https://theses.gla.ac.uk/

Theses Digitisation:

https://www.gla.ac.uk/myglasgow/research/enlighten/theses/digitisation/

This is a digitised version of the original print thesis.

Copyright and moral rights for this work are retained by the author

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

This work cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

Enlighten: Theses
https://theses.gla.ac.uk/
research-enlighten@glasgow.ac.uk

INVESTIGATION OF A COMPLEX PHENOTYPE IN *ESCHERICHIA COLI* K-12

A thesis submitted for the degree of Doctor of Philosophy at the University of Glasgow

by

Fiona J Hundley

Institute of Genetics
University of Glasgow

April 1991

ProQuest Number: 10984124

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

ProQuest 10984124

Published by ProQuest LLC (2018). Copyright of the Dissertation is held by the Author.

All rights reserved.
This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

The research reported in this thesis is my own work, except where otherwise indicated. This work has not been submitted for any other degree.

Fiona J Hundley

For Steve and Ulrich

CONTENTS

ABBREVIATIONS

amp - ampicillin
ATCase - aspartate transcarbamoylase
ATP - adenosine triphosphate
bp - base pairs
°C - degrees centigrade
CAT - chloramphenicol acetyl transferase
cm - centimetres
cm - chloramphenicol
cut - citrulline utilizing
DNA - deoxyribonucleic acid
dNTP - deoxynucleotide triphosphate
DTT - dithiothreitol
EDTA - ethylenediamine tetra-acetic acid
EthBr - ethidium bromide
FIS - factor for inversion stimulation
g - grammes
g - centrifugal force equal to gravitational acceleration
Hfr - High frequency recombination (strain carrying a conjugal plasmid integrated into its' chromosome)
hr - hours
IS - insertion sequence
kan - kanamycin
kb - kilobase/kilobase pairs
kd - kilodalton
l - litres
M - Molar
min - minutes
ml - millilitres
mM - milliMolar
mRNA - messenger RNA
ng - nanogrammes
nt - nucleotides
OD - optical density
ORF - open reading frame
OTCase - ornithine transcarbamoylase
pH - acidity ($-\log_{10}$[Molar concentration of H^+ ions])
r-det - resistance determinant (of R plasmids)
REP - repeated extragenic palindromic (sequence)
RNA - ribonucleic acid
rpm - revolutions per minute
rRNA - ribosomal RNA
RTF - resistance transfer factor (of R plasmids)
SDS - sodium dodecylsulphate
strep - streptomycin
TBE - Tris-borate-EDTA
TE - Tris-EDTA
tet - tetracycline
Tn - transposon
Tris - tris (hydroxymethyl) amino ethane

u - units
UV - ultraviolet
vol - volume
λdv - lambda derivative
µg - micrograms
µl - microlitres

ACKNOWLEDGEMENTS

Thank you to:
All those who comprised the Genetics Department of Glasgow University from 1986-1991 for their friendship, enthusiasm, helpfulness and moral support.
My supervisor, Adrienne Jessop, for allowing me to work in her laboratory and providing me with strains, plasmids and helpful advice.
Dave Sherratt, Kim Kaiser and Iain Hunter for sharing equipment and reagents.
Flora Rodgers and Mary Burke for showing me how to use aforementioned equipment and for always having whatever I'd forgotten to make up.
The Prep Room Ladies for saving Flora and Mary from having to lend me all that media.
Joyce Moore who set up at least two of the ligations described in this thesis - unfortunately I was too tired and emotional at the time to remember now which ones they were.
Members of the Jessop, Sherratt and Kaiser groups for sharing their data with me and looking at mine.
Mike Ashburner and the Genetics Department of Cambridge University for use of their library and word-processing facilities.
My husband Steve Russell for all of the above and more.
This work was supported by an SERC studentship.

SUMMARY

This study investigates the ability of *Escherichia coli* K-12 strains to utilize citrulline as a source of carbamoyl phosphate. One response of strains to this selection procedure is the amplification of the *arg*F gene. This response had only previously been observed when the *E. coli* sex factor, F, was integrated adjacent to Tn2901, the transposon like structure that carries the *arg*F gene. I demonstrate here that tandem amplification of Tn2901 can occur in an F^- strain after long incubation of the strain on selective medium at 30°C. In addition stimulation of *arg*F amplification in F^- cells, in the presence of multicopy plasmids is shown. The nature of this amplification is not investigated. Mutants able to utilize citrulline are shown to cross-feed non-mutant cells offering a potential explanation for a class of non-amplified mutants. The role of the F factor in stimulating amplification of Tn2901 is investigated. Conjugal DNA transfer and the genes of the *tra* operon are shown to be unnecessary for this stimulation. F factor DNA is cloned and a system for returning cloned F fragments is described. The potential for replication initiated by the F factor to stimulate Tn2901 amplification is discussed. Vectors for replacing the IS1 elements of Tn2901 with mutated IS1 or non-homologous sequences are designed. DNA required for constructing these vectors is cloned. The construction of the vectors is initiated. Three different mutations are made in a cloned IS1 sequence.

CHAPTER ONE

INTRODUCTION

Introduction

The study of mutant phenotypes is the bedrock of genetic research. Classically the isolation of mutants unable to perform specific processes is used as the means by which the mechanisms giving rise to those processes are understood. More recently molecular biology has allowed the cloning and characterization of genes whose function is unknown resulting in the need for reverse genetics; the disruption *in vitro* of DNA sequences and their subsequent return to the chromosome. However mutant phenotypes may be studied in their own right. This is of particular importance in medicine where relief from the phenotype is sought. In addition the investigation may uncover hitherto unrecognised cellular processes or unsuspected links between different processes leading to a greater understanding of cellular biology.

The ability of mutants of *E.coli* K-12 to utilize citrulline as a source of carbamoyl phosphate is an interesting phenotype, described in detail below. *arg*F and *arg*I constitutive operator mutants and *arg*G bradytrophs all display this phenotype. The number of mutants produced by a strain is stimulated enormously by the *E.coli* sex factor F. One aspect of this stimulation involves the amplification of the *arg*F gene on an IS1-flanked segment of DNA. In Hfr strains mutants start to appear after 2-3 days and continue to appear thereafter suggesting that they arise on the plates as a consequence of slow parental growth on citrulline. In *E.coli* K-12 *arg*F and *arg*I are duplicate genes encoding ornithine transcarbamoylase.

Genetic rearrangements, and amplification in particular, can be found in many cell processes. In some processes they appear to occur in a controlled fashion. For instance variation of the major pilus in *E. coli* (Klemm 1986) or, in *Drosophila*, amplification of chorion genes in the ovary during egg development (Spradling and Mahowald 1980). They can also be observed in transformed cell lines, suggesting a role in cancer (Cairns 1981). Insertion sequences are known to cause rearrangements and can effect gene expression by creating promoters following transposition (Prentki *et al.* 1986). They are thought to be important in

evolution, as are duplicate genes where one may be mutated without loss of function in the other (Ohno 1970). The study of genetic rearrangements and factors that stimulate them is thus of both medical and evolutionary importance. This type of investigation is far from straight forward. Therefore a simple prokaryotic system where much is known about the components offers many advantages over those in more complex higher organisms.

In this introduction I review a number of elements that are known to play a role in this system. There are almost certainly others. I start with recombination which by necessity must be only a brief outline. I then describe the F factor, IS1, OTCase encoding genes and finally the citrulline utilizing phenomenon itself.

Recombination

Classically two types of recombination have been described in *E.coli*; general or homologous recombination and site-specific recombination. General recombination will occur between any two homologous DNA sequences and is mediated by a range of enzymes described below. Site-specific recombination occurs, as it's name suggests, at specific DNA regions and is generally dependent on a specialized recombinase. Good reviews of homologous recombination include those of Cox and Lehman (1987) and Weinstock (1987) while site-specific recombination has recently been dealt with by Craig and Kleckner (1987).

Many of the enzymes involved in general recombination also have roles in DNA repair and it has been suggested that recombination itself is merely an added bonus resulting from the need to repair DNA (Berstein *et al.* 1985). Indeed a major stimulus for initiation of recombination in *E.coli* is either single or double strand DNA breakage. Thus mutations that result in DNA discontinuities, for instance *pol*A mutants which affect the sealing of Okazaki fragments, exhibit a stimulatory effect on recombination (Konrad 1977). This has been taken as an

indication that initial strand breakage is the limiting step in recombination. Strand breakage may provide a site for loading recombination enzymes onto DNA. For example, Seifert and Porter have shown that the F factor *ori*T site (see below) can stimulate recombination at distal sites in the presence of the *tra*Y-Z endonuclease that specifically nicks *ori*T (Seifert and Porter 1984). Discontinuities could also stimulate resolution of the cross-over region if branch migration brings it into contact with a nick (Weinstock 1987). Recombinants are formed at high frequency following conjugal DNA transfer suggesting that recombination is stimulated in recipients in this process. This may reflect the fact that a single strand is transferred in conjugation or may be the result of an F encoded recombination stimulator; the postulated product of the *rsf* locus (Chernin *et al.* 1978). In yeast transcriptionally active DNA has been shown to have elevated levels of recombination. Two possible explanations have been advanced to explain this: Transcription may alter chromatin structure allowing access to recombination enzymes. Alternatively the stimulation of recombination may result from the action of one of the enzymes in the transcription complex, for example, a topoisomerase (Thomas and Rothstein 1989). Replication, transcription and recombination share the mechanistic problems resulting from the need to wind and unwind DNA helices and the resulting changes in DNA supercoiling (Honigberg and Radding 1988). The control of DNA supercoiling is the province of the topoisomerases. Topoisomerases can catalyse strand transfer *in vivo* and this activity may be responsible for the genetic rearrangements observed when the rejoining transesterification of topoisomerase action is blocked *in vitro* (Lui 1989). Topoisomerases may also act to suppress recombination by dissociating paired strands and thus disrupting recombination intermediates (Wang *et al.* 1990). Indeed the study of supercoiling, topoisomerases and their effects on recombination is likely to add greatly to the basic understanding of recombination (Wang *et al.* 1990).

The first recombination mutants isolated were in the genes encoding the RecA protein and the RecBCD enzyme in *E. coli* (Clark and Margulies 1965). RecA is a DNA-dependent ATPase

which unwinds DNA in the presence of ATP. It binds single stranded DNA in a co-operative manner and catalyses the homologous pairing of duplex molecules with single stranded or partially single stranded DNA to form the heteroduplex crossover region (Radding 1982). This strand transfer reaction is stimulated by single strand binding proteins encoded by *E.coli*, plasmid F or bacteriophage T4 (Egner *et al.* 1987). RecA has also been shown to promote the branch migration phase of recombination (Cox and Lehman 1987). In addition RecA is responsible for catalysing the cleavage of LexA and phage λ repressors. It thus plays a central role in regulation of the SOS response to agents that damage DNA or interfere with replication (Walker 1987). RecA protein appears to be involved in all homologous recombination reactions in *E.coli* with virtually no recombination apparent in *rec*A strains (Weinstock 1987). A possible exception to this is microhomologous recombination, for instance the precise excision of transposons. I will deal with transposition as a site specific reaction, however precise excision does not require the activity of the transposon encoded recombinase. It is thought to occur by recombination between the small repeated sequences generated by transposon insertion. Precise excision of IS1 therefore requires recombination to occur between 9bp target duplications (Ohtsubo *et al.* 1981a). RecA protein does not act efficiently on sequences with less than 40bp of homology (Gonda and Radding 1983)(Smith 1988). Thus it's role in this process has been called into question. However a study by Lu *et al.* has demonstrated that a phage P1 function that stimulates precise excision of IS1 does so in a RecA dependent manner (Lu *et al.* 1989). REP (repeated extragenic palindromic) sequences may also be sites of microhomologous recombination. These 35bp inverted repeat sequences have been found at the novel joints of chromosomal duplications in the *his*G-D operon of *Salmonella* (Shyamala *et al.* 1990). Duplications in this operon were observed in *rec*A strains, although at one sixth of the frequency that they appear in *rec*$^+$ strains. Shyamala and colleagues analysed the end points of the RecA independent duplications. Many of these occurred in REP sequences and the authors suggest that, as REP sequences may

specifically bind DNA gyrase (Yang and Ames 1988), this could be the result of a site specific reaction catalysed by DNA gyrase. RecA independent duplications with end points outside REP sequences were also observed, however, one apparently resulting from recombination between only 7bp of homology (Shyamala *et al.* 1990). It has been suggested that microhomologous recombination may occur by a copy choice mechanism during DNA replication. It is envisaged that DNA polymerase slips or jumps from one single stranded region of homology to the other, by-passing the intervening DNA (Albertini *et al.* 1982) and that looping out of the intervening DNA by flanking inverted repeats could stimulate this process (Schaaper *et al.* 1986). Microhomologous recombination is thus a complex phenomena that may occur by a variety of mechanisms some of which may depend on or be stimulated by RecA.

Exonuclease V, the product of the *rec*B, *rec*C and *rec*D genes, catalyses the best characterized general recombination pathway in *E.coli*. The RecBCD enzyme shows DNA dependent ATPase activity, ATP dependent exonuclease and unwinding activity on double stranded DNA and endonuclease and exonuclease activities on single stranded DNA (Weinstock 1987). It's preferred substrate is linear duplex DNA with blunt ends although it will act on nicked circular DNA provided the nick is greater than 5bp in length (Roman and Kowalczykowski 1989). It has been suggested that the RecB polypeptide is responsible for the unwinding activity and the RecD subunit for the nuclease activity of exonuclease V (Amundsen *et al.* 1986).
Recombination via the RecBCD pathway is stimulated by the presence of chi sites at which the enzyme nicks. The stimulation is greatest near the site, but can still be measured up to 10 kb away. Work on purified enzyme suggests that after cutting at a first chi site, RecBCD does not cut at subsequent sites (A.F. Taylor, cited in Smith, 1991). In the presence of linear DNA RecBCD probably acts early in recombination by unwinding and cutting DNA to provide a substrate for RecA. It may also play a role in resolution of the crossover junction (Weinstock 1987)(Cox and Lehman 1987). Mahan and Roth (1989) have analysed the the role of RecBCD in chromosomal rearrangements. They found

that recombination between direct repeats of Tn10 to form a deletion was independent of RecBC function while circle integration and inversion were substantially reduced in a *rec*B C mutant. They propose a two-step model for recombination. The first stage, independent of RecBCD, generates one recombinant product and two double stranded ends. RecBCD can then load onto the chromosome at the double strand break catalysing the second step; joining the second pair of flanking markers (Mahan and Roth 1989). Given it's action on double stranded breaks, it is not surprising that RecBCD is the major recombination pathway during conjugation, transduction, transformation, and the repair of chromosomal breaks. G.R. Smith has recently reviewed early data on conjugal recombination. He suggests that 80% of recombination following conjugation occurs as a result of the RecBCD enzyme acting on the ends of the transferred fragment with resulting "long chunk integration". Thus the number of cross-overs would be dependent on the number of double strand breaks with one RecBCD molecule acting at each cross-over site. From this one would predict that if, during an Hfr cross, a marker on the donor molecule distal to the origin of transfer is selected, any proximal marker located between the selected marker and the origin of transfer will be derived from the donor molecule in 80% of the recombinants examined. Smith ascribes the remaining 20% of events to "short chunk integration" where recombination occurs near the selected marker and suggests that this could occur by a mixture of RecBCD and RecF action (Smith 1991).

In *rec*BCD mutants near wild-type levels of recombination can be observed provided the *sbc*B gene product, exonuclease I, is inactive. This is the result of the RecF recombination pathway. A number of genes; *rec*F, *rec*J, *rec*N, *rec*O, *rec*Q, *uvr*D and *ruv* are thought to be involved in this pathway. Mutations in *rec*F, *rec*J or *rec*O also reduce recombination between plasmids in otherwise wild type strains. The *rec*N, *uvr*D and *ruv* genes are induced by the SOS response (Weinstock 1987).

The *rec*E gene is carried on a defective lambdoid prophage (RAC) present in the chromosome of most *E.coli* K-12 strains and is normally repressed. Mutations in *sbc*A which map close to

RAC allow expression of the *rec*E gene product, exonuclease VII (Kaiser and Murray 1979). This pathway may be related to the RecF pathway although its affects on plasmid recombination are quantitatively different (Weinstock 1987).

Thus general recombination in *E.coli* is a complex subject. The major players, RecA and RecBCD, have been analysed extensively at the physical, biochemical and genetic levels, but much remains to be discovered of their roles in specific processes; while the elucidation of the *rec*F pathway, with its inducible components linking recombination to cell physiology and it's interaction with the main *rec*BC pathway, remains an intriguing prospect.

A variety of systems in *E.coli* exhibit site-specific recombination. Transposable elements and insertion sequences encode a recombinase called a transposase that acts specifically on the ends of the element. Some transposons, for example Tn7, have a preferred target site. Others, such as IS1 (see below), require no specific sequence in the target molecule. Elements that transpose replicatively form a transposition intermediate or cointegrate such that donor and target are fused by duplicate copies of the element. These transposons, epitomized by Tn3, encode a second site-specific recombinase, resolvase, that acts to resolve cointegrates leaving a single copy of the element on each molecule (Craig and Kleckner 1987). ColE1 multicopy plasmids carry a site at which a host encoded recombinase acts to resolve plasmid dimers (Colloms *et al.* 1990). Resolution of phage P1 dimers results from the action of a phage encoded recombinase. In the absence of RecA protein, this recombinase also acts to circularize the terminally redundant P1 genome (Sternberg and Hoess 1983). P1 encodes a second site-specific recombination system that catalyses the inversion of a DNA segment that lies between the two recombination sites allowing alternate expression of two sets of tail fibre proteins. A similar inversion system is used by bacteriophage Mu for control of expression of tail fibre proteins. Four bacterial systems that rely on the inversion of a DNA segment for alternate gene expression have so far been recognised (Craig and Kleckner 1987). The inversion systems are particularly interesting in that efficient inversion

requires the presence of an enhancer sequence distal to the recombination crossover site. These enhancers are binding sites for the host encoded protein FIS (factor for inversion stimulation). Cellular levels of FIS are a function of growth phase providing a mechanism whereby recombination in these systems may be linked to the physiological state of the cell (Thompson *et al.* 1987). FIS has also been implicated in regulation of the phage λ site-specific recombination system (Thompson *et al.* 1987). The integration of lambdoid phages into the host genome and their excision are catalysed by phage encoded integrase. These integrase reactions are subject to a vast array of regulatory interactions that couple integration and excision to the phage life cycle. An easy to read review of the lambda life cycle is Ptashne's "A Genetic Switch" (Ptashne 1986). A second edition of this book is due this year and I will not attempt to pre-empt it here.

These various site-specific recombination systems have proved ideal for mechanistic studies of recombination and are likely to become the benchmark against which our understanding of any recombination system is measured.

The F factor

Conjugal transfer was the first recognized and most obvious function of the *E. coli* F factor. The name F (for fertility) derives from this fact (Cavalli-Sforza *et al.* 1953). Over 30% of the F genome is given over to genes encoding functions required for efficient transfer of F DNA from one cell to another (Willetts and Skurray 1987). The genes of the transfer (*tra*) operon and the process of conjugal transfer have been extensively studied (for reviews see Willetts and Skurray, 1987; Ippen-Ihlier and Minkley, 1986). The *tra* operon of F has been defined as the region from *ori*T at 66.6 min. on the map to the start of IS3a at 0/100 min. (Willetts and Wilkins 1984). In fact IS3a is an insertion into the last gene of the operon; *fin*O (Cheah and Skurray 1986). In related conjugal plasmids (R1, R100) the *fin*O

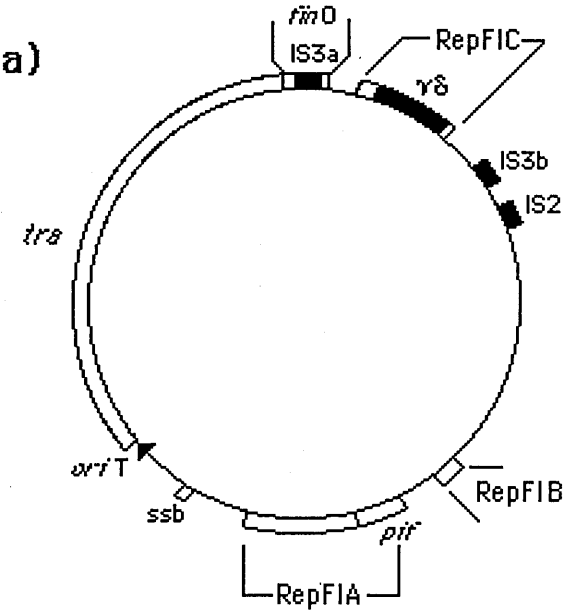
Figure 1.1

a) Map of the F factor (after Willetts and Skurray, 1987). Insertion sequences IS2, IS3a, IS3b and $\gamma\delta$ are shown as black boxes. The location of the three known replication regions RepFIA, RepFIB and RepFIC are shown. The position of the F factor single strand binding protein (ssb) on the leading edge is marked. The origin of conjugal DNA transfer (*ori*T) and the direction of transfer are given by the arrow head. The position of the transfer operon (*tra*) is indicated with the final gene of the operon (*fin*O), interrupted by IS3a, shown separately.

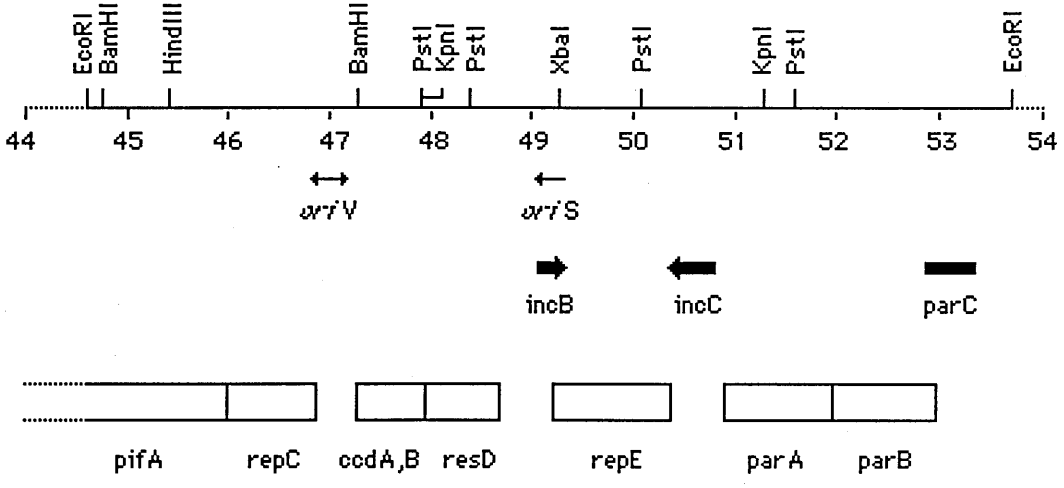
b) Restriction map of RepFIA (mini F) after Willetts and Skurray, 1987. Sites for EcoRI, BamHI, HindIII, PstI, KpnI and XbaI are shown. The numbers are F co-ordinates on the 94.5 kb map of the F factor. The position of *ori*V and *ori*S and the direction of replication initiated from these sites are shown by small arrows. *inc*B and *inc*C are shown by large arrows and *par*C by a thick line. The position of the *pif*A, *rep*C, *ccd*A and B, *res*D, *rep*E, *par*A and *par*B genes are indicated.

Figure 1.1

a)


finO
IS3a
RepFIC
γδ
IS3b
IS2
traI
oriT
ssb
RepFIB
pif
RepFIA

b)


EcoRI
BamHI
HindIII
BamHI
PstI
KpnI
PstI
XbaI
PstI
KpnI
PstI
EcoRI
44
45
46
47
48
49
50
51
52
53
54
oriV
oriS
incB
incC
parC
pifA
repC
ccdA,B
resD
repE
parA
parB

and *fin*P gene products are both required to inhibit expression of TraJ the positive regulator of *tra* transcription. The F factor is therefore naturally derepressed for conjugation unless it is repressed *in trans* with the *fin*O gene product from another plasmid (Willetts 1977). The *fin*P gene product is an anti-sense RNA that complements the untranslated portion of the *tra*J transcript causing premature termination (Mullineaux and Willetts 1985). FinO may be a 21 kDa protein that is mutated in the derepressed plasmid R100-1drd (Yoshioka *et al.* 1987). However deletion analysis of R100 suggests that FinO may be more complex than this; early frame shift mutations do not affect FinO activity (McIntyre and Dempsey 1987). Hybridization analysis detects two short transcripts encoded on the opposite strand, but nothing from the ORF. It has therefore been suggested that FinO is also an RNA species (Dempsey 1987). The FinO gene product has been shown to stabilize the FinP RNA and may play a role in catalysing the interaction of FinP with the *tra*J mRNA (Frost *et al.* 1989).

The genes of the transfer operon have been mapped by complementation and polypeptide analyses. Sequence analysis of a number of genes is in progress. To date 23 *tra* genes required for conjugation and 6 *trb* genes of unknown function have been identified (Ippen-Ihler and Minkley 1986)(Willetts and Skurray 1987)(Ham *et al.* 1989). The *tra* genes fall into four functional groups: The regulatory genes *tra*J, *fin*O and *fin*P have been discussed above. The second group are genes involved in the formation of the F pili and in establishing intercellular contacts, the first stage of conjugation. *tra*A encodes pilin, the major if not only constituent of pili and the *tra*Q gene product is thought to be involved in the processing of *tra*A (Wu and Ippen-Ihler 1989). At least ten other *tra* gene products are required for assembly or function of F pili while *tra*N and *tra*G are responsible for the subsequent stabilization of mating pairs observed when donor and recipient cells come into close physical contact (Willetts and Skurray 1987). The third class of *tra* genes, *tra*S and *tra*T, are not required for conjugal transfer but mediate the surface exclusion effect that causes cells to act as poor recipients thus limiting unproductive mating between two F

bearing cells. The remaining *tra* genes (M, Y, D, I and Z) are involved in DNA transfer. This process begins by nicking at the origin of transfer *ori*T. Nicking is dependent on *tra*Y and *tra*Z and it is possible that these gene products form an endonuclease (Everett and Willetts 1980). The *tra*Z activity may arise as a translation reinitiation product of *tra*I; DNA helicase I. The helicase activity of *tra*I is associated with the carboxy terminal of the protein and mis-sense mutations in this region do not interfere with nicking however the amino terminal region may encode *tra*Z activity (Traxler and Minkley 1987). A *tra*Y-*tra*IZ complex may therefore be responsible for coordinate nicking at *ori*T and unwinding of the DNA strands (Willetts and Skurray 1987). Nicking at *ori*T does not require synthesis of Pili or formation of mating pairs and it is likely that nicked and closed forms of F exist in equilibrium (Everett and Willetts 1980). Mating pair formation may activate strand unwinding by *tra*I via a conformational change in *tra*M (Everett and Willetts 1980). The nicked strand is unwound in a 5' to 3' direction (away from the *tra* genes) and passed single stranded into the recipient. The "leading edge" or first DNA to enter the recipient on transfer encodes a single strand binding protein that is not part of the *tra* operon. Whether this protein plays any role in conjugation has yet to be determined (Willetts and Skurray 1987). DNA transfer through the donor cell envelope may be the role of *tra*D and ATP hydrolysis by DNA helicase I (*tra*I) may serve to energize the process (Willetts and Wilkins 1984)(Ippen-Ihler and Minkley 1986). After initiation conjugative DNA synthesis is carried out by host encoded proteins with complementary strand synthesis occuring in the recipient and replacement strand synthesis in the donor. Both processes are catalysed by DNA polymerase III (Willetts and Skurray 1987).

With the exception of *fin*P all of the genes described above are transcribed from the strand that is transferred during conjugation. Five transcription units have been proposed: *tra*M and *tra*J have their own promotors producing single gene transcripts. The *tra*Y promotor provides a transcript that encompasses the entire 32kb length of the *tra*YZ operon including all the other *tra* and *trb* genes. Additionally *tra*I and

traS/*traT* have weak internal promotors (Willetts and Skurray 1987). The *traM* and *traY* promotors have upstream regions of homology that may serve as binding sites for TraJ (Thompson and Taylor 1982)(Mullineaux and Willetts 1985). Two upstream oriented promotors (*artA* and *artB*) have also been recognised (Ippen-Ihler and Minkley 1986) with the *artA* promotor capable of directing synthesis of an *ArtA-lacZ* fusion protein (Wu and Ippen-Ihler 1989).

Adjacent to the *tra* region lies the leading edge. Like the transfer region it is conserved among F-like plasmids. In addition to the single strand binding protein mentioned above, the 13kb leading region of F encodes seven polypeptides of unknown function. Three loci have been mapped to this region; *parL*, *rsf* and *prt*. The *parL* locus increases plasmid stability when cloned into the vector pACYC184. Functional studies suggest that *parL* is a *cis*-acting site but as yet there is no evidence to suggest that it is involved in maintenance of F (Willetts and Skurray 1987). *rsf* specifies a *trans*-acting factor involved in stimulation of recombination in the recipient following conjugation (Goldfarb *et al.* 1973)(Chernin *et al.* 1978). The *prt* locus has both *cis* and *trans* components involved in providing the host cell with protection against DNA damaging agents (Goldfarb *et al.* 1974). A further locus, *chr*, has been mapped to a region between co-ordinates 52.2 and 55.8 kb. This locus is required for integrative suppression of a temperature sensitive replication mutant of HfrC (Chernin *et al.* 1983). Since these co-ordinates overlap with the primary replication region of F *chr* may be an allele of *oriV* (see below), but it is possible that the leading edge contains the remnants of a fourth replication region.

The F factor carries four insertion elements; gamma delta, IS2 and two copies of IS3. These are clustered in an A-T rich region between 0 and 20 minutes on the F map (Figure 1.1a). The insertion sequences serve as attachment sites for recombination between F and the *E. coli* chromosome leading to the formation of Hfr strains. Insertion of F into the chromosome results in duplicate insertion elements flanking the F factor in direct repeat (Davidson *et al.* 1975). Initiation of conjugal transfer in

an Hfr strain, such as Jef8, can mobilize the entire *E.coli* chromosome. Excision of F from the chromosome of Hfr strains is not always a precise event and can result in the formation of F-prime plasmids; F plasmids carrying a fragment of the bacterial genome. The amount of chromosomal DNA carried can vary from 1 min (about 46 kb) up to 30% of the *E.coli* genome (Holloway and Low 1987). F-primes may or may not contain a deletion of F DNA (Scaife 1967). A subclass of F' plasmids; F'Δ*tra* plasmids are formed by aberrant excision of F from the bacterial chromosome such that the plasmid carries a piece of chromosomal DNA but is deleted for the entire transfer region. This may occur during conjugal DNA transfer in an Hfr strain if the *tra*YZ endonuclease attached at *ori*T aberrantly nicks and religates in a chromosomal sequence. Horowitz and Deonier have shown that the sequence of the F-chromosomal DNA joint in some F' plasmids is consistent with an *ori*T nick-ligation site and that the original chromosomal sequence involved in forming these F primes contains a short region of homology with *ori*T (Horowitz and Deonier 1985).

The F factor contains at least three replication regions. RepFIA and RepFIB are capable of functioning as autonomous replicons. RepFIC is the remains of a replicon that is interrupted by the transposon γδ. The insertion of γδ may also have been responsible for the deletion of the majority of RepFIC sequences. The leading edge may contain the remnants of a fourth replicon (Willetts and Skurray 1987). Autonomously replicating RepFIB plasmids are inherently unstable and no evidence exists to suggest that this replicon contributes to normal F maintenance. The *inc*E site on RepFIB however is responsible for the observed incompatibilty of F with plasmids ColV3-K30 and pHH507 (Willetts and Skurray 1987).

RepFIA is the primary replicon of plasmid F. It is responsible for control of F replication, copy number and incompatibility with other F plasmids. It can be isolated on a single EcoRI fragment called mini-F. Analysis of mini-F has provided a great deal of detail on this region. A map of the known loci is shown in Figure 1.1b. Two replication origins have been identified on mini-F; *ori*V (*ori*I) and *ori*S (*ori*II). Replication from *ori*V is

predominantly bidirectional. It requires *rep*C function and may also require the proteins RepE and RepD or RepG *in trans*. RepC protein autoregulates it's own synthesis and is the first gene of the *pif* operon responsible for inhibition of phage T7 infection. The other genes of the *pif* operon do not appear to be involved in replication (Willetts and Skurray 1987). Replication initiated at *ori*S is only apparent in mutants deleted for *ori*V. This replication is unidirectional and to the left. Replication from *ori*S requires RepE protein and four 19 bp repeats that form the *inc*B locus (Kline 1985). RepE transcription is mediated by RNA polymerase containing sigma32 factor, better known for it's role in regulating the heat shock response (Wada *et al.* 1987). RepE protein is a repressor of it's own synthesis. It binds to repeats of *inc*B and to the five 19 bp repeats that constitute the *inc*C locus. *inc*C is required for control of *ori*S and for regulation of copy number. This control may be achieved by titration of RepE. Together *inc*B and *inc*C are responsible for F incompatibilty. Current favoured models suggest that incompatibility is the result of titration of RepE protein to levels below that required for initiation of replication (Willetts and Skurray 1987). Initiation at *ori*S, like initiation of phage P1 or λ replication, requires the host heat shock proteins DnaK, DnaJ and GrpE. These proteins may be involved in activation or stabilization of RepE (Kawasaki *et al.* 1990). It has recently been shown that DnaK and DnaJ proteins interact with the P1 initiator protein activating it for *ori*P1 binding (Wickner *et al.* 1991). The *ori*S region also contains two *dna*A boxes and DnaA protein is among the other host encoded functions required for mini-F replication (Kline 1985)(Murakami *et al.* 1987). Both the F factor and mini-F are able to integratively suppress *dna*A temperature sensitive mutants. This may reflect leakiness of these mutations (Kogoma and Kline 1985b) or it might suggest use of a *dna*A-independent origin during integrative suppression. Which origin is normally responsible for replication of F is as yet unclear. Shields *et al.* have measured origin concentration as a function of growth rate. They found that plasmids containing only *ori*S mimicked host chromosomal origin control in maintaining origin concentration independent of growth rate. Plasmids driven only by *ori*S, but

which contained a transposon insertion in *inc*C showed a concentration that was independent of growth rate but double that of plasmids without the insertion. F'*lac* and mini-F plasmids containing both *ori*V and *ori*S however increased their concentration as growth rates fell (Shields *et al.* 1987).

Adjacent to and possibly overlapping with *ori*V is the *rfs*F locus. This area shows sequence homology with *ori*C and is required for RecA-independent cointegrate formation between *ori*V containing plasmids and F-primes. It is also likely to be responsible for site-specific integration of F at *ori*C. The RepC protein may be involved in these reactions (Lane *et al.* 1984). Cointegrates formed at *rfs*F are unstable, but can be stabilized by mutations in *res*D. Resolution mediated by ResD occurs at a region called *fcr* about 80 bp from *rfs*F which includes a DNA gyrase cleavage site (Willetts and Skurray 1987). This site-specific recombination system is probably responsible for ensuring that F remains in monomeric form thus enhancing it's stability.

Between the *rfs*F/*fcr* loci and the *res*D gene lie two genes, *ccd*A and *ccd*B that couple F replication to cell division. The *ccd*B encoded polypeptide (RepG) is believed to inhibit host cell division and the *ccd*A polypeptide (RepH) to repress this function. In addition *ccd*B can induce the host SOS response if F factor replication or partitioning is blocked and may be involved in replication from *ori*V (Willetts and Skurray 1987).

The locus involved in partitioning mini-F, ParFIA, is composed of a *cis* -acting site, *par*C, containing eleven 43 bp repeats and two genes *par*A and *par*B. The *par*B gene product has been shown to bind to *par*C which may constitute a site for interaction of F with the cell membrane or host chromosome (Willetts and Skurray 1987). Thus F is a large and complicated plasmid. While conjugation and replication from RepFIA have been extensively studied, much remains to be learned. The leading edge, partitioning locus and secondary replication region, RepFIB, are known but little understood and at least 20kb remains completely undescribed.

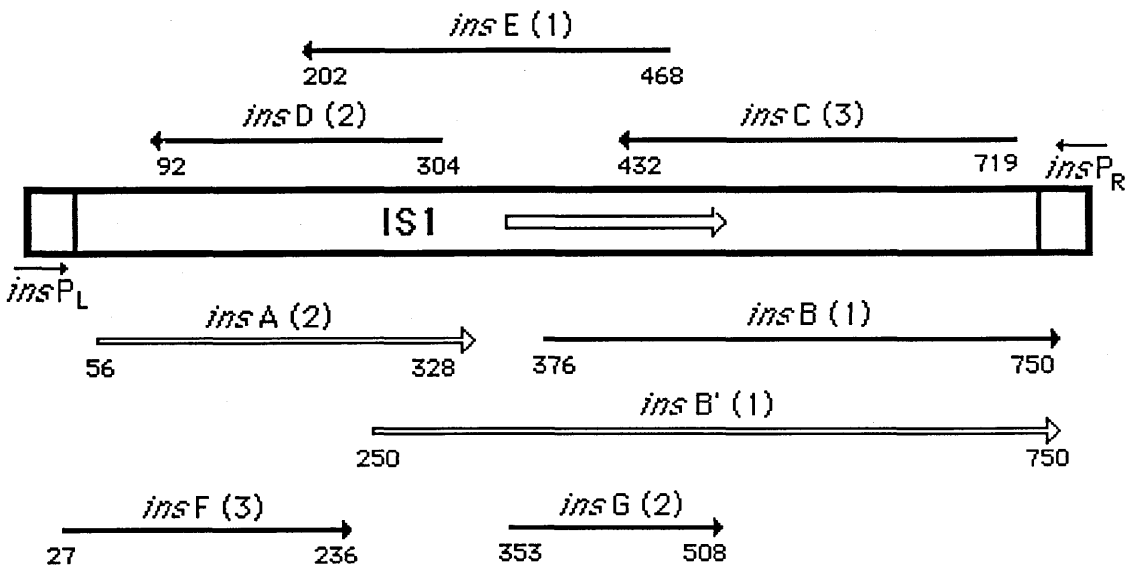
Figure 1.2

a) Map of IS1 after Jakowec *et al.* 1988. The eight open reading frames are shown by large arrows that point in the direction of transcription. The two ORFs responsible for production of the IS1 transposase (*ins*A and *ins*B') are given by open arrows. The reading frame of each is given in parentheses. The IS1 co-ordinates for each ORF are shown. Small arrows indicate the position and orientation of the two promoters $ins P_L$ and $ins P_R$ located in the terminal inverted repeats (boxes).

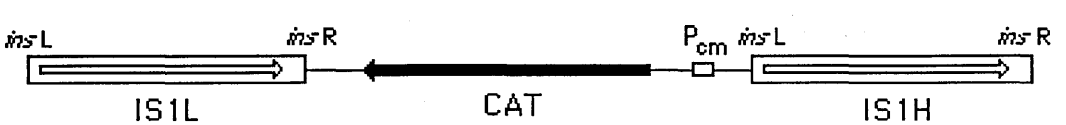
b) Diagram of the chloramphenicol resistance transposon Tn9 after Machida *et al.* 1983. Open arrows inside the IS1 elements indicate their orientation. The left and right hand ends of the elements are called *ins*L and *ins*R respectively. The position and orientation of the chloramphenicol acetyl transferase gene is given by the thick arrow and and it's promoter is shown by a small box.

Figure 1.2

a) IS1



b) Tn9



DEPT. OF GENETICS
UNIVERSITY. GLASGOW.

IS1

IS1 is the smallest active IS element known in bacteria, being only 768bp in length (Hirsch *et al.* 1972)(Fiandt *et al.* 1972)(Ohtsubo and Ohtsubo 1978). IS1 shares the tendency of many insertion sequences to promote DNA rearrangements. It can transpose, producing a new copy of itself onto the same or a different DNA target. Intermolecular transposition often results in the fusion of donor and target replicons, called cointegrates (Shapiro 1979)(Arthur and Sherratt 1979). IS1 can mediate cointegration of two different plasmids leaving a duplication of IS1 at the cointegrate junctions (Ohtsubo *et al.* 1980)(Iida and Arber 1981). Alternatively IS1 can transpose to leave a simple insertion (Galas and Chandler 1982)(Biel and Berg 1984a). It has been suggested that both products arise from a single intermediate: cointegrates whenever the element replicates and simple insertions if it does not (Biel and Berg 1984a). Cointegrates are stabilized in *rec*A mutants suggesting that host encoded functions provide the primary route for resolution. However transposition may proceed mainly by simple insertion. The fate of donor replicons following simple insertions is not yet clear (Craig and Kleckner 1987). IS1 can also stimulate deletions (Reif and Saedler 1975)(Schwartz *et al.* 1988) and, when present in direct repeat, inversions and amplification (Cornelis and Saedler 1980)(Iida *et al.* 1987)(Braedt 1988). Repeats of IS1 flanking an otherwise nontransposable DNA segment can give rise to composite transposons capable of transposing as a unit; the best studied of these being the chloramphenicol resistance transposon Tn9 (MacHattie and Jackowski 1977). The *arg*F gene of *E. coli* is contained on an 11kb DNA segment flanked by directly repeated IS1 elements that has been termed Tn2901 although it's transposition has yet to be recorded (York and Stodolsky 1981)(Hu and Deonier 1981a)(Clugston 1986).

IS1 is found naturally in the chromosome of a wide range of gram negative bacteria (Nyman *et al.* 1981). The number of

copies per chromosome varies widely: In *Salmonella typhimurium* from 0 to 9 copies can be found while in strains of *Shigella* from 2 to 56 copies have been seen (Ohtsubo *et al.* 1981b)(Nyman *et al.* 1981)(Bustos-Martinez and Gomez-Eichelmann 1987). *E. coli* strains can carry from 0 to 40 copies (Nyman *et al.* 1983) and *E. coli* K-12 laboratory strains generally show 6 to 10 copies (Nyman *et al.* 1981) although D7-8, one of the strains used in this work, may contain only 2 or 3 copies (Adrienne Jessop, unpublished data). A statistical analysis of IS1 distribution resulted in a model implying weak regulation of IS transposition and host fitness that decreases linearly with copy number (Hartl and Sawyer 1988). Transposition frequencies and recombination events mediated by IS1 also vary between strains. In general these events occur at much higher frequencies in *S. typhimurium* strains than in *E. coli* or *Shigella sonneri* suggesting that differences in genetic background may play a role in determining copy number and distribution of IS1 (Watanabe *et al.* 1982)(Bustos-Martinez and Gomez-Eichelmann 1987).

IS1 insertion shows no sequence specificity *per se* however certain sequence environments for instance AT-rich regions are preferred (Galas *et al.* 1980)(Zerbib *et al.* 1985). This may reflect topological constraints imposed by the mechanism of transposition. Each end of IS1 contains a single site for *E. coli* integration host factor (IHF), a histone-like protein that is involved in several site-specific recombination systems, gene expression and DNA replication (Leong *et al.* 1985)(Gamas *et al.* 1986)(Gamas *et al.* 1987a). pBR322 contains a region with four IHF binding sites that constitutes a hotspot for IS1 insertion (Zerbib *et al.* 1985)(Gamas *et al.* 1987b). IHF has been shown to bend the DNA at the ends of IS1 and in the pBR322 insertion hotspot (Prentki *et al.* 1987) suggesting that the presence of IHF-induced bends may result in a hotspot for IS1 insertion. Cointegration and transposition of IS1 results in a 9bp target duplication (Ohtsubo *et al.* 1981a). Base substitutions which result in a single amino acid change in IS1 transposase (see below) can give rise to abnormal target duplications ranging from 7bp to 14bp in size. It is possible that these mutations

alter the specificity of transposase resulting in the introduction of staggered nicks in the target molecule that are separated by abnormal distances (Machida and Machida 1987).

Analysis of the IS1 sequence (Johnsrud 1979) shows eight overlapping open reading frames (ORFs) potentially capable of encoding proteins of greater than 50 amino acids in length. These have been called *ins*A, B, B', and *ins*C to G (Figure 1.2a). Mutational analysis has shown that *ins*A and B (B') are essential for transposition and cointegrate formation and together encode the IS1 transposase (Machida *et al.* 1982)(Machida *et al.* 1984b)(Jakowec *et al.* 1988). The *ins*A ORF has been shown to direct synthesis of a 9.8kd protein which binds specifically to the ends of IS1. Only one or two monomers of InsA protein are bound per end and the binding does not produce any major conformational change in the DNA (Zerbib *et al.* 1987). The IS1 transposase is probably produced as an InsA-B'-InsB fusion protein by a frameshifting event from the *ins*A reading frame to the *ins*B' frame (Sekine and Ohtsubo 1989). The sequence AAAAAC found in the overlap between *ins*A and B' reading frames has been proposed as a frameshift signal in retroviruses (Jacks *et al.* 1987) and an IS1 mutant containing a one adenine insertion into this sequence was able to mediate cointegrate formation at 80 times higher frequency than wild type IS1 (Sekine and Ohtsubo 1989). In addition IS1 shares with retroviruses a region following the proposed frameshift signal that is capable of forming stem-loop structures. If the structure of wild-type transposase resembles Sekine and Ohtsubo's mutant transposase then frameshifting occurs by slippage of the tRNA that is reading the 0 frame codon back one nucleotide to the -1 frame. Alternatively the tRNA reading the 0 frame may undergo a 2bp translocation or mRNAs containing a 1bp addition at a frameshifting site may be produced to give an active transposase (Sekine and Ohtsubo 1989). If frameshifting does not occur then InsA protein is produced and probably competes with transposase for the ends of IS1 resulting in inhibition of transposition.

Jakowec and colleagues examined the behaviour of mutants interrupted in each IS1 open reading frame in a strain of

Salmonella which carries no chromosomal copies of IS1. They found that nonsense mutants in *ins*E, and possibly *ins*G, reduced cointegrate formation in *Salmonella* but not in *E. coli*. They suggest that one or both of these ORFs could encode accessory proteins that are provided *in trans* from chromosomal IS1 elements in *E. coli* (Jakowec *et al.* 1988). Mutations in *ins*A and *ins*B are not efficiently complemented *in trans* (Machida *et al.* 1982) suggesting that their protein products act preferentially on the IS1 sequence from which they are produced. The transposase of IS903 also acts preferentially *in cis* and this cis preference has been correlated with transposase instability (Derbyshire *et al.* 1990). An element that transposes by a simple insertion mechanism not involving replication leaves behind a broken chromosome. Thus a cis acting transposase ensures that a silent copy of the element in a target genome is not trans-activated resulting in a potentially lethal excision (Derbyshire *et al.* 1990).

Mutations in *ins*C do not affect cointegrate formation however studies by Braedt suggest that *ins*C may catalyse an exchange reaction similar to homologous recombination. This IS1 dependent exchange was independent of *rec*A or transposase and occurred between direct repeats of DNA not necessarily of IS1 origin. The exchange reaction was seen in an *E. coli* W strain which contains no chromosomal IS1 elements, but not in *E. coli* K-12 suggesting that a repressor or inhibitor of IS1 may be present in cells that contain copies of IS1 (Braedt 1988). Mutants in *ins*C were unable to perform the exchange reaction while maintaining the ability to promote deletions diagnostic of transposase activity. It is possible that *ins*C encodes a protein that resolves cointegrates (Braedt 1988). Close to the unique PstI site of IS1 is a sequence that shows homology to the ends of IS1 and could be an internal resolution site (Reif and Arber 1980). It would be interesting to examine mutants in which this site was altered without disrupting the *ins*A reading frame to determine if it functions as a hotspot for *ins*C-dependent exchange reactions.

The 35bp terminal repeats (*ins*R and *ins*L) have also been shown to be essential for translocation of IS1 (Ohtsubo *et al.*

1981a)(Machida *et al.* 1982). The terminal repeat sequences are promoters, with the *ins*L promoter (*ins*P_L) being responsible for synthesis of mRNA for the *ins*A or *ins*A-B'-*ins*B gene products. The promoter located at *ins*R (*ins*P_R) directs synthesis of mRNA that could encode an InsC protein. It may also negatively regulate synthesis of InsA/transposase by interfering with the transcript from *ins*P_L or prevent the interaction of transposase with *ins*P_L (Machida *et al.* 1984a). If the frameshifting event required for production of transposase is the result of ribosome stalling induced by mRNA secondary structure then anti-mRNA from *ins*P_R could interfere with or enhance this event.

The two ends of IS1 show a functional difference in that *ins*R is more competent than *ins*L in promoting transposition and cointegration (Ishizaki and Ohtsubo 1984). It has been suggested that transposases interact first with nearest IS ends to form a transposase-end complex that then interacts with the second, more distal, end (Derbyshire *et al.* 1987). Insertions between the 3' end of IS903 transposase and the nearest terminal repeat result in a 100 fold drop in transposition frequency while an increase in overall transposon length has only a small effect (Weinert *et al.* 1984)(Derbyshire *et al.* 1990). If the cis preference shown by IS1 transposase is also related to instability then *ins*R being closest to the 3' end of the *ins*A*ins*B transcription unit would interact first with transposase and may therefore be under stronger selective pressure than *ins*L resulting in the observed functional difference. Derbyshire *et al.*, propose an interesting consequence of cis acting transposase in relation to composite transposons (Derbyshire *et al.* 1990). If we consider the composite transposon Tn9 shown in Figure 1.2b we can see that a cis-acting transposase encoded from IS1L (the left hand IS1 element) would interact first with an inside end of Tn9. This complex could then interact with an *ins*L site either on IS1L resulting in transposition only of IS1 or on IS1H resulting in inverse transposition. By comparison cis-acting transposase encoded by IS1H (the right hand IS1 element) would interact first with an outside end of Tn9. The complex formed could then interact with *ins*L of IS1H (IS1 transposition) or the far outside end of the composite transposon resulting in transposition of

Tn9. Thus if IS1 encodes a cis-acting transposase the integrity of Tn9 is more likely to be maintained if transposase is encoded by IS1H. Interestingly although the IS1 elements of Tn9 have identical nucleotide sequences IS1L is 20 times less efficient in promoting cointegrate formation than IS1H (Machida *et al.* 1982). This has been shown to be the result of transcriptional read-through from the adjacent chloramphenical resistance gene (Machida *et al.* 1983)(Ahmed 1984). Transcriptional inhibition of the IS1 elements in Tn9 has also been observed from external promoters regardless of the orientation of IS1 with respect to the incoming transcript (Chandler and Galas 1983a)(Biel *et al.* 1984b). It would therefore be interesting to observe the effect on Tn9 transposition of reversing the orientation of the CAT gene with respect to the IS1 elements.

ArgF and Tn2901

In *E.coli* the genes involved in arginine biosynthesis form a scattered operon under negative control of the *arg*R gene product. In the presence of arginine ArgR represses transcription of *arg* genes by binding at a consensus arginine operator sequence (arg box) and preventing productive RNA polymerase binding (Cunin *et al.* 1983).

The sixth step in arginine biosynthesis, the production of citrulline and phosphate from ornithine and carbamoyl phosphate, is catalysed by the enzyme ornithine transcarbamoylase (OTCase). In *E.coli* K-12 the equilibrium of OTCase does not favour the reverse reaction (the equilibrium constant for the carbamoylation of ornithine at pH8 is 2×10^5), but a low level phosphorolysis of citrulline does occur *in vivo* (Legrain and Stalon 1976)(Legrain *et al.* 1976a). OTCase is encoded by two genes in *E.coli* K-12; *arg*F and *arg*I, situated at 7.5 and 96.5 minutes on the chromosome map respectively (Legrain *et al.* 1976a). Sequence analysis has revealed a 78.1% overall homology at the nucleotide level and 86% identity at the amino acid level (van Vliet *et al.* 1984). The two gene products

associate randomly to form the trimeric OTCase resulting in four isoenzymes designated FFF, FFI, FII and III. The homogeneous isoenzymes are distinguishable from each other only by their thermolability and elution patterns from diethylaminoethyl-Sephadex columns (Legrain *et al.* 1972)(Legrain *et al.* 1976a). No *arg*F gene has been detected in *E.coli* B or *E.coli* W (Legrain *et al.* 1972), nor has an equivalent been found in related enterobacter, including *Salmonella typhimurium* . Legrain *et al.* have suggested that *arg*F may have been inherited from some related species via a transposition event, a theory strengthened by the finding that *arg*F is flanked by two IS1 elements in *E.coli* K-12 (Hu and Deonier 1981a)(York and Stodolsky 1981).

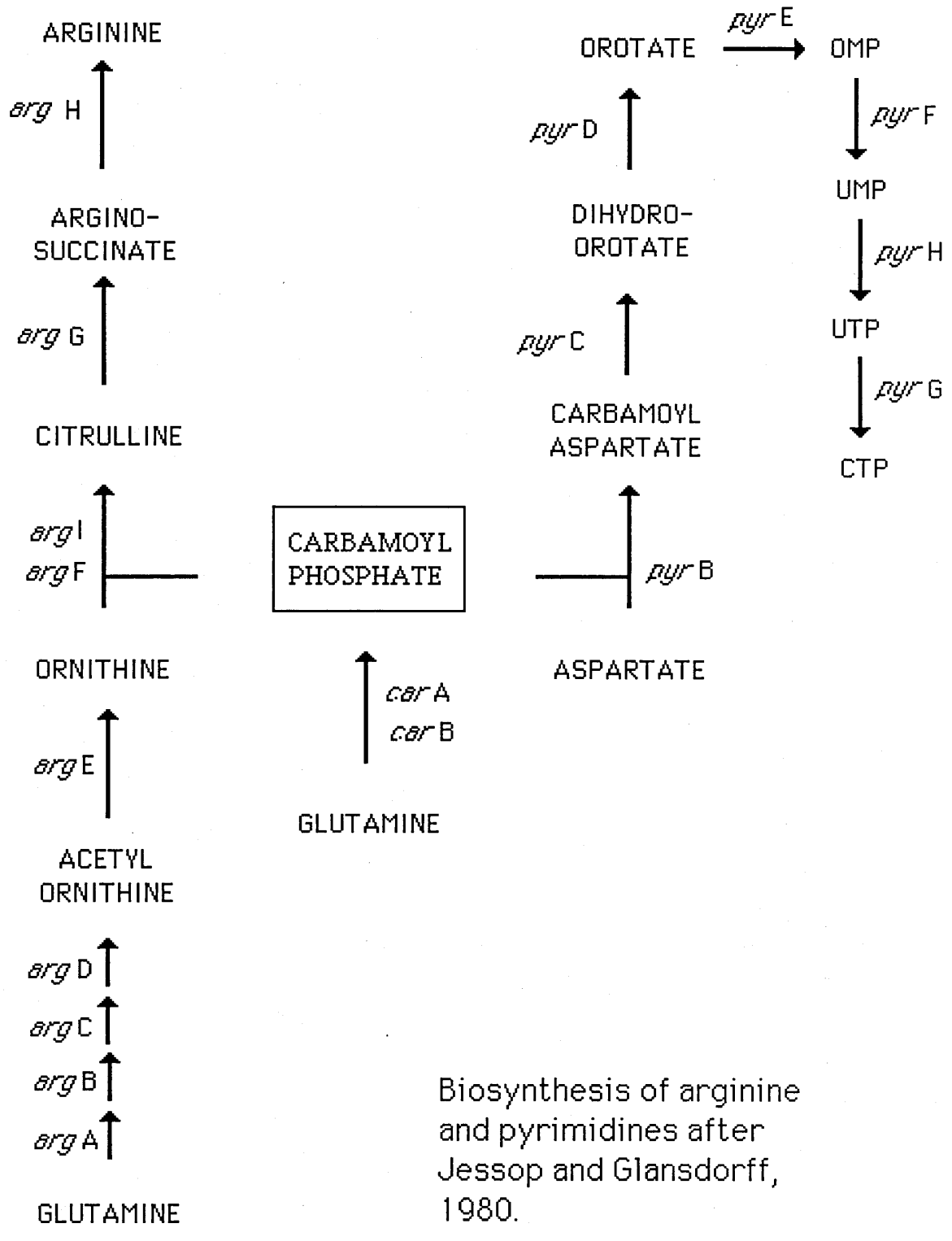
Aspartate transcarbamoylase (ATCase), encoded by *pyr*B, catalyses the carbamoylation of aspartate (Figure 1.3). The *pyr*B gene lies close to *arg*I in *E.coli* and the overall amino acid homology between the two genes is around 40%. The equatorial domain of ATCase is responsible for substrate specificity and Houghton *et al.* have produced an *arg*I/*pyr*B fusion protein that functions as an ATCase when only the equatorial domain is derived from *pyr*B (Houghton *et al.* 1989). Thus the two proteins appear to have a common origin and may have arisen from an ambiguous carbamoyltransferase by duplication (Glansdorff 1987).

