Tc^R as appropriate by patching onto a selective TSB plate then a plain TSB plate.

3.2.2 Mini-lysates of S.aureus

This method was a modification of that of Gonzalez and Carlton (1980) as described by Wilson et al. (1981).

A single colony was picked with the broad end of a sterile toothpick and streaked on a selective TSB plate. After incubation at 37°, 1 to 2 mm^3 of cell mass were picked up with the broad end of a toothpick and resuspended in 100 µl of TES (30 mM Tris-HCl, pH 8.0, 5 mM EDTA, 50 mM sodium chloride) containing 20% (w/v) sucrose in an Eppendorf tube (1.5 ml capacity). After a 15 min. incubation with 20 µg of lysostaphin at 37°, the cells were lysed by the addition of 200 µl of TES containing 8% (w/v) SDS and incubation at 60° to 65° for 10 min., followed by incubation in an ice-bath for 20 min. Most of the chromosomal DNA and SDS were removed by centrifugation in a microfuge (5 min. at 11,000 x g). The supernatant obtained was incubated for 20 min. at 37° with 30 µg of "DNase-free" RNase (Chapter 2, 2.8.5) before being mixed with 0.25 vol of loading mix (50% (v/v) glycerol and

0.025% (w/v) bromophenol blue). Sixty to eighty μl were applied to a 0.7 to 1.0% (w/v) agarose gel in TAE or TBE electrophoresis buffer (Chapter 2, section 2.6.1). Electrophoresis was performed overnight at 15 to 20 V, or, for TAE only, for 3 hr at 60 V, as convenient.

3.2.3 Construction of Tc^R derivatives of pCW6.

Two μg of HindIII-digested pCW6 were ligated with 9 μg of HindIII-digested pCW3 in a total volume of 35 μl. About 5 μg of ligated DNA were used to transform WS 2001 (3.2.5), selecting on TSB plates containing 10 μg/ml each of Cm and Tc. To check for competence, 0.5 μg of CCC pCW6 was used to transform similarly treated cells to Cm^R, and negative (no DNA) controls were also performed. Transformants were analysed by the mini-lysate technique (3.2.2).

3.2.4 Filling in the BstEII site of pSES19.

Four μg of BstEII-digested pSES19 DNA were incubated in 50 μl of NTB (see Chapter 6, 6.2.3.3) with 80 μM dATP, dCTP, dGTP and TTP and 4 U of polymerase I (Klenow fragment, BRL) for 30 min at 20°. The reaction was terminated by extraction with an equal volume of phenol (Chapter 2, 2.8.2) which was then back-extracted with 50 μl of TE (2.8.6). The combined aqueous layers were extracted once with 2 to 3 vols of diethyl ether before ethanol precipitation (2.8.3). The recovered material was incubated overnight with 6 U of ligase in 32 μl of ligation buffer. A second 4 μg portion of BstEII-cut pSES19 was incubated with 1.2 U of ligase under the same conditions: 20 μl (containing about 2.5μg of DNA) of either ligation reaction were used to transform WS 2001 (3.2.5)

selecting on TSB plates containing Tc (10 μg/ml). Cm^R and Tc^R were scored by patching with toothpicks onto TSB plates containing 10 μg/ml of Cm or Tc.

3.2.5 Transformation of S.aureus.

(After Wilson and Baldwin, 1978).

All glassware was thoroughly rinsed before sterilisation to remove any traces of detergent.

A single colony of the chosen host strain was used to inoculate a slope of BHI agar which was incubated for 18 or 30 hr. at 37°. (The longer time was used for rec⁻ strains.) Three ml of TSB were used to resuspend this growth and 2 ml of this suspension were mixed with a further 3 ml of TSB. One or two ml of this suspension were used to inoculate 100 to 200 ml of TSB which were incubated at 37° with vigorous shaking for 2 to 3 hr. (rec⁺ strains) or 4 to 6 hr. (rec⁻ strains) until the A_{600} was between 0.20 and 0.23. Calcium chloride solution (0.5 M) was added to give a final concentration of 1.25 mM, and at least 2×10^{10} pfu of phage 55 (propagated and titrated as described in section 3.2.6) were added.

After standing for 15 min. at room temperature the cells were collected by centrifugation, either for 15 min. at 9,000 rpm at 20° (6 x 250 ml rotor, MSE 18) or in 10 ml portions for 5 min. at 2,000 rpm in a bench centrifuge (MSE Minor). The cell pellets were resuspended in 0.5 ml of 75 mM calcium chloride per 10 or 20 ml of original culture, and chilled in an ice-bath. DNA (0.5 to 5 μg) was added and the suspension was vortexed for 30 s. Incubation at 0° continued for 50 min. followed by a

heat-shock (6 min. at 47° for plastic tubes, 42° for glass tubes) then at least 25 min. longer on ice. The cells were collected by centrifugation and resuspended in 0.5 ml of TSB per tube. The suspensions were incubated at 37° for 90 min. before being spread on TSB plates containing appropriate antibiotics. (The incubation at 37° could be omitted when transforming to Cm^R.)

3.2.6. Propagation and titration of phage 55.

All equipment was thoroughly rinsed before sterilisation to remove any traces of detergent.

Approximately 3 x 10^8 pfu of phage 55 were added to an exponentially growing culture (A_{600} of about 0.25) of propagating strain 55 (= WS 2064) in 100 ml of TSB which contained 2 mM calcium chloride, and incubation at 37° was continued, the A_{600} being monitored. After one hour the turbidity had ceased to increase and 3 hr. after the addition of the phage the A_{600} had decreased to 0.2. Most of the surviving cells and cell debris were removed by centrifugation (6 x 250 ml rotor, MSE 18, 5°, 20 min. at 10,000 rpm) and the remainder by filtration through a Sartorius cellulose nitrate filter (pore-size = 0.45 μm). The filtrate was stored at 4° until required. Preparations contained 10^{10} to 10^{11} pfu per ml.

The phage preparation was titrated using soft agar overlays containing 0.1 ml exponential (A_{600} = 0.3) WS 2064 and 0.1 ml of appropriate dilutions of the phage preparation (10^6 to 10^9 fold). Both the top (0.5% agar, w/v) and bottom (2% agar) layers contained TSB and 1.5 mM calcium chloride. The preparations were also checked for bacterial contamination by spreading 0.1 ml on TSB plates.

3.3 Results

3.3.1 Comparison of pCW8 and pC194.

Although the restriction maps of pCW8 and pC194 reported by Wilson and Baldwin (1978) and Gryczan et al. (1978) appeared very similar no side-by-side comparison of the two plasmids had been reported. They were therefore subjected to digestion by various restriction enzymes and the resulting fragments analysed by agarose gel electrophoresis.

AccI cut both pCW8 and pC194 only once (gel not shown) as predicted from the nucleotide sequence of pC194 (Horinouchi and Weisblum, 1982). Figs. 3A and 3B show TaqI and HinfI digests of pC194 and pCW8 (fig. 3A, tracks 2 and 3; fig. 3B, tracks 5 and 6). Their AluI patterns (not shown) are also identical. From the DNA base-sequence of pC194 Sau3A should cut pC194 twice; however, complete digests of pC194 by this enzyme were never obtained, even when 0.7 μg of DNA was incubated with 8 U of Sau3A at 37^{o} overnight (fig. 3C, tracks 7 and 8). Fainter bands appeared in addition to the expected 0.9, 2.0 and 2.9 kb bands under these conditions; these could have been due to trace contamination of the Sau3A preparation by a second endonuclease activity which would have been negligible under more normal conditions. It is unclear why Sau3A should fail to digest completely preparations of the plasmids which were scissile by other enzymes (see above), however, as can be seen in fig. 3C, pCW8 and pC194 give the same pattern after incubation under the same conditions. Possible causes of incomplete digestion would include heterogeneity in base-sequence, the presence of tightly bound protein, abnormal

local secondary structure, or modified bases in the -GATC- recognition sequence (other than methylated A, which Sau3A recognises).

3.3.2 Incompatibility relationships of derivatives of pC221.

A $Cm^{S}Tc^{R}$ derivative of pC221, pCW54, constructed by Dr. C. R. Wilson (described in table 3.2) was used for incompatibility tests. No CAT (< 0.001 U/mg) was detectable in extracts made from S.aureus (pCW54) after incubation with 3-deoxyCm. Fig. 3D shows mini-lysates of the double plasmid strains, prepared from cells grown on TSB plates containing both Cm and Tc. Table 3.4 shows the data obtained from the segregation experiments with these strains; data from rec^{-} strains carrying the individual plasmids are presented in table 3.3. While pCW54 co-exists stably with pCW6 or pCW7 when selection is for only one plasmid of the pair, a significant loss of the unselected plasmid is observed in strains carrying pCW54 and pC221 or pCW41 (which, like pCW54, is derived from pC221, see table 3.2). In WS 2086 pCW54 and pC221 segregate away from each other when selection for either is released; i.e., pCW54 is Inc4 like pC221. WS 2087, carrying pCW54 and pC221 (the latter isolated from WS 2009) gave similar results (not shown). The results obtained with WS 2088, where the segregation was more marked, show that pCW41 is also Inc4.

3.3.3 Incompatibility relationships of pCW66, a derivative of pCW7.

As indicated in table 3.2, pCW66 is a $Cm^{S}Tc^{R}$ derivative of pCW7 constructed by Dr. C. R. Wilson (Wilson et al., 1981). Figs. 3F and 3G show mini-lysates of the double plasmid strains

at the start of the incompatibility tests. While pCW66 is strongly incompatible with its parent, pCW7, it is compatible with all the other plasmids tested (table 3.5). Incompatibility relationships with examples of plasmids from other Inc groups (1-3, 5-7, 11-13) were not examined.

3.3.4 Incompatibility relationships of pSES20, a derivative of pCW6.

To establish the incompatibility relationships of pCW6, pSES20 (table 3.2 and section 3.3.6) was used. Mini-lysates of the double plasmid strains are shown in fig. 3F, tracks 5-9. The data presented in table 3.6 indicate that pSES20 is incompatible not only with pCW6, from which it was derived, but also with pUB112 (Inc group 9; Iordanescu et al., 1978). On its own, pSES20 was stable without selection (table 3.3), and was compatible with pC221, pC223 and pC194 (table 3.6).

To determine how similar pCW6 and pUB112 were, restriction digests were performed on both plasmids. Like pCW6, pUB112 has a unique BstEII site (data not shown). MboI and HinfI digests (figs. 3C, tracks 2 and 3, and 3H, tracks 3 and 4) show that the plasmids are very similar. Table 3.7 gives the sizes of the restriction fragments determined from these gels.

3.3.5 Inducibility of CAT in strains of S. aureus.

Preliminary experiments (data not shown) indicated that strains of S.aureus and B.subtilis SB 634 carrying pC221, pUB112, pC223 and pC194 synthesised CAT inducibly. This agreed with the observations of Iordanescu et al. (1978) and Kono et al. (1978). The results of an induction experiment using S.aureus carrying

pCW6, pCW7, pCW8 or pC221 are shown in table 3.8. Only small amounts of CAT were detectable in the extracts unless the cultures had been incubated with 3-deoxyCm.

3.3.6. Construction of Tc^R derivatives of pCW6 and interruption of the BstEII site.

Fig. 3K outlines this experiment. WS 2001 (the *recA*$^+$ *S.aureus* host strain) transformed with ligated, HindIII-digested pCW3 and pCW6 gave two Cm^RTc^R transformants. One hundred Cm^R transformants were obtained using 0.5 μg of CCC pCW6 in the same experiment (method described in section 3.2.3). No colonies appeared on CmTc or Cm plates if DNA was omitted from the transformation incubations. The two Cm^RTc^R transformants obtained, WS 2118 and WS 2119, contained plasmids with different electrophoretic mobilities. Both were larger than pCW6; pSES18 was about 7.1 kb and pSES19 about 6.5 kb. A HindIII digest of a mini-lysate of WS 2118 (gel not shown) suggested that pSES18 might be made up of the 2.4 kb (Tc^R) and 0.6 kb fragments of pCW3 inserted into pCW6: this strain was not studied further. Plasmid pSES19 carries the 2.4 kb Tc^R fragment of pCW3 (fig. 3I) and transformed WS 2065 (the *recA*$^-$ host strain) to both Cm^R and Tc^R whether selection was for Cm^R or Tc^R (data not shown).

Transformation of WS 2001 with pSES19 DNA which had been ligated after filling in the BstEII site with polymerase I (see section 3.2.4) produced eight Tc^R transformants, all of which were sensitive to Cm. (About sixty-five Tc^R transformants were obtained if the filling-in procedure was omitted: of the eight tested, all were also Cm^R.) The "filled-in" derivatives of

pSES19 varied slightly in electrophoretic mobility (fig. 3E). It is possible that small DNA fragments in the preparation of pSES19 could have been inserted during the ligation: this would have produced the plasmids slightly larger than pSES19. The most likely cause of the plasmid smaller than pSES19 is exonuclease activity during the polymerase incubation (deletions were not observed when this step was omitted).

WS 2120, an isolate containing pSES20, very similar in mobility to pSES19 (as were three others out of the eight, fig. 3E), was chosen for further study. DNA isolated from this strain transformed WS 2065 to Tc^R. Fig. 3J shows that BstEII failed to cut pSES20 and that the fragments produced by HindIII digestion corresponded in size with linear pCW6 and the 2.4 kb (Tc^R) fragment of pCW3. (Although HpaII-digested pSES19 and pSES20 ran differently on this gel, this difference was not reproducible.) The data are consistent with the introduction of a small change at the BstEII site of pSES19.

Table 3.9 shows data from an induction experiment: pSES19, like pCW6, encodes CAT inducibly, but CAT is no longer detectable after interruption of the BstEII site to form pSES20.

3.4 Conclusions and discussion

The incompatibility studies described here established that, of the previously unclassified Cm^R plasmids, pCW6 was in Inc group 9 (prototype, pUB112). A Tc^R derivative of pCW7 was compatible with plasmids of Inc groups 4, 8, 9, and 10, while incompatible with its parent, pCW7. In the absence of information about its behaviour in the presence of plasmids of other previously described staphylococcal Inc groups it would, of course, be premature to assign pCW7 to a new Inc group; however, so far, plasmids carrying different solitary resistance-determinants have been compatible (Ruby and Novick, 1975; Iordanescu et al., 1978; Iordanescu and Surdeanu, 1980.)

The Cm^STc^R derivative of pCW6, pSES20, was used to establish the Inc group of pCW6, with which it was shown to be incompatible (table 3.6). While pUB112 displaced pSES20 (as did pCW6), pSES20 appeared to be much less effective in the displacement of pUB112 than of pCW6. This indicates a possible difference between the replication and/or maintenance functions of pCW6 and pUB112; for instance, pCW6, and hence pSES20, could be sensitive to a negative regulator of copy-number produced by pUB112, while pUB112 was markedly more resistant to the equivalent product of pCW6. Such a phenomenon has been described by Som and Tomizawa (1983). ColE1 encodes a trans-acting function (a 63 amino-acid protein) which, in vitro, increases hybrid formation between RNA I and the pre-primer RNA, thus increasing the inhibitory activity of RNA I on formation of the primer for replication (see General Introduction, section 1.2.2). The analogous protein encoded by pMB1 and

its derivatives acts against ColE1 but not *vice versa*.

By restriction digests, however, pCW6 and pUB112 did appear very similar, although isolated in Maryland (Wilson and Baldwin, 1978) and Great Britain (Lacey and Grinsted, 1973) respectively, showing that plasmid type is not restricted geographically. Similarly, pCW8 was isolated in Connecticut (Wilson and Baldwin, 1978) and the apparently identical pC194 in Roumania (Iordanescu, 1976). This agreed with previous observations on Tc^R and Sm^R plasmids, compared by Iordanescu *et al*. (1978) (sources listed by Novick, 1976.)

It was confirmed that expression of the *cat* gene of the four Cm^R plasmids studied here is inducible. The *Bst*EII site of pCW6 must lie in or near the *cat* gene (or its regulatory elements) since the interruption of this site abolished detectable CAT activity: pCW6, therefore, resembles pC221 and pCW7 (Wilson *et al*., 1981) in this respect. In addition, insertion of DNA at the *Hind*III sites of pCW6 (section 3.3.6) and of pC221 and pCW7 (Wilson *et al*., 1981) did not prevent the inducible synthesis of CAT.

Figs. 3A-3J (cont.)

Fig. 3F Samples were mini-lysates of : 1, WS 2076; 2, WS 2092; 3, WS 2093; 4, WS 2094; 5, WS 2099; 6, WS 2098; 7, WS 2096; 8, WS 2097; 9, WS 2095; 10, WS 2085. The gel was 0.7% agarose in TAE.

Fig. 3G Samples were mini-lysates of: 1, WS 2072; 2, WS 2090; 3, WS 2090 after 3 transfers in the absence of Cm; 4, WS 2091; 5, WS 2091 after 3 transfers in the absence of Cm; 6, WS 2076; 7, WS 2065, the rec host strain. Electrophoresis was for 17 hr at 28 V on a 1% gel in TBE.

Fig. 3H Samples were: 1, pCW6; 2,λ/HindIII; 3, pCW6/HinfI; 4, pUB112/HinfI; 5, pBR322/HinfI; 6, pSES3/Sau3A; 7, pCW8/Sau3A; 8, pCW8. The gel was 1.2% agarose in TAE.

Fig. 3I Samples were: 1, pSES19; 2, pSES19/BstEII; 3, pCW8/HindIII; 4, pSES19/HindIII; 5, pCW6/HindIII; 6, pCW6. Electrophoresis was for 2.6 hr at 70 V on a 1.2% gel in TAE.

Fig. 3J Samples were: 1, pSES20; 2, pSES20 incubated without BstEII; 3, pSES19/BstEII; 4, pSES20/BstEII; 5, pSES20/ HpaII; 6, pSES19/HpaII; 7, pCW3/HindIII; 8, pSES20/HindIII; 9, pCW6/HindIII; 10, pSES19/HindIII (partial digest). The gel was 0.8% agarose in TAE.

Figs. 3A-3J

Electrophoresis was for 3 hr at 60 V except for the gels shown in figs. 3G and 3I. TAE and TBE buffers are described in Chapter 2, section 2.6.1., p. 31. The λ digest used in figs. 3C and 3H was prepared by I.G. Charles. Size-markers (see section 2.6.2, p. 31, and Chapter 7) were (in kb):

λ/HindIII, 23.1, 9.4, 6.8, 4.36, 2.32, 2.03, 0.564;
pBR322/AluI, 0.91, 0.659 & 0.655, 0.521, 0.403, 0.281, 0.257, 0.226, 0.136, 0.10 and smaller;
pBR322/HinfI, 1.63, 0.517 & 0.506, 0.396, 0.344, 0.298, 0.221 & 0.220 and smaller;
pBR322/TaqI, 1.444, 1.307, 0.475, 0.368, 0.315, & 0.312, 0.141;
pSES3/Sau3A, 2.0, 1.37, 0.358, 0.341, 0.317, 0.272, 0.258, 0.207, 0.107 and smaller.

Fig. 3A Samples were: 1, pBR322/AluI; 2, pCW8/TaqI; 3, pC194/TaqI; 4, pBR322/TaqI. The gel was 2% agarose in TAE.

Fig. 3B Samples were: 1, pSES3/Sau3A; 2, pBR322/TaqI; 3, pCW8/MboI; 4, pC194/MboI; 5, pCW8/HinfI; 6, pC194/HinfI; 7, pBR322/AluI. The gel was 1.2% agarose in TAE.

Fig. 3C Samples were: 1, pCW6; 2, pCW6/MboI; 3, pUB112/MboI; 4, λ/HindIII; 5, pCW41/MboI; 6, pSES3/Sau3A; 7, pC194/Sau3A; 8, pCW8/Sau3A; 9, pCW8. The gel was 1.2% agarose in TAE.

Fig. 3D Samples were mini-lysates of: 1, WS 2071; 2, WS 2089; 3, WS 2081; 4, WS 2086; 5, WS 2070; 6, WS 2087; 7, WS 2075; 8, WS 2088; 9, WS 2074; 10, WS 2041; on a 0.7% gel in TAE.

Fig. 3E Samples 1 and 11 were purified pSES19 DNA. Sample 6 was a mini-lysate of WS 2119; sample 5, of WS 2120. The remaining tracks contained mini-lysates of other isolates from the same experiment (section 3.3.6). The gel was 0.8% agarose in TAE.

Figs. 3A-3J

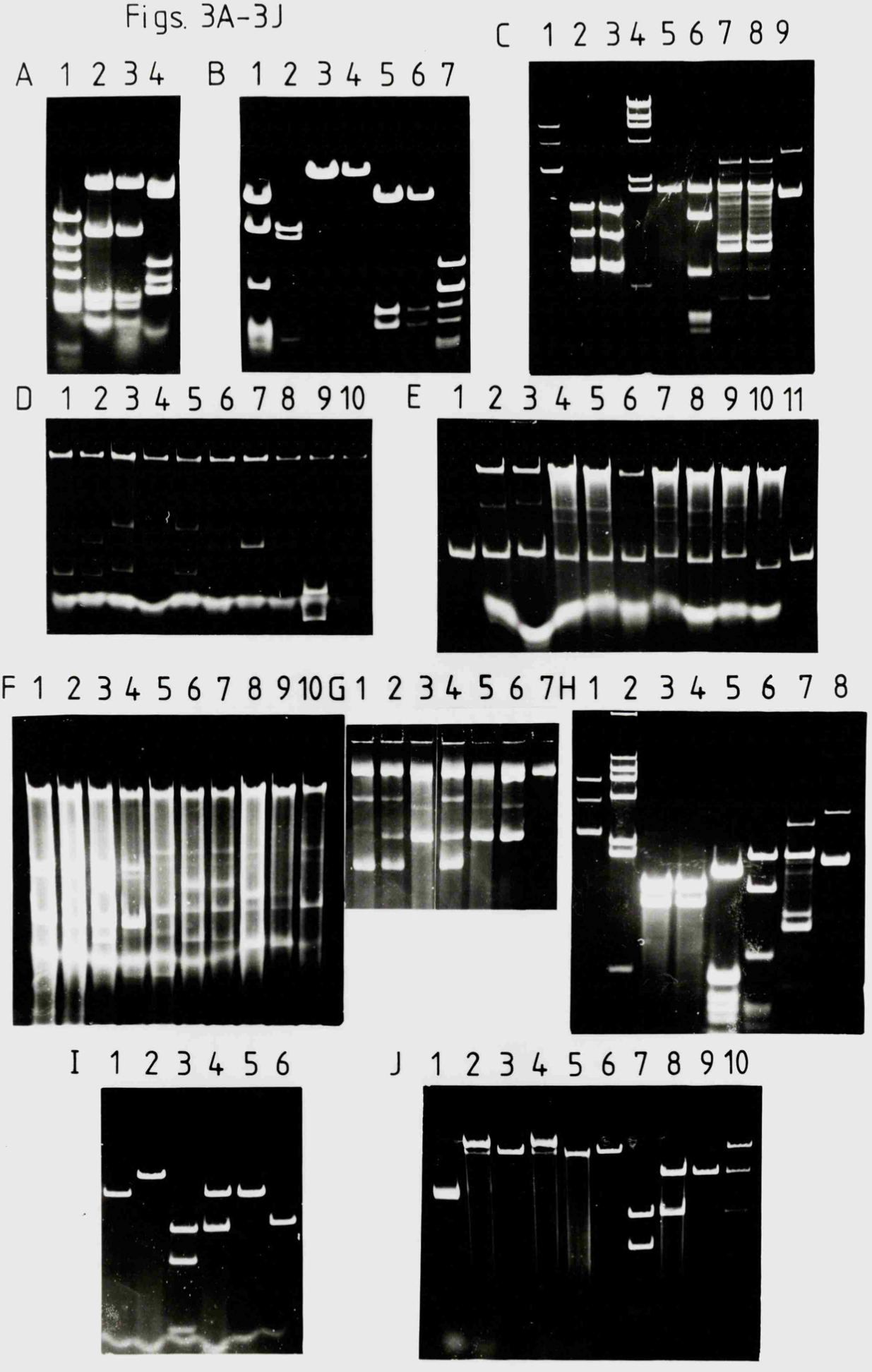
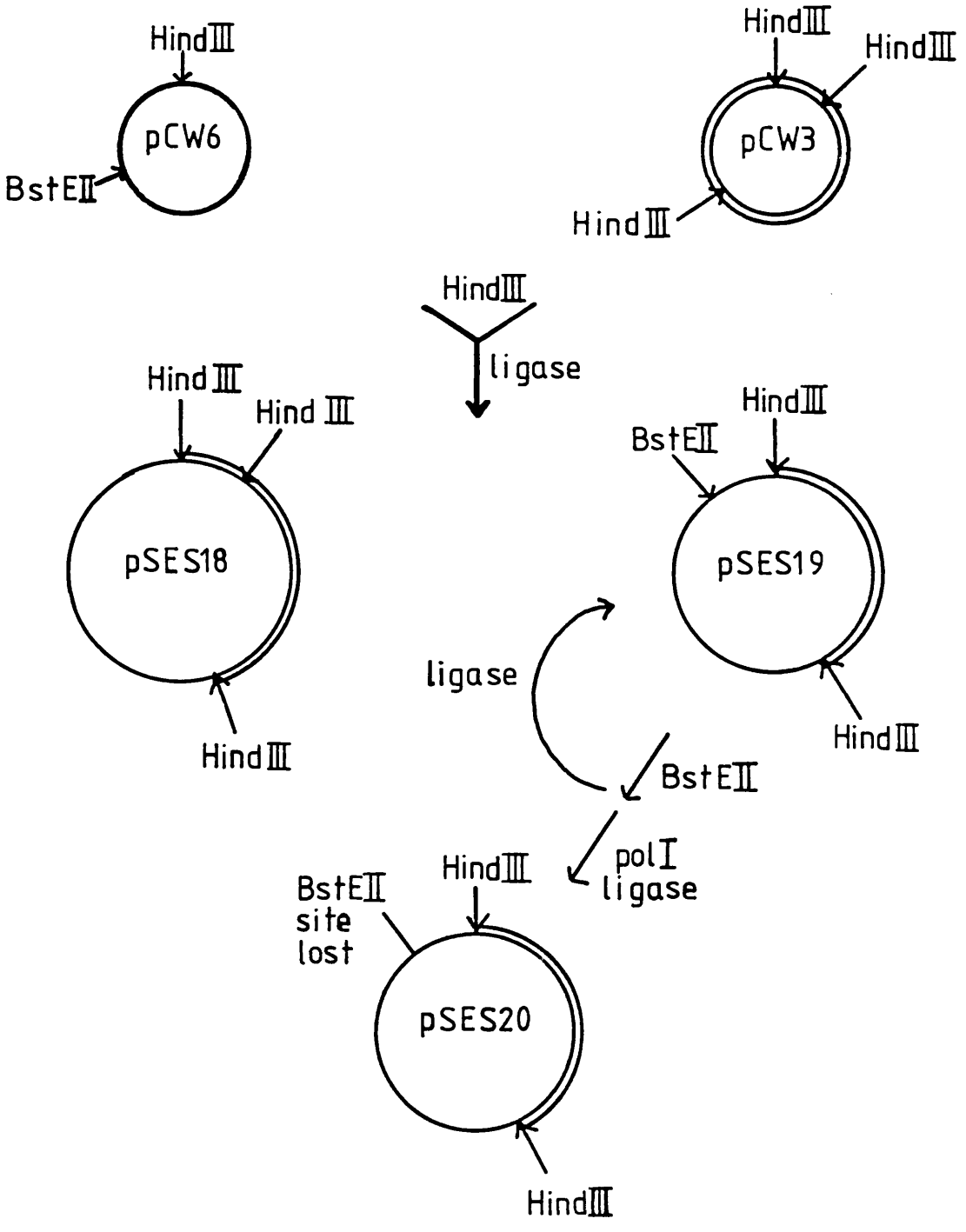

A 1 2 3 4
B 1 2 3 4 5 6 7
C 1 2 3 4 5 6 7 8 9
D 1 2 3 4 5 6 7 8 9 10
E 1 2 3 4 5 6 7 8 9 10 11
F 1 2 3 4 5 6 7 8 9 10
G 1 2 3 4 5 6 7
H 1 2 3 4 5 6 7 8
I 1 2 3 4 5 6
J 1 2 3 4 5 6 7 8 9 10

Fig. 3K Construction of pSES18, pSES19 and pSES20.



Plasmids are described in tables 3.1 and 3.2. Restriction sites in pCW3 and pCW6 are from Wilson and Baldwin (1978).

polI: incubation with polymerase I and dNTPs to fill in ends.

——— from pCW6 ═══ from pCW3

Notes to table 3.1:

a) Sizes of pCW2 and pCW3 from Wilson and Baldwin (1978); pC223 from Novick (1976); pC194 from DNA sequence (Horinouchi and Weisblum, 1982). All others from this chapter and Chapter 5.

b) Inc groups determined by Ruby and Novick (1975) and Iordanescu *et al*. (1978) or in this chapter (pCW6). Figures in parentheses indicate presumed Inc group.
"?": pCW7 is shown here not to be Inc 4, 8, 9, or 10.

c) Dates of isolation may have been earlier than those given here in some cases.

d) References:
1 Wilson and Baldwin, 1978
2 Chabbert *et al*., 1964
3 Lacey and Grinsted, 1973
4 Iordanescu, 1976

Table 3.1 Origins of naturally occurring plasmids referred to in Chapter 3.

Plasmid	Size[a] (kb)	Resistance-determinants	Inc[b] group	Obtained from:	Place and date of isolation[c]	Ref.[d]
pCW2	4.5	Tc^R	(3)	C.R.Wilson	Maryland 1972	1
pCW3	4.5	Tc^R	"	C.R.Wilson	New York 1972	1
pCW6	4.2	Cm^R	9	C.R.Wilson	Maryland 1972	1
pCW7	4.4	Cm^R	?	C.R.Wilson	New York 1972	1
pCW8	2.9	Cm^R	(8)	C.R.Wilson	Connecti-cut 1975	1
pC221	4.6	Cm^R	4	C.R.Wilson R.P.Novick	Paris 1964	2
pUB112	4.2	Cm^R	9	R.P.Novick	Britain 1970	3
pC223	~4.5	Cm^R	10	R.P.Novick	Paris 1964	2
pC194	2.9	Cm^R	8	R.P.Novick	Roumania 1975	4

Table 3.2 Derivative plasmids referred to in Chapter 3.

Plasmid	Size (kb)	R dets.[b]	Description of plasmid	Ref./ source
pCW15	8.3	$Cm^R Tc^R$	HindIII fragments[a] A and B of pCW3 in the HindIII site of pC221	Wilson et al. (1981)
pCW16	6.8	" "	HindIII fragment A of pCW2 in the HindIII site of pC221	Wilson (1978)
pCW41	2.0	Cm^R	Largest MboI fragment of pC221, via pCW15 (see Chapter 6)	Wilson et al. (1981)
pCW54	6.7	Tc^R	BstEII site of pCW16 filled in: about 0.3kb deletion from pC221 region	C.R. Wilson (unpublished)
pCW59	5.3	$Cm^R Tc^R$	HindIII fragment A of pCW3 in HindIII site of pCW7: 1.3 kb lost from pCW7 region	Wilson et al. (1981)
pCW66	5.3	Tc^R	BstEII site of pCW59 filled in	"
pSES18	~7.1	$Cm^R Tc^R$	HindIII fragments A and ? C of pCW3 in HindIII site of pCW6	This chapter
pSES19	6.5	" "	HindIII fragment A of pCW3 in HindIII site of pCW6	" "
pSES20	6.5	Tc^R	BstEII site of pSES19 filled in	" "

Notes a) HindIII cuts both pCW2 and pCW3 into three fragments: A, 2.4 kb (Tc^R); B, 1.5 kb; C, about 0.6 kb (Wilson and Baldwin, 1978).

b) R dets.: resistance phenotype conferred on S.aureus carrying these plasmids.

Table 3.3 Stability of individual plasmids in WS 2065.

Strain	Plasmid	% resistant patches
WS 2071	pCW6	100
WS 2072	pCW7	100
WS 2074	pCW41	100
WS 2075	pCW54	100
WS 2076	pCW66	100
WS 2076	pCW66	100[a]
WS 2081	pC221	100[b]
WS 2082	pUB112	100
WS 2083	pC223	100
WS 2084	pC194	100
WS 2085	pSES20	100

Notes: Plasmids are described in tables 3.1 and 3.2. Experiments were performed as in section 3.2.1. Scoring was normally done after a single transfer in the absence of antibiotics.

a) Scored after three transfers in the absence of Tc.

b) pC221 from WS 2206 (Novick's strain)

Table 3.4 Incompatibility tests using pCW54.

Strain	selection	selected plasmid	unselected plasmid	% resistant patches
WS 2086	Tc	pCW54	pC221	77
	Cm	pC221	pCW54	48
WS 2088	Tc	pCW54	pCW41	37
	Cm	pCW41	pCW54	0
WS 2089	Tc	pCW54	pCW6	100
	Cm	pCW6	pCW54	99
WS 2077	Tc	pCW54	pCW7	100
	Cm	pCW7	pCW54	100

Procedures are described in section 3.2.1 and plasmids in tables 3.1 and 3.2.

Table 3.5 Incompatibility tests using pCW66.

Strain	selection	selected plasmid	unselected plasmid	% resistant patches
WS 2090	Tc	pCW66	pCW7	10
	Cm	pCW7	pCW66	0[a]
WS 2092	Tc	pCW66	pUB112	100
	Cm	pUB112	pCW66	100
WS 2093	Tc	pCW66	pC223	100
	Cm	pC223	pCW66	100
WS 2094	Tc	pCW66	pC194	100
	Cm	pC194	pCW66	100
WS 2080	Tc	pCW66	pCW6	100
	Cm	pCW6	pCW66	100[a]

Procedures are described in section 3.2.1 and plasmids in tables 3.1 and 3.2.

Note: a) These results were obtained after three successive single-colony isolations in the absence of Tc.

Table 3.6 Incompatibility tests using pSES20.

Strain	selection	selected plasmid	unselected plasmid	% resistant patches
WS 2095	Tc	pSES20	pCW6	50
	Cm	pCW6	pSES20	61
WS 2096	Tc	pSES20	pC221	100
	Cm	pC221	pSES20	100
WS 2097	Tc	pSES20	pUB112	90
	Cm	pUB112	pSES20	54
WS 2098	Tc	pSES20	pC223	100
	Cm	pC223	pSES20	100
WS 2099	Tc	pSES20	pC194	100
	Cm	pC194	pSES20	100

Procedures are described in section 3.2.1 and plasmids in tables 3.1 and 3.2.

Table 3.7 Restriction digests of pCW6 and pUB112.

Restriction enzyme	Sizes (kb) of observed fragments[a]	Total (kb)[b]
MboI	0.72, 0.77, 1.12, 1.6	4.2
HinfI	1.20, 1.40, 1.47	4.1

Notes: a) Sizes of fragments were obtained from the gels shown in figs. 3C and 3H.

b) Linear pCW6 ran at 4.2 kb.

Table 3.8 Induction of CAT in S.aureus carrying Cm^R plasmids.

Strain	Plasmid	Concentration of 3-deoxyCm (μM)	Specific activity of CAT (U/mg)
WS 2006	pCW6	0	0.001
		6	0.42
WS 2007	pCW7	0	0.009
		6	0.54
WS 2008	pCW8	0	0.067
		6	2.3
WS 2009	pC221	0	0.004
		6	0.59

Notes: For each strain, two 50 ml portions of TSB were each inoculated with 1 ml of an overnight culture and incubated with shaking at 37^{o}. At A_{600} = 0.1, 3-deoxyCm was added to one flask and incubation continued for five hours. Extracts were prepared from 10 ml of each culture and assayed for protein and CAT as described in Chapter 2 (section 2.4).

Table 3.9 Induction of CAT in S. aureus carrying pCW6 or its derivatives.

Strain	Plasmid	Concentration of 3-deoxyCM (μM)	Specific activity of CAT (U/mg)
WS 2001	none	6	< 0.001
WS 2006	pCW6	0	0.001
		6	0.43
WS 2119	pSES19	0	0.002
		6	0.35
WS 2120	pSES20	6	< 0.001

Notes: Methods as for table 3.8, except that incubation after the addition of inducer was only four hours.

CHAPTER 4

HETERODUPLEXES OF Cm^R PLASMIDS

4.1 Introduction

Comparison of the restriction maps of pC221, pCW6 and pCW7 constructed by Wilson and Baldwin (1978) suggested that these plasmids might share a region of homology at least 1.7 kb and including restriction sites for XbaI, HpaII and BstEII; pCW8 did not appear to share this homology, however. Iordanescu et al. (1978) observed 68% hybridisation between a radioactive pC221 probe and pUB112 (which is similar to pCW6, see Chapter 3) but less than 10% hybridisation between pC221 and pC194 (which, by the criteria used in Chapter 3, appeared to be identical with pCW8). Apart from some homology between the cat genes there was little similarity evident between the nucleotide sequences of pC194 and pCW41, the 2.0 kb derivative of pC221 (Horinouchi and Weisblum, 1982; Hawkins et al., unpublished, see App. 1).

Electronmicroscopy of heteroduplexes was chosen as a suitable technique for the investigation of the apparent structural homologies between these plasmids since the position and extent of any homologous region observed could be compared with the locations of restriction sites and genes (where known). In order to provide structural reference-points the plasmids were to be cut at unique restriction sites before the hybridisations were performed. Additionally, for mechanical stability and ease of interpretation, it was desirable that either end of the linear heteroduplexes should terminate in at least 0.3 kb of ds DNA and that the two ends be readily distinguishable from each other.

It was decided to insert the Cm^R plasmids by their unique HindIII sites into the HindIII site of pBR322. This approach had

several advantages. The positions of restriction sites for many enzymes were known from the nucleotide sequence (Sutcliffe, 1979). This made it easy to select a restriction enzyme which would cut each of the hybrid plasmids asymmetrically in the common section (pBR322) while leaving intact the region derived from the Cm^R plasmid. The construction of such a series was more easily performed in E.coli than in S.aureus (transformation frequencies were 10^5 to 10^6 per μg of CCC pBR332 for E.coli, 50 to 200 per μg of similarly sized plasmids for S.aureus) and rapid techniques were already available for restriction analysis of plasmids in mini-lysates of the former host (e.g. that of Birnboim and Doly, 1979). Direct selection for Cm^R in E.coli for pBR322 carrying staphylococcal cat genes was known to be possible (Ehrlich, 1978; Goze and Ehrlich, 1980; Goursot et al., 1982; Chapter 7, this work). Furthermore, the use of E.coli as a source of the plasmid DNA for electronmicroscopic studies circumvented a practical problem encountered during preliminary experiments using plasmids prepared from S.aureus; such preparations were frequently contaminated with small fragments of nucleic acids, which would have had to be removed. In addition, if a staphylococcal plasmid had been used as the source of the region common to the hybrid plasmids, the choice of a suitable restriction fragment and the interpretation of results might well have been complicated by homologies between the cloned fragment and the Cm^R plasmids. For example, Iordanescu et al. (1978) found that pC221 gave 40% hybridisation with pT127 (Tc^R, Inc3) and 60% with pS194 (Sm^R, Inc5).

4.2 Methods

Preparation of media, restriction digests, ligations, transformations and mini-lysate techniques have been described in Chapter 2 (sections 2.3, 2.6 and 2.7). The plasmids used are listed in table 4.1.

4.2.1 Construction of derivatives of pBR322.

E.coli was transformed either with 0.2 μg of CCC pBR322 or with 0.1 μg of HindIII - cut pBR322 ligated with one of the following: 0.3 μg of pC221, pCW6 or pCW7 or 0.2 μg of pCW8 (each having first been linearised by HindIII). In all cases the total volume was about 0.2 ml. Some of the cells transformed with pBR322 were spread on LCm10 (0.1 ml) and LAp40 plates (0.1 ml of a 10^4-fold dilution). Each of the remaining four transformation mixtures was divided approximately equally between LCm10 and LCm25 plates.

Twelve representative Cm^R colonies from each transformation were streaked for single-colony isolation on LCm10 or LCm25 plates as appropriate, and single colonies from these plates were screened for resistance to Ap (40 μg/ml), Tc (10 μg/ml) and Cm (10 μg/ml) and for nutritional requirements. Several isolates were examined by mini-lysate analysis. For re-transformation tests 5 μl of mini-lysate preparations were generally used.

In the construction of pSES14, 0.4 μg of a re-ligated HindIII digest of pSES4 was used to transform E.coli in a total volume of 0.2 ml which was divided equally between LCm10 and LCm25 plates. Transformations using 0.24 μg of pBR322 or 0.2 μg of pSES4 were also included as positive controls. Representative

Cm^R transformants were screened as above.

4.2.2 Preparation of linear plasmid DNA for heteroduplex studies.

Plasmid DNA was isolated from WS 2305, WS 2314, WS 2317, WS 2318, WS 2319 and WS 2320 as described in Chapter 2 (section 2.5) using the Beckman VTi50 rotor for the CsCl/EthBr centrifugation step, which was repeated to remove residual RNA. The plasmid DNAs were concentrated by precipitation with ethanol, their concentrations estimated from the A_{260} of diluted samples (Chapter 2, section 2.8) and 2.5 μg of each were digested with PstI.

4.2.3 Hybridisation of pBR322-derivatives and electronmicroscopy of heteroduplexes.

I am indebted to Mrs. P. McTurk for carrying out the procedures described in this section, which were essentially those of Durnam et al. (1980) as described by McDonald et al. (1983). I am also grateful to Mr. G. Sharpe and Dr. A. R. Hawkins for preparing the OC pBR322 and ss M13mp9 DNAs used as markers.

The PstI-digested DNAs (section 5.2.2) were combined in equimolar amounts at 1 to 3 μg/ml in the pairs indicated in table 4.5. The reaction mixes also contained 70% (v/v) formamide (Sigma), 300 mM sodium chloride, 10 mM Tris-HCl (pH 8.5) and 1 mM EDTA. They were incubated for 5 min. at 75°, then for 30 min. at 25°. They were then adjusted to 0.3 to 0.9 μg/ml DNA in 50% (v/v) formamide, 0.1 M Tris-HCl (pH 8.5), 0.01 M EDTA and 100 μg/ml cytochrome c, giving the hyperphase solutions. M13mp9 and OC pBR322 were added to each as size-markers.

Each hyperphase solution was spread on a hypophase of

deionised water and picked up on collodion-coated grids, then stained with uranyl acetate and shadowed using a Polaron micro-sputter module with a gold target and a low-angle shadowing attachment. The electronmicroscope used was a Joel 100cx (accelerating voltage, 60 kV). The contour lengths of twenty to thirty molecules of each sort were measured using a Kontron digitising tablet and computer, and the lengths of the different regions were converted to kb by comparison with the appropriate size-marker, M13mp9 (7599 b: Messing and Vieira, 1982; van Wezenbeek et al., 1980) for ss regions, and OC pBR322 (4,362 bp; Sutcliffe, 1979) for ds regions.

4.3 Results

4.3.1 Transformation of E.coli by pBR322 ligated with pC221.

Table 4.2 shows the number of Cm^R transformants obtained and the results of screening representative isolates for Tc^R. Fig. 4.2 shows mini-lysates from five $Cm^R Tc^S$ isolates. Since the host strain, WS 2301, is recA$^+$ the multiplicity of bands is probably due to multimeric forms of the plasmids (Bedbrook and Ausubel, 1976) and is also seen in track 6 for pSES 3 (which is described in Chapter 7). By comparison of mobilities with the pSES3 bands, the fastest-moving band would represent SC monomer of about 9 kb and the next, SC dimer of about 17.3 kb.

ClaI-digested mini-lysates of all five isolates gave two bands (about 1.1 and 7.8 kb, as in tracks 4 and 5 in fig. 4.9) consistent with the orientation (A) of pC221 shown in fig. 4.10(a). One such isolate was designated WS 2304, and its plasmid, pSES4. HindIII digestion of a mini-lysate of this strain gave a broad band of similar mobility to those of HindIII-cut pC221 and pBR322 (fig. 4.5, tracks 10, 11 and 9). These data are consistent with WS 2304 containing multimeric forms of pSES4.

A mini-lysate of WS 2304 after two further single-colony isolations on LCm10 plates gave the same ClaI and HindIII digestion patterns and transformed WS 2301 to the same phenotype as WS 2304. The three representative transformants tested contained plasmids identical with pSES4 in their multimer and digestion patterns. One of these isolates (WS 2317) was retained for further use.

4.3.2 Transformation of E.coli by pBR322 ligated with pCW6.

The results of this transformation are given in table 4.2. Only one of the six Cm^R isolates studied appeared to contain a single type of plasmid. This isolate, WS 2305, contained a plasmid, pSES5, with SC monomers of about 8.2 kb and SC dimers of about 16.5 kb (by comparison with pSES3 multimers, see fig. 4.3). A WS 2305 mini-lysate digested with EcoRI gave a single fragment of about 8.8 kb which was cut by BstEII to 2.8 kb and about 5.7 kb (gel not shown), consistent with the orientation (B) of pCW6 indicated in fig. 4.10(b). The HindIII fragments of pSES5 were of similar mobility to those of pCW6 and pBR322 (4.2 and 4.4 kb, see fig. 4.6). Three Cm^R isolates of E.coli transformed with a mini-lysate of WS 2305 contained multimers identical with those from WS 2305 and showed the same pattern of antibiotic-resistances.

The other five isolates studied each appeared to contain two types of plasmids, one similar in size to pSES5 and one of about 12.6 kb (fig. 4.3). From HindIII, EcoRI and EcoRI + BstEII digestion patterns (table 4.3) these strains were thought to contain pBR322 with both single and double inserts of pCW6.

4.3.3 Transformation of E.coli by pBR322 ligated with pCW7.

Table 4.2 shows the results of this transformation. Three isolates from LCm10 plates, all Tc^S, contained plasmids of about 8.6 kb (dimers about 17 kb); an example is shown in fig. 4.4 (track 1). EcoRI digests of mini-lysates gave the same single fragment (8.7 kb), which was cut by BstEII to about 0.8 and 8.0 kb (gel not shown). These results are consistent with the orientation (A) shown in fig. 4.10(c). One isolate was designated

WS 2306 and its plasmid, pSES6. Fig. 4.5 (track 7) shows a HindIII digest of a mini-lysate of this strain. A mini-lysate prepared from WS 2306 after two additional single-colony isolations on LCm10 plates gave results similar to those described above and was used to transform E.coli to Cm^R. The Cm^R transformant tested, WS 2318, was indistinguishable from WS 2306 by antibiotic-resistance pattern and nutritional requirements, and contained a plasmid identical with pSES6 in multimer and HindIII digestion patterns. Fragments of the expected size were also obtained after EcoRI and BstEII digestion (not shown).

Four isolates selected with 25 μg/ml Cm in the original transformation (table 4.2), all Tc^S, appeared to contain, in addition to pSES6, larger plasmids. From the results of HindIII, EcoRI and EcoRI + BstEII digests (table 4.4) these were probably pBR322 carrying two (and possibly three) pCW7 inserts. A mini-lysate of one such isolate appears in fig. 4.4, track 2.

4.3.4 Transformation of E.coli by pBR322 ligated with pCW8.

The results of this transformation are shown in table 4.2. Mini-lysates from six Cm^R isolates (all Tc^S) contained plasmids of about 7 kb (fig. 4.7). ClaI-digested mini-lysates of two isolates (both selected with 25 μg/ml Cm) contained two fragments (1.6 and 5.2 kb), consistent with the orientation (A) shown in fig. 4.10 (d). The smallest fragment predicted (0.15 kb) would not have been seen on this gel. The remaining four isolates gave fragments of 1.0, 1.6 and 4.4 kb, in agreement with the opposite orientation (B) of pCW8, shown in fig. 4.10 (e). ClaI-digested mini-lysates of these isolates are shown in

fig. 4.8. Mini-lysates of WS 2307 (containing pSES7, with pCW8 in orientation A) and WS 2308 (containing pSES8, with pCW8 in orientation B) gave HindIII fragments identical in mobility with those from pCW8 and pBR322 (fig. 4.5, tracks 5, 4, 6 and 3).

Mini-lysates of WS 2307 and WS 2308 after another two single-colony isolations on LCm plates gave the same results and transformed E.coli to the same antibiotic-resistance and auxotrophic phenotype. Mini-lysates of three transformants from either experiment showed the expected multimer, HindIII and ClaI digestion patterns. One of either class was retained for further use (WS 2319 and WS 2320, carrying pSES7 and pSES8, respectively).

4.3.5 Transformation of E.coli with religated HindIII-digested pSES4.

Table 4.2 shows the results of this transformation. Mini-lysates from Cm^R isolates which were Tc^S, like WS 2317 (from which the pSES4 DNA used in the construction had been purified), contained plasmids identical with pSES4 in multimer and ClaI digestion patterns (data not shown). Of the five Cm^RTc^R isolates studied, two contained, in addition to a plasmid of similar size to pSES4, another larger plasmid and were not studied further. The other three gave the same multimer pattern as WS 2317. On ClaI digestion of mini-lysates (gel shown in fig 4.9) one (from a strain only weakly Tc^R) gave a pattern identical with that of WS 2317 and was discarded. The other two mini-lysates gave ClaI fragments of 3.6 and 5.5 kb, indicating that pC221 was inserted into pBR322 in orientation B (see fig. 4.10 (f)). HindIII digestion gave a single band of the same mobility as HindIII-

digested pC221 and pBR322 (not shown). A mini-lysate prepared from one isolate (WS 2314, carrying pSES14) transformed E.coli to the same antibiotic-resistance pattern as WS 2314.

4.3.6 Electronmicroscopy of heteroduplexes.

Table 4.5 shows measurements made from electronmicrographs of heteroduplexes formed from the pBR322-derivatives of four staphylococcal Cm^R plasmids. The measured sizes of the ds pBR322 arms (regions a and g in table 4.5 and fig. 4.11) are very close to those predicted from the nucleotide sequence (Sutcliffe, 1979), so there does not appear to have been hybridisation between the inserts in the regions around the HindIII sites (the junctions between pBR322 and the Cm^R plasmids). Average sizes of 4.7, 4.3, 4.4 and 2.9 kb were obtained from the contour lengths of the inserted pC221, pCW6, pCW7 and pCW8. While the last value agrees with that from the nucleotide sequence of pC194 (Horinouchi and Weisblum, 1982) which appeared to be the same as pCW8 (Chapter 3), the other values obtained here are slightly larger than those of 4.4, 3.9 and 4.2 kb reported by Wilson and Baldwin (1978). More recently, however, values of 4.6, 4.2 and 4.4 kb were obtained (Chapters 3 and 5, this work). Allowance was made for these slight discrepancies when plotting the data shown in table 4.5 on the maps of the plasmids.

About 2 kb of pCW6 and pC221 are homologous (pSES5 X pSES14, fig. 4.13): this region includes the XbaI, HpaII and BstEII sites common to pC221 and pCW6 (fig. 4.12). No homology was observed between pC221 and pCW6 in the opposite orientation (pSES5 X pSES4; table 4.5) nor between pCW6 and pCW8 in either orientation

(pSES5 X pSES7, pSES5 X pSES8). Measurement of pSES6 X pSES4 heteroduplexes (e.g. fig. 4.14) indicated that pCW6 and pCW7 are homologous with approximately the same 2 kb region of pC221 (fig. 4.12), although, in this case, ten out of the twenty-six heteroduplexes examined showed regions of non-homology within this (e.g. fig. 4.15). Table 4.6 gives approximate measurements for the "bubbles" observed; bubbles 0.1 kb or smaller would have been overlooked. No homology was observed between pC221 and pCW7 in the opposite orientation (pSES6 X pSES14). Although no homology was observed between the inserts of pSES6 and pSES7, about 0.28 kb of pCW7 and pCW8 hybridised in pSES6 X pSES8 (fig. 4.16) immediately beyond the region of pCW7 homologous with pC221 (fig. 4.12). No homology was observed between pC221 and pCW8 in either orientation (e.g. fig. 4.17; and see table 4.5, pSES4 X pSES7 and pSES4 X pSES8). Although they were not measured, for pSES4 X pSES14 and pSES7 X pSES8 some molecules were observed aligned so that the inserts (the Cm^R plasmids) hybridised and the long and short "arms" of pBR322 were ss (e.g. fig. 4.18).

4.4 Conclusions and discussion

Electronmicroscopy of heteroduplexes between pCW6 or pCW7 and pC221 indicated that there was a 2 kb region of homology, including restriction sites for XbaI, HpaII and BstEII, whose location and extent corresponded with the largest MboI fragment of pC221, pCW41. From the nucleotide sequence of pCW41 (Hawkins et al., unpublished, see App. 1) could be inferred the position and orientation of the cat gene of pC221 and hence of pCW6 and pCW7 (see fig. 4.14). In the case of pCW7 the homology was only partial in places since two ss bubbles were visible within this area in some heteroduplexes (39% of those examined); one lay within the cat gene region, the other outside it. Hyman et al. (1973) observed regions of partial homology between coliphages: when the hybridisation conditions were changed the same region was ss, but in different numbers of molecules.

The failure to observe homology between pC221 and pCW8 agreed with the data of Iordanescu et al. (1978), referred to in section 4.1. The similarities between the DNA sequences of these plasmids in the cat gene region must have been insufficient to produce an observable region of homology under the conditions used here. No homology was observed between pCW6 and pCW8: between pCW7 and pCW8, however, there exists a short (0.28 kb) region of homology such that molecules bearing the cat genes in opposite orientations hybridised. The shared region (which is just outside that part of pCW7 which hybridised with pC221) was required by pC194 (= pCW8) neither for expression of Cm^R (being beyond the 3' end of the cat gene) nor for plasmid replication in

B.subtilis (Horinouchi and Weisblum, 1982). However, it probably contains the recombination sequence, RS-C_A of pC194 (Novick et al., 1981; Grandi et al., 1981).

When small staphylococcal plasmids are cotransduced they may undergo site-specific recombination (Novick et al., 1981). Recombination sequences (RS) probably occur on pC221, pUB112 and pC223 as well as on pC194 since Iordanescu et al. (1978) were able to obtain derivatives of these plasmids in vivo by cotransduction. It is, therefore, quite possible that the 0.28 kb region of pCW7 homologous with pCW8 contains an RS. It may be relevant to note that this region lies within, or very near to, the region deleted spontaneously during the construction of pCW59 (Wilson et al., 1981); however, the effect of phage-mediated transformation on the activity of RS has not been examined, so no conclusion can be drawn.

Fig. 4·1

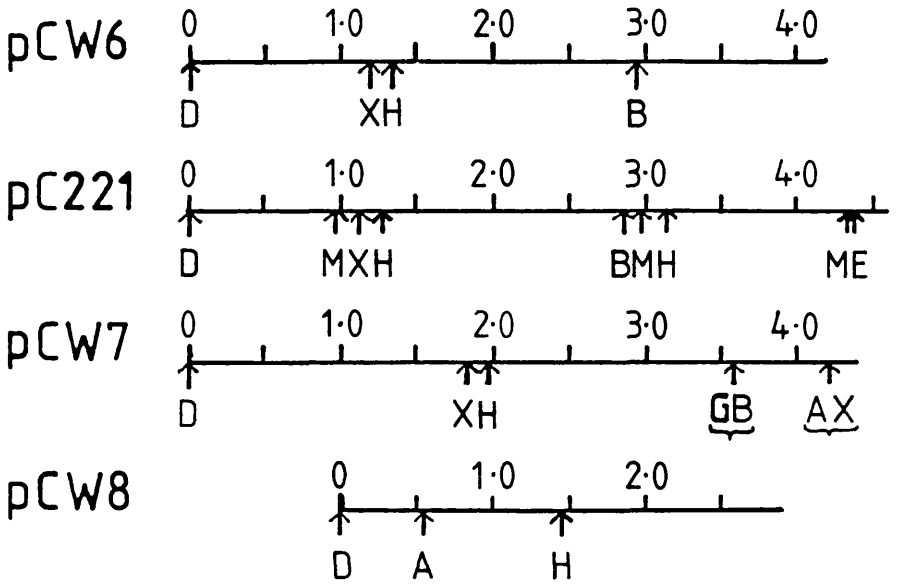


Fig. 4.1 Restriction maps of pCW6, pCW7, pCW8 and pC221

Restriction maps of pCW6 (4.2 kb), pC221 (4.6 kb), pCW7 (4.4 kb) and pCW8 (2.9 kb), modified from those constructed by Wilson and Baldwin (1978) using data from Horinouchi and Weisblum (1982) and Chapters 3 & 5 of this work. The position of the XbaI site shown for pC221 is from the nucleotide sequence of pCW41 (Hawkins et al., unpublished; App. 1); the presence of additional XbaI sites has not been investigated. MboI sites are only shown for pC221. The numbers indicate the distance (in kb) from the HindIII site (0).

Key:					
A	HaeIII	E	EcoRI	M	MboI
B	BstEII	G	BglII	X	XbaI
D	HindIII	H	HpaII		

Figs. 4.2-4.9

Electrophoresis was overnight (12 to 18 hr) at 15 to 20 V on 1% agarose in TAE except for the gels shown in figs. 4.5, 4.8 and 4.9. Size-markers used (see section 2.6.2, p. 31) were (in kb): λ/HindIII, 23.1, 9.4, 6.8, 4.36, 2.32, 2.03, 0.564; pBR322/TaqI, 1.444, 1.307, 0.475, 0.368, 0.315 and 0.312, 0.141; pBR322, 4.36; pSES3 , 6.4 (Chapter 7).

Fig. 4.2 Tracks 1-5, mini-lysates of WS 2304 and four other isolates from the same transformation (section 4.3.1); 6, WS 2303 mini-lysate (pSES3).

Fig. 4.3 Tracks 1 and 8, purified pSES3; 2, WS 2305 mini-lysate; 3-7, mini-lysates of five other isolates from the same transformation (section 4.3.2).

Fig. 4.4 Track 1, WS 2306 mini-lysate; 2, mini-lysate of another isolate from the same transformation; (section 4.3.3); 3, pSES3.

Fig. 4.5 Track 1, EcoRI digest of WS 2306 mini-lysate; Tracks 2-11, HindIII digests of: 2, λ; 3, pBR322; 4, WS 2308 mini-lysate; 5, WS 2307 mini-lysate; 6, pCW8; 7, WS 2306 mini-lysate; 8, pCW7; 9, pBR322; 10, WS 2304 mini-lysate; 11, pC221. Electrophoresis was for 2.3 hr at 80 V on 0.8% agarose in TAE.

Fig. 4.6 HindIII digests of: 1, λ; 2, pBR322; 3, WS 2305 mini-lysate; 4-6, three other mini-lysates from the same transformation (section 4.3.2); 7, pCW6 (incomplete).

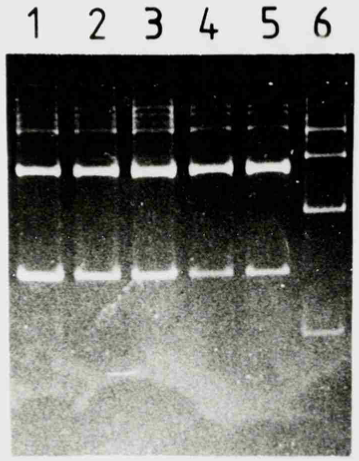
Fig. 4.7 Track 1, pSES3; 2-7, mini-lysates of isolates from the transformation described in section 4.3.4.

Fig. 4.8 Track 1, pBR322/TaqI (incomplete); 8, λ/HindIII; tracks 2-7, ClaI digests of mini-lysates of isolates from the transformation described in section 4.3.4; WS 2307 in track 7, WS 2308 in track 2. Electrophoresis was at 50 V for 4 hr on 1.2% agarose in TAE.

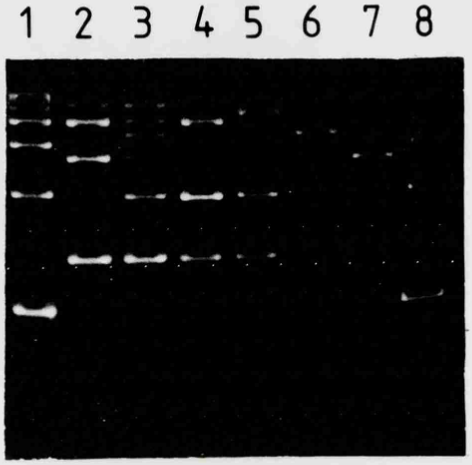
Fig. 4.9 Track 1, pSES3/ClaI and pBR322/ClaI; 6, pBR322/TaqI; 2-5, ClaI digests of mini-lysates of isolates from the transformation described in section 4.3.5; WS 2314 is in track 3, WS 2317 in track 5. Electrophoresis was for 2.6. hr at 70 V on 1.2% agarose in TAE.

Figs. 4·2 – 4·9

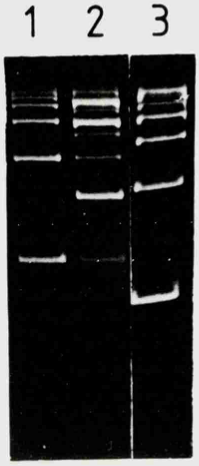
4·2



4·3

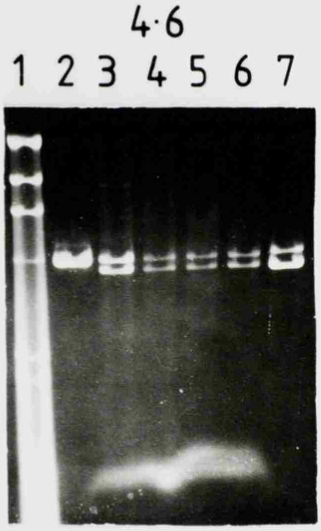


4·4

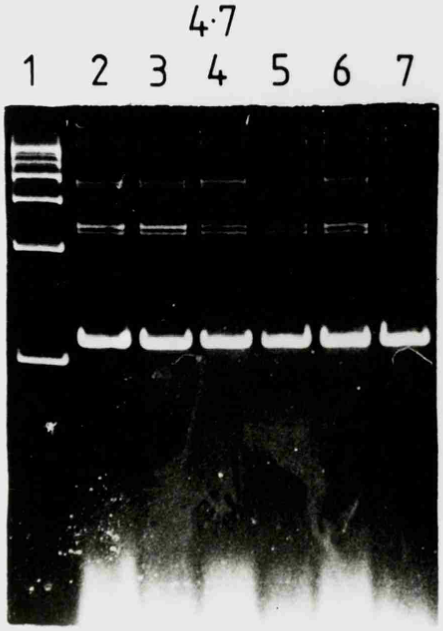


4·5

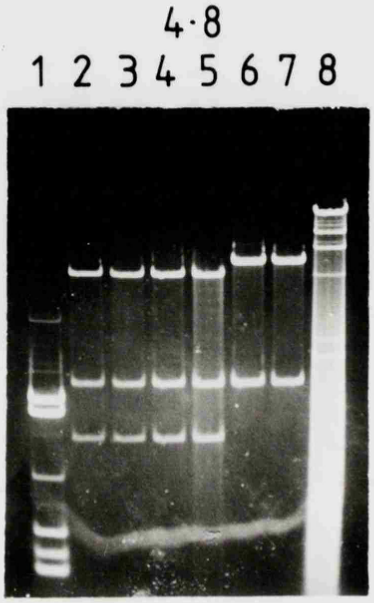
4·6



4·7



4·8



4·9

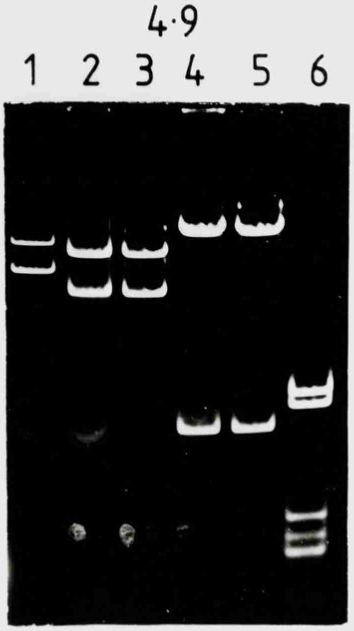


Fig. 4.10 Restriction maps of pSES4, 5, 6, 7, 8 and 14

The positions shown for the restriction sites are those predicted from the nucleotide sequences of pBR322 (Sutcliffe, 1979) and pC194 (Horinouchi and Weisblum, 1982) and the restriction maps of pC221, pCW6 and pCW7 (Wilson and Baldwin, 1978; Chapters 3 and 5, this work). The identity of the insert is indicated in parentheses in each case. The position and orientation of the coding-region for CAT is shown for the pC221 and pCW8 inserts (see text: orientation A, right to left; orientation B, left to right). In each case only part of the region derived from pBR322 is shown.

Key: ——— inserted Cm^R plasmid ═══ pBR322

Scale: 0 0·5 1·0 kb

A HaeIII (shown for pCW8 inserts only)

B BstEII

C ClaI

D HindIII

E EcoRI

H HpaII (shown for inserts only)

P PstI

Fig. 4·10

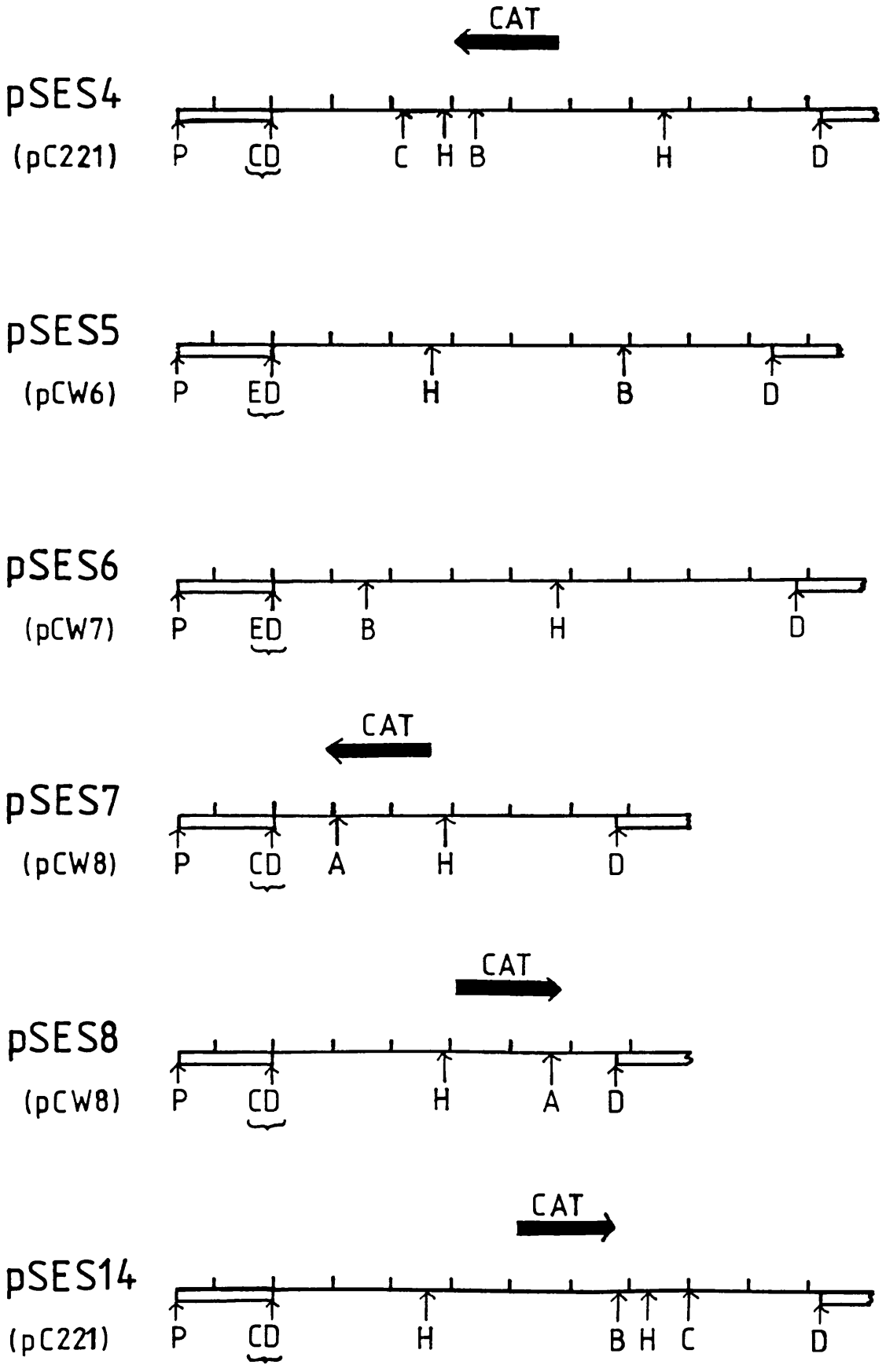

CAT
pSES4
(pC221)
P
CD
C
H
B
H
D
pSES5
(pCW6)
P
ED
H
B
D
pSES6
(pCW7)
P
ED
B
H
D
CAT
pSES7
(pCW8)
P
CD
A
H
D
CAT
pSES8
(pCW8)
P
CD
H
A
D
CAT
pSES14
(pC221)
P
CD
H
B
H
C
D

Fig. 4·11

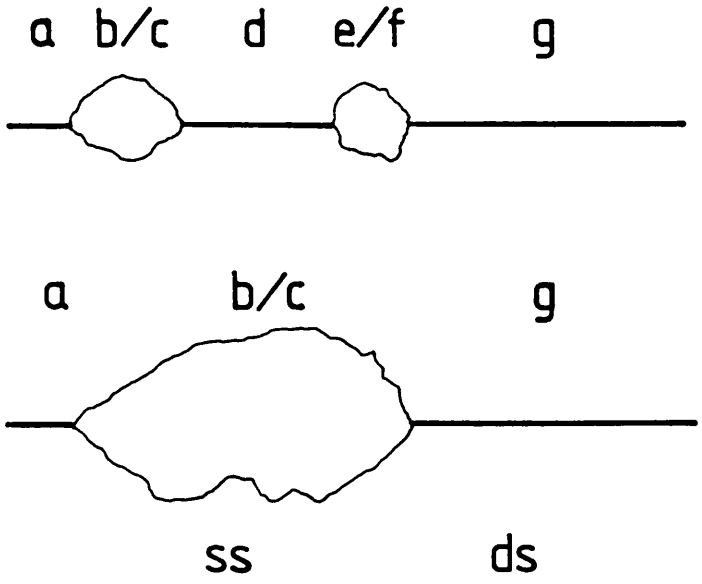


Fig 4.11 shows sketches of the two types of molecules examined in the heteroduplex experiments described in section 4.3.6. Measurements of segments a-g appear in table 4.5. Plasmids were linearised with PstI before hybridisation and electronmicroscopy (described in section 4.2.3), thus the ds regions (thicker lines) at either end (segments a and g) are sequences derived from pBR322, while segments b and c (and d, e and f, if observed) are from the inserts.

Fig. 4·12

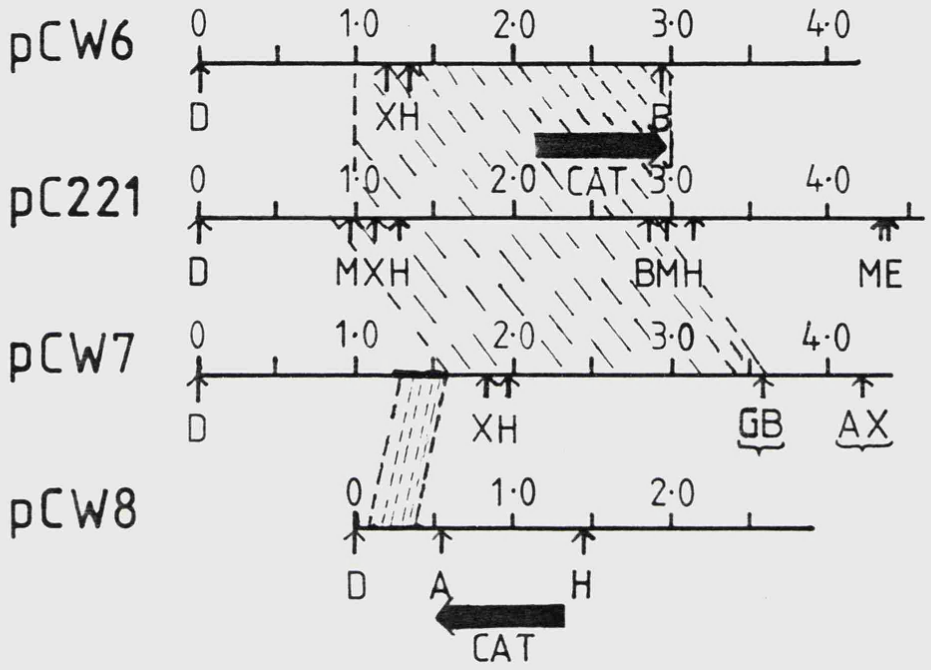


Fig. 4.12 Regions of homology between Cm^R plasmids.

The regions of homology deduced from the results of heteroduplex experiments (section 4.3.6) are shown as shaded areas plotted against restriction maps of pCW6, pC221, pCW7 and pCW8 (references as for fig. 4.1). The position and orientation of the coding-region for CAT are shown for pC221 and pCW8 (inferred from the nucleotide sequences of pCW41, Hawkins et al., unpublished, see App. 1, and pC194, Horinouchi and Weisblum, 1982). Numbers indicate the distance (in kb) from the HindIII site.

Key: A HaeIII E EcoRI M MboI

B BstEII G BglII X XbaI

D HindIII H HpaII

Fig.4·13

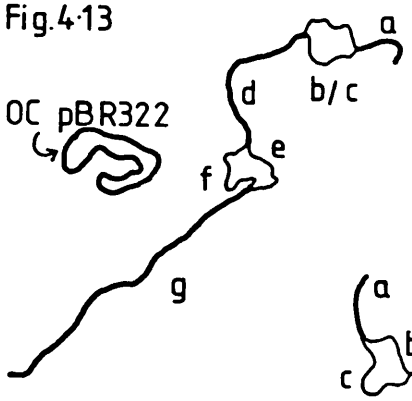
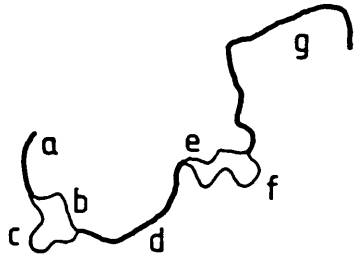


Fig.4·14



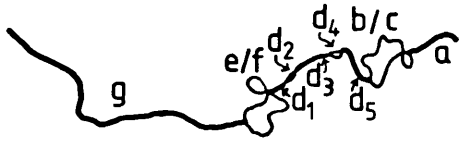


Fig.4·15

Fig.4·16

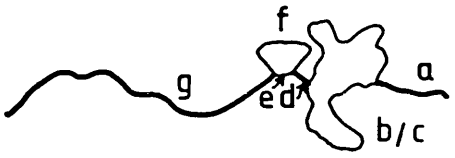


Fig.4·17

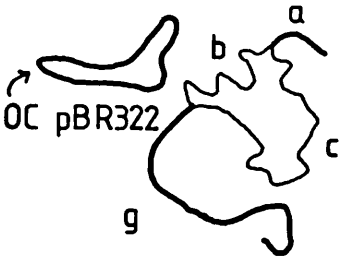
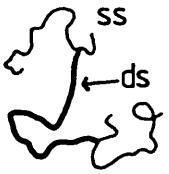


Fig.4·18



Figs. 4.13-4.18 Electronmicroscopy of heteroduplexes

Heteroduplexes of PstI-digested plasmids were prepared as described in section 4.2.3. The letters on the sketches above refer to the measurements of the different segments (given in tables 4.5 and 4.6). In figs. 4.13 and 4.17 the size-marker for ds DNA, OC pBR322 (4362 bp), is also visible. Fig. 4.18 is at a lower magnification than the others.