A final interesting relationship among OTCases is that of the OTCases of *Pseudomonas aeruginosa*. *P. aeruginosa* encodes two OTCases: *arg*F(P) is an anabolic OTCase catalysing the same reaction as the *E.coli* OTCases while *arc*B encodes a catabolic OTCase that catalyses the reverse reaction (no catabolic OTCase exists in *E.coli*). Both *P. aeruginosa* enzymes function unidirectionally although an *arc*B mutant that functions as an anabolic OTCase has been isolated (Haas *et al.* 1979). The *arg*F(P) gene has 52% nucleotide homology with *arc*B, 47% homology with the *E.coli arg*F gene and 44% homology with *arg*I suggesting that all four genes have evolved from a common ancestor (Itoh *et al.* 1988). Interestingly the catabolic enzyme *arc*B shows greater homology (63% nucleotide, 57% amino acid) with the *E.coli* anabolic *arg*F gene than with *arg*F(P) from *P. aeruginosa* (Itoh *et al.* 1988). In addition antisera raised against the

catabolic OTCase of *P. putida* cross-reacted strongly with the *arg*F products of *E.coli* K-12, *Klebsiella aerogenes, Proteus Vulgaris,* and *S. typhimurium,* but only weakly with the *E.coli* K-12 *arg*I gene product and OTCases from *E.coli* B and W. The antisera failed to react with anabolic OTCases from seven *Pseudomonas* species or with those from a number of other bacterial genera (Falmagne *et al.* 1985). Finally the codon usage of the *E. coli arg*F gene resembles that used in *Pseudomonas* rather than *E. coli* (Nicholas Glansdorff, personal communication). This strongly suggests a link in the ancestry of *E. coli* and *Pseudomonas* OTCase genes.

The transposon-like nature of the DNA containing the *arg*F gene in *E.coli* K-12 was first recognized by York and Stodolsky. Infecting *E.coli* with temperate bacteriophage P1 and selecting for specialized transducing derivatives (P1std) they found P1*arg*F formed with 10,000 times greater frequency than P1*lac* or P1*pro*AB. Analysis of P1*arg*F phages showed an 11kb insert into the IS1 element on the P1 chromosome resulting in *arg*F flanked by directly repeated IS1 sequences. This structure is typical of composite transposons and was given the name Tn2901 (York and Stodolsky 1981). Confirmation that Tn2901 exists in the *E.coli* chromosome was provided by hybridization studies and heteroduplex analysis (Hu and Deonier 1981a). P1*arg*F formation is greatly reduced in *rec*A strains and all Tn2901 insertions observed in P1 are at the IS1 locus. Thus the transposition of Tn2901 has yet to be recorded (York and Stodolsky 1981). A number of mechanisms likely to be involved in control of IS1 promoted transposition have been discussed above. In addition Chandler and colleagues, have suggested that the frequency of transposition of IS1 flanked transposons may be a function of their size (Chandler *et al.* 1982). At 11kb Tn2901 is a relatively large transposon therefore it would not be surprising if it's transposition is an extremely rare event.

Figure 1.3



Biosynthesis of arginine and pyrimidines after Jessop and Glansdorff, 1980.

Jef8 and the Citrulline Utilizing Phenotype

E.coli K-12 mutants unable to synthesis carbamoyl phosphate (*car*$^-$) have an absolute requirement for arginine and uracil; no growth is seen if only one of these supplements is provided. When *car*$^-$ strains are grown on citrulline the cells are starved of pyrimidines and exhibit very slow growth dependant on the phosphorlysis of citrulline by OTCase. This property was used by Legrain *et al.*, as a screen for isolating *arg*F constitutive mutants. These mutants will display high OTCase activity and have the ability to utilize citrulline as a source of carbamoyl phosphate. Three types of mutant are expected to display this citrulline utilizing (cut) phenotype; constitutive *arg*I and *arg*F operator mutants unable to bind the arginine repressor ArgR and leaky *arg*G mutants which accumulate citrulline and show derepression of OTCase (Legrain *et al.* 1976b). Note that *arg*R mutants are not efficient citrulline utilizers as all the enzymes of the *arg* regulon are derepressed resulting in removal of available citrulline (Figure 1.3). The strains chosen by Legrain *et al.* for this experiment were Hfr Jef8, Hfr3000XIII*car*B8 and P678-14(*car*B8) (Legrain *et al.* 1976b).

Jef8, a derivative of the well known Hfr P4X, contains a deletion in *car*B8 and thus lacks the heavier subunit of carbamoylphosphate synthase. It is both *arg*F$^+$ and *arg*I$^+$ (a geneology of Jef8 is shown at the start of Chapter 3). Growth of Jef8 on citrulline resulted in the isolation of a large number of citrulline utilizing colonies most of which were unstable. A wide range of OTCase specific activities was observed among the unstable mutants with the highest being three or four times that observed in an *arg*R (genetically derepressed) mutant and ten times that of a stable *arg*F constitutive mutant. The elution pattern and thermolability of the OTCase encoded by these mutants is that of the FFF isoenzyme, while the I isoenzyme remains repressible (Legrain *et al.* 1976b).

P678-14 carries the *car*B8 mutation but produces about twenty-five fold less cut mutants than Jef8 when plated on citrulline. Both *arg*I and *arg*F constitutive mutants were been

isolated from this strain, but no unstable mutants(Legrain *et al.* 1976b).

Hfr3000XIII*car*B8 is deleted for the *pro-arg*F-*lac* chromosomal region. The *car*B8 and *thi*$^+$ loci at least are inherited from Jef8. When plated on citrulline it produces about one third of the cut mutants that Jef8 does. None of these mutants are unstable and all fall into one of two classes as assessed by OTCase specific activity; *arg*G bradytrophs being about twice as common as *arg*I constitutive mutants (Legrain *et al.* 1976b).

Why are citrulline utilizing mutants produced in far larger numbers in the two Hfrs than in the F$^-$ strain? The unstable mutants produced from Jef8 are the most obviously interesting and amenable to investigation. Thus the initial interest in this system, of which this thesis is a continuation, has centred on them. It is, however, worth bearing in mind that there may be other factors, not addressed here, that can enhance a strain's ability to utilize citrulline as a source of carbamoyl phosphate.

The unstable mutation is strongly linked to the site of the *arg* F gene as judged by conjugation and co-transduction experiments (Legrain *et al.* 1976b)(Jessop and Glansdorff 1980) and hybridization analyses have shown it to be the result of a 45-fold amplification of Tn2901 (Jessop and Clugston 1985). Jessop and Glansdorff showed that production of citrulline utilizing colonies at high frequencies in Jef8 and the amplification of Tn2901 is dependent on the presence of an integrated F factor adjacent to Tn2901. Transduction experiments showed that the *arg*F genes of Jef8 and P678-14 are not functionally different. Crosses between Jef8 and F$^-$ P678-14 pinpointed the F factor in Hfr Jef8 as the primary cause of the increased frequency of cut mutants in this strain (Jessop and Glansdorff 1980).
Introduction of the F plasmid to P678-14 and to D7-8F$^-$ (isolated from a P678-14 x Jef8 cross) resulted in a very small increase in numbers of cut mutants observed. Some Hfrs isolated from these strains gave high frequencies of cut mutants at least 50% of which were unstable. These Hfrs, like Jef8, transfer *arg*F and *pro*A proximally in a clockwise direction. Two Hfrs that transfer *pro*C proximally in an anticlockwise direction gave cut mutants only at the frequency of F$^+$ strains (Jessop and Glansdorff 1980).

In Hfr Jef8 the F factor is integrated at an IS3 element about 20kb downstream of Tn2901 (Figure 1.4b). Studies are currently in progress to determine if the site of F integration affects the ability to produce citrulline utilizers. Preliminary southern analyses, using IS3 probes, of Hfrs, isolated as citrulline utilizers from D7-8F$^+$ strains, have identified only one type of strain (Adrienne Jessop, personal communication). This suggests that the regional location is important, but, recalling that F requires a region of homology (usually IS2, IS3 or gamma delta) to form an Hfr, does not eliminate the possibility that alternate integration sites in that region could be equally effective (Adrienne Jessop, personal communication).

When strains carrying an amplification of Tn2901 (unstable cut mutants) are grown in the absence of selection, that is in arginine and uracil (for about 20 generations) non cut segregants are isolated in proportions ranging from 10%-70%. However these segregants do not appear to be identical to the original non cut strain as they yield cut mutants at extremely high frequencies (Jessop and Glansdorff 1980). Co-transduction frequencies of *arg*F with proximal markers from segregants resemble those seen from unstable strains rather than than from Jef8 or F$^-$ strains suggesting that these segregants retain some rearrangement that allows rapid amplification of Tn2901 (Jessop and Glansdorff 1980). Clugston and Jessop have suggested that the amplification is a two step process with the F factor required only for the initial event. They constructed F$^-$ strains that carry several copies of Tn2901 and have shown that these strains can yield many citrulline utilizing colonies, most containing amplified Tn2901 DNA (Clugston and Jessop 1990). An Hfr strain, AJ318, that produces few citrulline utilizing colonies from a single copy of Tn2901, but can amplify several copies of Tn2901 has been isolated. It seems likely that this Hfr contains a mutation that interferes with the initial event in amplification. The mutation does not map in the F factor nor adjacent to Tn2901 (Clugston and Jessop 1990).

Southern analyses of the amplification in unstable cut mutants has shown: About 45 copies of Tn2901 are present in tandem array. No extrachromosomal Tn2901 DNA has yet been isolated

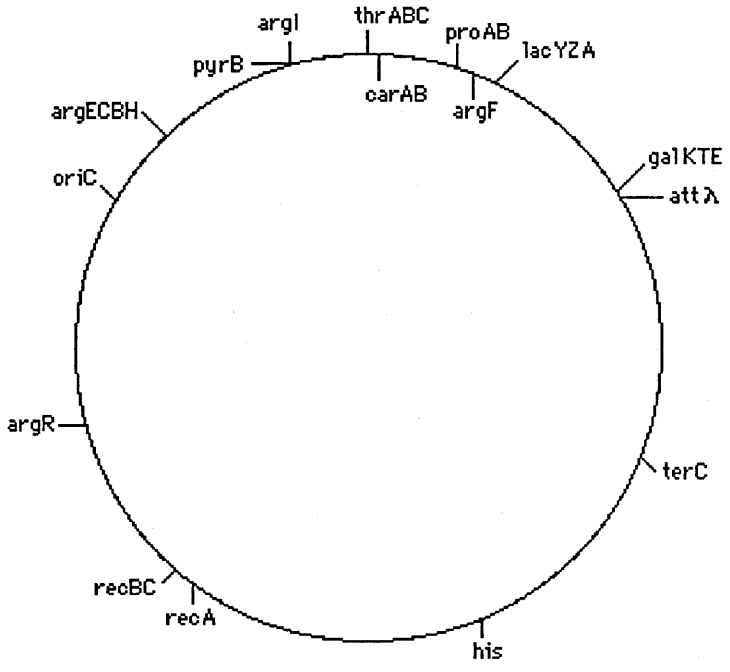
Figure 1.4

a) Map of the *Escherichia coli* chromosome showing the relative positions of the *arg*F gene and a number of other loci discussed in this thesis

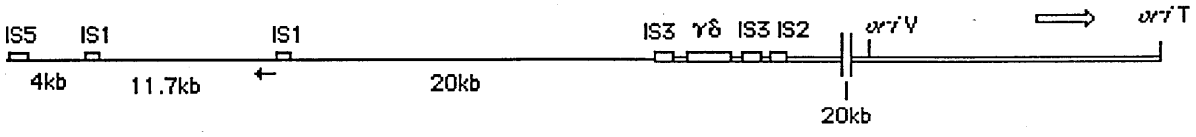
b) Map showing the position of the F factor in Jef8 relative to Tn2901, after Clugston, 1986. Insertion sequences are represented by small boxes. Approximate distances between insertion sequences IS5-IS1, IS1-IS1 (Tn2901) and IS1-IS3 are given. The small arrow denotes the position and orientation of *arg*F. F factor sequences are shown by a double horizontal line. The position of *ori*V and *ori*T are given. The large arrow indicates the direction of conjugal transfer. Vertical lines mark a compression of the diagram

Figure 1.4

a)


argI
thrABC
proAB
lacYZA
pyrB
carAB
argF
argECBH
galKTE
oriC
attλ
argR
terC
recBC
recA
his

b)


IS5
IS1
IS1
IS3
γδ
IS3
IS2
oriV
oriT
4kb
11.7kb
20kb
20kb

from the unstable cut mutants. A single IS1 element is present at the novel joint between each amplified unit (Jessop and Clugston 1985)(Clugston 1986)(Clugston and Jessop 1990) suggesting that the initial event catalysed by the F factor is a recombination between the IS1 elements of Tn2901 (Clugston and Jessop 1990).

Clugston and Jessop have also analysed a number of Hfrs derived from a cross between Jef8 and P678-14 for their ability to produce cut mutants. Interestingly some of these Hfrs have been found to produce ten-fold fewer colonies containing amplified Tn2901 than Jef8 while the total number of cut mutants remains the same. The authors conclude that amplification is not the only mutation stimulated by the presence of the F factor. These other mutants have been termed class II citrulline utilizers (Clugston and Jessop 1990). Upon introduction of several copies of Tn2901 to AJ318, the mutant defective in the initial event leading to amplification, described above, both amplified and class II cuts are produced suggesting that class II cut colonies are derived from or dependent on the event(s) that lead to the amplification of Tn2901 (Clugston and Jessop 1990).

Studies of the affects of recombination mutants on the amplification of Tn2901 are made considerably more difficult by the lack of an independent screen for the F-dependent first stage of amplification (Adrienne Jessop, personal communication). The introduction of a *rec*A allele to amplified strains results in considerable stabilization with relatively few segregants being produced from *rec*A strains (Legrain *et al.* 1976b). F^- strains that are *rec*A or *rec*BC are unable to further amplify several copies of Tn2901 (Adrienne Jessop, unpublished data). Thus the second stage of the amplification appears to require these host encoded recombination functions (Clugston and Jessop 1990). The double mutation *rec*B C*sbc*B or mutations in *rec*F or *pol*A do not appear to affect amplification of Tn2901 from several copies in F^- strains suggesting that the RecF pathway can substitute for the RecBCD pathway in the second stage of amplification (Adrienne Jessop, personal communication). A recent interesting result is that the *pol*A mutation which does not affect

amplification from many copies reduces the ability of Hfr strains to produce the amplification from a single copy of Tn2901 (Adrienne Jessop, unpublished data).

F prime plasmids have been used by Clugston and Jessop, and in this thesis to investigate the role of conjugation in formation of cut mutants (Chapter 4). F primes are easily isolated by conjugation of an Hfr with an F$^-$ *rec*A strain (Miller 1972). When made *rec*$^+$ they show a substantial increase in numbers of cut colonies compared with F$^-$ or F$^+$ strains (around 10-fold) but not the levels seen in Hfr strains. F primes generally share extensive homology with the chromosome (see above). Thus the levels of cut stimulation seen probably reflects that proportion of cells, at time of selection, which contain an integrated F prime (Adrienne Jessop, personal communication). On average about 50% of citrulline utilizing colonies derived from F primes contain amplified Tn2901 DNA suggesting that F primes also stimulate the production of class II citrulline utilizers (Adrienne Jessop, unpublished data).

My main aim in this thesis has been to further the work already undertaken in characterizing the unstable mutants of Jef8. In particular I have looked at the role that the F factor plays in the initial event in the amplification process. I have tried to establish systems that will allow this event to be examined both from the perspective of F factor function and at the level of events occuring at Tn2901. In doing so, I have had to take account of some other factors that might stimulate utilization of citrulline in *car*$^-$ cells and these are discussed at the start of the thesis, in Chapter 3.

CHAPTER TWO

MATERIALS AND METHODS

Table 2.1

Strain	Genotype/Description	Source/Reference
Jef8	Hfr relA1 metB1 thrB carB spoT1 PO3 λ+	A. Jessop
Hfr13	Hfr derivative of Jef8 deleted for Tn2901	A. Jessop
Cut1	Hfr derivative of Jef8 with amplification of Tn2901	A. Jessop
Cut8	Hfr derivative of Jef8 with amplification of Tn2901	A. Jessop
P4Xλ-	Hfr Jef8 cured of λ	N. Glansdorff
P4Xλ+	Hfr P4Xλ- lysogenized with λ	Chapter 4
NGX2	F- argI pyrB argF proA leu lac gal xyl strep^r	A. Jessop
DT6	F- argI argF pro thr xyl lac gal Strep^r nal^r	A. Jessop
DS902	F- recA thr leu his pro strep^r λ-	D. Sherratt
CB51	F- dam3 ara thi Δlac-pro	C. Boyd
NM621	F- hsdR mcrA mcrB supE44 recD1009	Whittaker *et al.* 1988
AB1157	F- pro metB carB thi xyl his gal lac strep^r λ+	A. Jessop
AB1157::pCC7	F- AB1157 λ+ with pCC7 integrated at Tn2901	Chapter 4
AB1157::pFJH96	F- AB1157 λ+ with pFJH96 integrated adjacent to Tn2901	Chapter 4
D7-8	F- metB1 thr carB xyl7 lacY1 strep^r λ-	A. Jessop
D7-8λ+	F- D7-8 lysogenized with λ	Chapter 4
D7-8λ+::pFJH96	F- D7-8λ+ with pFJH96 integrated adjacent to Tn2901	Chapter 4
D7-8recA	F- D7-8 recA from KL14 λ-	A. Jessop
D7-8recAλ+	F- D7-8recA lysogenized with λ	Chapter 4
D7-8prorecA	F- D7-8pro recA from KL14 λ-	A Jessop

D7-8prorecAλ$^+$	F$^-$ D7-8prorecA lysogenized with λ	Chapter 4
D7-8F$^+$	D7-8 F$^+$ from ED79	A. Jessop
D7-8F$^+$λ$^+$	D7-8F$^+$ lysogenized with λ	Chapter 4
D7-8prorecA/ F'Δ trapro$^+$	F' plasmid in D7-8prorecA	Chapter 4
D7-8prorecA/ F'Δ traΔ Tn2901pro$^+$	F' plasmid in D7-8prorecA	Chapter 4
D7-8cI	F$^-$ D7-8 transformed with pcI857	Chapter 3/Chapter 4
FJH10	D7-8cI with pFJH121 integrated adjacent to Tn2901	Chapter 4
FJH11	D7-8cI with pFJH135 integrated adjacent to Tn2901	Chapter 4
FJH12	D7-8cI with pFJH136 integrated adjacent to Tn2901	Chapter 4
FJH13	D7-8cI with pFJH96 integrated adjacent to Tn2901	Chapter 4

Escherichia coli K-12 strains used in this work.

Table 2.2

Name	Information	Reference
19A	derivatve of pCB101	Chris Boyd
pCC7	2.3kb BamHI fragment from pCC1 in pCB101	Clugston, 1986
pCC18	2.7kb HindIII fragment from pCC21 in 19A	Clugston, 1986
pFR10	3.7kb PstI fragment from pFJH87 in 19A	Flora Rodgers
pFJH20	exoIII deletion of pCC18	Chapter 5
pFJH50	2.3kb PstI fragment from pCC18 in 19A	Chapter 4
pFJH53	F fragments f6+f15 in pFJH50	Chapter 4
pFJH54	F fragment f6 in pFJH50	Chapter 4
pFJH55	F fragments f10+f16 in pFJH50	Chapter 4
pFJH56	pFJH50 but opposite orientation	Chapter 4
pFJH57	F fragment in pFJH50	Chapter 4
pFJH58	F fragment in pFJH50	Chapter 4
pFJH59	F fragment in pFJH50	Chapter 4
pFJH95	1.5 min exoIII deletion of pFJH56	Chapter 4
pFJH96	1 min exoIII deletion of pFJH56	Chapter 4
pFJH110	8.2kb HindIII fragment from pFJH92 in 19A (oriS)	Chapter 4
pFJH121	4.3kb XbaI fragment from pFJH91 in 19A (oriV-rep$^-$)	Chapter 4
pFJH122	8.2kb HindIII fragment from pFJH92 in 19A (oriS)	Chapter 4
pFJH123	pFJH122 but opposite orientation in.19A (oriS)	Chapter 4
pFJH135	3.5kb XbaI fragment from pFJH134 in 19A (oriV-rep$^+$)	Chapter 4
pFJH136	pFJH135 but opposite orientation in 19A (oriV-rep$^+$)	Chapter 4
pFJH137	IS1 ΔHaeII + flanking chromosomal λ regions in 19A	Chapter 5
pFJH138	APH gene + flanking chromosomal λ regions in 19A	Chapter 5

λdv plasmids used in this work. All confer resistance to chloramphenicol.

Table 2.3

Name	Information	Reference
pUC19	Derivative of pBR322	Yanish-Perron *et al.* 1985
pUC71K	APH gene in pUC	Vieira and Messing, 1982
pCC1	11.7kb EcoRI fragment from cut1 in pUC9	Clugston, 1986
pCC15,16	9.4kb BglII fragment from cut1 in pUC8	Clugston, 1986
pIC20H/R	pUC based vectors with extended polylinkers	Marsh *et al.* 1984
pICmodX	pIC20R modified to remove one XbaI site	Chapter 5
pFJH30	EcoRI deletion of pCC15	Chapter 3
pFJH40	EcoRI deletion of pCC16	Chapter 3
pFJH41	BamHI deletion of pFJH40	Chapter 3
pFJH44	Omega factor in pFJH41	Chapter 3
pFJH60	HaeII deletion of IS1 in pUC19	Chapter 5
pFJH70	IS1 as EcoRI fragment in pIC20R	Chapter 5
pFJH71	ΔPstI in IS1 of pFJH70	Chapter 5
pFJH72	ΔMluI in IS1 of pFJH70	Chapter 5
pFJH83	2.1kb EcoRI fragment from λ5A5 in pUC19	Chapter 5
pFJH87	7.4kb EcoRI fragment from λ5A5 in pUC19	Chapter 5
pFJH88	7.4kb + 5.9kb EcoRI fragments from λ5A5 in pUC19	Chapter 5
pFJH90	15.9kb EcoRI fragment from λ3C7 in pUC19	Chapter 5
pFJH91	oriV-rep$^-$ in pFJH97	Chapter 4
pFJH92	oriS in pFJH97	Chapter 4
pFJH93	1.2kb EcoRI fragment from λ3C7 in pUC19	Chapter 5
pFJH97	1.5kb EcoRI/EcoRV fragment from pFJH96 in pIC20H	Chapter 4
pFJH120	4.5kb SalI fragment from pB4 (argI) in pUC19	Chapter 4

pFJH126	1.3kb HindIII/BamHI fragment from pFJH90 in pICmodX	Chapter 5
pFJH127	1.3kb EcoRI/BamHI fragment from pFJH126 in pFJH97	Chapter 5
pFJH129	APH gene in pFJH127	Chapter 5
pFJH130	HaeII mutant of IS1 in pFJH127	Chapter 5
pFJH131	Opposite orientation to pFJH130	Chapter 5
pFJH132	Opposite orientation to pFJH129	Chapter 5
pFJH134	oriV-rep$^+$ in pIC20H	Chapter 4
pFJH140	APH gene in pIC20H	Chapter 4
pFJH113	1.15kb BglII/KpnI fragment from pFJH87 in pIC20R	Chapter 5

pUC based plasmids used in this work. All confer resistance to ampicillin. Those containing the APH gene also confer resistance to kanamycin and neomycin.

Table 2.4

Name	Description	Phenotype	Reference
pBR322	Cloning vector	Amp^r/Tc^r	Sutcliffe, 1979
pMC23	*arg* F in pBR322	Amp^r	Crabeel *et al.* 1979
pPE14	*rec* A clone	Amp^r	David Sherratt
pHP45Ω	Omega factor in pBR322 derivative pHP45	$Amp^r/Sm^r/Sp^r$	Prentki and Kirsch 1984
pSH6	F DNA (f6) in ColE1	amp^r	Achtman *et al.* 1978
pB4	*arg* I^+ cosmid	amp^r	Knott *et al.* 1989
pcI857	cI gene in pACYC184 based vector	Kan^r	Remaut *et al.* 1983
pFJH133	cI gene deleted from pcI857	Kan^r	Chapter 3
pFJH98	mini F-Kan	Kan^r	Chapter 4

Other plasmids used in this work

Table 2.5

Name	Reference
M13	Vieira and Messing, 1982
λ^+	Mary Burke
λ^{vir}	Mary Burke
λ^{cI}	Mary Burke
λ3C7	Kohara *et al.* 1987
λ5A5	Kohara *et al.* 1987

Bacteriophage used in this work

2.1 Bacterial strains.

The bacterial strains used and constructed in this work are all derivatives of *Escherichia coli* K-12 and are listed in Table 2.1. Genotype and phenotype symbols are those recommended (Bachmann 1987b)(Novick *et al.* 1976).

2.2 Plasmids and bacteriophage

The plasmids used and constructed in this study are listed in Tables 2.2-2.4, using the nomenclature of Novick *et al.* (1976). The 'phage are shown in Table 2.5.

2.3 Other materials

a) Enzymes: Restriction Endonucleases, T4 DNA ligase, E.coli DNA polymerase I, Mungbean exonuclease and S1 nuclease from BRL. Klenow fragment of E.coli DNA polymerase I, calf intestinal phosphatase and E.coli exonuclease III from Boeringer Mannheim.

b) Specific reagents: Sephadex G50, pd(N_6) and dNTPs from Pharmacia, Hybond-N membrane from Amersham, radiochemicals from NEN, universal sequencing primer (5'-TCCCAGTCACGACGT-3') from Boeringer Mannheim, GENECLEAN from Bio 101(Stratech), media from Difco and Oxoid.

c) General chemicals: Chemicals, biochemicals and organic solvents were purchased from generally available sources (BRL, BDH, May and Baker, Sigma).

d) Buffers and Media: Buffers for restriction and modifying enzymes were prepared according to the manufactures recommendations. Common buffer and media recipes were obtained from the Cold Spring Harbour Molecular Cloning manual (Maniatis *et al.* 1982) with the following exceptions;

R-agar: 11g tryptone, 11g yeast extract, 11g NaCl, 15g agar, to 1 litre in distilled water and adjusted to pH7.5 with NaOH.

Davis-Mingioli Salts (4X): 28g K_2HPO_4, 8g KH_2PO_4, 4g $(NH_4)_2SO_4$, 1g Sodium Citrate, 0.4g $MgSO_4.7H_2O$ to 1 litre in distilled water.

10X E buffer: 242g Tris, 82g Sodium acetate, 18.6g $Na_2EDTA.H_2O$ to 5litres in tap water (except where otherwise stated) and adjusted to pH8.2 with glacial acetic acid.

Single Colony Gel loading buffer: 2% Ficoll, 1% SDS, 0.01% bromophenol blue, 0.01% orange G in E buffer.

e) Media Supplements: concentration of supplements added to water agar and antibiotics for both liquid and plate selection were as detailed in Clugston, 1986.

2.4 Nucleic Acid Isolation

a) Plasmids: Large scale isolation was carried out by the alkaline lysis method (Birnboim and Doly 1979) as described in Maniatis *et al.* (1982) or on a small scale by the boiling method (Holmes and Quigley 1981).

b) F plasmid: F plasmid DNA was isolated using a minor modification of the alkaline lysis method (Birnboim and Doly 1979), such that the initial concentration step from LB-medium to glucose-tris-EDTA buffer was only five fold. This appears to reduce shearing of high molecular weight DNA.

c) Lambda: Large scale isolation of Lambda DNA was as described in Maniatis *et al.*(1982) using host strain NM621 (Whittaker 1988).

d)*E.coli* DNA: Small scale isolation of chromosomal DNA from *E.coli* was performed as follows.
1.5ml of liquid culture at stationary phase (except where otherwise stated) was spun at 15,000g for 3-5mins. to ensure a

firm cell pellet. Culture medium was completely removed using a drawn pasteur pipette. The cells were resuspended in 150µl of 25% sucrose in 0.25M Tris-HCl pH8 and chilled on ice. Lysozyme (1mg/ml final concentration) then EDTA (50mM final concentration) were added on ice. The cells were lysed at 4°C by addition of an equal volume of 1% Triton X-100, 50mM Tris-HCl pH8, 60mM EDTA pH8. The lysate was incubated with Ribonuclease A at 50µg/ml for 15-30 mins. at 37°C. Proteinase K was added to a final concentration of 100µg/ml and the incubation continued for 2-12 hours. The DNA was gently extracted, once with phenol, once with phenol/chloroform and once with chloroform. An equal volume of isopropanol was added and thoroughly mixed by rocking. The DNA was immediately pelleted (15,000g, 5mins.) and the isopropanol completely removed using a drawn pasteur pipette. The DNA pellet was washed twice with 70% ethanol, allowed to dry at room temperature and resuspended in 1xTE buffer. On the rare occasion when DNA prepared in this fashion failed to restrict it was necessary to dialyse it against several changes of 1xTE.

2.5 DNA manipulation

a) Restriction endonuclease digestion of DNA and the removal or filling in of resulting 3' or 5' terminal overhangs were as described in Maniatis *et al.* (1982). Production of staggered deletions in plasmid DNA using Exonuclease III and SI nuclease followed exactly the procedure of Henikoff (1984).

b) Isolation of DNA from agarose gels was generally by the electroelution method as follows. Restricted DNA was electrophoresed in low melting point agarose gels and E buffer to avoid contaminants that might affect subsequent manipulations. After staining with ethidium bromide (0.6µg/ml) the DNA was visualized using a longwave U.V. transilluminator (300-360nm) to minimize nicking. An agarose slice containing the band of interest was excised and transferred to a piece of dialysis tubing. Electroelution was at 4°C in E buffer (Maniatis *et al.* 1982). After removal of the DNA solution the gel slice and dialysis bag were

washed with a small volume of 1xTE. Solution and wash were pooled, made 0.3M NaOAc and the DNA precipitated with Analar absolute ethanol pre-chilled to -20°C. The precipitation was continued on ice for 15mins then the DNA was pelleted (30-40mins, 15,000g, 4°C), washed twice with 70% ethanol, dried and resuspended in distilled water. Where large quantities of small DNA fragments (<1Kb) were required, DNA was extracted from agarose gels using GENECLEAN following the manufacturers instructions.

c) Ligation of DNA fragments to yield circular products was as follows. Whenever symmetrically linearized vector was used it was first treated with calf intestinal phosphatase (CIP) to remove 5' phosphate groups and prevent recircularization. CIP was added directly to restriction digests at 1u per 50pm of 5' ends and incubated at 37°C for 20mins. The CIP was inactivated by incubation at 70°C for 10mins. When vector DNA was asymmetrically linearized for a forced cloning CIP treatment was unnecessary, however the two restriction digests were performed sequentially to optimize cutting efficiency. As it appears that BamH1 endonuclease can remain bound to DNA after restriction (Cathy Ives personal communication) it's use in this procedure was followed by phenol extraction before further manipulation. Linear DNA fragments were generally purified by isolation from agarose gel prior to ligation. Ligations were in 20µl at 16°C in 1x ligation buffer, 1mM ATP and 1u T4 ligase for at least 8 hours. Vector concentration was generally 2µg/ml or less. A 3:1 insert:vector molar ratio of fragments was used except for ligations involving large inserts, more than one insert or blunt ends where a 10:1 molar ratio was used. Aliquots of the ligation reaction were diluted 1:4 in distilled water prior to being used to transform competent cells.

d) Plasmid DNA sequencing was by the method of Chen and Seeburg (1985) as described in "Guidelines for quick and simple plasmid sequencing" supplied by Boehringer Mannheim with the exception of "Denaturating buffer"; 0.2M NaOH (not 0.2mM NaOH), 0.2mM EDTA pH8 (Chen and Seeburg 1985).

2.6 Genetic Manipulation of E.coli

a) Plasmid transformation of *E.coli* was carried out using the protocols developed by Hanahan (Hanahan 1985). For routine preparation of plasmids the *rec*A strain DS902 was found to reliably yield good transformation efficiencies (10^5-10^6 cells/μg DNA) whilst minimizing recombination. The presence of plasmid in transformed colonies was confirmed by single colony gel analysis: A single colony was patched onto selective medium and grown overnight. Cells from about $1cm^2$ area were resuspended in 200μl single colony gel buffer and allowed to lyse at room temperature. Cell debris was pelleted (15,000g, 4°C, 30mins.) and 50μl of plasmid containing supernatant was loaded directly onto a gel.

b) Transduction using P1 phage, isolation of F-prime strains, production of F^- phenocopies and bacterial matings using Hfr or F-prime donors were as described in Miller(1972) except that culture matings were performed on a 33rpm rotor at 37°C to optimize aeration without disrupting mating pairs. The recombination status of strains was ascertained using a UV sensitivity test: colonies to be tested were patched onto fresh R-agar plates and immediately exposed to UV light (254nm 42cm from source, approximately 120uW cm^{-2}) for 2 minutes, then replicated onto fresh R-agar plates and incubated overnight. $RecA^+$ strains are seen to grow while $recA^-$ strains do not (Miller 1972).

2.7 Electrophoresis, photography and blotting

a) Gel electrophoresis of sequencing reactions was carried out in 6% polyacrylamide TBE wedge gels (Biggin *et al.* 1983) as described in "Guidelines for quick and simple plasmid sequencing" supplied by Boeringer Mannheim.

b) Electrophoresis of DNA through agarose gels was in 1x E buffer. Vertical gels were used for single colony gel analysis.

Horizontal gels were used for restriction mapping or Southern analysis. Gel size and agarose percentage varied according to usage (Maniatis *et al.* 1982). Fragment sizes were calculated according to the method of Helling *et al.* (Helling *et al.* 1974) using restriction digested cI857 s7 DNA as a standard (Sanger *et al.* 1982). Gels were stained in 0.6μg/ml ethidium bromide.

c) Ethidium bromide stained gels were viewed on a 254nm U.V. transilluminator and photographed using Polaroid type 67 land film or using a Pentax 35mm SLR and Ilford HP5 film. Both cameras were fitted with a Kodak Wratten filter No.23A.

d) For hybridization gels were blotted to Hybond-N membranes using a modification to the procedure of Southern (1975) as described in "Blotting and hybridization protocols for Hybond membranes" supplied by Amersham (Southern 1975). Hybridization and washing conditions were also as recommended by Amersham. Blots were exposed to Kodak Xomat-S film at -70°C with intensifying screens.

e) DNA was radiolabelled with ^{32}P either by nick translation of plasmid DNA (Rigby *et al.* 1977) as described in Maniatis *et al.* (1982) or by random priming of gel purified restriction fragments (Feinberg and Vogelstein 1983). All probes were purified by Sephadex G50 chromatography in columns prepared in disposable 1ml syringes.

2.8 Testing car⁻ strains of E.coli for ability to produce cut mutants

Strains to be tested were grown from an overnight culture to log phase (except where otherwise stated) in 2x D+M salts with arginine, uracil , other required supplements and where necessary selective antibiotics. The cultures were titrated on arginine and uracil to obtain viable cell counts, on arginine, uracil and valine to estimate the background mutation rate, on

citrulline to test for ability to produce cut mutants and without arginine, uracil or citrulline to check for car$^+$ revertants. Viable cell counts were scored after 1 or 2 days and discarded. On those occasions when viable cell scoring was continued up to 20 days no change in colony numbers was observed. The other titrations were scored after 5 days and at varying times thereafter up to a maximum of 28 days. Generally no attempt was made to maintain antibiotic selection on plates however resulting cut colonies were subsequently tested for retention of antibiotic markers.

2.9 Testing cut mutants for amplification of Tn2901 and instability.

a) Citrulline utilizing colonies to be tested for amplification of Tn2901 were patched directly onto 5 cm^2 pieces of Hybond-N membrane on agar plates containing citrulline and other required supplements. Three control strains; Jef8cut1(unstable cut), Jef8cut12(stable cut) and D7-8cut (F$^-$ stable cut) were also included. Colonies were grown to 3-5mm in diameter (generally 2 days growth) then processed as described in "Blotting and hybridization protocols for Hybond membranes" supplied by Amersham. Membranes were hybridized with radiolabelled pCC1 (Clugston 1986). After autoradiography colonies were classified as either amplified : colonies showing high levels of hybridization to pCC1 as compared with Jef8cut1 or as unamplified : colonies showing little or no hybidization to pCC1 as seen with D7-8cut. In some cases colonies classified as 'amplified' were subsequently confirmed as containing an amplification of Tn2901 by preparation of chromosomal DNA and Southern blotting.

b) Colonies were tested for instability of the citrulline utilizing phenotype as follows. Cells were taken from a cut colony and resuspended in 2ml of 2x D+M salts with arginine, uracil and other required supplements and grown to stationary phase. A 10^{-4} dilution of this culture was used to inoculate 10ml of D+M salts with arginine and uracil. The new culture was grown to

stationary phase and the dilution procedure repeated. The final culture was grown to log phase and titrated on agar with arginine and uracil to give single colonies. When these attained about 2mm size they were replicated to agar with citrulline and agar with arginine and uracil and scored for non-citrulline utilizing segregants after 24 hours.

2.10 Word processing

This thesis was written on a Commordore Amiga 2000 Computer running the Readysoft AMAX II Macintosh emulator. Microsoft Word version 4.0 was used for text and tables. Superpaint version 1.1 from Silicon Beach Software was used for figures. The bibliography was compiled using Endnote Plus version 1.0 from Niles and Associates.

CHAPTER THREE

CONCERNING STRAINS AND MUTATIONS

3.1 Introduction

The experiments presented in this first chapter are an oddball collection. While some were designed to answer specific questions regarding the nature of the mutations apparent in the citrulline-utilizing (cut) phenotype, some started life as controls for experiments presented later in the thesis. I have included them here because the results obtained have, to some degree, shaped my thinking on the system in general.

The Hfr strain Jef8, a *car* B8 derivative of Hfr P4X, produces many times more cut mutants than F^- strains. Various F^- strains have been used in this system but, to reduce the complications of strain differences, I decided to concentrate on one. AB1157 initially seems an attractive choice as it is a well characterized strain. Unfortunately on rare occasions AB1157 can produce surprisingly large numbers of citrulline utilizing colonies (Adrienne Jessop unpublished data), an interesting effect which is as yet uncharacterised. While not approaching the number of citrulline utilizers seen with Jef8 this occasional effect could well confuse the interpretation of results. Thus a less well characterized strain D7-8 was chosen. This strain has several advantages: Firstly it has a very low yield of citrulline utilizers. Secondly it has only ever been seen to produce large numbers of cuts when the F factor has been introduced *in cis* to Tn2901 either by conjugation or by production of Hfrs from D7-8F^+ cells (Jessop and Glansdorff 1980). Finally a number of useful mutants have been isolated from it (Adrienne Jessop unpublished data). Limited strain pedigrees are shown in Figure 3.1 and the derivation of D7-8 strains in Figure 3.2.

In Chapter 4 the forced integration of λdv plasmids into F^- strain D7-8 using temperature sensitive cI repressor is described. As the cut phenotype has previously been examined only at 37°C, I tested cut production from D7-8 at 30°C. These results are presented in this chapter as they allowed the isolation of amplified DNA from an F^- strain for the first time. This is interesting as we had previously described the action of the F factor as "activating" or "allowing" the amplification of

Figure 3·1 Strain Pedigrees

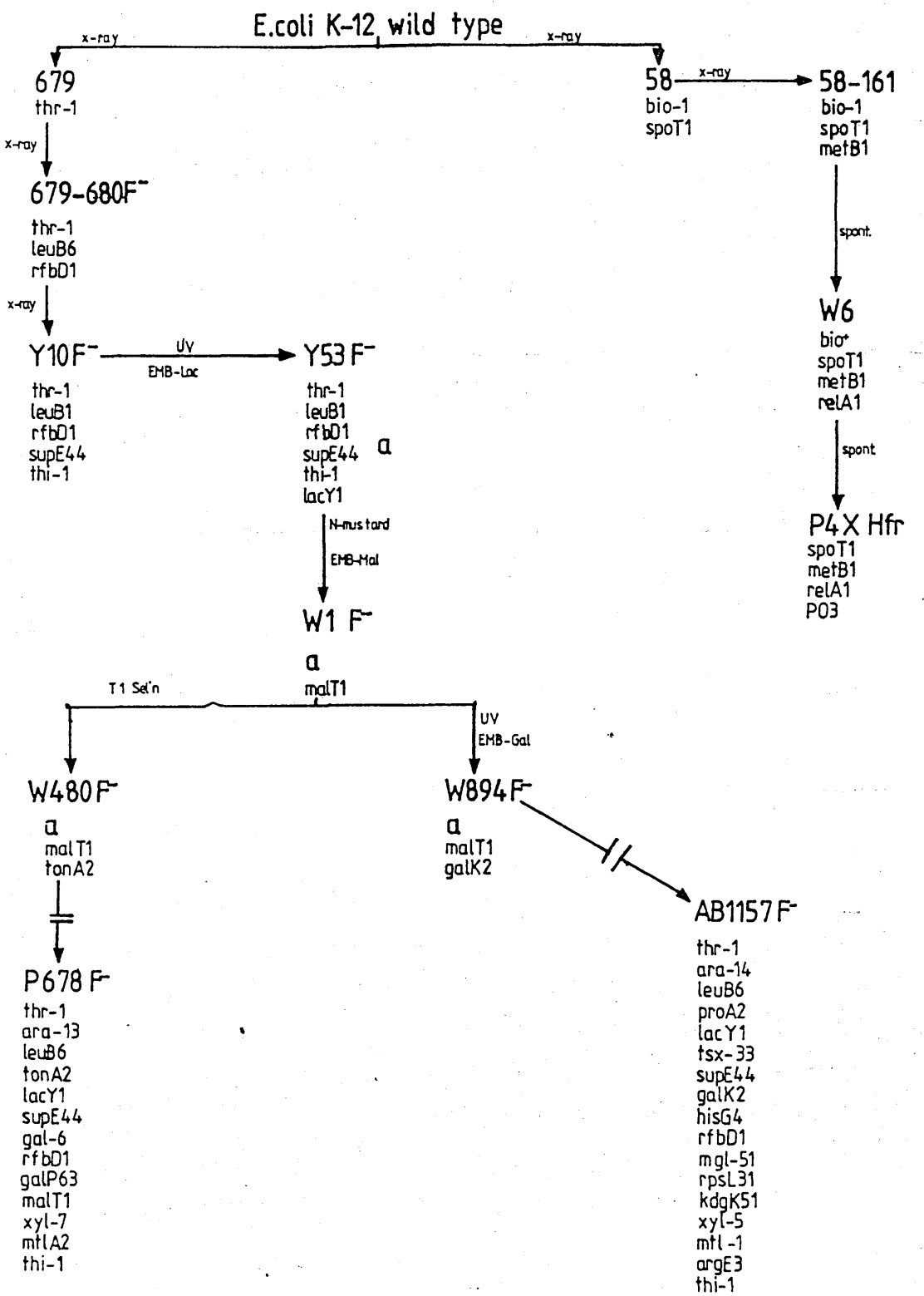
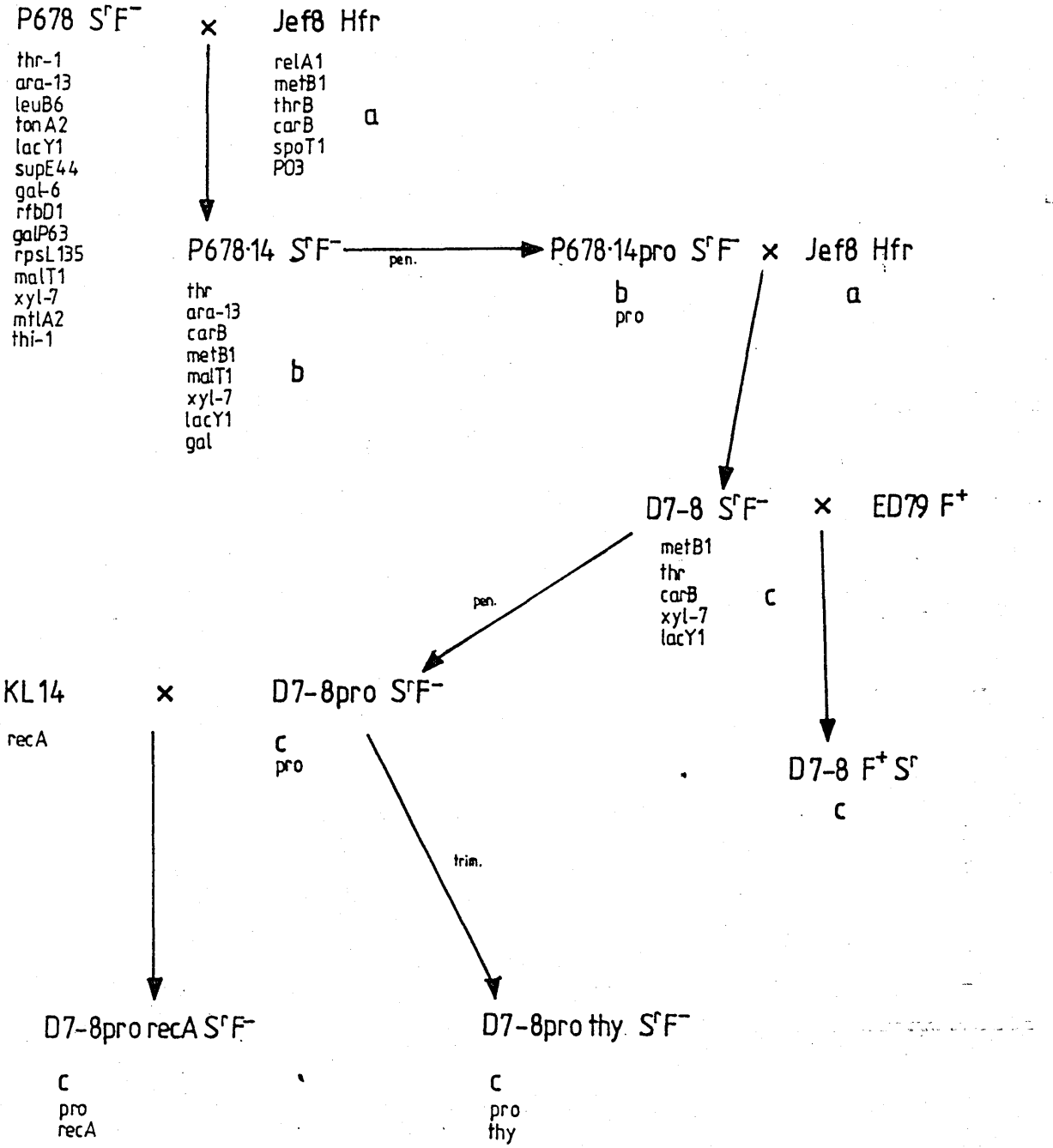


Figure 3·2 Derivation of D7-8 strains



Tn2901 while it now appears that amplification can occur in the absence of F, although it does so very rarely. This temperature affect was investigated further using both D7-8 and Jef8.

A second experiment that started life as a control for the use of temperature sensitive cI repressor is the effect of plasmids on the cut phenotype. The cI repressor is expressed from λpR on plasmid pcI857. This plasmid and plasmids pBR322 and pUC19 are shown to stimulate cut production.

When Carol Clugston first cloned amplified Tn2901 DNA from the strain cut1 (an amplified derivative of Jef8), she chose two different restriction fragments (Clugston 1986): An 11.7kb EcoRI fragment was cloned in both orientations as pCC1 and pCC5 and a 9.4kb BglII fragment was cloned as pCC15/pCC16 (Figure 3.7). When pCC15 and pCC16 are used to transform the *arg*F$^-$, *arg*I$^-$ strain NGX2 they are able to complement these mutations whereas pCC1 and pCC5 are incapable of complementing the arginine requirement in NGX2. Two possible explanations were offered to account for this; either the start of the *arg*F gene is left behind when an EcoRI fragment is cloned or pCC1 and pCC5 carry a non-functional copy of *arg*F (Clugston 1986). The published sequence of *arg*F (Piette *et al.* 1982)(van Vliet *et al.* 1984) shows the transcriptional start site to be 200bp from this EcoRI site with the promoter and operator sequences contained entirely within the EcoRI fragment (Figure 3.8), however the sequence also shows an upstream BamHI site not found in our restriction mapping (see Chapter 5 for mapping of pCC18 or Clugston, 1986 for mapping of pCC1/pCC5 and pCC15/pCC16). Jef8 or strains such as cut1 deriving from it may therefore contain a rearrangement or a restriction fragment polymorphism with respect to this EcoRI site. It would be interesting to know if this was the case as it could bear on the nature of the cut mutants produced by these strains. Alternatively, amplification of Tn2901 may have resulted in a rearrangement involving the EcoRI site or a non-functional copy of *arg*F. This chapter includes experiments that address this problem.

During the course of this thesis a debate which had been 'bubbling under' in genetics circles was made public: Namely that while the classic experiments of Luria and Delbruck (1943)

lead to the generally held principle that mutations are pre-existing and do not arise as a consequence of selection, there are, never-the-less, some systems in which it appears that certain mutations arise at far lower frequencies in the absence of selection (Cairns *et al.* 1988). The systems presented by Cairns *et al.* as potentially exhibiting "directed mutation" differ by necessity from the T1 phage-resistance mutants selected by Luria and Delbruck in that the selection applied does not immediately kill non-mutant cells (Luria and Delbruck 1943)(Cairns *et al.* 1988). This allows cells to respond to the environmental pressure applied. Whether, in some systems, this response is directed is still a matter of contention (Hall 1990). Car$^-$ cells exhibit slow growth on citrulline (Legrain *et al.* 1976b). When the car$^-$ strain Jef8 is plated on citrulline the first mutant colonies appear after two days, subsequently colonies continue to appear until at five days after plating the number of cut mutants is significantly higher than is expected from argI/argF constitutive mutants and argG bradytrophs (Jessop and Glansdorff 1980). A large proportion of the mutants isolated from Jef8 contained an amplification of an IS1-flanked element, Tn2901, containing argF (Jessop and Clugston 1985). The classic explanation for this observation is that the amplification of Tn2901 is selected by plating on citrulline. However, in the absence of selection, amplification in Jef8 has only been observed on one occasion over seven years (Adrienne Jessop and Carol Clugston personal communication, own observations). If we wished to test the hypothesis of directed mutation more detailed comparative experiments would need to be undertaken. However an alternative explanation is that the original cut mutants feed non-mutant cells allowing faster growth and thus greater opportunity for mutation. Experiments presented in this chapter confirm that cut mutants from Jef8 and from the F$^-$ strain D7-8 do indeed feed non-mutant cells and that mutant colonies do frequently arise on patches of poorly growing non-mutant cells.

The final experiment presented in this chapter was designed as the first in an analysis of the relationship between amplified or unstable mutants and the stable mutations seen in F$^-$ strains.

Legrain *et al.*, 1976b, have shown that the *arg*I gene in unstable mutants is still under control of the arginine repressor. This has led Nicholas Glansdorff to suggest that one or more copies of *arg*F that are not under control of the arginine repressor may be present in amplified Tn2901 DNA (N. Glansdorff, personal communication). Clugston and Jessop, 1990, have identified Hfr strains derived from Jef8 that produce ten-fold fewer colonies containing amplified Tn2901 than Jef8 while the total number of cut mutants remains the same. They have called this excess of non-amplified citrulline-utilizing mutants Class II mutations. One simple explanation for some Class II mutations could be that they arise as segregants from an overexpressing *arg*F copy in amplified DNA. I sequentially examined a set of Jef8 cut mutants to see if I could detect segregation to a stable phenotype.

3.2 Results

3.2.1 Cut production at 30°C

Isolation of amplified DNA from D7-8

Since I was using temperature sensitive cI repressor to maintain λdv plasmids in the chromosome at 30°C, D7-8 was tested to determine if growth at this temperature affected ability to produce citrulline utilizing mutants. The results are shown in Table 3.1. Growth at 30°C reproducibly resulted in greater numbers of D7-8 cuts, and as the experiment progressed the scatter between identical plates increased and the colonies on all test medium became more mucoid. The data in Table 3.1 were taken from plates with undiluted cells which ceased to grow after about day 20. Higher dilutions continued to show growth up to about day 28. Citrulline utilizing colonies isolated on day 18 from D7-8 grown at 30°C were tested for the presence of amplified Tn2901 by colony hybidization. 6 out of 56 colonies tested showed strong hybridization with the pCC1 probe (Figure 3.3a). This is surprising as amplified Tn2901 DNA has never before been observed in F^- strains. Of these six colonies, one failed to grow and three were too mucoid to allow isolation of single colonies when streaked out. Chromosomal DNA was prepared from the two remaining colonies, digested with EcoRI or EcoRI/BamHI and run on a 0.7% agarose gel. As can be seen in Figure 3.3b amplified DNA is present in a band of about 11.7kb in EcoRI digested DNA and in four bands of 4.7kb, 3.1kb, 2.3kb and 1.8kb in the EcoRI/BamHI digest. These band sizes are diagnostic of Tn2901 amplification. The DNA on this gel was blotted to Hybond-N membrane and probed with ^{32}P labelled, nick translated pCC1. This probe hybridizes strongly to all the amplified bands (Figure 3.3c) suggesting that these two isolates do indeed contain amplified Tn2901 DNA. Neither of the isolates were able to support the growth of the male-specific phage M13 confirming that they are F^- strains.

Table 3.1

Testing D7-8 for ability to produce cut colonies at 30°C

Strain	$val^r/10^7$	$cut/10^7$	Time(days)
D7-8 37°C	49	2	8
		3	17
		4	18
D7-8 30°C	32	2	8
		13	17
		23	18

Strains were grown to mid log phase (about 10^7 cells/ml.) in minimal medium supplemented with glucose, arginine, uracil and other required amino acids. Cultures were plated on citrulline plates and controls as described in materials and methods. Numbers are mean values for three plates scored on the day shown from plates containing about 50 colonies.

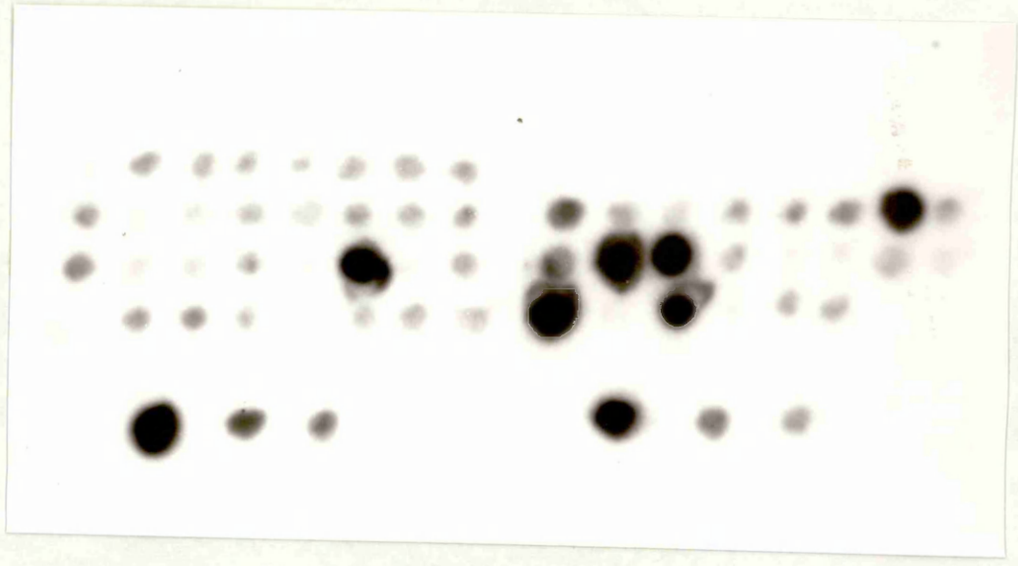
Figure 3.3

a)
Autoradiograph from blots of cut colonies from strain D7-8 grown at 30°C probed with nick translated pCC1. The three control strains at the bottom are from left to right; Cut1 which usually contains amplified Tn2901 DNA, Cut12 which occasionally contains amplified DNA and D7-8cut which has not been seen to amplify Tn2901. The blot was washed at high stringency and exposed for four hours.

b)
DNA prepared from two of the isolates that showed strong hybridization to pCC1 in a) digested with EcoRI or EcoRI/BamHI and run in E-buffer on 0.7% agarose slab gels. The gels were stained with EthBr.

c)
Autoradiographs from the Southern blots of the gels in b) probed with nick translated pCC1. The blots were washed at high stringency and exposed for four hours.

Figure 3.3


a)

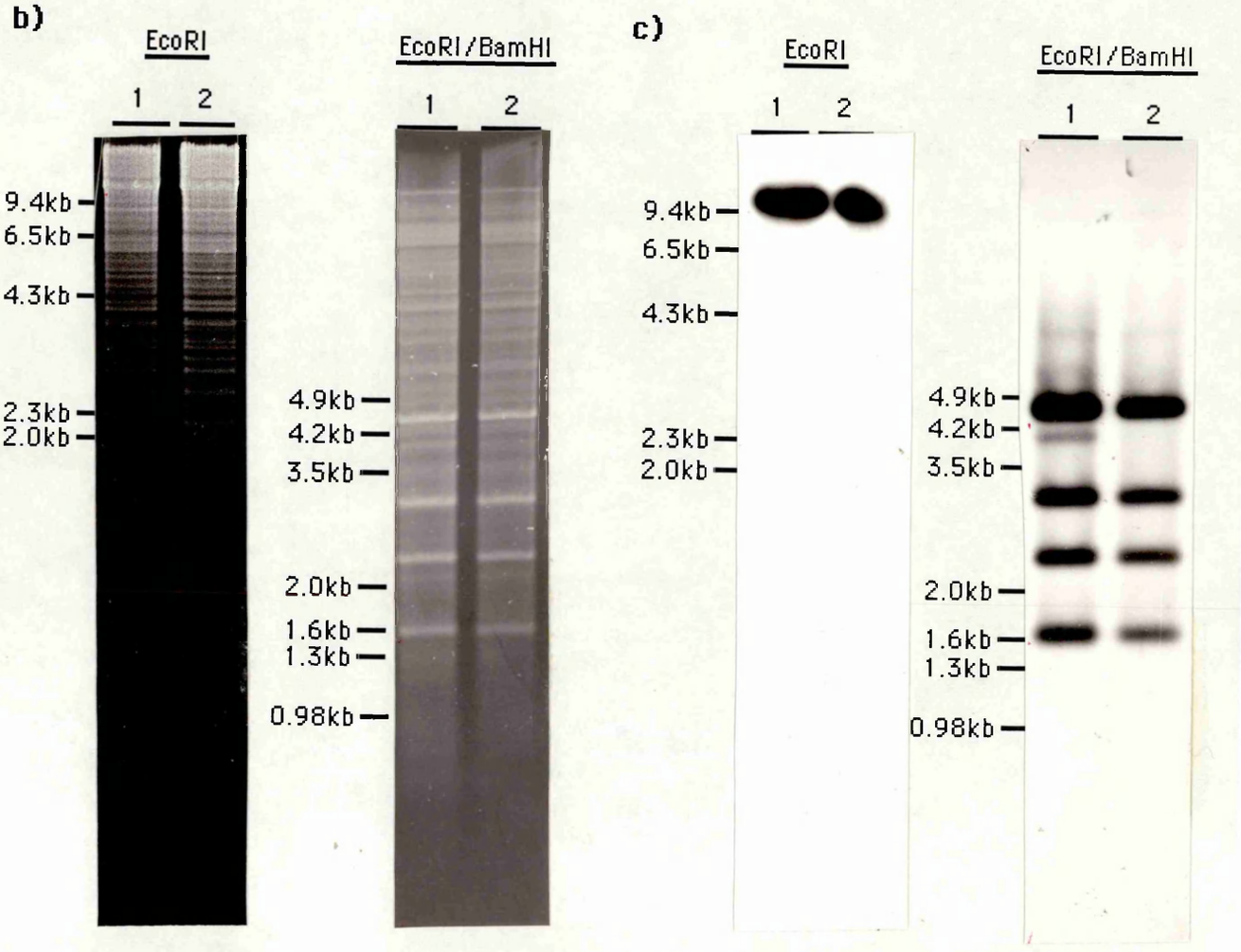

b)
EcoRI
1
2
9.4kb
6.5kb
4.3kb
2.3kb
2.0kb
EcoRI/BamHI
1
2
4.9kb
4.2kb
3.5kb
2.0kb
1.6kb
1.3kb
0.98kb
c)
EcoRI
1
2
9.4kb
6.5kb
4.3kb
2.3kb
2.0kb
EcoRI/BamHI
1
2
4.9kb
4.2kb
3.5kb
2.0kb
1.6kb
1.3kb
0.98kb

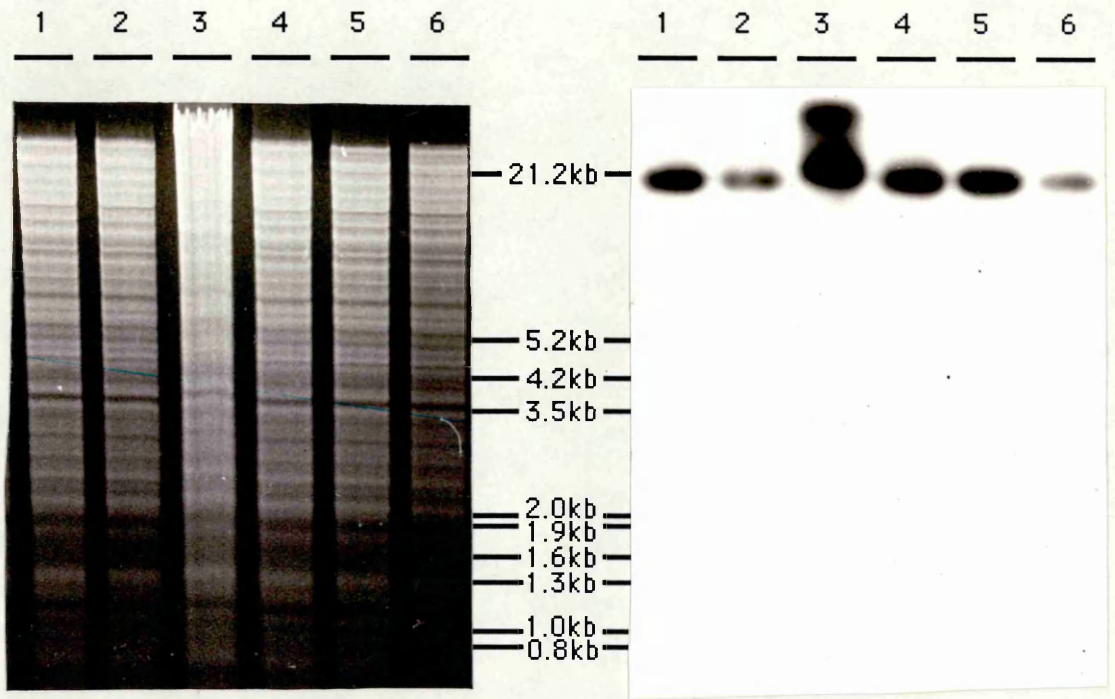
Figure 3.4

a)
DNA from strain Jef8 grown
1- to stationary phase at 37°C in complete medium
2- to log phase at 37°C in complete medium
3- to stationary phase at 30°C in complete medium
4- to stationary phase at 25°C in complete medium
5- to stationary phase at 30°C in minimal medium with supplements
6- to stationary phase at 25°C in minimal medium with supplements
digested with EcoRI and run in E-buffer on a 0.7% agarose slab gel. The gel was stained with EthBr. Plus an autoradiograph of a Southern blot of the gel probed with the random primed, *arg* F containing fragment from pMC23. The blot was washed at high stringency and exposed for four hours.

b)
DNA from strain D7-8 grown
1- to stationary phase at 37°C in complete medium
2- to log phase at 37°C in complete medium
3- to stationary phase at 30°C in complete medium
4- to stationary phase at 25°C in complete medium
5- to stationary phase at 30°C in minimal medium with supplements
6- to stationary phase at 25°C in minimal medium with supplements
digested with EcoRI and run in E-buffer on a 0.7% agarose slab gel. The gel was stained with EthBr. Plus an autoradiograph of a Southern blot of the gel probed with the random primed, *arg* F containing fragment from pMC23. The blot was washed at high stringency and exposed for four hours.

Figure 3.4

a) Jef8



b) D7-8

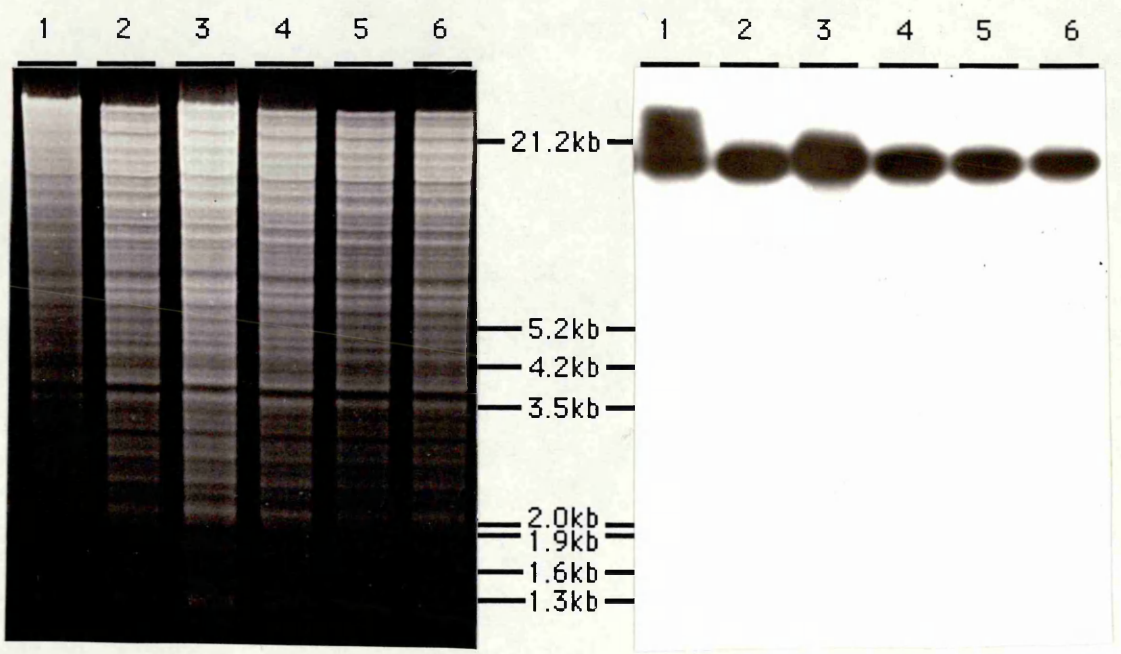


Figure 3.5

DNA from pFJH133 and pcI857 digested with ClaI or ClaI/BglI and run in E-buffer on a 0.8% agarose slab gel. The markers are λ DNA cut with EcoRI/HindIII. The gel was stained with EthBr.

Figure 3.6

a)
Autoradiograph from blots of cut colonies from strain D7-8/pBR322 grown at 30°C probed with nick translated pCC1. The three control strains at the bottom are from left to right; Cut1 which usually contains amplified Tn2901 DNA, Cut12 which occasionally contains amplified DNA and D7-8cut which has not been seen to amplify Tn2901. The blot was washed at high stringency and exposed for four hours.

b)
Autoradiograph from blots of cut colonies from strain D7-8/pBR322 grown at 37°C probed with nick translated pCC1. The three control strains at the bottom are from left to right; Cut1 which usually contains amplified Tn2901 DNA, Cut12 which occasionally contains amplified DNA and D7-8cut which has not been seen to amplify Tn2901. The blot was washed at high stringency and exposed for four hours.

Figure 3.5

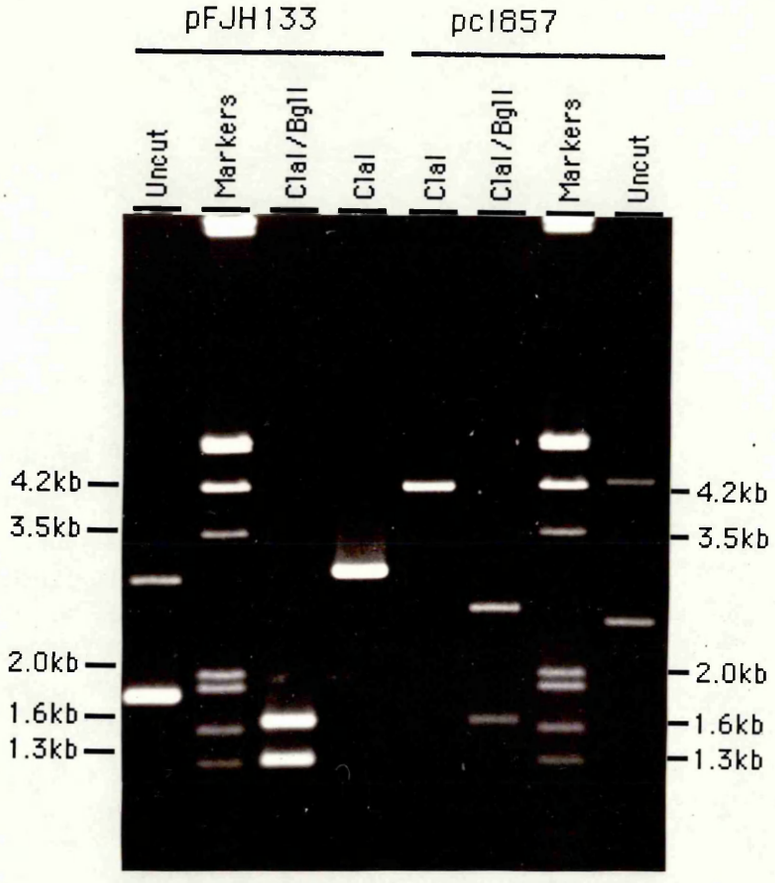
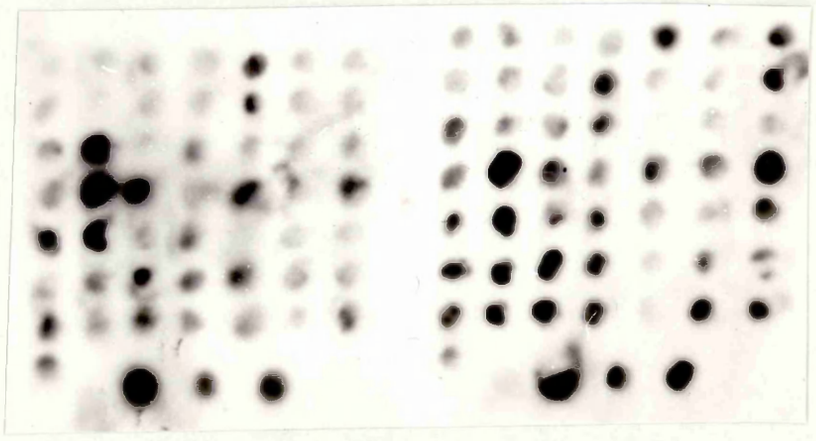


Figure 3.6

a)



b)

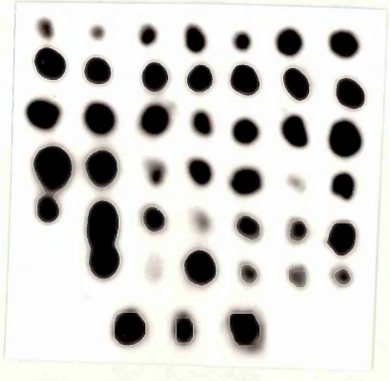


Table 3.2

Testing D7-8 strains carrying various multicopy plasmids for ability to produce cut colonies

Strain	$val^r/10^7$	$cut/10^7$	Time(days)
D7-8	11	4	10
D7-8/pC_I857	4	12	12
D7-8/pFJH133	19	20	12
D7-8/pUC19	16	37	12
D7-8/pBR322[1]	1	14	10
D7-8/pBR322[2]	1	40	10
Jef8	3	417	10

Strains were grown to mid log phase (about 10^7 cells/ml.) in minimal medium supplemented with glucose, arginine, uracil and other required amino acids. Cultures were plated on citrulline plates and controls as described in materials and methods. Numbers are mean values for three plates scored on the day shown from plates containing about 10^3 colonies.

---

[1] Incubation on citrulline plates at 30°C

[2] Incubation on citrulline plates at 37°C

Growth of Jef8 and D7-8 at 25 and 30°C

Incubation of D7-8 plated on citrulline at 30°C stimulated production of cut mutants and allowed us to observe amplified Tn2901 in an F^- strain for the first time. Southern analysis has shown the presence of amplified Tn2901 DNA in Jef8 in the absence of selection on one occasion (Clugston, unpublished data). I grew Jef8 and D7-8 in L-broth or in minimal medium supplemented with glucose, methionine, threonine, arginine, uracil and in the case of D7-8 aneurine/HCl at 25 or 30°C and in L-broth at 37°C to early-log, mid-log or stationary phase. Chromosomal DNA prepared from strains grown under these various growth conditions was subjected to southern analysis and probed with a ^{32}P-labelled, random-primed argF fragment from pMC23. The results are shown in Figure 3.4a and b. In the absence of selection none of these growth conditions stimulated amplification of Tn2901 DNA sufficiently to allow detection by southern analysis.

3.2.2 Effect of multicopy plasmids on cut production

D7-8 was transformed with plasmid pcI857 and the resulting strain D7-8/pcI857 was tested for its ability to produce citrulline utilizing mutants. When scored it was found to produce three times more cut colonies than the D7-8 control (Table 3.2, lines 1 and 2). To determine if this was due to the presence of λ repressor the cI gene was removed from the plasmid as follows: pcI857 DNA was digested with PstI and BstEII. The restricted DNA was Klenow treated to produce blunt ends, religated and used to transformed DS902. Three colonies examined were all smaller in size than pcI857. One of these pFJH133 was examined by digestion with ClaI and ClaI/BglI. ClaI digestion of pcI857 is expected to give a linear band of 4.122kb while removal of the cI gene should result in a linear band of about 2.9kb. The ClaI/BglI double digest of pcI857 gives two bands (2.5kb and 1.6kb) and removal of the cI gene should result in reduction of

the larger band to 1.2kb. Figure 3.5 shows these results. pFJH133 is thus the basic pACYC-based vector of pcI857 deleted for the cI gene. D7-8 was transformed with either pFJH133, pUC19 or pBR322 and these strains were tested for their ability to produce citrulline utilizing mutants. The results are shown in Table 3.2. Clearly the increased cut production of D7-8/pcI857 is not a result of the over-expression of λ repressor but rather the presence of multicopy plasmids in the cells. 100 citrulline utilizing colonies from D7-8/pBR322 at 30°C and 50 from the same strain at 37°C were patched out and subjected to colony hybridization using nick-translated pCC1 as a probe (Figure 3.6). Six of the 100 colonies isolated and grown at 30°C show strong hybridization to the pCC1 probe whilst at least eleven of the 50 colonies isolated and grown at 37°C would appear to contain amplified Tn2901 DNA. This phenomenon has been investigated further by Tom Dunlop in this laboratory. It is most obvious with pBR322 which when present in a strain results in an order of magnitude more colonies hybridizing strongly to the pCC1 probe than when other plasmids are present. Initial attempts to analyse some of these colonies suggest that transposition of Tn2901 or some rearrangement resulting in translocation of Tn2901 sequences to pBR322 may be responsible (Tom Dunlop and Adrienne Jessop unpublished data).

3.2.3 Non-functional copies of *arg*F are present in the amplified DNA of cut1

Amplified Tn2901 DNA cloned as an EcoRI fragment from Hfr cut1 is unable to complement the *arg*F$^-$ *arg*I$^-$ mutations of strain NGX2, while amplified DNA cloned as a BglII fragment from cut1 does complement this defect. It should be possible to test if all copies of *arg*F isolated from cut1 carry a restriction fragment polymorphism at the EcoRI site by removing DNA up to that site in a plasmid known to be able to complement the arginine requirement in NGX2. pCC15 and pCC16 contain the BglII

Figure 3.7

Restriction map showing the tandem amplification of Tn2901 after Clugston, 1986. Three copies of Tn2901 are shown for convenience. Insertion sequences are shown as open boxes. The arrows represent the position and orientation of the *arg* F gene. The positions of the 9.4kb BglII fragment cloned as part of pCC15/16 and the 11.7kb EcoRI fragment cloned as part of pCC1/5 relative to the amplification are also shown.

Figure 3.8

Nucleotide sequence of the control region of the *arg* F gene after Van Vliet *et al.* , 1984 and Glansdorff, 1987. Restriction sites and the promotor -35 and -10 regions are underlined and labelled. The two *arg* boxes (operators) are framed and the transcriptional start sites are indicated by vertical arrows. The ribosome binding site and translational start codon are shown by wavy lines.

Figure 3.7

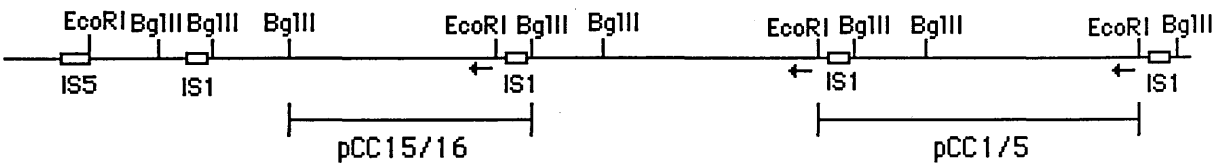


Figure 3.8

GGATCCAATCATTCTCATTTCTGACTCGACCTAGTTGTAGAATTCGATCC

BamHI EcoRI

AATGTCTTTCTGCTTCTGCAGAGAATCGGAGGCAGATACGATTATTTTCA

PstI

CACACGGACGGGTTTGCCTCCACCTTTGTAAGAAAGAATTGTGAAATGGG

GTTGCAAATGAATAATTACACATATAAAGTGAATTTTAATTCAATAAGTG

-35 -10

GCGTTCGCCATGCGAGGATAAAATGTCCGATTTATACAAAAAACACTTTC

TGAAACTGCTCGACTTTACCCCTGCACAGTTCACTTCTCTGCTGACCCTT

GCCGCACAGCTCAAAGCCGATAAAAAAAAATGGCAAGGAAGTACAGAAGCT

HindIII

TACCGGTAAAAACATCGCGCTCATCTTCGAAAAAGACTCGACTCGT

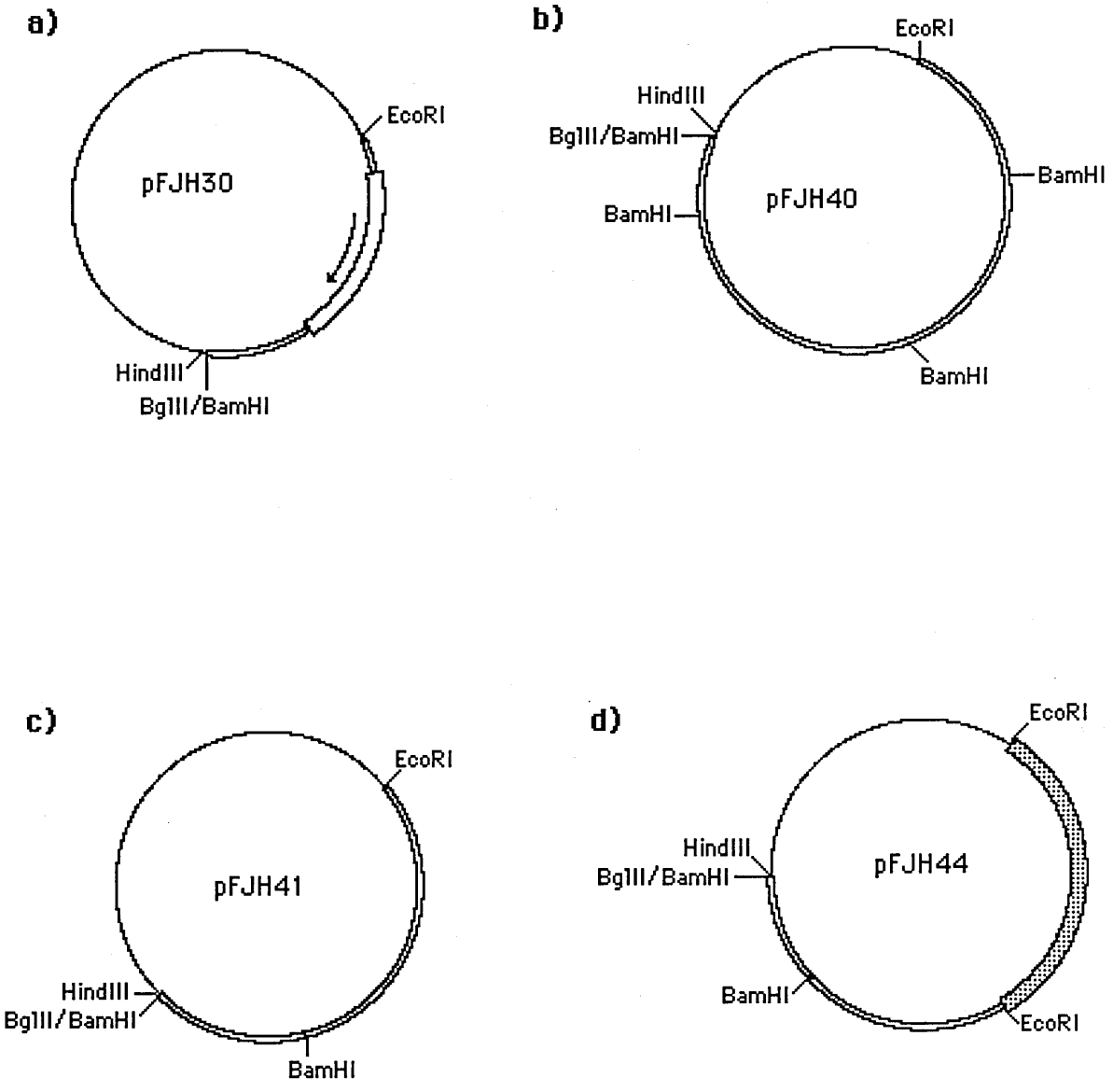
Figure 3.9

Restriction maps of;

a) pFJH30
b) pFJH40
c) pFJH41
d) pFJH44

Vector sequences are defined by a single line. IS1 is represented by a large open box and it's orientation as defined by nucleotide sequence is shown by the arrow. Chromosomal DNA is shown as small boxed regions and the omega factor by a large stippled box.

Figure 3.9


a)
pFJH30
EcoRI
HindIII
Bglll/BamHI
b)
EcoRI
HindIII
Bglll/BamHI
BamHI
pFJH40
BamHI
BamHI
c)
pFJH41
EcoRI
HindIII
Bglll/BamHI
BamHI
d)
EcoRI
HindIII
Bglll/BamHI
pFJH44
BamHI
EcoRI

fragment from amplified Tn2901 (Clugston 1986) cloned into pUC8 in opposite orientations (Figure 3.7). Digestion of pCC15 with EcoRI gives two bands of about 11kb and 1kb while digestion of pCC16 with EcoRI gives bands of around 8kb and 4kb. pCC15 and pCC16 were digested with EcoRI and half of the restriction reaction was used directly as the basis for a ligation reaction. Since the kinetics of ligation favours the joining of ends on the same molecule over the joining of ends on different molecules this provides a simple way to remove restriction fragments. The ligation reactions were used to transform DS902 and plated on R medium supplemented with ampicillin. Resulting colonies were screened by single colony gel analysis to identify plasmids that were smaller than the starting material. pFJH30 was isolated from the pCC15 reaction and pFJH40 from pCC16. On digestion with EcoRI pFJH30 gives a single band of about 4kb and pFJH40 shows a single band of around 11kb. Restriction maps of these plasmids are shown in Figure 3.9a and b. To eliminate the possibility of readthrough transcription from the *lac*Z promoter of pUC8 the omega factor (containing transcriptional and translational stop sites, (Prentki and Krisch 1984)) from pHP45Ω was introduced into the remaining EcoRI site. Firstly the two BamHI fragments in pFJH40 which are unnecessary for production of functional OTCase and merely increase the size of the plasmid were removed, as described above, by cutting with BamHI and religating. A restriction map of the resulting plasmid of 5.5kb, pFJH41, is shown in Figure 3.9c. pFJH41 and pHP45Ω were cut with EcoRI and ligated. Plasmid pFJH44 was isolated from single colony screening and contains the omega factor inserted into the EcoRI site of pFJH41 (Figure 3.9d). NGX2 was transformed with pFJH40, pFJH41 or pFJH44 and cells plated on R medium containing ampicillin. Resulting colonies were patched onto minimal medium supplemented with leucine, proline and uracil. All three plasmids expressed *arg*F. Thus it would appear that non-functional copies of *arg*F are present within amplified DNA.

3.2.4 Cut colonies crossfeed

Very rarely are cut colonies seen in the first 5 days of incubation on citrulline (Adrienne Jessop, personal communication; own observations). Thereafter they continue to appear until the plates are exhausted, as estimated by cessation of growth in all colonies. In the experiment reported above, 3.2.1 "cut production at 30°C", for instance, new D7-8 cut colonies arose more than three weeks after first plating. When these colonies are patched to fresh citrulline plates there appears to be no correlation between time of appearance on the original plate and rate of colony growth. It would seem therefore that mutation to the cut phenotype also occurs on the citrulline plates rather than merely being selected by them, a pattern of growth that is similar to those investigated by Cairns *et al.*, 1989. Phosphorolysis of citrulline by OTCases occurs at a low but measurable rate and in *E.coli* allows very slow growth of *car* mutants on citrulline (Legrain *et al.* 1976b). While conducting plating experiments I had observed that new colonies often appeared close to existing cut colonies and checked, firstly Jef8, for crossfeeding. Jef8 was grown to early log phase and titrated on minimal medium containing glucose, methionine, threonine and either arginine and uracil or citrulline. The titre calculated from arginine and uracil plates was about 5×10^4. After six days incubation citrulline plates containing high dilutions of Jef8 ($10^{-2, -3 \text{ and } -4}$) were bisected with plastic inserts and seeded with a cut colony isolated from a citrulline plate containing undiluted Jef8. Five days after seeding the seeded colony had grown and small patches of background growth or 'pseudocolonies' were observed on the same side of the plate. Ten days after seeding the plates were finally scored (Table 3.3). All but one citrulline utilizing colony grew on or from a pseudocolony. This one colony was the only growth observed on an unseeded side of a plate. Some large pseudocolonies gave rise to more than one cut colony. Control plates that had not been seeded showed no colonies or pseudocolonies. Remembering that only half a plate is scored it is interesting to note that the

Table 3.3

Hfr Jef8 was grown from an overnight culture to an OD_{600} of 0.45 and plated on minimal medium containing:

1) Glucose, methionine, threonine, arginine and uracil for viable cell counts. The titre after 2 and 16 days was $5x10^4$

2) Glucose, methionine and threonine to measure the rate of reversion to *car*$^+$. No colonies were apparent after 16 days.

3) Glucose, methionine, threonine, arginine, uracil and valine. The rate of reversion to *val*^{r} was $6x10^{-5}$

4) Glucose, methionine, threonine and citrulline. The number of colonies per plate after 6 days incubation is shown below

[a]

Dilution on citrulline	10^{-2}	10^{-3}	10^{-4}
	0	0	0
	33	0	0
	8	0	0
	7	0*	0*
	1*	0*	0*

Plates marked with an asterisk were bisected with a plastic insert. The -2 dilution set was then reincubated. The bisected plates from the -3 and -4 dilution sets were seeded with a citrulline utilizing colony (from a plate containing undiluted Jef8) on one side of the insert. The plates from these two dilution sets were then reincubated.

Table 3.3

The plates were finally scored 10 days later after a total of 16 days incubation. The number of colonies per plate is shown below

b

Dilution on citrulline	**10^{-2}**	**10^{-3}**	**10^{-4}**
	37	0	0
	162	0	0
	109	0	0
	107	29pc/17c*	3pc/3c*
	102*+53	17pc/8c*	2pc/2c*+1

The bisected plate in the -2 dilution set had 102 colonies on the side of the plate that had contained a colony when it was bisected(*) and 53 colonies on the other side of the plate. There were a great many pseudocolonies.

In the -3 dilution set no colonies were observed on unseeded plates. On the two seeded plates one had 29 pseudocolonies (pc) and 17 colonies and the other 17 pseudocolonies and 8 colonies all on the the side of the the plate that had received the seed colony (*).

In the -4 dilution set no colonies were observed on unseeded plates. On the two seeded plates one had 3 pseudocolonies (pc) and 3 colonies and the other 2 pseudocolonies and 2 colonies on the the side of the the plate that had received the seed colony (*). In addition one colony was observed on the side of the plate that had not been seeded.

number of pseudocolonies is of the same order as the number of cells plated. Figure 3.10 shows one of these plates, which were very difficult to photograph. This experiment was repeated and similar results using strains D7-8 and D7-8*recA* (not shown). It seems therefore that if a cut colony exists on a plate it will feed other cells on that plate.

3.2.5 Sequential examination of Jef8 cut derivatives

One question that springs to mind when considering the origins of citrulline utilizing mutants in Jef8 is whether it is possible to derive stable non-amplified cut colonies from colonies previously showing an amplification of Tn2901. I have attempted to answer this question by plating Jef8 on citrulline as previously described and monitoring the resulting colonies on a regular basis. Cells were taken in a line from the centre to the outside of a colony (so that an even sample of the colony was taken) and used to test for amplification of Tn2901 by colony hybridization. The results are shown in Figure 3.11. This is a 'dirty' experiment as it is impossible to control for slight differences in growth rate and no attempt has been made to measure hybridization quantitatively. One colony, colony 8, is a citrulline utilizer that contains no amplified DNA. It shows weak hybridization to the pCC1 probe in all five blots (note that the array in blot (a) is **5**x2 whereas in all other blots it is **6**x3, 4 or 5). Colony 1 shows strong hybridization to the probe in blots (a), (c) and (e), weak hybridization in blot (b) and intermediate hybridization in blot (d). Blot (d) suffered from some background and has therefore been slightly underexposed so should perhaps be discounted. A single instance of non-hybridization could be the result of a hybridization artefact as appears to be the case with colony 30 on blot (e). In future experiments of this type it would be wise to use duplicate filters. In general, however, most colonies exhibited roughly equivalent hybridization to the probe throughout the course of the

experiment. In no case was hybridization lost and the citrulline utilizing phenotype maintained without further amplification.

3.3 Discussion

The isolation of amplified DNA from F$^-$ strains grown at 30°C is interesting. It had previously been speculated that the F factor was required for amplification of Tn2901 by allowing the expression of an IS1 specific recombinase or in some other fashion 'activating' the IS1 sequences (Clugston 1986). If this is the case then it is necessary to postulate some other origin for the amplification in these F$^-$ stains. After incubation at 30°C D7-8 cells have a mucoid appearance not apparent after growth at 37°C. This may make the cells more susceptible to transformation or may allow them to uptake (or lose) nutrients more easily. It is possible that cell death and subsequent cannibalistic transformation of remaining colonies could result in duplicate copies of Tn2901 within a cell.

Alternatively it is possible that the function provided by the F factor is not an activation but an enhancement. The isolation of amplified DNA from F$^-$ strains only occurred after long incubation (18 days) and may even then have been fortuitous; no further amplified F$^-$ strains have been isolated (Adrienne Jessop, personal communication). Thus if the amplification is not dependent on the presence of the F factor it is clearly an extremely rare event. Growth at 30°C may not directly stimulate amplification; certainly none of the environmental conditions tested in this chapter stimulated amplification in either D7-8 or Jef8 in the absence of selection although amplification had previously been observed in Jef8 without selection (Carol Clugston, personal communication). Growth of D7-8 at 30°C did result in greater numbers of citrulline utilizers than growth at 37°C and this observation has been confirmed (Adrienne Jessop, unpublished data). *car*$^-$ cells plated on citrulline do not immediately die and may exhibit slow growth (Legrain *et al.* 1976b). Thus growth at 30°C may simply facilitate uptake of citrulline from the plates or of other nutrients from other cells on the plates allowing longer survival and thus greater opportunity for amplification to occur.

Six amplified colonies were visible on the blots in this experiment although only two were sufficiently healthy to allow further analysis. Given the mucoid nature of D7-8 colonies after 18 days at 30°C, I cannot eliminate the possibility that all six colonies are representatives of the same strain. Certainly four of these colonies are clustered in one region of the blot. It is also possible that a common progenitor of the colonies has suffered a rare mutation or rearrangement that allows amplification of Tn2901 in the absence of the F factor. This possibility could be tested by further Southern analysis of the two 'healthy' isolates.

The stimulation of cut production and apparent amplification seen when multicopy plasmids are present in cells is also of interest. Although Tn2901 has, for ease of description, been assigned a transposon number it has not as yet been shown to be capable of transposition (York and Stodolsky 1981)(Hu and Deonier 1981a). Thus it would be of some general interest if Dunlop and Jessop's investigations were to reveal transposition to multicopy plasmids. Fortunately the stimulation of cut production seen in D7-8/pcI857 is only four-fold even after twelve days incubation which should not interfere with screening for fragments capable of reproducing the stimulation seen in Hfr Jef8 which is of the order of 100-fold after five days.

Plasmids pCC15 and pCC16 carry functional *arg*F genes, while plasmids pCC1 and pCC5 do not. As the *arg*F gene in both sets of plasmid is derived from the same amplified strain, cut1, I tested pCC15 and pCC16 to determine if cut1 contained a rearrangement or restriction fragment polymorphism prior to amplification. Removal of the EcoRI fragment from these plasmids did not affect their ability to complement the *arg*F$^-$ *arg*I$^-$ mutations in NGX2. Transcription across the cloned fragment from the pUC8 *lac*Z promoter was prevented by use of a symmetrical insert containing transcription and translation stop sites (Prentki and Krisch 1984). This confirms that transcription of *arg*F in pCC15 and pCC16 originates from the *arg*F promoter and that the promoter lies distal to the EcoRI site. The loss of *arg*F function in pCC1 and pCC5 must therefore have occurred during amplification. No further analysis of this defect was undertaken.

The finding that cut colonies from either Jef8 or the F- strain D7-8 can feed non cut cells suggests an origin for the class II cut mutants described by Clugston and Jessop (1990). In Hfr Jef8 the majority of cut mutants are unstable, but in related Hfrs which produce cut mutants at frequencies similar to those observed in Jef8, the proportion of unstable mutants can be as low as 20%. This is a ten-fold greater yield of stable cut mutants than is observed in F- strains (Clugston and Jessop 1990). Large numbers of stable cut mutants are also found in F' strains (Chapter 4). These stable class II cut mutants are not observed in an Hfr deleted for Tn2901, but they do arise when multiple copies of Tn2901 are introduced into an Hfr defective only in the initial stage of Tn2901 amplification (Clugston and Jessop 1990). Nicholas Glansdorff has suggested that in amplified strains at least one copy of *arg*F will not be under control of the *arg* repressor (N. Glansdorff, personal communication). Amplified strains should therefore be very effective cross-feeders. Cross-feeding and subsequent continued growth of non-mutant cells will allow greater opportunity for mutation to a cut phenotype. What the nature of these mutations may be remains a matter for speculation. Certainly we would expect *arg*F and *arg*I constitutive mutants and *arg*G bradytrophs as predicted by Legrain *et al.* (1976b). However this system may allow the identification of rarer or more unusual mutations. Clugston and Jessop (1990) have suggested that stable mutations could arise from minor rearrangements in the right hand IS1 of Tn2901 that create a strong promoter from which *arg*F is transcribed (Clugston and Jessop 1990).

Interestingly Mittler and Lenski (1990) have shown that the excision of bacteriophage Mu from strain MCS2 in minimal lactose-arabinose medium also has a cross-feeding component that enhances the appearance of directed mutation. In MCS2 Mu is inserted between the araC regulatory gene and the lacYZ genes. Thus growth on lactose, in the presence of arabinose as inducer, occurs only on excision of Mu (Mittler and Lenski 1990). By pre-incubating cells without sugars Mittler and Lenski have shown that induction of Mu excision increases as a function of length of starvation prior to plating. The selection of cut

mutants requires incubation on plates for about 5 days. It has been suggested that physiological conditions in old colonies could induce bursts of transposition activity (Symonds 1989). Such an event could result in rearrangements that affect the right hand IS1 of Tn2901.

If this sort of rearrangement does occur it may be relatively simple to detect by southern analysis of class II cut mutants. PstI cuts in IS1 and very frequently at the right hand end of Tn2901 (Kohara *et al.*, 1987; results Chapter 5). If PstI digested chromosomal DNA is probed with IS1 and with sequences lying between IS1 and the EcoRI site of Tn2901 (ie between IS1 and the start of *arg*F) it may be possible to observe differences between class II cut mutants and Jef8 that result from these rearrangements.

A second unusual type of mutation that could result in a cut phenotype is suggested by the cross-reactivity of antisera raised against the catabolic OTCase of *Pseudomonas putida* with the *arg*F OTCase. It is possible that only a few simple point mutations would be required to change the equilibrium of the *arg*F OTCase in favour of the phosphorolysis of citrulline. Thus the initial analysis of class II cuts should be a biochemical investigation of the OTCase encoded by these mutants. This will allow the elimination of leaky *arg*G and *arg*I constitutive mutants, the pinpointing of *arg*F mutants that could be screened for rearrangements as described above and the identification of any mutants with altered OTCase equilibrium constants.

The sequential analysis of a handful of cut mutants derived from Jef8 did not suggest that stable mutants arise as segregants from amplified strains at any great frequency. While this is only a preliminary analysis of a very small number of mutants it confirms more detailed analyses of unstable mutants by other workers. These suggest that segregants retain some rearrangement, possibly a tandem duplication of Tn2901, that allows rapid amplification (Jessop and Glansdorff 1980)(Clugston and Jessop 1990). This in turn suggests that the stable class II cut mutants are indirectly dependent on the amplification of Tn2901, possibly by the cross-feeding mechanism described above. This idea is strengthened by preliminary results with an

Hfr deleted for *argI* which seem to imply an *argI* contribution to the class II cut mutants (Adrienne Jessop, personal communication).

CHAPTER FOUR

THE ROLE OF THE F FACTOR

4.1 Introduction

F- strains AB1157, P678.14 and D7-8 produce few citrulline utilizing (cut) colonies and are generally not seen to amplify Tn2901 DNA. The introduction of the F factor *in cis* to Tn2901 enables all these strains to produce excess cut colonies, many of which contain an amplification of Tn2901 (Jessop and Glansdorff 1980)(Jessop and Clugston 1985). The F factor is required only for the initial step in the amplification process; a recombination event between the IS1 elements of Tn2901 (Clugston and Jessop 1990)(Clugston 1986). This chapter examines the F factor and the mechanisms that might be involved in F-directed stimulation of this recombination. Two F factor functions, conjugal DNA transfer and replication from RepFIA, have been studied in detail by other investigators. Conjugation and replication are discussed and experiments designed to discover their role, if any, are reported. Other possible F functions, for instance the provision of a protein binding site, could also be important. A system that could be used to investigate other F functions is described here, but the reader is referred to Clugston, 1986, for a full discussion of their potential for stimulating recombination at IS1. While all these functions are dealt with separately it should be remembered that the function provided by the F factor need not reside in a single locus but may be the product of a number of stimulatory events.

Conjugal transfer may represent a mechanism that would lead to the stimulation of either homologous recombination or an IS-mediated event. Hopkins and colleagues showed that the F factor stimulated both precise (rec-independent) excision of Tn5 and Tn10 from the bacterial or F chromosome and *recA-dependant* recombination between IS3 elements on F (Hopkins *et al.* 1980). Interestingly the F factor used in these experiments is F'128 (Rubens *et al.* 1976). This large F' plasmid derives from Hfr P4X (the parental strain of Jef8) and carries both *lac* and *pro*AB chromosomal regions. Mutations in the F *tra* region were isolated that either reduced or enhanced the stimulatory effect on recombination.

Berg *et al.*, (1983), examined the excision of Tn5 from the *lac* operon. They found that excision from sites on the *E.coli* chromosome was at most twice as frequent in F^+ as in F^- strains. However excision from haploid F'*lac* ::Tn5 strains was two to three orders of magnitude greater than from the same sites in the chromosomes of F^- cells. In *rec*A F' partial diploid strains carrying distinguishable Tn5 elements excision was one to two orders of magnitude more frequent from episomal than from chromosomal sites (Berg *et al.* 1983). Stimulation of excision was also seen with a Tn5 element carrying direct instead of inverted terminal repeats (Hirschel and Berg 1982), but at a reduced rate. Finally *Lac*$^+$ revertants were found at a 50-fold higher frequency among cells that had just received an F' plasmid than in those in which the F' plasmid had been established. Based on these data Berg *et al.*, (1983), proposed that this *cis*-acting stimulation of Tn5 excision was due to the formation of single-stranded regions during conjugal transfer. These would allow the complementary ends of Tn5 to anneal such that DNA synthesis using the single strand as a template produces a new strand lacking the Tn5 element. In order to produce a duplication by a similar mechanism one would need to postulate slippage and recombination after DNA synthesis leaving a single-stranded duplicated region. This type of mechanism is easier to envisage if the terminal repeats are in direct rather than inverted orientation. The ends of Tn2901 are composed of directly repeated IS1 elements and in the progeny of Hfr Jef8 amplification of Tn2901 is frequently seen while by comparison deletion of the unit is a fairly infrequent event (Adrienne Jessop unpublished results).

In 1986 Syvanen *et al.*, published a continuation of the earlier work by Hopkins *et al.*, (1980). Characterization of F*tra* mutations that failed to stimulate recombination showed these mutations to be defective in conjugal transfer and a mutation that enhanced stimulation of recombination was shown to be *traS*. The *traS* gene product is required for surface exclusion and null *traS* mutants behave as recipients during conjugation. In addition Syvanen *et al.* were able to show that F'128*traS* males produced four times more sex pili than F'128 males suggesting

that conjugal transfer is more frequent in this cell population. Using genetically distinct male and female cells, precise excision of Tn5 was seen to occur in the recipient during conjugal transfer, supporting the model proposed by Berg *et al.*, (1983). However, in addition to this *cis*-acting stimulation of precise excision, the authors also demonstrated a conjugation-dependent, six-fold stimulation of either *recA-dependant* recombination or precise excision *in trans*. Not all F plasmids showed *trans*-acting stimulation of recombination. Neither wild-type F+ nor an F+ plasmid derived from F'128 had any *trans*-acting stimulatory effect. Interestingly F' plasmids of increasing size showed an increasing stimulation of recombination. *Trans*-acting stimulation of recombination was dependant on conjugal transfer, leading the authors to propose that the presence of single-stranded DNA entering the recipient after conjugal transfer signals a general response to increase genetic recombination (Syvanen *et al.* 1986).

The most obvious potential contribution that conjugal transfer could make to the duplication of Tn2901 is that it will, at least transiently, provide a second copy of the transposon in the cell. Add this to the increase in general recombination predicted above and it is not difficult to imagine mechanisms that would result in a chromosomal duplication of Tn2901.

If conjugal transfer can offer an explanation for the amplification of Tn2901 seen in Jef8, can it also lead to increased cut yield? Recall that repression of the *arg* operon is apparently unaffected in strains carrying amplified Tn2901 DNA. Conjugal DNA synthesis replaces the transferred strand in the donor and replicates the incoming single-stranded DNA in the recipient (Willetts and Skurray 1987). Like vegetative DNA synthesis these reactions are catalysed by *E.coli* DNA polymerase III (Willetts and Skurray 1987) however some differences have been observed. Unlike vegetative DNA synthesis, conjugal synthesis is not blocked by UV or ionizing irradiation of donor and recipient (Green *et al.* 1971), *dnaB* mutations (Bresler *et al.* 1973) or adenine starvation (Gross and Caro 1966). It has also been shown to be inherently less accurate, with a calculated 300-fold increase in the rate of base substitutions per round of

replication leading to an observed (1.8-fold) increase in the overall mutation rate (Kunz and Glickman 1983). This infidelity of replication during conjugal transfer could indeed contribute to the production of citrulline utilizing mutants either through mutation to the promoters or structural genes encoding OTCase, or by creating a leaky *arg*G strain (Legrain *et al.* 1976b). Indeed a cut mutant resulting from a single G:C to A:T transition in the *arg*F control region has been described (van Vliet *et al.* 1984).

Initial attempts by Clugston to address the contribution of conjugation to production of cuts in Jef8 started with the use of SDS in growth medium. While the presence of SDS reduced conjugation between Jef8 and a suitable donor 12 to 500-fold, cut production by Jef8 was only reduced 1.7-fold. The author found this result difficult to interpret in that little information exists regarding the effects of SDS on uptake of nutrients and citrulline (Clugston 1986). The second approach taken was to construct a conjugation deficient strain of Jef8. The plasmid pBE51 contains a *tra*J gene carrying an amber mutation cloned into a pSC101 vector (Kennedy *et al.* 1977). Strains carrying a nonsense mutation in *tra*J, the positive regulator of the *tra* operon, are not able to undergo conjugation. Clugston introduced pBE51 into Jef8 by transformation and subjected the resulting clone to a 2 to 3 second UV pulse to induce gene conversion. An isolate from this procedure was shown to be both transfer deficient as determined by it's inability to transfer the *arg*$^+$ phenotype and proficient in production of cuts as compared with Jef8 (Clugston 1986). The first results presented in this section describe the further characterization of this isolate; Jef8tra.

We felt that this experiment, while suggestive, neglected one possibility; Jef8 may undergo conjugation at any time with other Jef8 cells. Hfr cells that behave as recipients are called F$^-$ phenocopies. F$^-$ phenocopies are easily produced by starvation conditions, (Miller 1972), that could be reproduced on storage. In liquid culture, the frequency of F' transfer observed when two distinguishable F' strains are grown together to stationary phase, is 4-7%. When grown on an agar surface this frequency of transfer rises to nearly 30% (Berg *et al.* 1983). Thus a Jef8 colony could already contain many cells that had experienced

conjugation and were capable of amplifying Tn2901 DNA. Amplified Tn2901 DNA is easily distinguishable on Southern analysis by it's different migration from wild-type Tn2901 DNA (Clugston 1986). On at least one occasion amplified Tn2901 DNA has been observed in Jef8 in the absence of selection by Southern analysis (Clugston, unpublished data).

I therefore decided to switch from a dissection of Jef8 and tried to reproduce the cut behaviour in an F^- strain. F'Δtra plasmids isolated from Jef8 can be shown to confer cut behaviour on F^- strains (Clugston and Jessop 1990)(Results, this chapter). These plasmids are made from Jef8 by selection for pro^+ into pro^- recipients and therefore introduce the Tn2901 region from Jef8 into the recipient. Hfr13 is a derivative of Jef8 that will be shown to be deleted for Tn2901, probably by recombination across the IS1 elements. Hfr13 is the ideal donor in this experiment as the F factor DNA is derived from Jef8, but the chromosomal DNA that will undergo conjugal transfer to generate the F prime cannot itself introduce cut ability.

Replication initiated by the F factor could be another source of increased cut yield. In eukaryotes it has been suggested that repeated initiation of replication from a single origin would lead to the amplification of adjacent sequences (Stark and Wahl 1984). Replication initiation in eukaryotes occurs at many origins and is temporally regulated, however the nature of this regulation is a poorly understood. In *E.coli* replication is initiated at *ori*C and is positively regulated by transcription from the adjacent *mio*C promoter and by DnaA protein. Initiation occurs when a certain cell mass per origin, "initiation mass", is reached (Donachie 1968). It seems likely that DnaA protein is responsible for determining the initiation mass and that autoregulation of the *dna*A gene maintains this relationship (Lobner-Oleson *et al.* 1989). It has recently been suggested that DnaA protein acts through the formation of an *ori*C-membrane complex to allow initiation of replication (Gayama *et al.* 1990). In addition re-initiation at *ori*C is actively prevented, possibly by topological constraints, the need for DNA methylation and sequestration of the origin by the cell membrane (Von Meyenberg and Hansen 1987)(Landoulsi *et al.* 1990)(Boye and

Lobner-Olesen 1990). While RNaseH is not required for normal initiation at *ori*C, RNaseH mutants deleted for *ori*C can initiate "stable DNA replication" at many *ori*K sites around the chromosome. This replication is independent of DnaA and appears to be random (de Massy and Fayet 1984)(Von Meyenberg and Hansen 1987). Stable DNA replication is also induced as part of the cell's SOS response to agents that damage DNA or interfere with replication (Walker 1987). Broad cell size distribution in cells deleted for *ori*C and rescued by integrative suppression by plasmids F or R1 suggests that initiation of replication in these strains may also be random (Von Meyenberg and Hansen 1987).

Hfr strains may be considered as replicon cointegrates and it has been suggested that replication may be initiated from F when the average concentration of the integration site falls below the normal concentration of the autonomous F factor (Pritchard *et al.* 1975)(Lycett and Pritchard 1986). Lycett and Pritchard have calculated that in exponentially growing cells relative gene dosage at *lac* would be similar to that of autonomous F. Thus cointegrates between *lac* and *ori*C should rarely, if ever, initiate replication, while Fs integrated between *lac* and *ter*C would be expected to show considerable evidence of replication from *ori*F. Lycett and Pritchard demonstrate replication initiated by F integrated between *pyr*C and *pyr*D near the terminus of replication in exponentially growing cells. Previous work (Berg and Caro 1967) had failed to show replication from F in Hfr OR11 where the site of integration is between *pro*AB and *lac* (similar to Jef8, but in the opposite orientation).

Chandler and co-workers have examined chromosome replication in LC480, a derivative of Hfr OR11 marked with λ and Mu prophages. They observed replication only or mainly from *ori*C in cells growing exponentially in rich medium. However if cells were first starved for thymine in the presence of amino acids, then initiation from both F and chromosomal origins was observed on release. As thymine starvation would result in an increase in cell mass without increasing the copy

number of either origin the authors conclude that this data fits the hypothesis outlined above (Chandler *et al.* 1976).

When Jef8 is plated on citrulline cells are presumably starved of pyrimidines. It is possible to imagine that under these growth conditions cell mass to F origin ratio might change sufficiently to cause initiation of replication from F. Measurements of origin concentration for an autonomously replicating mini-F plasmid have shown that F copy number increases as cell growth rate decreases. It has been suggested that at slow growth rates *ori*V*rep*C sequences are responsible for this rise in F plasmid concentration with respect to *ori*C concentration (Shields *et al.* 1987). Thus the F factor could provide a local origin of replication that fires more frequently than the chromosomal origin due to the slow growth on citrulline.

Initiation of DNA replication in the vicinity of an amplifiable unit leads to amplification in the *thy*B region of *B. subtilis* (Petit *et al.* 1986). This is an artificial amplifiable structure in the *subtilis* chromosome created from 3.9kb repeats of pBR322 sequences flanking an 2.2kb DNA segment containing a cm^R gene. By inserting a temperature-sensitive replicon in the vicinity Petit *et al.* were able to show that amplification occurred in cells at the permissive temperature, but was not observed at the restrictive temperature. Analysis of the amplification products using a restriction enzyme for which there are no sites in the unit showed that amplification occurred in a proportion of the cells only (the single unit was still visible) and that it must occur by saltatory replication as no intermediates were found. The authors propose that saltatory replication involving a rolling circle mechanism (see Figure 4.0) may be a common mechanism for amplification in prokaryotes (Petit *et al.* 1986).