The pairs of plasmids are as follows:-

4.13	pSES5 x pSES14
4.14 and 4.15	pSES6 x pSES4
4.16	pSES6 x pSES8
4.17	pSES4 x pSES7
4.18	pSES4 x pSES14

Fig. 4·13

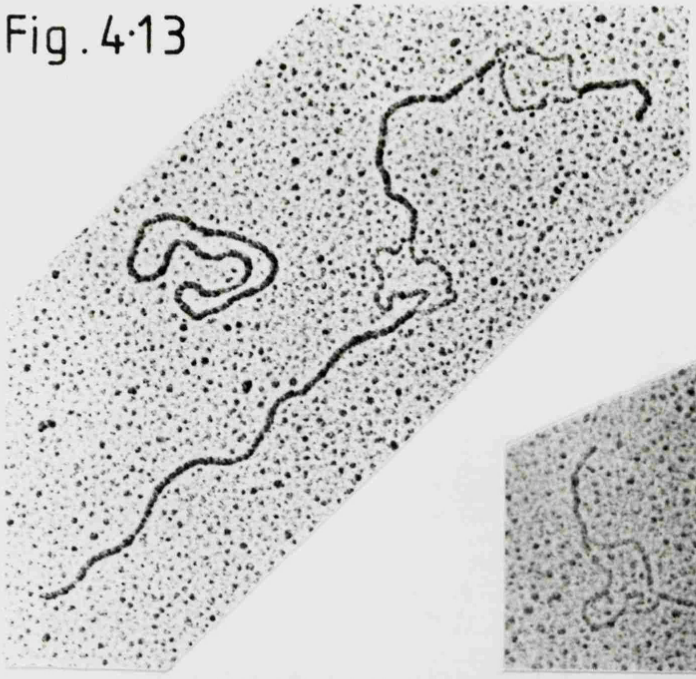
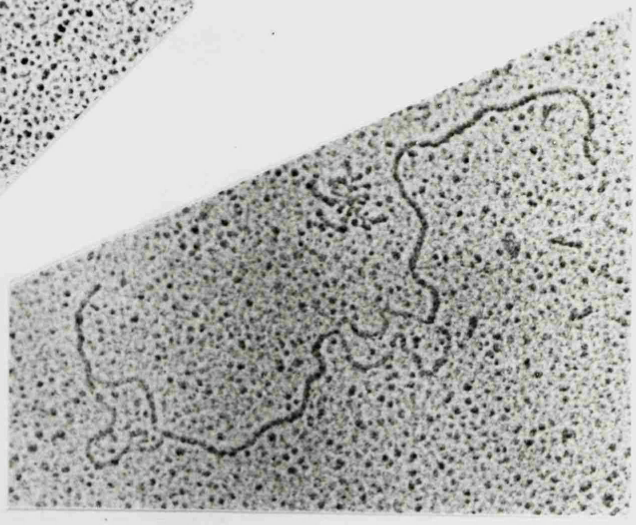


Fig. 4·14



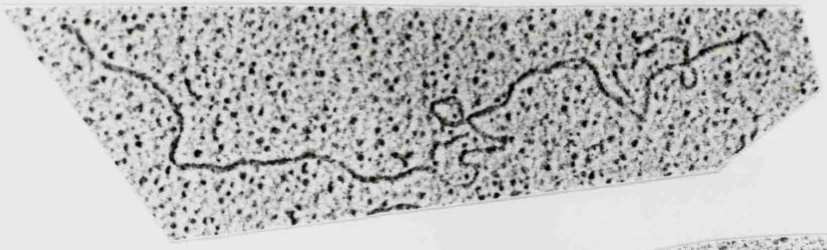


Fig. 4·15

Fig. 4·16

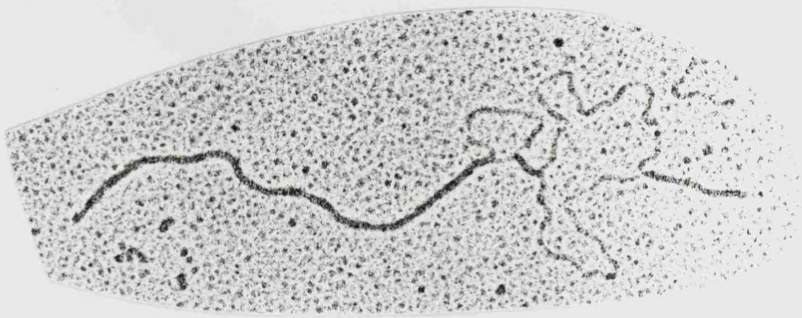


Fig. 4·17

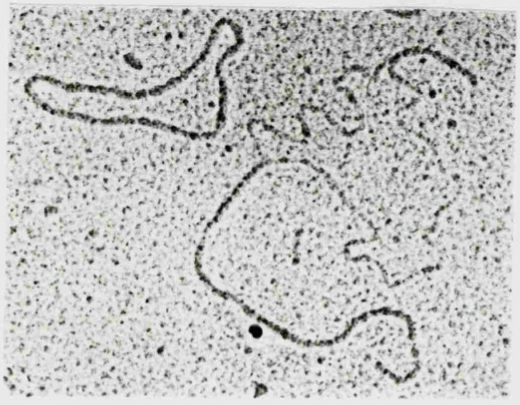


Fig. 4·18

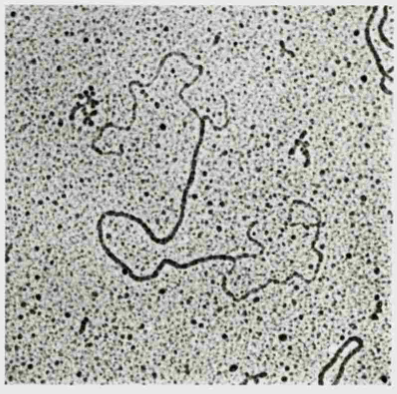


Table 4.1 Plasmids used in Chapter 4.

Plasmid	Insert	Orientation of insert	Strains
pBR322	--	--	WS 2302
pSES4	pC221	A	WS 2304 WS 2317
pSES5	pCW6	B	WS 2305
pSES6	pCW7	A	WS 2306 WS 2318
pSES7	pCW8	A	WS 2307 WS 2319
pSES8	pCW8	B	WS 2308 WS 2320
pSES14	pC221	B	WS 2314

With the exception of pBR322 (Bolivar et al., 1977), the construction of these plasmids is described in this chapter. The orientation is that inferred for the cat gene (see text). WS 2301 is the host strain in each case.

Notes to table 4.2

Procedures are explained in section 4.2.1

a) All Cm^R transformants tested were Ap^R and resembled WS 2301 in their nutritional requirements.

b) "n.t." indicates not tested.

c) These strains were very weakly resistant to Tc (10 μg/ml) compared with WS 2301 as the sensitive control and gave variable results.

Table 4.2 Transformation of WS 2301

Expt	DNA	selection	colonies per 0.1 ml	no. Tc^S per no. tested[a]
1	--	Cm10	0	--
	pBR322	Ap40	4×10^5	n.t.[b]
	pBR322	Cm10	300	0/6
	+pC221	Cm25	20	5/6
	pBR322	Cm10	144	[c]
	+pCW6	Cm25	27	
	pBR322	Cm10	145	4/6
	+pCW7	Cm25	20	5/6
	pBR322	Cm10	252	6/6
	+pCW8	Cm25	125	3/6
2	--	Cm10	7	n.t.
	pBR322	Tc20	4×10^4	n.t.
	pSES4	Cm10	1×10^4	n.t.
	re-ligated	Cm10	550	7/11
	pSES4	Cm25	0	--

Table 4.3 Restriction analysis of pCW6-pBR322 hybrids.

Isolates	Approx. sizes (kb) of restriction fragments	
	EcoRI	EcoRI + BstEII
A, C, D, E	8.8, 13	1.3, 4.2, ≥7
B	8.8, 13	2.8, 4.2, 5.7, >7[a]
F (WS 2305)	8.8	2.8, 5.7

Notes: a) A 1.3 kb fragment may not have been observed due to insufficient DNA.

HindIII digestion of mini-lysates of isolates A, B, C and F (not done for D and E) gave only 4.2 and 4.4 kb fragments (fig. 4.6).

Table 4.4 Restriction analysis of pCW7-pBR322 hybrids.

Isolates	Approx. sizes (kb) of restriction fragments	
	EcoRI	EcoRI + BstEII
A, B, C, D	9, 13[a], 18	0.8, 4.4, ~8
E, F, G[b]	9	0.8, ~8

Notes: a) This fragment was the major product

b) Isolate G was WS 2306

HindIII fragments for WS 2306 and isolate A co-migrated with those of pCW7 and pBR322.

Notes to table 4.5

Measurements of contour lengths of heteroduplexes between the pairs of plasmids indicated were converted to kb as described in 5.2.3. Columns a-g represent the segments of the structures as indicated in fig. 4.11. The figures in parentheses are the standard deviations of the measurements.

Table 4.5 Measurements of heteroduplexes

Pairs of plasmids	Size (kb) of segment						
	a	b	c	d	e	f	g
pSES5x pSES4	0.75 (0.06)	4.23 (0.21)	4.70 (0.18)	-	-	-	3.56 (0.09)
pSES5x pSES14	0.73 (0.03)	1.07 (0.09)		2.04 (0.07)	1.11 (0.07)	1.61 (0.09)	3.60 (0.11)
pSES5x pSES7	0.76 (0.03)	2.92 (0.10)	4.31 (0.23)	-	-	-	3.57 (0.08)
pSES5x pSES8	0.77 (0.03)	2.89 (0.20)	4.45 (0.19)	-	-	-	3.56 (0.09)
pSES6x pSES4	0.75 (0.05)	0.76 (0.09)	1.68 (0.16)	2.15 (0.13)	0.95 (0.21)	1.73 (0.16)	3.62 (0.09)
pSES6x pSES14	0.75 (0.03)	4.40 (0.23)		-	-	-	3.52 (0.07)
pSES6x pSES7	0.73 (0.05)	2.87 (0.17)	4.39 (0.24)	-	-	-	3.54 (0.13)
pSES6x pSES8	0.74 (0.02)	2.71 (0.11)		0.28 (0.03)	0.12 (0.03)	1.31 (0.07)	3.57 (0.09)
pSES4x pSES14	0.76 (0.04)	4.69 (0.23)		-	-	-	3.69 (0.10)
pSES4x pSES7	0.71 (0.04)	2.94 (0.31)	4.65 (0.31)	-	-	-	3.54 (0.18)
pSES4x pSES8	0.71 (0.04)	2.94 (0.12)	4.66 (0.32)	-	-	-	3.47 (0.08)
pSES7x pSES8	0.75 (0.03)	2.90 (0.20)		-	-	-	3.55 (0.11)

Table 4.6 Measurements of detail within segment "d" for pSES6 X pSES4.

Sizes (kb) of segments					Total (kb)
d_1	d_2	d_3	d_4	d_5	
0.47	0.17	0.30	0.19	0.86	2.0
(0.13)	(0.06)	(0.08)	(0.04)	(0.11)	(0.4)

Measurements were obtained from 10 heteroduplexes (pSES6 X pSES4) in which bubbles of homology were observed in segment "d" e.g. fig. 4.15. See also notes to table 4.5.

CHAPTER 5

RESTRICTION MAPS OF pC221 AND pCW7

5.1 Introduction

To facilitate further studies on pC221 such as the construction of deletions *in vitro* and the determination of its nucleotide sequence it was decided to construct a map of the cleavage sites of various restriction enzymes. The restriction maps of pC221 and pCW7 constructed by Wilson and Baldwin (1978) (upon which those in fig. 4.1 were based) had revealed a similar pattern of sites in one region of the plasmids. Construction of a detailed restriction map was therefore also undertaken for pCW7, for comparison. Further information about restriction sites on pCW7 might also be useful in the construction of Gram-positive cloning vehicles utilising the Cm^R gene of pCW7, which has been shown to be a target for insertional inactivation at the unique *Bst*EII and (more versatile) *Bgl*II sites (Wilson *et al.*, 1981).

Although maps of the cleavage sites of restriction endonucleases which cut a plasmid once or twice can be built up rapidly from the results of double digests (i.e. incubation of DNA with two restriction enzymes simultaneously or sequentially), this method may not always provide sufficient information to distinguish between possible arrangements of three or more fragments generated by the same enzyme. Analysis of partial digests is possible but may depend on the highly accurate measurement of sizes of restriction fragments over a wide size-range. The method of Smith and Birnstiel (1976) is often more convenient and provides, in addition, the relative order of sites for the various restriction enzymes. In this technique partial digests are performed on linear DNA which is radio-labelled at only one

end, so that after gel electrophoresis, the sizes of the fragments visualised by autoradiography provide the distance of the corresponding restriction sites from the labelled terminus.

5.2 Methods

Purification of staphylococcal plasmid DNA (from WS 2007 and WS 2009), restriction digests and agarose gel electrophoresis (using TAE or TBE buffer) etc. were as described in Chapter 2 (sections 2.5, 2.6 and 2.8). The fragments of pCW7 used for the Smith-Birnstiel procedure were purified by Dr. C. R. Wilson (up to and including extraction from the gel slices as described in 5.2.1).

5.2.1 Extraction of DNA fragments from gel slices by the syringe method.

This procedure, which was based on that of Jeffreys et al. (1980), was used for the extraction of the pC221 and pCW7 used for the Smith-Birnstiel procedure (5.2.2 - 5.2.4). The slice of agarose gel containing the required DNA fragment was broken up by repeated passage through a disposable syringe (Becto-Dickison) in 5 to 10 vols of water until the particles of gel were sufficiently small to remain in suspension for at least 10 min. The suspension was incubated overnight at 5^{o}, then most of the agarose was removed by centrifugation (10 min at 18,000 rpm, MSE 18, 8 x 50 ml rotor, 4^{o}). The volume of the supernatant was reduced to about 400 μl by repeated extractions with iso-butyl alcohol. After centrifugation in a microfuge to remove more agarose (which tended to come out of suspension during the concentration steps) the DNA was precipitated with ethanol, and TE buffer was added to dissolve the DNA pellet. Yields were 30 to 50% when about 50 μg of linear plasmid DNA had been applied to the preparative gel.

5.2.2 Labelling the 5'-termini of DNA fragments.

The procedure used was that of Smith and Birnstiel (1976) with the modifications of Carlton and Brown (1979). DNA (10 pmol of 5'-termini) in 20 μl of 10 mM Tris-HCl (pH 7.6) containing 10 mM magnesium chloride and 5 mM DTT was incubated with 1 U of bacterial alkaline phosphatase (Bethesda Research Labs) for 30 min at 65^{o} to remove the 5'-phosphate groups. After transfer to ice, 2 μl of 10 mM sodium phosphate buffer (pH 7.6) (to inhibit the phosphatase) and 1 μl of 1 M NaCl were mixed in thoroughly. The solution was transferred to a tube in which 20 μCi of [γ-^{32}P]ATP (5 to 25 pmol, 3000 Ci/mmol on reference date; Radiochemical Centre, Amersham) had been dried down *in vacuo*. After thorough mixing to dissolve the ATP, the whole was incubated with 3 to 5 U of T4 polynucleotide kinase (New England Biolabs) for 60 min at 37^{o}. After the kinase reaction had been inhibited by the addition of 20 μl of 3 M ammonium acetate, and 60 or 120 μg of phenol-extracted tRNA (*E.coli*, Sigma) had been added as carrier, the volume was made up to 100 μl with TE (7.6) buffer (Chapter 2, section 2.8.6). The solution was then extracted with 50 μl of phenol (saturated with 10 mM Tris-HCl, pH 8.0). The phenol layer was back-extracted with 100 μl of TE (7.6) and the unincorporated label was removed from the combined aqueous layers by passage through a 10 ml column of Sephadex G-50 equilibrated with TE (7.6): fractions of ten drops each (0.5 to 0.6 ml) were collected. Those fractions containing the peak of excluded material were located by liquid scintillation spectrophotometry in the absence of added

scintillant ("Cerenkow counting").

Samples (10 to 50 μl) of fractions were subjected to gel electrophoresis and autoradiography (as described in 5.2.3) to identify those fractions of the excluded peak (usually the first two) which contained the desired labelled fragments and were relatively free from unincorporated ATP and inorganic phosphate. The selected fractions were pooled, and concentrated by ethanol precipitation. The yields of useful radio-labelled DNA varied from 0.2 to 3 μCi depending on the substrate.

5.2.3 Digests of 5'-labelled DNA.

The procedure described in the previous section produces DNA fragments labelled at both 5'-termini; digestion with a second enzyme to give two fragments, each labelled at only one end, was necessary before partial digests were performed. Kinased linear plasmid was digested with the second enzyme as indicated: pC221/HindIII with EcoRI; pC221/EcoRI with HindIII; pCW7/HindIII with HaeIII; pCW7/BstEII with BglII. In each case the second enzyme cleaved the linear plasmid 0.2 kb (pC221) or less (pCW7) from one (labelled) end. To avoid incurring losses in purification steps it was decided to use the unfractionated mixtures for partial digests.

It was necessary to check that the second enzyme had cut to completion. Samples of the DNAs were taken after incubation with the second enzyme and digested completely with MboI (pC221) or HpaII (pCW7). In some cases (see 5.3.2 and 5.4.1), even after repeated redigestion by the second enzyme about 10% of the DNA remained uncut; this had to be taken into account when

interpreting the data.

5.2.4 Partial digests of 5'-labelled DNA.

Partial digests were performed using 1 to 2 μg of DNA (30,000 to 70,000 Cerenkow cpm) for frequently cutting enzymes, e.g. AluI (0.1 or 0.25 U), HinfI (0.1 U), TaqI (1.4 to 2.0 U). The amount of DNA was halved for enzymes which had fewer sites, e.g. MboI (0.04 U). For total digests, 1 to 2 U of enzyme were incubated with 0.5 μg of DNA in 60 μl for at least 60 min. After the addition of the enzyme (the last component added) on ice and brief mixing, 12 μl aliquots were incubated at 37° (65° for TaqI) for 2, 5, 10, 30 or 60 min. The reactions were stopped by the addition of 3 μl of stop mix (5% SDS, w/v; 50% glycerol, v/v; 0.025% bromophenol blue, w/v) and heated at 65° for 10 to 15 min, except for TaqI incubations which were chilled in an ice-bath as soon as the stop mix had been thoroughly mixed in.

Following the procedure of Carlton and Brown (1979) the samples incubated for different times with the same enzyme were pooled before electrophoresis. The pooled digests were usually divided equally between low (0.7 to 1.0) % and high (1.6 to 2.2) % agarose gels (in TAE) and size-markers (λ/HindIII, pBR322/TaqI, pBR322/AluI, labelled as in 4.2.2) were also loaded on the gels. After electrophoresis the gels were dried onto DE81 paper (Whatman) using absorbent paper (as described by Smith and Birnstiel, 1976). Autoradiography was at room temperature under weights for 14 hr to 12 days (as necessary) on Kodak "No-Screen" 5T film.

5.2.5 Size-markers for DNA fragments.

In addition to the size-markers already described in Chapter 2 (section 2.6.2), for some of the experiments in sections 5.3.1 and 5.3.3 HindIII-digested PM2 was used as the source of size-markers. The values taken for the sizes were determined by comparison with digests of λ and pBR322 (not shown), and were 0.27, 0.425, 0.45, 1.05, 2.2 and 5.2 kb. These sizes agree closely with the mean of those published by Parker et al. (1977) and Kovacic and Wang (1979).

5.3 Restriction map of pC221

5.3.1 Mapping the MboI sites of pC221.

The results of HpaII + BstEII double digests and a triple (HpaII + BstEII + HindIII) digest of pC221 (not shown) were consistent with the map constructed by Wilson and Baldwin (1978, see fig. 4.1). The data presented in table 5.1 were used to establish the positions of the three MboI sites (fig. 5.1). The use of revised sizes for the marker digests of λ and PM2 (see 2.6.2 and 5.2.5) led to a revised estimate of the total size of pC221; 4.6 kb, rather than 4.4 kb as reported in Wilson et al. (1981). The map in fig. 5.1 was used as a starting-point for interpreting the data presented in the following sections.

5.3.2 Smith-Birnstiel mapping of pC221.

Initial attempts to label linear pC221 using kinase and [γ-^{32}P]ATP were frustrated by the incorporation of the ^{32}P into small ($\leq$0.2 kb) fragments of nucleic acids present in the preparation, rather than into the full-sized pC221. In the experiments described here the linear DNA was extracted from preparative agarose gels as described in section 5.2.1 to select for the required size-class before proceeding to the phosphatase and kinase reactions.

Linear pC221, cut and labelled at the EcoRI site, was subjected to digestion by HindIII. Fig. 5.2(a) shows a sample from this digest after incubation with BstEII. The faint band (arrowed) in track 2 represents the proportion of pC221 uncut by HindIII (judged by eye to be about 5%); molecules cut by HindIII give the smaller EcoRI/HindIII fragment (0.2 kb). Other

samples were subjected to partial digestion by AluI, HinfI, MboI or TaqI; autoradiograms are shown in figs. 5.2(b) and (c). The strong band (0.2 kb) is the smaller EcoRI/HindIII fragment of pC221, which would obscure any other bands having approximately the same mobility. Similarly, bands corresponding to sites just clockwise of the HindIII site would not be resolved from the band produced by the larger EcoRI/HindIII fragment. The restriction map shown in fig. 5.3(a) is derived from measurements of autoradiograms from this experiment.

Faint bands on the autoradiograms (dotted arrows on the diagram, fig. 5.3(a)) could have been caused either by cutting of the fraction of the material (approximately 5%) which was labelled at both ends (in which case the size of the radioactive fragment observed would correspond to the distance of a site clockwise from the EcoRI site) or by different susceptibilities of restriction sites to a given enzyme. Berkner and Folk, 1983, reported that the EcoRI sites of λ were cleaved at different rates. Similarly the autoradiograms presented by Smith and Birnstiel, 1976, who purified fragments labelled at only one end, show bands of different intensities in a given track.

Fig. 5.2(c) shows a band in track 5 corresponding to a HinfI site about 0.14 kb from the EcoRI site. As the intensity of the band at 0.2 kb (produced by the smaller HindIII/EcoRI fragment of pC221) is significantly less in this track than in the others, this would indicate that this HinfI site lies within the 0.2 kb fragment, about 0.14 kb from the labelled (EcoRI) end.

To confirm these results, and to obtain better resolution

of the sites from 0 to 1 kb on the map, pC221 was cut and labelled at the HindIII site, then subjected to digestion by EcoRI. Samples of this material were subjected to partial digestion by AluI, HinfI, MboI or TaqI (fig. 5.2(d)). Some of the faint bands (for example, at 1.6 kb in track 2) could have been due to the presence of material uncut by EcoRI (about 10% of the total in this preparation, data not shown). The map shown in fig. 5.3(b) was constructed using measurements of autoradiograms from this and a similar experiment.

5.3.3 Digests of unlabelled pC221.

Tables 5.2 to 5.5 summarise the data obtained from various restriction digests of pC221 and pCW41 (the largest MboI fragment of pC221, Wilson et al., 1981). Figs. 5.4(a) - (c) show examples of the gels from which the measurements were made. In addition, the sizes of the products of partial digestion of pCW41 by HinfI were determined (table 5.6) in order to differentiate between two possible arrangements of HinfI fragments. From the data in tables 5.2 to 5.6 many restriction fragments could be aligned with respect to the restriction sites shown in fig. 5.1, generating the partial restriction maps shown in fig. 5.5. Comparison with the maps shown in fig. 5.3 resolved the remaining ambiguities. Fig. 5.6 shows the restriction map of pC221 most consistent with the combined data.

5.4 Restriction map of pCW7

5.4.1 Smith-Birnstiel mapping of pCW7.

The map of pCW7 constructed by Wilson and Baldwin (1978) and re-drawn in fig. 5.7 was used as a starting-point for the construction of a more detailed restriction map. Linear pCW7 was purified by preparative agarose gel electrophoresis (and extracted as in 5.2.1) before being end-labelled (5.2.2).

HindIII-digested pCW7 was end-labelled with ^{32}P then cut with HaeIII. Samples of this material were subjected to total digestion by AluI, HinfI, MboI or TaqI, and analysed by gel electrophoresis and autoradiography (figs. 5.8(a) and (b)). The absence of a band in the HpaII track (track 8) at 2.3 to 2.4 kb indicated that the HaeIII digest was essentially (>95%) complete. Incomplete digestion by HaeIII would also have led to bands at 0.7 to 0.8 kb in BglII and BstEII tracks (tracks 5 and 6). Although bands are visible in these tracks at about 0.74 kb there are other faint bands in these and other tracks, including material migrating more slowly than linear pCW7 (particularly noticeable in track 9 of fig. 5.8(a)). This suggests that, despite preparative agarose gel electrophoresis prior to the labelling reactions, this preparation of HindIII-cut pCW7 was still contaminated by other DNA.

Data from this experiment were used to construct the restriction map shown in fig. 5.9(a). (A total size of 4.4 kb, indicated by the data in section 5.4.2, was used for pCW7 in this and the following maps.) It should be noted that the band at about 0.16 kb, produced by the smaller HindIII/HaeIII fragment,

would obscure any other bands in this part of the autoradiograms.

To establish the order of the sites between 3.6 and 4.4 kb, and for confirmation of the above data, pCW7 was cut and labelled at the BstEII site and then cut with BglII. Samples of this material were subjected to partial digestion (as in the previous experiment) or to total digestion by HaeIII, HindIII or HpaII (figs. 5.8(c) and (d)). As is indicated by the presence of faint bands at about 3.55, 3.45 and 1.4 kb in tracks 5, 6 and 8 of fig. 5.8(c) (produced by incubation with HaeIII, HindIII and HpaII respectively) about 10% of the BstEII-cut pCW7 remained uncut by BglII. This means that some of the faint bands in the other tracks may also be derived from molecules labelled at both 5'-termini. In addition, this preparation of linear pCW7, like that described above, appeared to be contaminated by material other than BstEII-cut pCW7.

The map shown in fig. 5.9(b) was constructed from measurements of the autoradiograms in figs. 5.8(c) and (d). The AluI site in the HindIII recognition sequence must be a poor substrate for AluI relative to the other sites for this enzyme on pCW7, as the relevant band (at about 0.8 kb in track 4 of figs. 5.8(c) and (d)) is faint. This site was also cut slowly in AluI digests of unlabelled pCW7 (fig. 5.4(d)). A similar phenomenon was observed for an AluI site in pC221 (section 5.3.2).

5.4.2 Digests of unlabelled pCW7.

Tables 5.7 to 5.10 show data from digestion of pCW7 by various combinations of restriction enzymes. Examples of the gels from which measurements were made are shown in figs. 5.4(d)-

(f). From these data partial restriction maps could be drawn for AluI, HinfI and MboI (fig. 5.10). The positions of TaqI fragments B, D and G relative to the unique restriction sites could also be inferred (not shown).

Comparison of these partial maps with the maps in figs. 5.9 (a) and (b) resolved most of the ambiguities. The presence of restriction sites at positions on pCW7 corresponding to many of the faint bands visible on the autoradiograms (section 5.4.1) was ruled out by the double-digest data. The positions of some AluI and TaqI sites were confirmed by experiments (not shown) using pCW59 (table 3.2 in Chapter 3; Wilson et al., 1981) cut and labelled at the BglII site, then cut with BstEII before being subjected to partial digests (as in section 5.4.1). The map of pCW7 most consistent with the data is shown in fig. 5.11. The positions of HinfI fragments F, G and H, and TaqI fragments H - M are somewhat arbitrary owing to their small size.

5.5 Conclusions and discussion

Fig. 5.6 shows the map of pC221 most consistent with the data from Smith-Birnstiel mapping and double-digest experiments. Total sizes of 2.0 and 4.6 kb were obtained for pCW41 and pC221 respectively, somewhat larger than those previously reported (1.8 and 4.4 kb, Wilson et al., 1981) owing to the use of different sizes for the standards. The total size found for pCW7, 4.4 kb, was also larger than that of 4.2 kb reported in the same paper, and the order of some restriction fragments on the map (fig. 5.11) was also revised.

Comparison of the detailed restriction maps of pC221 and pCW7 (figs. 5.6 and 5.11) revealed similarities between them, in addition to those already discussed in the Introduction to Chapter 4. The distances between the MboI and HpaII sites (0.98 to 1.28 kb on pC221; 1.69 to 2.0 kb on pCW7) are nearly identical. The HinfI and TaqI sites between them (at about 1.18 kb in pC221; 1.87 kb in pCW7) are in approximately the same positions relative to these sites. A TaqI site lies 0.8 kb anticlockwise of the BstEII site on both (at 2.81 kb on pCW7; 2.08 kb on pC221); similarly, the possible HinfI site at 3.45 kb (pCW7) would be equivalent to that at 2.72 kb on pC221. AluI fragment F of pCW7 (2.18 to 2.35 kb) appears to correspond with AluI fragment G of pC221 (1.49 to 1.65 kb) in size, and position relative to the BstEII site (1.23 or 1.26 kb anticlockwise of it). It should be noted, however, that the MboI site included in the BglII site of pCW7 (3.56 kb) is not equivalent to that at 2.98 kb on the map of pC221.

Thus, although some restriction sites are shared by the two plasmids there are many differences even in the regions which appeared very similar on the preliminary restriction maps (Wilson and Baldwin, 1978) and which are contained in a 2 kb sequence partially homologous by the criterion of electronmicroscopy of heteroduplexes (Chapter 4).

Fig. 5.1 Restriction map of pC221

The data shown in table 5.1 were used to map the MboI sites of pC221 and revise the map of pC221 constructed by Wilson and Baldwin (1978). Numbers give the distance (in kb) from the HindIII site (0).

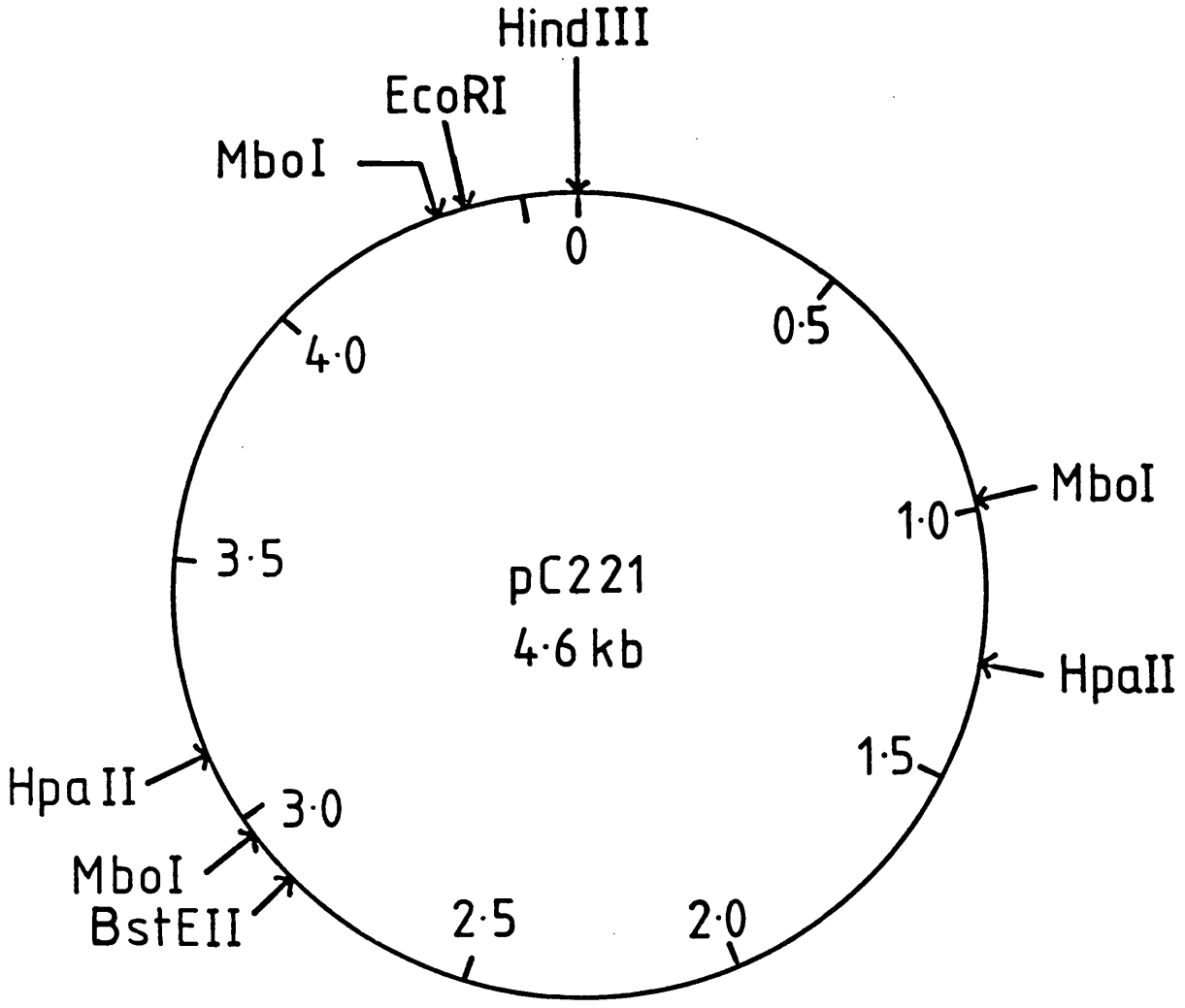


Fig. 5.2 Autoradiograms from pC221 mapping experiments

Procedures are described in section 5.2. Some faint bands have been lost during photographic reproduction. Size-markers were (in kb):

λ/HindIII, 23.1, 9.4, 6.8, 4.36, 2.32, 2.03, 0.564;
pBR322/TaqI, 1.444, 1.307, 0.616, 0.368, 0.315 and 0.312;
pBR322/AluI, 0.91, 0.659 and 0.655, 0.521, 0.403, 0.281, 0.257, 0.226, 0.136 and smaller.

(a), (b) and (c): EcoRI-digested pC221 was end-labelled with ^{32}P and cut with HindIII (referred to as pC221/*EcoRI/HindIII).

a) Track 1, pC221/*EcoRI/HindIII; 2, BstEII digest of material in track 1; 3, pBR322/TaqI. The arrow points to the band in track 2 which indicates the proportion of pC221 still uncut by HindIII. Electrophoresis was for 50 min. at 60 V then 35 min. at 90 V on 0.8% agarose in TAE.

b) Samples were: track 1, λ/HindIII; 2, pBR322/TaqI; 3, pC221/*EcoRI/HindIII; tracks 4-7, partial digests of pC221/*EcoRI/HindIII by AluI (track 4), HinfI (track 5), MboI (track 6) and TaqI (track 7). Electrophoresis was for 2 hr at 80 V on 0.8% agarose in TAE.

c) Track 1 was pBR322/AluI, tracks 2-7 as for b. Electrophoresis was for 2.5 hr at 60 V on 2.2% agarose in TAE.

d) HindIII-digested pC221 was end-labelled with ^{32}P and cut with EcoRI; a sample is shown in track 5. Tracks 1-4 contain partial digests of this material by TaqI (track 1), MboI (track 2), HinfI (track 3) and AluI (track 4). Track 5 contains pBR322/TaqI and track 6, λ/HindIII. Electrophoresis was for 3 hr at 60 V on 1% agarose in TAE.

Fig. 5·2

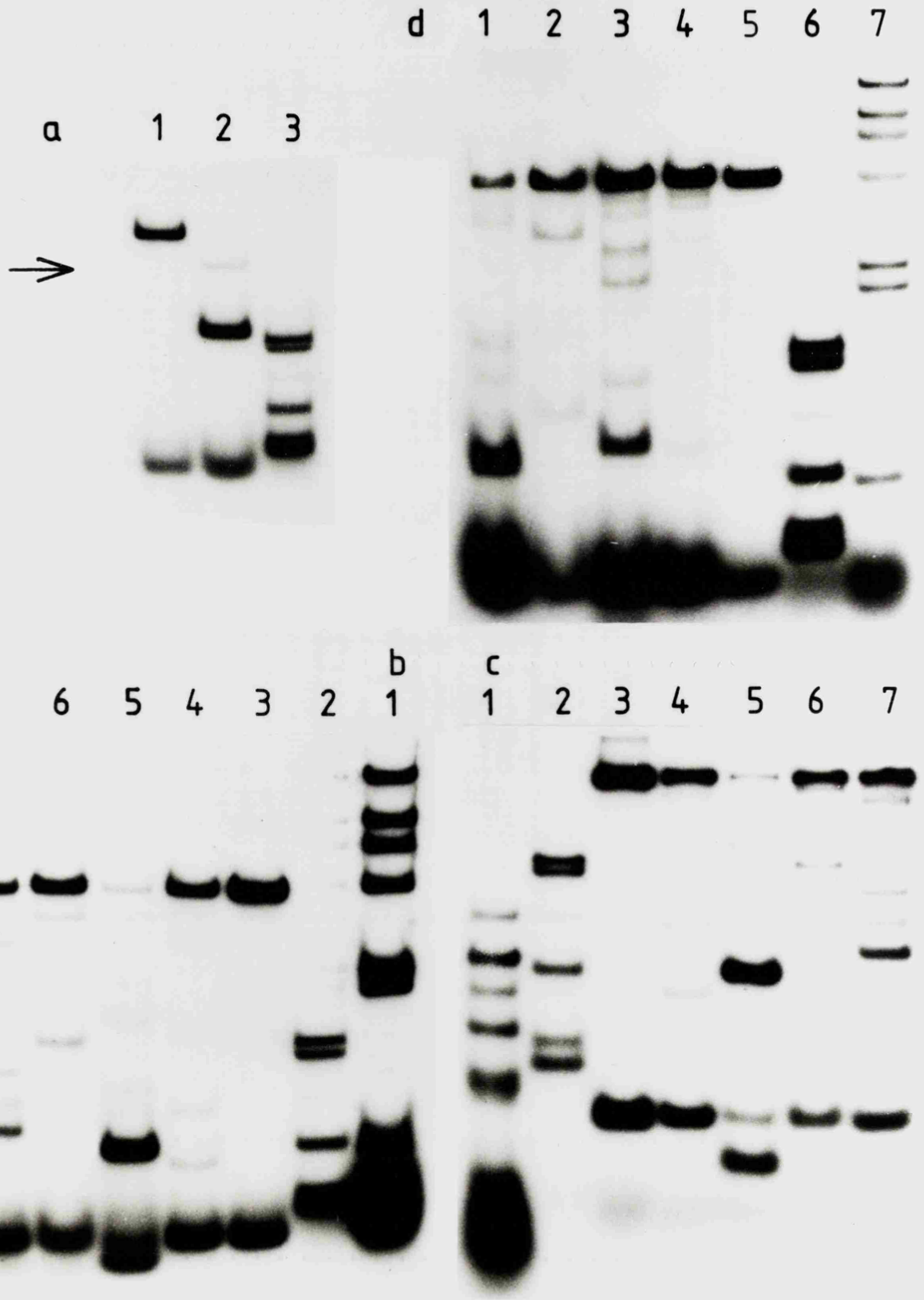


Fig. 5.3(a) and (b) Results of Smith-Birnstiel mapping of pC221
The mapping experiments are described in section 5.3.2. Dotted arrows correspond to relatively faint bands on the autoradiograms. Numbers indicate distances (in kb) from labelled termini (——————●).

Key: B BstEII L AluI

D HindIII M MboI

E EcoRI T TaqI

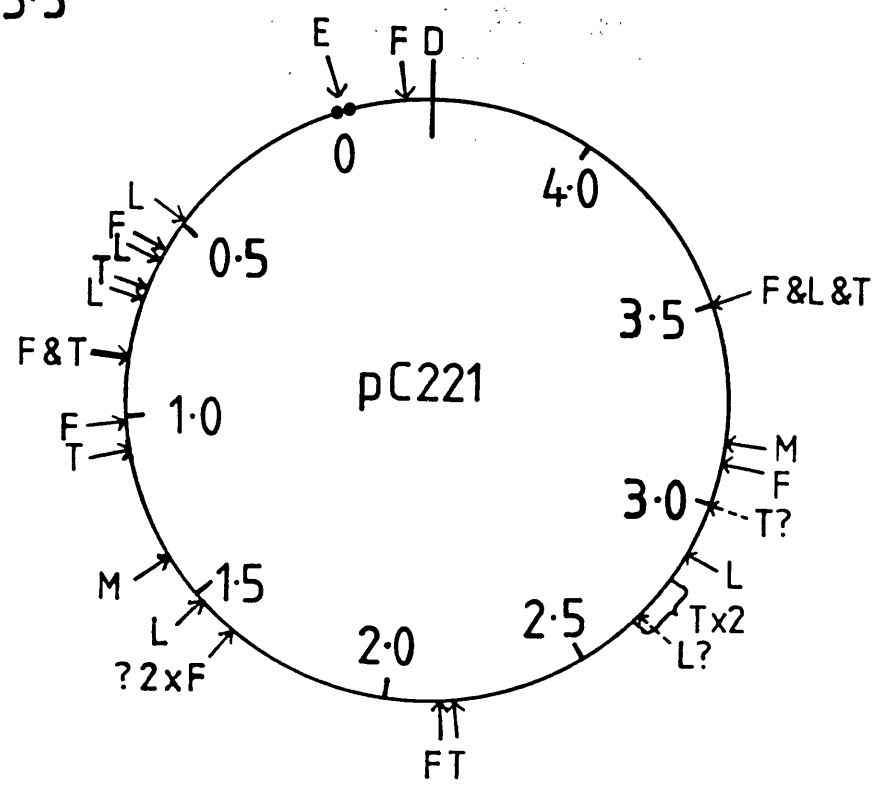
F HinfI

(a) Map constructed from experiments using EcoRI-digested pC221 end-labelled with ^{32}P then cut with HindIII before the digests indicated were performed (see fig. 5.2, (a), (b) and (c)). Distances are measured anticlockwise from the EcoRI site.