Replication from the F factor rather than from *ori*C could result in a topological environment more favourable to high levels of transcription from tandemly repeated copies of *arg*F. Brewer (1988) has noted that the majority (74%) of known transcription units on the *E.coli* chromosome are oriented so that transcription runs parallel to replication. In highly transcribed genes (those encoding components of the cell's protein synthesis machinery) this figure rises to 92% and the exceptional 8% is comprised

Figure 4.0

Amplification of a chromosomal region flanked by direct repeats by a rolling circle mechanism (after Young, 1987). Direct repeats are shown as open boxes, the region between them by a thick line and the DNA external to the amplifiable unit as a thin line.

a) Passage of a replication fork through the first direct repeat into the intervening DNA

b) Recombination between flanking elements

c) Amplification precursor with intact chromosome arm passing through the loop

d) Amplification by rolling circle replication

e) Reassimilation of the replication fork by homologous recombination and **f)** products

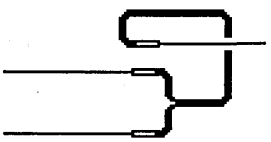
Alternatively

g) Reassimilation of the replication fork by illegitimate or microhomologous recombination and **h)** possible products

Figure 4.0

a)

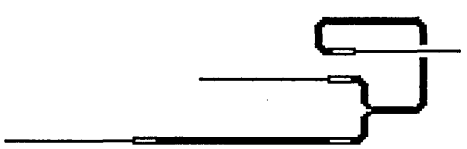
b)



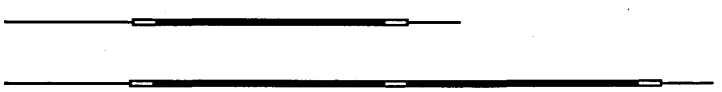
c)

d)

e)

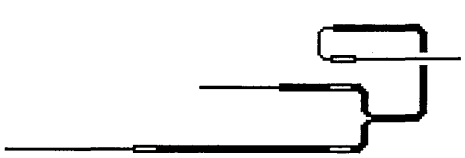


f)

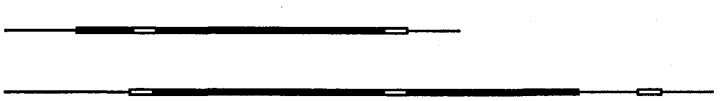


OR

g)



h)



entirely of genes encoding small ribosomal proteins that are not part of large transcription units (Brewer 1988). Transcription results in substantial changes in DNA supercoiling. Negative supercoils accumulate behind and positive supercoils in front of the transcription complex (Wu *et al.* 1988). Replication forks also generate positive supercoils in the DNA ahead of the fork (McMacken *et al.* 1987). Thus the meeting of opposing replicative and transcribing polymerases could result in a topology that is prohibitive to DNA replication. Data reviewed by Brewer suggests that *E.coli* has no efficient mechanism for resolving this conflict of polymerases and that replication is stalled by actively transcribed sequences (Brewer 1988). The *arg*F gene is oriented so that it's 3' end lies towards *ori*C. Therefore transcription of *arg*F will oppose replication from *ori*C. Measurements of OTCase levels in amplified strains suggest that one or more copies of *arg*F are transcriptionally derepressed (Legrain *et al.* 1976b). Thus if the formation of the amplification is dependent on a replicative mechanism it could be inhibited in strains where replication is from *ori*C (F^- strains) and stimulated by opposing replication from an integrated F factor.

If amplification is dependent on or contributed to by replication from F then it should be possible to stimulate it in F^- strains by introducing an F replication origin. The F factor can be forced to replicate the *E.coli* chromosome by integrative suppression of lethal replication mutations. However this creates a very different situation from that observed in Hfr strains where both *E.coli* and F origins are present and functional. If, for instance, amplification results in capture of a replication fork, then amplification in a strain with a single functional origin could be lethal. The interaction of origins in Hfr strains is a subject about which little is known and as such is of considerable interest. It would therefore be far more satisfying to introduce F origins to replication-competent *E.coli*. The F factor contains a number of replicons, however the prime candidate for replication initiated by F must be the mini-F replicon and it's two component origins *ori*V and *ori*S. Lane *et al.*, (1984), have shown that a cloned *ori*V fragment is capable of initiating replication if a *trans*-acting gene product, probably

RepE, is provided. In addition this fragment carries the site responsible for *ori*V catalysed site-specific recombination (Lane *et al.* 1984). This site could also be involved in stimulating amplification of Tn2901, possibly by interaction with a related site on or near the IS1 elements.

In concert with their role in replication *ori*S sequences are also responsible for F incompatibility. The *inc*B and *inc*C loci consist of 19 bp repeat sequences that bind RepE protein (Willetts and Skurray 1987). An interaction of these sequences with related sequences on the *E.coli* chromosome either directly or via RepE could be envisaged. This might result in the disruption of a gene that inhibits amplification of Tn2901, for instance a gene who's transcription runs across IS1 reducing IS1 transposition.

Transposons and insertion sequences are able to cause large scale rearrangements (Craig and Kleckner 1987) that might explain the requirement for the F factor *in cis*. For instance a deletion resulting from recombination between an IS element on F and an adjacent region of the chromosome could generate one of a range of novel sequences capable of stimulating IS1-IS1 recombination. Carol Clugston has shown this is not the case. She isolated DNA from Jef8, from cut mutants carrying an amplification of Tn2901 and from F$^-$ strains. These DNA samples were digested with a variety of restriction enzymes and probed with IS2, IS3, $\gamma\delta$ and IS5 (which does not exist on the F factor but is present in the *E.coli* chromosome adjacent to Tn2901). She found no evidence for major rearrangements in any of these elements, or in the chromosomal DNA adjacent to them (Clugston 1986). This analysis does not rule out minor rearrangements induced by insertion elements, for instance an inversion falling entirely within a restriction fragment that might stimulate amplification. An F factor derivative devoid of insertion elements, pOX38, exists (Chandler and Galas 1983b). An Hfr strain constructed with this plasmid could tell us if the F factor's insertion elements are required for the increased cut production seen in Hfrs. Hfr production however requires an area of homology with the chromosome, a feature usually provided by the insertion elements (Deonier and Hadley 1980). The insertion of a non-IS or transposon derived area of homology into pOX38

could be achieved by using the unique NotI site of F (Smith and Kolodner 1988). Assuming that a workably stable Hfr could be produced using pOX38, and that it failed to produce large numbers of citrulline utilizing colonies, it would then be of interest to return cloned insertion elements to the chromosome. A system for returning cloned F factor fragments (replication origins, insertion sequences or random cloned fragments) to the chromosome would be of great benefit. It would allow a two-pronged attack on the features of the F factor responsible for cut stimulation: Firstly F factor fragments could be returned to the chromosome of F^- strains and their effect on cut production observed, and secondly if F fragments are inserted into the chromosome of Hfr Jef8 it may be possible to use them to generate *in vivo* deletions stretching into the resident F factor DNA (Joyce and Grindley 1984). If one or more fragments of F capable of stimulating cut production in F^- strains can be found then a variety of experiments suggest themselves; *In vitro* mutagenesis could further define the function provided, and placement of the fragment at various chromosomal sites or in different orientations with respect to Tn2901 would allow an investigation of positional effects. Deletions in Jef8 could be screened for cut producing ability and those showing reduced ability by comparison with Jef8 would be characterized.

A number of systems can be used for the directed integration of cloned DNA into the *E. coli* chromosome. The basic requirements are an area of homology with the chromosome, *E. coli*'s homologous recombination system and a plasmid who's replication is conditional on some manipulable factor. The classic system has utilized ColEI based replicons which require host encoded DNA polymerase I (Kingsbur and Helinski 1973). In a *pol*A mutant ColEI-type plasmids carrying a region of chromosomal homology can survive by integration at the site of homology (Greener and Hill 1980). Recently a temperature sensitive pSC101 replicon was used to select for integration of the plasmid into the chromosome at 44°C (Hamilton *et al.* 1989). A third method utilizes λdv plasmids which are unable to replicate in the presence of cI repressor supplied either from a

resident λ lysogen or from an overexpressing plasmid (Joyce and Grindley 1984)(Boyd and Sherratt 1986).

As no temperature sensitive plasmid replicons are immediately available in the laboratory we have not considered this as a method of forced integration. Carol Clugston constructed a *pol*A I derivative of Jef8 and attempted to force integration of the pUC based plasmid pCC1. After transformation plasmid DNA was still visible on single colony gels indicating that integration of pCC1 had not occurred although the strain retained the *pol*A I phenotype (Clugston 1986). It is possible that the *pol*A allele used is leaky or that the *pol*A phenotype observed (sensitivity to UV light) is the result of some quite different mutation, however these experiments demonstrate the problems likely to be encountered in constructing and characterizing a number of strains in the absence of a transposon marked *pol*A allele. In addition *pol*A mutations are known to stimulate recombination in their own right, (Konrad 1977), and thus could contribute to the amplification of Tn2901.

Using a λdv plasmid constructed by Chris Boyd, (Boyd and Sherratt 1986), as a basic replicon, Clugston constructed pCC7, a plasmid containing 2.3kb of DNA isolated from Tn2901, and was able to force integration into Hfr λ^+ strains Cut1 and Jef8. Integration occurs at low frequency resulting in few transformants and in some cases transformants are seen to contain plasmid DNA by single colony gel analysis (Clugston 1986). Integration may also occur into the genome of the lambda lysogen via the sequences shared with the λdv plasmid (Boyd and Sherratt 1986). Thus careful screening is necessary. Screening for site of integration can be simply achieved either by genetic mapping, (Clugston 1986), or by Southern analysis. The advantages of Southern analysis being the likelihood of detecting unwanted rearrangements or multiple insertions. The second part of this chapter details the development of the λdv system and the cloning of various EcoRI fragments of the F factor for use in this system. The λdv system is used to insert cloned origin regions into the D7-8 chromosome in an attempt to assess their contribution to cut yield.

Finally, if replication is initiated by the F factor in Jef8, then it may be possible to measure a resulting gene dosage effect in a gene bank constructed from Jef8 DNA. Carol Clugston has constructed a gene bank from Jef8 (Clugston 1986) and an experiment of this type is reported in the last part of this chapter.

4.2 Results

4.2.1 The role of conjugal transfer

Characterization of Jef8tra

Jef8tra is unable to transfer the *arg*$^+$ phenotype when mated with NGX2 (*arg*F$^-$, *arg*I$^-$) however it is possible that this is due to the brief exposure to UV light that was required to produce the strain and not to a *tra*J mutation (Clugston 1986). Plasmid pSH6 (Achtman *et al.* 1978) carries a functional cloned *tra*J gene. If Jef8tra is a *tra*J mutant it should be possible to complement it with pSH6. Jef8tra was transformed with pSH6 and plated on L-agar containing ampicillin. Transformants were streaked out for single colonies, grown overnight in the presence of ampicillin and mated in liquid culture with AB1157. AB1157 carries *pro*$^-$ and *gal*$^-$ mutations and the gene encoding resistance to streptomycin. Conjugal mixtures were plated on minimal agar containing methionine, threonine, arginine, uracil, aneurine HCl, histidine, glucose and streptomycin to select for *pro*$^+$ recombinants or on minimal agar supplemented with methionine, threonine, arginine, uracil, aneurine HCl, histidine, proline, galactose and streptomycin to select for *gal*$^+$ recombinants. The control cross, untransformed Jef8tra mated with AB1157, was plated on identical medium. The results are shown in Table 4.1a. After 5 days Jef8tra mated with AB1157 produced no recombinants, while in the presence of pSH6 carrying the functional *tra*J gene, Jef8tra was able to function as a normal Hfr. Thus Jef8tra is clearly a *tra*J mutant. Jef8 and Jef8tra were tested for their ability to produce citrulline utilizers. As can be seen from the data in Table 4.1b Jef8tra shows no decrease in ability to produce cuts compared with Jef8. From this data it would appear that Jef8tra is more competent than Jef8 in cut production, however this is not always the case (Clugston and Jessop 1990). In any case we can conclude that, unless the initial activation event has already occurred in Jef8,

Table 4.1a

Cross	Pro+ recombinants	Gal+ recombinants
Jef8*tra*/pSH6 X AB1157	2.3 x 10^4	3.5 x 10^1
Jef8*tra* X AB1157	0	0

Bacterial crosses were carried out in liquid culture. Donor and recipient at mid log phase (about 10^8 cells/ml) were mixed in a ratio of 1:10 in a total volume of 5ml, and gentle aerated during mating. Mating for pro+ selection was for 60mins and for gal+ selection was for 110mins on media described in text. Numbers are mean values for 4 plates per cross.

Table 4.1b

Strain	$val^r/10^7$	$cut/10^7$
Jef8	0	57
Jef8*tra*	0	109

Strains were grown to mid log phase (about 10^7 cells/ml.) in minimal medium supplemented with glucose, arginine, uracil and other required amino acids. Cultures were plated on citrulline plates and controls as described in materials and methods. Numbers are mean values for three plates scored after 5 days from plates containing about 500 colonies.

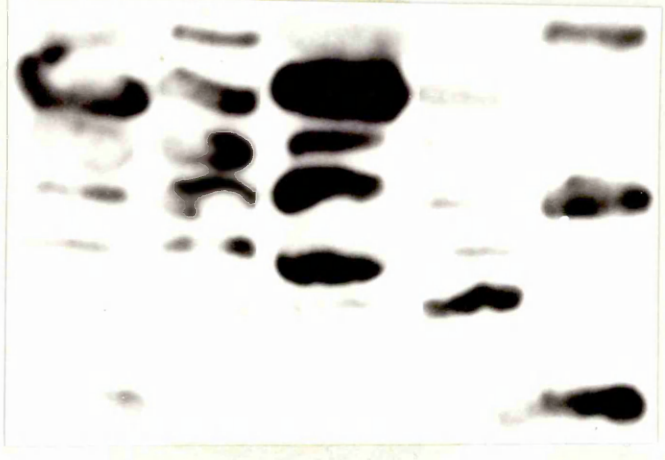
Figure 4.1

a)

Autoradiograph of cut colonies from strain D7-8::F'Δtra1/pPE14 probed with nick translated pCC1.

Autoradiograph of cut colonies from strain D7-8::F'Δtra2/pPE14 probed with nick translated pCC1.

b)



Autoradiograph of cut colonies from strain D7-8::F'ΔtraΔTn2901 /pPE14 probed with nick translated pCC1.

Table 4.2a

Cross	30mins	40mins	50mins
Jef8 X D7-8recA	1.3 x 10^2	4.2 x 10^2	8.6 x 10^2

Cross was carried out in liquid culture. Donor and recipient at mid log phase (about 10^8 cells/ml) were mixed in a ratio of 1:10 in a total volume of 5ml, and gentle aerated during mating. 1ml samples were removed at times shown, agitated to interrupt mating and plated on selective media. as described in text. Numbers are mean values for 4 plates per cross.

Table 4.2b

Strain	$val^r/10^7$	$cut/10^7$
D7-8/F'Δtra1	NT	21
D7-8/F'Δtra2	NT	29

Strains were grown to mid log phase (about 10^7 cells/ml.) in minimal medium supplemented with glucose, arginine, uracil and other required amino acids. Cultures were plated on citrulline plates and controls as described in materials and methods. Numbers are mean values for three plates scored after 10 days from plates containing about 30 colonies.

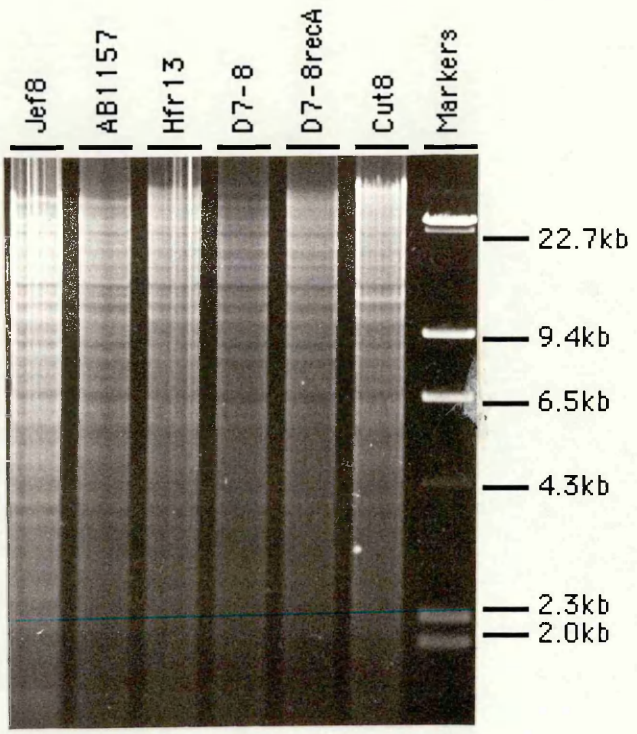
Figure 4.2

a)
DNA prepared from strains Jef8, AB1157, Hfr13, D7-8, D7-8recA and Cut8 digested with EcoRI and run in E-buffer on a 0.7% agarose slab gel. The markers are HindIII cut λ DNA ended labelled with Klenow. The gel was stained with EthBr.

b)
Autoradiograph of a Southern blot of the gel in a) probed with the random primed, *arg* F' containing fragment from pMC23. The blot was washed at high stringency and exposed overnight.

Figure 4.2

a)


Jef8
AB1157
Hfr13
D7-8
D7-8recA
Cut8
Markers
22.7kb
9.4kb
6.5kb
4.3kb
2.3kb
2.0kb

b)

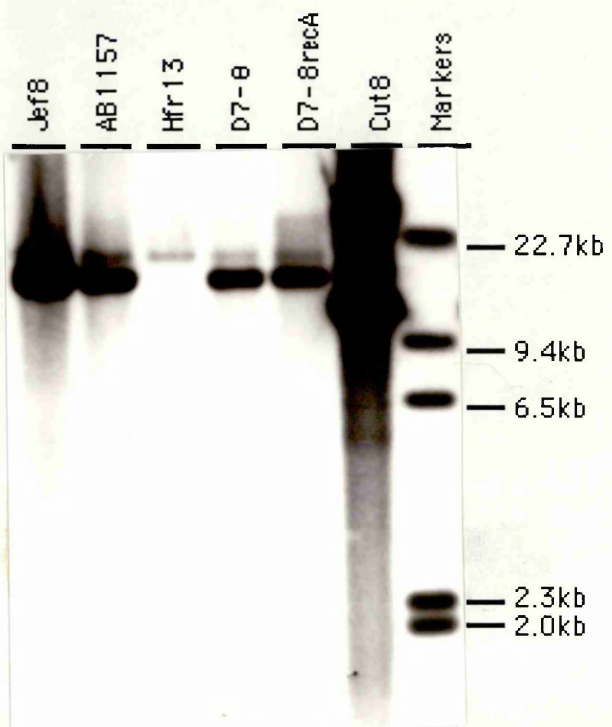

Jef8
AB1157
Hfr13
D7-8
D7-8recA
Cut8
Markers
22.7kb
9.4kb
6.5kb
2.3kb
2.0kb

Figure 4.3

a)
DNA from cosmid pB4 digested with a variety of restriction enzymes and run in E-buffer on a 0.8% agarose slab gel. The markers are λ DNA cut with EcoRI/HindIII on the left and HindIII on the right. The gel was stained with EthBr.

b)
Autoradiograph of a Southern blot of the gel in a) probed with the random primed, *arg* F containing fragment from pMC23. The blot was washed at high stringency and exposed overnight.

Figure 4.3

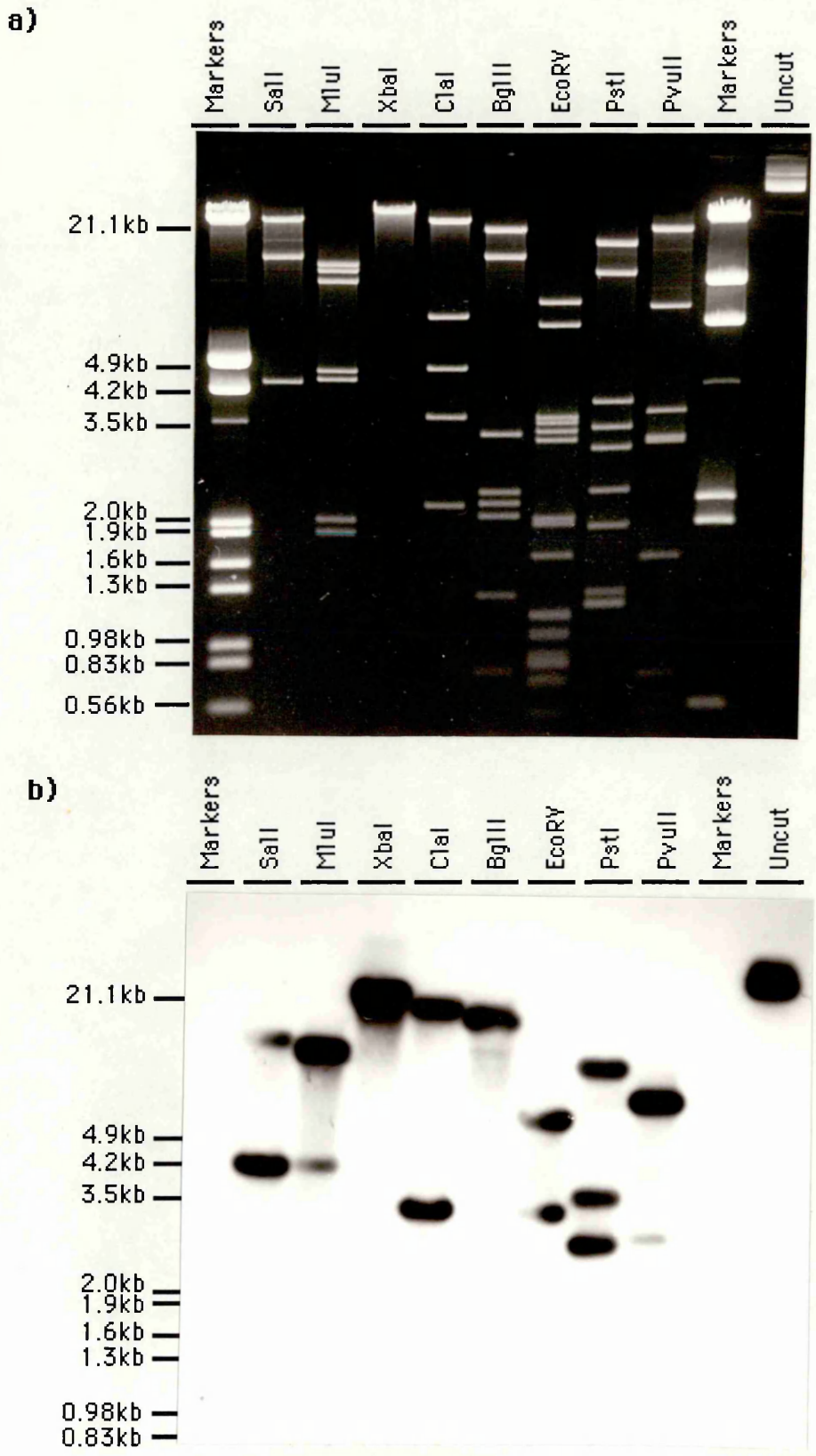

a)
Markers
SalI
MluI
XbaI
ClaI
BglII
EcoRV
PstI
PvuII
Markers
Uncut
21.1kb
4.9kb
4.2kb
3.5kb
2.0kb
1.9kb
1.6kb
1.3kb
0.98kb
0.83kb
0.56kb
b)
Markers
SalI
MluI
XbaI
ClaI
BglII
EcoRV
PstI
PvuII
Markers
Uncut
21.1kb
4.9kb
4.2kb
3.5kb
2.0kb
1.9kb
1.6kb
1.3kb
0.98kb
0.83kb

Figure 4.4

a)
Autoradiograph of a Southern blot from a gel containing EcoRI cut DNA from strains Cut12, Cut1, Hfr13, AB1157 and D7-8 probed with the random primed, *arg* F containing fragment from pMC23. The blot was washed at high stringency and exposed overnight.

b)
Autoradiograph of the Southern blot described in a) after probe removal and reprobing with nick translated pFJH120 containing an *arg* I gene fragment. The blot was washed at high stringency and exposed overnight.

c)
Autoradiograph of a Southern blot from a gel containing EcoRI cut DNA from strains D7-8, AB1157, Hfr13 and Cut1 probed with nick translated pFR10 (a λdv plasmid carrying DNA external to Tn2901 but within the 15.9kb EcoRI fragment containing Tn2901). The markers are HindIII/EcoRI cut λ DNA ended labelled with Klenow. The blot was washed at high stringency and exposed overnight.

Figure 4.4

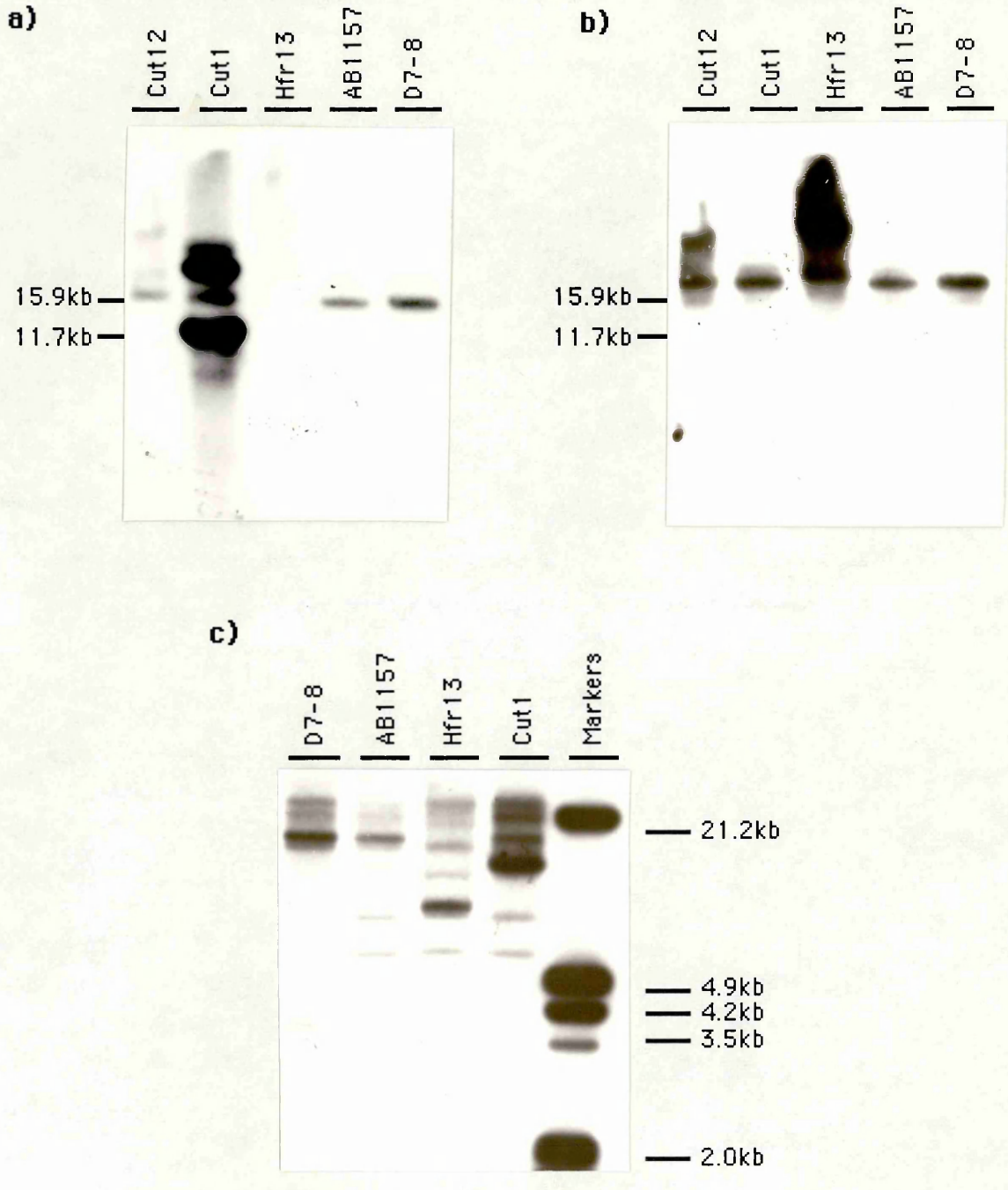

a)
Cut12
Cut1
Hfr13
AB1157
D7-8
15.9kb
11.7kb
b)
Cut12
Cut1
Hfr13
AB1157
D7-8
15.9kb
11.7kb
c)
D7-8
AB1157
Hfr13
Cut1
Markers
21.2kb
4.9kb
4.2kb
3.5kb
2.0kb

Table 4.3

Testing D7-8/F'ΔtraΔTn2901 for ability to produce cut colonies

Strain	$val^r/10^7$	$cut/10^7$
D7-8recA	7.0	0.4
Hfr13	10.0	0
F'ΔtraΔTn2901/pPE14[1]	0	37.5
F'ΔtraΔTn2901/pPE14[2]	1.5	14.0

Strains were grown to mid log phase (about 10^7 cells/ml.) in minimal medium supplemented with glucose, arginine, uracil and other required amino acids. Cultures were plated on citrulline plates and controls as described in materials and methods. Numbers are mean values for three plates scored after 8 days from plates containing about 25 colonies.

---

1 chloramphenicol added to plates.

2 Without chloramphenicol

conjugal transfer is not the main stimulus provided by the F factor.

Isolation of F'Δtra plasmids

Jef8 was mated with D7-8proRecA in liquid culture, samples were taken at 30, 40 and 50 minutes, agitated to disrupt mating complexes and plated on minimal agar containing methionine, threonine, arginine, uracil, glucose and streptomycin to select for F'Δ*trapro*$^+$ (Table 4.2a). *Pro*$^+$, *strep*^{r} colonies from 30 and 40 minute matings were first tested for transfer ability by mating with DT6 (*pro*$^-$, *nal*^{r}) in liquid culture and plated on minimal agar supplemented with methionine, threonine, arginine, uracil, glucose and nalidixic acid to select for *pro*$^+$ recombinants. After 5 days no recombinants were found. The same colonies were tested for the recA phenotype by sensitivity to UV light. All were found to be *rec*A. Thus the pro$^+$ phenotype did not arise as the result of recombination into the recipient chromosome and must be carried on an F factor derivative. When these strains were plated on citrulline no colonies were observed. Two colonies were transformed with pPE14 (*rec*A$^+$) and tested for ability to produce citrulline utilizing colonies. The results are shown in Table 4.2b. 102 cut colonies were tested for amplification of Tn2901 by colony hybridization. 24 were positive (Figure 4.1a). F'Δtra plasmids will therefore support the amplification of Tn2901.

Characterization of Hfr13

Hfr13 is an *arg*F$^-$*arg*I$^+$ derivative of Jef8 that could be deleted for the Tn2901 unit. Figure 4.2a shows chromosomal DNA preparations from Hfr13, Jef8, Cut8 (a citrulline utilizing derivative of Jef8 containing amplified Tn2901 DNA) and various F$^-$ strains digested with EcoRI restriction endonuclease. Figure

4.2b shows the same DNA, after southern transfer, probed with the *arg*F containing fragment of pMC23 radiolabelled by random priming. Hfr13 clearly lacks the 15.0Kb band produced from EcoRI digestion of DNA containing Tn2901. Only the Cut8 strain carries the 11.7kb band derived from amplified Tn2901 DNA (Clugston 1986). On longer exposure a weakly hybridizing band is seen in all lanes running above the 15kb *arg*F-containing band. It seems likely that this band corresponds to the *arg*I gene of *E.coli* which shares 78.1% sequence homology with *arg*F (van Vliet *et al.* 1984). As no cloned *arg*I gene was available in this laboratory it was necessary to isolate one in order to test this theory. The cosmid pB4 (Knott *et al.* 1989) contains about 45kb of DNA spanning the chromosomal region around 96.5mins on the *E.coli* map and should therefore contain the *arg*I gene (Stirling *et al.* 1988a). pB4 DNA was digested with a variety of restriction enzymes, blotted to Hybond-N membrane and probed with the radiolabelled *arg*F fragment from pMC23. The results are shown in Figures 4.3a and b. A 4.5kb SalI fragment showing strong hybridization with *arg*F was isolated from a low melting point agarose gel and ligated to SalI digested phosphatased pUC19 DNA. The resulting plasmid pFJH120 is a useful *arg*I probe although it does not contain the entire *arg*I gene and is unlikely to complement *arg*I$^-$ mutants. A southern blot containing EcoRI digested DNA from Hfr13, Cut1 and two F$^-$ strains is shown in Figure 4.4 hybridized to: a)the radiolabelled *arg*F fragment from pMC23 and b)nick-translated pFJH120, confirming that the larger band seen on EcoRI digests probed with *arg*F is indeed the *arg*I gene. This southern blot was subsequently probed with nick-translated pFR10 (a λdv plasmid containing DNA external to Tn2901 but within the 15.0kb EcoRI fragment containing Tn2901). Using previously generated chromosomal maps (Hadley *et al.* 1983)(Kohara *et al.* 1987)(results, chapter5) it can be calculated that the deletion of Tn2901 across the flanking IS1 elements would leave an EcoRI restriction fragment of between 6 and 7kb. The probe pFR10 hybridizes strongly to a band of 6.8kb in EcoRI digested Hfr13 DNA (Figure 4.4c lane 3). Weak hybridization is seen to a number of other bands using this probe. These may result from

hybridization of the λdv vector to λ sequences or hybridization to other IS1 sequences due to the 595bp of IS1 carried on pFR10. The difference in pattern of the weak bands in D7-8 (lane 1) compared with the three λ lysogens; AB1157, Hfr13 and Cut1 (lanes 2, 3 and 4) would favour the former explanation. Thus Hfr13 is deleted for the *arg*F gene and this deletion probably occurred as a result of recombination between the IS1 elements of Tn2901.

Isolation of F'ΔtraΔTn2901

Hfr13 was mated with D7-8*prorec*A in liquid culture. Mating was interrupted after 40 minutes by agitation and cells were plated on minimal agar supplemented with methionine, threonine, arginine, uracil, glucose and streptomycin to isolate F'ΔtraΔTn2901*pro*$^+$. 50 colonies were first tested for transfer ability by plate mating with DT6 (*pro*$^-$, *nal*^{r}) and replicated onto minimal agar supplemented with methionine, threonine, arginine, uracil, glucose and nalidixic acid to select for *pro*$^+$ recombinants. After 5 days no recombinants were found. The same colonies were tested for the *rec*A phenotype by sensitivity to UV light. All but 2 were found to be *rec*A$^-$. One of the *rec*A$^-$ strains was transformed with pPE14 (*rec*A$^+$) and tested for ability to produce citrulline utilizing colonies. The results are shown in Table 4.3. 64 cut colonies were tested for amplification of Tn2901 by colony hybridization. 14 were positive (Figure 4.1b). F'Δ*tra*ΔTn2901 plasmids will therefore support the amplification of Tn2901. I have not tested F'*tra*$^+$ plasmids for ability to produce citrulline utilizing colonies however data from other workers has shown that these plasmids produce about 10 cut mutants per 10^7 cells (for instance see Clugston and Jessop, 1990). Clearly F'Δtra plasmids are equally competent in stimulation of cut production.

4.2.2 A system for returning cloned F fragments to the *E.coli* genome

Constructing a control vector

In Jef8 the F factor is inserted at an IS3 element about 20kb from Tn2901. Ideally F fragments would be inserted at this site however the number and distribution of IS3 sequences in D7-8 are not accurately known. This could result in severe screening difficulties. As very few isolates containing integrated plasmid are expected (Clugston 1986) it is preferable to use an area of homology that will result in a single site of integration. A 2.3kb PstI fragment from plasmid pCC18 contains DNA adjacent to Tn2901 (see Chapter 5). This fragment contains 173bp of IS1 sequence which might be better removed. Unfortunately there appear to be no sites adjacent to this IS1 sequence for common restriction enzymes. Exonuclease III is a 3' to 5' double-strand specific exonuclease. If DNA is digested with two restriction enzymes that cut between the vector and insert, one leaving a 3' protruding end adjacent to the vector and the other a 5' overhang adjacent to the insert, exonuclease III will shorten only the insert sequence as 3' protruding ends are not a suitable substrate for the enzyme (Henikoff 1984). The 2.3kb PstI fragment from pCC18 was ligated to PstI digested, phosphatased 19A as described in materials and methods. The ligation was used to transform competent DS902 and the plasmid pFJH50 was isolated. The 2.3kb fragment from pCC18 contains a single HindIII site originating from the pCC18 polylinker which can be used to determine the inserts' orientation with respect to the HindIII polylinker site in 19A (Figure 4.5a and c). EcoRV digestion of pFJH50 gives three bands of 3.8kb, 2.8kb and 0.5kb while digestion with EcoRV and PstI shows four bands of 2.7kb, 1.95kb, 1.45kb and 0.5kb. Figure 4.5c shows a restriction map of pFJH50. To remove the IS1 sequence with Exonuclease III this sequence must lie adjacent to the polylinker sites. In order to obtain the PstI fragment in the opposite orientation pFJH50 DNA was cut with PstI and religated. Colonies isolated from

Figure 4.5

a)
DNA from pFJH50 digested with a variety of restriction enzymes and run in E-buffer on a 0.8% agarose slab gel. The gel was stained with EthBr.

b)
DNA from pFJH50 and pFJH56 digested with EcoRV or EcoRV/BamHI and run in E-buffer on a 0.8% agarose slab gel. The gel was stained with EthBr.

c) and **d)**
Restriction maps of pFJH50 and pFJH56 respectively. The 19A vector sequences are shown by a single line, the insert chromosomal DNA is represented by small boxed regions and the 173bp of IS1 by a large box.

Figure 4.5

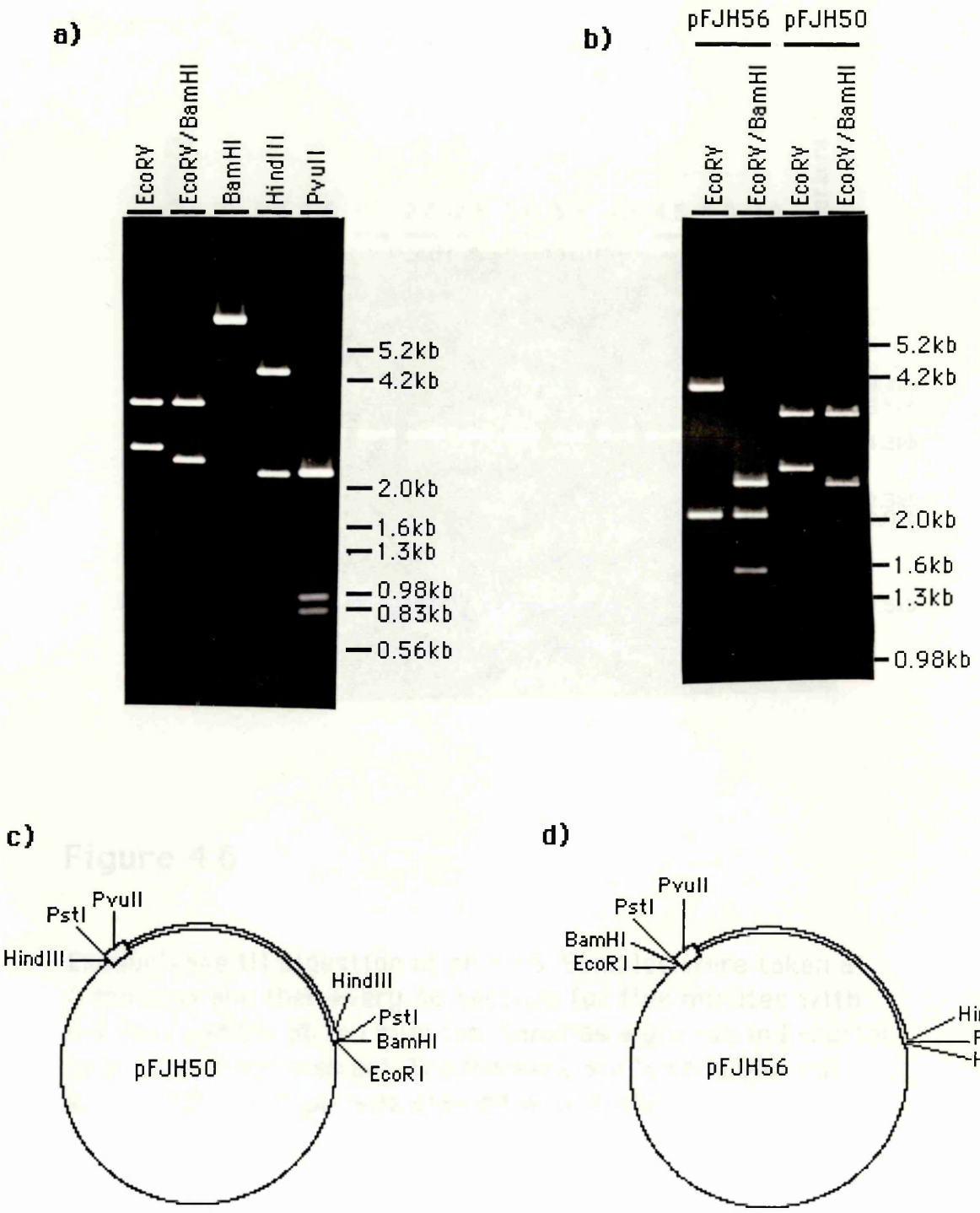

a)
EcoRV
EcoRV/BamHI
BamHI
HindIII
PvuII
5.2kb
4.2kb
2.0kb
1.6kb
1.3kb
0.98kb
0.83kb
0.56kb
b)
pFJH56
pFJH50
EcoRV
EcoRV/BamHI
EcoRV
EcoRV/BamHI
5.2kb
4.2kb
2.0kb
1.6kb
1.3kb
0.98kb
c)
PvuII
PstI
HindIII
HindIII
PstI
BamHI
EcoRI
pFJH50
d)
PvuII
PstI
BamHI
EcoRI
HindIII
PstI
HindIII
pFJH56

Figure 4.6

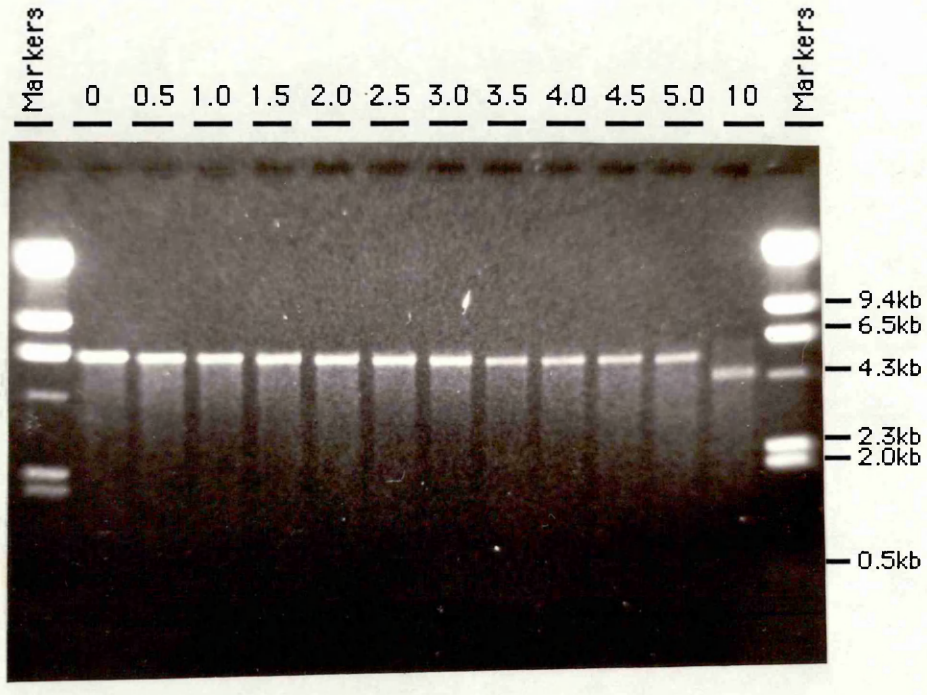


Figure 4.6

Exonuclease III digestion of pFJH56. Samples were taken at 0 minutes and then every 30 seconds for five minutes with one last sample at ten minutes. Samples were run in E-buffer on a 1% agarose slab gel. The markers are lambda DNA cut with HindIII. The gel was stained with EthBr.

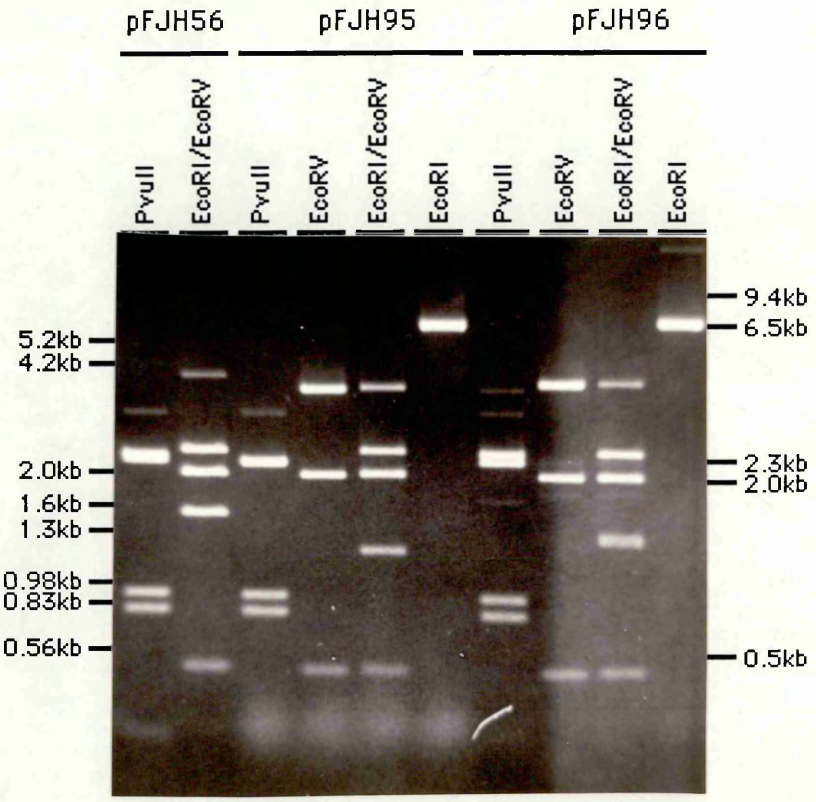
Figure 4.7

a)
DNA from pFJH56, pFJH95 and pFJH96 digested with PvuII or EcoRI/EcoRV and run in E-buffer on a 0.8% agarose slab gel. The gel was stained with EthBr.

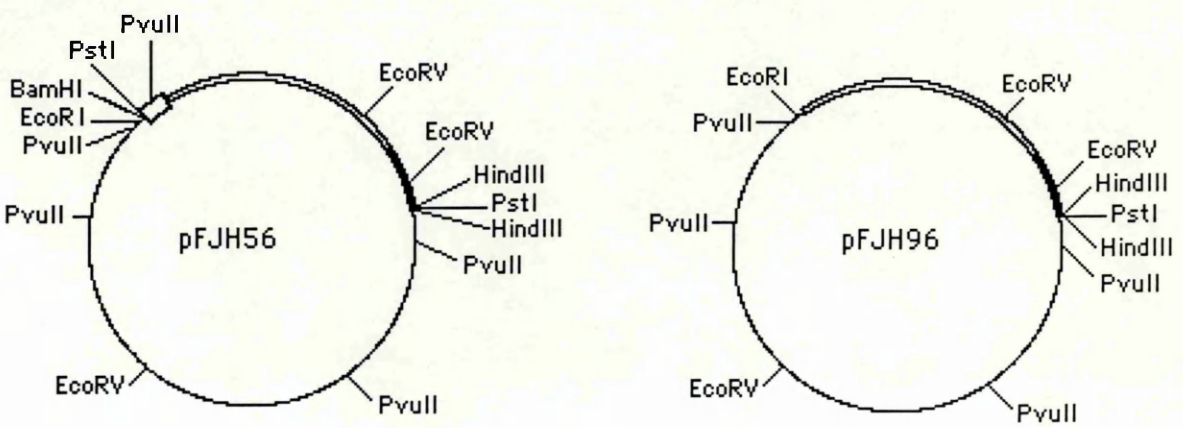
b)
Restriction maps of pFJH56 and pFJH96. The 19A vector sequences are shown by a single line, the insert chromosomal DNA is represented by small boxed regions and the 173bp of IS1 by a large box. Shaded boxed areas represent DNA originally derived from pBR322 via pCC18 (Clugston, 1986).

Figure 4.7

a)


pFJH56
pFJH95
pFJH96
PvuII
EcoRI/EcoRV
PvuII
EcoRV
EcoRI/EcoRV
EcoRI
PvuII
EcoRV
EcoRI/EcoRV
EcoRI
5.2kb
4.2kb
2.0kb
1.6kb
1.3kb
0.98kb
0.83kb
0.56kb
9.4kb
6.5kb
2.3kb
2.0kb
0.5kb

b)


PvuII
PstI
BamHI
EcoRI
PvuII
EcoRV
EcoRV
HindIII
PstI
HindIII
PvuII
PvuII
pFJH56
EcoRV
PvuII
EcoRI
PvuII
EcoRV
EcoRV
HindIII
PstI
HindIII
PvuII
PvuII
pFJH96
EcoRV
PvuII

transformed DS902 were screened by single colony gel analysis to eliminate those that had lost the insert. Three colonies containing inserts were subjected to restriction analysis with EcoRV and BamHI. One of these, pFJH56, is shown in Figure 4.5b. The 0.5kb EcoRV fragment was present on this gel however it has not reproduced well on photography. pFJH56 shows the restriction pattern that would be expected from a clone containing the pFJH50 insert in the opposite orientation (Figure 4.5d). pFJH56 DNA was digested sequentially with KpnI and XbaI and subjected to exonuclease III digestion as described by Henikoff. Samples were taken every 30 seconds for 5 minutes and one last sample at 10 minutes. Small aliquots from these samples are shown run on a 0.6% agarose gel (Figure 4.6). Although it is not possible to accurately state sizes from this gel the 10 minute time point shows a band slightly greater than 4.3kb in size. As the starting plasmid pFJH56 is 6.6kb in size this represents a rate of about 200bp per minute which is within the range expected (Henikoff 1984). Samples were religated and used to transform DS902. Figure 4.7a shows a restriction analysis of pFJH96 (plasmid isolated from the 1 minute time point), pFJH95 (plasmid isolated from the 1.5 minute time point) and pFJH56 (parental plasmid). The 215bp PvuII fragment seen in pFJH56 (Figure 4.7a, lane1) is generated from the PvuII site adjacent to the polylinker and the PvuII site at position 75bp in IS1 (Figure 4.7b); it is absent in both pFJH95 and pFJH96. All three plasmids share 4.0kb, 2.4kb, 2.1kb and 0.5kb bands on digestion with EcoRI and EcoRV. The 4.0kb band is a partial digestion product corresponding to an EcoRV fragment that has not been cut with EcoRI. Since this is present in pFJH56 it is probably the result of imperfect reaction conditions rather than loss of the EcoRI site during Exonuclease III treatment (sequence analysis has shown this to be the case for pFJH96, not shown). In addition to these common bands pFJH56 has a 1.6kb band, pFJH96 a 1.4kb band and pFJH95 a 1.2kb band corresponding to the DNA shortened by Exonuclease III digestion. pFJH96 has the maximum length of chromosomal DNA without any IS1 sequence and is in this sense a better vector than pFJH50 however Exonuclease III treatment has removed most of the polylinker

sites rendering it useful only for cloning of EcoRI fragments. pIC20H and pIC20R are high copy number pUC based vectors. They carry large semi-symmetrical polylinkers making them ideal for subcloning DNA fragments (Marsh *et al.* 1984). The 1.5kb EcoRI/EcoRV fragment from pFJH96 was isolated by gel purification. This leaves behind the remaining pBR322 DNA carried over from pCC18 (see Figure 4.7b). The 1.5kb fragment was ligated to EcoRI/EcoRV cut and phosphatased pIC20H to produce pFJH97. This chromosomal fragment is now free of IS1 and pBR322 sequences and can be isolated on a wide range of restriction fragments.

Cloning F factor DNA

The entire F plasmid has been previously cloned (Skurray *et al.* 1976)(Achtman *et al.* 1978). Unfortunately many of the authors concerned had moved laboratory or left the field entirely so that obtaining these clones would be inconvenient and time consuming. It seemed simpler to repeat their experiments and isolate our own F factor subclones. The F factor present in D7-8F$^+$ has previously been used to construct Hfrs capable of stimulating cut production (Jessop and Glansdorff 1980) and thus must contain the sites or sequences necessary for stimulation of Tn2901 amplification. D7-8F$^+$ was therefore used as the substrate for the isolation of F DNA and subsequent subcloning reported in this chapter. pFJH50 is probably not an ideal cloning vector as it is already 6.6kb in size and no information exists on the maximum size of insert possible with λdv plasmids. However it would be very convenient to clone F factor DNA directly into a vector that could be used to return that DNA to the chromosome without the need for subcloning. In their favour λdv plasmids have a medium range copy number (Boyd and Sherratt 1986)(personal observation). Total F factor DNA isolated from D7-8F$^+$ was digested with EcoRI, and ligated to EcoRI cut, phosphatased pFJH50. Cut and phosphatased pFJH50 DNA was religated without the addition of F DNA as a

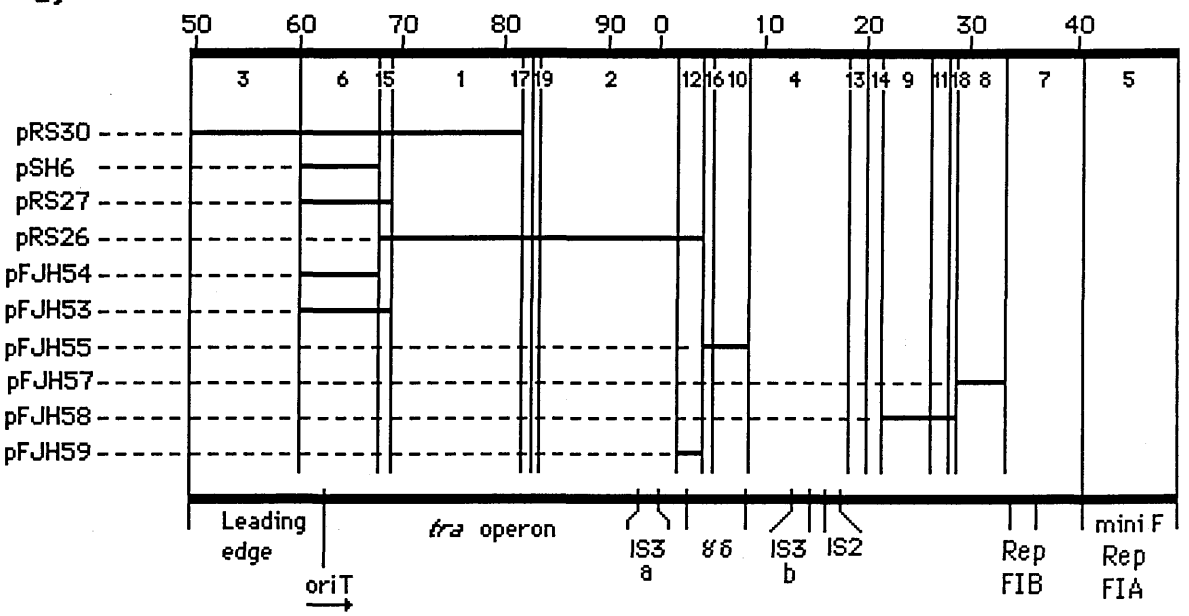
Figure 4.8

a)
Map of the F factor after Achtman *et al,* 1978. Kilobase coordinates are given in the original 94.5kb system with 0/94.5 placed at the end of IS3a and are shown above the heavy horizontal lines. The vertical lines represent EcoRI sites and the fragment numbers are given between them. The horizontal lines define inserts carried by various plasmids discussed in the text. The arrow under *ori* T shows the direction of conjugal DNA transfer with the leading edge entering the recipient bacteria first and the *tra* operon last.

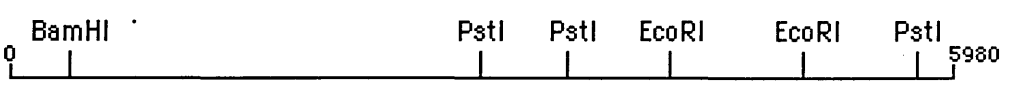
b)
Restriction maps of the transposon gamma delta (horizontal line) and plasmid pFJH55. F factor derived DNA is shown as a thick stippled box, the 19A vector sequences are shown by a single line, the insert chromosomal DNA is represented by a small boxed region and the 173bp of IS1 by a large unfilled box.