(b) Map constructed from experiments using pC221 cut and labelled at the HindIII site then digested by EcoRI before partial digestion by the enzymes indicated (see fig. 5.2 (d)). Distances are measured clockwise from the HindIII site.

Fig.5·3

a


E
F
D
0
4·0
0·5
3·5
F&L&T
pC221
F&T
1·0
3·0
M
F
T?
1·5
2·5
2·0
Tx2
L?
M
L
?2xF
FT

b

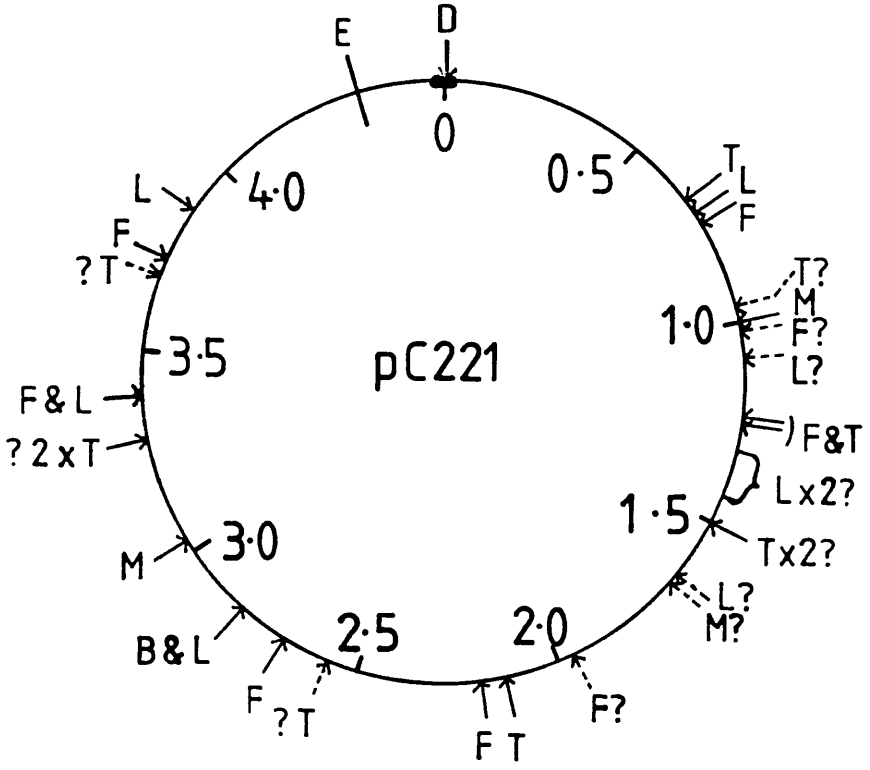

E
D
0
0·5
4·0
T
L
F
L
F
?T
T?
M
1·0
F?
3·5
pC221
L?
F&L
F&T
?2xT
L x2?
1·5
3·0
Tx2?
M
L?
M?
B&L
2·5
2·0
F
?T
F?
FT

Fig. 5.4 Restriction digests of pC221, pCW41 and pCW7

Sizes of markers (see sections 2.6.2 and 7.3.1) were (in kb):
λ/HindIII, 23.1, 9.4, 6.8, 4.36, 2.32, 2.03, 0.564;
pBR322/TaqI, 1.444, 1.307, 0.616, 0.368, 0.315 and 0.312;
pBR322/AluI, 0.91, 0.659 and 0.655, 0.521, 0.403, 0.281, 0.257, 0.226, 0.136, 0.10 and smaller.

(a) Samples were: 1, pBR322/TaqI; 2, pCW41/AluI; 3, pCW41/AluI/BstEII; 4, pCW41/AluI/HpaII; 5, pCW41/AluI/MboI; 6, pC221/AluI/MboI; 7, pC221/AluI/HpaII; 8, pC221/AluI/EcoRI; 9, pC221/AluI/BstEII; 10, pC221/AluI; 11, pBR322/AluI. The sodium chloride concentration in the samples on this gel was adjusted to 75 mM before loading. Electrophoresis was for 2.6 hr at 60 V on 1.8% agarose in TAE.

(b) Samples were: pBR322/AluI; 2, pC221/HinfI/BstEII; 3, pC221/HinfI/EcoRI; 4, pC221/HinfI/HindIII; 5, pC221/HinfI/HpaII; 6, pC221/HinfI/MboI; 7, pC221/HinfI; 8, pBR322/TaqI. Electrophoresis was for 2.5 hr at 60 V on 1.6% agarose in TAE.

(c) Samples were: 1, pBR322/TaqI; 2, pC221/TaqI; 3, pC221/TaqI/MboI; 4, pC221/TaqI/HpaII; 5, pC221/TaqI/HindIII; 6, pC221/TaqI/EcoRI; 7, pC221/TaqI/BstEII; 8, pBR322/AluI. Electrophoresis was for 2.5 hr at 60 V on 1.8% agarose in TAE.

(d) Samples were: 1, pBR322/AluI; 2, pCW7/AluI/HpaII; 3, pCW7/AluI/HaeIII; 4, pCW7/AluI/BglII; 5, pCW7/AluI/BstEII; 6, pCW7/AluI; 7, pBR322/TaqI; 8, λ/HindIII. Electrophoresis was for 2.5 hr at 50 V through a 1.2% agarose gel in TAE.

(e) Samples were: 1, pBR322/AluI; 2, pCW7/HinfI; 3, pCW7/HinfI/HpaII; 4, pCW7/HinfI/BglII; 5, pCW7/HinfI/BstEII; 6, pCW7/MboI; 7, pCW7/MboI/HpaII; 8, pCW7/MboI/HindIII; 9, pCW7/MboI/HaeIII; 10, pCW7/MboI/BglII; 11, pCW7/MboI/BstEII; 12, λ/HindIII. Electrophoresis was for 2.8 hr at 60 V on 1.2% agarose in TAE.

(f) Samples were: 1, pBR322/HinfI; 2, pCW7/HinfI; 3, pCW7/HinfI/HindIII; 4, pCW7/HinfI/HaeIII; 5, pCW7/TaqI; 6, pCW7/TaqI/HpaII; 7, pCW7/TaqI/HindIII; 8, pCW7/TaqI/HaeIII; 9, pCW7/TaqI/BstEII; 10, pCW7/TaqI/BglII; 11, pBR322/AluI. The tracks on this gel were overloaded to make the smaller fragments show up better. Electrophoresis was for 1.7 hr at 120 V on 2.4% agarose in TBE.

Fig. 5·4

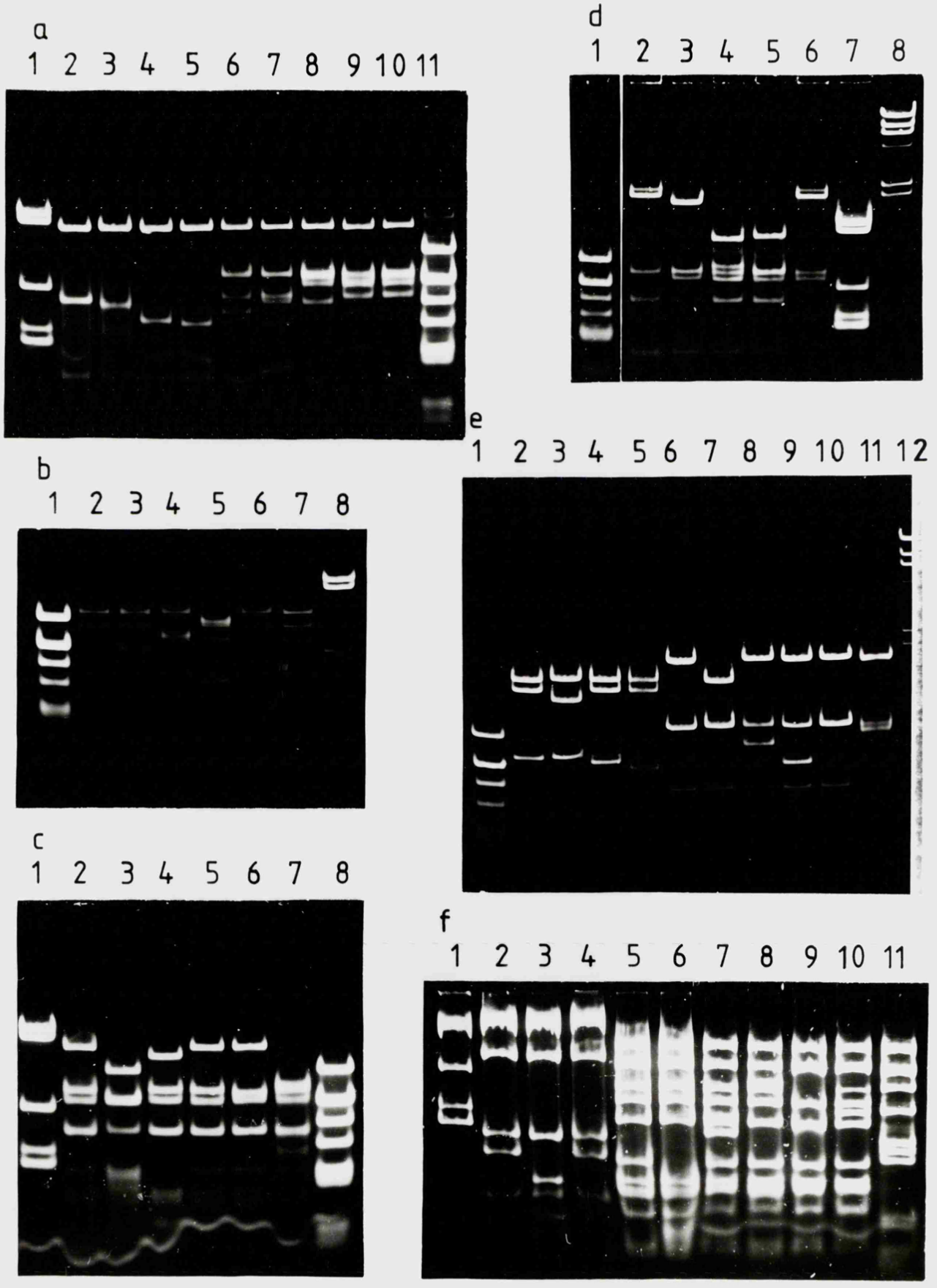
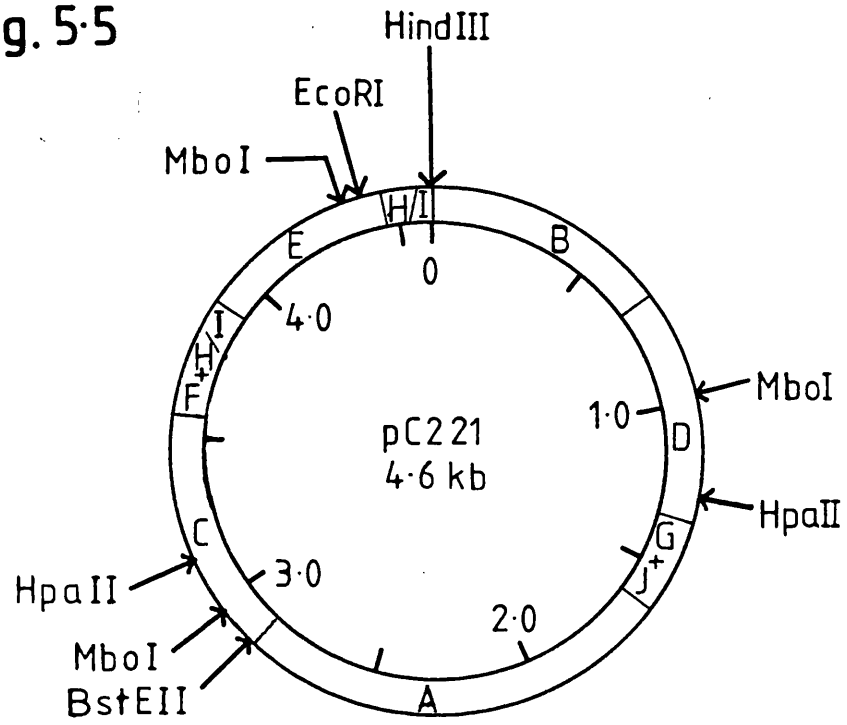
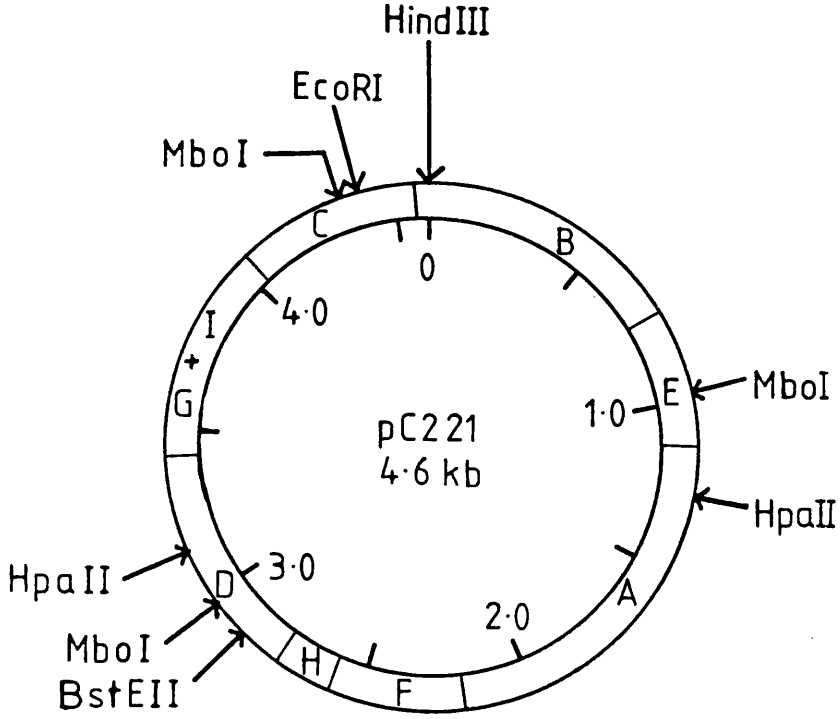

a
1 2 3 4 5 6 7 8 9 10 11
d
1 2 3 4 5 6 7 8
e
1 2 3 4 5 6 7 8 9 10 11 12
b
1 2 3 4 5 6 7 8
c
1 2 3 4 5 6 7 8
f
1 2 3 4 5 6 7 8 9 10 11

Fig. 5·5

a


HindIII
EcoRI
MboI
H/I
B
E
0
4·0
I
H+
F
pC221
4·6 kb
1·0
D
MboI
HpaII
G
J+
C
3·0
HpaII
2·0
MboI
BstEII
A

b


HindIII
EcoRI
MboI
C
B
0
4·0
I
+
G
pC221
4·6 kb
1·0
E
MboI
HpaII
HpaII
3·0
D
A
2·0
MboI
H
BstEII
F

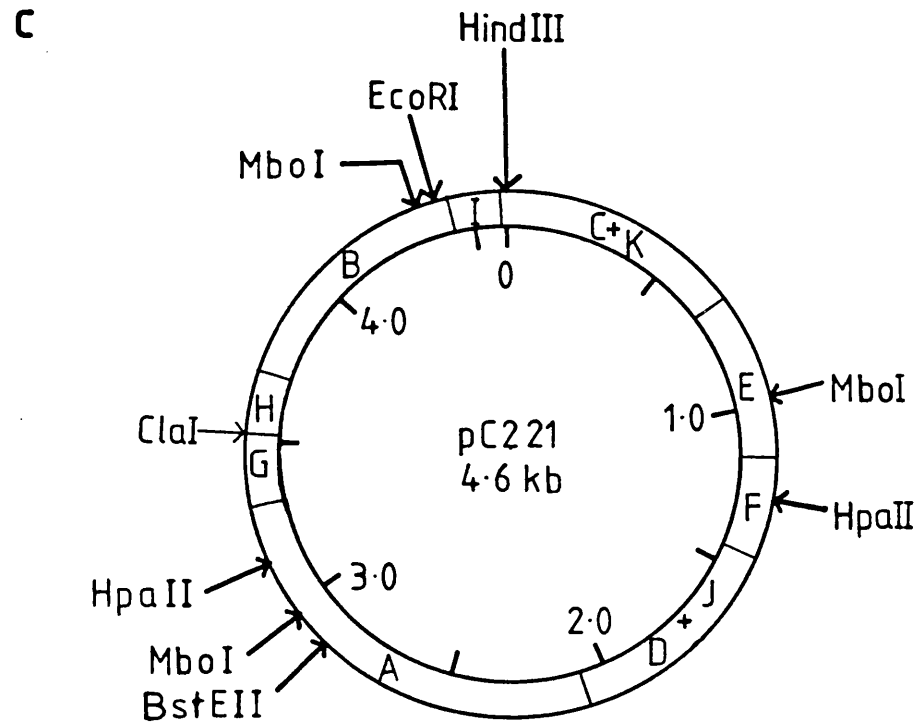


Fig. 5.5 Partial restriction maps of pC221

Positions of restriction fragments were deduced from the data shown in tables 5.2-5.6. Numbers indicate the distance (in kb) from the HindIII site (0).

a) AluI fragments

b) HinfI fragments

c) TaqI fragments

Fig.5·6

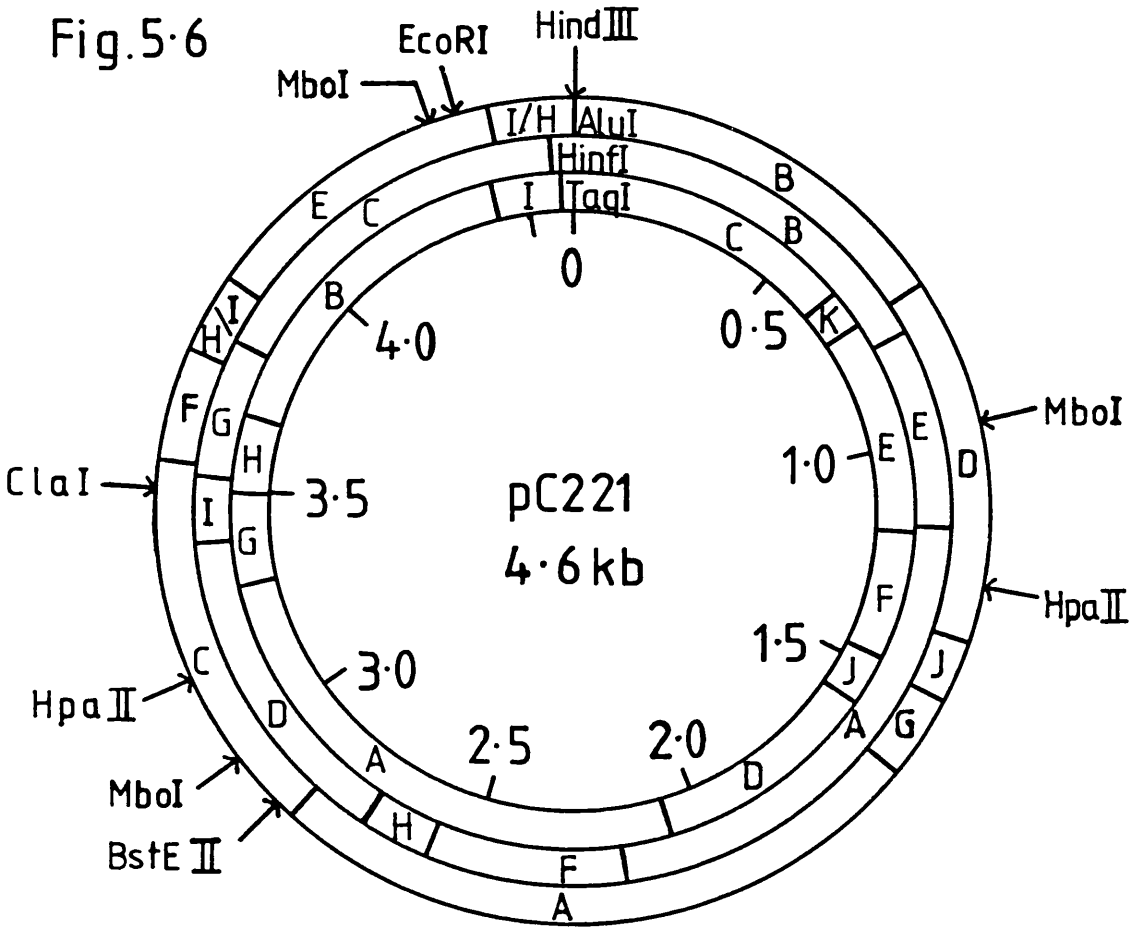


Fig. 5.6 Detailed restriction map of pC221

This map summarises the results presented in section 5.3.
Numbers indicate the distance (in kb) from the HindIII site (0).

Fig. 5·7

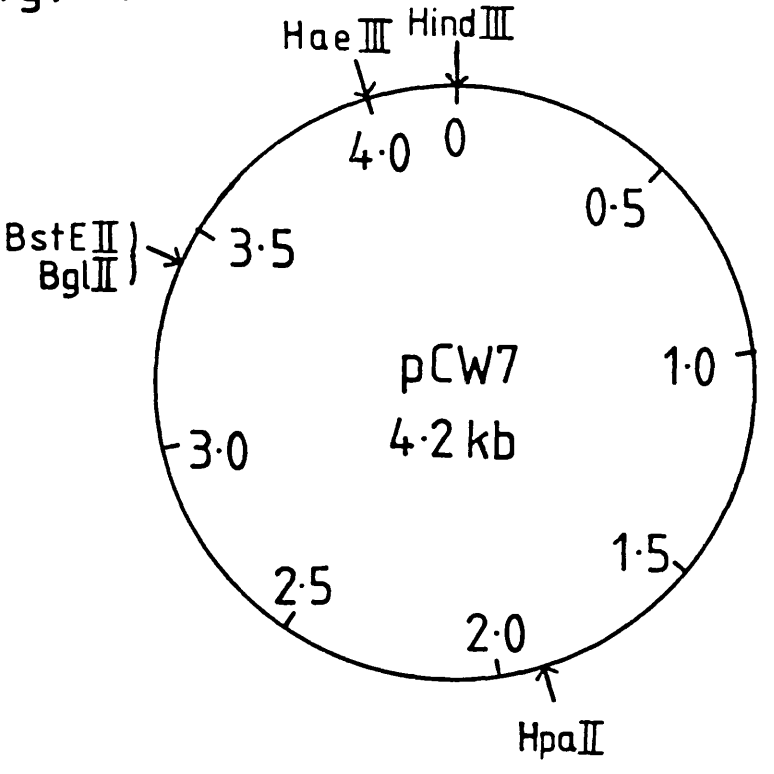


Fig. 5.7 Restriction map of pCW7
This map was drawn using data from Wilson and Baldwin (1978).
The XbaI sites have been omitted.

Fig. 5.8 Autoradiograms from pCW7 mapping experiments

Procedures are described in section 5.2.1. Experiments are described in section 5.4.1. As in fig. 5.2, some faint bands have reproduced poorly.

Size-markers were (in kb):

in track 1, λ /HindIII, 23.1, 9.4, 6.8, 4.36, 2.32, 2.03, 0.564;

in track 2, pBR322/TaqI, 1.444, 1.307, 0.616, 0.368, 0.315 and 0.312;

in track 3, pBR322/AluI, 0.91, 0.659 and 0.655, 0.521, 0.403, 0.281, 0.257, 0.226, 0.136 and smaller.

(a) and (b): pCW7 was linearised with HindIII before end-labelling with ^{32}P, then cut with HaeIII; samples of this material apppear in track 3 on either gel. Tracks 4-10 contained digests of this material by the following enzymes:

4, AluI, partial; 5, BglII, total; 6, BstEII, total; 7, HinfI, partial; 8, HpaII, total; 9, MboI, partial; 10, TaqI, partial.

(a) Electrophoresis was for 3 hr at 60 V on 0.8% agarose in TAE.

(b) Electrophoresis was for 2.5 hr at 60 V on 1.6% agarose in TAE.

(c) and (d): pCW7 was cut and labelled at the BstEII site, then cut with BglII; samples of this material appear in track 3 on either gel. Tracks 4-10 contained digests of this material by the following enzymes: 4, AluI, partial; 5, HaeIII, total; 6, HindIII, total; 7, HinfI, partial; 8, HpaII, total; 9, MboI, partial; 10, TaqI, partial. Electrophoresis was for 2.3 hr at 60 V in TAE on (c) 0.8% or (d) 2% agarose.

Fig. 5·8

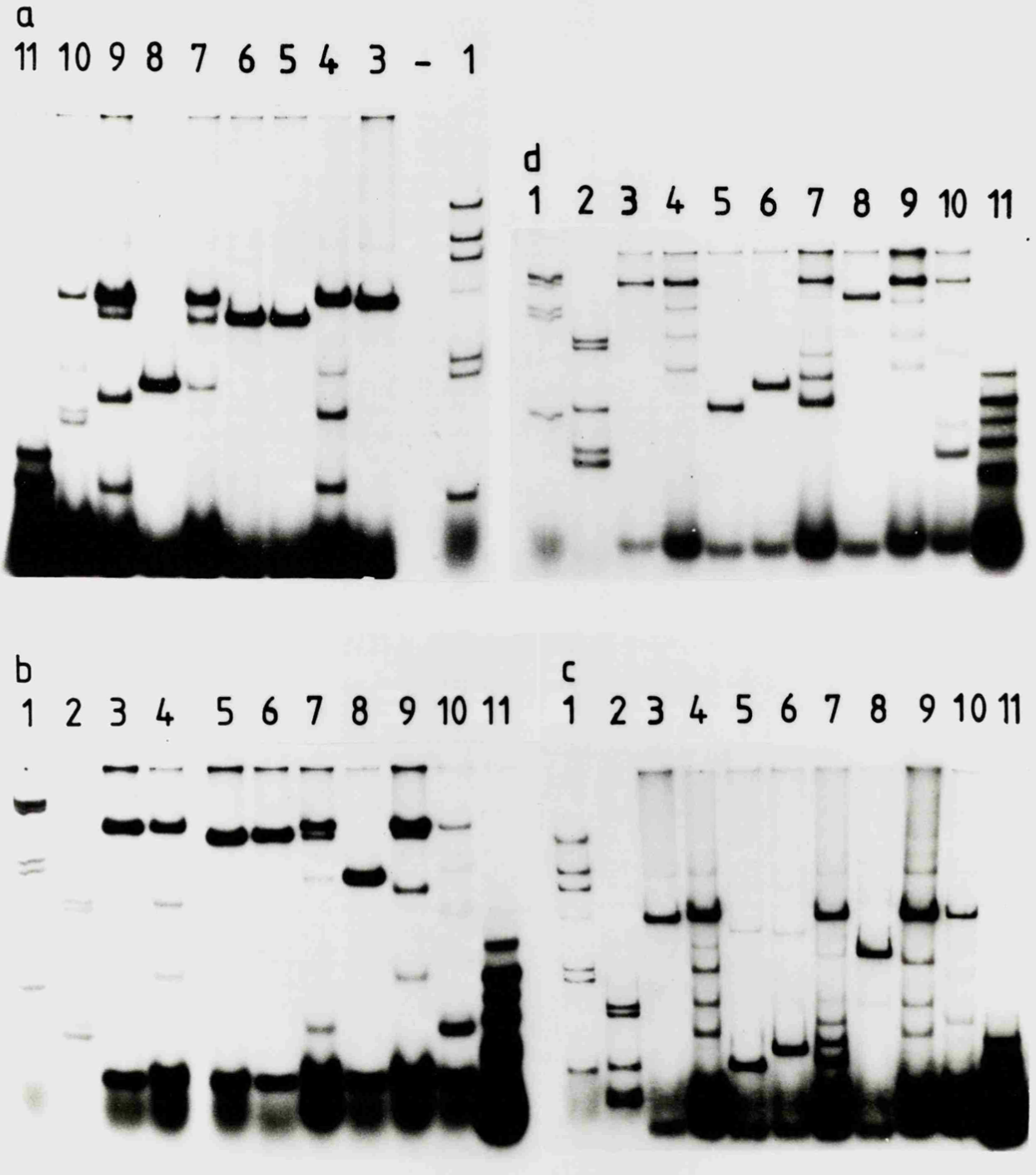

a
11 10 9 8 7 6 5 4 3 - 1
d
1 2 3 4 5 6 7 8 9 10 11
b
1 2 3 4 5 6 7 8 9 10 11
c
1 2 3 4 5 6 7 8 9 10 11

Fig. 5.9 (a) and (b) Results of Smith-Birnstiel mapping of pCW7
The mapping experiments are described in section 5.4.1. Dotted arrows correspond to relatively faint bands on the autoradiograms. Numbers indicate distances (in kb) from labelled termini (——————●).

Key:

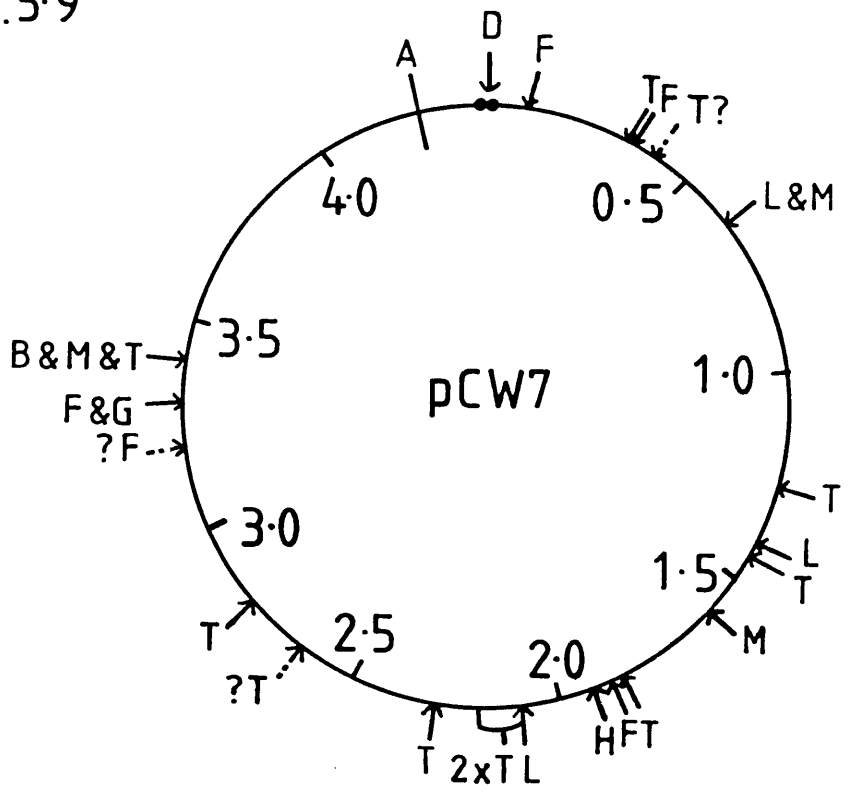
A	HaeIII	G	BglII
B	BstEII	H	HpaII
D	HindIII	L	AluI
F	HinfI	M	MboI
		T	TaqI

(a) Map constructed from experiments using HindIII-digested pCW7 end-labelled with ^{32}P then cut with HaeIII before the digests indicated were performed (see fig. 5.8, (a) and (b)). Distances are measured clockwise from the HindIII site.

(b) Map constructed from experiments using pCW7 cut and labelled at the BstEII site then digested by BglII before partial digestion by the enzymes indicated (see fig. 5.8, (c) and (d)). Distances are measured clockwise from the BstEII site.

Fig.5·9

a


A
D
F
TF
T?
L&M
0·5
1·0
T
L
T
1·5
M
2·0
HFT
T 2xTL
2·5
T
?T
3·0
B&M&T
F&G
?F
3·5
4·0
pCW7

b

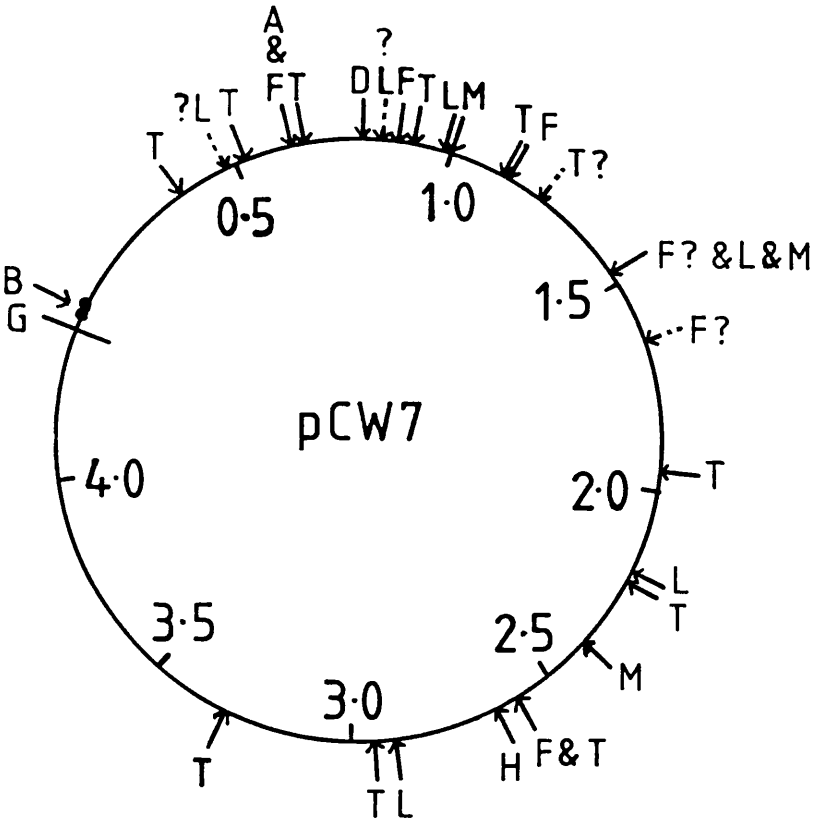
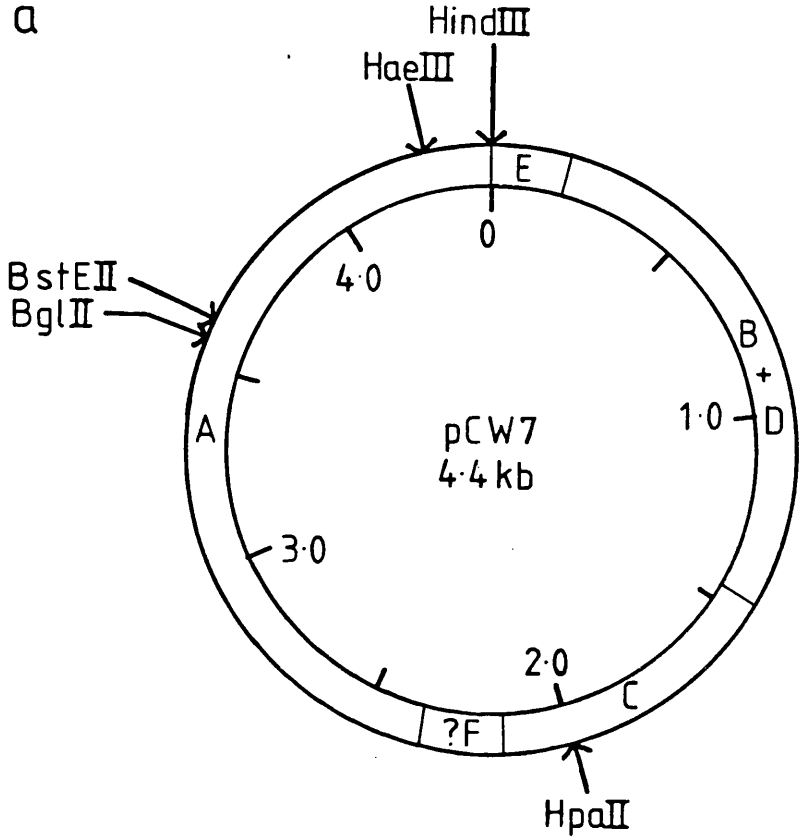
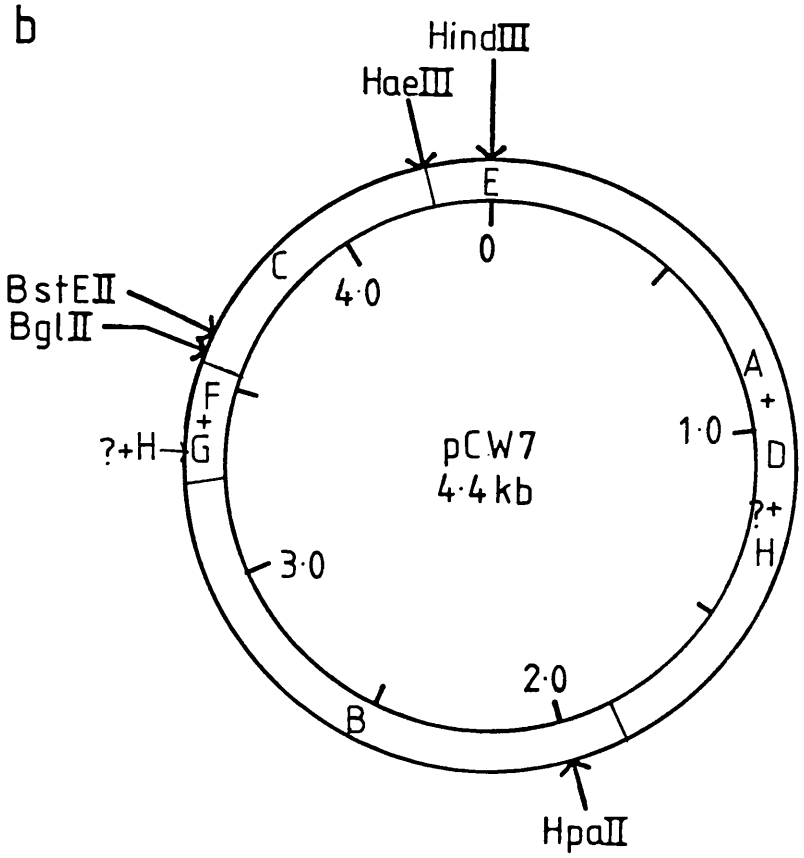