Figure 4.8

a)


50
60
70
80
90
0
10
20
30
40
3
6
15
1
17
19
2
12
16
10
4
13
14
9
11
18
8
7
5
pRS30
pSH6
pRS27
pRS26
pFJH54
pFJH53
pFJH55
pFJH57
pFJH58
pFJH59
Leading edge
oriT
tra operon
IS3 a
86
IS3 b
IS2
Rep FIB
mini F Rep FIA

b)


BamHI
PstI
PstI
EcoRI
EcoRI
PstI
0
5980

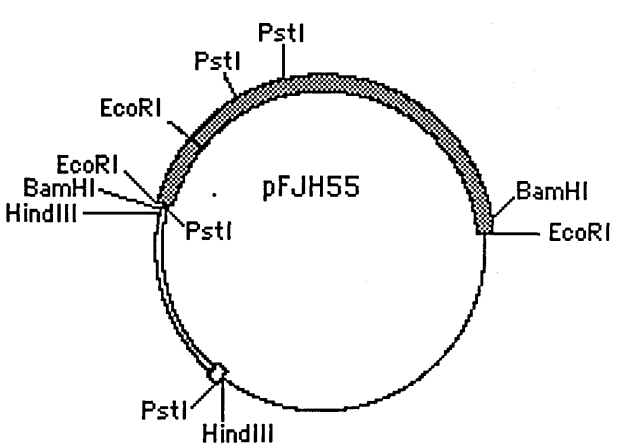

PstI
PstI
EcoRI
EcoRI
BamHI
HindIII
PstI
pFJH55
BamHI
EcoRI
PstI
HindIII

Figure 4.9

DNA from plasmids containing F factor derived inserts digested with a variety of restriction enzymes and run in E-buffer on 0.8% agarose slab gels. The gels were stained with EthBr.

a) pFJH54

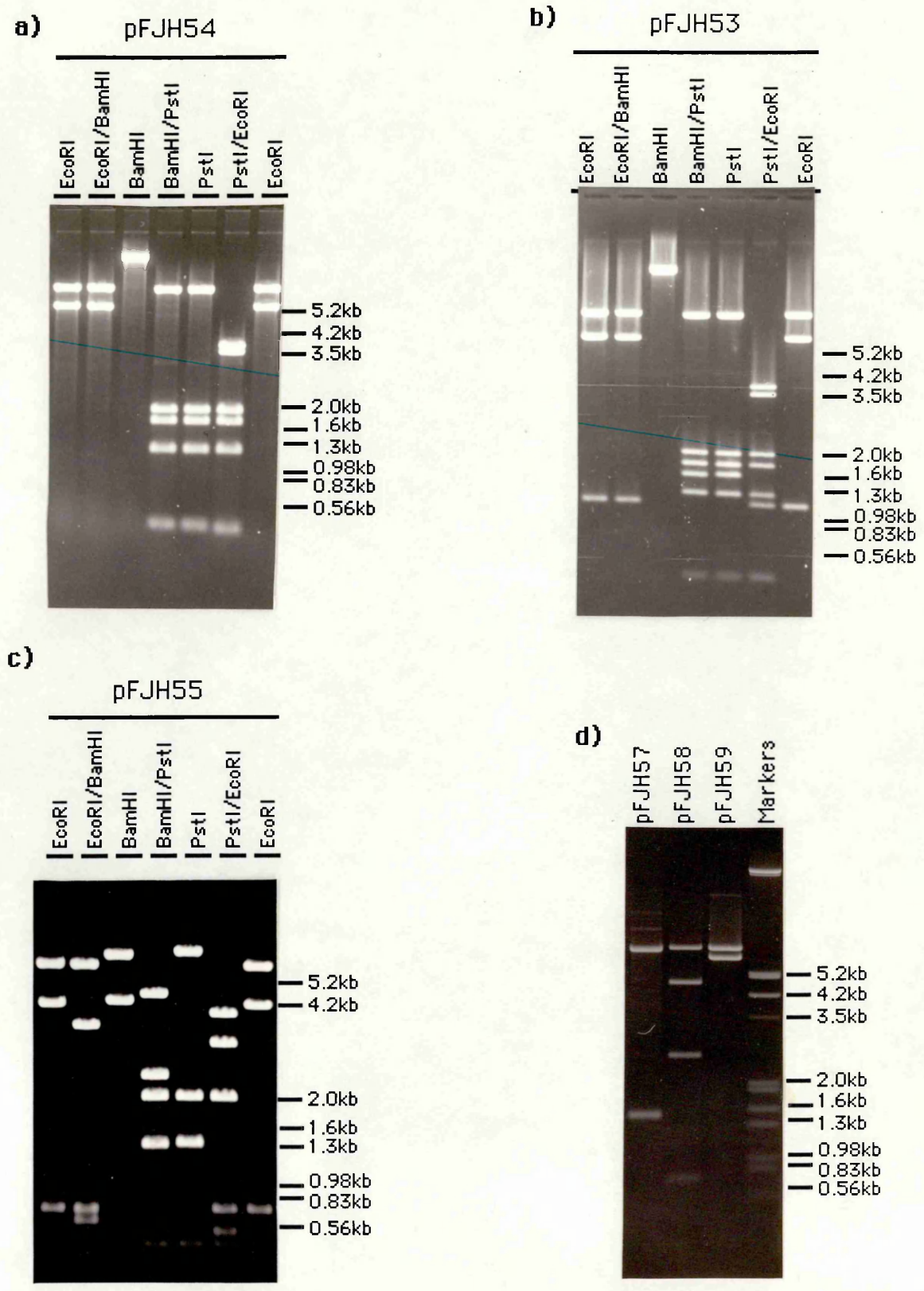
b) pFJH53

c) pFJH55

d)

DNA from pFJH57, pFJH58 and pFJH59 digested with EcoRI and run in E-buffer on a 0.8% agarose slab gel. The markers are λ DNA cut with HindIII. The gel was stained with EthBr.

Figure 4.9


a)
pFJH54
EcoRI
EcoRI/BamHI
BamHI
BamHI/PstI
PstI
PstI/EcoRI
EcoRI
5.2kb
4.2kb
3.5kb
2.0kb
1.6kb
1.3kb
0.98kb
0.83kb
0.56kb
b)
pFJH53
EcoRI
EcoRI/BamHI
BamHI
BamHI/PstI
PstI
PstI/EcoRI
EcoRI
5.2kb
4.2kb
3.5kb
2.0kb
1.6kb
1.3kb
0.98kb
0.83kb
0.56kb
c)
pFJH55
EcoRI
EcoRI/BamHI
BamHI
BamHI/PstI
PstI
PstI/EcoRI
EcoRI
5.2kb
4.2kb
2.0kb
1.6kb
1.3kb
0.98kb
0.83kb
0.56kb
d)
pFJH57
pFJH58
pFJH59
Markers
5.2kb
4.2kb
3.5kb
2.0kb
1.6kb
1.3kb
0.98kb
0.83kb
0.56kb

control. The ligation mixtures were used to transform competent DS902. The transformation efficiency with uncut pFJH50 was $5x10^5$ colonies per μg of DNA. 350 colonies potentially containing F DNA were isolated (equivalent to $2x10^3$/μg of DNA) and one transformant resulted from the control ligation (5/μg of DNA). The four most slowly growing colonies were examined on the grounds that they would probably contain the largest inserts. One failed to yield plasmid DNA, the other three (pFJH53, pFJH54 and pFJH55) are described below. The F factor contains nineteen sites for EcoRI and the nineteen resulting fragments are named in order of decreasing size f1 (largest) to f19 (smallest). These are shown in Figure 4.8a. Figures 4.9a and b show restriction digests of pFJH54 and 53 respectively. These are overlapping clones with insert sizes 8.4kb (pFJH54) and 9.6kb ie 8.4kb + 1.2kb (pFJH53). These sizes correspond to the f6 fragment (8.4kb) previously cloned as pSH6 (Achtman *et al.* 1978) and the f6 + f15 fragments (8.4kb + 1.2kb) previously cloned as pRS27 (Achtman *et al.* 1978). Restriction digest of pSH6 with PstI shows a similar pattern of bands (Flora Rodgers, personal communication). Since pSH6 contains the oriT site and traJ, L, E, K and B genes it should be possible to complement the *traJ* mutation in Jef8tra with pFJH53 and 54 to confirm these provisional identifications. Figure 4.9c shows restriction digests of pFJH55. The insert size is 5.2kb ie 4.4kb + 0.8kb. These sizes correspond to the f10 and f16 fragment sizes and the PstI and BamHI restriction pattern of pFJH55 corresponds very well with the known restriction map of γδ (from EMBL database) which constitutes the bulk of the f10 and f16 fragments (Figure 4.8b). Figure 4.9d shows EcoRI digests of three other isolates (pFJH57, pFJH58 and pFJH59) which have not been characterized further. pFJH57 contains a 5.8kb insert that may correspond to the f8 fragment. pFJH58 carries three EcoRI fragments of 4.7kb, 2.5kb and 0.68kb (f9+f11+f18?) and pFJH59 has a 1.35kb insert (f12?). The f12 fragment has previously been cloned as part of pRS26 (Achtman *et al.* 1978). It contains the remaining γδ DNA not isolated on pFJH55. Further restriction mapping of pFJH59 should determine if the 1.35kb insert is indeed f12. The f8, f9, f11 and f18 fragments of F have not been further described in

the literature. Confirmation that pFJH57 and pFJH58 carry these fragments can be most easily obtained by radiolabelling the plasmids and hybridizing them to EcoRI digested F DNA. These data are summarized in Figure 4.8a. pFJH50 already contains an insert of 2.3kb, pFJH53 contains an additional 9.6kb of DNA thus the maximum insert size in λdv plasmids must be of the order of 12kb. Further analysis of clones resulting from this experiment should allow the isolation of f6 to f19 fragments. The largest fragments f1 and f2 lie within the tra operon. Their involvement in this system has been eliminated by the experiments described above and in Jessop and Clugston, 1990. The f3 fragment however is of more interest as it contains the 'leading edge' of F. It encodes among other things the F factor single-strand binding protein. It has been previously cloned as pRS30 (Achtman *et al.* 1978) and it is possible that these authors would be able to supply this plasmid. The f4 fragment spans the IS2 and one of the IS3 insertions into the F factor while the f5 fragment constitutes the mini F replicon discussed below. Thus nine of the EcoRI fragments of F have been isolated in this cloning experiment.

Lysogeny in D7-8

Lysogeny in AB1157 is easily achieved and a number of AB1157 lysogens exist in our laboratory however D7-8 lysogens have proved more elusive and this section describes attempts to isolate them. As D7-8 is able to grow with maltose as sole carbon source (not shown) and therefore has not inherited the malT mutation from P678 (see Chapter 3, Figure 3.1) it should be capable of harbouring a λ lysogen. Four D7-8 strains (D7-8, D7-8F$^+$, D7-8*pro* and D7-8*prorec*A) and a derivative of Jef8 that has been cured of λ (P4Xλ$^-$, N. Glansdorff) were tested for their ability to produce lysogens. Strains were grown to mid log phase in L-broth, resuspended in one tenth volume 10mM $MgSO_4$ and 150μl were added to L-broth containing 10mM $MgSO_4$ and 0.6% agarose and spread on fresh R-agar plates. For each strain 10μl

Table 4.4

Testing various D7-8 strains for ability to produce cut colonies

Strain	$val^r/10^7$	$cut/10^7$	Time(days)
P4Xλ^-	1.0	80.0	8
P4Xλ^+	1.0	20.0	8
D7-8λ^-	2.5	0.7	6
D7-8λ^-	3.8	0.9	12
D7-8λ^+	4.0	1.0	6
D7-8λ^+	4.8	1.7	12
D7-8F$^+\lambda^-$	4.8	5.6	6
D7-8F$^+\lambda^-$	6.5	13.8	12
D7-8F$^+\lambda^+$	3.1	4.7	6
D7-8F$^+\lambda^+$	5.6	12.9	12

Strains were grown to mid log phase (about 10^7 cells/ml.) in minimal medium supplemented with glucose, arginine, uracil and other required amino acids. Cultures were plated on citrulline plates and controls as described in materials and methods. Numbers are mean values for three plates scored on the day shown from plates containing about 25 colonies.

spots of a dilution series of each of the following phages was applied: wild-type λ^+, λ^{vir} and λ^{cI}. All five strains showed turbid plaques with λ^+ and clear plaques with λ^{vir} and λ^{cI}. Thus D7-8 strains do not appear to be resistant to λ infection. Potential lysogens were streaked out on fresh R-agar from the centre of turbid λ^+ plaques. Six isolates from each strain were tested as follows: Fresh R-agar plates were streaked crosswise with each of the λ phages. Each isolate was then streaked crosswise at right angles to the phage streaks such that it intersected each of them. Isolates that showed no growth only at the intersection with λ^{vir} were then tested fully as described above. By this procedure it was possible to isolate λ lysogens from all 5 strains, although those isolated from D7-8 strains on occasion gave rise to one or a few spontaneous plaques when streaked out. Lysogens isolated from D7-8, D7-8F$^+$ and P4Xλ^- were tested for their ability to produce citrulline utilizing colonies. The results are shown in Table 4.4. The presence of λ lysogens in D7-8 strains appears not to significantly affect the numbers of cuts produced. Restoring strain P4Xλ^- to λ^+ status appears from this one experiment to reduce the number of cuts produced however there may be trivial reasons that can explain this result. It would be interesting to repeat this experiment unfortunately time did not allow me to do this.

Forced integration of λdv plasmids by lysogens

In a preliminary experiment AB1157λ^+ was transformed with the λdv plasmid pCC7. pCC7 carries the 2.3kb BamHI fragment from Tn2901 (Clugston 1986). Forced integration of this plasmid will result in the duplication of the 2.3kb BamHI fragment and the inclusion of the λdv plasmid into Tn2901. Eight transformants were isolated and mapped by conjugation and Southern analyses. Two isolates were found to have pCC7 integrated into Tn2901. In three of the other six isolates pCC7 was shown to be stably integrated into the λ genome (not shown). D7-8λ^+ was transformed with the λdv plasmid pFJH96

and with the pUC based plasmid pFJH87 (Chapter 5) as a transformation control. 1μg of pFJH87 DNA resulted in about 5 x 10^5 transformants whereas 1μg of pFJH96 DNA gave about 50 colonies. 16 colonies from D7-8λ::pFJH96 were tested for the presence of free plasmid by single colony gel analysis. No free plasmid was observed. Chromosomal DNA was prepared from two of these isolates, digested with EcoRI and subjected to southern analysis. pFJH96 contains 1.5kb of *E.coli* chromosomal DNA adjacent to the right hand IS1 element of Tn2901 and one EcoRI site. The same 1.5kb of chromosomal DNA is also available as an XbaI fragment in plasmid pFJH97. When the 1.5kb XbaI fragment from pFJH97 is used as a probe in southern analysis of EcoRI digested D7-8λ::pFJH96 isolates the following results can be expected. If pFJH96 is not integrated in the *E.coli* chromosome at any site a 6.2kb band representing linear pFJH96 and a 3.7kb band representing the EcoRI fragment from *E.coli* that spans the right hand IS1 element of Tn2901 and adjacent DNA should be seen (Figure 4.10a). If pFJH96 is integrated adjacent to Tn2901 two novel bands of 7.1kb and 2.8kb will be observed (Figure 4.10b). If however pFJH96 has integrated into the λ genome then one would expect to see the 3.7kb chromosomal band and a novel band larger than 6.2kb. The results of this experiment are shown in Figure 4.10c. In the first D7-8λ+ isolate strong hybridization to bands of 3.7kb and 6.2kb is observed. The second D7-8λ+ isolate shows a strongly hybridizing band of 3.7kb and a weakly hybridizing band of 6.2kb. Poor hybridization to the 6.2kb plasmid band in this second isolate suggests that the plasmid has been lost from many of the cells in this population. At least in these two isolates it would appear that pFJH96 is not stably integrated into the D7-8λ+ chromosome. In four isolates examined from an AB1157::pFJH96 strain none were found integrated adjacent to Tn2901. The third track of Figure 4.10c shows pFJH96 integrated into the λ genome in one of these isolates. The use of wild-type λ to force integration of λdv plasmids will therefore require the screening of a number of isolates.

Figure 4.10

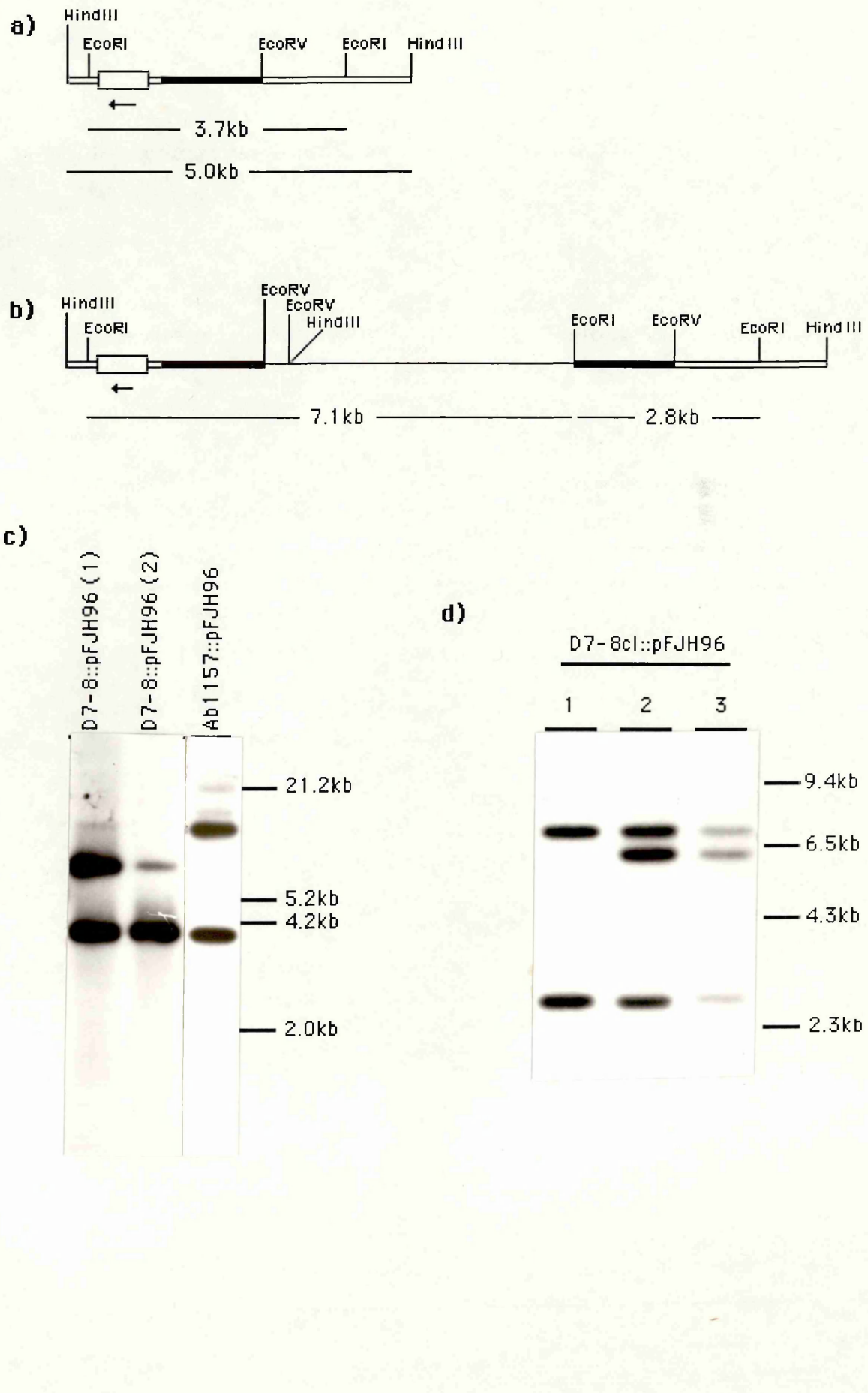
a)
A restriction map of the HindIII fragment that spans the right hand IS1 element of Tn2901. IS1 is represented by an open box and it's orientation as defined by nucleotide sequence is shown by the arrow below. Sizes of restriction fragments are indicated. The shaded region corresponds to the length of homology shared with plasmid pFJH96.

b)
The novel restriction map that will be generated by the integration of pFJH96 into the chromosomal region shown in a). Vector sequences are defined by a single line. The shaded areas of homology now constitute direct repeats. Sizes of EcoRI fragments are indicated.

c)
Autoradiograph of Southern blots from gels containing EcoRI cut DNA from strains D7-8::pFJH96 (1), D7-8::pFJH96 (2) and AB1157::pFJH96 probed with a random primed fragment from plasmid pFJH97 that is homologous to the shaded areas shown in diagrams a) and b). The blots were washed at high stringency and exposed overnight.

d)
Autoradiograph of a Southern blot from a gel containing EcoRI cut DNA from three isolates from strain D7-8cI::pFJH96 probed with a random primed fragment from plasmid pFJH97 that is homologous to the shaded areas shown in diagrams a) and b). The blot was washed at high stringency and exposed overnight.

Figure 4.10


a)
HindIII
EcoRI
EcoRV
EcoRI
HindIII
3.7kb
5.0kb
b)
HindIII
EcoRI
EcoRV
EcoRV
HindIII
EcoRI
EcoRV
EcoRI
HindIII
7.1kb
2.8kb
c)
D7-8::pFJH96 (1)
D7-8::pFJH96 (2)
Ab1157::pFJH96
21.2kb
5.2kb
4.2kb
2.0kb
d)
D7-8cl::pFJH96
1
2
3
9.4kb
6.5kb
4.3kb
2.3kb

Forced integration of λdv plasmids by overexpressing cI repressor

Overproduction of the λ cI repressor from a plasmid source can be sufficiently uncomfortable for *E.coli* that large scale propagation of the plasmid results in selection for deletions in the cI gene (Mary Burke, personal communication; Own data, not shown). A plasmid overexpressing the temperature sensitive λ cI857 repressor (pcI857(Remaut *et al.* 1983)) was kindly supplied by Noreen Murray. When grown at 37°C the repressor is inactivated and large quantities of plasmid can be isolated, while growth at 30°C results in production of active repressor that will force integration of λdv plasmids. D7-8cI (D7-8 carrying the pcI857 plasmid) was transformed with pFJH96. Three isolates from strain D7-8cI::pFJH96 were analysed. EcoRI digested DNA from these isolates probed with the 1.5kb XbaI fragment from pFJH97 will show bands of 7.1kb and 2.8kb when pFJH96 is integrated adjacent to Tn2901 or bands of 6.2kb and 3.7kb if it remains unintegrated (Figure 4.10a and b). All three isolates show bands of 7.1kb and 2.8kb (Figure 4.10d) however isolates 2 and 3 (lanes 2 and 3) also show a band of about 6.4kb hybridizing with equal intensity. This could represent an unintegrated plasmid band, but it appears slightly larger and no chromosomal fragment of 3.7kb is present. Since pFJH96 carries only one EcoRI site integration into pcI857 might produce a band of unexpected size. In either of these cases however one might expect this band to be of greater intensity than the, presumably, single copy bands resulting from integration into the chromosome and this, although not dramatic, would appear to be the case. The first isolate (lane 1) does not show this spurious band and was therefore chosen as the control strain (FJH13) for further experiments. The forced integration of λdv plasmids using a plasmid overexpressing the cI repressor rather than a λ lysogen appears to be cleaner and will require less screening of transformants.

4.2.3 The role of replication

Cloning the F origin of replication

Origins of replication have classically been cloned by marker rescue (Lovett and Helinski 1976) and this proved to be a simple and effective method. Plasmid pUC71K carries the aminoglycoside 3'-phosphotransferase (APH) gene from Tn903 conferring resistance to kanamycin, neomycin and G418 on a symmetrical polylinker (Vieira and Messing 1982). The APH gene was gel purified as a 1.5kb EcoRI fragment from pUC71K and ligated to EcoRI cut F DNA. On transformation of DS902 with this ligation reaction only four colonies were isolated however all contained the mini F replicon carrying the APH gene as determined by restriction digestion (not shown). This plasmid (mini F::APH) has been called pFJH98.

The mini F plasmid contains two BamHI sites. These sites conveniently divide mini F into a small (2695bp) *ori*V containing fragment and a large (6706bp) *ori*S fragment. pFJH98 was digested with BamHI. The resulting 2.7kb and 6.7kb fragments were gel purified and ligated to BamHI cut and phosphatased pFJH97. These ligations were used to transform DS902 and the plasmids pFJH91 (*ori*V) and pFJH92 (*ori*S), shown in Figure 4.11, were isolated. To obtain an *ori*V clone capable of functioning as a replication origin pFJH98 was digested with EcoRI and KpnI. The 3629bp fragment resulting from this digest was purified and ligated to EcoRI/KpnI cut and phosphatased pIC20H. This ligation was used to transform DS902 and plasmid pFJH134 was isolated (Figure 4.11). These three plasmids form the basis for all the constructs described below.

Returning *ori*V sequences to the chromosome

The *ori*V site on pFJH91 should not be able to initiate replication as it is missing required *cis* acting sequences, but should still

Figure 4.11

Restriction maps of
a) pFJH98
b) pFJH92
c) pFJH91
d) pFJH134
The vector sequences are shown by a single line, the insert chromosomal DNA is represented by small boxed regions, F factor DNA by large dark-stippled boxed areas and the Kanamycin resistance cassette by a large light-stippled boxed region.

Figure 4.11

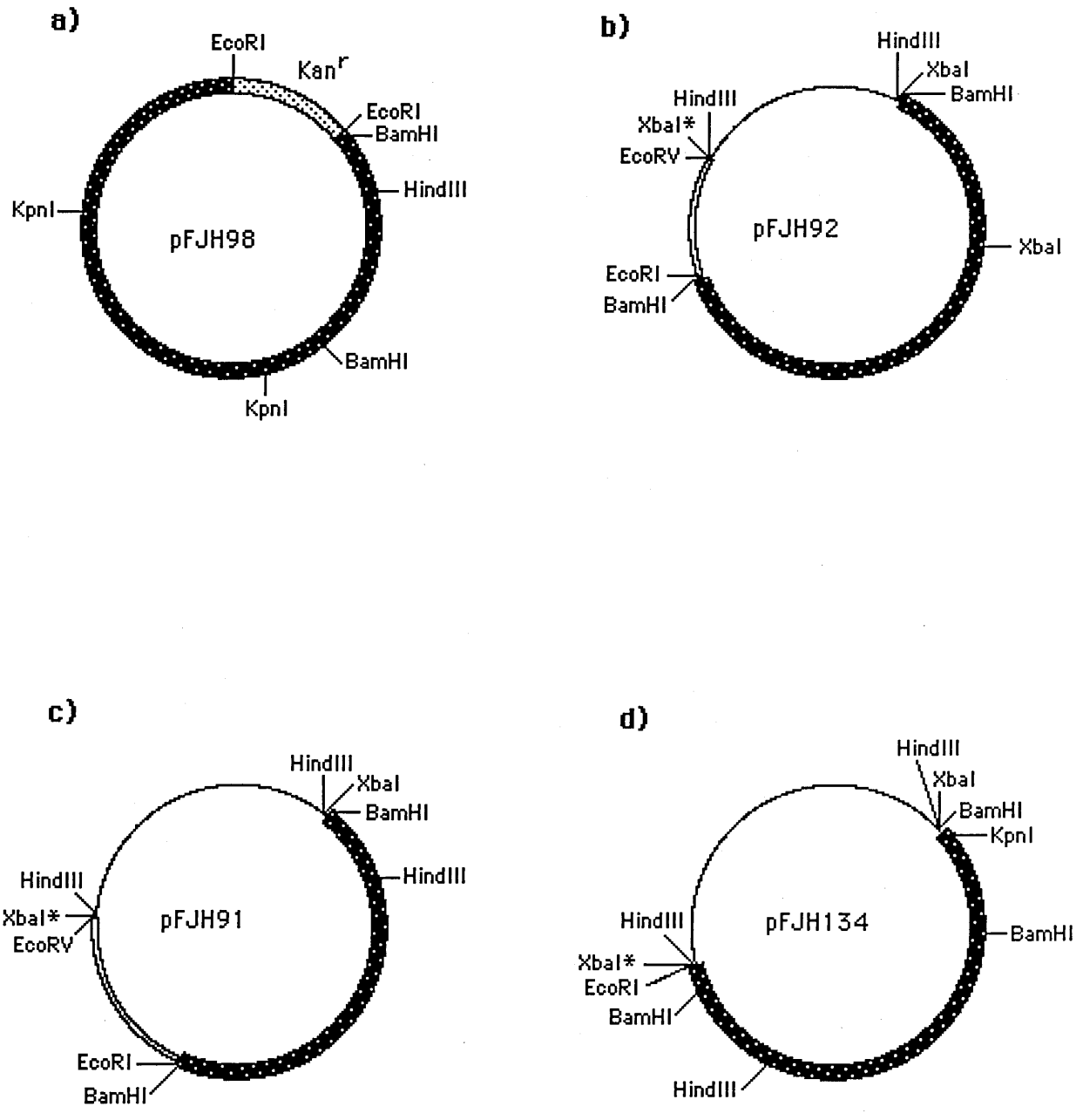

a)
EcoRI
Kan^r
EcoRI
BamHI
HindIII
KpnI
pFJH98
BamHI
KpnI
b)
HindIII
XbaI
BamHI
HindIII
XbaI*
EcoRV
pFJH92
XbaI
EcoRI
BamHI
c)
HindIII
XbaI
BamHI
HindIII
HindIII
XbaI*
EcoRV
pFJH91
EcoRI
BamHI
d)
HindIII
XbaI
BamHI
KpnI
HindIII
pFJH134
BamHI
XbaI*
EcoRI
BamHI
HindIII

Figure 4.12

Vector sequences are defined by a single line and F factor DNA by large dark-stippled boxed areas. IS1 is represented by large open boxes and it's orientation as defined by nucleotide sequence is shown by the arrows below. Chromosomal DNA is shown as small boxed regions with the shaded area corresponding to the length of homology shared between plasmid and chromosme. The arrows beneath *ori* V indicate the direction of replication initiated from this origin.

a)
Restriction map of pFJH121

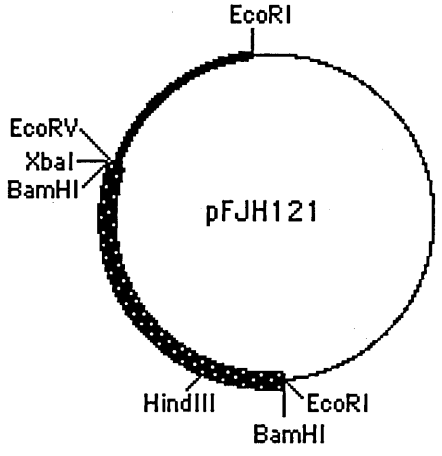
b)
The novel restriction map that will be generated by the integration of pFJH121 into the chromosomal region adjacent to Tn2901. Sizes of EcoRI fragments are indicated.

c)
Restriction map of pFJH135

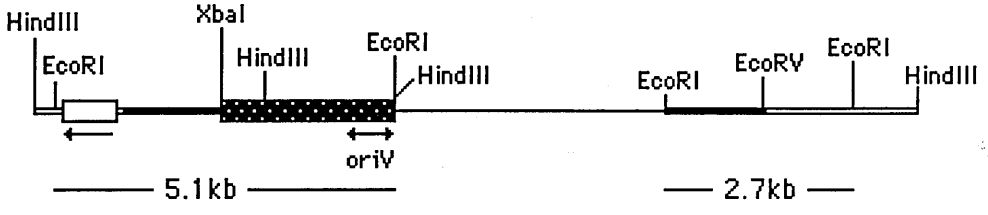
d)
The novel restriction map that will be generated by the integration of pFJH135 into the chromosomal region adjacent to Tn2901. Sizes of EcoRI fragments are indicated.

Figure 4.12

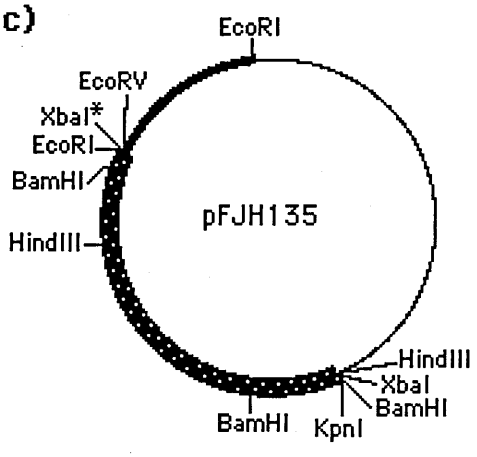
a)



b)



c)



d)

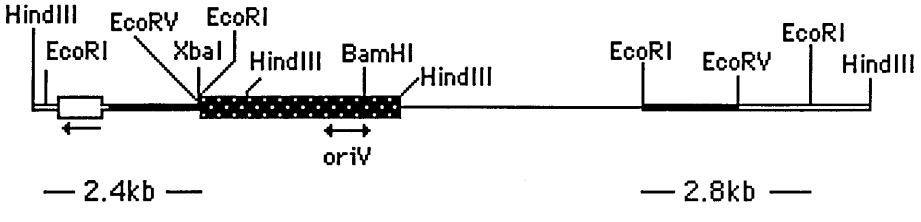


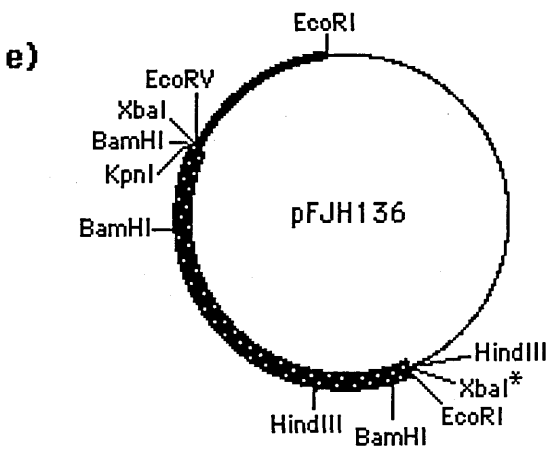
Figure 4.12

Vector sequences are defined by a single line and F factor DNA by large dark-stippled boxed areas. IS1 is represented by large open boxes and it's orientation as defined by nucleotide sequence is shown by the arrows below. Chromosomal DNA is shown as small boxed regions with the shaded area corresponding to the length of homology shared between plasmid and chromosome. Asterisks denote a restriction site that is sensitive to *dam* methylation. The arrows beneath *ori* V indicate the direction of replication initiated from this origin.

e)
Restriction map of pFJH136

f)
The novel restriction map that will be generated by the integration of pFJH136 into the chromosomal region adjacent to Tn2901. Sizes of EcoRI fragments are indicated.

Figure 4.12


e)
EcoRI
EcoRV
XbaI
BamHI
KpnI
BamHI
pFJH136
HindIII
XbaI*
EcoRI
HindIII
BamHI

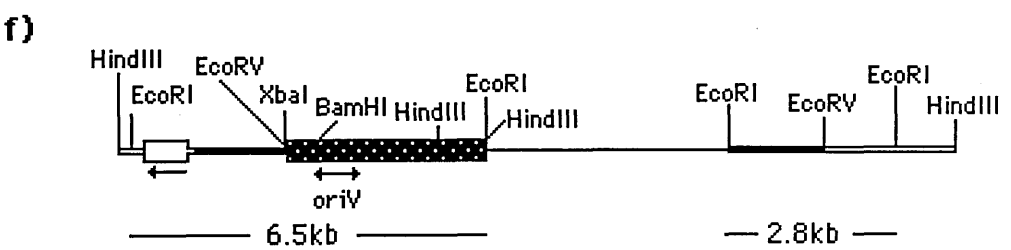

f)
HindIII
EcoRI
EcoRV
XbaI
BamHI
HindIII
EcoRI
HindIII
EcoRI
EcoRV
EcoRI
HindIII
oriV
6.5kb
2.8kb

Figure 4.13

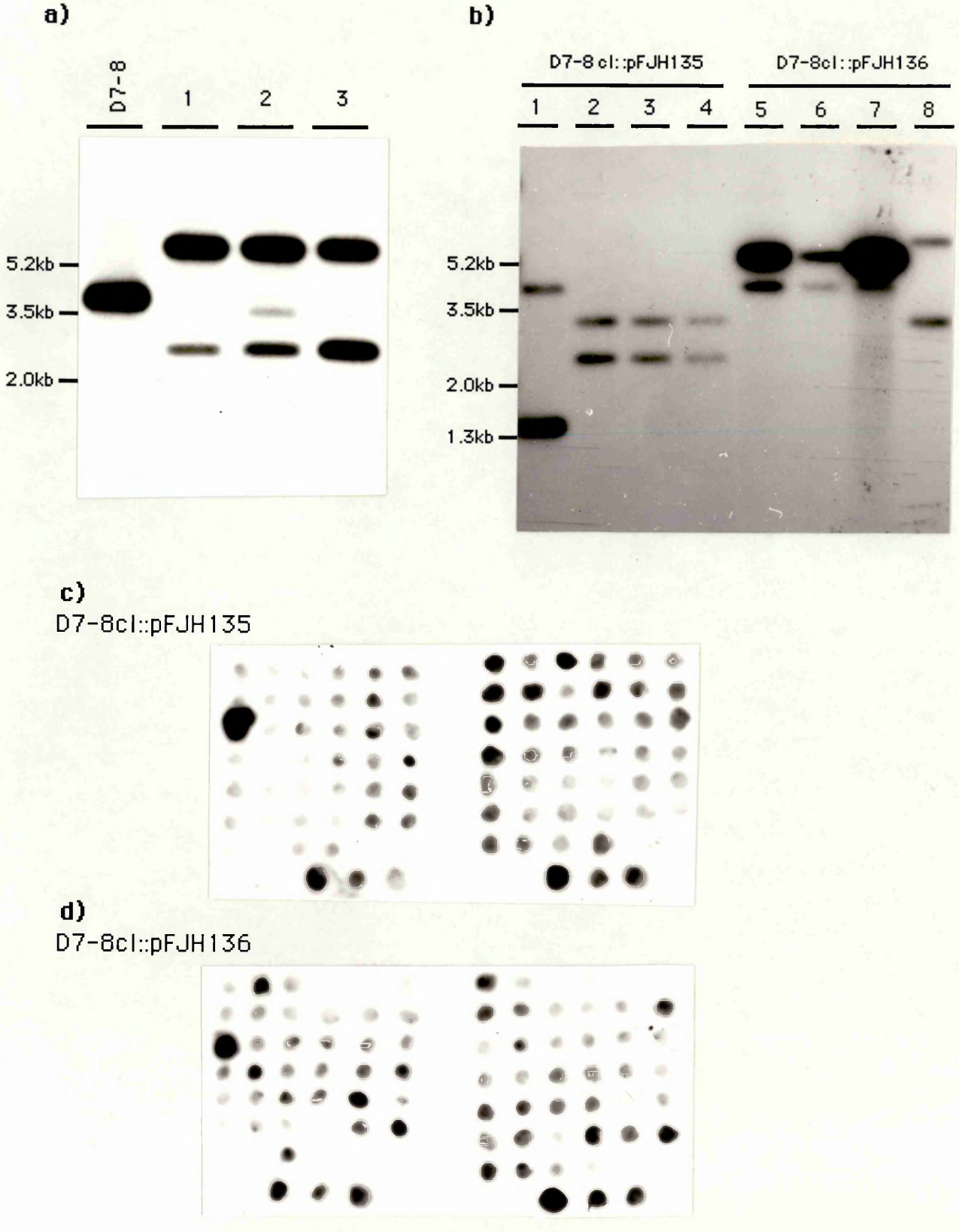
a)
Autoradiograph of a Southern blot from a gel containing EcoRI cut DNA from D7-8 and three isolates from strain D7-8cI::pFJH121 probed with a random primed fragment from plasmid pFJH121 that is described in the text and is homologous to the small shaded boxes shown in Figure 4.12. The blot was washed at high stringency and exposed overnight.

b)
Autoradiograph of a Southern blot from a gel containing EcoRI cut DNA from four isolates each from strains D7-8cI::pFJH135 and D7-8cI::pFJH136 probed with a random primed fragment from plasmid pFJH121 that is described in the text and is homologous to the small shaded boxes shown in Figure 4.12. The blot was washed at high stringency and exposed overnight.

c)
Autoradiograph from blots of cut colonies from strain D7-8cI::pFJH135 isolate 2 (FJH11) probed with nick translated pCC1. The three control strains at the bottom are from left to right; Cut1 which usually contains amplified Tn2901 DNA, Cut12 which occasionally contains amplified DNA and D7-8cut which has not been seen to amplify Tn2901. The blot was washed at high stringency and exposed for four hours.

d)
Autoradiograph from blots of cut colonies from strain D7-8cI::pFJH136 isolate 4 (FJH12) probed with nick translated pCC1. The three control strains at the bottom are from left to right; Cut1 which usually contains amplified Tn2901 DNA, Cut12 which occasionally contains amplified DNA and D7-8cut which has not been seen to amplify Tn2901. The blot was washed at high stringency and exposed for four hours.

Figure 4.13


a)
D7-8
1
2
3
5.2kb
3.5kb
2.0kb
b)
D7-8 cl::pFJH135
D7-8cl::pFJH136
1
2
3
4
5
6
7
8
5.2kb
3.5kb
2.0kb
1.3kb
c)
D7-8cl::pFJH135
d)
D7-8cl::pFJH136

provide a site for the site-specific recombination attributed to *ori*V. On the other hand the extended *ori*V site on pFJH134 should be capable of functioning as an origin of replication, but only if the repE protein is provided *in trans* (Lane *et al.* 1984). To move *ori*V sequences to a λdv plasmid unmethylated pFJH91 was digested with XbaI and the resulting 4.3kb fragment (*ori*V and 1.5kb chromosomal region) was gel purified. The 4.3kb XbaI fragment was cut with EcoRI and ligated to EcoRI cut and phosphatased 19A. The ligation reaction was used to transform DS902 and plasmid pFJH121 was isolated. pFJH121 gives two bands of 4.3kb on digestion with EcoRI, two bands of 5.8kb and 2.8kb with BamHI and on EcoRI/BamHI double digestion three bands of 4.3kb, 2.8kb and 1.5kb are obtained. Figure 4.12a shows a restriction map of pFJH121. To move the extended *ori*V sequence to a λdv plasmid the 3.5kb XbaI fragment was isolated from unmethylated pFJH134 DNA and ligated to the 5.8kb XbaI fragment (λdv plus 1.5kb chromosomal region) from pFJH121. The ligation reaction was used to transform DS902 and plasmids pFJH135 and pFJH136 were isolated. When pFJH135 is digested with EcoRI two bands of 7.8kb and 1.5kb are obtained while digestion of pFJH136 with EcoRI gives bands of 5.0kb and 4.3kb. Restriction maps of pFJH135 and pFJH136 are shown in Figures 4.12c and e. D7-8cI (D7-8 carrying the cI overexpressing plasmid pcI857) was transformed with pFJH121, pFJH135 and pFJH136. Chromosomal DNA prepared from three isolates from D7-8cI::pFJH121 was digested with EcoRI and subjected to Southern analysis. The probe for this experiment is the 1.5kb EcoRI/BamHI fragment from pFJH121 containing *E.coli* chromosomal DNA. It will hybridize to bands of 4.3kb and 3.7kb if integration has not occurred and to bands of 5.1kb and 2.7kb if integration is adjacent to Tn2901 (Figure 4.12b). The result of this Southern analysis is shown in Figure 4.13a. Isolates 1 and 2 (lanes 2 and 3) show reduced hybridization of the probe to the 2.7kb band compared with the 5.1 kb band suggesting that some rearrangement has ocurred. Lane 1 contains EcoRI digested D7-8 DNA to which the probe shows hybridization only to the 3.7kb chromosomal band. Isolate 3 (lane 4), FJH10, shows hybridization to bands of 5.1kb and 2.7kb and hence was

chosen for further analysis. Four isolates each from D7-8cI::pFJH135 and D7-8cI::pFJH136 were subjected to a similar analysis. Chromosomal DNA was prepared, digested with EcoRI, blotted to Hybond-N and probed with the probe described above. If pFJH135 is not integrated into the chromosome bands of 3.7kb and 1.5kb are expected while integration of this plasmid adjacent to Tn2901 should produce bands of 2.8kb and 2.4kb (Figure 4.12d). If pFJH136 is not integrated the expected band sizes are 5.0kb and 3.7kb, but integrated adjacent to Tn2901 bands of 6.5kb and 2.8kb should be seen (Figure 4.12f). Figure 4.13b shows the results of these experiments. Of the four isolates from D7-8cI::pFJH135, one is unintegrated (lane 1) while three are integrated adjacent to Tn2901 (lanes 2-4). Isolate 2 (FJH11) was chosen for further analysis. Of the four D7-8cI::pFJH136 isolates three are unintegrated and show far stronger hybridization to the 5.0kb plasmid band than to the 3.7kb chromosomal band (lanes 5-7). There are three possible explanations for this; the λdv plasmids are not being repressed by the cI repressor, *ori*V is functioning without RepE protein or integration has occurred into the pcI857 plasmid. The last of these seems the most likely explanation. The fourth isolate from D7-8cI::pFJH136 (lane 8) is integrated adjacent to Tn2901 and was chosen for further analysis (FJH12).

Do *ori*V sequences stimulate cut production?

Strains FJH10, 11, 12 and 13 were tested for ability to produce citrulline utilizing colonies as previously described. Although *ori*V *cis*-acting sequences are present none of these strains should be able to initiate replication from them. It should however be possible to activate the *ori*V origins in FJH11 and 12 by supplying RepE protein *in trans* (Lane *et al.* 1984). pFJH92 carries the *ori*S region and DNA encoding RepE. pFJH92 is a pUC based plasmid and as such is compatible with pcI857 (pACYC based) and the λdv plasmids already resident in these strains. FJH10, 11 and 12 were transformed with pFJH92 and the

resulting strains were tested for ability to produce cut mutants. The results are shown in Table 4.5. No increase in cut production was apparent in FJH11 and 12 compared with the control strain FJH10.

In order to determine if the *ori*V sequences in FJH11 and 12 are capable of initiating replication in the presence of RepE protein provided by pFJH92, I attempted to 'marker rescue' them using the APH gene from pUC71K. The F factor DNA used to construct FJH11 and 12 is contained in pFJH134 and is most easily isolated on an XbaI fragment. To obtain the APH gene on an XbaI fragment pUC71K was digested with EcoRI and the 1.5kb APH containing fragment was gel purified. The 1.5kb EcoRI fragment was ligated to EcoRI cut and phosphatased pIC20H and the reaction used to transform the *dam*$^-$ strain CB51. The resulting Kan^r plasmid pFJH140 gave bands of 2.7kb and 1.5kb on digestion with XbaI. The 1.5kb XbaI fragment was ligated to the 3.5kb phosphatased XbaI fragment isolated from unmethylated pFJH134. The ligation reaction was used to transform competent D7-8/pFJH92 (D7-8 carrying pFJH92) and plated on L-agar containing kanamycin. Four colonies were isolated. All four were able to grow on ampicillin and when digested with HindIII or BamHI produced a band of 2.7kb diagnostic of pUC-based vectors. Two contained inserts of 1.5kb (probably the APH gene) and two showed 5kb inserts (F DNA and APH gene). This suggests that the experiment was contaminated with low levels of pUC at some stage. When the experiment was repeated using twice gel purified fragments and new (unused) reaction tubes no colonies were obtained.

Failure to *trans*-activate the *ori*V sequences with pFJH92 in this assay suggests that they will not be active in strains FJH11 and 12. Lane *et al.* (1984) showed *trans*-activation of *ori*V sequences ligated to a pBR322 derivative in a *pol*A strain. At non-permissive temperatures and in the presence of mini-F sequences equivalent to those contained in pFJH92, *ori*V reduced ten-fold the number of plasmid free cells observed. However in the absence of any F sequences their pBR derived vector was maintained in around 60% of cells. It is therefore possible that *trans*-activation of *ori*V does not occur frequently enough to

produce an autonomous plasmid, but may occur sporadically in the chromosome.

I tested 80 citrulline utilizing colonies from FJH11 and 12 for the presence of pFJH92, pcI857 and F::λdv plasmids by patching to plates containing ampicillin, kanamycin or chloramphenicol respectively. While all colonies retained the λdv plasmid and the cI overexpressing plasmid, none were able to grow on ampicillin suggesting that pFJH92 had been lost. Since *ori*V sequences are *trans*-activated infrequently (if at all) and pFJH92 is lost during growth on citrulline the data in Table 4.5 can not be used to assess the contribution of replication to the citrulline utilizing phenomena. It can however be said that *ori*V sequences *per se* do not contribute significantly to the number of cuts produced. This does not eliminate the involvement of the site-specific recombination system attributed to *ori*V as resolution of *ori*V-induced dimers requires the product of the *res*D gene *in trans*. Whether this system contributes to the amplification of Tn2901 could be determined by supplying ResD from a low copy number plasmid when plating on citrulline.

The *ori*S origin of replication

The *ori*S site overlaps the coding region for the repE protein and cannot be separated from it. The 6.7kb fragment cloned as part of pFJH92 should therefore be capable of autonomous replication. pFJH92, however, is isolated in large quantities when plasmid DNA is prepared from cells harbouring it suggesting that in this plasmid replication is under the control of the pUC derived replicon. To move *ori*S to a λdv vector the 8.2kb HindIII fragment from pFJH92 (carrying the *ori*S region and the 1.5kb chromosomal fragment) was ligated to HindIII cut and phosphatased 19A. The resulting plasmid pFJH110 was isolated from transformed DS902 cells. Methylated pFJH110 is cut twice by XbaI, giving fragments of 10.5kb and 2.1kb[1] , twice

[1] pFJH110 contains a third site for XbaI, but it forms part of a *dam* methylation site and is not cut in methylated DNA.

Table 4.5

Strain	Integrated plasmid	$val^r/10^7$	$cut/10^7$
FJH10	pFJH121	2	8
FJH10/pFJH92	pFJH121	1	14
FJH11	pFJH135	11	14
FJH11/pFJH92	pFJH135	1	2
FJH12	pFJH136	4	16
FJH12/pFJH92	pFJH136	3	14
FJH13	pFJH96	9	21
D7-8cI	None	1	10
Jef8	F	3	200

Strains were grown to mid log phase (about $5x10^6$ cells/ml.) in minimal medium supplemented with glucose, arginine, uracil and other required amino acids. Cultures were plated on citrulline plates and controls as described in materials and methods. Numbers are mean values for three plates scored after 8 days from plates containing about 30 colonies except in the case of Jef8 where plates scored contained about 300 colonies.

Figure 4.14

Vector sequences are defined by a single line and F factor DNA by large dark-stippled boxed areas. IS1 is represented by large open boxes and it's orientation as defined by nucleotide sequence is shown by the arrows below. Chromosomal DNA is shown as small boxed regions with the shaded area corresponding to the length of homology shared with the plasmids. Asterisks denote a restriction site that is sensitive to *dam* methylation. The arrow beneath *ori* S indicates the direction of replication initiated from this origin.

a)
Restriction map of pFJH110

b)
The novel restriction map that will be generated by the integration of pFJH110 into the chromosomal region adjacent to Tn2901. Sizes of HindIII fragments are indicated.

c)
The novel restriction map that will be generated by the integration of pFJH122 into the chromosomal region adjacent to Tn2901.

d) and **e)**
Restriction maps of pFJH122 and pFJH123 respectively.

Figure 4.14

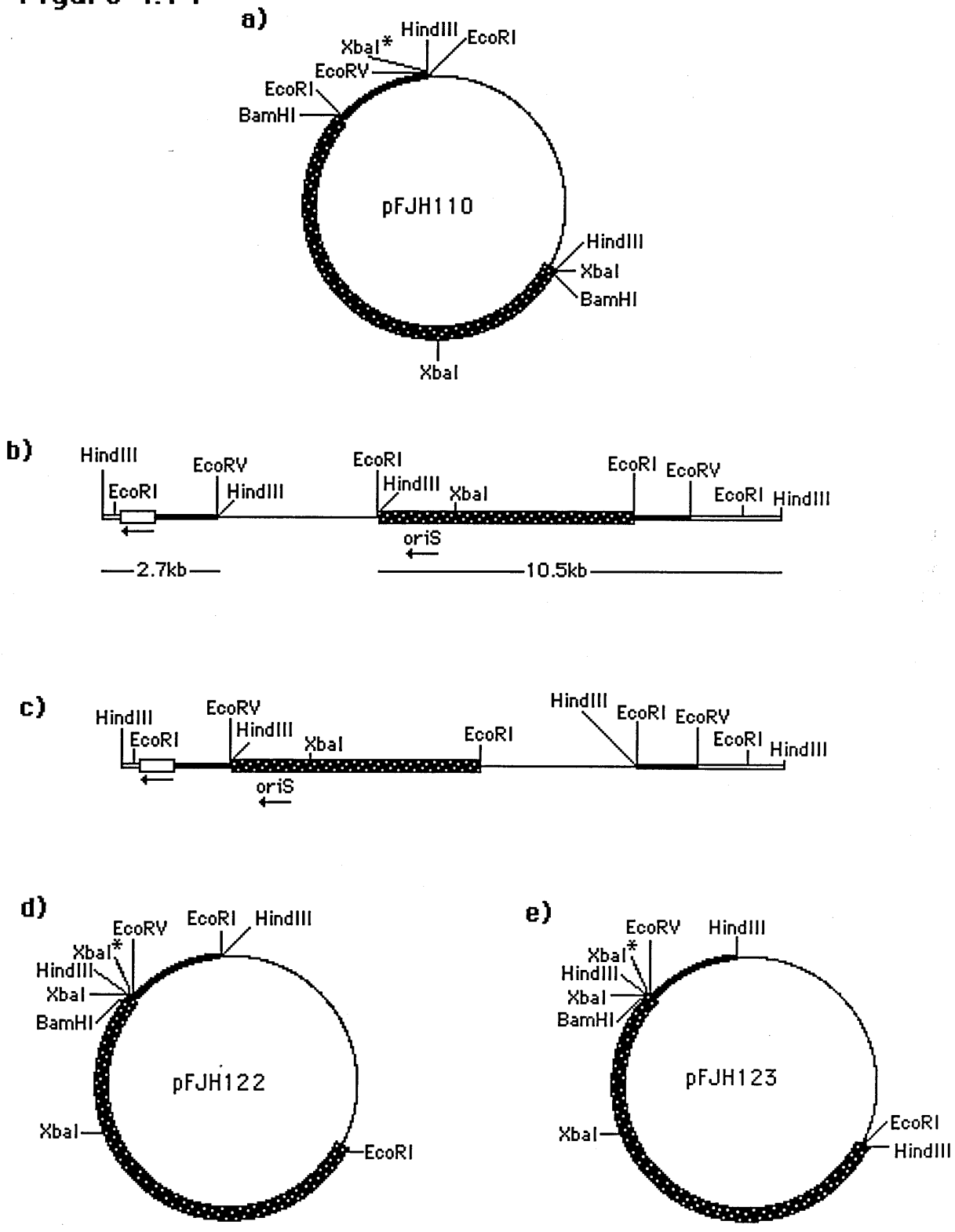
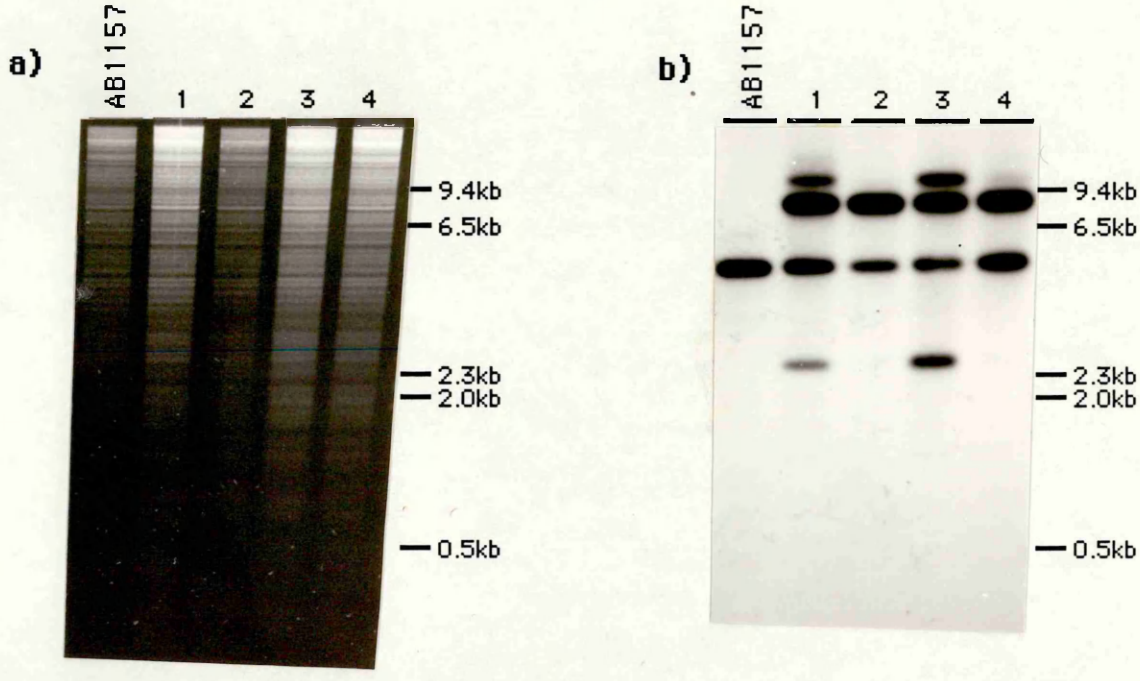

a)
XbaI*
HindIII
EcoRI
EcoRV
EcoRI
BamHI
pFJH110
HindIII
XbaI
BamHI
XbaI
b)
HindIII
EcoRV
EcoRI
EcoRI
HindIII
HindIII
XbaI
EcoRI
EcoRV
EcoRI
HindIII
oriS
2.7kb
10.5kb
c)
HindIII
EcoRV
EcoRI
HindIII
XbaI
EcoRI
HindIII
EcoRI
EcoRV
EcoRI
HindIII
oriS
d)
EcoRV
EcoRI
HindIII
XbaI*
HindIII
XbaI
BamHI
pFJH122
XbaI
EcoRI
e)
EcoRV
HindIII
XbaI*
HindIII
XbaI
BamHI
pFJH123
XbaI
EcoRI
HindIII

Figure 4.15

a)
DNA prepared from strain AB1157 and four isolates from strain AB1157::pFJH110 digested with HindIII and run in E-buffer on a 0.7% agarose slab gel. The gel was stained with EthBr.

b)
Autoradiograph from the Southern blot of the gel in a) probed with a random primed fragment from plasmid pFJH97 that is described in the text and is homologous to the small shaded boxes shown in Figure 4.14. The blot was washed at high stringency and exposed overnight.