A
&
FT
?
DLFTLM
?L T
T
TF
T?
0·5
1·0
F? &L&M
1·5
F?
B
G
T
2·0
L
T
2·5
M
F&T
H
3·0
TL
T
3·5
4·0
pCW7

Fig. 5·10

a


HindIII
HaeIII
E
0
4·0
BstEII
BglII
B
+
1·0
D
A
pCW7
4·4 kb
3·0
2·0
C
?F
HpaII

b


HindIII
HaeIII
E
0
C
4·0
BstEII
BglII
A
+
F
+
1·0
D
?+H
G
pCW7
4·4 kb
?+
H
3·0
2·0
B
HpaII

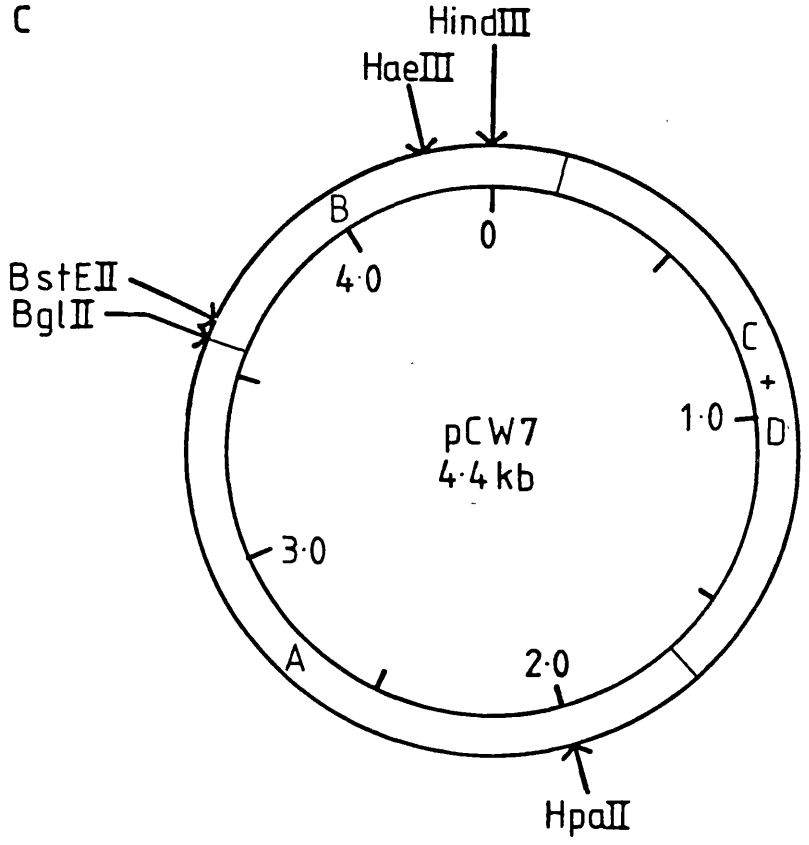


Fig. 5.10 Partial restriction maps of pCW7

Positions of restriction fragments were deduced from the data shown in tables 5.7-5.9. Numbers indicate the distance (in kb) from the HindIII site (0).

a) AluI fragments

b) HinfI fragments

c) MboI fragments

Fig. 5·11

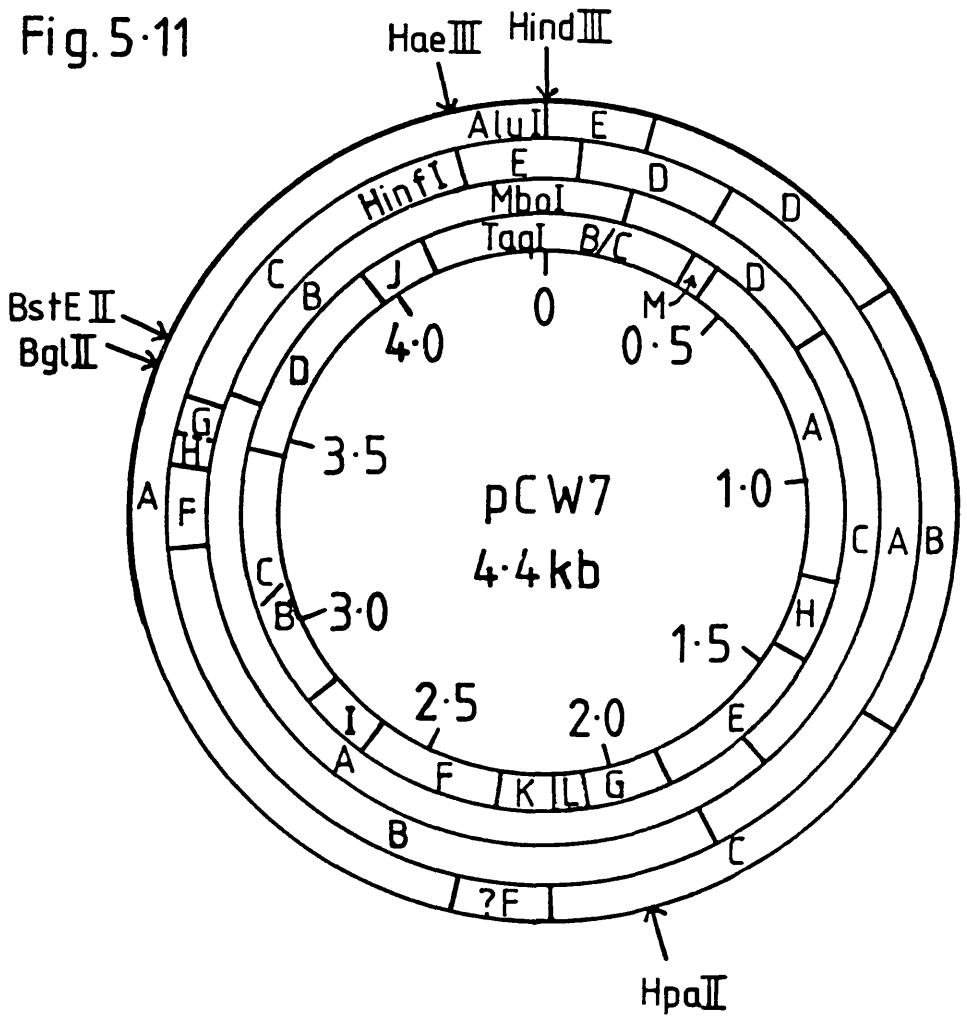


Fig. 5.11 Detailed restriction map of pCW7

This map summarises the results presented in section 5.4. Numbers indicate the distance in kb from the HindIII site (0). The positions shown for HinfI fragments F, G and H and TaqI fragments H-M are those most consistent with available data.

Table 5.1 Restriction digests of pC221

Restriction enzyme(s)	Sizes (kb) of observed fragments	Total (kb)
MboI	1.23, 1.38, 2.04	4.65
MboI + HindIII	~0.25, 0.97, 1.37, 2.0	4.59
MboI + EcoRI	1.16, 1.33, 1.98*	4.47
MboI + BstEII	1.24, 1.41, 1.90*	4.55
MboI + HpaII	~0.18, ~0.28, 2 x ~1.2, 1.69	~4.6
HpaII	1.87, 2.77	4.64
BstEII + HpaII	~0.26, 1.63, 2.73	4.62
	Average	4.61

Note: *: indicates that a small fragment was predicted but not observed under the conditions used.

Table 5.2 AluI fragments of pC221 and pCW41

Sizes (kb) of pC221 fragments:

A	B	C	D	E	F	G	H	I	J
1.26	0.72	0.70	0.64	0.55	0.21	0.19	0.14	0.14	~0.10

Sizes (kb) of pCW41 fragments:

A	G	J	Z
1.23	0.19	~0.10	0.54

Double digests: AluI as first enzyme

Plasmid	Second enzyme	Fragment(s) cut	Sizes (kb) of products observed[a]
pC221	BstEII	C	0.66
	EcoRI	E	0.51
	HpaII	C, D	0.29, 0.39, 0.52
	MboI	C, D, E	0.25, 0.39, 0.45, 0.55
pCW41	BstEII	Z	0.51
	HpaII	Z	~0.13, 0.40
	MboI	Z	~0.16, 0.39

Note: a) Only changes in the pattern are recorded here.

Table 5.3 HinfI fragments of pC221 and pCW41

Sizes (kb) of pC221 fragments:

A	B	C	D	E	F	G	H	I
1.02	0.85	0.74	0.65	0.41	0.41	0.26	0.13	~0.10

Sizes (kb) of pCW41 fragments:

A	F	H	Z
0.99	0.40	0.13	0.47

Double digests: HinfI as first enzyme

Plasmid	Second enzyme	Fragment(s) cut	Sizes (kb) of products observed[a]
pC221	BstEII	D	0.49
	EcoRI	C	0.58
	HindIII	B	0.78
	HpaII	A, D	0.25, 0.41, 0.89
	MboI	C, D, E	~0.19, 2 x ~0.21, 0.26, 0.39, 0.53
pCW41	BstEII	Z	0.32
	HpaII	A	0.90
	MboI	Z	~0.20, 0.26

Note: a) see note to table 5.2

Table 5.4 TaqI fragments of pC221 and pCW41

Sizes (kb) of pC221 fragments:

A	B	C	D	E	F	G	H	I
1.20	0.75	0.66	0.46	0.46	0.28	0.20	0.18	0.17

pC221 cont'd.		Sizes (kb) of pCW41 fragments			
J	K	D	F	J	Z
~0.12	⩽0.10	0.47	0.28	~0.12	1.17

Double digests: TaqI as first enzyme

Plasmid	Second enzyme	Fragment(s) cut	Sizes (kb) of products observed[a]
pC221	BstEII	A	0.365, 0.78
	EcoRI	B	0.73
	HindIII	-	-
	HpaII	A, F	~0.14, 0.20, 1.03
	MboI	A, B, E	~0.21, ? 2 x 0.26
			0.67, 0.91
pCW41	BstEII	Z	0.32, 0.79
	HpaII	F	0.18
	MboI	Z	0.21, 0.90

Note: a) see note to table 5.2

Table 5.5 Digests of ClaI-cut pC221

Second enzyme	Sizes (kb) of fragments observed	Total (kb)
BstEII	0.55, 4.0	4.55
EcoRI	0.84, 3.8	4.64
HindIII	1.03, 3.5	4.53
HpaII	0.355, 1.8[b], 2.4	4.56
MboI	0.51, 0.81, 1.2[c], 2.1[a]	4.62

Notes: a and c: co-migrated with MboI fragments A and C respectively

b: co-migrated with HpaII fragment B

ClaI - digested pC221 was subjected to digestion by the enzymes shown and the products were resolved on a 1% agarose gel (in TAE) for 2.5 hr at 60 V. Sizes over 1.5 kb are less reliable.

Notes to table 5.6

About 12 μg of pCW41 were incubated with 4 U of HinfI at 37°. Samples were heat-inactivated after 5, 10, 20, 40 or 90 min and electrophoresed through a 1% agarose gel (in TAE) for 3 hr at 60 V.

a) Order I (A-H-F-Z) should give A + H (1.12 kb) and Z + F (0.87 kb); order II (A-F-H-Z) should give A + F (1.39 kb) and Z + H (0.60 kb) (sizes from table 5.3)

b) CCC pCW41 (running on this gel as if a linear fragment of 1.25 kb) disappeared most rapidly with time.

c) By 90 min >95% of the DNA was in these bands and one containing a smaller fragment (presumably H), whose size was not measured.

Table 5.6 Partial digestion of pCW41 by HinfI

Sizes (kb) of fragments observed after:			Interpretation[a]
5 or 10 min[b]	20 or 40 min	90 min[c]	
1.8 to 2.0	1.8 to 2.0		linear pCW41 A + Z + F
1.55	1.55		A + Z + H
1.45	2 x ~1.5		A + F + H A + Z
	1.35		A + F
0.96	0.96	0.96	Z + F + H A
	0.60		Z + H
	0.54		F + H
0.48	0.48	0.48	Z
0.40	0.40	0.40	F

Table 5.7 AluI fragments of pCW7

Sizes (kb) of fragments:					
A	B	C	D	E	F?
2.05	0.80	0.69	0.50	~0.18	~0.18

Double digests: AluI as first enzyme

Second enzyme	Fragment cut	Sizes (kb) of products observed[a]
BglII	A[b]	0.84, 1.18
BstEII	A	0.80, 1.23
HaeIII	A	0.16, 1.9
HpaII	C	0.16, 0.52

Notes: a) Only the changes observed are recorded here.

b) A product of partial digestion (about 2.22 kb), deduced to be (A + E), was cut by BglII to 1.0 kb, BstEII to 0.95 kb and HaeIII to 0.35 kb (see fig. 5.4(d)).

Table 5.8 HinfI fragments of pCW7

Sizes (kb) of fragments:

A	B	C	D	E	F	G	H
1.48	1.35	0.72	0.30	0.23	0.15	~0.08	~0.06

Double digests: HinfI as first enzyme

Second enzyme	Fragments cut	Sizes (kb) of products observed[a]
BglII	C	0.67
BstEII	C	0.63
HindIII	E	~0.18
HpaII	B	1.25
HaeIII	-	-

Note: a) only the changes in pattern are recorded here.

Table 5.9 MboI fragments of pCW7

Sizes (kb) of fragments:

A	B	C	D
1.85	1.01	1.01	0.50

Double digests: MboI as first enzyme

Second enzyme	Fragment cut	Sizes (kb) of products observed
BstEII	B/C	0.96
HaeIII	B/C	0.345, 0.67
HindIII	B/C	~0.17, 0.84
HpaII	A	0.31, 1.79

Note: see note (a) of table 5.8

Table 5.10 TaqI fragments of pCW7

Sizes (kb) of fragments:							
A	B	C	D	E	F	G	H
0.83	0.65	0.64	0.50	0.43	0.34	0.21	0.19

I	J	K	L	M
0.16	0.16	0.14	~0.07	~0.07

Double digests: TaqI as first enzyme

Second enzyme	Fragment cut	Sizes (kb) of products observed[a]
BglII	D	0.40
BstEII	D	~0.15, 0.33
HaeIII	B/C	0.54
HindIII	B/C	0.30, 0.36
HpaII	G	? 2 x ~0.1[b]

Notes: a) Only the changes in pattern are recorded here.

b) These products were not clearly visible.

CHAPTER 6

INDUCTION OF CAT IN *S.AUREUS*

6.1 Introduction

Some of the mechanisms already described for regulation of expression of structural genes of prokaryotes have been outlined in Chapter 1 (section 1.4.1). Positive or negative control may be exerted at the level of either transcription or translation by the interaction of activator or repressor proteins (in some cases, ribosomes) with DNA or mRNA.

Previous studies by Shaw and his colleagues on the induction of staphylococcal CAT had centred on regulation of the *cat* gene of pC221 (for references see General Introduction, section 1.4.2). To study the organisation of this gene at the DNA level a series of derivatives of pC221 was constructed. By the creation of deletions *in vitro* and the interruption of restriction sites it was hoped to determine which regions of pC221 were involved in the regulation of CAT synthesis and so define regions to be studied by nucleotide sequence analysis. It was hoped that if a plasmid-encoded repressor or activator protein was involved in the regulatory mechanism its gene might be deleted or inactivated (but not the structural gene for CAT) leading to constitutively high or low synthesis of CAT.

It was of interest to determine whether the level of RNA from the *cat* gene increased or remained constant on induction. An increase in the amount of *cat* RNA after induction would suggest that regulation occurred at initiation or completion of transcription, whereas a post-transcriptional mechanism would be implicated if the level remained unchanged.

In earlier studies 3-deoxyCm had been used to induce CAT

synthesis; the suitability of the 3-fluoro analogue of Cm (Hahn, 1980) for use in future studies was also investigated.

6.2 Methods

Purification of plasmid DNA from S.aureus and E.coli, and DNA techniques have been described in Chapter 2 (sections 2.5 and 2.6). Fig. 6.1 outlines the construction of pCW41, pCW46, pCW48 and pCW55 (Wilson et al., 1981). [^{32}P] RNA (23S RNA from Bacillus stearothermophilus) was a gift from Dr. R. H. Skinner; Bentonite (Serva) was a gift from Dr. E. Cundliffe.

6.2.1 Induction and measurement of CAT activity.

This was performed as described in Chapter 2 (section 2.4). In the experiments indicated the D,L,threo mixture of 3-deoxyCm was used. Rifampicin (if used) was added at the same time as the 3-deoxyCm, as a 5 mg/ml solution in 0.1 M hydrochloric acid. The host strain for all the experiments described in this chapter was S.aureus WS 2001.

6.2.2 Preparation of RNA from S.aureus

(Modified from Zouzias et al., 1973)

Three different sets of preparations (A, B, and C) were made; differences in procedure are indicated here and in table 6.1(a). To avoid contamination by RNase, glassware and solutions (where possible) were heat-sterilised before use, and gloves were worn.

Portions of TSB (100 ml in 250 ml flasks) were inoculated with 1 ml of an overnight culture of WS 2001 (one flask) or of WS 2009, i.e. WS 2001 carrying pC221, (two flasks) and incubated at 37^{o} with shaking. In preparation B duplicate cultures were set up and treated separately. After exponential

growth had started, 3-deoxyCm (final concentration, 6 to 8 μM) or ethanol was added to each flask at the A_{600} shown in table 6.1(a). After 50 to 90 min, 10 ml of each culture were removed for the measurement of CAT activity (Chapter 2, section 2.4); sodium azide was added to the remainder of each culture (as a 50 mM aqueous solution) to a final concentration of 2.5 mM (B) or 5 mM (A and C) and the cultures were chilled in ice-baths.

Cultures were harvested by centrifugation (10 min, 9,000 rpm, 4°, MSE 18, 6 x 250 ml rotor) and washed and resuspended in 10 ml of 50 mM Trizma base, 50 mM EDTA, 100 mM NaCl (pH adjusted to 7.5 with Trizma base). In preparation C this buffer also contained 1 mM sodium azide. The suspensions were transferred to conical flasks and 0.5 mg of lysostaphin was added to each: in preparations A and C incubation was for 10 min on ice; in preparation C the flasks were swirled for 1 min in a 37° water-bath half-way through the incubation on ice.

The preparations were extracted with 10 ml of hot phenol by swirling the flasks in a 60° water-bath for 3 to 4 min, and the phases were separated by centrifugation. In preparation A, the resulting aqueous phase was extracted once with 10 ml of chloroform /iso-amyl alcohol (24:1, v/v). In preparation B, 10 ml of chloroform/iso-amyl alcohol were added before centrifugation and the flasks were swirled at room temperature for 2 min before separating the phases. In preparation C, SDS and Bentonite were added to each flask (to 1% and 0.2%, w/v, respectively) together with the phenol. The aqueous phases were extracted again with phenol and Bentonite at 60° and once with

chloroform/iso-amyl alcohol. In all the preparations the nucleic acids in the aqueous layers were precipitated at -70° overnight as described in Chapter 2 (2.8.3).

The nucleic acids were collected by centrifugation at 15,000 rpm for 15 to 30 min (0° , MSE 18, 8 x 50 ml rotor). In preparations A and B, each pellet was resuspended in 1 ml of water; in preparation C, to remove residual SDS, the nucleic acids were reprecipitated from 6 ml then from 1 ml of 20 mM Tris-HCl, pH 8.0, as above, and finally resuspended in 200 µl of water. In all three preparations, Tris-HCl (pH 7.8 or 8.0) and magnesium chloride were added to 8 mM each and the samples were incubated at 37° with DNase as indicated in table 6.1(a) (the DNase used for preparations A and B was Sigma EP grade; for preparation C, Merck DNase "guaranteed free from RNase" was used).

The nucleic acid solutions were treated once with Bentonite (0.2%, w/v) then precipitated by the addition of sodium acetate (to 0.2 M) and 2.5 vols of ethanol and storage at -70° overnight. The precipitates were collected by centrifugation at 11,000 x g for 5 min in a microfuge and resuspended in water. The yields, which are shown in table 6.1(a), were estimated from the A_{260} of diluted samples. The A_{260}/A_{280} ratio was at least 1.8. The solutions were stored at -20° until required.

6.2.3 Preparation of the radioactive probe.

6.2.3.1 Electro-elution of fragments.

Restriction fragments of plasmid DNA were electro-eluted

from slices of 1% agarose gels into 0.2 to 0.4 ml of TAE in dialysis bags (prepared as described in Chapter 2, 2.8.4) at 60 V for 40 to 60 min, and concentrated by extraction with iso-butyl alcohol (if necessary) and ethanol precipitation at -70°. Fragments for nick-translation were purified either by centrifugation through a Sephadex G-50 "spin-column" (0.3 ml in TE buffer, section 2.8.6; Maniatis et al., 1982) or by passage through an Elutip-D (Schleicher and Schuell) according to the manufacturers' instructions. Yields were 5 to 50%; substantially greater losses were obtained with smaller amounts of DNA.

6.2.3.2 Low melting-point agarose gels.

(After Wieslander, 1979).

Fragments were extracted from slices of low melting-point agarose gels (1.0 or 1.2% in TAE) by melting the slices at 68° and extracting once or twice with an equal volume of pre-heated redistilled phenol (saturated with 10 mM Tris-HCl, pH 8.0). The phenol layers were back-extracted with water, and the combined aqueous phases for each slice were extracted with 1 vol of phenol/chloroform/iso-amyl alcohol (25:24:1). The aqueous layers from this step were concentrated by repeated extractions with iso-butyl alcohol, extracted once with diethyl ether, and precipitated with ethanol at -70°. Yields were similar to those in 6.2.3.1.

6.2.3.3 Nick-translation: method A.

(After Maniatis et al., 1975).

The DNA fragment (50 to 150 ng), isolated by preparative

agarose gel electrophoresis, was incubated with 2 to 3 U of DNA polymerase I (BRL) in 100 µl of nick-translation buffer (NTB; 50 mM Tris-HCl, pH 7.8, 5 mM magnesium chloride, 10 mM BME and 50 µg/ml BSA) containing dCTP, dGTP and TTP (25 µM each) and 25 µCi of [α-^{32}P] dATP (1,000 to 3,000 Ci/mmol; Radiochemical Centre, Amersham). After incubation for 45 min at room temperature the enzyme was heat-inactivated (10 min at 65°) and unincorporated radioisotope separated from DNA by passage through a 10 ml Sephadex G-100 column equilibrated with 10 mM Tris-HCl (pH 8.0). Fractions of ten drops each (0.5 to 0.6 ml) were collected. The peak fractions of the excluded material were located by Cerenkow counting (see Chapter 5, section 5.2.2) and pooled. The specific radioactivities obtained were 20 to 60 µCi/µg.

6.2.3.4 Nick-translation: method B.

(After Rigby et al., 1977).

About 140 ng of fragment were incubated with 6 to 8 U of DNA polymerase I and 1 to 8 pg of DNase I (BRL) in 25 µl of NTB containing dCTP, dGTP and TTP (50 µM each), 8 mM spermidine and 20 to 25 µCi of [α-^{32}P] dATP at room temperature for about 60 min. The reactions were terminated by the addition of 25 µl of quench mix (10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.5% SDS, w/v) and extraction with 1 vol of phenol/chloroform/iso-amyl alcohol (25:24:1). The organic layer was back-extracted with 10 mM Tris-HCl, pH 8.0, and tRNA (50 µg) was added as carrier. Unincorporated dATP was removed by two precipitations with sodium acetate (added to give 0.2 M) and ethanol (2.5 vols), the pellet

having been washed with 70% ethanol before resuspension. The final pellet was resuspended in 200 µl of 10 mM Tris-HCl (pH 8.0) and 2 µl were taken for liquid scintillation spectrophotometry in Fisofluor no. 1 fluid (Fisons). Efficiency of counting was assumed to be 90%. The specific radioactivities obtained were 10 to 20 µCi/µg.

6.2.4 Preparation of RNA "dot-blots".

6.2.4.1 Preparation and use of DBM-paper.

(After Alwine et al., 1977).

NBM-paper (a gift from J. Mullins) was reduced to ABM-paper by incubation of 8 or 16 cm^2 at 60^o in 100 ml of 20% (w/v) sodium dithionite (Fisons, SLR) for 30 min. The paper was then washed at room temperature in water (5 changes of 100 ml), 30% (v/v) acetic acid (100 ml) and water (5 changes of 100 ml).

Freshly prepared sodium nitrite solution (1.3 ml of 10 mg/ml) was added to 50 ml of 10% (v/v) hydrochloric acid (ice-cold). The presence of nitrous acid was tested for with starch-iodide (1% starch, w/v; 3% potassium iodide, w/v). The strip of ABM-paper (from the procedure above) was incubated in the nitrous acid preparation for 30 to 40 min with occasional agitation. The presence of nitrous acid was checked at the end of the incubation.

The paper was washed quickly in ice-cold water (5 changes of 100 ml each) and in ice-cold 0.1 x sodium acetate (100 ml), blotted briefly on Whatman no. 1 paper and cut into 1 cm squares. (1 x sodium acetate was 0.2 M in sodium ions, pH

adjusted to 4.0 with glacial acetic acid.) The RNA samples had previously been adjusted to 0.1 x sodium acetate and 10 µl of each were loaded onto the paper squares as soon as possible. (In experiment 1, 10 µl contained 8 µg, in experiments 2 and 4, 10 µg). Blanks (0.1 x sodium acetate) were treated identically. The paper squares were washed in 1 x sodium acetate (2 x 100 ml, 5 min.) before pre-hybridisation (section 6.2.5).

6.2.4.2 Preparation and use of DPT-paper.

APT-paper (Seed, 1982), obtained from Schleicher and Schuell, was incubated with nitrous acid, washed and blotted as described for ABM-paper (in the previous section) except that 1 x sodium acetate was 0.2 M in acetate ions (3.0 g/l of anhydrous sodium acetate and 0.5 ml/l of glacial acetic acid), about 0.18 of the concentration of that used in section 6.2.4.1. After the DPT-paper had been cut up, RNA samples (10 µg in 10 µl of 0.05 x sodium acetate) were applied and incubated as before. The paper squares were washed twice for 5 min in two 100 ml changes of 1 x sodium acetate before pre-hybridisation, and were yellow by this stage.

6.2.4.3 Preparation and use of nitrocellulose.

(Thomas, 1980)

Nitrocellulose (Schleicher and Schuell, 0.45 µm pore-size) was floated on distilled water to wet thoroughly, then soaked in 20 x SSC for 10 min. (20 x SSC was 3 M sodium chloride, 0.3 M trisodium citrate.) After the filter had been dried under a heat-lamp for 20 min, 10 µg samples of RNA (in 5 µl of water)

were applied at 2 cm intervals. Blanks (5 μl of water) were treated identically. The filter was dried again for 10 min under the heat-lamp then baked at 80^{o} for 4 hr before pre-hybridisation.

6.2.5 Hybridisation of RNA-papers with radioactive probe.

Pre-hybridisation and hybridisation were performed in a heat-sealed bag (Rhinoflex) and were in H buffer (0.75 M sodium chloride, 0.075 M trisodium citrate, 0.024 M sodium phosphate buffer (pH 6.5), 0.2% SDS,(w/v) containing 50% formamide, 0.2% (w/v) yeast RNA (Sigma) and 5 or 10 X Denhardt's solution at 36^{o} or 42^{o} as indicated in table 6.5. (10 x Denhardt's solution was 0.2% (w/v) each of BSA, PVP 360 and Ficoll; Denhardt, 1966). Formamide was deionised by stirring it with BDH MB-1 resin until the conductivity was below 70 μmho. Glycine (1%, w/v) was added to the prehybridisation solution in experiments 1-4 to inactivate any remaining diazonium groups. (The DBM- and DPT-papers became orange at this stage.) In experiment 6, dextran sulphate was added to 10% (w/v). Solutions were degassed by autoclaving or at the vacuum pump before use.

Radioactive probe (1 to 5 μCi) was heated in a boiling-water bath for 10 to 15 min and transferred immediately to an ice-bath before adding it to the hybridisation solution and filters in the bag. In all cases the probe used was fragment X (0.9 kb, see fig. 7.4 in Chapter 7) carrying the structural gene for CAT (Chapters 3, 7, and App. 1). Apart from experiment 1, where this fragment was purified after digestion of

pCW41 with MboI and TaqI, fragment X was isolated after TaqI digestion of the largest Sau3A fragment of pSES3 (which corresponds to MboI-cut pCW41, see Chapter 7). After preparative agarose gel electrophoresis the DNA fragments were extracted from gel slices by electro-elution (section 6.2.3.1) or from low melting-point agarose by heating and phenol extraction (6.2.3.2). Fragment X was labelled with ^{32}P by nick-translation (6.2.3.3 or 6.2.3.4). Table 6.1(b) indicates which methods were used to prepare the probe for a given experiment.

After hybridisation for the times indicated in table 6.5 the filters were washed, either in H buffer containing 50% formamide (HF50) four to six times at the hybridisation temperature, or, for experiment 3, twice in 2 x SSPE, 0.1% (w/v) SDS and once in 0.1 x SSPE, 0.1% (w/v) SDS, all at room temperature. (5 x SSPE was 0.9 M sodium chloride, 5 mM EDTA, 50 mM sodium phosphate buffer, pH 7.3.) After the final wash excess solution was removed from the filters by blotting with Whatman no. 1 paper (the NC strips were cut up at this stage) and they were dried at 60^{o} for 30 min. Radioactivity was measured in 3 ml either of toluene containing 2,5,diphenyloxazole (4 g/l) and 1,4,bis(5-phenyloxazolyl) benzene (50 mg/l) or of Fisofluor no. 1 fluid (Fisons) by liquid scintillation spectrophotometry at 80% efficiency of counting. Each sample was counted for 10 or 20 min.

6.3 Results

6.3.1 Induction of CAT encoded by smaller derivatives of pC221.

Fig. 6.1 outlines the procedure used by Dr. C. R. Wilson to construct pCW41 and pCW46 (Wilson et al., 1981). MboI cuts pC221 into three fragments, A (2.0 kb), B (1.4 kb) and C (1.2 kb) (Chapter 5); pCW46 consists of fragments A and B (oriented as in pC221, data not shown), while pCW41 corresponds to fragment A.

The data presented in table 6.2 indicate that pCW41 encodes CAT in an inducible manner, although the specific activity attained was only one quarter of that observed for pC221. (Similar results were obtained with cultures incubated with 16 μM D,L,threo-3-deoxyCm for 3.5 hr.) Fig. 6.2(a) shows the increase in the specific activity of CAT in the first 100 min after the addition of inducer to cultures of S.aureus carrying either pC221 or pCW41. While the zero-time points were virtually identical, the average rate of increase in the specific activity of CAT in extracts of S.aureus (pC221) during the first 60 min after the addition of inducer was 3.3 times that measured for S.aureus (pCW41). In contrast, the time-course for S.aureus (pCW46) was indistinguishable from that for S.aureus (pC221) (see fig. 6.2(b)).

The nature of the function(s) encoded by fragment B of pC221 is unknown, however the CAT proteins purified from S.aureus (pC221) and S.aureus (pCW41) appeared identical in mobility on SDS-PAGE and very similar in specific activity (150 and 130 U/mg respectively) and amino-acid analysis (C. R.

Wilson and J. W. Keyte, personal communication). On PAGE under non-denaturing conditions the proteins were also indistinguishable (A. J. Corney and S.E.S., data not shown). In Ouchterlony immunodiffusion experiments using anti-serum to purified pC221 CAT, complete identity (no spur) was observed between the two CAT preparations. Thus, by these criteria, no change in the structural gene for CAT appeared to have occurred during the construction of pCW41.

6.3.2 Interruption of HpaII and BstEII sites of pCW41.

As can be seen in fig. 6.3, when pCW3 was inserted into the HpaII site of pCW41 expression of the cat gene of the recombinant plasmid, pCW48, was still inducible. In the first hour after induction the average rate of increase in the specific activity of CAT in extracts of S.aureus (pCW48) was 0.7 of that measured for S.aureus (pC221); however, the specific activity of CAT when the inducer was added (time = 0 min) for S.aureus (pCW48) was 2.4-fold that for S.aureus (pC221). Interruption of the BstEII site to form pCW55 (fig. 6.1) abolished CAT activity (table 6.3).

6.3.3 RNA and the induction of CAT.

When rifampicin was added at the same time as inducer, little or no change in CAT levels was observed (table 6.4). This was in agreement with the observation by Horinouchi and Weisblum (1982) that rifampicin prevented the induction of Cm^R conferred by pC194.

Experiments were performed to compare the levels of RNA complementary with fragment X (which contains the cat gene) in cultures of S.aureus (pC221) incubated with and without

3-deoxyCm (table 6.5). RNA from the plasmid-free host strain was used to estimate non-specific binding. As no one set of data appeared typical, those from several experiments are presented. CAT activity was measured in samples of the same cultures to check that induction of CAT had occurred.

The highest apparent increase in cat RNA on induction is seen in the data from experiment 1. In contrast, the levels of hybridisation observed in experiment 6 are the same in induced and uninduced cultures. Small differences were obtained in experiments 2-5. Variation was also seen within a given experiment. Comparison of lines 5 and 6 of either experiment 2 or experiment 3 reveals that although practically identical activities of CAT were measured in extracts of the duplicate cultures the amounts of probe hybridising to RNA from these cultures were noticeably different.

The levels of hybridisation observed in experiments 2 and 3 were considerably lower than expected. To examine whether the RNA applied was binding to the DBM- and DPT-papers, 10 μg portions of 23S rRNA (labelled with ^{32}P) were loaded onto DBM- and DPT-papers using the procedures described in section 6.2.4 and washed twice in sodium acetate (pH 4.0, 0.2 M in sodium ions): more than 90% of the cpm applied were retained. After incubation of the RNA-DPT-paper in H buffer containing 50% formamide for 20 hr at 42^{o} and washing with SSPE solutions as for experiment 3 (see section 6.2.5) more than 50% of the applied cpm were still bound.

Neither the degree of denaturation of the probe nor the

efficiency of hybridisation was determined. Although gel electrophoresis of RNA samples from preparation B had indicated that most of the RNA was larger than tRNA, the expected pattern for the rRNA was not observed, i.e. some degradation of the rRNA had occurred. This suggested that the levels of the less stable mRNA population might also be affected.

In preparation C additional precautions were taken against RNase activity; no heat-pulse during the incubation of the cells with lysostaphin, the addition of SDS and Bentonite at the phenol extraction stage, less DNase (of higher quality.) Otherwise, experimental conditions resembled preparation A rather than B (see table 6.1(a)). In spite of this, little hybridisation to RNA from preparation C was observed in experiments 4-6 and little or no difference between induced and uninduced levels of *cat* RNA was detected. (CAT activity in the induced culture was almost 60-fold that in the uninduced culture.) It may be relevant that the preparation of fragment X used to prepare radioactive probes for experiments 4-6 was contaminated to an unusually high extent by low molecular-weight material (gel not shown); this might indicate that an excessive amount of degradation had occurred during preparation or storage of the fragment. The use of DNase I in nick-translation method B (6.2.3.4) might also have decreased the ss length of the probe (compared with those used in experiments 1-3). To allow shorter DNA-RNA hybrids to be detected the hybridisation temperature was lowered by 6^{o} in experiments 5 and 6. In experiment 6, dextran sulphate was included to increase the rate of association (Wahl *et al.*, 1979). The overall level of hybridisation was unaffected.

6.3.4 Induction of CAT by 3-fluoroCm.

Fig. 6.4 shows the specific activities of CAT in extracts of S.aureus (pC221) incubated with different concentrations of 3-fluoroCm: the maximum induction was observed at 1 μM. In another experiment, in which 1 μM 3-fluoroCm was added to a culture at an A_{600} of 0.17 the specific activity of CAT in an extract made 3 hr later was 0.95 U/mg, which is similar to the maximum specific activities obtained using 3-deoxyCm (compare table 6.2, for example). The value for the control (uninduced) culture was 0.005 U/mg. At concentrations above 1 μM inhibition of growth was observed: the doubling-times (A_{600} from 0.2 to 0.4) were one, two and three times that of the control when exponential cultures of S.aureus (pC221) were incubated with 1, 3 and 10 μM 3-fluoroCm respectively.

6.4 Conclusions and discussion

The largest MboI fragment of pC221 (pCW41; 2.0 kb) contained the information necessary for the inducible synthesis of CAT (section 6.3.1), although the rate of synthesis and the specific activity achieved after prolonged incubation with 3-deoxyCm were one quarter to one third of those for pC221 itself. A plasmid containing MboI fragments A and B (pCW46) gave a time-course of induction indistinguishable from that for pC221. The CAT proteins encoded by pC221 and pCW41 were very similar by a variety of criteria.

Several explanations for the difference in expression are possible: the copy-number of pCW41 could be lower than that of pC221 (and pCW46); fragment B could contain stop signals which prevented transcription or translation extending into the cat gene in the "wrong" direction; if transcription of cat RNA from pC221 and pCW46 extended into fragment B such transcripts might be more stable than those from pCW41, due, for instance,to some 3'-terminal secondary structure; fragment B could encode some effector (RNA or protein) for the induction process. As removal of fragment B appeared to affect only the induced level of CAT a reduction in copy-number seems the least likely explanation.

The induction experiment using pCW48 (fig. 6.3), in which the HpaII site of pCW41 had been interrupted (and sequences in its vicinity had undergone some rearrangement and deletion) showed that the integrity of this site was unnecessary for the inducible synthesis of CAT. The specific activity of CAT in

uninduced cultures of S.aureus (pCW48) was 2.4-fold that in uninduced cultures of S.aureus (pC221); it is possible, therefore, that the copy-number of pCW48 is 2.4 times that of pC221. Assuming that this is the case, the rate of synthesis of CAT per gene-copy would become 0.29 relative to that for pC221, a value very close to that of 0.30 for pCW41.

This hypothesis might also be extended to explain the apparent discrepancy between the relative levels of expression seen in the induction experiments described here, and the MIC values determined by Wilson et al. (1981). The MICs of Cm for S.aureus carrying the following plasmids were reported to be: pC221, 70 μg/ml; pCW41, 40 μg/ml; pCW48, about 40 μg/ml. The copy-number of pCW48 relative to those of pC221 and pCW41 might be different under the different conditions used for the induction experiments and the MIC determinations. Measurements of the copy-numbers of pC221, pCW41 and pCW48 under different conditions would be necessary to clarify the contribution of gene-dosage effects.

Interruption of the BstEII site of pCW48 abolished detectable CAT activity. Consideration of the size of the CAT subunit and the relative positions of the MboI and BstEII sites allowed an estimate to be made by Wilson et al. (1981) of the position of the structural gene for CAT. More recently, analysis of the nucleotide sequence of pCW41 has confirmed that the BstEII site lies within the coding-region for CAT (Hawkins et al., unpublished; see App. 1). No plasmid determining the constitutive synthesis of CAT was isolated in this series of

experiments.

Rifampicin, which inhibits the initiation of transcription, prevented the induction of CAT synthesis in S.aureus (pC221). This contrasted with its effect on the induction of the Em^R gene of pE194: after the addition of rifampicin (or streptolygidin, which inhibits the elongation of transcripts) induction of the Em^R methylase by Em could still be detected in B.subtilis mini-cells (Shivakumar et al., 1980). This implies that if the control-point for regulation of expression of the cat gene is post-transcriptional, as is the case for the Em^R gene (see General Introduction, section 1.4.1) CAT mRNA is not unusually stable.