Figure 4.15


a)
AB1157
1
2
3
4
9.4kb
6.5kb
2.3kb
2.0kb
0.5kb
b)
AB1157
1
2
3
4
9.4kb
6.5kb
2.3kb
2.0kb
0.5kb

by HindIII to give 8.2kb and 4.3kb fragments and twice by BamHI producing 6.8kb and 5.7kb fragments. A restriction map of pFJH110 is shown in Figure 4.14a. Notice that in this orientation *ori*S will initiate replication that will travel through the λdv vector before reaching Tn2901 (Figure 4.14b). To isolate *ori*S in the opposite orientation with respect to the vector the 8.2kb HindIII fragment from pFJH92 was cut with EcoRI and the two resulting fragments were ligated to EcoRI cut phosphatased 19A. When this ligation reaction was used to transform DS902 plasmids pFJH122 and pFJH123 were isolated. pFJH122 produces bands of 8.2kb and 4.3kb on digestion with EcoRI and bands of 11kb and 1.5kb on digestion with either BamHI or HindIII. Digestion of pFJH123 with EcoRI also gives fragments of 8.2kb and 4.3kb while digestion with BamHI or HindIII leaves bands of 6.7kb and 5.8kb. Restriction maps of these two plasmids are shown in Figure 4.14c and d. AB1157 was transformed with pFJH110. Resulting colonies were subjected to single colony gel analysis. No DNA corresponding to pFJH110 was visible. Chromosomal DNA prepared from these isolates was digested with HindIII, run on a 0.8% agarose gel and subjected to Southern analysis. The blot was probed with the 1.5kb XbaI fragment from pFJH97 radiolabelled by random priming. This fragment is derives from chromosomal DNA adjacent to Tn2901 and will hybridize to a chromosomal band of 5.0kb[2] and a plasmid band of 8.2kb if pFJH110 is not integrated or is integrated into the λ genome. If pFJH110 is integrated into the chromosome adjacent to Tn2901 then novel bands of 10.5kb and 2.7kb should be seen (Figure 4.14b). Figure 4.15 shows chromosomal digests of four isolates from strain AB1157::pFJH110 and the autoradiograph resulting from Southern analysis. In two of the isolates (1 and 3) bands of 10.5kb and 2.7kb are seen. It is therefore possible to force the integration of the *ori*S region using the λdv system, but this insertion appears to be unstable. It may be possible to stabilize this insertion in a *rec*$^-$ background. Unfortunately the amplification of Tn2901, our only measure of events stimulated

[2] Calculated using the restriction map of the *E.coli* chromosome generated by Kohara *et al* , 1988.

by the F factor, is *rec*A dependent. My final experiment concerning replication is described below.

Transformation of NGX2 with a gene bank constructed from Jef8

If replication from the F factor is important in the cut phenotype then it should occur in Jef8 and it may be possible to observe it's effects in a Jef8 gene bank. Carol Clugston constructed her gene bank by ligating size selected Sau3A digested genomic DNA from Jef8 into the BamHI site of pBR322. The strain NGX2 has the genotype: *arg*F, *arg*I, *pyr*B, *pro*A, *leu*[1], *lac* , *gal*[2] , *xyl*. NGX2 was transformed with gene bank DNA and plated on; a) complete medium or minimal medium with glucose and all required supplements to determine the number of viable cells, b) complete medium with ampicillin to calculate the transformation efficiency and c) minimal medium with glucose and all but one required supplement or with all required supplements and either xylose or galactose to measure the ability of gene bank DNA to complement each of the mutations in NGX2. Untransformed NGX2 was plated on each of these media to check for reversion to wild type. The results are shown in Table 4.6. After transformation the number of viable cells is seen to be $7x10^6$ on complete medium or $2x10^5$ on minimal medium. The transformation efficiency is $1x10^5$ colonies/lg DNA. Pyr^+ and xyl^+ transformants are seen at about the same frequency. Arg^+ transformants are seen at double this frequency which is most easily explained by the presence of two genes (*arg*F and *arg*I) capable of complementing the arg^- phenotype. Gal^+ and leu^+ transformants are seen at about two thirds of this frequency which may be within the plating error range of the experiment. Pro^+ and lac^+ transformants are seen at about three times this

[1] This mutation maps at 2 minutes ie within the *leu* ABCD operon.
[2] This mutation maps at 17 minutes ie within the *gal* EKT operon.

Table 4.6

Supplements added to media	A	B	C
glucose, uracil, leucine, proline	arg^+	60	0.2
glucose, arginine, leucine, proline	ura^+	33	0.4
glucose, arginine, uracil, proline	leu^+	20	0.2
glucose, arginine, uracil, leucine	pro^+	83	0
lactose, arginine, uracil, leucine, proline	lac^+	93	0
xylose, arginine, uracil, leucine, proline	xyl^+	32	0
galactose, arginine, uracil, leucine, proline	gal^+	24	0

Strain NGX2 (*arg*F, *arg*I, *pyr*B, *pro*A, *leu*, *lac*, *gal*, *xyl*) was made competent as described in materials and methods transformed with a gene bank that had been constructed from Jef8 DNA and plated on minimal medium with the supplements shown above. The phenotype selected is shown in column A and the number of colonies per 10^5 cells obtained is shown in column B. Column C shows the number of colonies obtained from untransformed NGX2 plated on the same media. Numbers are mean values for five plates.

frequency, a result which I am confident can not be attributed to plating error. Hence pro$^+$ and lac$^+$ bearing plasmids appear to be overrepresented in this gene bank.

A DNA segment may be under-represented in this type of experiment if it's presence on multicopy plasmids is deleterious to the cell. Since all the genes selected here have previously been cloned on multicopy plasmids this problem is unlikely to affect the frequency of transformation seen here.
Transformation frequencies may also be skewed if a gene contains an excess or paucity of Sau3A sites. This can not be discounted as an explanation for the range of frequencies observed here although an examination of the restriction map of the *E.coli* chromosome (Kohara *et al.* 1987) shows no unusual clustering of BamHI sites in the regions encompassing these genes[3]. The Kohara map was generated using the K-12 strain W3110 in lambda vectors. Knott *et al.*, were able to fill the 8 gaps in the Kohara map using cosmid clones constructed on strain K-12 803 (Knott *et al.* 1989). Their analysis of the distribution of cosmid clones at and adjacent to these gaps shows that 7 of these regions are difficult to clone in either type of vector (perhaps not surprisingly in the case of *ori*C or *rrn*D and *rrn*E which are end-points of a large inversion in W3110). They found that the eighth gap at *pyr*B was bridged by the number of clones expected assuming an even distribution of clones in their cosmid library. Furthermore while the gap adjacent to *xyl* was underrepresented, the clone distribution spanning *xyl* itself was normal. There was no gap in the Kohara map in the *pro-lac* region however *E.coli* 803 has suffered an inversion and deletion here. Analysis of clone distribution in this region shows that *lac*I is preferentially cloned in cosmids, with nearly twice as many clones apparent as would be expected in a normal distribution while clones spanning the *pro*AB region are represented at or slightly less than the number expected (Knott *et al.* 1989). While it would be naive to extrapolate from a system using a different strain and cosmid vectors containing large inserts, the random distribution of clones in the *pyr*B and *xyl* regions is reassuring.

---

[3] Sau3A cuts at the sequence GATC which forms the core of the sequence recognised by BamHI (GGATCC).

A number of other factors could affect clone distribution, for instance, the generation of favourable/unfavourable local topologies or the presence of small regions of homology that result in deletion. Thus an accumulation of trivial factors could explain the distribution observed here, however an interesting possibility exists; that the increase in frequency observed for Pro^+ (and possibly Lac^+) transformants in NGX2 is the result of replication initiated from the F factor in Jef8.

4.3 Discussion

The experiments and results described at the start of this chapter complement those of other workers in this laboratory, now in press (Clugston and Jessop 1990). We have shown that a derivative of Jef8; Jef8tra carries a mutation in the *traJ* gene and is still capable of producing large numbers of citrulline utilizing mutants. In the absence of traJ few, if any, of the *tra* gene products will be made. In particular *traJ* mutants do not produce the *tra*YZ endonuclease required for nicking at *ori*T.

While it is possible that the Jef8 strain used to construct Jef8tra was already capable of amplifying Tn2901 we feel this is unlikely. When Jef8 is plated on citrulline cut colonies do not arise immediately. A few colonies appear after 2 days and thereafter new colonies continue to appear until the plates are exhausted (Chapter 3). This has led us to believe that the majority of cut mutants are not pre-existing, but arise on the plates. Jef8tra shows a similar growth pattern on citrulline and since it is not capable of undergoing conjugation on the citrulline plates we feel that this is strong evidence against the hypothesis that the stimulation of cut production by the F factor is due to conjugation or events at *ori*T. In support of this we have conducted experiments using a different system; introduction of F' plasmids into F^- strains that produce few citrulline utilizers. The introduction of F' plasmids to F^- strains significantly increases cut production although not to the levels seen in Jef8 (Clugston and Jessop 1990). The simplest explanation for this difference is that the F factor is required *in cis* for stimulation of cut production and that F' plasmids must first integrate into the chromosome of the recipient strain thus reducing the efficiency of cut production compared with Hfr Jef8. The F' system also differs from the use of Hfrs in that it is necessary to introduce a multicopy plasmid that expresses RecA protein. Failure to maintain selection for the recA encoding plasmid pPE14 on citrulline results in a reduction of cut yield (Table 4.3). This reduction may represent the contribution to cut stimulation resulting from the presence of a multicopy plasmid (see Chapter

3). Alternatively the reduction in cut yield may result from the loss of RecA function, in line with our suspicion that cut production is a dynamic process that continues after plating. As pPE14 is present in all F' strains tested this does not affect our conclusions, but it might be interesting to test the F- strain containing only pPE14 as a control in future F' experiments. We have shown that F'Δ*tra* plasmids are as capable as ordinary F' plasmids of supporting increased cut production and amplification of Tn2901. In addition, by using as a donor a strain shown to be deleted for Tn2901, we have eliminated the possibility that the cut phenotype is introduced on the F' plasmid itself. While the end points of these F'Δ*tra* plasmids are not known we can safely assume that they do not contain any DNA encoding genes of the transfer operon. Thus if conjugal transfer itself or any of the *tra* gene products are involved in stimulating the amplification of Tn2901 their contribution is minor.

Having eliminated conjugation as the major player in cut production, we were interested in developing a system of molecular analysis that could be used to answer a variety of questions. The insertion of cloned F fragments and the replacement of the IS1 elements using insertion vectors is such a system and λdv plasmids were chosen as the vectors. λdv plasmids carrying F fragments were forced to integrate into F- strains using either λ lysogens or cI overexpressing plasmids. The use of lysogens is marred by the sometimes incomplete suppression, resulting in free plasmid, and the number of isolates that need to be screened to eliminate those containing insertions into the λ genome. However, by careful screening, strains carrying stably integrated plasmid can be obtained. By comparison the use of a cI overexpressing plasmid appears to be cleaner and to require less screening. A second advantage of pcI857 over lysogens is the ability to inactivate the repressor by growth at 37°C. This in conjunction with removal of selection should result in loss of both overexpressing and λdv plasmid from the cell allowing the λdv plasmids to be used as either replacement or deletion vectors (Joyce and Grindley 1984). This system should prove extremely useful not only in the analysis of the F factor function that stimulates cut production but also in

examining events occuring at Tn2901. Strains carrying amplified Tn2901 DNA have been shown to arise by an initial recombination event between the IS1 elements of Tn2901 (Clugston 1986). Whether this event requires IS1 encoded functions or is a result of the cells normal recombination systems is not yet known. The replacement of the IS1 elements with a non transposable DNA fragment could answer this question and this is addressed further in Chapter 5.

In retrospect the hope that cloned replication origins could be inserted into the chromosome and then switched on seems a little naive considering the complexity of the initiation process. The insertion of origin fragments into the chromosome was successful. However, *ori*V sequences did not significantly increased the cut yield of F$^-$ strains by comparison with controls. The *ori*V the insertion was stable and it can be said that these sequences are insufficient to stimulate cut production. Plasmid pFJH92 was introduced to this strain to supply trans-acting proteins, for instance RepE and RepD/G (required for replication) or ResD (required for resolution of *ori*V induced cointegrates). This had no appreciable effect on cut production. Subsequently the supply of trans-acting replication proteins from pFJH92 was shown to be insufficient to support *ori*V driven replication of an autonomous plasmid. Thus, unfortunately, these experiments supply insufficient data to allow the elimination of either *ori*V driven replication or ResD promoted resolution as factors involved in the amplification process, although the latter has not been tested directly.

The copy number of the λdv-*ori*S construct suggests that replication is under control of the λ origin and it's insertion into the chromosome using cI repressor confirms this. This is consistent with previous observations and the hypothesis that F origin replication is regulated by sensing plasmid concentration (Tsutsui and Matsubara 1981). There are a number of possible explanations for the apparent instability of the *ori*S insertion. A mutation or inherently unstable structure could have been generated during the cloning procedure. The λdv-*ori*S construct was difficult to isolate but once isolated it did not appear to suffer deletions during large scale preparation as might be

expected in an unstable structure. The λ prophage may not supply sufficient repressor. Clugston (1986) was unable to insert λdv plasmids into AB1157. I had previously obtained a stable insertion of the λdv plasmid pCC7 in AB1157, however it is possible that it is difficult to introduce λdv plasmids into AB1157. Alternatively it is possible that initiation of replication by F stimulates excision of the plasmid from the chromosome. Clearly excision and F replication must be linked in some fashion. Hfr P4X and it's derivative Hfrs including Jef8 are unusually stable. This is unlikely to be due to a defect in either the host or plasmid replication systems as F prime plasmids have been isolated from both P4X and Jef8. It is possible that some minor rearrangement has occurred in the IS3 sequences flanking the F factor in P4X such that excision to reform an F^+ strain is inhibited. In this context it is interesting that the first F prime plasmid to be recognised derived from P4X (Adelberg and Burns 1960).

The possibility that replication was observed in a gene bank made with Jef8 DNA is intriguing. The gene bank used in this final experiment was constructed by Carol Clugston. While we cannot know the exact conditions she used for the growth of cells from which the DNA was isolated, the protocols I inherited from her recommend the isolation of chromosomal DNA from stationary overnight cultures grown in minimal medium (Clugston 1986). This could lead to a condition under which replication from F was initiated. If this is the case then the fact that amplification was not detected in stationary cultures in the experiments described in Chapter 3 could be explained by the rapid loss of amplified DNA in the absence of selection. Alternatively initiation of replication from F may occur in only some cells. Chandler *et al.* estimated that they observed initiation from *oriC* at twice the frequency of initiation from F. They were unable to differentiate between concomitant initiation within a cell and sub-populations of cells using different origins (Chandler *et al.* 1976). Thus if a small proportion of cells give rise to amplified DNA these would outgrow wild-type cells under selection but could be outgrown themselves in its absence. Clearly if one wished to show conclusively that replication was initiated by F

in Jef8 it would be better to mark the Jef8 chromosome at a number of sites with different sequences for which probes exist and measure the gene dosage quantitatively by Southern hybridization. While lambda sequences in Jef8 could be used to quantitate gene dosage in the region adjacent to F, sequences in the *ori*C region and opposite F would need to be engineered. The gene bank experiment was quick and used materials available in the laboratory. Although it is inconclusive it does suggest that the involvement of replication from F in the amplification of Tn2901 is a function which merits further investigation.

A variety of modifications to the experiments described in this chapter could be suggested to test the replication hypothesis. For instance the expression of RepE protein from an inducible promoter may allow the "switching on" of *ori*V sequences. The use of the other *ori*S plasmids, pFJH122 and pFJH123 with the cI overexpressing system may result in stable integration. However the success of these experiments is made doubtful by the complexity of the initiation process and the need to maintain multicopy plasmids in the cell during selection on citrulline. Nor is it obvious from the literature that there is any consensus opinion on whether *ori*V, *ori*S or both origins together are responsible for replication of F, particularly in the unusual situation of an Hfr. A more thorough analysis of gene dosage using quantitative Southern hybridization on Jef8 growing exponentially in rich medium or slowly in poor medium compared with F^- strains under the same conditions could determine if these conditions did indeed induce replication initiation from F but would not link replication to amplification. The deletion of F origin regions from Jef8 currently under way in this laboratory may certainly implicate replication if these deletions are unable to amplify Tn2901, however very careful mapping of the deletion end points would be required before the involvement of other F functions could be eliminated.

The recent cloning of *ter*C, the *E.coli* terminus of replication, suggests an alternative method of examining this hypothesis. *ter*C is composed of four sequences each of which, in the presence of the Tus protein, stall replication forks passing in one direction only. *ter*C consists of two of these sequences in each

orientation (Kuempel *et al.* 1989). It should be possible to place a strong consensus copy of this sequence between the F factor and Tn2901 in Jef8 such that replication from F is stalled but replication from *ori*C is unhindered. Examination of the terminus region from *E. coli*, R100 and R6K has allowed identification of a 23bp consensus sequence. In vitro studies of Tus binding have confirmed in vivo observations that this consensus is a strong terminator (Kuempel *et al.* 1989). Oligonucleotides containing this sequence arrest replication in an orientation specific manner in ColE1 plasmids (Hill *et al.* 1988). In vitro replication extracts from *E. coli* contain sufficient endogenous contaminating Ter protein to cause replication termination in plasmids (Khatri *et al.* 1989), suggesting that it would not be necessary to supply Ter protein *in trans.*

Recently Noreen Murray has kindly supplied our laboratory with a transposon marked temperature sensitive *pol*A allele and Jef8::*pol*A has been constructed (Adrienne Jessop personal communication). Thus a pUC18/19 based plasmid containing DNA from the right of Tn2901 into which had been inserted the *ter* oligonucleotide and a novel restriction site to aid mapping could be constructed and inserted into the Jef8::*pol*A chromosome. Obviously care would have to be taken to ensure that the *ter* sequence was correctly oriented during construction. Removal of the pUC plasmid by growth at the permissive temperature should leave 50% of the cells containing a *ter* sequence between Tn2901 and F. Removal of the plasmid is preferable as the *pol*A allele may itself affect amplification (Adrienne Jessop, personal communication). As this experiment is to be done in Jef8, cloned F DNA could be used as the site of insertion. For instance the gamma delta sequences on F lie between Tn2901 and the F replication regions. The advantage of this is that the sequence of gamma delta is known and it's sequence organization is better understood than that of the local chromosomal DNA. If replication from F plays a role in the amplification of Tn2901 then amplification should be considerably reduced in a strain carrying a polar replication terminus compared with a strain carrying an identically placed "junk" insertion. It may not be abolished completely as

amplification has been observed in F^- cells (Chapter 3) and even a strong *ter* sequence may not stop the passage of every replication fork. If replication is not the primary function supplied by the F factor then F factor fragments can be returned to the chromosome of an F^- strain to see if any of them are responsible for stimulating the amplification of Tn2901. Alternatively, if particular sites on the F factor are thought to be involved, these could be insertionally inactivated using the F factor clones generated in this study. Indeed the insertion of a *ter* oligonucleotide sequence could be combined with such an experiment provided appropriate controls were undertaken.

CHAPTER FIVE

A SYSTEM TO INVESTIGATE THE ROLE OF THE IS1 ELEMENTS

5.1 Introduction

The initial event in the amplification of Tn2901 is a recombination between the IS1 elements that form this composite transposon (Clugston 1986). At present it is not clear whether this recombination event is simply a product of the cell's normal homologous recombination mechanisms or if it involves some site-specific recombination system encoded, for instance, by IS1. A greater understanding of the events occuring at IS1 would facilitate the further investigation of the role played by the F factor. If for instance a particular site-specific recombination system is implicated then likely F interactions with that system can be targeted for further study and conversely if it appears that the IS1 elements act as sites for homologous recombination then F stimulation of this process can be investigated. However the role played by IS1 has significance in its own right. The contribution that insertion sequences carried on plasmids might make to host evolution is a question amenable to investigation using a number of systems, but examples where this question might be addressed in a chromosomal context are less abundant. The amplification of Tn2901 is one system where this may be possible.

The best characterized case of IS1 dependent amplification is that of the drug resistance genes on the R plasmid NR1 in *Proteus mirabilis*. Resistance genes for chloramphenicol, fusidic acid, streptomycin, spectinomycin, sulphonamides and mercuric ions on this plasmid are part of a 20.6kb sequence flanked by direct repeats of IS1 in a structure known as the resistance determinant (r-det). The remainder of the plasmid, the resistance transfer factor (RTF), encodes the transfer genes, the replication origin, a locus required *in cis* for plasmid stability and carries a copy of Tn10 which includes a tetracycline resistance gene (Hu *et al.* 1975)(Rownd 1986). When *P. mirabilis* containing NR1 is grown in the presence of one of the drugs encoded by the r-det, amplification of r-det DNA is seen. This amplification is found both as tandem copies of r-det in NR1 and as autonomous multimeric copies of r-det. Removal of selection

results in the loss of autonomous r-det molecules and return of the plasmid to one r-det per RTF (Rownd 1986). Dissociation of r-det and RTF, or amplification of r-det, can be found in *P. mirabilis* cells that have not been subject to antibiotic selection (Peterson and Rownd 1985b).

When NR1 is cultured in *E. coli* (where it is known as R100-1) no amplification of the r-det is observed on selection for high levels of antibiotic resistance. Instead copy number mutants which have an increased number of NR1 copies per cell are found (Huffman and Rownd 1984). Plasmids carrying deletions in the r-det that remove the site of a cointegrate resolution system do amplify the remaining r-det DNA in *E. coli* (Rownd 1986). Rownd has suggested that this resolution system is active in *E. coli*, deleting extra copies of r-det, but quiescent in *P. mirabilis*. Strains of *E. coli* carrying a ts *dna*A mutation cannot replicate at the non-permissive temperature. This phenotype can be integratively suppressed by NR1. Hfrs created in this fashion can give rise to autonomous r-det DNA (Chandler *et al.* 1977). Production of autonomous r-det is abolished if a *rec*A allele is introduced into these strains. Chandler and colleagues suggest that r-det DNA is replicated continuously in situ by abortive replication from NR1 and then excised. They note that this replication would need to be aborted after replication of the r-det DNA (about 20kb) but before the entire NR1 plasmid (about 90kb total) was replicated. In this context abortive replication could include the capture of a replication fork by autonomous r-det DNA resulting from recombination between the IS1 sequences. They suggest that that unidirectional replication from the NR1 origin could be responsible for the abortive replication while bidirectional replication is responsible for replication of the host chromosome (Chandler *et al.* 1979).

Although the initial event in amplification of the r-det is a recombination between the IS1 elements (Peterson and Rownd 1985a) it appears that any region of homology can serve as a site for this event. When the r-det in NR1 is replaced by an artificial construct composed of a kanamycin resistance gene flanked by homologous sequences not derived from IS elements,

amplification of the kan resistance gene is observed (Peterson and Rownd 1983).

This system has features in common with the amplification of Tn2901. The initial step is a recombination event between directly repeated IS1 elements. It has been suggested, but not demonstrated that that this initial event is stimulated in the chromosome by abortive replication. There are however some differences. Removal of selection for r-det amplification results in reappearance of the wild-type NR1 plasmid containing a single copy of the r-det. Removal of strains carrying amplified Tn2901 DNA from selection does not appear to restore them to their pre-amplified state (Jessop and Glansdorff 1980). A second major difference is that autonomous r-det DNA can easily be isolated in *P. mirabilis* while it has not yet been possible to isolate autonomous Tn2901 sequences. Finally amplification of Tn2901 is not commonly observed in the absence of selection.

This chapter sets out to investigate other potential similarities between these two systems. The IS1 sequences in r-det amplification are dispensable and I describe here the construction of vectors that will allow the replacement of the Tn2901 IS1 elements with regions of non-IS derived homology.

Rownd has postulated that failure to observe r-det amplification in *E. coli* is due to the presence of a cointegrate resolution system that does not function in *P. mirabilis*. It is possible that the integration of the F factor results in inhibition or loss of resolvase action. For instance F might provide a binding site for a repressor of such a system. The two binding sites required for repression of the *deo* operon can be separated by 4kb with only a four fold drop in co-operative repression (Dandanell *et al.* 1987). At 39kb no cooperative repression is observed. By extrapolation the authors conclude that cooperativity will be lost in this system at distances greater than 10kb (Dandanell *et al.* 1987). The F factor in Jef8 is situated about 20kb from Tn2901 however the postulated resolution system need not be encoded on Tn2901 itself. Braedt has suggested that the *ins*C reading frame of IS1 encodes a resolvase function, but reports evidence that this is inhibited or redundant in *E. coli* K-12 (Braedt 1988).

If a resolution system acts on IS1 it may be possible to interrupt or destroy its site of action. Insertions into the PstI site on IS1 result in reduced transposition in *rec*A strains and accumulation of cointegrates and it has been suggested that this represents a resolution site (Reif and Arbor 1980). Should we find that an IS encoded function is required for the initial event in the amplification, then IS1 elements in which point mutations have been created, would prove useful in investigating which function is required. Mutations at the PstI site of IS1 will interrupt the *ins*A reading frame only while mutations at the MluI site will occur only in the *ins*B reading frame (Braedt 1988). Mutations that destroy restriction sites on IS1 would also be useful to differentially mark the two IS1 sequences on Tn2901. This would enable us to determine if a single crossover site is used in the initial event or if the recombination leading to amplification is random.

The vectors used for replacing the IS1 elements with non-IS homology can also be used for replacing them with mutated sequences. This will be additionally useful if it found that the IS1 sequences themselves are required for the initial event. Cloned IS1 DNA was available in the laboratory as part of pCC18 (Clugston 1986). The characterization of this plasmid and creation of IS1 mutations is described first. I initially attempted to isolate DNA external to Tn2901, required for construction of the vectors, by chromosome walking in a Jef8 library constructed by Carol Clugston (Clugston 1986). This proved to be complicated by the large number of insertion sequences present in this region of the chromosome and the small insert size of the library. Thus when Kohara and colleagues published their restriction map of the *E. coli* chromosome (Kohara *et al.* 1987) I decided it would be simpler to clone this DNA "by post". Unfortunately time did not permit me to complete the construction of the vectors or to attempt the experiments described above, these are however currently underway in this laboratory.

DEPT. OF GENETICS
UNIVERSITY, GLASGOW.

5.2 Results

5.2.1 Mapping '19A' and pCC18

pCC18 was constructed from the 3.2kb HindIII fragment that spans the right hand IS1 element of Tn2901 (Clugston 1986) and a λdv vector called 19A; a recent modification of an older vector pCB101 (Boyd and Sherratt 1986). The details of the construction of 19A are known, but no restriction map exists. The known restriction map of pCB101 is shown in Figure 5.1a. 19A was constructed by deleting part of the cI gene from pCB101 thus removing two unwanted HindIII sites and grown in a mutator strain to remove two unwanted EcoRI sites. The pCB101 polylinker was replaced by the HaeII polylinker containing fragment from pUC19. The overall size is 4289bp and the HindIII and EcoRI polylinker sites are now unique (Chris Boyd, personal communication). The orientation of the HaeII fragment in 19A is unknown. pCC18 was constructed by ligating the 3.2kb HindIII fragment from pCC21 into the HindIII site of 19A (Clugston 1986). The 3.2kb HindIII fragment contains the chromosomal region shown in Figure 5.1c and some pBR322 DNA. It should contain a single EcoRI site (Clugston 1986). EcoRI digestion of pCC18 gives two bands of 4.4kb and 3.0kb (Figure 5.2a lane4). Thus the orientation of the HindIII fragment in pCC18 is that shown in Figure 5.1d. Digestion of pCC18 with KpnI and NcoI gives two bands of 7.0kb and 0.5kb (Figure 5.2a lane3) confirming that the orientation of the HaeII polylinker fragment with respect to the rest of the plasmid is that shown in Figure 5.1b. Digestion of 19A with PvuII gives 4 bands, the smallest of which is cut by PstI (Figure 5.2b). Thus 19A has gained an extra PvuII site by comparison with pCB101 (Figure 5.1 a and b) due to the removal of the PvuII polylinker from pCB101 and the insertion of the HaeII polylinker fragment from pUC19 which contains two PvuII sites. The single EcoRV site in pCB101 is maintained in 19A (Figure 5.2b lane1). PstI digestion of pCC18 gives three bands of 4.4kb, 2.2kb and 0.7kb

Figure 5.1

a)
Restriction map of the λdv plasmid pCB101

b)
Restriction map of the λdv plasmid 19A

c)
Restriction map of the chromosomal region containing Tn2901. Insertion sequences are represented by large open boxes and their orientation as defined by nucleotide sequence is shown by the arrow below. The small boxed area represents the region of the chromosome cloned as part of pCC18.

d)
Restriction map of the λdv plasmid pCC18. The 19A vector sequences are shown by a single line. Chromosomal DNA is represented by small boxed regions and IS1 by a large box. The orientation of IS1 as defined by nucleotide sequence is shown by the arrow below.

Figure 5.1

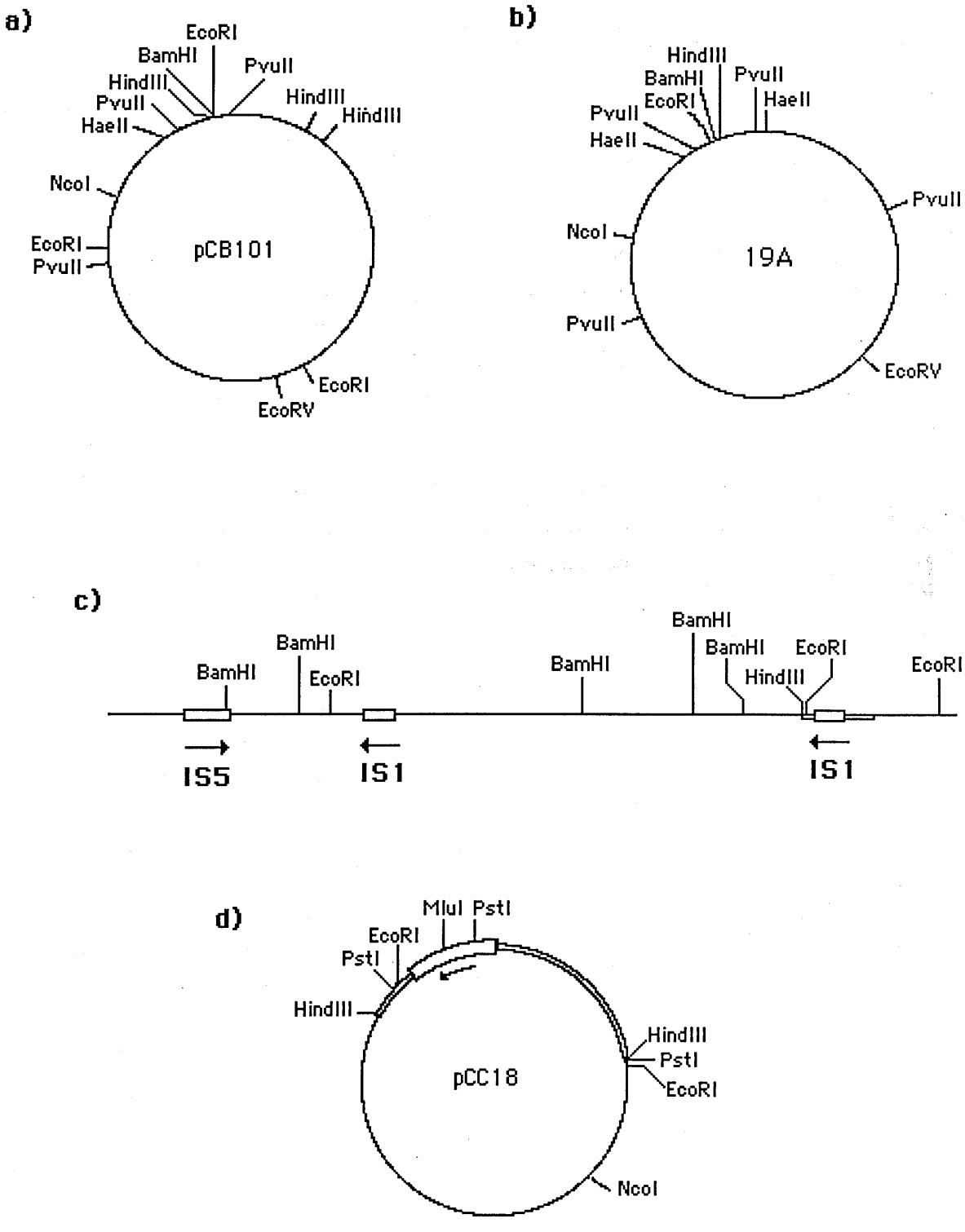

a)
EcoRI
BamHI
PvuII
HindIII
PvuII
HaeII
HindIII
HindIII
NcoI
EcoRI
PvuII
pCB101
EcoRI
EcoRV
b)
HindIII
BamHI
PvuII
EcoRI
HaeII
PvuII
HaeII
PvuII
NcoI
19A
PvuII
EcoRV
c)
BamHI
BamHI
EcoRI
BamHI
BamHI
BamHI
EcoRI
HindIII
EcoRI
IS5
IS1
IS1
d)
MluI
PstI
EcoRI
PstI
HindIII
HindIII
PstI
EcoRI
pCC18
NcoI

Figure 5.3

Vector sequences are defined by a single line. IS1 is represented by large open boxes and it's orientation as defined by nucleotide sequence is shown by the arrows below. Chromosomal DNA is shown as small boxed regions.

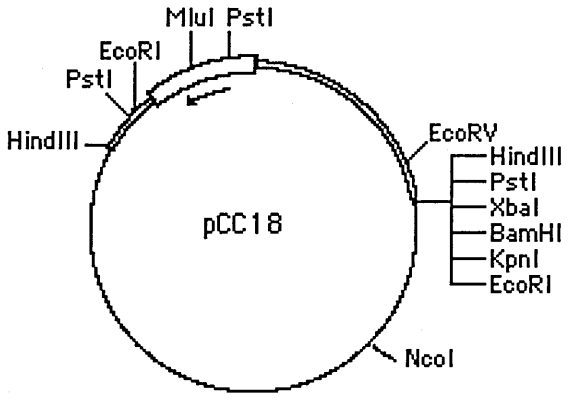
a)
Restriction map of pCC18.

b)
The HaeII fragment and polylinker from pUC19 that constitute the polylinker sequences in pCC18.

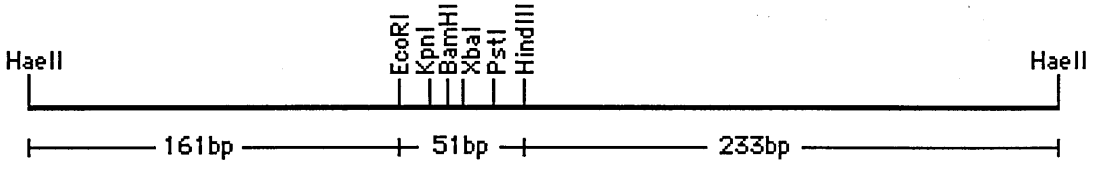
c)
Restriction map of the pFJH20 insert including IS1. Known fragment sizes are marked.

Figure 5.3

a)



b)



c)

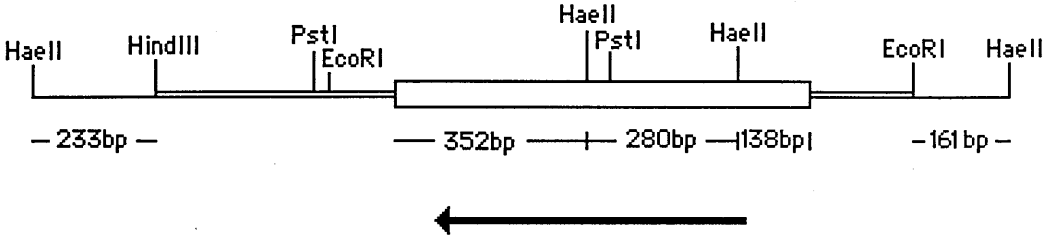


Figure 5.4

a)
DNA from pFJH20 digested with a variety of restriction enzymes and run in TBE buffer on a 2.5% agarose slab gel. The markers are HindIII/EcoRI cut λ DNA. The gel was stained with EthBr.

b)
Restriction map of pFJH20. Vector sequences are defined by a single line. IS1 is represented by a large open box and it's orientation as defined by nucleotide sequence is shown by the arrow. Chromosomal DNA is shown as small boxed regions.

c)

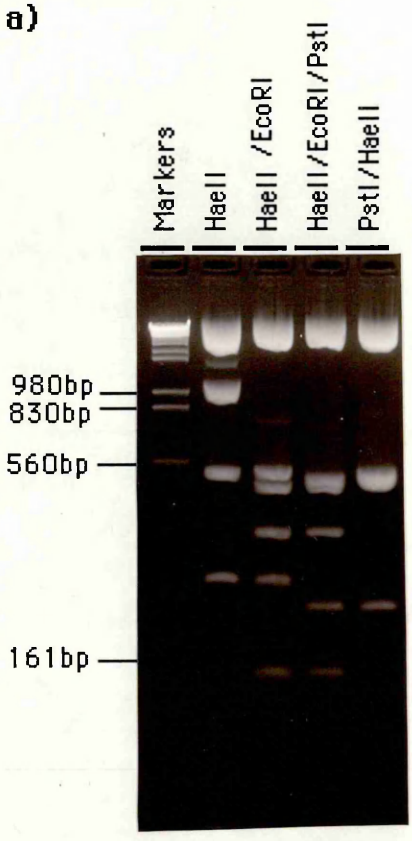
HaeII		HaeII/EcoRI		HaeII/EcoRI/PstI		HaeII/PstI	
obs	exp	obs	exp	obs	exp	obs	exp
490bp		500bp		490bp		460bp	
260bp	280bp	460bp		460bp		245bp	240bp
		340bp		340bp			
		260bp	280bp	245bp	240bp		
		150bp	161bp	150bp	161bp		

Table showing the fragment sizes observed on the gel in a). Note that large (greater than 800bp) fragments are not well resolved on high percentage gels and have been ignored. Expected sizes are from the data shown in Figure 5.3

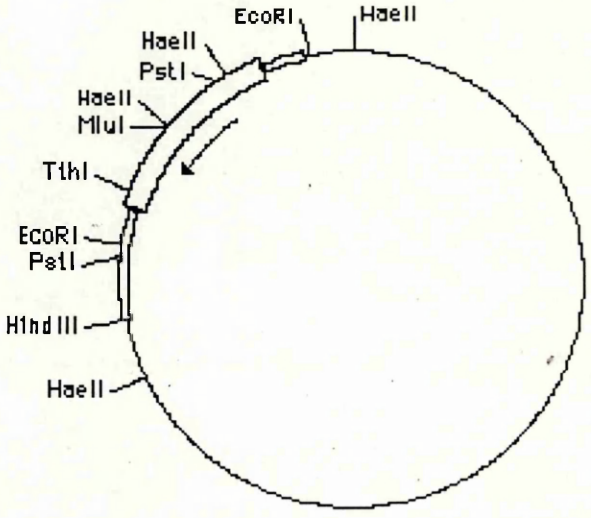
d)
Restriction map of the pFJH20 insert including IS1. Fragment sizes including the data shown in c) are marked. Vector sequences are defined by a single line. IS1 is represented by a large open box and it's orientation as defined by nucleotide sequence is shown by the arrow. Chromosomal DNA is shown as small boxed regions.

Figure 5.4

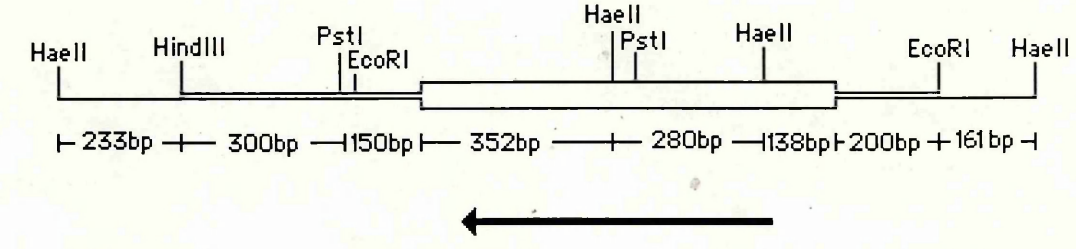
a)



b)



d)



(Figure 5.2a lane2), MluI digestion linearizes the plasmid (not shown) while double digestion with PstI and MluI gives four bands of 4.4kb, 2.2kb and two smaller fragments derived from the 0.7kb PstI band (Figure 5.2a lane 1). Since the orientation of the IS1 element and the position of the PstI and MluI sites in it are known the third PstI site must be adjacent to the EcoRI site (Figure 5.1d). This confirms Carol Clugston's restriction mapping of pCC21 (Clugston 1986). The 3.2kb HindIII fragment of pCC18 contains no sites for the following restriction enzymes; BamHI, SphI, ClaI[1], KpnI, AccI, AvaI, NcoI, XbaI[2] or BglII (Not shown).

5.2.2 Creating mutations in IS1 sequences

The IS1 sequence in pCC18 can be isolated on a HindIII/EcoRV fragment of about 2.4kb, but IS1 lies at one end of this fragment (Figure 5.3) making it difficult to manipulate. To remove non-IS1 sequence, pCC18 was sequentially digested with XbaI and KpnI then subjected to Exonuclease III treatment as described by Henikoff (1984). Samples were taken after 3, 6, 9 and 12 minutes, ligated and used to transform DS902. pFJH20 was isolated from the 12 minute reaction. This plasmid is cut twice by EcoRI giving bands of 4.3kb (vector) and 1.1kb (insert). Detailed restriction mapping of pFJH20 is shown in Figure 5.4. Since the sequence of IS1 and the *lac*Z gene containing the pCC18 polylinker are known (Johnsrud 1979)(Yanish-Perron *et al.* 1985), expected as well as calculated sizes are shown for some fragments. Briefly; HaeII cuts pFJH20 to four fragments of about 4.0kb, 1.0kb, 500bp and 280bp. PstI cuts the 280bp HaeII fragment to 240bp and 40bp (not seen). EcoRI cuts the 500bp HaeII fragment to 340bp and 161bp. The 1.0kb HaeII fragment

---

1 The ClaI recognition sequence (ATCGAT) can form a *dam* methylation site when adjacent to a cytosine residue (ATCGATC) inhibiting ClaI digestion. ClaI digestion was tested only with methylated DNA and there is therefore a possibility that a ClaI site was not observed.

2 The XbaI recognition sequence (TCTAGA) can form a *dam* methylation site when adjacent to a thymine and cytosine residue (TCTAGATC) inhibiting XbaI digestion. XbaI digestion was tested on unmethylated DNA.

is cut by HindIII (750bp and 233bp) not shown, PstI (520bp and 510bp) and EcoRI (533bp and 500bp). The first IS1 mutant, a deletion of the 280bp HaeII fragment, was constructed as follows. The 340bp fragment from the HaeII/EcoRI digest contains the start of IS1 and is easily isolated from a 3% agarose gel. The 500bp HaeII/EcoRI fragment contains the end of IS1, but is not easily separated from the 533bp fragment. This fragment was isolated from a HaeII/EcoRI/HindIII triple digest which cuts the 533bp fragment to 300bp and 233bp leaving the 500bp fragment free. The 340bp and the 500bp EcoRI/HaeII fragments were ligated to EcoRI cut and phosphatased pUC19. The ligation reaction was used to transform DS902 and pFJH60 was isolated. Figure 5.5 shows pFJH60 cut with a number of restriction endonucleases and the resulting derived map. As the entire sequence of pUC19 is known (Yanish-Perron *et al.* 1985), expected as well as observed sizes are shown for all fragments. EcoRI digestion of pFJH60 gives two fragments, the smaller of which is calculated to be 800bp. This is close to the 840bp expected from the ligation of the two pFJH20 HaeII/EcoRI fragments. HaeII digestion of pFJH60 gives four fragments. The largest (1871bp) and smallest (370bp) derive exclusively from pUC19 DNA (Yanish-Perron *et al.* 1985). The 640bp fragment is the result of ligation of the pFJH20 500bp fragment to the pUC19 161bp fragment while the 600bp fragment derives from 338bp of pFJH20 and 284bp of pUC19. In confirmation PstI/MluI digestion of pFJH60 results in a small fragment of calculated size 440bp which is close to the 392bp expected from an IS1 element deleted for the internal HaeII fragment. pFJH60 therefore contains an IS1 element that is *ins*A$^-$, *ins*B$^-$ but *ins*C$^+$ (Braedt 1988) and should be deleted for the postulated internal resolution site (Reif and Arbor 1980). To create small mutations in IS1 the 1.1kb IS1 containing EcoRI fragment from pFJH20 was first isolated and ligated to EcoRI cut and phosphatased pIC20R. The resulting plasmid pFJH70 was isolated from transformed DS902. It contains single sites for PstI and MluI (Figure 5.6a). pFJH70 was digested with either PstI or MluI, blunt ended with DNA polymerase I and religated. pFJH71 was isolated from DS902 cells transformed with the reaction originally digested

Figure 5.5

a)
DNA from pFJH60 digested with a variety of restriction enzymes and run in TBE buffer on a 2.5% agarose slab gel. The markers are HindIII/EcoRI cut λ DNA. The gel was stained with EthBr.

b)
Restriction map of pFJH60. Vector sequences are defined by a single line. IS1 is represented by a large open box and it's orientation as defined by nucleotide sequence is shown by the arrow. Chromosomal DNA is shown as small boxed regions

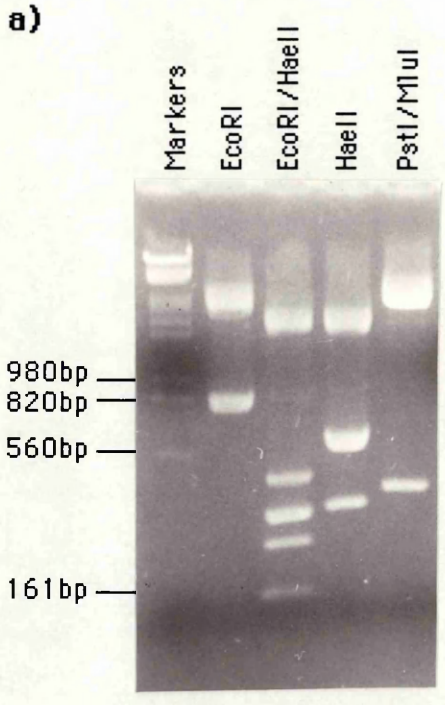
c)

EcoRI		HaeII/EcoRI		HaeII		PstI/MluI	
obs	exp	obs	exp	obs	exp	obs	exp
800bp	838bp	470bp	500bp	640bp	661bp	440bp	392bp
		365bp	370bp	600bp	622bp		
		340bp	338bp	365bp	370bp		
		280bp	280bp				
		180bp	161bp				

Table showing the fragment sizes observed on the gel in a). Note that large (greater than 800bp) fragments are not well resolved on high percentage gels and have been ignored. Expected sizes are from the data shown in Figure 5.3

Figure 5.5

a)


Markers
EcoRI
EcoRI/HaeII
HaeII
PstI/MluI
980bp
820bp
560bp
161bp

b)

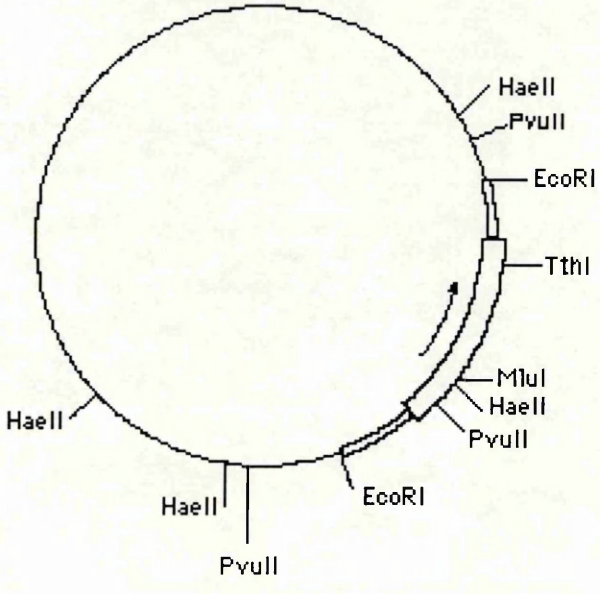

HaeII
PvuII
EcoRI
TthI
MluI
HaeII
PvuII
EcoRI
HaeII
PvuII
HaeII

Figure 5.6

Vector sequences are defined by a single line. IS1 is represented by large open boxes and it's orientation as defined by nucleotide sequence is shown by the arrows. Chromosomal DNA is shown as small boxed regions.

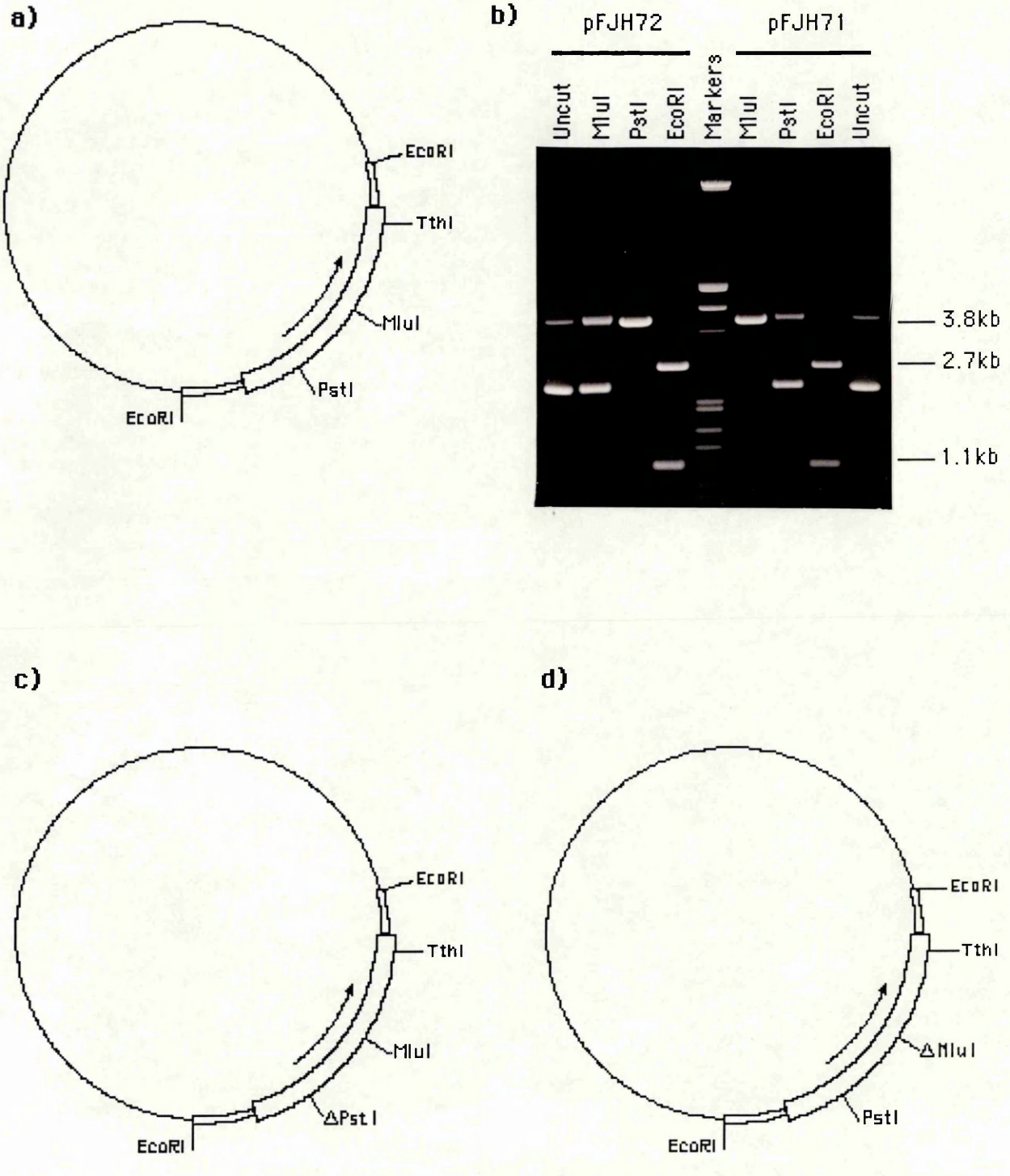
a)
Restriction map of pFJH70

b)
DNA from pFJH71 and pFJH72 cut with EcoRI, PstI or MluI and run on a 0.8% agarose slab gel. The markers are HindIII/EcoRI cut λ DNA. The gel was stained with EthBr.

c)
Restriction map of pFJH71

d)
Restriction map of pFJH72

Figure 5.6


a)
EcoRI
TthI
MluI
PstI
EcoRI
b)
pFJH72
pFJH71
Uncut
MluI
PstI
EcoRI
Markers
MluI
PstI
EcoRI
Uncut
3.8kb
2.7kb
1.1kb
c)
EcoRI
TthI
MluI
ΔPst I
EcoRI
d)
EcoRI
TthI
ΔMluI
PstI
EcoRI

with PstI. It is not cut by PstI, but is linearized by MluI and gives fragments of 2.7kb and 1.1kb when cut with EcoRI. DS902 transformed with the reaction originally cut by MluI yielded plasmid pFJH72. pFJH72 is not cut by MluI, is linearized by PstI and cuts to fragments of 2.7kb and 1.1kb with EcoRI (Figure 5.6a and b). The exact nature of these mutations has not been checked by sequencing. It is therefore possible that they contain three base pair insertions that will still allow functional *ins*A (pFJH71) or *ins*B (pFJH72) gene products to be produced. If used to replace the wild-type IS1 elements in the chromosome however, they will serve as useful markers that will allow the differentiation of DNA derived from left and right IS1 elements within the amplification of Tn2901.

5.2.3 Mapping Kohara clones λ3C7 and λ5A5

The λ clones kindly supplied by Yuji Kohara are two of a set that covers the entire *E.coli* chromosome. Both contain >15kb of DNA spanning the Tn2901 region cloned as Sau3A fragments into the BamHI sites of λ EMBL4 (Kohara *et al.* 1987). The inserts can be excised with EcoRI. The relevant portion of the restriction map provided with the phage is shown in Figure 5.7a. λ3C7 DNA was restricted with EcoRI and ligated to EcoRI cut and phosphatased pUC19. Colonies resulting from DS902 transformation were analysed by single colony gel analysis. Two clonable fragments should result from EcoRI digestion of 3C7 DNA and these were isolated as pFJH90 (pUC19 plus about 17kb insert) and pFJH93 (pUC19 plus 1.2kb insert). The known restriction map of this region of DNA, derived from mapping of pCC21, pCC1 (Clugston 1986) and Hadley *et al.* (1983) is shown in Figure 5.8b. Digestion of pFJH90 with ClaI[1] gives two fragments of 9.8kb and 8.0kb (Figure 5.9). The smaller ClaI fragment is cut with EcoRI to 4.2kb, 2.7kb (vector) and 1.5kb. HindIII cuts pFJH90 to three

[1] The ClaI recognition sequence (ATCGAT) can form a *dam* methylation site when adjacent to a cytosine residue (ATCGATC) inhibiting ClaI digestion. ClaI digestion was tested only with methylated DNA and there is therefore a possibility that some ClaI sites were not observed

fragments of 8.4kb 7.2kb and 3.0kb. Only the 3.0kb fragment is not cut by ClaI and must span the vector sequences. The 8.4kb HindIII fragment is cut to 4.2kb and 4.0kb by ClaI, while the 7.2kb fragment is cut to 5.8kb and 1.1kb. As the HindIII site at the right hand end of Tn2901 is known to to lie 1.1kb from a ClaI site (Clugston 1986) the relative positions of these fragments must be that shown in Figure 5.9. BamHI digestion of pFJH90 releases six fragments; 6.4kb, 4.3kb, 3.1kb, 2.3kb, 1.15kb and 0.95kb. The 4.3kb fragment is cut by EcoRI and must contain the 2.7kb pUC19 sequence. The 1.15kb fragment is 'shaved' by EcoRI and thus lies adjacent to the pUC19 polylinker. The position of the 2.3kb and 3.1kb fragments are known (Clugston 1986). The 6.4kb BamHI fragment is cut to 6.0kb and 0.4kb[2] by HindIII. The full deduced restriction map of pFJH90 is shown in Figure 5.9. DNA from λ5A5 was digested with EcoRI and ligated to EcoRI cut and phosphatased pUC19. Colonies resulting from DS902 transformation were analysed by single colony gel analysis. Three EcoRI fragments of 7.4kb, 5.9kb and 2.1kb make up the insert of λ5A5 (Figure 5.8). The 2.1kb fragment was cloned as pFJH83 and the 7.4kb fragment as pFJH87. The 5.9kb fragment was not isolated on it's own from this experiment, but is present with the 7.4kb fragment in pFJH88. The 7.4kb fragment is calculated to span the left hand end of Tn2901, thus further restriction mapping concentrated on pFJH87. Since this plasmid should contain the left hand IS1 element of Tn2901 it was restricted with a range of restriction enzymes and subjected to Southern analysis. The 1.1kb EcoRI fragment from pFJH70 contains an IS1 element and was used as a random primed probe in this experiment. The results are incorporated in Figure 5.10. Extensive restriction mapping of pFJH87 was carried out. The results are summarized in Figure 5.10. BglII cuts pFJH87 three times giving fragments of 6.3kb, 2.4kb and 1.6kb (IS1 containing). EcoRI cuts the largest BglII fragment to 3.1kb, 2.7kb and 0.5kb. ClaI cuts pFJH87 once. From mapping pFJH90 which overlaps with this plasmid the ClaI site is known to be external to Tn2901. ClaI cuts the smallest BglII fragment to 0.86kb and 0.77kb and the 7.4kb EcoRI fragment to 4.1kb and 3.5kb. Thus the relative positions of

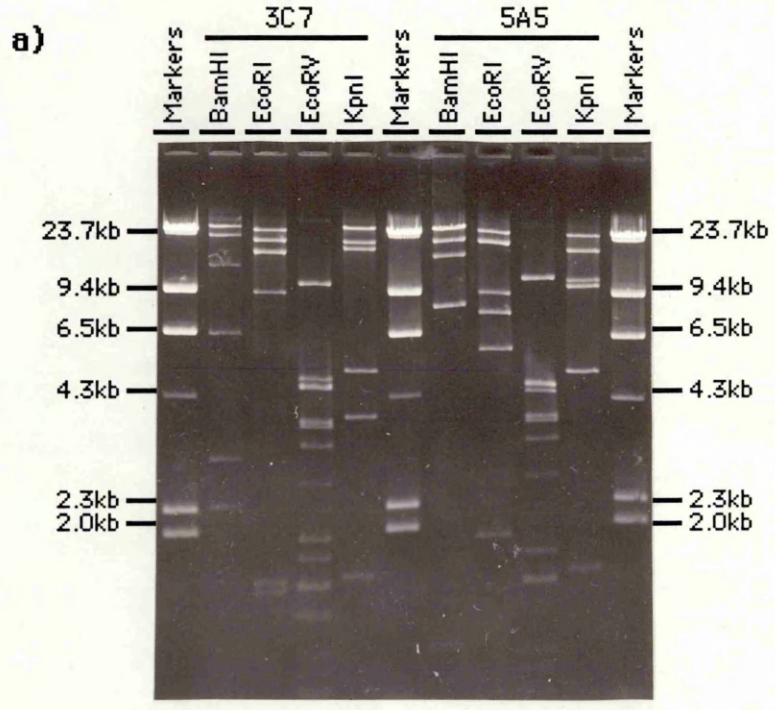
Figure 5.7

a)
DNA from λ clones 3C7 and 5A5 cut with a variety of restriction enzymes and run on a 0.8% agarose slab gel. The markers are HindIII cut λ DNA. The gel was stained with EthBr.

b)
Restriction map of λ3C7 from data supplied with the clone (Kohara *et al.*, 1987).

c)
Restriction map of λ3C7 by restriction mapping. An additional EcoRI fragment is visible in my restriction mapping at site expected from the map of Hadley *et al.*, 1983. Thus the clone extends further than realised by Kohara *et al.*

Figure 5.7


a)
3C7
5A5
Markers
BamHI
EcoRI
EcoRV
KpnI
Markers
BamHI
EcoRI
EcoRV
KpnI
Markers
23.7kb
9.4kb
6.5kb
4.3kb
2.3kb
2.0kb
23.7kb
9.4kb
6.5kb
4.3kb
2.3kb
2.0kb


b)
BamHI
KpnI
BamHI
KpnI
HindIII
BamHI
BamHI
BamHI
HindIII
EcoRI

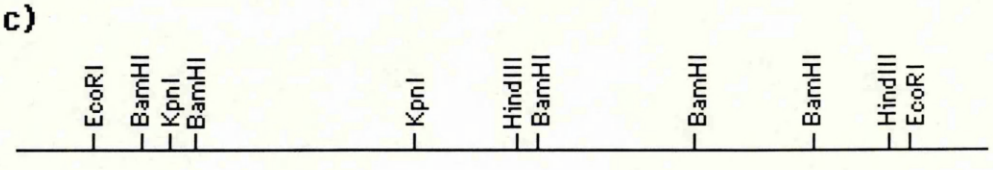

c)
EcoRI
BamHI
KpnI
BamHI
KpnI
HindIII
BamHI
BamHI
BamHI
HindIII
EcoRI

Figure 5.8

a)
Restriction map of λ5A5, which spans the left hand end of Tn2901
i)From data supplied with the clone (Kohara *et al.*, 1987).
ii)From Hadley *et al.*, 1983. Insertion sequences are shown by small boxes.

b)
Restriction map of λ3C7, which spans Tn2901
i)From data supplied with the clone (Kohara *et al.*, 1987). The likely position of the extra EcoRI fragment is shown by a heavy line (see Figure 5.7).
ii)From Hadley *et al.*, 1983. Insertion sequences are shown by small boxes. The arrow marks the position and orientation of the *arg* F gene.

Figure 5.8

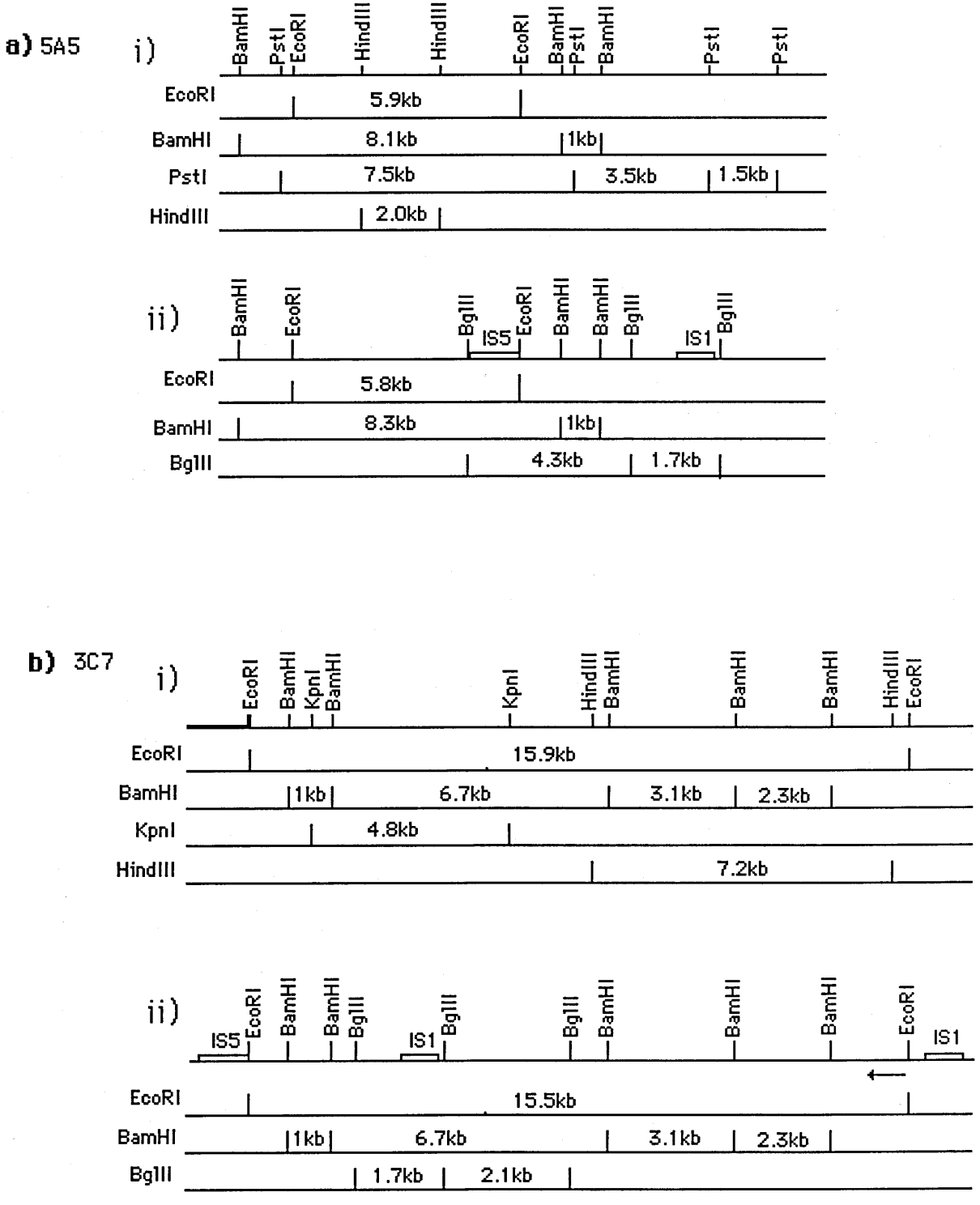

a) 5A5
i)
BamHI
PstI
EcoRI
HindIII
HindIII
EcoRI
BamHI
PstI
BamHI
PstI
PstI
EcoRI
5.9kb
BamHI
8.1kb
1kb
PstI
7.5kb
3.5kb
1.5kb
HindIII
2.0kb
ii)
BamHI
EcoRI
BglII
IS5
EcoRI
BamHI
BamHI
BglII
IS1
BglII
EcoRI
5.8kb
BamHI
8.3kb
1kb
BglII
4.3kb
1.7kb
b) 3C7
i)
EcoRI
BamHI
KpnI
BamHI
KpnI
HindIII
BamHI
BamHI
BamHI
HindIII
EcoRI
EcoRI
15.9kb
BamHI
1kb
6.7kb
3.1kb
2.3kb
KpnI
4.8kb
HindIII
7.2kb
ii)
IS5
EcoRI
BamHI
BamHI
BglII
IS1
BglII
BglII
BamHI
BamHI
BamHI
EcoRI
IS1
EcoRI
15.5kb
BamHI
1kb
6.7kb
3.1kb
2.3kb
BglII
1.7kb
2.1kb

Figure 5.9

a)
DNA from pFJH90 digested with a variety of restriction enzymes and run in E-buffer on a 0.8% agarose slab gel. The markers are λ DNA cut with HindIII on the left and EcoRI/HindIII on the right. The gel was stained with EthBr.

b)
Restriction map of pFJH90. Vector sequences are defined by a single line. IS1 is represented by a large open box and it's orientation as defined by nucleotide sequence is shown by the arrow. Chromosomal DNA is shown as small boxed regions.

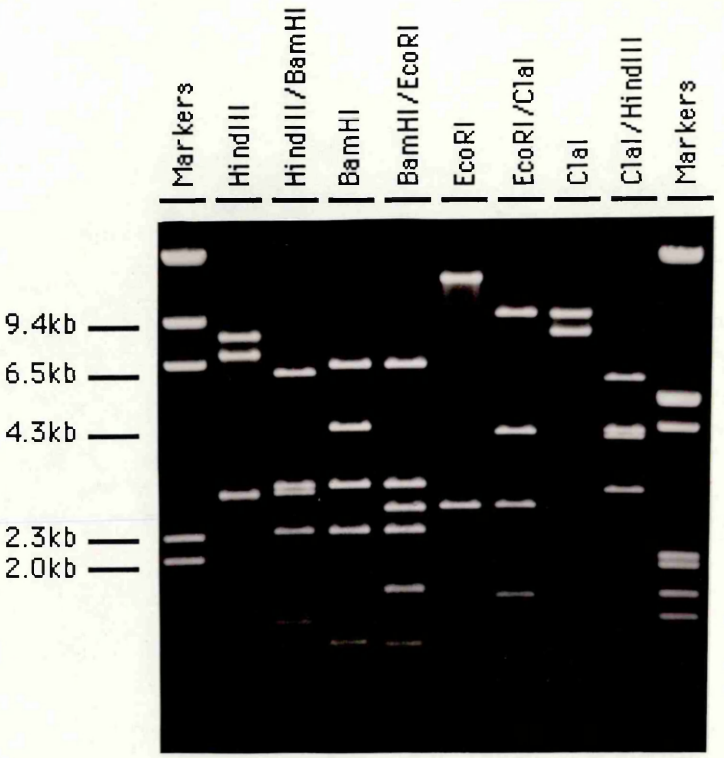
c)

HindIII	HindIII /BamH I	BamHI	BamHI /EcoRI	EcoRI	EcoRI/ ClaI	ClaI	HindII I/ClaI
8.4kb 7.2kb 3.0kb	6.0kb 3.15kb 3.05kb 2.3kb 1.3kb 1.15kb 0.95kb 0.39kb	6.4kb 4.3kb 3.1kb 2.3kb 1.15kb 0.95kb	6.4kb 3.1kb 2.7kb 2.3kb 1.6kb 1.15kb 0.95kb	>13kb 2.7kb	9.8kb 4.2kb 2.7kb 1.5kb	9.8kb 8.0kb	5.8kb 4.2kb 4..0kb 3.0kb 1.1kb
18.6kb	17.8kb	18.2kb	18.2kb	>15.7kb	18.3kb	17.8kb	18.1kb

Table showing the fragment sizes observed on the gel in a). The bottom row of numbers represents the total plasmid size derived by adding fragment sizes for each digest.

Figure 5.9

a)


Markers
HindIII
HindIII/BamHI
BamHI
BamHI/EcoRI
EcoRI
EcoRI/ClaI
ClaI
ClaI/HindIII
Markers
9.4kb
6.5kb
4.3kb
2.3kb
2.0kb

b)

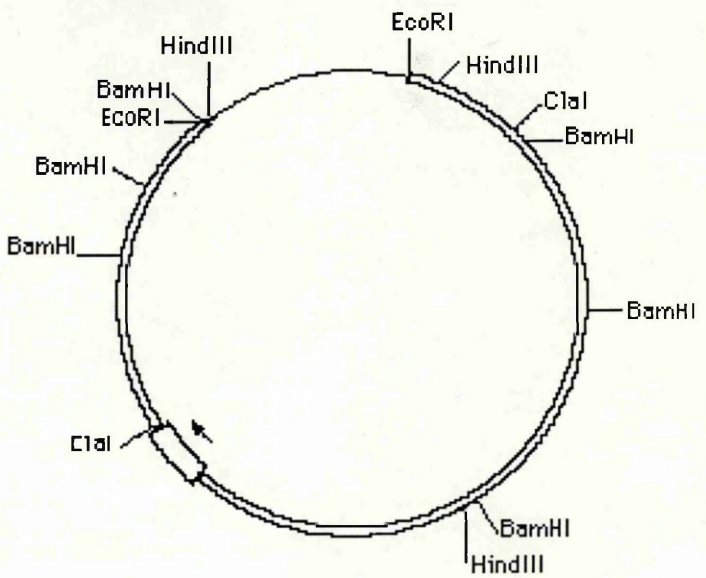

EcoRI
HindIII
HindIII
BamHI
EcoRI
ClaI
BamHI
BamHI
BamHI
BamHI
ClaI
BamHI
HindIII

Figure 5.10

Restriction maps of pFJH87 derived from the data shown below. Vector sequences are defined by a single line. IS1 is represented by large open boxes and it's orientation as defined by nucleotide sequence is shown by the arrows. Chromosomal DNA is shown as small boxed regions. Fragments showing hybridization to an IS1 probe are marked by an asterisk.

a)

BglII	BglII /EcoRI	EcoRI	EcoRI/ClaI	ClaI	ClaI/BglII
6.3kb	3.1kb	7.4kb*	4.1kb	10.1kb*	6.3kb
2.4kb	2.7kb	2.7kb	3.5kb		2.4kb
1.6kb*	2.4kb		2.7kb		0.86kb
	1.6kb				0.77kb
	0.5kb				

b)

KpnI	KpnI /EcoRI	KpnI /BglII
5.2kb*	5.2kb	3.1kb
4.2kb	2.7kb	2.4kb
0.96kb	1.5kb	1.6kb
	0.96kb	1.15kb
		1.05kb
		0.96kb

c)

MluI	MluI /EcoRI	MluI /ClaI	MluI /BglII
6.2kb	3.1kb	6.2kb	3.7kb
2.5kb	2.7kb	2.5kb	2.5kb
1.6kb*	2.5kb	1.3kb	2.4kb
	1.6kb	0.3kb	1.0kb
	0.4kb		0.6kb
			0.5kb

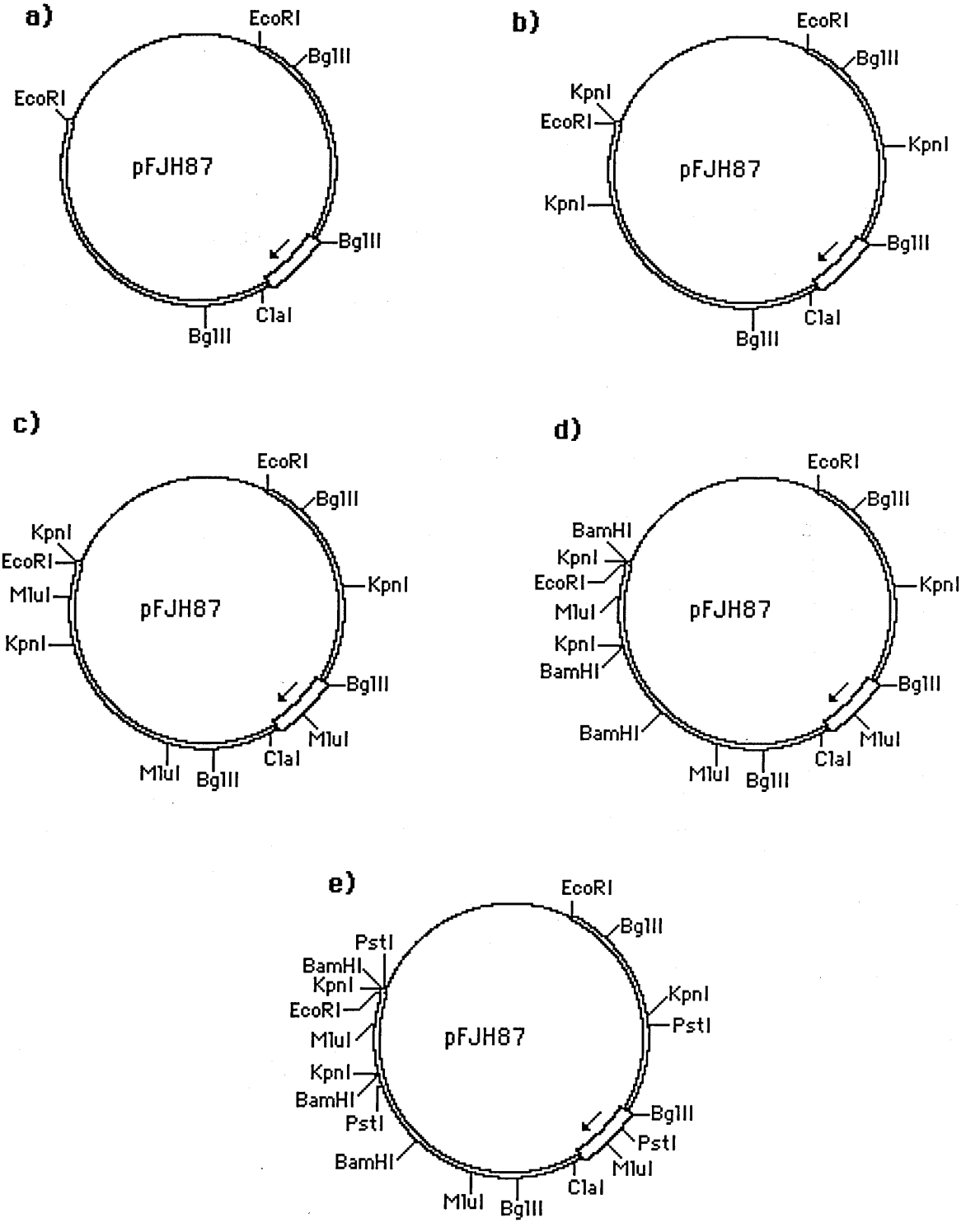
d)

BamHI	BamHI /EcoRI	BamHI /MluI
8.2kb*	5.5kb	5.5kb
1.1kb	2.7kb	1.6kb
0.85kb	1.1kb	0.95kb
	0.85kb	0.85kb
		0.6kb
		0.5kb

e)

PstI	PstI/ClaI	PstI /BglII
4.3kb	4.3kb	3.0kb
3.6kb*	3.0kb	2.2kb
1.3kb*	1.3kb	1.4kb
1.1kb	1.1kb	1.3kb
	0.6kb	1.1kb
		1.0kb
		0.3kb

Figure 5.10


a)
EcoRI
BglII
EcoRI
pFJH87
BglII
ClaI
BglII
b)
EcoRI
BglII
KpnI
EcoRI
KpnI
pFJH87
KpnI
BglII
ClaI
BglII
c)
EcoRI
BglII
KpnI
EcoRI
MluI
KpnI
pFJH87
KpnI
BglII
MluI
ClaI
MluI
BglII
d)
EcoRI
BglII
BamHI
KpnI
EcoRI
MluI
KpnI
BamHI
pFJH87
KpnI
BglII
MluI
ClaI
BamHI
MluI
BglII
e)
EcoRI
BglII
PstI
BamHI
KpnI
EcoRI
MluI
KpnI
BamHI
PstI
pFJH87
KpnI
PstI
BglII
PstI
MluI
ClaI
BamHI
MluI
BglII

these fragments can be deduced to be that shown in Figure 5.10a. KpnI cuts pFJH87 three times (once in the pUC19 polylinker) to fragments of 5.2kb, 4.2kb and 0.96kb. EcoRI cuts the 4.2kb KpnI fragment to 2.7kb and 1.5kb while BglII cuts this KpnI fragment to 3.1kb and 1.15kb. BglII cuts the the large 5.2kb (IS1 containing) KpnI fragment to 2.4kb, 1.6kb and 1.05kb allowing the map shown in Figure 5.10b to be deduced. MluI cuts pFJH87 to three fragments of 6.2kb, 2.5kb and 1.6kb. EcoRI cuts the 6.2kb MluI fragment to 3.1kb, 2.7kb and 0.4kb. ClaI cuts the 1.6kb MluI fragment to 1.3kb and 0.3kb. MluI/BglII digest produces six bands; 3.7kb (containing the vector sequences), 2.5kb (an intact MluI fragment), 2.2kb (an intact BglII fragment), 1.0kb, 0.6kb and 0.5kb. The map derived from these digests is shown in Figure 5.10c. BamHI cuts pFJH87 three times (once in the pUC19 polylinker) producing fragments of 8.2kb, 1.1kb and 0.85kb. EcoRI cuts the largest (IS1 containing) BamHI fragment to 5.5kb and 2.8kb. MluI cuts the 1.1kb BamHI fragment to 0.6kb and 0.5kb and the 8.2kb fragment to 5.5kb, 1.6kb and 0.95kb. These data are shown in Figure 5.10d. PstI cuts pFJH87 four times (once in the pUC19 polylinker and once in IS1) giving fragments of 4.3kb, 3.6kb, 1.3kb and 1.1kb. As expected two bands (3.6kb and 1.3kb) show hybridization to the IS1 probe. ClaI cuts the 3.6kb PstI fragment to 3.0kb and 0.6kb. BglII cuts the 4.3kb PstI fragment to 3.0kb and 1.3kb, the 3.6kb fragment to 2.2kb and 1.4kb and the 1.3kb fragment to 1.0kb and 0.3kb. BglII does not cut the 1.1kb PstI fragment. These data allow the placement of PstI sites in the complete restriction map of pFJH87 shown in Figure 5.10e. The PstI fragments from pFJH87 have subsequently been subcloned by Flora Rodgers. Restriction mapping of these subclones has confirmed the data presented here.

5.2.4 Constructing vectors to replace the wild-type IS1s of Tn2901

Gutterson and Koshland, using the *pol*A/ColE1 system, were able to replace chromosomal sequences at high frequency (Gutterson and Koshland 1983). Using the λdv system the *E.coli xer* genes have been replaced with non-functional copies (Flinn *et al.* 1989). The basic requirement in this system is sufficient regions of homology on either side of the mutation to allow homologous recombination in either direction (Figure 5.11). Using the pIC20 plasmids (Marsh *et al.* 1984) as building blocks I have designed a system that will allow the replacement of Tn2901's IS1 elements with either IS1 mutants or simply an antibiotic gene. The scheme for the construction of the right hand vector is shown in Figure 5.12. The starting point for this vector is pFJH97 which is described in Chapter 4. The 1.3kb HindIII/BamHI fragment from pFJH90 would be ideal homology from inside Tn2901. Unfortunately the HindIII sites in pFJH97 are symmetrical and would in any case insert the fragment in the wrong orientation. The HindIII end of the 1.3kb fragment was changed to an EcoRI end by cloning it into pICmodX. pICmodX is derived from pIC20R by cutting with NruI and EcoRV, both of which produce blunt ends, and religating. pICmodX is thus deleted for EcoRV, XbaI, BglII, XhoI and NruI sites. The 1.3kb HindIII/BamHI fragment from pFJH90 was ligated to HindIII/BamHI cut and phosphatased pICmodX. pFJH126 was isolated from transformed DS902 cells. The 1.3kb fragment was re-isolated as a EcoRI/BamHI fragment from pFJH126 and ligated to EcoRI/BamHI cut and phosphatased pFJH97. The ligation reaction was used to transform DS902 and pFJH127 was isolated. When pFJH127 is digested with BamHI/EcoRV fragments of 2.8kb and 2.7kb are produced, with BamHI/EcoRI fragments of 4.3kb and 1.3kb, with EcoRV/EcoRI fragments of 4.0kb and 1.5kb and with HindIII fragments of 2.7kb, 1.5kb and 1.3kb are seen (Figure 5.13). The single EcoRI site on pFJH127 can be used for any of the IS1 mutants described above. In addition wild-type or mutant IS1 elements

Figure 5.11

Replacement of wild type chromosomal regions by sequences altered *in vitro*. The scheme shows the replacement of the right hand IS1 of Tn2901. It is, however, applicable to any region for which roughly equivalent amounts of cloned flanking DNA from both sides is available. Recombination can occur on either side of IS1 resulting in integration, in this case forced by the use of a λdv replicon in the presence of cI repressor and chloramphenicol. Recombination to the right of IS1 and the resulting integrated structure are shown in the diagram. Recombination to the left of IS1 will result in a similar structure with the mutant IS1 sequence lying to the left of the wild type. Removal of chloramphenicol from the growth medium should result in resolution. If this occurs on the opposite side of IS1 to the integration event then the mutant sequence will replace the wild type in the chromosome. Vector sequences are defined by a single line and the CAT gene (cm^r) by a double line. IS1 is represented by large open boxes and the *in vitro* altered sequences by an inverted black triangle. Its orientation as defined by nucleotide sequence is shown by the arrows. Chromosomal DNA is shown as small boxed areas with regions of homology shaded.

Figure 5.11

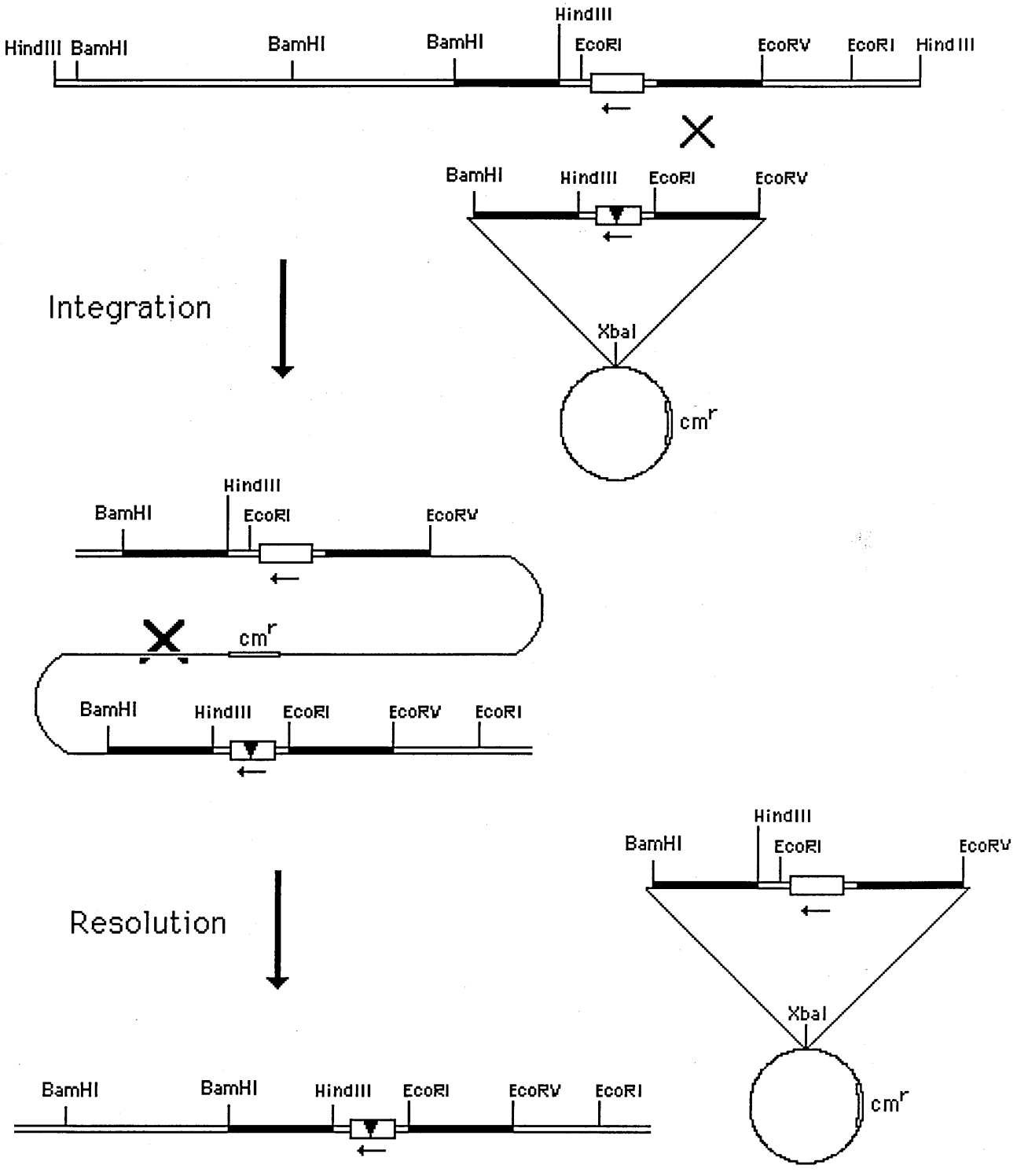

HindIII
HindIII BamHI
BamHI
BamHI
EcoRI
EcoRV
EcoRI
Hind III
BamHI
HindIII
EcoRI
EcoRV
Integration
XbaI
cm^r
HindIII
BamHI
EcoRI
EcoRV
cm^r
BamHI
HindIII
EcoRI
EcoRV
EcoRI
HindIII
BamHI
EcoRI
EcoRV
Resolution
XbaI
cm^r
BamHI
BamHI
HindIII
EcoRI
EcoRV
EcoRI

Figure 5.12

Scheme for building a replacement vector for the right hand IS1 of Tn2901. Details in the text. Vector sequences are defined by a single line. IS1 is represented by a large open box and it's orientation as defined by nucleotide sequence is shown by the small arrow below. Chromosomal DNA is shown as small boxed regions. Asterisks denote a restriction site that is sensitive to *dam* methylation.

a)

The right hand end of Tn2901 with suitable IS1-flanking DNA underlined.

b)

The pIC20R polylinker with the region deleted in pIC20RmodX underlined. This deletion removed an XbaI site that would have fallen between the two regions of homology, adjacent to the EcoRI site in g).

c)

The pIC20RmodX polylinker with the region deleted by the insertion of the BamHI/HindIII chromosomal region to produce pFJH126 underlined.

d)

The polylinker and insert of pFJH126 with the EcoRI/BamHI fragment cloned as part of pFJH127 underlined.

e)

The pIC20H polylinker with the region deleted by the insertion of the chromosomal region from plasmid pFJH96 (Chapter 4) underlined.

f)

The polylinker and insert of pFJH97 (Chapter 4). The region deleted by insertion of the EcoRI/BamHI fragment from pFJH126 is underlined.

g)

The polylinker and inserts of pFJH127. The IS1-flanking DNA is present in the same relative orientation as that in the chromosome. The unique EcoRI site can be used to insert either mutant IS1 elements or any other piece of DNA isolated on an EcoRI fragment that one wishes to replace the wild type IS1 with. The entire construction can then be moved using the XbaI sites to a λdv vector for insertion into the chromosome.

Figure 5.12

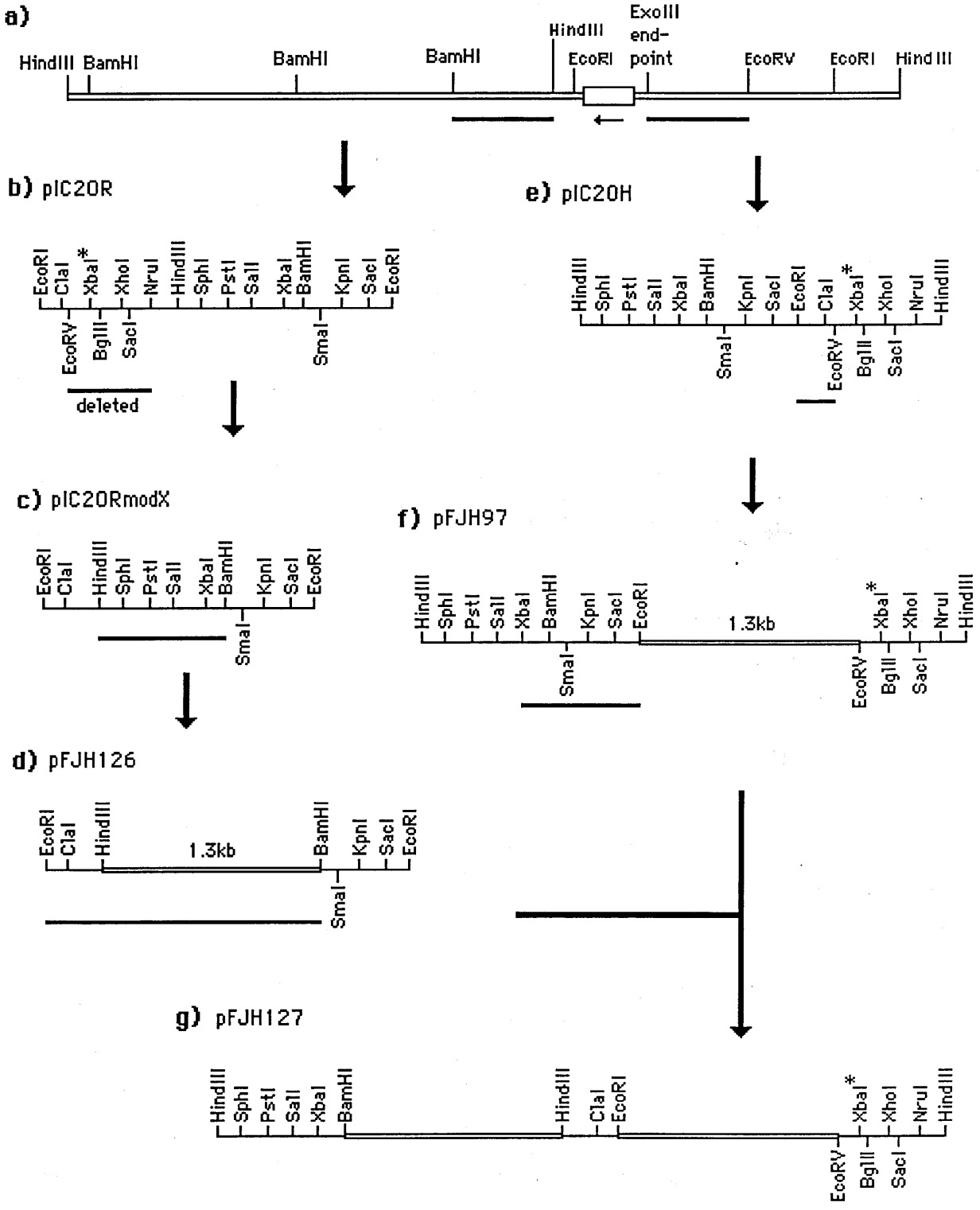

a)
HindIII BamHI
BamHI
BamHI
HindIII
EcoRI
ExoIII end-point
EcoRV
EcoRI
HindIII
b) pIC20R
EcoRI ClaI XbaI* XhoI NruI HindIII SphI PstI SalI XbaI BamHI KpnI SacI EcoRI
EcoRV BglII SacI
SmaI
deleted
e) pIC20H
HindIII SphI PstI SalI XbaI BamHI KpnI SacI EcoRI ClaI XbaI* XhoI NruI HindIII
SmaI
EcoRV BglII SacI
c) pIC20RmodX
EcoRI ClaI HindIII SphI PstI SalI XbaI BamHI KpnI SacI EcoRI
SmaI
f) pFJH97
HindIII SphI PstI SalI XbaI BamHI KpnI SacI EcoRI
1.3kb
XbaI* XhoI NruI HindIII
SmaI
EcoRV BglII SacI
d) pFJH126
EcoRI ClaI HindIII
1.3kb
BamHI KpnI SacI EcoRI
SmaI
g) pFJH127
HindIII SphI PstI SalI XbaI BamHI
HindIII ClaI EcoRI
XbaI* XhoI NruI HindIII
EcoRV BglII SacI

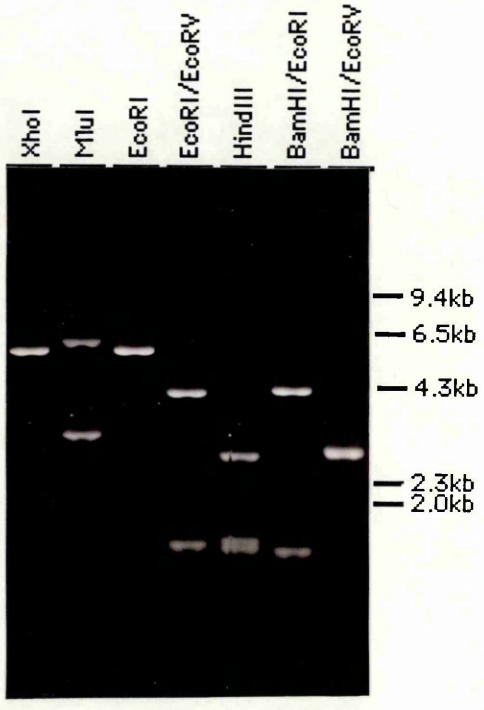
Figure 5.13

a)
DNA from pFJH127 digested with a variety of restriction enzymes and run in E buffer on a 0.8% agarose slab gel. The gel was stained with EthBr.

b)
Restriction map of pFJH127. Vector sequences are defined by a single line. Chromosomal DNA is shown as small boxed regions. Asterisks denote a restriction site that is sensitive to *dam* methylation.

Figure 5.13

a)



b)

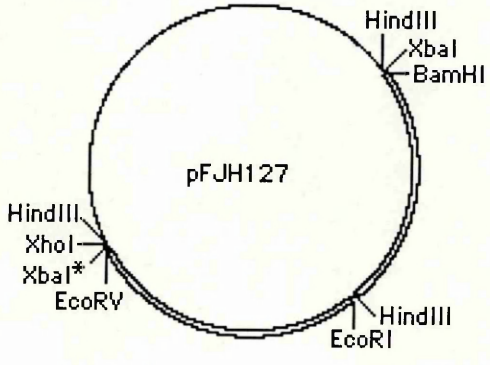


Figure 5.14

a)
DNA from pFJH130 digested with EcoRI, EcoRI/MluI or HindIII/MluI and run in TBE buffer on a 2.5% agarose slab gel. The markers are HindIII cut λ DNA. The gel was stained with EthBr.

b)
DNA from pFJH132 digested with HindIII or HindIII/XhoI and run in TBE buffer on a 2.5% agarose slab gel. The markers are HindIII/EcoRI cut λ DNA. The gel was stained with EthBr.

c)
A 2.5% agarose slab gel run in TBE buffer showing STET DNA from:
Lane1; pFJH129, Lane 2; pFJH132, Lane 3; an isolate identical to pFJH132, all cut with HindIII/XhoI
Lane 4; pFJH130, Lane 5; pFJH131, Lane 6; an isolate identical to pFJH130, all cut with HindIII/MluI.
The markers are pFJH130 cut with EcoRI/MluI. The gel was stained with EthBr.

Figure 5.14

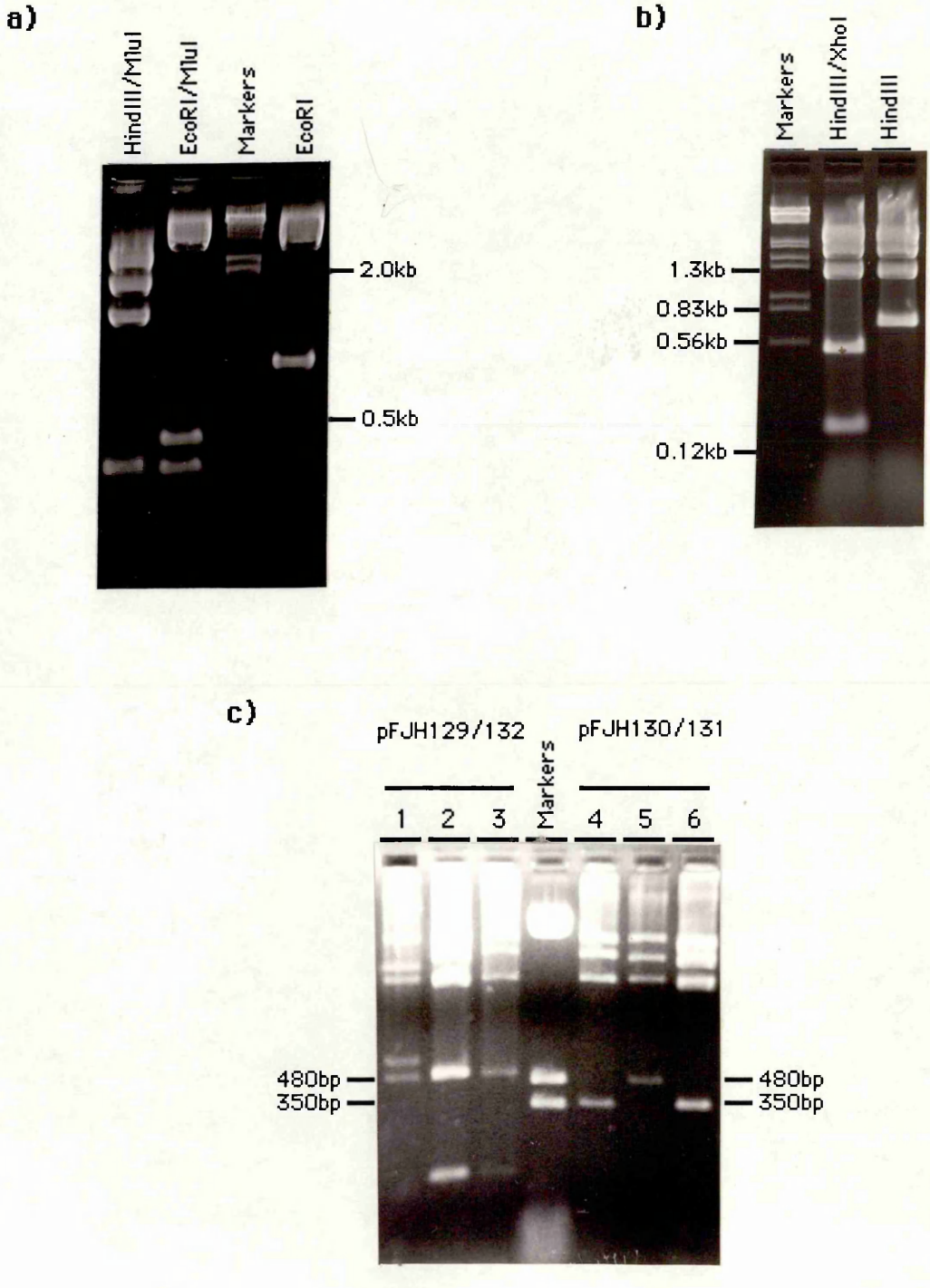

a)
HindIII/MluI
EcoRI/MluI
Markers
EcoRI
2.0kb
0.5kb
b)
Markers
HindIII/XhoI
HindIII
1.3kb
0.83kb
0.56kb
0.12kb
c)
pFJH129/132
pFJH130/131
1 2 3
Markers
4 5 6
480bp
350bp
480bp
350bp

Figure 5.15

Vector sequences are defined by a single line. The *in vitro* altered IS1 sequences are represented by large open boxes with an inverted black triangle. It's orientation as defined by nucleotide sequence is shown by the arrows. The APH gene is shown as large stippled boxes and chromosomal DNA by small boxed areas.

a)

Restriction maps showing the orientation of the *in vitro* altered IS1 with respect to the internal HindIII site of pFJH127 in

i) pFJH131

ii) pFJH130

Fragment sizes are shown. pFJH131 carries the IS1 element in the chromosomal orientation with respect to the flanking DNA.

b)

Restriction maps showing the orientation of the APH gene with respect to the internal HindIII site of pFJH127 in

i) pFJH132

ii) pFJH129

HindIII and HindIII/XhoI fragment sizes are shown.

Figure 5.16

Restriction maps of the λdv plasmids derived from pFJH130 and pFJH132. Vector sequences are defined by a single line. The *in vitro* altered IS1 sequence is represented by a large open box with a black triangle. It's orientation as defined by nucleotide sequence is shown by the arrow. The APH gene is shown as a large stippled box and chromosomal DNA by small boxed areas. Asterisks denote a restriction site that is sensitive to *dam* methylation.

a) pFJH137 from pFJH130

b) pFJH138 from pFJH132

Figure 5.15

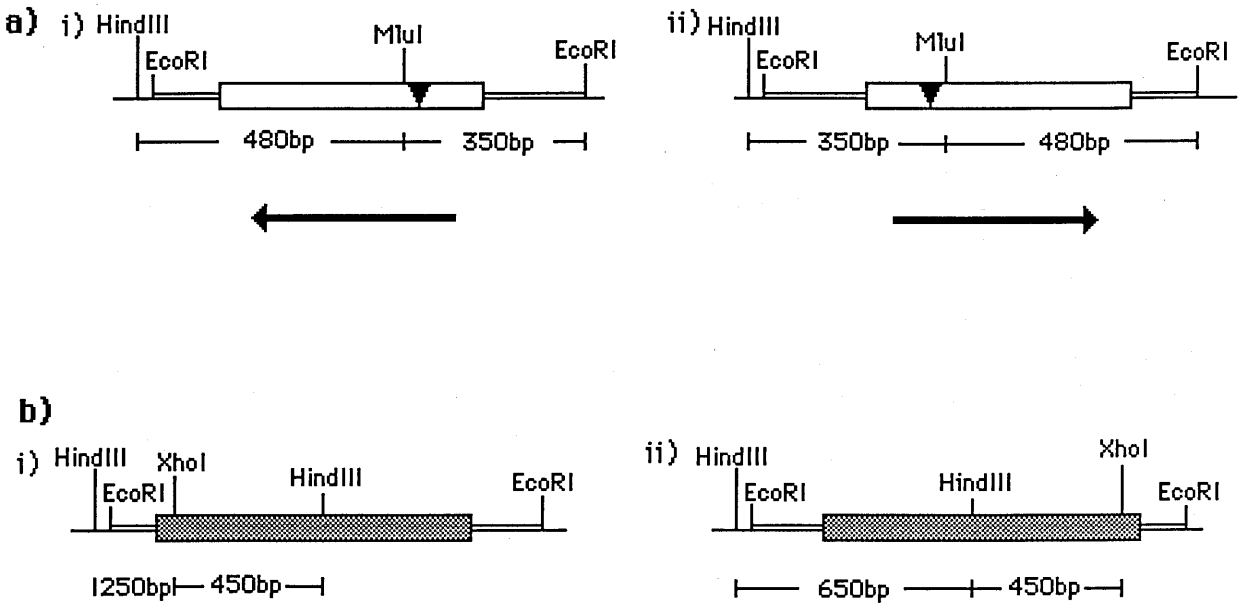
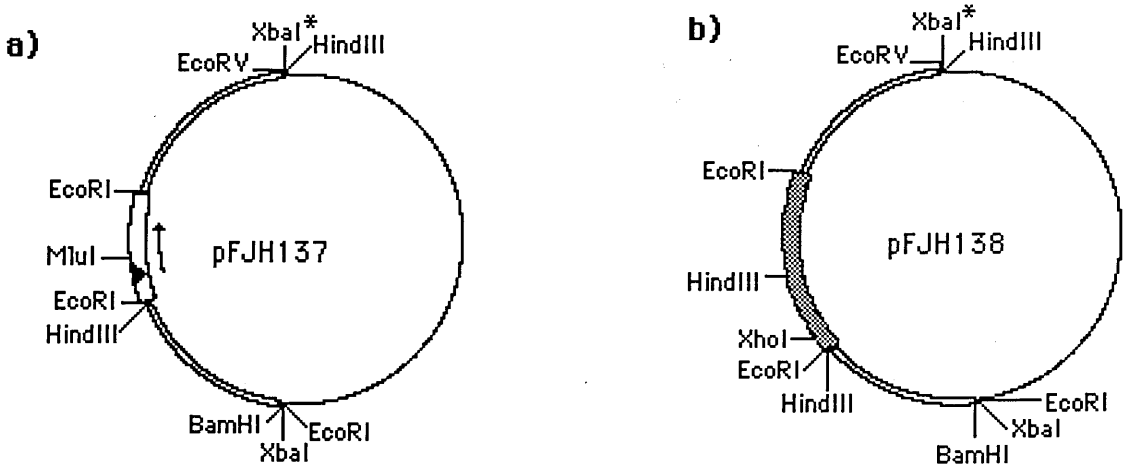


Figure 5.16



can be inserted in an opposite orientation to that found in the chromosome. The HaeII deletion mutant of IS1 isolated as a 850bp EcoRI fragment from pFJH60 and the 1.5kb EcoRI fragment from pUC71K carrying the APH gene were separately ligated to EcoRI cut and phosphatased pFJH127. pFJH130 and pFJH131 contain the HaeII deletion mutant inserted in pFJH127 in opposite orientations. When cut with EcoRI/MluI pFJH130 produces small fragments of about 450bp and 400bp and when cut with HindIII/MluI only the 400bp fragment is seen (Figure 5.14a). Thus pFJH130 contains the mutant IS1 element in the opposite orientation to the IS1 elements in the chromosome. Conversely pFJH131 produces a 450bp small fragment when cut with HindIII/MluI and must contain the HaeII deletion mutant in the same orientation as the chromosomal IS1 elements (Figure 5.14c). pFJH132 and pFJH129 contain the APH gene from pUC71k inserted in pFJH127 in opposite orientations. When pFJH132 is digested with HindIII a small fragment of about 0.7kb is seen. This fragment is cut by XhoI to two small fragments of 450bp and 250bp (Figure 5.14b). When pFJH129 is digested with HindIII/XhoI the 0.7kb fragment is not cut, instead a second small fragment of about 750bp is released (Figure 5.14c). This data is summarized in Figure 5.15. To replace the right hand IS1 of Tn2901 with either the APH gene or the HaeII deletion mutant they and the flanking chromosomal DNA must be moved to a λdv plasmid. Unmethylated DNA was prepared by growing pFJH130 and pFJH132 in CB51. The 3.5kb XbaI fragment from pFJH130 and the 4.1kb XbaI fragment from pFJH132 were isolated and each ligated to XbaI cut and phosphatased 19A. Plasmids pFJH137, carrying the IS1 HaeII deletion mutant, and pFJH138, carrying the APH gene, were isolated from DS902 (Figure 5.16). These plasmids carry a 300bp deletion at the start of the *arg*F gene. The deletion extends from the EcoRI site (Figure 3.8, line 1) lying to the right of *arg*F to the first HindIII site in the gene (Figure 3.8, line 7) and includes the entire promotor region. This fragment can be isolated from a HindIII/EcoRI digest of pFJH20 (Figure 4.5d). The 300bp fragment, or mutated promotor fragments, can be returned to the constructs as follows; The EcoRI end of the fragment can be converted to a HindIII end using linkers. This will not effect *arg*F (Recall that inserts at this site did not affect expression of *arg*F, Chapter 3, Results, paragraph 3). The 3.5kb XbaI

Figure 5.17

Scheme for building a replacement vector for the left hand IS1 of Tn2901. Details in the text. Vector sequences are defined by a single line. IS1 is represented by a large open box and it's orientation as defined by nucleotide sequence is shown by the small arrow below. Chromosomal DNA is shown as small boxed regions. Asterisks denote a restriction site that is sensitive to *dam* methylation.

a)
The left hand end of Tn2901 with suitable IS1-flanking DNA underlined.

b)
The pIC20H polylinker with the region that will be deleted by the insertion of the BglII/ClaI chromosomal region underlined.

c)
The polylinker and insert of the plasmid that will be produced from b) with the region that will be deleted by the insertion of the EcoRI/KpnI fragment from pFJH113 (e) underlined.

d)
The pIC20R polylinker with the region deleted by the insertion of the BglII/KpnI chromosomal region underlined.

e)
The polylinker and insert of pFJH113 with the region that will be cloned as part of the final plasmid underlined.

f)
The polylinker and inserts expected in the final plasmid. The IS1-flanking DNA is present in the same relative orientation as that in the chromosome. The unique EcoRI site can be used to insert either mutant IS1 elements or any other piece of DNA isolated on an EcoRI fragment that one wishes to replace the wild type IS1 with. The entire construction can then be moved using either the HindIII sites, or in the case of the APH gene which contains an internal HindIII site the KpnI and SacI sites, to a λdv vector for insertion into the chromosome.