In order to determine whether regulation of the cat gene occurred during transcription or afterwards, the levels of cat RNA in induced and uninduced cultures of S.aureus (pC221) were compared. Although in one experiment (out of three) the RNA and CAT levels appeared to increase approximately in parallel on induction, there was much uncertainty in the uninduced level of CAT measured. In the other experiments the discrepancy between the 60-fold difference in CAT activity in induced and uninduced cultures and the small (at most, six-fold) changes measured for cat RNA levels in the same cultures argued against regulation occurring solely at the initiation of transcription.

It should be noted that hybridisation data may give an inaccurate reflection of the concentration of functional mRNA; Shivakumar et al. (1980) noticed a difference between the functional concentration of mRNA for the Em^R methylase and its concentration as determined by hybridisation with DNA-filters.

A necessary caveat in interpretation of the data from the experiments in section 6.3.3 (table 6.5) is that the amounts of cat RNA detected were insufficient to account for the rate of increase in CAT activity observed on induction. Only in one experiment did the amounts of hybridisation detected approach those expected. Some of the cat RNA may have been lost (relative to the more stable rRNA) due to degradation during the extraction of the RNA from S.aureus and its purification. A positive control was not included in these experiments, so it is unclear whether inefficient hybridisation caused the low values obtained. Additional precautions against degradation by RNase and some measure of the efficiency of hybridisation would, therefore, be useful in any similar studies of this system. It would probably be easier to extract intact mRNA from B.subtilis than from S.aureus.

The efficacy of 3-fluoroCm as an inducer of CAT was examined. The maximum specific activity achieved (at 1 μM) was similar to that observed when 3-deoxyCm was used as inducer. At concentrations greater than 1 μM, 3-fluoroCm inhibited the growth of S.aureus (pC221) and the specific activity of CAT in cell extracts fell sharply. Approximately 50% inhibition of growth rate was produced by 3 μM 3-fluoroCm.

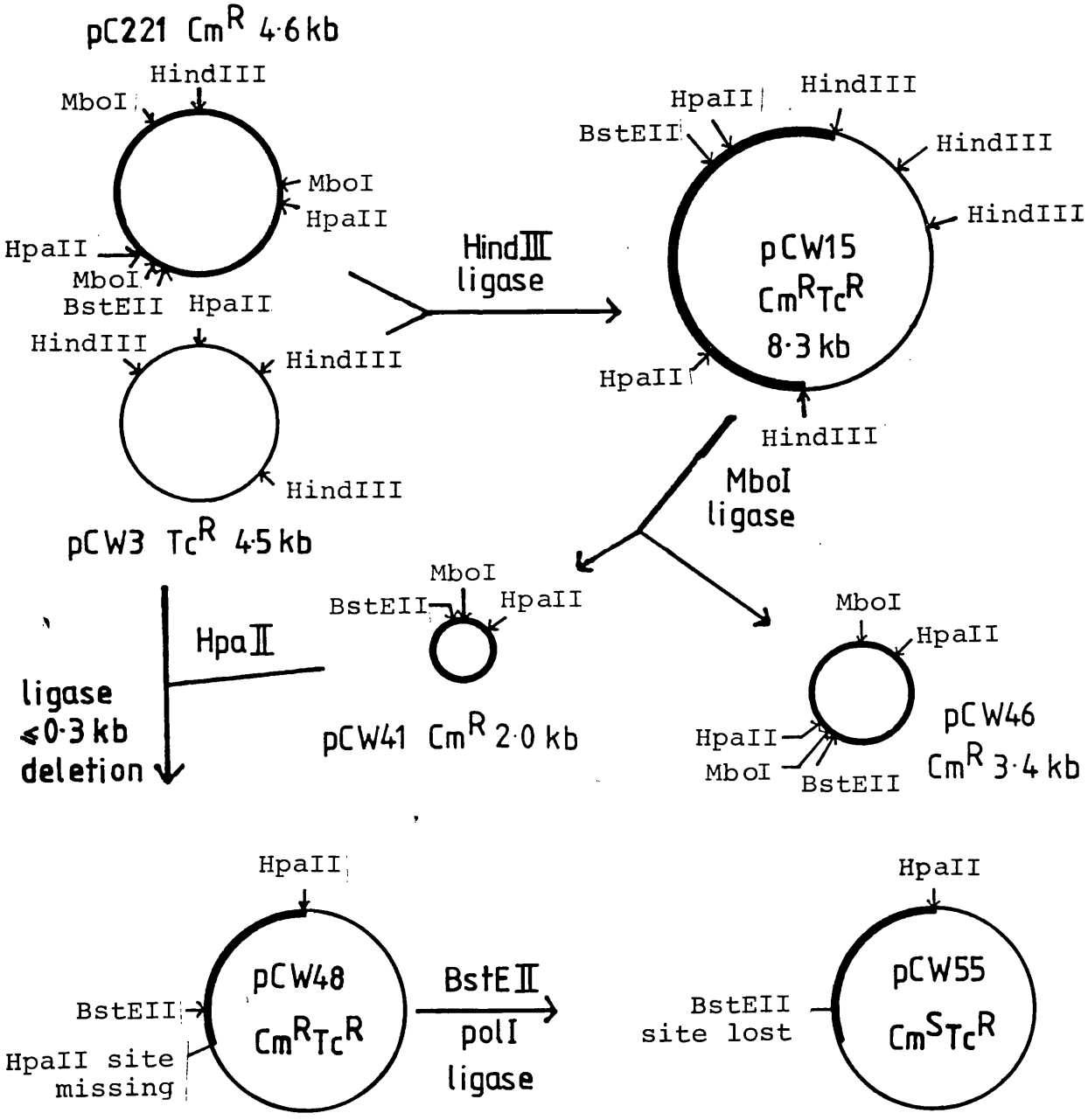


Fig. 6.1 Construction of pCW41, pCW46, pCW48 and pCW55

Outline of the strategy followed by C.R. Wilson to construct the plasmids used in sections 6.3.1 and 6.3.2. This figure was drawn using data from Wilson et al. (1981), where the construction of these plasmids was reported, Novick et al. (1982) and Chapter 5.

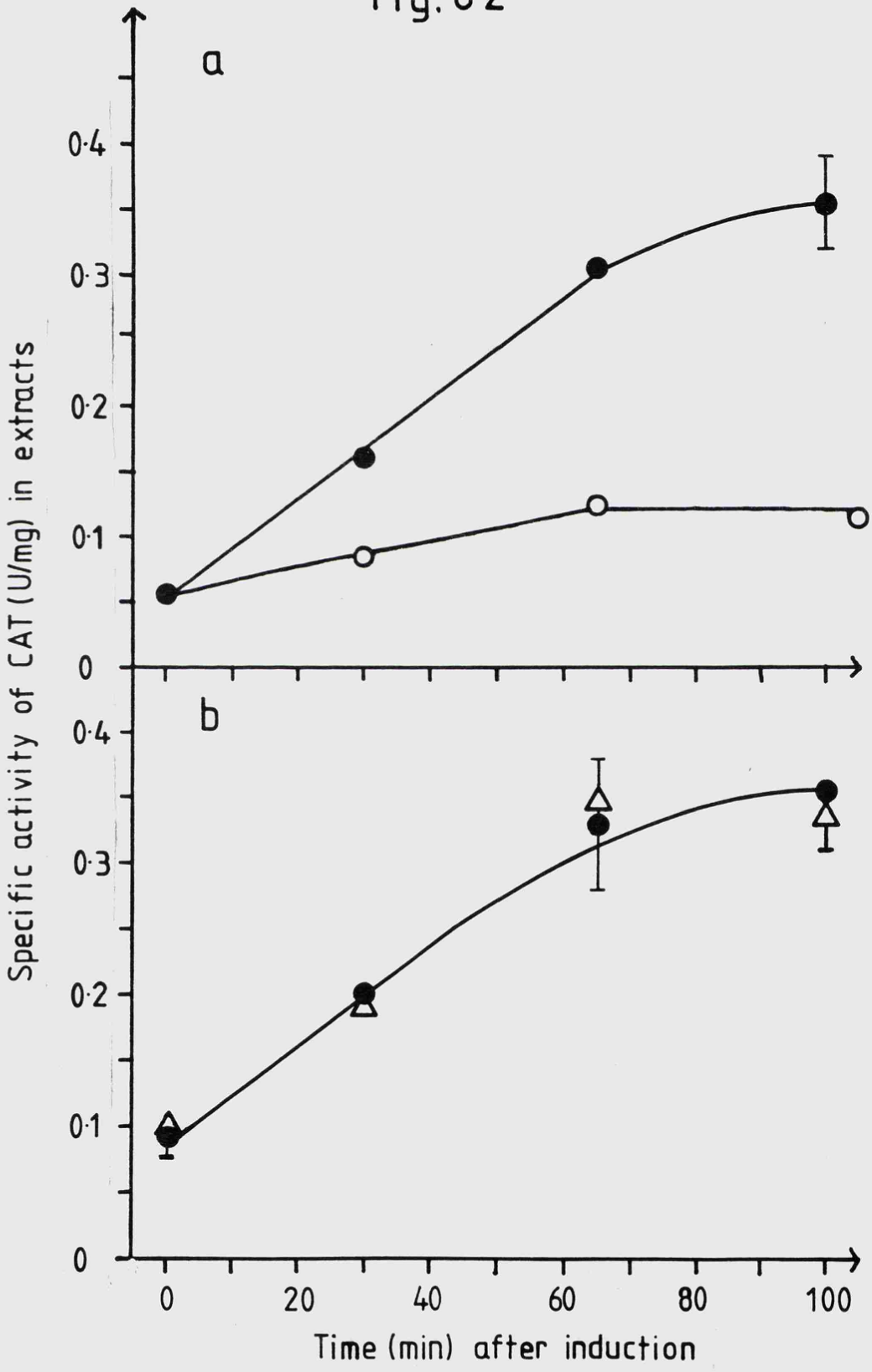
Fig. 6.2 Induction of CAT in S.aureus

Duplicate 125 ml cultures of S.aureus (A_{600}= 0.6 to 0.7) carrying the plasmids indicated below were induced with 27 μM D,L,threo-3-deoxyCm. At each time-point 10 ml samples were removed and extracts were prepared and assayed for CAT and protein (Chapter 2, section 2.4). The average specific activity of CAT in these extracts (± range) is plotted against time after addition of inducer.

(a) pC221 ● and pCW41 ○

(b) pC221 ● and pCW46 △

Fig. 6·2


a
b
0·4
0·3
0·2
0·1
0
0
20
40
60
80
100
Specific activity of CAT (U/mg) in extracts
Time (min) after induction

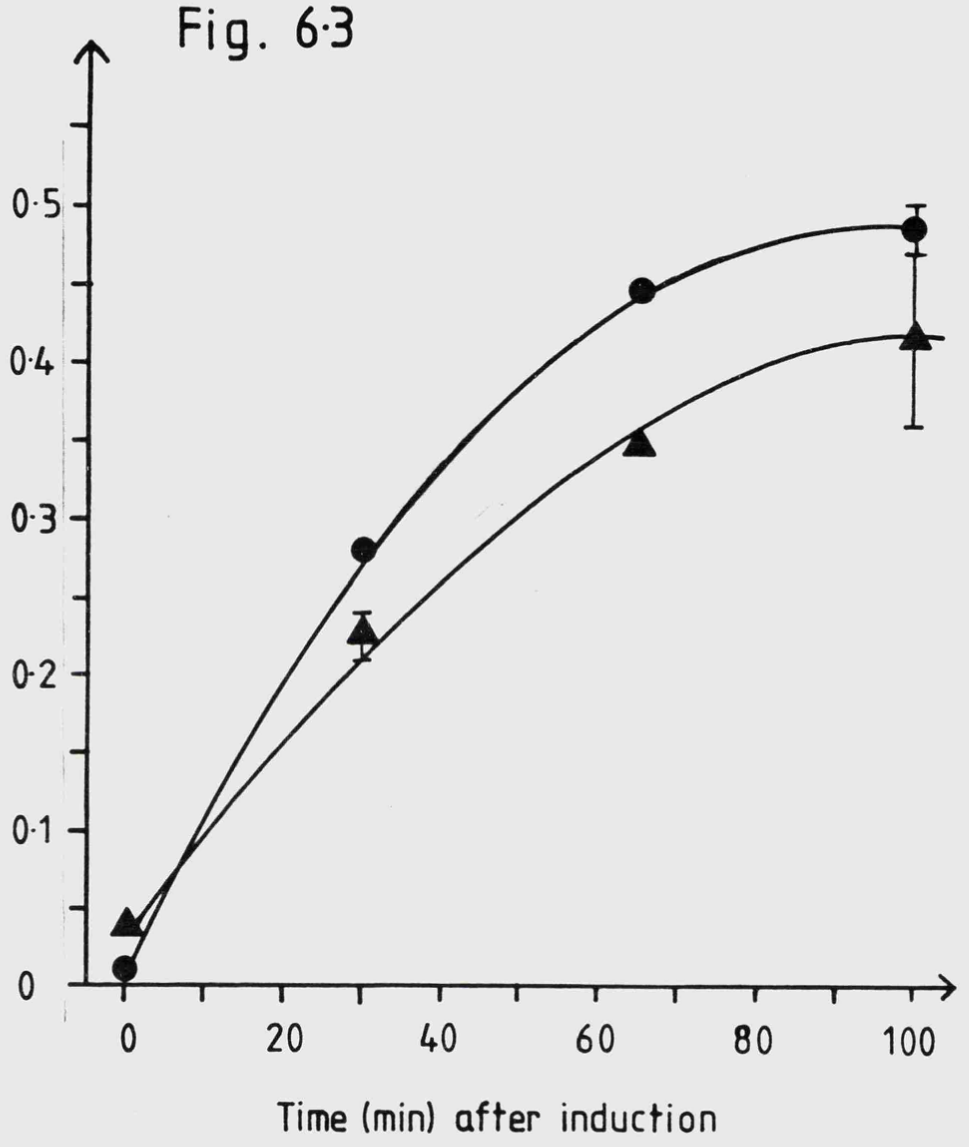


Fig. 6.3 Induction of CAT in S.aureus (pCW48)
3-deoxyCm was added to a final concentration of 6 μM to cultures of S.aureus carrying pC221 (A_{600}= 0.8) or pCW48 (A_{600}= 0.6). Extracts were made from 10 ml samples at the times indicated and the specific activity of CAT in these extracts was determined (Chapter 2, section 2.4). The average specific activity (± range) from duplicate cultures is plotted against time after addition of inducer.

S.aureus (pC221) ●
S.aureus (pCW48) ▲

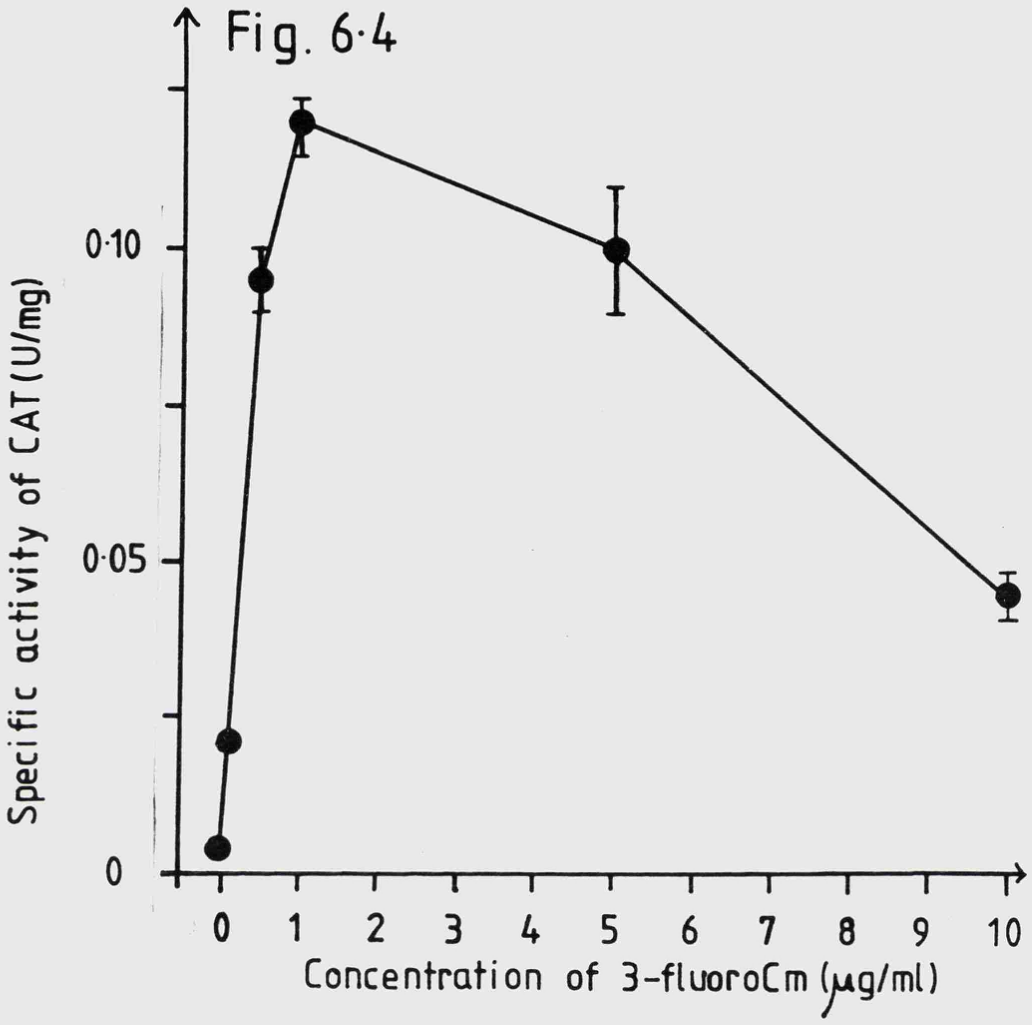


Fig. 6.4 Induction of CAT by 3-fluoroCm

3-fluoroCm was added to cultures of S.aureus (pC221) (A_{600}= 1.0) to the final concentrations indicated. After 40 min. incubation at 37° extracts were prepared and assayed for CAT and protein as described in Chapter 2 (section 2.4). CAT assays were performed in triplicate (range, ±6%), protein determinations in duplicate (range, ±2%).

Table 6.1(a) Preparations of RNA used for hybridisation experiments.

Variables	RNA preparations		
	A	B	C
A_{600} at addition of inducer	0.10	0.18	0.12
A_{600} at addition of azide	0.33	0.6	0.27
DNase incubation: µg added	50	50	4
duration (hr)	1.0	4.5	0.5
Yield (mg) per 90 ml of culture	0.025 to 0.98	2.0	0.2 to 0.4

Differences in the methods used to prepare RNA from S.aureus (see section 6.2.2)

Table 6.1(b) Preparations of radioactive fragment X

Experiment	Source of DNA	Isolation from gel	Nick-translated method
1	pCW41	1	A
2	pSES3	2	A
3	pSES3	1	A
4-6	pSES3	1	B, B, B

See section 6.2.3:

1 electro-elution (6.2.3.1)

2 low melting-point gel (6.2.3.2)

A 6.2.3.3

B 6.2.3.4

Table 6.2 Induction of pCW41 CAT

Strain	Plasmid	Specific activity of CAT (U/mg)	
		control	induced
WS 2009	pC221	0.011 (± 0.001)	0.88 (± 0.02)
WS 2041	pCW41	0.010 (± 0.002)	0.22 (± 0.01)

The average specific activity of CAT (± range) in duplicate extracts prepared as described in Chapter 2 (section 2.4) is shown. Cultures (A_{550} = 0.13) were induced by incubation with 33 μM D,L,threo-3-deoxyCm for 6 hr. The final A_{550} was 3.6.

Table 6.3 The effect of interrupting the BstEII site of pCW48

Strain	Plasmid	Specific activity of CAT (U/mg)
WS 2001	none	<0.001
WS 2009	pC221	0.90 (± 0.04)
WS 2055	pCW55	<0.001

3-deoxyCm was added (to a final concentration of 6 μM) to duplicate cultures of each strain at $A_{600} \simeq 0.27$ and incubation was continued for 3 hr. Extracts were prepared as described and assayed for CAT and protein (Chapter 2, section 2.4). The average specific activity is shown for each strain (± range for WS 2009).

Table 6.4 The effect of rifampicin on induction

Concentration of rifampicin (μg/ml)	Specific activity of CAT (U/mg)	
	control	induced
0	0.004	0.06
0.5	0.005	0.009
5.0	0.005	0.007

Rifampicin and 3-deoxyCm (final concentration, 6 μM) were added as shown to 100 ml cultures of S.aureus (pC221) at a Klett reading of 150. Cultures were harvested after 40 min incubation and extracts were prepared as described in Chapter 2 (section 2.4). CAT assays were performed in triplicate, protein assays in duplicate. The specific activity is shown; ranges were ±10%.

Table 6.5 Hybridisation experiments

expt no.	paper	duration (hr) of prehybr.	hybr.	temp.	[D]	probe (μCi)	washes
1	DBM	4	12	42°	10X	1.2	HF50
2	DBM	7	11	42°	5X	3.7	HF50
3	DPT	8	11	42°	5X	4.7	SSPE
4	DBM	28	16	42°	10X	2.7	HF50
5	NC	24	20	36°	10X	2.7	HF50
6	NC	24	20	36°	10X +d.s.	1.1	HF50

RNA prep.	Strains	+/- 3-deoxyCm	cpm bound	net cpm	CAT in extract (U/ml)
A	host	-	390±27	0	<0.004
	pC221	-	646±10	256	0.02 ± 0.01
	pC221	+	2748±80	2358	0.26 ± 0.04
blank			490±155		
B	host	-	300±30	23	<0.004
	host	-	253±12	-24	<0.004
	pC221	-	262±15	-15	0.012 ± 0.001
	pC221	-	253±5	-24	0.009 ± 0.002
	pC221	+	292±7	15	0.69 ± 0.02
	pC221	+	391±9	114	0.71 ± 0.02
blank			241±6		
B	host	-	287±38	+2	
	host	-	283±5	-2	
	pC221	-	350±12	65	
	pC221	-	351±10	66	
	pC221	+	472±54	187	see expt 2
	pC221	+	694±61	409	
blank			385±66		
C	host	-	147±12	0	<0.004
	pC221	-	168±11	21	0.004 ± 0.002
	pC221	+	197±13	50	0.23 ± 0.01
blank			114±3		
C	host	-	102±0	0	
	pC221	-	125±13	23	
	pC221	+	195±19	93	see expt 4
blank			128±27		
C	host	-	128±15	0	
	pC221	-	204±16	76	
	pC221	+	191±19	63	see expt 4
blank			117±10		

Notes to table 6.5

RNA (8 μg in expt 1, 10 μg in expts 2-6), prepared from S.aureus as described in section 6.2.2, was loaded onto DBM- or DPT-paper or nitrocellulose (NC), prepared and used as described in section 6.2.4. The filters were prehybridised and hybridised under the conditions indicated (for details see section 6.2.5), the radioactive probe being a 0.9 kb DNA fragment containing the structural gene for CAT (fragment X in fig. 7.4) which was isolated and labelled as described in section 6.2.3 and table 6.1(b). After hybridisation the filters were washed either in H buffer containing 50% formamide (HF50) at the hybridisation temperature or in different concentrations of SSPE buffer (containing 0.1% SDS) at room temperature (section 6.2.5).

[D]: indicates concentration of Denhardt's solution used in prehybridisation and hybridisation incubations.

+d.s.: dextran sulphate (10%, w/v) was also added in this experiment

RNA preps. A, B, and C: the differences in preparation are described in section 6.2.2 and table 6.1(a)

Blanks: sodium acetate buffer or water (as appropriate) was loaded instead of RNA solution

Strains: the host was S.aureus WS 2001; WS 2001 (pC221) is WS 2009

+/- 3-deoxyCm: 3-deoxyCm (6 to 8 μM final concentration) or an equal volume of ethanol was added to the cultures 50 to 90 min before harvesting the cells

Notes to table 6.5 (continued)

cpm bound: average ± range for duplicate filters

net cpm: the average value for binding to host RNA was subtracted from total cpm bound

CAT in extract: CAT was assayed in extracts prepared from 10 ml of each culture (Chapter 2, section 2.4).

CHAPTER 7

EXPRESSION OF pC221 *cat* IN *E.COLI*

7.1 Introduction

The largest MboI fragment of pC221, pCW41, had been shown to contain sufficient information for inducible CAT synthesis (Chapter 6 and Wilson et al., 1981). It was hoped that study of fragments smaller than this would give a more precise definition of the areas involved in the regulation of CAT synthesis and hence allow correlations to be made between function and the nucleotide sequence of pCW41 (Hawkins et al., unpublished; App. 1). Fragments of pC221 smaller than pCW41 (2.0 kb), however, would probably not have included regions necessary for their replication and maintenance as autonomous replicons, so these functions would need to be supplied by a vector plasmid in the new constructions.

Although both B.subtilis and E.coli provided good transformation efficiencies, and a variety of cloning vehicles was available for both, knowledge of the complete nucleotide sequence of pBR322 (Sutcliffe, 1979) and ease of restriction analysis of mini-lysates of E.coli carrying recombinant plasmids (Birnboim and Doly, 1979) suggested the choice of this vector-host system (as in Chapter 4). Previous experience of this system in this laboratory was also an advantage. No barrier to the expression in E.coli of staphylococcal Cm^R genes carried on pBR322-derivatives had been reported by previous workers (Ehrlich, 1978; Goze and Ehrlich, 1980).

In a cell-free transcription-translation system (made from E.coli), TaqI-digested pCW41 had directed synthesis of a polypeptide of the same mobility as CAT on SDS-PAGE (data not shown). This suggested the use of TaqI to obtain CAT-encoding fragments.

7.2 Methods

General methods (preparation of media, CAT assays, purification of plasmid DNA from S.aureus and E.coli, DNA techniques, transformation of E.coli and screening of transformants) were as described in Chapter 2 (sections 2.3 to 2.7). The plasmids used in the induction experiments described in this chapter are indicated in table 7.4. The host strain for all the experiments was E.coli WS 2301. The construction of pSES4 and pSES14 has been described in Chapter 4; the strain carrying pSES4 was WS 2317.

7.2.1 Construction of pSES3, 9, 10, 11 and 13.

Competent E.coli were transformed with the following ligation mixtures (contained in 2 to 3 μl of ligation buffer):

in experiment 1,

a) 0.12 μg of pCW41/MboI and 0.09 μg of pBR322/BamHI,

b) 0.24 μg of pC221/HpaII and 0.08 μg of pBR322/ClaI,

c) 0.12 μg of pC221/TaqI and 0.04 μg of pBR322/ClaI;

in experiment 2,

0.18 μg of pC221/TaqI and 0.06 μg of pBR322/ClaI, taken from the same ligation incubation as that used in experiment 1(c);

in experiment 3,

a) 0.23 μg of pCW41/MboI/TaqI and 0.16 μg of pBR322/BamHI/ClaI,

b) 0.34 μg of pCW41/MboI/TaqI and 0.24 μg of pBR322/BamHI/ClaI.

In each experiment 0.2 μg of CCC pBR322 was used as a positive control, and a negative control (no DNA added) was also performed.

Transformation mixtures (0.2 ml) were spread on LCm10 plates (except for 10 μl spread on LAp40 plates for pBR322 transformation). Representative Cm^R transformants were streaked for single colonies on LCm10 plates, and single colonies from these plates were tested for resistance to Ap (40 μg/ml), Tc (20 μg/ml) and Cm (10 μg/ml), and for nutritional requirements. Mini-lysates of representative isolates were analysed by restriction digestion and agarose gel electrophoresis: 5 to 10 μl of mini-lysates were used to check that the plasmids would retransform E.coli to the same phenotype.

7.2.2 Construction of pSES15 and pSES16.

To ensure that only a single TaqI fragment was inserted into the ClaI site of pBR322 the 5'-phosphate groups of TaqI-digested pSES13 were removed by treatment with phosphatase prior to ligation (Maniatis et al., 1982).

Plasmid DNA was purified from WS 2313 using a vertical rotor for the CsCl/EthBr centrifugation. As the preparation contained an appreciable amount of RNA (though free from chromosomal DNA) this was taken into account in calculating the amount of phosphatase required. TaqI-digested pSES13 (2 μg) in 50 μl of 50 mM Tris-HCl, pH 9.0, 1 mM magnesium chloride, 0.1 mM zinc chloride and 1 mM spermidine was treated with 0.6 U of calf intestinal phosphatase (Boehringer-Mannheim) at 37° for 30 min. Another 0.5 U was added and incubation was continued for another 30 min. Phosphatase was inactivated by the addition of 5 μl of 10% (w/v) SDS, 10 μl of 10 x STE (0.1 M Tris-HCl, pH 8.0, 1 M sodium chloride, 10 mM EDTA) and water (to 100 μl), and

heating at 68° for 20 min. After two extractions with equal volumes of phenol/chloroform/iso-amyl alcohol (25:24:1) and two extractions with equal volumes of chloroform/iso-amyl alcohol (24:1) the sample was passed through a 1 ml Sephadex G-50 "spin-column" equilibrated with TE. (Centrifugations were for 1 min. at 2,000 rpm in the MSE Minor centrifuge). Of the 140 μl eluted, 40 μl were subjected to gel electrophoresis to check the recovery. By comparison of band intensity with pBR322/TaqI (0.9 μg), the yield was judged to be >50%.

The remainder of the phosphatased material was ligated with pBR322/ClaI (0.125 μg) in a volume of 30 μl. E.coli was transformed with 10 μl of this ligation mixture, selecting on LCm10 plates (controls as in 7.2.1). Mini-lysates of Cm^R transformants were analysed for plasmid content and orientation of the inserts, and 2 μl were used to transform E.coli to Cm^R.

7.2.3 Induction experiments.

Two 100 ml portions of L broth (pre-warmed) in 250 ml flasks were inoculated with 1 to 2 ml of overnight cultures of each strain and shaken at 37°. When the A_{600} of the cultures was approximately 0.1, 5 to 10 μM 3-deoxyCm was added to one flask of each pair. (This was the concentration range giving maximal induction in S.aureus; Winshell and Shaw, 1969). The same volume of ethanol was added to the other (control) flask of each pair.

Incubation at 37° was continued for a further 2 to 3 hr. (to the final A_{600} indicated) and the cultures were harvested by centrifugation (9,000 rpm, 10 min., 4°, MSE 18, 6 x 250 ml

rotor). Pellets were washed once with 10 ml of 10 mM Tris-HCl, 10 mM EDTA, pH 8.0, 50 mM glucose, containing 2 or 4 mg/ml lysozyme. The suspensions were incubated at 37° for 30 to 60 min. and usually frozen and thawed 3 to 4 times. DNase (20 to 40 μg) was added to reduce the viscosity before the cell debris was removed by centrifugation (5 to 10 min., 11,000 x g). The supernatants were stored at -20° until assayed for CAT and BLA activities.

7.2.4 BLA assay.

The procedure was based on that of Sawai et al. (1978). A portion of each extract (10 μl) was diluted one hundred-fold in sodium phosphate buffer (0.1 M, pH 7.0) and 5 to 200 μl samples of the diluted extracts (containing 0.02 to 0.10 U of BLA) were made up to 500 μl with the same buffer ("sample tubes"). After pre-incubation of the tubes at 30° for 5 min., 100 μl of 20 mM ampicillin (freshly dissolved in water) were added to each: exactly 10 min. afterwards, 1 ml of iodine reagent (8 mM iodine, 60 mM potassium iodide, in 0.5 M sodium acetate, pH 4.0) was added to each tube and incubation continued at room temperature for 10 min. Tubes "A" (at least two per run) contained 0.5 ml of phosphate buffer only and were treated as for the sample tubes. Tubes "B" (one per sample tube) had samples of diluted extract added after the iodine reagent instead of before the ampicillin.

The units of activity in each sample tube were calculated using the formula:

$$\frac{8}{A_{540}(A)} \times \frac{\Delta 540}{3.7} \times \frac{1}{10}$$

where $\Delta 540$ is the difference in A_{540} between tube B and the sample tube, $A_{540}(A)$ is the average A_{540} of tubes A (for a given run), and 3.7 is the number of moles of iodine reduced by 1 mole of penicilloic acid (to which penicillin is degraded by BLA) (Sawai et al., 1978; Sargent, 1968). (Eight μmol of iodine were present in each tube and the incubations were for 10 min.)

One unit of BLA activity was defined as that activity which hydrolysed 1 μmol of ampicillin per minute at 30° under the assay conditions described above.

In fig. 7.1 the number of units of BLA observed was plotted against the volume of diluted E.coli (pBR322) extract added. The plateau value of 0.2 U (representing 2 μmol of ampicillin hydrolysed in 10 min.) corresponded to a change in A_{540} of about 0.57.

7.3 Results

7.3.1 Ligation of pBR322 with pCW41.

As indicated in table 7.1 (experiment 1(a)), all six Cm^R transformants tested were Tc^S as expected if insertion into pBR322 had occured at the BamHI site, which is in the Tc^R gene. Mini-lysates of four of the isolates contained plasmids of similar mobility, cut by HindIII to a single fragment of about 6.4 kb. (The other two isolates contained larger plasmids and were discarded.) Sau3A digests appeared identical with that of pBR322 with the addition of a 2.0 kb fragment (as expected) (Sau3A, rather than MboI, was used since it recognises -GATC-, the Dam^+ methylation site, when A is methylated; see Maniatis et al, 1982). TaqI digests contained a band at about 1.1 kb indicating that pCW41 was inserted in orientation B (see fig. 7.2 (a); gels not shown). One isolate, WS 2303, was retained for further study. A mini-lysate from this strain transformed E.coli to the same phenotype.

Figs. 7.3(a) and (b) show Sau3A digests of pSES3, purified from WS 2303 (which is dam^+); pSES3 contains all the Sau3A fragments of pBR322 with the addition of a 2 kb fragment co-migrating with linear pCW41. TaqI digests of pSES3, pBR322 (from the dam^- host, GM48) and pCW41 (from S.aureus) are shown in fig. 7.3(c). TaqI failed to cut some molecules of pSES3 at 1125 (position on Sutcliffe's map, 1978) due to the overlapping Dam^+ methylation site (McClelland, 1981). The 0.312 kb fragment, which contains the BamHI site has been replaced in pSES3

by fragments of 0.255 and 1.16 kb (the latter coincidentally co-migrates with the largest TaqI fragment of pCW41) and fragments of 0.12 and 0.47 kb from pCW41 (TaqI fragments J and D of pC221).

7.3.2 Ligation of pBR322 with HpaII-cut pC221.

Table 7.1 shows the results of transformation of E.coli with this ligation mixture (experiment 1(b)). Mini-lysates from five Cm^R isolates were digested with HindIII. From the data in table 7.2, isolate A (WS 2311) alone might contain one copy of the 1.86 kb HpaII fragment of pC221 (fragment Z) per pBR322 moiety. The data for isolates D and E are consistent with the insertion of pC221 (cut at 3.14 on the map shown in fig. 7.4) with the cat gene in orientation B (clockwise). The HindIII fragments observed for isolate B would have been observed if pC221 (cut at the HpaII site at 1.28 on the map) and an additional copy of fragment Z had both been inserted into pBR322. Similarly, isolate C might have been formed by the insertion of two copies of fragment Z per pBR322 moiety (or four per two pBR322 moieties). Further studies were confined to WS 2311.

HinfI digestion of a mini-lysate of WS 2311 (fig. 7.3(a)) indicated that the HpaII fragment was inserted into pBR322 with the cat gene in orientation A (as shown in fig. 7.2(b)). The other additional bands (about 0.13 and 0.41 kb) in this track co-migrated with HinfI fragments H and F of pC221. In a HpaII digest of the same mini-lysate a fragment of about 2.45 kb replaced the 0.622 kb fragment, indicating that the insert was about 1.8 kb as expected. All the other fragments appeared

identical with those of pBR322/HpaII, giving a total size of 6.2 kb for the plasmid, pSES11. E.coli transformed with a WS 2311 mini-lysate showed the same antibiotic-resistance pattern and nutritional requirements as WS 2311.

7.3.3 Ligation of pBR322 with TaqI-digested pC221.

Table 7.1 shows the results of transforming E.coli with this ligation mixture (experiments 1(c) and 2). Mini-lysates from one Cm^R isolate from experiment 1(c), WS 2310, and six from experiment 2 were examined: WS 2310 contained a plasmid of about 5.5 kb (pSES10); the smallest plasmid from experiment 2, pSES13 (in WS 2313) was 0.2 to 0.4 kb larger than this (gels not shown). TaqI digests of mini-lysates (figs. 7.3(d) and (e)) showed that while both pSES10 and pSES13 contained the 1.2 kb TaqI fragment of pC221 (fragment Y, fig. 7.4) in addition to those from pBR322, pSES13 also contained fragments of about 0.12 and 0.18 kb (co-migrating with TaqI fragments J and H of pC221, fig. 7.3(d)).

As indicated in figs. 7.2(c) and (d), insertion into the ClaI site of pBR322 interrupts the 0.622 kb HpaII fragment. HpaII digests of WS 2310 mini-lysates lacked this fragment and gained a 1.5 to 1.6 kb fragment (fig. 7.3(f)). The other new fragment apparently runs slightly behind the 0.238 and 0.242 kb fragments from pBR322 (arrowed in fig. 7.3(h)). This is consistent with the structure shown for pSES10 in fig. 7.2(c), with the cat gene in orientation B. For WS 2313, new HpaII fragments of 0.92 and 1.13 kb replace the 0.622 kb fragment, which is consistent with the structure shown for pSES13 in

fig. 7.2(d), i.e. with the cat gene in orientation A and the two other TaqI fragments from pC221 at its 3' end. BstEII cut purified pSES13 to about 6.0 kb, and the smaller products of BstEII + EcoRI and BstEII + BamHI double digests were 0.73 and 1.0 kb respectively, which also agreed with this structure (gels not shown).

7.3.4 Ligation of pBR322 with pCW41 cut with MboI and TaqI.

Table 7.1 shows the results of transforming E.coli with this ligation mixture (experiment 3). Mini-lysates of eight typical isolates were examined: the four smallest plasmids were of similar mobility, the others were of different sizes and were studied no further. EcoRI or BstEII digests of the small plasmids gave single fragments of about 4.9 kb; TaqI digests showed the loss of the 0.312 and 0.315 kb fragments of pBR322 and an extra band at 1.15 kb; AluI digests contained new bands at 0.43 and 0.74 kb. The observations are consistent with the structure shown for pSES9 in fig. 7.2(e). A mini-lysate prepared from one isolate (WS 2309) transformed E.coli to the same phenotype, and transformants contained plasmids of the same size as pSES9. Fig. 7.3(g) shows TaqI, Sau3A and AluI digests of purified pSES9 compared with those of dam$^-$ pBR322. Just as for pSES3 (section 7.3.1 and fig. 7.3(c)) both 0.475 and 0.616 kb TaqI fragments were observed for pSES9 (track 3) and pSES13 (track 1).

7.3.5 Ligation of pBR322 with TaqI-cut pSES13.

Transformation with this ligation mixture produced the results shown in table 7.3 Mini-lysates prepared from six Cm^R isolates and WS 2310 contained plasmids of identical mobility

which were cut by TaqI to the same pattern: HpaII digests lacked the 0.622 kb fragment of pBR322 and contained, in addition to the remaining pBR322 fragments, either a 1.6 kb fragment (as for WS 2310) or 0.6 and 1.1 kb fragments (gel not shown). These data are consistent with the structures shown for pSES15 and pSES16 in fig. 7.2(d) and (c). Mini-lysates of one isolate from either class, WS 2315 and WS 2316, transformed E.coli to the same antibiotic-resistance patterns as WS 2315 and WS 2316.

Fig. 7.3(d) is a comparison of TaqI digests of mini-lysates of WS 2302, WS 2313, WS 2315 and WS 2316, and of purified pC221 and dam$^-$ pBR322. Fig. 7.3(f) shows HpaII digests of WS 2302, WS 2310, WS 2313, WS 2315 and WS 2316 mini-lysates. As is also evident in fig. 7.3(h), HpaII digests of WS 2310 and WS 2316 mini-lysates (which in this particular experiment were incomplete) are very similar.

7.3.6 Expression of the cat gene of pC221 in E.coli.

Preliminary experiments with E.coli carrying TaqI-digested pC221 ligated with ClaI-digested pBR322 had indicated that CAT activity increased seven- to twenty-fold on the addition of 10 μM 3-deoxyCm to cultures of this strain (data not shown). Table 7.4 shows data from experiments designed to test whether the cat gene of pC221 was inducible in E.coli. BLA activity was measured to check that no increase in plasmid copy-number had been caused by the addition of 3-deoxyCm. Insertion of DNA at the ClaI or HindIII site of pBR322 caused a significant reduction in the BLA activity measured in these extracts. Insertion of pCW41 at the BamHI site of pBR322 to form pSES3 had much less effect.

In contrast with the results of the preliminary experiments, the data from experiments 1 and 2 (table 7.4) showed that, while synthesis of CAT was still inducible in these strains, the induced level of CAT activity was apparently only 4 or 5 times that found in the uninduced cultures (compared with 50- to 100-fold increases seen for S.aureus (pC221) in Chapters 3 and 6). This ratio would, of course, have been affected by any errors in the measurement of the uninduced level: although the lower levels of CAT can be measured fairly precisely, the values obtained might be inaccurate. Both false positive values (e.g. for E.coli (pBR322) in experiments 3 and 4 of table 7.4) and false negative values (in strains producing very low levels of CAT, not shown) have been obtained. That less CAT was found in extracts of E.coli carrying pSES4 than of that carrying pSES3 or pSES11 is consistent with the lower level of Cm^R conferred in vivo by this plasmid (see table 7.5). Low Cm^R was also conferred by pSES14, which consists of pC221 inserted into pBR322 in the opposite direction.

The data from experiments 3 and 4 (table 7.4) reflected the differences in Cm^R observed in vivo between orientations A and B of fragment Y (compare E.coli carrying pSES10 and pSES13 in tables 7.4 and 7.5). Although for E.coli (pSES10) no difference in CAT levels was apparent on the addition of inducer, a small increase was observed for E.coli (pSES9); however, the magnitude of the change was difficult to quantify. A substantial increase (about 20-fold) in CAT levels was however observed for E.coli (pSES13), which confirmed the results of the preliminary experiments

mentioned above.

As pSES13 contains two other small fragments of pC221 in addition to fragment Y (see section 7.3.3), the construction of pSES15 (and pSES16) was undertaken (sections 7.2.2 and 7.3.5) using phosphatase treatment to prevent more than one TaqI fragment of pSES13 being inserted into each pBR322 molecule. The data from experiment 4 (table 7.4) confirm that it is the difference of orientation of the cat gene fragment rather than the presence of the two extra fragments which affects the expression of the cat gene (table 7.4) and the level of Cm^R conferred in vivo (table 7.5). The apparent difference in the levels of CAT observed for induced cultures of E.coli (pSES13) in experiment 3 and E.coli (pSES15) in experiment 4 may not be significant as the experimental conditions varied somewhat. (In preliminary experiments similar variations in the amount of induction observed had been obtained for a given strain, not shown). As shown in section 7.3.5, pSES10 and pSES16 are probably identical. As was the case for E.coli (pSES10) in experiment 3, no significant change in CAT levels was observed after incubation of E.coli (pSES16) with inducer in experiment 4. The expression of the cat gene in E.coli was always much lower than that in S.aureus; the induced specific activity of CAT in E.coli (pSES13) in experiment 3 was about 4% of that measured for S.aureus (pC221) under comparable conditions (data not shown).

7.3.7 The effect of the cloned fragments on Tc^R.

Table 7.5 shows the levels of Tc^R conferred on E.coli by various plasmids. As expected, insertion of pCW41 into the BamHI

site of pBR322, which lies in the structural gene for tet, abolished Tc^R, as did removal of part of the tet gene during the construction of pSES9. As reported by previous workers, however, not all inserts into the HindIII or ClaI site in the tet promoter caused Tc^S (e.g. Widera et al., 1978), although none of the plasmids conferred as high a level of Tc^R as did pBR322. From the data in table 7.5 certain deductions can be made about transcription reading through from the cloned fragments (fig. 7.5). Low Tc^R was conferred by pSES14, but not by pSES4, which indicated some transcription clockwise across the HindIII site of pC221. Moderate Tc^R was conferred by pSES11; this implied that there is transcription anticlockwise across the HpaII site at 1.28 kb in pC221. Thus, some sequences in several regions of pC221 can apparently be recognised as promoters by E.coli RNA polymerase.

Only marginal Tc^R was conferred by either pSES13 or pSES15; thus, there appeared to be little transcription anticlockwise across the TaqI site at 2.08 in pC221 (i.e. away from cat). Reversal of fragment Y, however, permitted moderate Tc^R (pSES10 and pSES16), indicating that transcription could proceed clockwise through the TaqI site at 2.29 kb. West et al. (1979) observed that expression of Tc^R depended on the orientation of promoter-bearing fragments inserted at the HindIII site of pBR322, which is adjacent to the ClaI site used here.

7.4 Conclusions and discussion

Varying levels of Cm^R were conferred on E.coli by the different plasmids described in this chapter; similarly, the amount of CAT activity detected in extracts of induced cultures of these strains differed by as much as twenty-fold in a given experiment. Assuming that the plasmids have similar copy-numbers, this indicates that expression of the cat gene carried by them differed.

One explanation for the low levels of CAT measured is that the pC221 cat promoter might be poorly recognised by E.coli RNA polymerase; in some cases transcription from a pBR322 promoter might then enhance expression. Fig. 7.6 shows the position and orientation of some of the promoters of pBR322 in relation to the restriction sites used for cloning the staphylococcal cat gene. Transcription of fragments inserted at the HindIII or ClaI site in orientation A (i.e. with the cat gene anticlockwise) could initiate at the anti-tet promoter. Increasing the distance from this promoter to the coding-region for CAT would increase the probability of attenuation by termination signals (compare pSES13 or pSES15 with pSES11 and pSES4). For pSES3, which carries pCW41 inserted into the tet gene of pBR322 at the BamHI site, some transcription initiating at the tet promoter might read through into the cat gene. As above, some attenuation might occur before the coding-region for CAT was reached. For the other plasmids carrying the cat gene in orientation B, pSES10 (or pSES16) and pSES9, interruption of the ClaI site would have abolished the activity of the tet promoter (see fig. 7.6(b)).

Removal of the anti-*tet* promoter, which was excised during the construction of pSES9, caused only a slight increase in the induced level of CAT activity; this suggests that transcription from the anti-*tet* promoter in the opposite orientation to *cat* transcription has only a small effect on *cat* expression. The twenty-fold difference in expression observed on changing the orientation of fragment Y in the ClaI site (pSES13 *versus* pSES10, or pSES15 *versus* pSES16) was reminiscent of the hundred-fold change in expression of a promoterless (*E.coli*) *cat* gene inserted into the HindIII site of pBR322 (West and Rodriguez, 1982).

The explanation given above does not, however, cover the different induction ratios observed for the different plasmids. Although the results presented in this chapter are generally in agreement with the report by Horinouchi and Weisblum (1982) that a 1 kb fragment of pC194 carried by pBR322 conferred inducible Cm^R on *E.coli*, the induction ratios measured here varied from one (i.e. uninducible) to twenty for plasmids containing different fragments of pC221. Low expression of Cm^R was associated with little or no induction; no constitutively high expression was observed. These results suggested that some property of *cat* transcripts initiating at pBR322 promoters was necessary for efficient induction of CAT in *E.coli*, either their greater quantity (if, indeed, transcription from the pC221 *cat* promoter is deficient in *E.coli*) or their greater length.

The former property could be important if, for instance, some translation product of *cat* RNA were essential for the induction process; there would then be a sigmoidal dependence of

expression on the amount of *cat* transcription. Initiation at a pBR322 promoter would only be necessary for good induction in this case if transcription from the *cat* promoter were insufficient. Promoters from Gram-positive bacteria are normally recognised by *E.coli* RNA polymerase; it would appear that some sequences in pC221 can act as promoters in *E.coli* since transcription of the *tet* gene probably initiates within fragments of pC221 in some of the plasmids constructed here. Whether the greater lengths of *cat* transcripts initiating at pBR322 promoters is important for the induction of *cat* in *E.coli* will be discussed in Chapter 8.

Presumably the failure to reproduce *in vitro* the induction of CAT synthesis observed in *S.aureus in vivo*, using a cell-free transcription-translation system from *E.coli* directed by pCW41 (experiments not shown), was related to the poor induction of *cat* observed *in vivo* in *E.coli* when no pBR322 promoter was available.

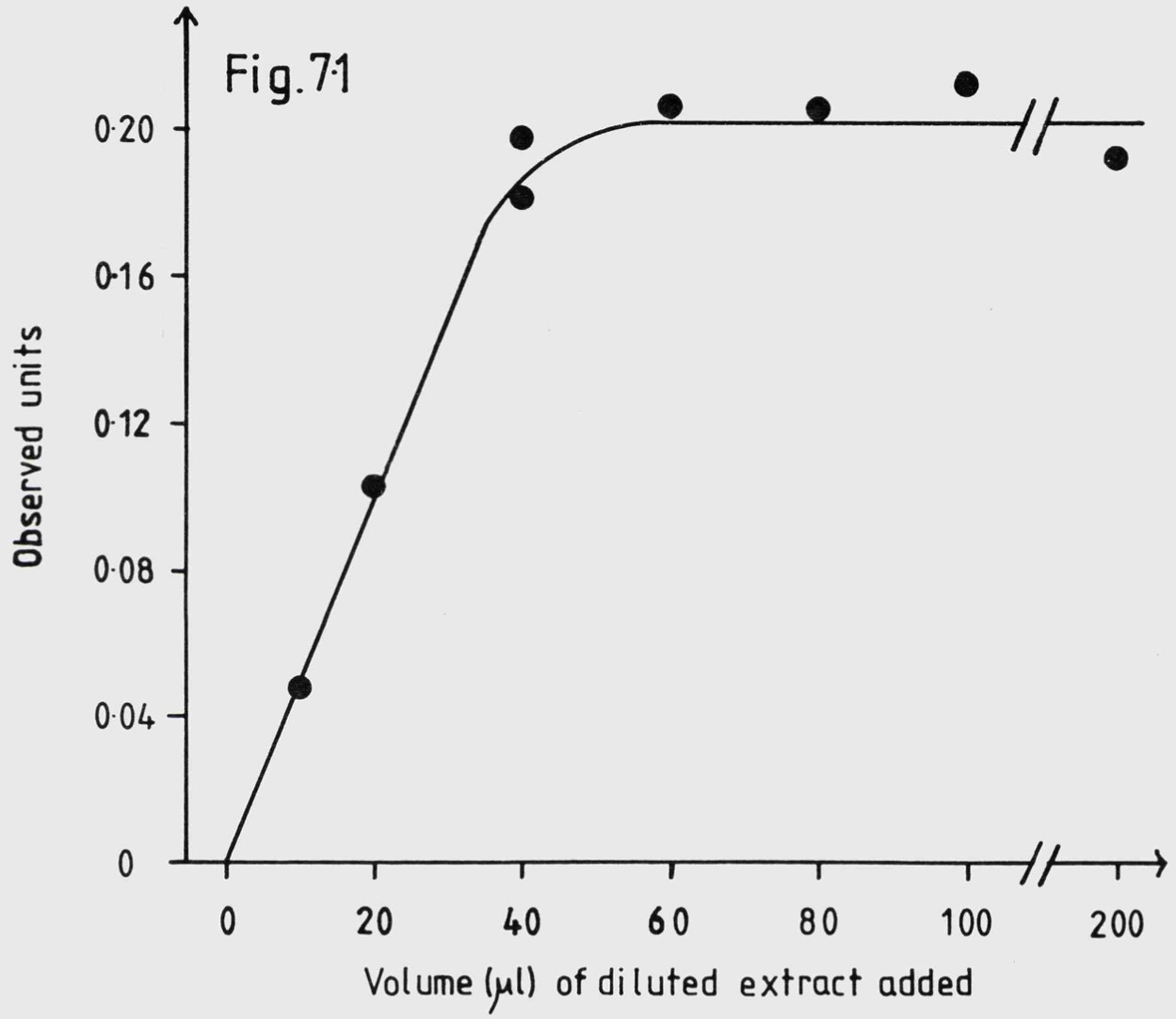


Fig. 7.1 BLA assay

An extract of E.coli (pBR322) was prepared, and a portion diluted 100-fold and assayed for BLA activity (see sections 7.2.3 and 7.2.4 for methods). The number of units of BLA observed was plotted against the volume of diluted extract added.

Fig. 7.2 Restriction maps of pSES3, 9, 10, 11, 13, 15 and 16

The positions shown for the restriction sites are those predicted from the nucleotide sequence of pBR322 (Sutcliffe, 1979) and the restriction map of pC221 (Chapter 5). In each case, only part of the region derived from pBR322 is drawn and only the restriction sites nearest to the insert are shown. In each case, only part of this region is drawn. The position and orientation of the coding-region for CAT are indicated.

Key:

B	BstEII	L	AluI
E	EcoRI	S	Sau3A
F	HinfI	T	TaqI
H	HpaII		

——— inserted fragment ═══ pBR322

Scale: 0 0·5 1·0 kb for a and b

0 0·2 0·4 kb for c, d and e

To construct pSES3 (a), pCW41 was inserted into the BamHI site of pBR322 (see section 7.3.1). To form pSES10, pSES11, pSES13, pSES15 and pSES16 (b-d) the ClaI site of pBR322 was used (sections 7.3.2, 7.3.3 and 7.3.5). The position of the additional fragments present in pSES13 (section 7.3.3) has been indicated in (d). During the construction of pSES9 (e), the 0.35 kb ClaI-BamHI fragment of pBR322 was replaced by the 0.9 kb TaqI-MboI fragment of pCW41 (fragment X in fig. 7.4; see section 7.3.4).

Fig.7·2

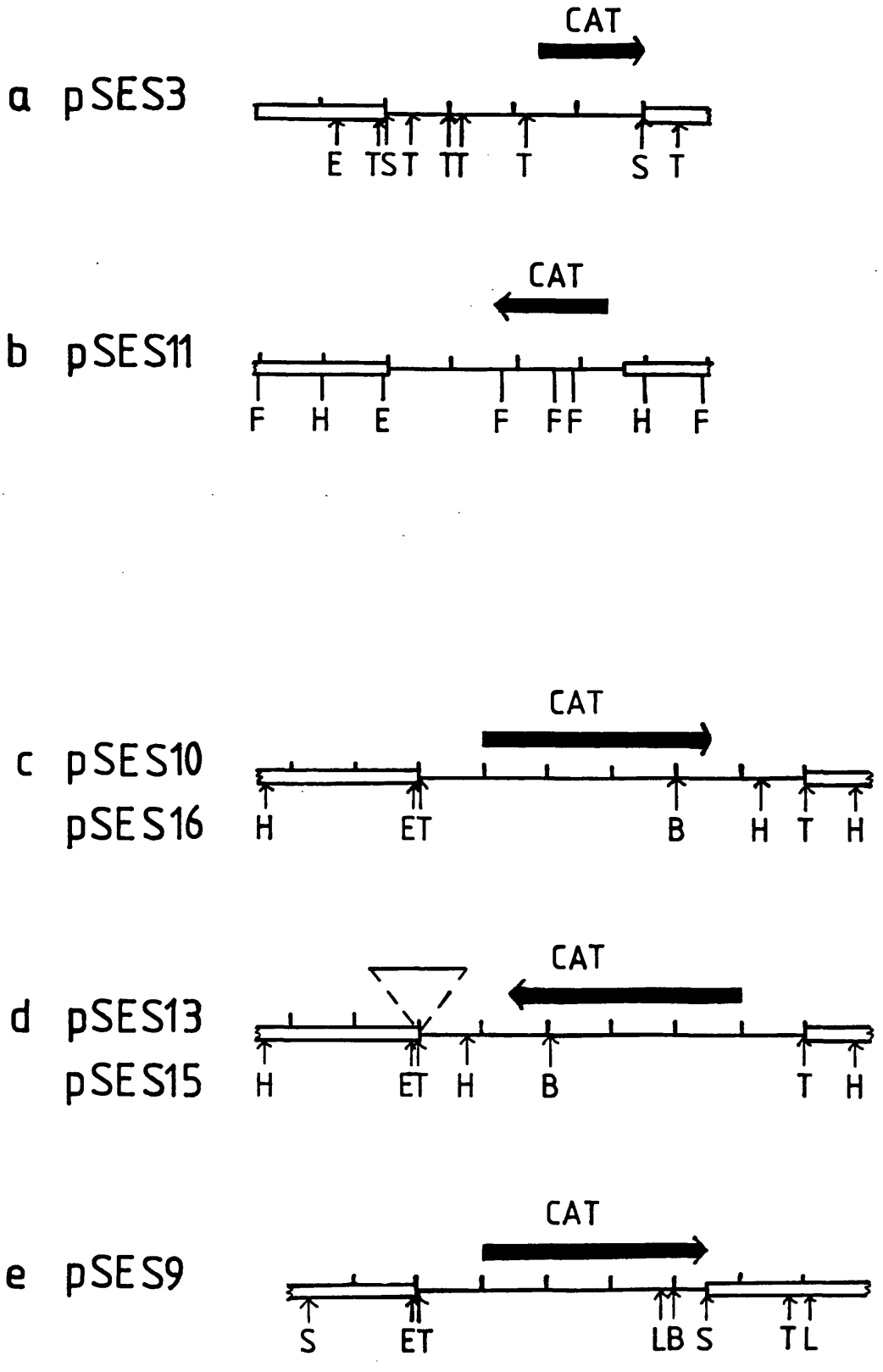

a pSES3
CAT
E TST T TT T S T
b pSES11
CAT
F H E F FF H F
c pSES10
pSES16
CAT
H ET B H T H
d pSES13
pSES15
CAT
H ET H B T H
e pSES9
CAT
S ET LB S TL

Fig. 7.3 Restriction digests

See sections 7.3.1. to 7.3.5. Electrophoresis was for 3 hr at 60 V in TAE except for the gels shown in c, d and g. The size-markers used were (sizes in kb):

λ/HindIII, 23.1, 9.4, 6.8, 4.36, 2.32, 2.03, 0.564;

pBR322/AluI, 0.91, 0.659 and 0.655, 0.521, 0.403, 0.281, 0.257, 0.226, 0.136 and smaller;

pBR322/HinfI, 1.63, 0.517 and 0.506, 0.396, 0.344, 0.298, 0.221 and 0.220, 0.154, 0.075;

pBR322/HpaII, 0.662, 0.527, 0.404, 0.309, 0.242 and 0.238, 0.217, 0.201 and smaller;

pBR322/Sau3A, 1.37, 0.665, 0.358, 0.341, 0.317, 0.272, 0.258, 0.207, 0.105, 0.091 and smaller;

pBR322/TaqI, 1.444, 1.307, 0.475, 0.368, 0.315 and 0.312, 0.141.

If Dam^{+} methylation has occurred a 0.616 kb fragment replaces all or some of the 0.475 and 0.141 kb fragments (section 7.3.1).

a) Samples were: 1, pBR322/HinfI; 2, HinfI digest of WS 2311 mini-lysate; 3, pBR322/Sau3A; 4, pSES3/Sau3A; 5, λ/HindIII, showing the 2.32 and 2.03 kb bands only. The gel was 2% agarose.

b) Samples were: 1, λ/HindIII; 2, pCW41/MboI; 3, pSES3/Sau3A. The gel was 1.2% agarose.

c) Samples were: 1, pBR322/TaqI; 2 and 4, pSES3/TaqI; 3, pCW41/TaqI. Electrophoresis was for 2.6 hr at 70 V on 1.6% agarose in TAE.

d) Samples were TaqI digests of: 1, pBR322; 2-5, mini-lysates of WS 2302, WS 2316, WS 2315 and WS 2313; 6, pC221. The gel was 1.8% agarose in TAE; electrophoresis was for 3.2 hr at 60 V.

e) Samples were TaqI digests of: 1, pC221; 2-4, mini-lysates of WS 2313, WS 2310, WS 2302, 6; dam⁻ pBR322. The gel was 1.6% agarose.

f) Samples were: 1, λ/HindIII; 2-5, HpaII digests of mini-lysates of WS 2313, WS 2316, WS 2315, and WS 2310. The gel was 0.9% agarose

g) Samples were: 1-3, TaqI digests of pSES13, dam⁻ pBR322, and pSES9; 4-6, Sau3A digests of pSES3, pBR322, and pSES9; 7, pBR322/AluI; 8, pSES9/AluI. Electrophoresis was for 2.6 hr at 60 V on 1.6% agarose in TAE.

h) Samples were HpaII digests of mini-lysates of: 1, WS 2313; 2, WS 2316, 3, WS 2302; 4, WS2310. The gel was 2% agarose. The 0.238 and 0.242 kb fragments from pBR322 are indicated with an arrow

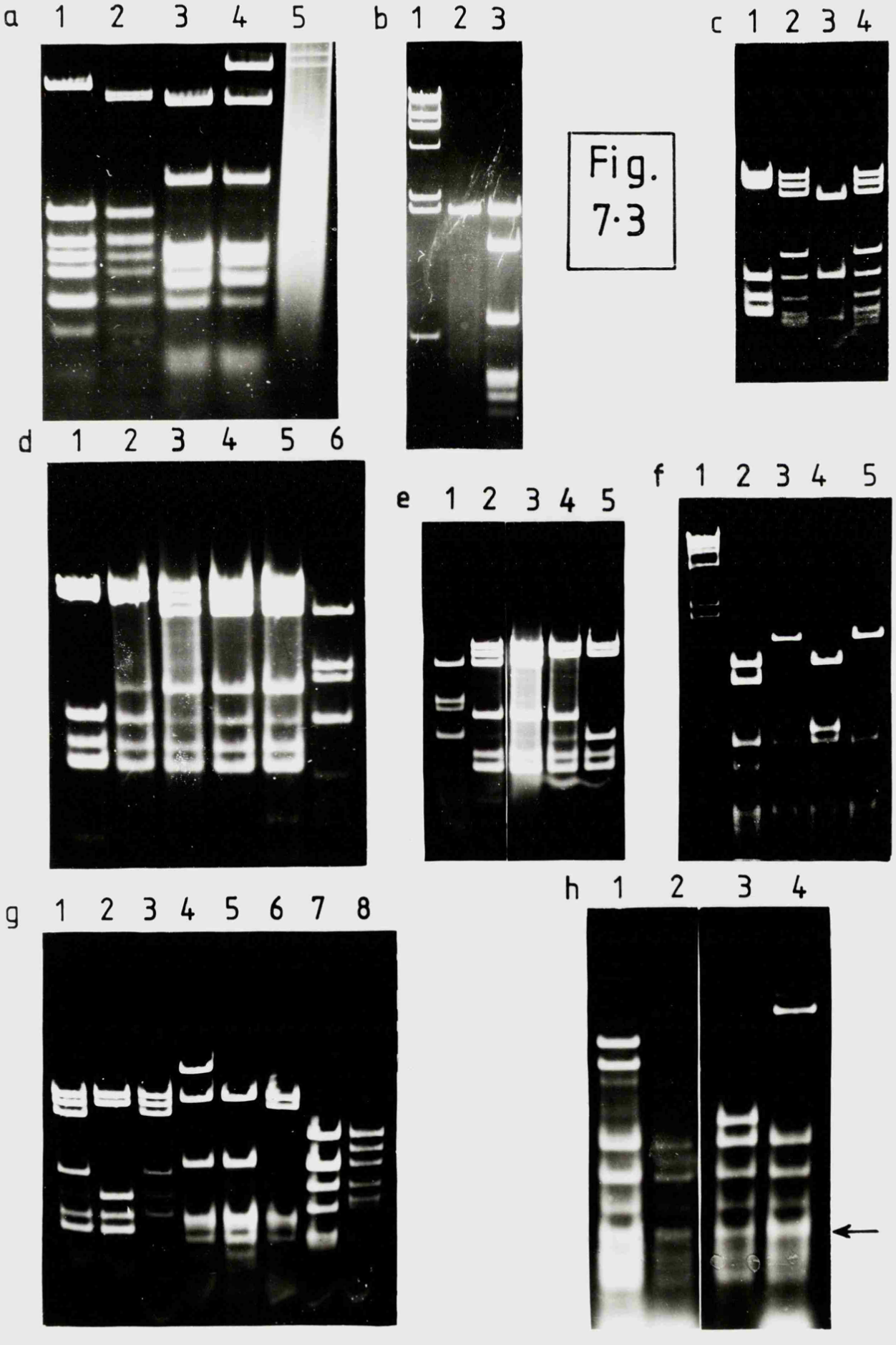


Fig. 7·3

Fig. 7·4

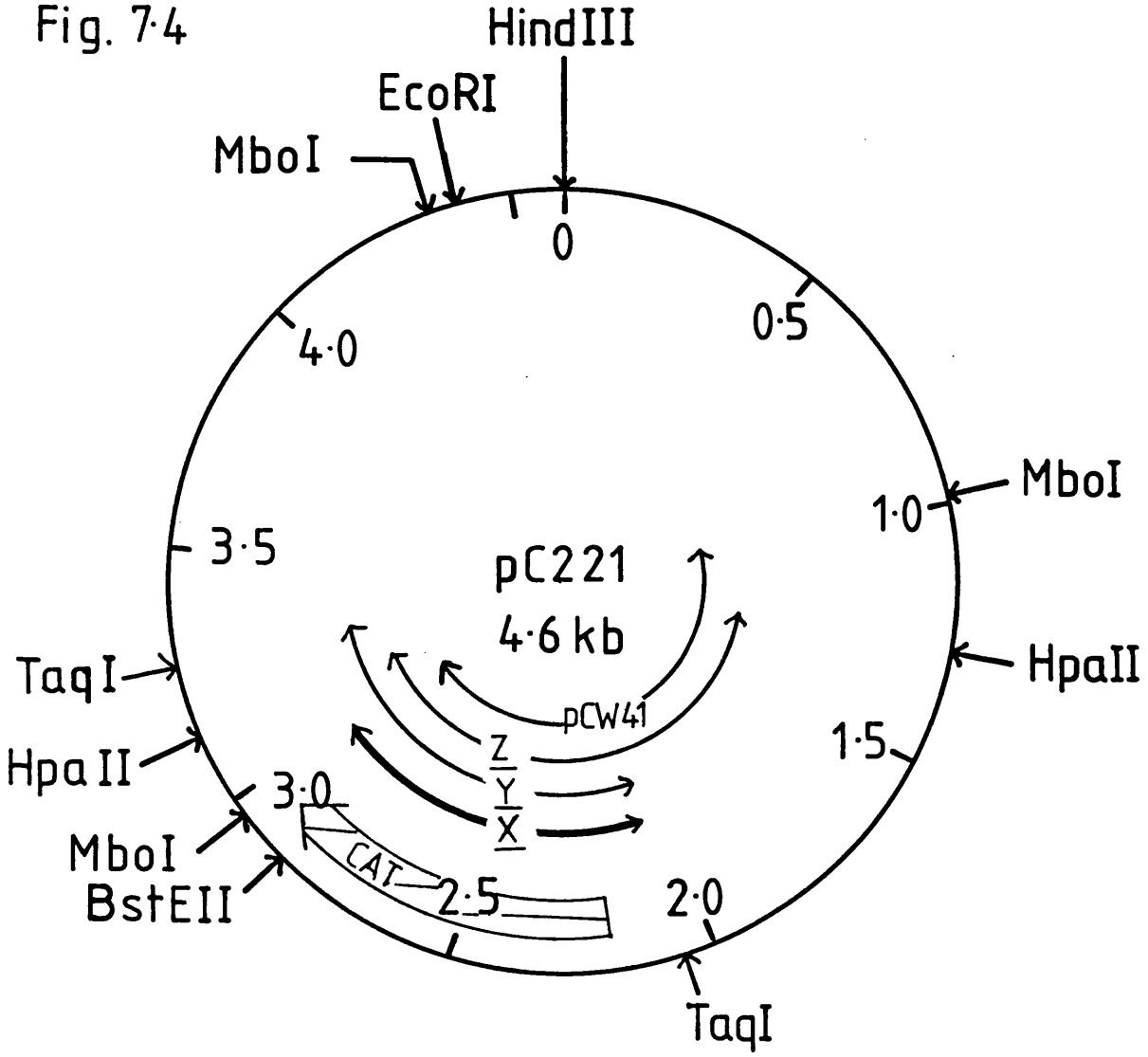


Fig. 7.4 Map of selected restriction sites on pC221
Only sites necessary for interpretation of data presented in this chapter are shown. The map is drawn from the data in Chapter 5. The position and orientation of the coding-region for CAT (see App. 1) is indicated. Fragments X, Y and Z and the MboI fragment corresponding to pCW41 are also indicated.

Fig. 7·5

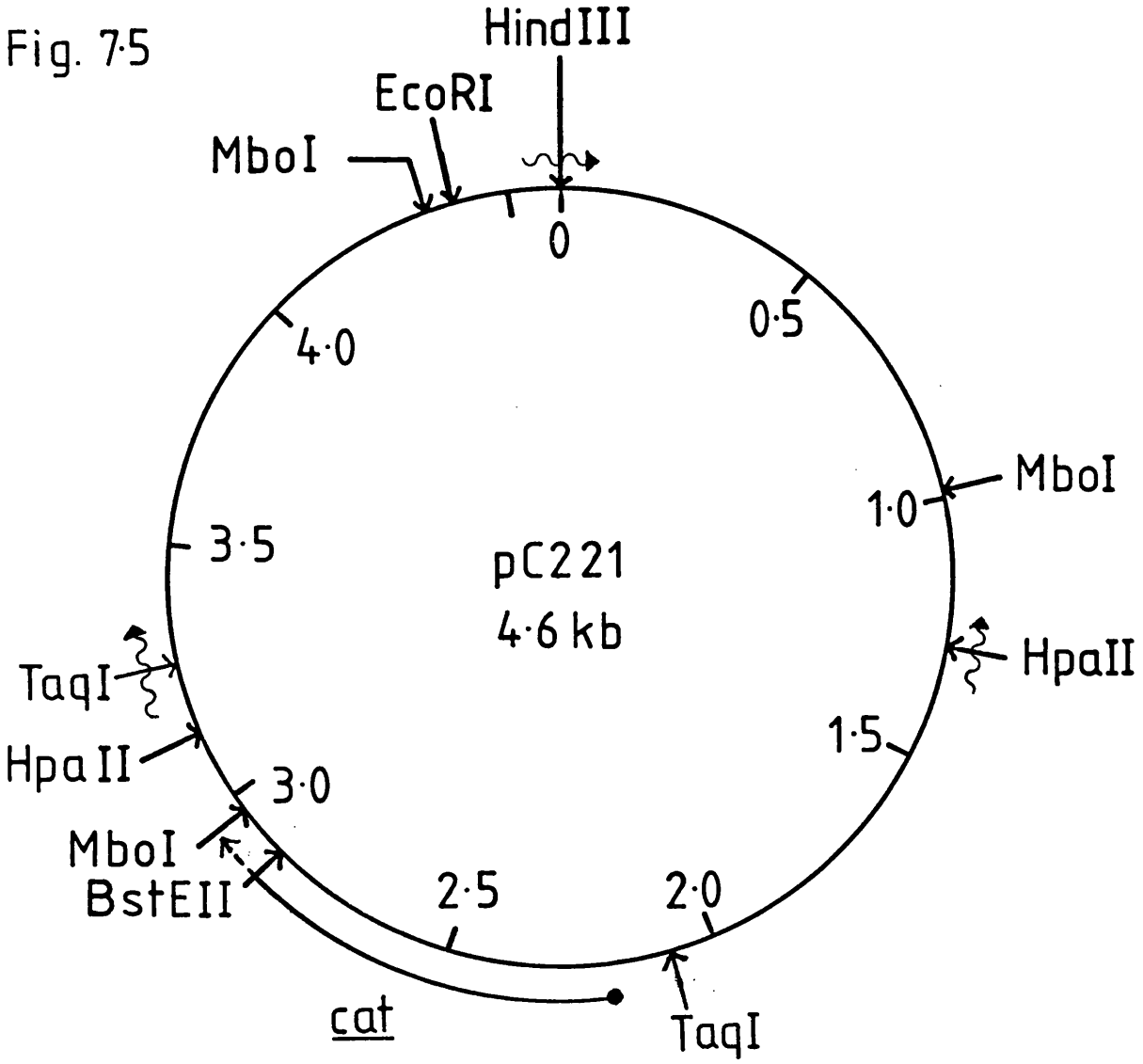


Fig. 7.5 Transcription of pC221 in E.coli

The observations made in section 7.3.7 are summarised on the map of pC221 (above). The position at which transcription of the cat gene is started by B.subtilis RNA polymerase in vitro (Le Grice, unpublished; see App. 2) is also shown.

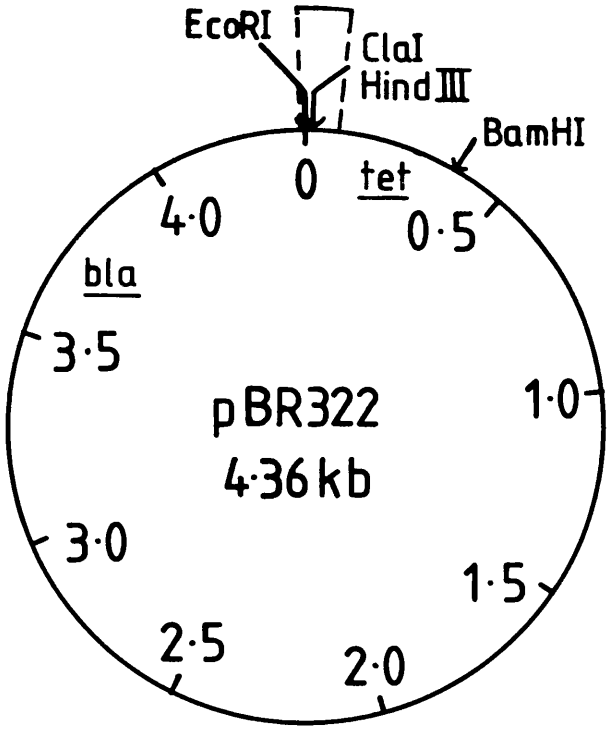
Fig. 7.6 Restriction sites and promoters on pBR322

a) Restriction map of pBR322:
From the data of Sutcliffe (1978 and 1979).
The region indicated is expanded in (b).

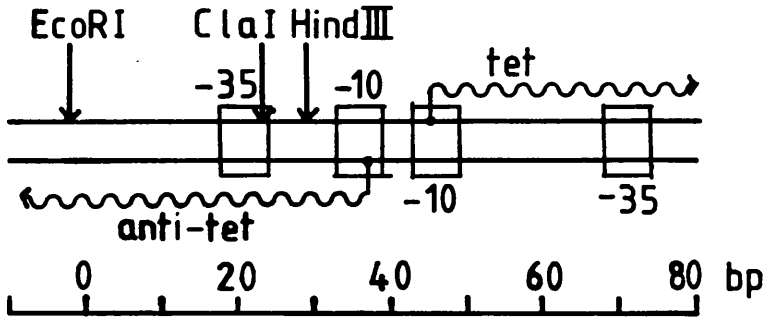
(b) Promoters in the tet gene region
The region of pBR322 from about -10 to +80 on map (a) is shown schematically. The positions of the transcriptional start-sites are indicated (from Brosius et al., 1982) as are the sites at which restriction enzymes cut the upper strand of DNA, and the -35 and -10 sequences of the tet and anti-tet promoters.

Fig. 7·6

a


EcoRI
ClaI
HindIII
BamHI
0
tet
0·5
1·0
1·5
2·0
2·5
3·0
3·5
4·0
bla
pBR322
4·36 kb

b


EcoRI
ClaI
HindIII
-35
-10
tet
-10
-35
anti-tet
0
20
40
60
80
bp

Notes to table 7.1

Procedures used are described in section 7.2.1 and in Chapter 2 (section 2.7.1).

All Cm^R transformants tested were also Ap^R and had the same nutritional requirements as WS 2301. The concentration (μg/ml) of antibiotics used for selection is indicated.

"n.t.": not tested

a : very small colonies

Table 7.1 Transformation of E.coli

expt		DNA	selection	colonies per 0.2 ml	no. Tc^R per no. tested
1	a	pCW41/MboI +pBR322/BamHI	Cm10	13	0/16
	b	pC221/HpaII +pBR322/ClaI	Cm10	13	2/4
	c	pC221/TaqI +pBR322/ClaI	Cm10	3	1/2
	d	CCC pBR322	Cm10	10^a	n.t
			Ap40	$\sim 10^4$	2/2
	e	none	Cm10	10^a	n.t.
2	a	pC221/TaqI +pBR322/ClaI	Cm10	23 (+ 5 to 10^a)	3/6
	b	CCC pBR322	Cm10	3^a	n.t.
			Ap40	$\sim 10^4$	n.t.
	c	none	Cm10	3^a	n.t.
3	a	pCW41/MboI/TaqI +pBR322/BamHI/ClaI (0.4 μg)	Cm10	37	0/13
	b	same (0.6 μg)	Cm10	60	
	c	CCC pBR322	Cm10	1	n.t.
			Ap40	$\sim 10^4$	n.t.
	d	none	Cm10	0	———
			none	5×10^7	n.t.

Table 7.2 Screening of pC221/HpaII derivatives of pBR322

Isolate	Sizes (kb) of HindIII fragments observed
A[a]	6.2
B	4.9, 5.7, (10 to 11)[b]
C	3.6[c], 8.0, 12.5, 16 to 18
D,E	3.0, 5.7, (8.8)

Notes: Digests were separated by electrophoresis on 0.8% gels (in TAE) with HindIII-digested λ as size-marker.

a) Isolate A was WS 2311

b) Parentheses indicate products of partial digestion by HindIII

c) The major products of this HindIII digest were about 3.6 and 12.5 kb. The smallest SC form observed was 8.0 kb.

Table 7.3 Transformation of *E.coli* with sub-cloned pSES13

DNA	selection	colonies per 0.1 ml
none	Cm10	0
	none	5×10^6
CCC pBR322	Tc20	4×10^5
pSES13/*TaqI*[a] +pBR322/*ClaI*	Cm10	116

Notes: a) 0.02 ml of this transformation mixture contained $\sim 10^3$ Ap^R transformants

Other notes as for table 7.1

Table 7.4 Induction of *cat* in *E.coli*

Expt	Plasmid	Insert	Site of insertion	Orientation of *cat*
1	pSES3	pCW41/*Mbo*I	*Bam*HI	B
2	pSES3	- - - - - - - - - - as above - - - - - - - - - -		
	pSES11	fragment Z	*Cla*I	A
	pSES4	pC221/*Hind*III	*Hind*III	A
3	pBR322	——	——	——
	pSES9	fragment X	*Cla*I-*Bam*HI[a]	B
	pSES10	fragment Y	*Cla*I	B
	pSES13	fragment Y and two others	*Cla*I	A
4[b]	pBR322	——	——	——
	pSES15	fragment Y	*Cla*I	A
	pSES16	fragment Y	*Cla*I	B

Final A_{600}	Concn. of 3-deoxyCm (μM)	CAT in extract (U/ml)	BLA in extract (U/ml)	$\frac{CAT}{BLA} \times 10^3$
0.85	0	0.017 ± 0.003	345 ± 5	0.05
	5.5	0.067 ± 0.003	330	0.20
0.7	0	0.021 ± 0.002	343 ± 3	0.06
	5.0	0.057 ± 0	358 ± 8	0.16
0.6	0	0.012 ± 0.002	30 ± 5	0.4
	5.0	0.048 ± 0.001	26 ± 1	0.9
0.5	0	0.005 ± 0.001	45 ± 5	0.11
	5.0	0.025 ± 0.002	46 ± 4	0.54
1.0	0	~0.003	583 ± 3	n.c.
	9	~0.003	575 ± 65	n.c.
	0	0.014 ± 0.004	26	0.54
	9	0.027 ± 0.005	19	1.4
	0	0.013 ± 0.001	34	0.4
	9	0.015 ± 0.001	38	0.4
	0	0.015 ± 0.003	42	0.36
	9	0.31 ± 0.01	47	6.6
0.6	0	~0.004	305 ± 35	n.c.
	7	0.004 ± 0.002	240 ± 20	n.c.
	0	0.023 ± 0.002	43 ± 4	0.5
	7	0.13 ± 0.01	35 ± 1	3.7
	0	0.002 ± 0.004	8.5 ± 1.0	~0.3
	7	0.005 ± 0.005	7.3 ± 1.3	~0.7

Notes to table 7.4

In all experiments the host strain was E.coli WS 2301. Strain numbers have been omitted for simplicity. The structures of the plasmids are shown in fig. 7.2. The locations of fragments X, Y and Z and pCW41 on pC221 are indicated in fig. 7.4. In orientation A the cat gene is anticlockwise relative to the restriction map of pBR322 (Sutcliffe, 1978); in orientation B, clockwise. CAT assays were usually performed in triplicate and are presented in U/ml of extract (± range). BLA assays were usually performed in duplicate (except in experiments 1 and 3).

a) In the construction of pSES9 the 0.35 kb ClaI-BamHI fragment of pBR322 was replaced by fragment X.
b) In experiment 4 the freezing and thawing steps were omitted from the lysis procedure.

n.c.: not calculated.

Table 7.5 Resistance to Cm and Tc

Strain	Plasmid	Growth On: Tc20	Tc10	Cm25	Cm10
WS 2301	none	-	-	-	-
WS 2302	pBR322	+++	+++	-	-
WS 2303	pSES3	-	-	+++	+++
WS 2309	pSES9	-	-	-	+
WS 2310	pSES10	++	+++	(+)	+
WS 2311	pSES11	+	+++	+++	+++
WS 2313	pSES13	-	(+)	+++	+++
WS 2314	pSES14	(+)	+	(+)	+
WS 2315	pSES15	-	(+)	+++	+++
WS 2316	pSES16	++	+++	(+)	+
WS 2317	pSES4	-	-	(+)	+

Notes: Most of the plasmids used here are described in table 7.4; pSES14 is HindIII-cut pC221 inserted into the HindIII site of pBR322 with the cat gene in orientation B (see Chapter 4). Observations from several experiments have been combined. Incubation was for 40 hr. at 37°.

(+): faint growth or one colony

+++: heavy growth

All patches grew heavily on L plates without antibiotics.

CHAPTER 8

GENERAL DISCUSSION

8·1 Evolution of Cm^R Plasmids

As indicated in the General Introduction (Section 1.3.1) both macro- and micro-evolution of plasmids can occur, the former by translocation of segments of DNA, the latter by base-changes in the nucleotide sequence. The heteroduplexes visualised by electronmicroscopy in Chapter 4 indicated that a 2 kb region of homology was shared by pC221, pCW6 and pCW7; as this region corresponded with pCW41, which has been shown to carry the functions necessary for replication, incompatibility and maintenance in addition to those for inducible synthesis of CAT (Wilson *et al.*, 1981; Chapters 3 and 6, this work), these functions are probably located here for pCW6 and pCW7, too. Interruption of the *Bst*EII sites of these plasmids abolished CAT activity, indicating that they were in or near the structural genes for CAT or their controlling elements. The DNA sequence of pCW41 (Hawkins *et al.*, unpublished; see App. 1) has confirmed this for pCW41; the *Bst*EII site is located very near the codons for the "active-site peptide," a highly conserved region of CAT which contains a histidine residue strongly implicated in the catalytic mechanism (see review by Shaw, 1983).

This similarity suggests that pC221, pCW6 and pCW7 have evolved from a hypothetical Cm^R plasmid module of about 2 kb which has become attached to other sequences forming the three different plasmids (macro-evolution). The homology observed in the 2 kb region between pC221 and pCW7 was only partial, however, and their restriction maps are only partly conserved

in this region (Chapter 5). Thus, some sequence divergence (micro-evolution) has taken place.

No hybridisation was detected between the cat genes of pC221, pCW6 or pCW7 and pCW8, though the amino-acid sequences predicted for the CATs encoded by pC221 and pC194 (=pCW8) show over 50% homology (Shaw, 1983). Although inspection of the DNA sequence of pC194 revealed a 5/6 base match with the recognition site for BstEII at the same relative position in the cat gene as the BstEII site in the cat gene of pCW41, the distance from this site to the HpaII site of pC194 was much smaller than in the other plasmids (0.87 kb in pC194; 1.6 kb in pC221). In addition to greater sequence divergence it would therefore be necessary to postulate the deletion of 0.7 kb from between these sites in order to fit pCW8 to the above model.

Behnke and Gilmore (1981) have demonstrated insertional inactivation of Cm^R at the BstEII site of a streptococcal $Cm^R Em^R$ plasmid, pGB301. Interruption of its HpaII site (about 1.5 kb away) affected the stability of the plasmid and caused deletions which abolished Cm^R (Behnke et al., 1981). It is possible that pGB301 is related to the staphylococcal Cm^R plasmids, as it appears to share the BstEII - HpaII motif, with the former site in the Cm^R determinant.

8.2 On the Induction of CAT

Several mechanisms for the regulation of prokaryotic gene expression have been outlined in the General Introduction (section 1.4.1) and can be categorised according to the level(s) at which regulation occurs; initiation or completion of the synthesis of mRNA or its translation product. In this section the data available for the inducible *cat* genes will be discussed in relation to various models for regulation. It should, of course, be borne in mind that regulation may occur at more than one level, as is the case for the *trp* operon, where both initiation and completion of transcription are controlled.

The data presented in Chapter 7 indicated that induction could still occur when transcription had probably initiated from a heterologous promoter. Since it seems highly unlikely that 3-deoxyCm could affect initiation at the anti-*tet* promoter of pBR322, this would rule out control of initiation of transcription as the mechanism causing the induction of CAT observed in these experiments. Similarly, expression of a *cat* gene from *B.pumilus* was shown to be inducible by Cm when transcription was dependent on the presence of a heterologous (phage SPO2) promoter (Duvall *et al.*, 1983). When a DNA fragment carrying a gene for dihydrofolate reductase (DHFR) was inserted into the 5' pre-coding region of the *cat* gene, DHFR was synthesised constitutively and CAT inducibly, apparently from the same transcript. This provided further evidence for regulation being at a later stage than initiation of transcription.

The failure to observe a large change in staphylococcal *cat* RNA levels (Chapter 6) under conditions where the activity of CAT increased sixty-fold agreed with this conclusion. The observation of a small change in *cat* RNA concentrations on induction is not, however, inconsistent with a model where the vast majority of transcripts terminate prematurely in the absence of inducer. Alternatively, in a model involving control of initiation of translation, these data could be explained in terms of protection of the CAT mRNA from degradation by the ribosomes translating it: the increase in *cat* RNA detected on induction would then be an effect rather than the primary cause of increased translation of CAT.

If premature termination of transcription occurred it might be possible to detect both long and short transcripts of *cat*. Experiments described in Appendix 2 (performed by Dr. S. LeGrice) examined transcription from pCW41 and its pBR322 derivative, pSES3, using purified *B.subtilis* RNA polymerase *in vitro*. Although a 92 b transcript was observed in addition to a 670 b run-off transcript (presumed to be from the *cat* gene) when *Bst*EII - cut pCW41 or pSES3 was the template, these RNAs were shown to start at different sequences. It could well be argued, however, that additional factors, absent from the RNA polymerase preparation, would be required for termination at an attenuation site.

The lengths and relative amounts of *cat* transcripts *in vivo* could be examined by Northern blots of RNA isolated from induced and uninduced cultures. In the *B.pumilus* *cat* system, although CAT levels increased thirty-fold on incubation with Cm, Northern

blots failed to show a large increase in the amount of sequences complementary with the coding-region for CAT (Duvall *et al.*, 1983), a finding consistent with translational control. Cell-free transcription-translation experiments using extracts from a Gram-positive bacterium such as *B.subtilis* in which pC221 *cat* is known to be expressed and induced well (Chapter 3) might also be profitable. Advantages of working with such a system include the ease with which products of transcription and translation can be labelled with radioisotopes and subsequently analysed. It might, for instance, be easier to detect labile RNA species *in vitro* than *in vivo*.

As the major control-point in *cat* regulation is after the initiation of transcription, the structure of the *cat* transcript may well be important. Comparison of the data in Appendix 2 (priming by dinucleotides of transcription *in vitro*) with the nucleotide sequence of pCW41 (App. 1) indicated the site of initiation of the *cat* transcript of pCW41 (Fig. 8.1). An inverted repeat is apparent in the transcribed pre-coding region, such that the putative ribosome binding-site (Shine and Dalgarno, 1974 and 1975) is part of a ds RNA stem. Similar structures can be drawn for the *B.pumilus* *cat* transcript (Duvall *et al.*, 1983) and for the predicted CAT mRNA of pC194 (Horinouchi and Weisblum, 1982). That such a structure (see Fig. 8.2) has been conserved when the DNA sequences of the coding-region for CAT differ (Harwood *et al.*, 1983; Horinouchi and Weisblum, 1982; App. 1) suggests that its function is important.

Unlike a simple transcriptional terminator (such as those involved in attenuation; see review by Kolter and Yanofsky, 1982) the G+C-rich stem is not followed by a U-rich tract. A role in a more complex termination or anti-termination mechanism might be possible. The sequestration in the stem structure of the presumed ribosomal binding-site for the translation of CAT suggests that initiation of translation may be controlled. The effects on cat expression of removal and/or alteration of the stem-loop structure and surrounding sequences should be informative.

Although such a structure is an obvious candidate for the target of the regulatory process, the identity of the sensor of Cm (or other inducers) is less apparent. Horinouchi and Weisblum (1982) showed that Cm^R was still inducible when CAT was apparently the only protein encoded by a cloned 1 kb fragment of pC194; similarly, in Chapter 7 of this work, at least some induction of CAT was observed when a 1.2 kb fragment of pC221 was inserted into pBR322. Thus, a separate plasmid-encoded regulatory protein can be ruled out. Duvall et al. (1983) showed that in the B.pumilus cat system no more than the first thirty codons for CAT and the 144 bp preceding them were necessary for the (eight-fold) induction by Cm of DHFR expression from a fragment inserted into codon 29 of the coding-region for CAT. This indicated that CAT could not be its own regulatory protein. Thus any protein(s) necessary for regulation of cat must be encoded by the host chromosome.

Apart from CAT the only other major binding-site for Cm is the ribosome. If this is the receptor for the inducer in the regulatory process all inducers of CAT should bind to ribosomes (in a manner similar to Cm) and would, therefore be expected to inhibit protein synthesis. In this case there should be a correlation between efficiency of induction of CAT and inhibition of protein synthesis by a given compound. Interpretation of the results of previous studies of the inducing efficiencies and antibacterial activities of analogues of Cm have been complicated by two factors in particular; acetylation by CAT of some of the compounds, which lowers their concentration, and the shape of the dose-response curves for induction (e.g.,fig. 6.4). Use of a hybrid system devoid of CAT activity where the coding-region for CAT was fused to that for another easily measurable enzyme, e.g. β-galactosidase would overcome the complication of acetylation. Measurements of inhibitory activity against protein synthesis could also be performed *in vivo* with the same strain. The concentration required for half-maximal induction by each compound could be compared with its inhibitory activity. A working hypothesis(suggested by Duvall *et al.*, 1983) is that a ribosome to which Cm (or another inducer) is bound interacts in some way with the *cat* RNA to open up the ds RNA stem. In an attenuation model this would prevent termination of transcription; in a translational model this would allow initiation of translation of CAT.

The rate of CAT synthesis depends on the concentration of functional RNA and the frequency of its translation. In a model where initiation of translation is regulated, stabilisation

of mRNA by translation might significantly increase its functional concentration, thereby amplifying the original induction signal. As already noted in Chapter 6, CAT mRNA is not unusually stable (in the uninduced state). Even in the case of the stable mRNA of the Em^R gene of pE194, the functional half-life (3 to 6 min.) increased seven to twenty-fold on induction by Em in B.subtilis mini-cells (Shivakumar, et al., 1980).

Different amounts of induction of CAT were observed for E.coli carrying the cat gene of pC221 on different plasmids (Chapter 7). Good expression of Cm^R and induction of CAT were only obtained when a nearby pBR322 promoter was available for initiation of transcription of the cat gene. Whether this was due to enhancement of cat transcription by the pBR322 promoter has already been discussed in Chapter 7. Transcripts of the cat gene which had initiated outside the cloned fragments would, of course, be longer than those starting at the natural promoter. It is possible that the functional region in such longer transcripts might be protected from 5'-3' exonuclease activity to some extent by its greater distance from the 5' end or even by (fortuitous) stable secondary structures in the "extra" part of the RNA. Any reduction in degradation would, however, affect only the level of expression and not the induction ratio, unless either the synthesis of CAT varied sigmoidally with the level of cat transcription (as discussed in Chapter 7) or the integrity of some sequence in the 5' precoding part of the mRNA was necessary for induction.

It may be relevant to note, in this respect, that the

cat transcripts of the pCW41 and *B.pumilus* *cat* genes (Fig. 8.1; Duvall, *et al.*, 1983) each include a potential coding-region for a hypothetical leader-peptide. The stalling of a ribosome while translating this (due to inhibition by Cm or some other inducer) would be expected to open up the ds RNA stem, which has been assumed in this discussion to be sufficient for induction. Against such a hypothesis, however, the suggested start-site of the *cat* transcript of pC194 (Horinouchi and Weisblum, 1982) would not include such a potential coding-region.

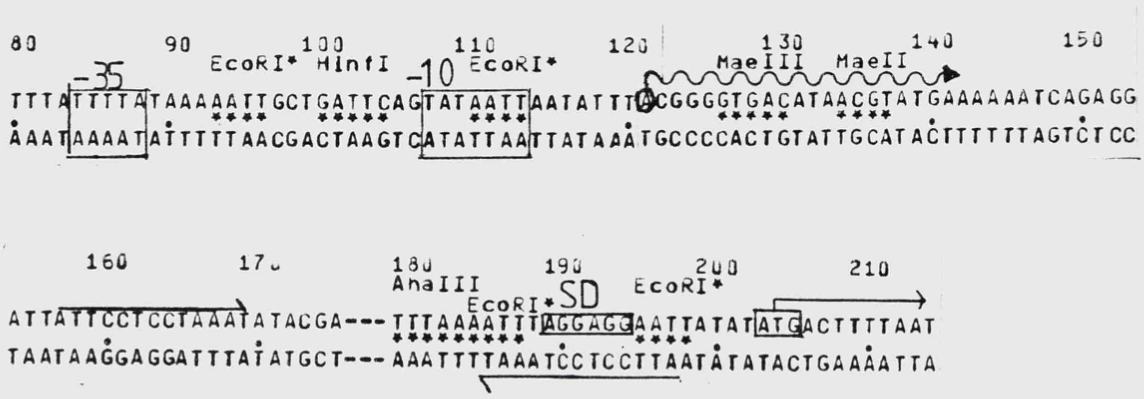
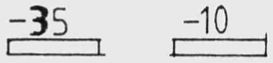


Fig. 8.1 Site of initiation of transcription of cat

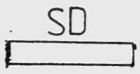
Part of the sequence of pCW41 as determined by Hawkins et al. (unpublished, see App. 1). From the data in App. 2 (Le Grice, unpublished) it was inferred that the most probable start-site for transcription of cat mRNA is 121. The potential coding-region mentioned in the text would start at 138-140 and terminate at 165-167, with the ribosome binding site at 123-128.

Key:



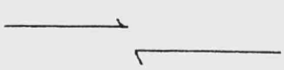
probable promoter sequences

transcriptional start-site



Shine-Dalgarno sequence

translational start-site for CAT



inverted complementary repeat sequences

Fig. 8·2

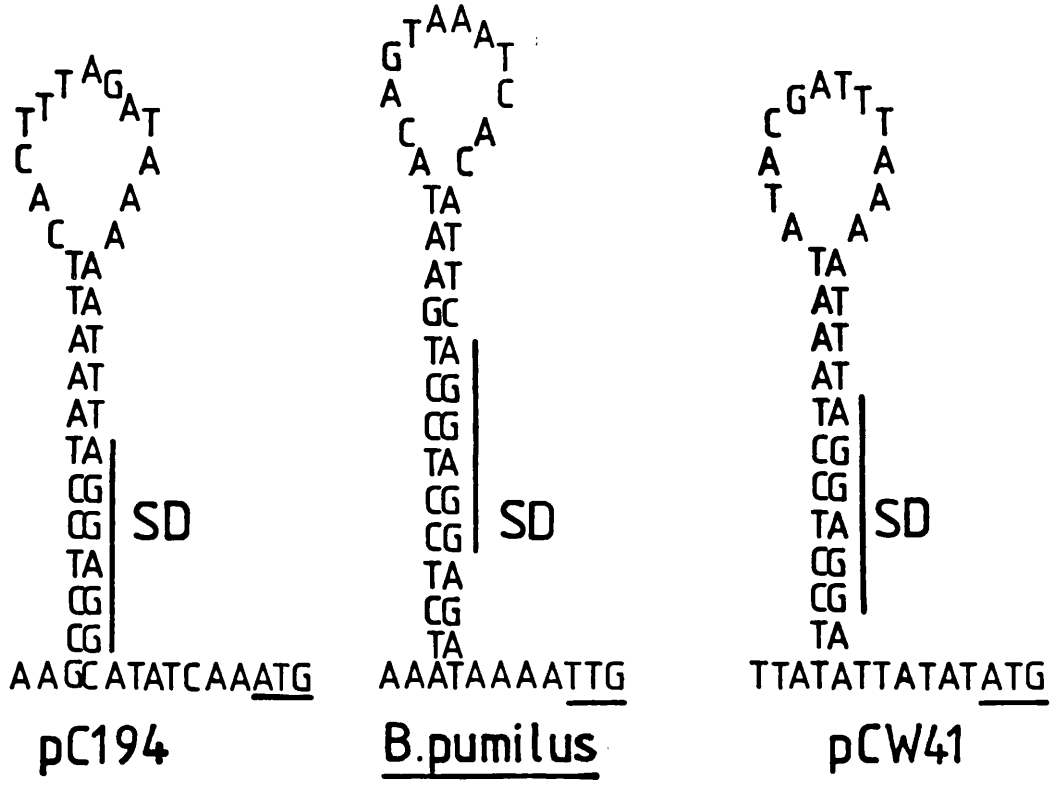


Fig. 8.2 Potential secondary structures in cat genes
The structures shown are from Horinouchi and Weisblum (1982), Duvall et al. (1983) and the data of Hawkins et al. (App. 1). The Shine-Dalgarno sequence and the start codon for CAT are shown in each case.

APPENDICES AND REFERENCES

Appendix 1

The nucleotide sequence of pCW41

Fig. A1(a) shows the nucleotide sequence of most of pCW41 (A.R. Hawkins, J.W. Keyte, D. Brenner and W.V. Shaw; personal communication, December 1983), obtained by the methods of Sanger *et al.* (1977), Schreier and Cortese (1979) and Maxam and Gilbert (1977). The sequence shown begins at the *Taq*I site at 0 kb on the map of pCW41 in fig. A1(b). (*Bst*EII is an isoschizomer of *Eca*I; *Sau*3A of *Mbo*I). The construction of pCW41 is schematised in fig. 6.1. The position of the *cat* gene was inferred from mapping data (Wilson *et al.*, 1981) and the partial amino-acid sequence of CAT (cited by Shaw, 1983). The Shine-Dalgarno sequence is at 189-194, the start codon at 203-205 and the stop codon at 851-853. The codons for the highly conserved "active-site peptide" are 767-781. From the data in App. 2 transcription of the *cat* gene starts at 121, implying that the -35 and -10 promoter sequences are at 84-89 and 107-113. Fragment X (Chapters 6 and 7) is nucleotides 1-895 in fig. A1(a), 0 to 0.9 kb in fig. A1(b).

Fig.A1(a) Nucleotide sequence of pCW41

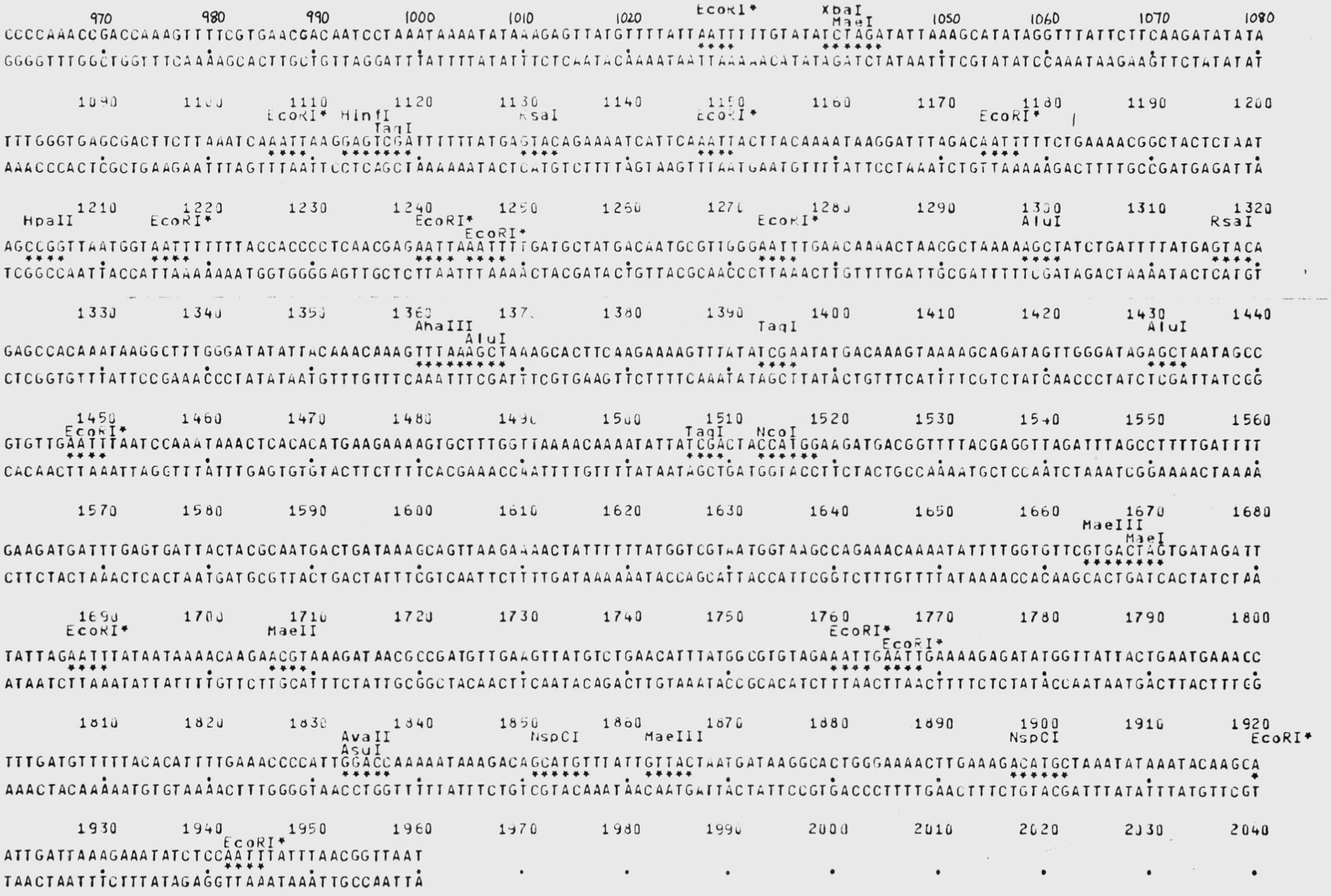

970 980 990 1000 1010 1020 1030 1040 1050 1060 1070 1080
EcoRI* XbaI MaeI
CCCCAAACCGACCAAAGTTTTCGTGAACGACAATCCTAAATAAAATATAAAGAGTTATGTTTTATTAATTTTTGTATATCTAGATATTAAAGCATATAGGTTTATTCTTCAAGATATATA
GGGGTTTGGCTGGTTTCAAAAGCACTTGCTGTTAGGATTTATTTTATATTTCTCAATACAAAATAATTAAAAACATATAGATCTATAATTTCGTATATCCAAATAAGAAGTTCTATATAT
1090 1100 1110 1120 1130 1140 1150 1160 1170 1180 1190 1200
EcoRI* HinfI TaqI PsaI EcoRI* EcoRI*
TTTGGGTGAGCGACTTCTTAAATCAAATTAAGGAGTCGATTTTTTATGAGTACAGAAAATCATTCAAATTACTTACAAAATAAGGATTTAGACAATTTTTCTGAAAACGGCTACTCTAAT
AAACCCACTCGCTGAAGAATTTAGTTTAATTCCTCAGCTAAAAAATACTCATGTCTTTTAGTAAGTTTAATGAATGTTTTATTCCTAAATCTGTTAAAAAGACTTTTGCCGATGAGATTA
1210 1220 1230 1240 1250 1260 1270 1280 1290 1300 1310 1320
HpaII EcoRI* EcoRI* EcoRI* EcoRI* AluI RsaI
AGCCGGTTAATGGTAATTTTTTTACCACCCCTCAACGAGAATTAAATTTTGATGCTATGACAATGCGTTGGGAATTTGAACAAAACTAACGCTAAAAAGCTATCTGATTTTATGAGTACA
TCGGCCAATTACCATTAAAAAAATGGTGGGGAGTTGCTCTTAATTTAAAACTACGATACTGTTACGCAACCCTTAAACTTGTTTTGATTGCGATTTTTCGATAGACTAAAATACTCATGT
1330 1340 1350 1360 1370 1380 1390 1400 1410 1420 1430 1440
AhaIII AluI TaqI AluI
GAGCCACAAATAAGGCTTTGGGATATATTACAAACAAAGTTTAAAGCTAAAGCACTTCAAGAAAAGTTTATATCGAATATGACAAAGTAAAAGCAGATAGTTGGGATAGAGCTAATAGCC
CTCGGTGTTTATTCCGAAACCCTATATAATGTTTGTTTCAAATTTCGATTTCGTGAAGTTCTTTTCAAATATAGCTTATACTGTTTCATTTTCGTCTATCAACCCTATCTCGATTATCGG
1450 1460 1470 1480 1490 1500 1510 1520 1530 1540 1550 1560
EcoRI* TaqI NcoI
GTGTTGAATTTAATCCAAATAAACTCACACATGAAGAAAAGTGCTTTGGTTAAAACAAAATATTATCGACTACCATGGAAGATGACGGTTTTACGAGGTTAGATTTAGCCTTTTGATTTT
CACAACTTAAATTAGGTTTATTTGAGTGTGTACTTCTTTTCACGAAACCAATTTTGTTTTATAATAGCTGATGGTACCTTCTACTGCCAAAATGCTCCAATCTAAATCGGAAAACTAAAA
1570 1580 1590 1600 1610 1620 1630 1640 1650 1660 1670 1680
MaeIII MaeI
GAAGATGATTTGAGTGATTACTACGCAATGACTGATAAAGCAGTTAAGAAAACTATTTTTTATGGTCGTAATGGTAAGCCAGAAACAAAATATTTTGGTGTTCGTGACTAGTGATAGATT
CTTCTACTAAACTCACTAATGATGCGTTACTGACTATTTCGTCAATTCTTTTGATAAAAAATACCAGCATTACCATTCGGTCTTTGTTTTATAAAACCACAAGCACTGATCACTATCTAA
1690 1700 1710 1720 1730 1740 1750 1760 1770 1780 1790 1800
EcoRI* MaeII EcoRI* EcoRI*
TATTAGAATTTATAATAAAACAAGAACGTAAAGATAACGCCGATGTTGAAGTTATGTCTGAACATTTATGGCGTGTAGAAATTGAATTGAAAAGAGATATGGTTATTACTGAATGAAACC
ATAATCTTAAATATTATTTTGTTCTTGCATTTCTATTGCGGCTACAACTTCAATACAGACTTGTAAATACCGCACATCTTTAACTTAACTTTTCTCTATACCAATAATGACTTACTTTGG
1810 1820 1830 1840 1850 1860 1870 1880 1890 1900 1910 1920
AvaII AsuI NspCI MaeIII NspCI EcoRI*
TTTGATGTTTTTACACATTTTGAAACCCCATTGGACCAAAAATAAAGACAGCATGTTTATTGTTACTAATGATAAGGCACTGGGAAAACTTGAAAGACATGCTAAATATAAATACAAGCA
AAACTACAAAAATGTGTAAAACTTTGGGGTAACCTGGTTTTTATTTCTGTCGTACAAATAACAATGATTACTATTCCGTGACCCTTTTGAACTTTCTGTACGATTTATATTTATGTTCGT
1930 1940 1950 1960 1970 1980 1990 2000 2010 2020 2030 2040
EcoRI*
ATTGATTAAAGAAATATCTCCAATTTATTTAACGGTTAAT
TAACTAATTTCTTTATAGAGGTTAAATAAATTGCCAATTA

Fig.A1(b)

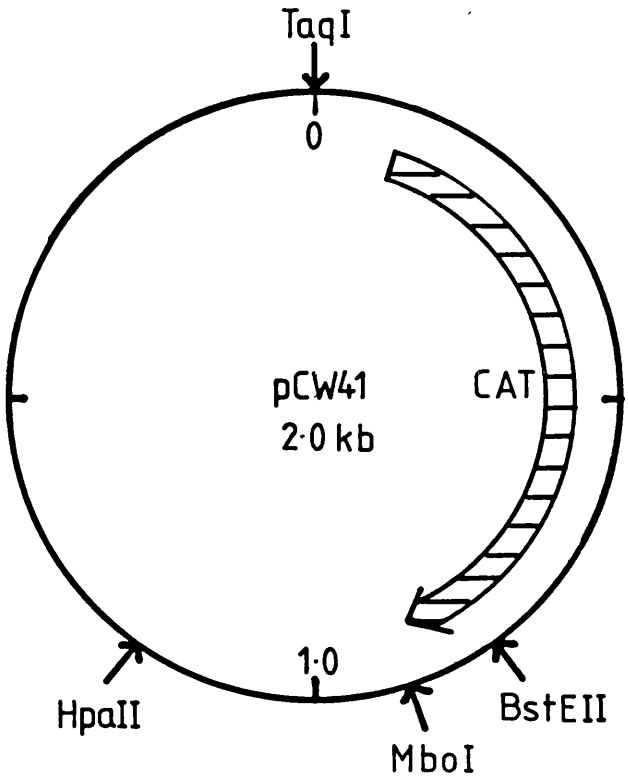


Fig. A1(b) : Restriction map of pCW41.
Positions of restriction sites are from data in Chapter 5. Only one of the TaqI sites is shown. The position of the coding-region for CAT is from the nucleotide sequence data of Hawkins et al. (fig. A1(a)).

Appendix 2

Transcription *in vitro* by *B.subtilis* RNA polymerase

I am grateful to Dr S.J.F. Le Grice for performing the experiments described here, using the procedures of Le Grice and Sonenshein (1982).

When *Bst*EII-cut pSES3 (Chapter 7) or pCW41 was used to direct transcription *in vitro* by *B.subtilis* RNA polymerase the two major products were 670 b (presumably a run-off transcript from the *cat* gene) and a 92 b RNA. The latter was also observed using CCC plasmid DNA. A small RNA (about 100 b) was transcribed from CCC pSES3, probably RNA I (see General Introduction, section 1.2.2). Transcription of *Bst*EII-cut pSES3 primed by different dinucleotides was used to establish the DNA sequences at which the RNA species initiated. From the data in fig. A2 the sequence at which transcription of the 670 b transcript (arrowed) started was inferred to be TT(T)AC, and for the 92 b RNA (bracketed), TAT. A 450 b RNA apparently starting at TGGA was also observed.

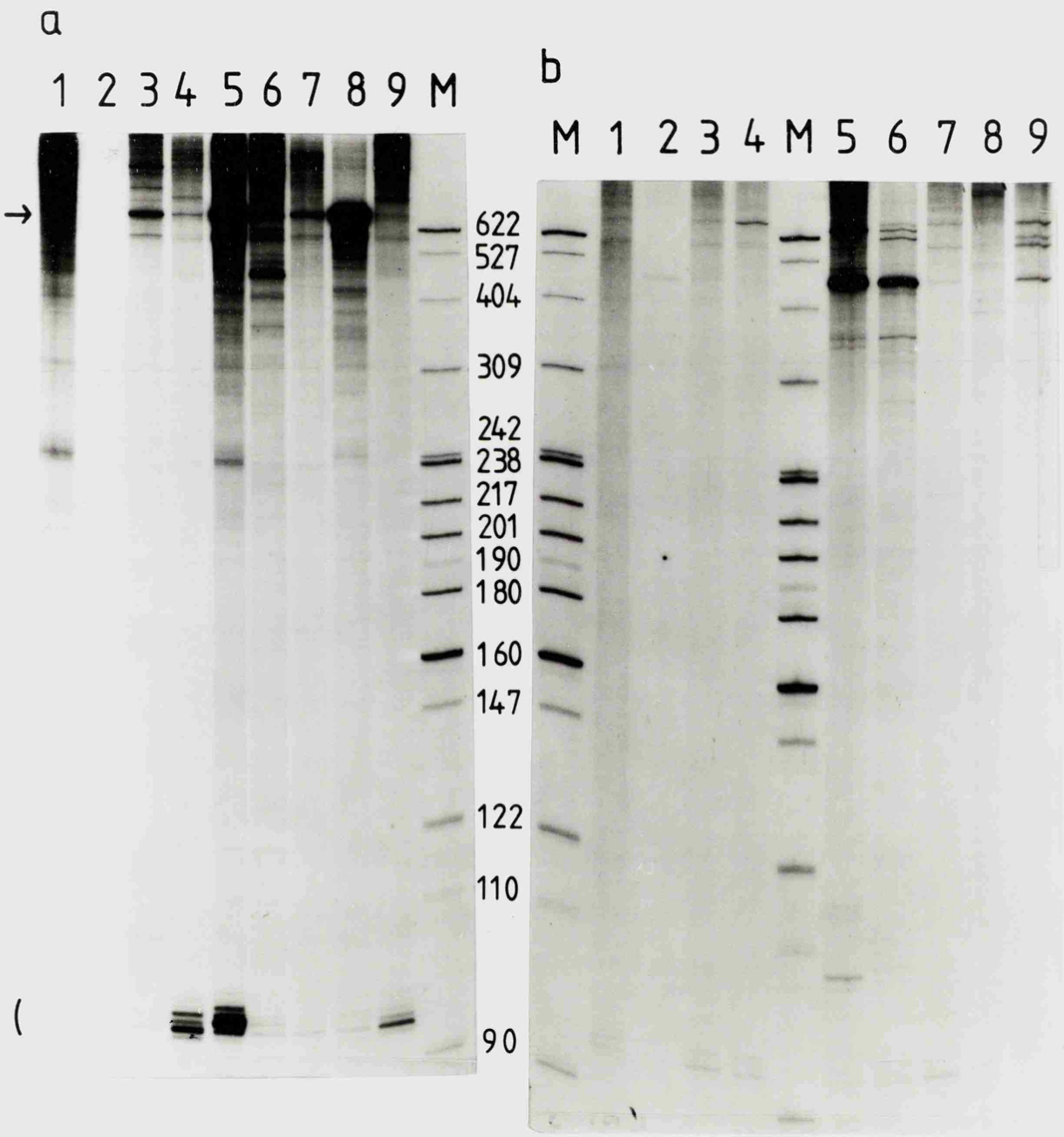
Fig. A2 : Dinucleotide-primed transcription *in vitro*.
Transcription of *Bst*EII-cut pSES3 by RNA polymerase purified from *B.subtilis* was carried out as described by Le Grice and Sonenshein (1982). The dinucleotide concentrations were 250 μM, the mononucleotide concentrations, 10 μM. After 10 min. incubation at 37^{o} the transcription products were precipitated, resuspended in loading buffer containing 5 M urea and applied to a 1.5 mm thick 8% polyacrylamide gel containing 8 M urea.
Markers (M) were *Hpa*II-digested pBR322 (sizes in bases).
Transcription was primed with the following dinucleotides:

a) 1, ApA; 2, ApG; 3, ApC; 4, ApU; 5, UpA; 6, UpG; 7, UpC; 8, UpU;

b) 1, CpA; 2, CpG; 3, CpC; 4, CpU; 5, GpA; 6; GpG; 7, GpC; 8, GpU.

Sample 9 of gel (a) was primed with high ATP, sample 9 of gel (b) with high GTP.
The arrow indicates the presumed *cat* run-off transcript; the bracket, RNA species of about 92 b.

Fig. A 2


a
1 2 3 4 5 6 7 8 9 M
622
527
404
309
242
238
217
201
190
180
160
147
122
110
90
b
M 1 2 3 4 M 5 6 7 8 9

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