Figure 5.17

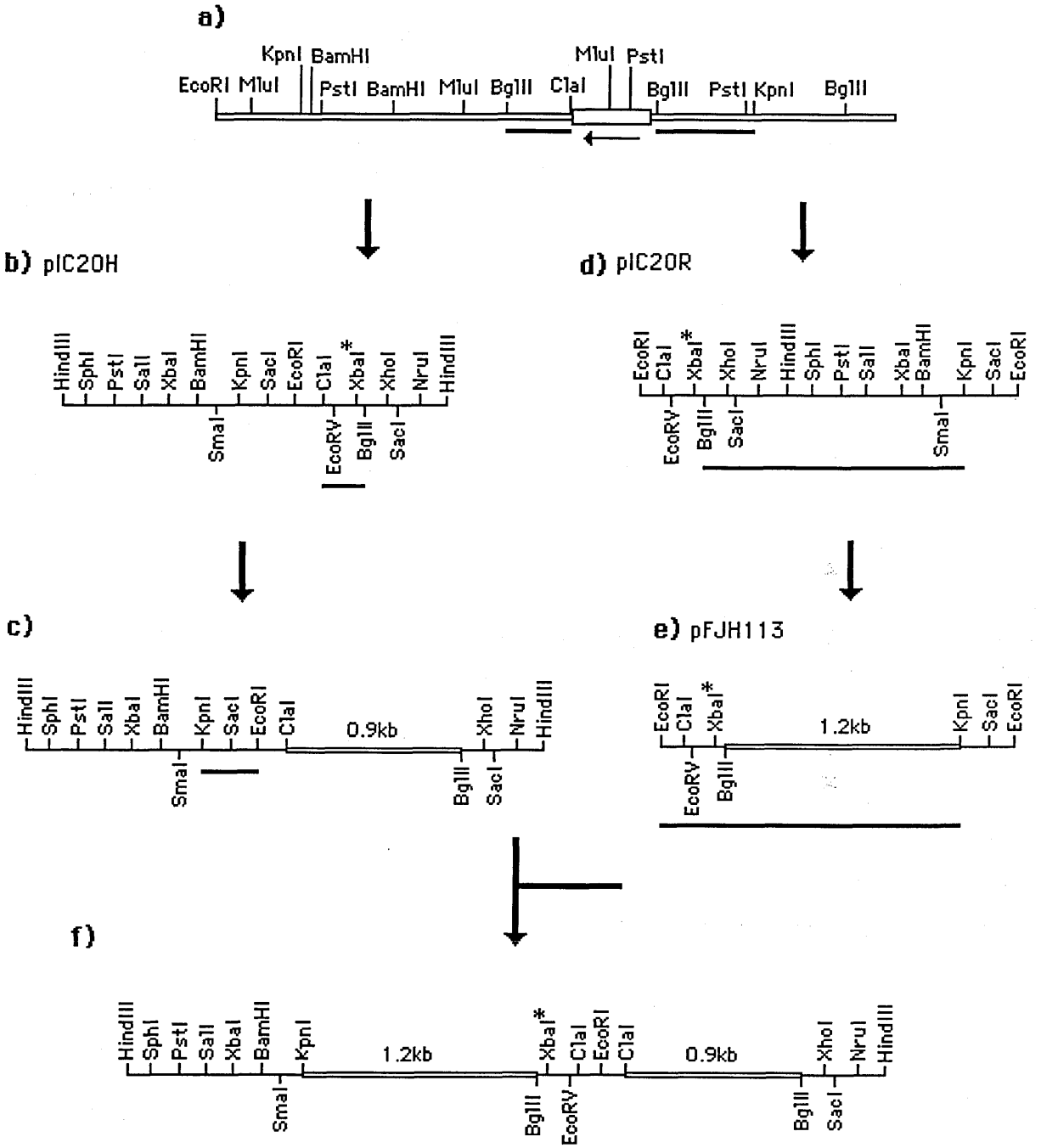

a)
KpnI BamHI
MluI PstI
EcoRI MluI
PstI BamHI MluI BglII ClaI
BglII PstI KpnI BglII
b) pIC20H
HindIII SphI PstI SalI XbaI BamHI KpnI SacI EcoRI ClaI XbaI* XhoI NruI HindIII
SmaI
EcoRV BglII SacI
d) pIC20R
EcoRI ClaI XbaI* XhoI NruI HindIII SphI PstI SalI XbaI BamHI KpnI SacI EcoRI
EcoRV BglII SacI
SmaI
c)
HindIII SphI PstI SalI XbaI BamHI KpnI SacI EcoRI ClaI 0.9kb XhoI NruI HindIII
SmaI
BglII SacI
e) pFJH113
EcoRI ClaI XbaI* 1.2kb KpnI SacI EcoRI
EcoRV BglII
f)
HindIII SphI PstI SalI XbaI BamHI KpnI 1.2kb XbaI* ClaI EcoRI ClaI 0.9kb XhoI NruI HindIII
SmaI
BglII
EcoRV
BglII SacI

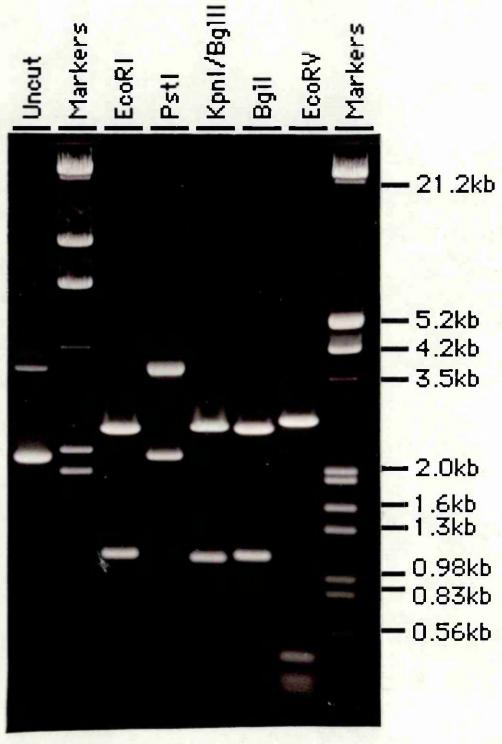
Figure 5.18

a)
DNA from pFJH113 digested with a variety of restriction enzymes and run in E buffer on a 0.8% agarose slab gel. The markers are λ DNA cut with HindIII on the left and HindIII/EcoRI on the right. The gel was stained with EthBr.

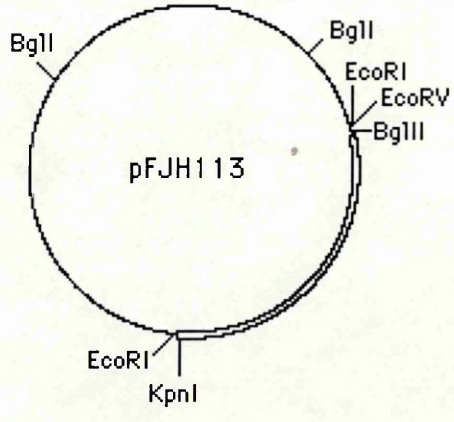
b)
Restriction map of pFJH113. Chromosomal DNA is shown as a small boxed region and vector sequences by a single line. The insert contains at least two EcoRV sites the position of which has yet to be accurately determined.

Figure 5.18

a)



b)



fragment from pFJH131 can be inserted into the XbaI site of a λdv plasmid deleted for the HindIII site by cutting, filling-in and blunt end ligating. The resulting plasmid will contain a single HindIII site into which the 300bp *arg*F fragment can be inserted. The orientation of the HindIII fragment can be determined by MluI/PstI digest (a 500bp fragment will be generated in the 'correct' orientation, but an 800bp will be seen when the fragment is in the wrong orientation with respect to the other chromosomal sequences).

5.2.5 Vector for replacing the left hand IS1 of Tn2901

The scheme for the construction of this vector is shown in Figure 5.17. The 1.15kb BglII/KpnI fragment from pFJH87 lies inside Tn2901. It was isolated and ligated to BglII/KpnI cut and phosphatased pIC20R. The resulting plasmid pFJH113 was isolated from transformed DS902 cells (Figure 5.18). Unfortunately time has not allowed me to complete the other steps in this procedure but they are being carried out in this laboratory by Flora Rodgers. Briefly; The 0.9kb ClaI/BglII fragment from pFR10 will be cloned to pIC20H. The 1.15kb fragment from pFJH113 will be isolated as a KpnI/EcoRI fragment and ligated to the new plasmid. This will result in a unique EcoRI site into which mutant IS1's or an antibiotic resistance gene may be inserted. The entire cassette can be transfered to a λdv plasmid using either the HindIII sites or as a SstI/KpnI fragment if the APH gene which contains a HindIII site is used.

5.3 Discussion

A vector for replacing the right hand IS1 of Tn2901 was successfully constructed. The IS1 deletion mutant and the kanamycin resistance cassette have been cloned into the vector. The IS1 deletion mutant has now been inserted into the chromosome and screening to identify a strain where the deletion has replaced the wild-type IS1 is under way (Adrienne Jessop, personal communication). Thus it seems likely that this family of vectors can be used successfully to replace wild type sequences with those altered *in vitro*.
Unfortunately Flora Rodgers who was completing the construction of the left hand replacement vector has left the laboratory, so that this work has suffered some delay. I am however confident that these vectors can be used to determine if the IS1 elements function merely as regions of homology in the amplification of Tn2901. If an IS encoded function is required for this amplification then the vectors, combined with the IS1s in which point mutations have been created will prove additionally useful in investigating which function is required. The IS1 elements could be placed in inverse orientation. *Arg*F promotor mutants may be inserted to see if promotor strength is related to number of units in the amplification.

CHAPTER SIX

CONCLUDING REMARKS

Concluding Remarks

I set out in this thesis to investigate the mechanisms by which *E. coli* cells utilize citrulline as a source of carbamoyl phosphate. In particular how the F factor allows the amplification of an IS1-flanked unit, Tn2901, in response to this selection. As is shown in Chapter 3, the F factor is not the only means of stimulating Tn2901 amplification. Incubation of F^- cells on citrulline at 30°C for a long period resulted in the appearance of amplified Tn2901 DNA. The presence of multicopy plasmids in cells during selection also resulted in some rearrangement that resembles amplification in colony hybridization tests. Preliminary results of other workers in the laboratory suggest that this may be the result of Tn2901 transposition to the multicopy plasmid. The observation that these events occur at greater frequency when pBR322 (which contains a hot-spot for IS1 insertion) rather than other multicopy plasmids is used strengthens this suggestion.

I have also demonstrated that citrulline utilizing mutants can cross-feed non mutant cells. This in conjunction with preliminary sequential hybridization studies suggests an origin for a class of stable (unamplified) mutants observed in some strains that also give rise to unstable (amplified) mutants. Stable class II cut mutants may be indirectly dependent on the amplification of Tn2901, but are they dependent on the presence of the F factor? If so are those that occur at *arg*F related to the same event(s) that lead to amplification? Since the initial event in the amplification is recombination between the IS1 elements of Tn2901, (Clugston 1986), then amplification could be prevented by replacing one of the IS1 elements by a region of non-homology. If the left hand IS1 is replaced then events at the right hand IS1 may still occur. A vector for replacing the left hand IS1 has been designed and the sequences required for its construction have been cloned (Chapter 5). Another question that must be raised about the class II cut mutants is why so few are observed when Jef8 is plated on citrulline compared with related Hfrs (Clugston and Jessop 1990). If the answer to this is

that the amplification occurs more readily in Jef8, then we would like to understand why that is. Both this question and events at Tn2901 will be more amenable to investigation once the major role of the F factor in amplification is known.

The results presented at the start of Chapter 4 complete and extend Carol Clugston's analysis of the role played by conjugal DNA transfer in the amplification process. We are convinced by these results that if conjugation plays any part in the amplification of Tn2901 then it is a minor one. The major contender for a role in the stimulation of amplification must be replication. Replication has been observed to stimulate amplification in other systems (Petit *et al.* 1986). Unfortunately experiments designed to demonstrate a role for replication using replication origins were unsuccessful. Convincing evidence for or against the replication hypothesis may be obtained by inserting a replication terminus between F and Tn2901 in Jef8 as described in the discussion to Chapter 4. Alternative approaches may however yield some useful data. If replication is involved it may proceed by a rolling circle mechanism as suggested by Petit *et al.* for the amplification of their artificial construct in *B. subtilis*. This could be tested by cutting amplified strains with a restriction enzyme for which there are no sites in Tn2901. Since Tn2901 is more than 11kb long and restriction mapping of plasmids into which portions of it have been inserted demonstrates site for many six base pair cutters (Chapter 5), it may be necessary to use a rare cutting enzyme such as NotI and analyse the results by pulse field electrophoresis. If amplification does not occur by a rolling circle mechanism it should be possible to observe amplification intermediates. One technical problem with this experiment is that selection for amplification must be maintained for all cells. Segregants losing the amplification by unequal crossing over will confuse the results. It is feasible given the cross-feeding observed that segregants exist even within colonies. This problem might be avoided by introducing a concentration sensitive antibiotic resistance gene into Tn2901. Such genes appear from the literature to be available (Meyer and Lida 1979), however workers in this laboratory have not yet identified one that

performs adequately in the amplification of Tn2901 (Adrienne Jessop, personal communication). If replication initiated by the F factor is the major stimulus for amplification then the roles of the *rel*A and *spo*T mutations carried by Jef8 should be examined in relation to the question "why does the amplification occur so readily in Jef8?". *rel*A and *spo*T gene products are involved in the stringent response to amino acid starvation in *E. coli* (Cashel and Rudd 1987). It has recently been shown that replication of plasmids derived from phage lambda is under stringent control. Replication of these plasmids is inhibited in stringent strains but proceeds for several hours in isogenic *rel*A strains (Wegrzyn *et al.* 1991). Replication of λ and F share a requirement for many host factors. Thus it would not be surprising if replication from F was under stringent control and was initiated more frequently under our selection procedure in a relaxed strain such as Jef8. It may in any case be interesting to examine the role of these mutations in the citrulline utilising phenomena as it has been reported that synthesis of *arg*I and *arg*F gene products is under positive stringent control (Cashel and Rudd 1987).

If replication is not involved in the amplification of Tn2901 then it may be possible to identify an F fragment that is able to stimulate amplification using the system described in Chapter 4 to insert cloned F fragments into F^- strains. Alternatively cloned F fragments could be disrupted *in vitro* and used to replace the corresponding F fragment in Jef8. Another interesting approach would be the characterization of the mutant described by Cluston and Jessop (1990) that is defective in the initial event leading to amplification. Detailed mapping of this mutant might suggest a likely locus in which characterized mutations exist. These could then be tested for their ability to interfere with the amplification of Tn2901. Failing this it may be necessary to clone the mutation to identify it. Since the mutation does not map in Tn2901 or F (Clugston and Jessop 1990), it is possible that it encodes a *trans*-acting factor required for the initial event. If this is the case it may be possible to clone the region responsible by complementation. This is likely to be difficult; recall that multicopy plasmids stimulate events of some nature at Tn2901 and that plasmids may be lost during incubation on

citrulline. In addition it would be wise to use a library made on a strain deleted for Tn2901 when conducting this experiment. Given these considerations it may be simpler to reisolate the mutant following transposon mutagenesis which would allow quick and easy cloning.

Biochemical analyses of the OTCase produced by stable class II mutants should allow them to be divided into mutations at *arg*I or *arg*F (by elution pattern from diethylaminoethyl-sephadex and thermolability) and those that are leaky *arg*G mutants (by OTCase specific activity). Mutations in *arg*F and *arg*I may be tested for a potential shift in the equilibrium of OTCase in favour of the phosphorolysis of citrulline. When data from these experiments are added to those from the genetic analyses of both stable and amplified mutants described above, a fascinating array of *E. coli*'s responses to this selection procedure will be apparent.

This process of dissecting the mutations has already been in progress for a number of years and the experiments described above will inevitably require a number more. An alternative and complementary approach to identifying the range of mutations or genetic activity produced in *E. coli* by particular environmental conditions may be the differential hybridization screen, beloved of eukaryotic geneticists. The recent publication by Kohara *et al.* of a complete map of the *E. coli* genome and the willingness of these authors to distribute full clone sets provides us with a perfectly representative library. cDNA probes made from cells under different environmental conditions could be used to screen an array of the entire genome for differential gene activity. The beauty of this type of experiment in *E. coli* is that much of the gene expression observed will derive from known loci allowing us to observe or deduce an overall pattern of cellular responses to the chosen environmental conditions.

BIBLIOGRAPHY

Achtman, M., Skurray, R.A., Thompson, R., Helmuth, R., Hall, S., Beutin, L. and Clark, A.J. (1978). "Assignment of *tra* cistrons to EcoRI fragments of F sex factor DNA." J. Bact. **133**: 1383-1392.

Adelberg, E.A. and Burns, S.N. (1960). "Genetic variation in the sex factor of *Escherichia coli*." J. Bact. **79**: 321-330.

Ahmed, A. (1984). "A deletion analysis of transposon Tn9." J. Mol. Biol. **173**: 523-529.

Albertini, A.M., Hofer, M., Calos, M.P. and Miller, J.M. (1982). "On the formation of spontaneous deletions: The importance of short sequence homologies in the generation of large deletions." Cell **29**: 319-328.

Amundsen, S.K., Taylor, A.F., Chaudhury, A.M. and Smith, G.R. (1986). "*recD*: the gene for an essential third subunit of Exonuclease V." Proc. Natl. Acad. Sci. USA **83**: 5558-5562.

Arthur, A. and Sherratt, D.J. (1979). "dissection of the transposition process: a transposon encoded site specific recombination system." Mol. Gen. Genet. **175**: 267-274.

Bachmann, B.J. (1987b). "Derivations and genotypes of some mutant derivatives of *Escherichia coli* K-12." *Escherichia coli and Salmonella typhimurium*: Cellular and Molecular Biology. Ed F. C. Neidhardt. American Society for Microbiology. (Washington, D.C.) First Edition, 1190-1219.

Berg, C.M. and Caro, L.G. (1967). "Chromosome replication in *E. coli*. I. Lack of influence of the integrated F factor." J. Mol. Biol. **29**: 419-431.

Berg, D.E., Egner, C. and Lowe, J.B. (1983). "Mechanism of F factor-enhanced excision of transposon Tn5." Gene **22**: 1-7.

Berstein, H., Byerly, H.C., Hopf, F.A. and Michod, R.E. (1985). "Genetic damage, mutation and the evolution of sex." Science **229**: 1277-1281.

Biel, S.W., Adelt, G. and Berg, D.E. (1984b). "Transcriptional control of IS1 transposition in *Escherichia coli*." J. Mol. Biol. **174**: 251-264.

Biel, S.W. and Berg, D.E. (1984a). "Mechanism of IS1 transposition in *E. coli*: choice between simple insertion and cointegration." Genetics **108**: 319-330.

Biggin, M.D., Gibson, T.J. and Hong, G.F. (1983). "Buffer gradient gels and [^{35}S]-label as an aid to rapid DNA sequence determination." Proc. Natl. Acad. Sci. USA **80**: 3963-3965.

Birnboim, H.C. and Doly, J. (1979). "Rapid alkaline extraction procedure for screening recombinant plasmid DNA." Nucl. Acid Res. **7**: 1513-1523.

Boyd, A.C. and Sherratt, D.J. (1986). "Polar mobilization of the *Escherichia coli* chromosome by the ColE1 transfer origin." Mol. Gen. Genet. **1986**: 496-504.

Boye, E. and Lobner-Olesen, A. (1990). "The role of *dam* methylation in the control of DNA replication in *E. coli*." Cell **62**: 981-989.

Braedt, G. (1988). "Different reading frames are responsible for IS1-dependent deletions and recombination." Genetics **118**: 561-570.

Bresler, S.E., Lanzov, V.A. and Likhachev, V.T. (1973). "On the mechanism of conjugation in *Escherichia coli* K12. III. Synthesis of DNA during the course of bacterial conjugation." Mol. Gen. Genet. **120**: 125-131.

Brewer, B.J. (1988). "When polymerases collide: Replication and transcriptional organization of the *E. coli* chromosome." Cell **53**: 679-686.

Bustos-Martinez, J.A. and Gomez-Eichelmann, M.C. (1987). "Frequency of IS1-mediated molecular events in different members of the family *Enterobacteriaceae*." J.Bact. **169**: 4946-4949.

Cairns, J. (1981). "The origins of human cancers." Nature **289**: 353-357.

Cairns, J., Overhaugh, J. and Miller, S. (1988). "The origin of mutants." Nature **335**: 142-145.

Cashel, M. and Rudd, K.E. (1987). "The stringent response." *Escherichia coli* and *Salmonella Typhimurium*: Cellular and Molecular Biology. Ed F. C. Neidhardt. American Society for Microbiology. (Washington, D.C.) First Edition, 1410-1438.

Cavalli-Sforza, L., Lederberg, J. and Lederberg, E. (1953). "An infective factor controlling sex compatibility in *E.coli*." J. Gen. Microbiol. **8**: 89-103.

Chandler, M., Clerget, M. and Caro, L. (1982). "The transposition frequency of IS1-flanked transposons is a function of their size." J. Mol. Biol. **154**: 229-243.

Chandler, M. and Galas, D.J. (1983a). "Cointegrate formation mediated by Tn9. II. Activity of IS1 is modulated by external DNA sequences." J. Mol. Biol. **170**: 61-91.

Chandler, M. and Galas, D.J. (1983b). "IS1-mediated tandem duplication of plasmid pBR322. Dependence on *rec*A and DNA polymeraseI." J. Mol. Biol. **165**: 183-190.

Chandler, M., Silver, L., Frey, J. and Caro, L. (1977). "Suppression of an *Escherichia coli dna*A mutattion by the integrated plasmid

R100.1: Generation of small plasmids after integration." J. Bact. **130**: 301-309.

Chandler, M., Silver, L., Lane, D. and Caro, L. (1979). "Properties of an autonomous r-Determinant from R100.1." CSHSQB **43**: 1223-1231.

Chandler, M., Silver, L., Roth, Y. and Caro, L. (1976). "Chromosome replication in an Hfr strain of *Escherichia coli*." J. Mol. Biol. **104**: 517-523.

Cheah, K.-C. and Skurray, R. (1986). "The F plasmis carries an IS3 insertion within FinO." J. Gen. Microbiol. **132**: 3269-3275.

Chen, E.J. and Seeburg, P.H. (1985). "Supercoil sequencing: a fast simple method for sequencing plasmid DNA." DNA **4**: 165-170.

Chernin, L.S., Gukova, L.A. and Ovadis, M.I. (1983). "Involvement of the F plasmid into chromosome replication of an Hfr strain of *Escherichia coli* K-12." Genetika **19**: 416-424.

Chernin, L.S., Ovadis, M.I. and Goldfarb, D.M. (1978). "F plasmid genes involved in the production of recombination-stimulating factor, contol of sensitivity to some injurious agents, and chromosome replication in *Escherichia coli* K-12 HfrC." J. Bact. **133**: 33-42.

Clark, A.J. and Margulies, A. (1965). "Isolation and characterization of recombination deficient mutants of *Escherichia coli* K-12." Proc. Natl. Acad. Sci. USA **53**: 451-459.

Clugston, C.K. (1986). Amplification of an IS1 flanked unit in Escherichia coli K-12. Ph.D. Thesis Glasgow University.

Clugston, C.K. and Jessop, A.P. (1990). "A bacterial position effect: When the F factor in *E. coli* K12 is integrated *in cis* to a chromosomal gene that is flanked by IS1 repeats the elements

are activated so that amplification and other regulatory changes that affect the gene can occur." Mut. Res. **In Press:**

Colloms, S.D., Sykora, P., Szatmari, G. and Sherratt, D.J. (1990). "Recombination at ColE1 *cer* requires the *Escherichia coli xer*C gene product, a member of the lambda integrase family of site-specific recombinases." In press :

Cornelis, G. and Saedler, H. (1980). "Deletions and an inversion induced by a resident IS1 of the lactose transposon Tn951." Mol. Gen. Genet. **178**: 367-374.

Cox, M.M. and Lehman, I.R. (1987). "Enzymes of general recombination." Ann. Rev. Biochem. **56**: 229-262.

Craig, N.L. and Kleckner, N. (1987). "Transposition and Site-Specific Recombination." *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology. Ed F. C. Neidhardt. American Society for Microbiology. (Washington, D.C.) First Edition, 1054-1070.

Cunin, R., Eckhardt, T., Piette, J., Boyen, A., Pierard, A. and Glansdorff, N. (1983). "Molecular basis for modulated regulation of gene expression in the arg regulon of *Escherichia coli* K-12." Nuc. Acid. Res. **11**: 5007-5019.

Dandanell, G., Valentin-Hansen, P., Larsen, J.E.L. and Hammer, K. (1987). "Long-range cooperativity between gene regulatory sequences in a prokaryote." Nature **325**: 823-826.

Davidson, N., Deonier, R.C., Hu, S. and Ohtsubo, E. (1975). "Electron microscope heteroduplex studies of sequence relations among plasmids of *Escherichia coli*. X. Deoxyribonucleic acid sequence organization of F and of F-primes, and the sequences involved in Hfr formation." Microbiology-1974. Ed D. Schlessinger. American Society for Microbiology. (Washington, D.C.) First Edition, 56-65.

de Massy, B. and Fayet, O. (1984). "Multiple origin usage for DNA replication in *sdr*A (*rnh*) mutants of *Escherichia coli* K-12." J. Mol. Biol. **178**: 227-236.

Dempsey, W.B. (1987). "Transcript analysis of the plasmid R100 TraJ and FinP genes." Mol. Gen. Genet. **209**: 533-544.

Deonier, R.C. and Hadley, R.G. (1980). "IS2-IS2 and IS3-IS3 relative recombination frequencies in F integration." Plasmid **3**: 48-64.

Derbyshire, K.M., Hwang, L. and Grindley, N.D.F. (1987). "Genetic analysis of the interaction of the insertion sequence IS903 transposase with its terminal inverted repeats." Proc. Natl. Acad. Sci. USA **84**: 8049-8053.

Derbyshire, K.M., Kramer, M. and Grindley, N.D.F. (1990). "Role of instability in the cis action of the insertion sequence IS903 transposase." Proc. Natl. Acad. Sci. USA **87**: 4048-4052.

Donachie, W.D. (1968). "Relationship between cell size and time of initiation of DNA replication." Nature **219**: 1077-1079.

Egner, C., Azhderian, E., Tsang, S.S., Radding, C.M. and Chase, J.W. (1987). "Effects of various single-stranded-DNA-binding proteins on reactions promoted by RecA protein." J. Bact. **169**: 3422-3428.

Everett, R. and Willetts, N. (1980). "Characterization of an *in vivo* system for nicking at the origin of conjugal DNA transfer of the sex factor F." J. Mol. Biol. **136**: 129-150.

Falmagne, P., Portetelle, D. and Stalon, V. (1985). "Immunological and structural relatedness of catabolic ornithine carbamoyltransferases and the anabolic enzymes of enterobacteria." J. Bact. **161**: 714-719.

Feinberg, A.P. and Vogelstein, B. (1983). "A technique for radiolabelling DNA restriction fragments to high specific activity." Anal. Biochem. **132**: 6-13.

Fiandt, M., Szybalski, W. and Malamy, M.H. (1972). "Polar mutations in *lac*, *gal* and phage consist of a few IS-DNA sequences inserted with either orientation." Mol. Gen. Genet. **119**: 223-231.

Flinn, H., Burke, M., Stirling, C.J. and Sherratt, D.J. (1989). "Use of gene replacement to construct *Escherichia coli* strains carrying mutations in two genes required for stability of multicopy plasmids." J. Bact. **171**: 2241-2243.

Frost, L., Lee, S., Yanchar, N. and Paranchych, W. (1989). "*fin*P and *fis*O mutations in FinP anti-sense RNA suggest a model for FinOP action in the repression of bacterial conjugation by the F*lac* plasmid JCFL0." Mol. Gen. Genet. **218**: 152-160.

Galas, D.J., Calos, M.P. and Miller, J.H. (1980). "Sequence analysis of Tn9 insertions in the *lac*Z gene." J. Mol. Biol. **144**: 19-41.

Galas, D.J. and Chandler, M. (1982). "Structure and stability of Tn9 mediated cointegrates: evidence for two pathways of transposition." J. Mol. Biol. **154**: 245-272.

Gamas, P., Burger, A.C., Linder, P., Churchward, G., Caro, L., Galas, D. and Chandler, M. (1986). "Replication of pSC101: effects of mutations in the *E. coli* DNA binding protein IHF." Mol. Gen. Genet. **204**: 85-89.

Gamas, P., Caro, L., Calas, D. and Chandler, M. (1987a). "Expression of F transfer functions depends on the *Escherichia coli* integration host factor." Mol. Gen. Genet. **207**: 302-305.

Gamas, P., Chandler, M., Prentki, P. and Galas, D. (1987b). "Escherichia coli Integration Host Factor binds specifically to the

ends of the insertion sequence IS1 and to its major insertion hotspot in pBR322." J. Mol. Biol. **195**: 261-173.

Gayama, S., Kataoka, T., Wachi, M., Tamura, G. and Nagai, K. (1990). "Periodic formation of the *ori*C complex of *Escherichia coli*." EMBO.J **9**: 3761-3765.

Glansdorff, N. (1987). "Biosynthesis of arginine and polyamines." *Escherichia coli* and *Salmonella Typhimurium*: Cellular and Molecular Biology. Ed F. C. Neidhardt. American Society for Microbiology. (Washington, D.C.) First Edition, 321-344.

Goldfarb, D.M., Golberg, G.I., Chernin, L.S., Gukova, L.A., Avdienko, I.D., Kuznetsova, V.N. and Kushner, I.K. (1973). "A protein produced by male strains of *Escherichia coli* which increases the yield of recombinants: its nature and mode of action." Mol. Gen. Genet. **120**: 211-226.

Goldfarb, D.M., Gukova, L.A., Chernin, L.S., Avdienko, I.D., Mnatsakanian, G.G., Kushner, I.K., Kuznetsova, V.N. and Strachova, T.S. (1974). "Rsf mutants of *Escherichia coli* HfrC defective in the production of the factor stimulating recombination in conjugation." Mol. Gen. Genet. **129**: 295-310.

Gonda, D.K. and Radding, C.M. (1983). "By searching processively, RecA protein pairs DNA molecules that share a limited stretch of homology." Cell **34**: 647-654.

Green, M.H.L., Bridges, B.A. and Riazuddin, S. (1971). "Effect of gamma-radiation on the donor ability of *rec*A and *rec*A$^+$ strains of *Escherichia coli*." J. Gen. Micro. **67**: 63-68.

Greener, A. and Hill, C.W. (1980). "Identification of a novel genetic element in *Escherichia coli* K-12." J. Bact. **144**: 312-321.

Gross, J.D. and Caro, L.G. (1966). "DNA transfer in bacterial conjugation." J. Mol. Biol. **16**: 269-284.

Gutterson, N.L. and Koshland, D.E. (1983). "Replacement and amplification of bacterial genes with sequences altered *in vitro*." Proc. Natl. Acad. Sci. USA **80**: 4894-4898.

Haas, D., Evans, R., Mercenier, A., Simon, J.-P. and Stalon, V. (1979). "Genetic and physiological characterization of *Pseudomonas aeruginosa* muants affected in the catabolic ornithine carbamoyltransferase." J. Bact. **139**: 713-720.

Hadley, R.G., Hu, M., Timmons, M., Yun, K. and Deonier, R. (1983). "A partial restriction map of the *pro*A-*pur*E region of the *Escherichia coli* K-12 chromosome." Gene **22**: 281-287.

Hall, B.G. (1990). "Spontaneous point mutations that occur more often when advantageous than when neutral." Genetics **126**: 5-16.

Ham, L.M., Firth, N. and Skurray, R. (1989). "Nucleotide sequence of the F plasmid transfer gene, *tra*H: Identification of a new gene and a promoter within the transfer operon." Gene **75**: 157-165.

Hamilton, C.M., Aldea, M., Washburn, B.K., Babitzke, P. and Kushner, S.R. (1989). "New method for generating deletions and gene replacements in *Escherichia coli*." J. Bact. **171**: 4617-4622.

Hanahan, D. (1985). "Techniques for transformation of *E.coli*." DNA cloning volume 1. Ed D. M. Glover. IRL Press Limited. (Oxford) First Edition, 109-135.

Hartl, D.L. and Sawyer, S.A. (1988). "Multiple correlations among insertion sequences in the genome of natural isolates of *Escherichia coli*." Transposition. Ed A. J. Kingsman, K. F. Chater and S. M. Kingsman. Cambridge University Press. (Cambridge) First Edition, 91-106.

Helling, R.B., Goodman, H.M. and Boyer, H.W. (1974). "Analysis of the R.E. *coli* fragments of DNA from the lamboid bacteriophage

and other viruses by agarose gel electrophoresis." J. Virol. **14**: 1235-1244.

Henikoff, S. (1984). "Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing." Gene **28**: 351-359.

Hill, T.M., Pelletier, A.J., Tecklenberg, M.L. and Kuempel, P.L. (1988). "Identification of the DNA sequence from the E.coli terminus region that halts replication forks." Cell **55**: 459-466.

Hirsch, H.J., Starlinger, P. and Brachet, P. (1972). "Two kinds of insertion in bacterial genes." Mol. Gen. Genet. **119**: 191-206.

Hirschel, B.J. and Berg, D.E. (1982). "A derivative of Tn5 with direct terminal repeats can transpose." J. Mol. Biol. **155**: 105-120.

Holloway, B. and Low, K.B. (1987). "F-prime and R-prime factors." *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology. Ed F. C. Neiddhardt. Ameican Society for Microbiology. (Washington, D.C.) First Edition, 1145-1153.

Holmes, D.S. and Quigley, M. (1981). "A rapid boiling method for the preparation of bacterial plasmids." Anal. Biochem. **114**: 193-197.

Honigberg, S.M. and Radding, C.M. (1988). "The mechanics of winding and unwinding helices in recombination: Torsional stress associated with strand transfer promoted by recA protein." Cell **54**: 525-532.

Hopkins, J.D., Clements, M.B., Liang, T.-Y., Isberg, R.R. and Syvanen, M. (1980). "Recombination genes on the *Escherichia coli* sex factor specific for transposable elements." Proc. Natl. Acad. Sci. USA **77**: 2814-2818.

Horowitz, B. and Deonier, R.C. (1985). "Formation of Δ*tra*F' plasmids: specific recombination at *ori*T." J. Mol. Biol. **186**: 267-274.

Houghton, J.E., O'Donovan, G.A. and Wild, J.R. (1989). "Reconstruction of an enzyme by domain substitution effectively switches substrate specificity." Nature **338**: 172-174.

Hu, M. and Deonier, R.C. (1981a). "Mapping of IS1 elements flanking the *arg* F gene region on the *Escherichia coli* K-12 chromosome." Mol. Gen. Genet. **181**: 222-229.

Hu, S., Ohtsubo, E., Davidson, N. and Saedler, H. (1975). "Electron microscope heteroduplex studies of sequence relationships among bacterial plasmids: Identification and mapping of the insertion sequences IS1 and IS2 in F and R plasmids." J. Bact. **122**: 764-771.

Huffman, G.A. and Rownd, R.H. (1984). "Transition of deletion mutants of the composite resistance plasmid NR1 in *Escherichia coli* and *Salmonella typhimurium*." J. Bact. **159**: 488-498.

Iida, S. and Arber, W. (1981). "On the role of IS1 in the formation of hybrids between bacteriophage P1 and the R plasmid NR1." Mol. Gen. Genet. **177**: 261-170.

Iida, S., Kulka, I., Meyer, J. and Arber, W. (1987). "Amplification of drug resistance genes flanked by inversely repeated IS1 elements: Involvement of IS1-promoted DNA rearrangements before amplification." J. Bact. **169**: 1447-1453.

Ippen-Ihler, K. and Minkley, E.G. (1986). "The conjugation system of F, the fertility factor of *Escherichia coli*." Ann. Rev. Genet. **20**: 593-624.

Ishizaki, K. and Ohtsubo, E. (1984). "Functional difference of the two ends of insertion sequence IS1 in transposition and cointegration." CSHSQB : 207-213.

Itoh, Y., Soldati, L., Stalon, V., Falmange, P., Terawaki, Y., Leisinger, T. and Haas, D. (1988). "Anabolic ornithine carbamoyltransferase of *Pseudomonas aeruginosa*: Nucleotide sequence and transcriptional control of the *arg*F structural gene." J. Bact. **170**: 2725-2734.

Jacks, T., Townsley, K., Varmus, H.E. and Majors, J. (1987). "Two efficient frameshift events are required for synthesis of mouse mammary tumor virus *gag*-related polypeptides." Proc. Natl. Acad. Sci. USA **84**: 4298-4302.

Jakowec, M., Prentiki, P., Chandler, M. and Galas, D., J, (1988). "Mutational analysis of the open reading frames in the transposable element IS1." Genetics **120**: 47-55.

Jessop, A.P. and Clugston, C. (1985). "Amplification of the *arg* F region in strain HfrP4X of *E.coli* K-12." Mol. Gen. Genet. **201**: 347-350.

Jessop, A.P. and Glansdorff, N. (1980). "Genetic factors affecting recovery of nonpoint mutations in the region of a gene coding for ornithine transcarbamylase: Involvement of both the F factor in its chromosomal state and the *rec*A gene." Genetics **96**: 779-799.

Johnsrud, L. (1979). "DNA sequencing analysis of the transposable element IS1." Mol. Gen. Genet. **169**: 213-218.

Joyce, C.M. and Grindley, N.D.F. (1984). "Method for determining whether a gene of Escherichia coli is essential: application to the polA gene." J. Bact. **158**: 636-643.

Kaiser, K. and Murray, N.E. (1979). "Physical characterization of the "rac-prophage" in *E.coli* K-12." Mol. Gen. Genet. **175**: 159-174.

Kawasaki, Y., Wada, C. and Yura, T. (1990). "Roles of Escherichia coli heat shock proteins DnaK, DnaJ and GrpE in mini-F plasmid replication." Mol. Gen. Genet. **220**: 277-282.

Kennedy, N., Beutin, L., Achtman, M. and Skurray, R. (1977). "Conjugation proteins encoded by the F sex factor." Nature **270**: 580-585.

Khatri, G.S., MacAllister, T., Sista, P.R. and Bastia, D. (1989). "The replication terminator protein of E. coli is a DNA sequence-specific contra-helicase." Cell **59**: 667-674.

Kingsbur, D.T. and Helinski, D.R. (1973). "Temperature sensitive mutants for replication of plasmids in Escherichia coli. Requirements for deoxyribonucleic acid Polymerase I in replication of Plasmid colE." J. Bact. **114**: 11-16.

Klemm (1986). "Two regulatory genes, himB and himE, control phase variation of type 1 fimbriae in *E. coli*." EMBO J **5**: 1389-1393.

Kline, B.C. (1985). "A review of mini-F plasmid maintenance." Plasmid **14**: 1-16.

Knott, V., Blake, D.J. and Brownlee, G.G. (1989). "Completion of the detailed restriction map of the *E.coli* genome by the isolation of overlapping cosmid clones." N.A.R. **17**: 5901-5912.

Kogoma, T. and Kline, B.C. (1985b). "Integrative suppression of *dnaA*^{ts} mutations mediated by plasmid F in *E. coli* is a dnaA dependent process." Mol. Gen. Genet. **200**: 103-109.

Kohara, Y., Akiyama, K. and Isono, K. (1987). "The physical map of the whole *E.coli* chromosome: Application of a new strategy for rapid analysis and sorting of a large genomic library." Cell **50**: 495-508.

Konrad, E.B. (1977). "Method for the isolation of *Escherichia coli* mutants with enhanced recombination between chromosomal duplications." J. Bact. **130**: 167-172.

Kuempel, P.L., Pelletier, A.J. and Hill, T.M. (1989). "Tus and the terminators: The arrest of replication in prokaryotes." Cell **59**: 581-583.

Kunz, B.A. and Glickman, B.W. (1983). "The infidelity of conjugal DNA transfer in *Escherichia coli*." Genetics **105**: 489-500.

Landoulsi, A., Malki, A., Kern, R., Kohiyama, M. and Hughes, P. (1990). "The *E. coli* cell surface specifically prevents the initiation of DNA replication at *ori*C on hemimethylated DNA templates." Cell **63**: 1053-1060.

Lane, D., Hill, D., Caughey, P. and Gunn, P. (1984). "The mini-F primary origin. Sequence analysis and multiple activities." J. Mol. Biol. **180**: 267-282.

Legrain, C., Halleux, P., Stalon, V. and Glansdorff, N. (1972). "The dual genetic control of ornithine carbamoyltransferase in *Escherichia coli:* a case of bacterial hybrid enzymes." Eur. J. Biochem. **27**: 93-102.

Legrain, C. and Stalon, V. (1976). "Ornithine carbamoyltransferase from Escherichia coli W: purification, structure and steady-state kinetic analysis." Eur. J. Biochem. **63**: 289-301.

Legrain, C., Stalon, V. and Glansdorff, N. (1976a). "E. coli ornithine carbamoyltransferase isoenzymes. Evolutionary significance and the isolation of lambda argF and lambda argI transducing phages." J. Bact. **128**: 35-38.

Legrain, C., Stalon, V., Glansdorff, N., Gigot, D., Pierard, A. and Crabeel, M. (1976b). "Structural and regulatory mutations allowing utilization of citrulline or carbamoylaspartate as a

source of carbamoylphosphate in *Escherichia coli* K-12." J. Bact. **128**: 39-48.

Leong, J., Nunes-Duby, S., Lessere, C., Youderian, P., Susskind, M. and Landy, A. (1985). "The phi80 and P22 attachment sites; primary structure and interaction with *Escherichia coli* Integration Host Factor." J. Biol. Chem. **260**: 4468-4477.

Lobner-Oleson, A., Skarstad, K., Hansen, F.G., von Meyerburg, K. and Boye, E. (1989). "The DnaA protein determines the initiation mass of *Escherichia coli* K-12." Cell **57**: 881-889.

Lovett, M.A. and Helinski, D.R. (1976). "Method for isolation of replication region of a bacterial replicon: Construction of a miniF'km plasmid." J. Bact. **127**: 982-987.

Lu, S.D., Lu, D. and Gottesman, M. (1989). "Stimulation of IS1 excision by bacteriophage P1 *ref* function." J. Bact. **171**: 3427-3432.

Lui, L.F. (1989). "DNA topoisomerase poisons as antitumor drugs." Ann. Rev. Biochem. **58**: 351-375.

Luria, S.E. and Delbruck, M. (1943). "Mutations of bacteria from virus sensitivity to virus resistance." Genetics **28**: 491-511.

Lycett, G.W. and Pritchard, R.H. (1986). "Functioning of thr F-plasmid origin of replication in an *Escherichia coli* K12 strain during exponential growth." Plasmid **16**: 168-174.

MacHattie, L.A. and Jackowski, J.B. (1977). "Physical structure and deletion effects of the chloramphenicol resistance element Tn9 in phage lambda." Plasmids and Episomes. Ed A. I. Bukari, J. Shapiro and S. Adhya. Cold Spring Harbor Laboratory. (Cold Spring Harbor, New York) First Edition, 219-228.

Machida, C. and Machida, Y. (1987). "Base substitutions in transposable element IS1 cause DNA duplication of variable

length at the target site for plasmid cointegration." EMBO.J **6**: 1799-1803.

Machida, C., Machida, Y. and Ohtsubo, E. (1984a). "Both inverted repeat sequences located at the ends of IS1 provide promoter functions." J. Mol. Biol. **177**: 247-267.

Machida, C., Machida, Y., Wang, H.-C., Ishizaki, K. and Ohtsubo, E. (1983). "Repression of cointegrate ability of insertion element IS1 by transcriptional readthrough from flanking regions." Cell **34**: 135-142.

Machida, Y., Machida, C. and Ohtsubo, E. (1984b). "Insertion element IS1 encodes two structural genes required for its transposition." J. Mol. Biol. **177**: 229-245.

Machida, Y., Machida, C., Ohtsubo, H. and Ohtsubo, E. (1982). "Factors determining frequency of plasmid cointegration mediated by insertion sequence IS1." Proc. Natl. Acad. Sci. USA **79**: 277-281.

Mahan, M.J. and Roth, J.R. (1989). "Role of *rec*BC function in formation of chromosomal rearrangements: A two step model for recombination." Genetics **121**: 433-443.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press.

Marsh, J.L., Erfle, M. and Wykes, E.J. (1984). "The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation." Gene **32**: 481-490.

McIntyre, S. and Dempsey, W.B. (1987). "Fertility inhibition genes of plasmid R100." Nuc. Acid. Res. **15**: 2029-2042.

McMacken, R., Silver, L. and Georgopoulos, C. (1987). "DNA Replication." *Escherichia coli* and *Salmonella Typhimurium*:

Cellular and Molecular Biology. Ed F. C. Neidhardt. American Society for Microbiology. (Washington, D.C.) Firstt Edition, 564-612.

Meyer, J. and Lida, A. (1979). "Amplification of chloramphenicol resistance transposons carried by phage P1cm in *E. coli*." Mol. Gen. Genet. **176**: 209-219.

Miller, J.H. (1972). Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press.

Mittler, J.E. and Lenski, R.E. (1990). "New data on excisions of Mu from *E.coli* MCS2 cast doubt on directed mutation hypothesis." Nature **344**: 173-175.

Mullineaux, P. and Willetts, N. (1985). "Promotors in the transfer region of plasmid F." Plasmids in Bacteria. Ed D. R. Helinski, S. N. Cohen, D. B. Clewell, D. A. Jackson and A. Hollaender. Plenum Publishing Corp. (New York) First Edition, 605-614.

Murakami, Y., Ohmori, H., Yura, T. and Nagata, T. (1987). "Requirement of the *Escherichia coli dnaA* gene function for *ori2*-dependent mini F plasmid replication." J. Bact. **169**: 1724-1730.

Novick, R.P., Clones, R.C., Cohen, S.N., Curtiss, R., Datta, N. and Falkow, S. (1976). "Uniform nomenclature for bacterial plasmids: A proposal." Bacteriol. Rev. **40**: 168-189.

Nyman, K., Nakamura, K., Ohtsubo, H. and Ohtsubo, E. (1981). "Distribution of the insertion element IS1 in gram-negative bacteria." Nature **289**: 609-612.

Nyman, K., Ohtsubo, H., Davidson, D. and Ohtsubo, E. (1983). "Distribution of insertion element IS1 in natural isolates of *Escherichia coli*." Mol. Gen. Genet. **189**: 516-518.

Ohno, S. (1970). Evolution by gene duplication. Springer Verlag. (New York)

Ohtsubo, E., Zenilman, M. and Ohtsubo, H. (1980). "Plasmids containing insertion elements are potential transposons." Proc. Natl. Acad. Sci. USA **77**: 750-754.

Ohtsubo, E., Zenilman, M., Ohtsubo, H., McCormick, M., Machida, C. and Machida, Y. (1981a). "Mechanism of insertion and cointegration mediated by IS1 and Tn3." CSHSQB **45**: 283-296.

Ohtsubo, H., Nyman, K., Doroszkiewicz, W. and Ohtsubo, E. (1981b). "Multiple copies of the iso-insertion sequence of IS1 in *Shigella dysenteriae* chromosome." Nature **292**: 640-643.

Ohtsubo, H. and Ohtsubo, E. (1978). "Nucleotide sequence of an insertion element IS1." Proc. Natl. Acad. Sci. USA **75**: 615-619.

Peterson, B.C. and Rownd, R.H. (1983). "Homologous sequences other than insertion elements can serve as recombination sites in plasmid drug resistance gene amplification." J. Bact. **156**: 177-185.

Peterson, B.C. and Rownd, R.H. (1985a). "Recombination sites in plasmid drug resistance gene amplification." J. Bact. **164**: 1359-1361.

Peterson, B.C. and Rownd, R.H. (1985b). "Drug resistance gene amplification of plasmid NR1 derivatives with various amounts of resistance determinant DNA." J. Bact. **161**: 1041-1048.

Petit, M.A., Mesas, J., Noirot, P. and Ehrlich, S.D. (1986). Mechanism of DNA amplification in *Bacillus subtilis* chromosome. Fifth International Symposium on the Genetics of Industrial Microorganisms,

Piette, J., Cunin, R., van Vliet, F., Charlier, D., Crabeel, M., Ota, Y. and Glansdorff, N. (1982). "Homologous control sites and DNA transcription starts in the related *arg*F gene and *arg*I gene of *Escherichia coli* K-12." EMBO J **1**: 853-857.

Prentki, P., Chandler, M. and Galas, D.J. (1987). "*Escherichia coli* integration host factor bends the DNA at the ends of IS1 and in an insertion hotspot with multiple IHF binding sites." EMBO J. **6**: 2479-2487.

Prentki, P. and Krisch, H.M. (1984). "In vitro insertional mutagenesis with a selectable DNA fragment." Gene **29**: 303-313.

Prentki, P., Teter, B., Chandler, M. and Galas, D.J. (1986). "Functional promoters created by the insertion of transposable element IS1." J. Mol. Biol. **191**: 383-393.

Pritchard, R.H., Chandler, M.G. and Collins, J. (1975). "Independence of F replication and chromosome replication in *Escherichia coli*." Mol. Gen. Genet. **138**: 143-155.

Ptashne, M. (1986). A Genetic Switch. Gene control and phage lambda. Cell and Blackwell Scientific Publications. (Oxford)

Radding, C.M. (1982). "Homologous pairing and strand exchange in genetic recombination." Annu. Rev. Genet. **16**: 405-437.

Reif, H., J. and Arber, W. (1980). "Analysis of transposition IS1-*kan* and its relatives." CSHSQB **45**: 40-43.

Reif, H.J. and Saedler, H. (1975). "IS1 is involved in deletion formation in the *gal* region of *E. coli* K-12." Mol. Gen. Genet. **137**: 17-28.

Remaut, E., Tsao, H. and Fiers, W. (1983). "Improved plasmid vectors with a thermoinducible expression and temperature-regulated runaway replication." Gene **22**: 103-113.

Rigby, P.W.J., Dieckmann, M., Rhodes, C. and Berg, P. (1977). "Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I." J. Mol. Biol. **113**: 237-251.

Roman, L.J. and Kowalczykowski, S.C. (1989). "Characterization of the adenine triphosphatase activity of the *Escherichia coli* RecBCD enzyme: relationship of ATP hydrolysis to the unwinding of duplex DNA." Biochemistry **28**: 2873-2881.

Rownd, R. (1986). Plasmid drug resistance gene amplification in *Enterobacteriaceae*. Fifth International Symposium on the Genetics of Industrial Microorganisms,

Rubens, C., Heffron, F. and Falkow, S. (1976). "Transposition of a plasmid deoxyribonucleic acid sequence in *Pseudomonas aeruginosa* which mediates resistance to gentimicin and other anti-microbial agents." J. Bact. **128**: 425-434.

Sanger, F., Coulson, A.R., Hong, G.F., Hill, D.F. and Petersen, G.B. (1982). "Nucleotide sequence of bacteriophage Lambda DNA." J. Mol. Biol. **162**: 729-773.

Scaife, J. (1967). "Episomes." Ann. Rev. Microbiol. **21**: 601-638.

Schaaper, R.M., Danforth, D.N. and Glickman, B.W. (1986). "Mechanisms of spontaneous mutagenesis: An analysis of the spectrum of spontaneous mutation in the *Escherichia coli lac*I gene." J. Mol. Biol. **189**: 273-284.

Schwartz, E., Herberger, C. and Rak, B. (1988). "Second-element turn-on of gene expression in an IS1 insertion mutant." Mol. Gen. Genet. **211**: 282-289.

Seifert, H.S. and Porter, R.D. (1984). "Enhanced recombination between λ*plac*5 and F42*lac*: identification of *cis*- and *trans*-acting factors." Proc. Natl. Acad. Sci. USA **81**: 7500-7504.

Sekine, Y. and Ohtsubo, E. (1989). "Frameshifting is required for production of the transposase encoded by insertion sequence 1." Proc. Natl. Acad. Sci. USA **86**: 4609-4613.

Shapiro, J.A. (1979). "Molecular model for the transposition and replication of bacteriophage Mu and other transposable elements." Proc. Natl. Acad. Sci. USA **76**: 1933-1973.

Shields, M.S., Kline, B.C. and Tam, J.E. (1987). "Similarities in control of Mini-F plasmid and chromosomal replication in *Escherichia coli*." J. Bact. **169**: 3375-3378.

Shyamala, V., Schneider, E. and Ames, G.F.-L. (1990). "Tandem chromosomal duplications: role of REP sequences in the recombination event at the join-point." EMBO.J **9**: 939-946.

Skurray, R.A., Nagaishi, H. and Clark, A.J. (1976). "Molecular cloning of DNA from F sex factor of *Escherichia coli* K-12." Proc. Natl. Acad. Sci. USA **73**: 64-68.

Smith, C.L. and Kolodner, R.D. (1988). "Mapping of *Escherichia coli* chromosomal Tn5 and F insertions by pulsed field gel electrophoresis." Genetics **119**: 227-236.

Smith, G.R. (1988). "Homologous recombination in prokaryotes." Microbiol. Rev. **52**: 1-28.

Smith, G.R. (1991). "Conjugal recombination in E. coli: Myths and Mechanisms." Cell **64**: 19-27.

Southern, E.M. (1975). "Detection of specific sequences among DNA fragments separated by gel electrophoresis." J. Mol. Biol. **98**: 503-517.

Spradling, A.C. and Mahowald, A.P. (1980). "Amplification of genes for chorion proteins during oogenesis in *Drosophila melanogaster*." Proc. Natl. Acad. Sci. USA **77**: 1096-2002.

Stark, G.R. and Wahl, G.M. (1984). "Gene Amplification." Ann. Rev. Biochem. **53**: 447-491.

Sternberg, N. and Hoess, R. (1983). "The molecular genetics of bacteriophage P1." Annu. Rev. Genet. **17**: 123-154.

Stirling, C.J., Szatmari, G., Stewart, G., Smith, M.C.M. and Sherratt, D.J. (1988a). "The arginine repressor is essential for plasmid-stabilizing site-specific recombination at the ColE1 *cer* locus." EMBO **7**: 4389-4395.

Symonds, N. (1989). "Anticipatory mutagenesis?" Nature **337**: 119-120.

Syvanen, M., Hopkins, J.D., Griffin, T.J., Liang, T.-Y., Ippen-Ihler, K. and Kolodner, R. (1986). "Stimulation of precise excision and recombination by conjugal proficient F' plasmids." Mol. Gen. Genet. **203**: 1-7.

Thomas, B.J. and Rothstein, R. (1989). "Elevated recombination rates in transcriptionally active DNA." Cell **56**: 619-630.

Thompson, J.F., de Vargas, L.M., Koch, C., Kahmann, R. and Landy, A. (1987). "Cellular factors couple recombination with growth phase: Characterization of a new component in the λ site-specific recombination pathway." Cell **50**: 901-908.

Thompson, R. and Taylor, L. (1982). "Promotor mapping and DNA sequencing of the F plasmid transfer genes *tra*M and *tra*J." Mol. Gen. Genet. **188**: 513-518.

Traxler, B.A. and Minkley, E.G. (1987). "Revised genetic map of the distal end of the F transfer operon: Implications for DNA Helicase I nicking at *ori*T and conjugal DNA transfer." J. Bact. **169**: 3251-3254.

Tsutsui, H. and Matsubara, K. (1981). "Replication and switch-off function as observed with a mini-F factor plasmid." J. Bact. **147**: 509-516.

van Vliet, F., Jacobs, A., Piette, J., Gigot, D., Lauwreys, M., Pierard, A. and Glansdorff, N. (1984). "Evolutionary divergence of genes for ornithine and aspartate carbamoyl-transferases: complete sequence and mode of regulation of the *Escherichia coli argF* gene; comparison of *argF* with *argI* and*pyrB*." Nucl. Acid. Res. **12**: 6277-6289.

Vieira, J. and Messing, J. (1982). "The pUC plasmids, a M13mp7 derived system for insertion mutagenesis and sequencing with synthetic universal primers." Gene **19**: 259-268.

Von Meyenberg, K. and Hansen, F.G. (1987). "Regulation of chromosome replication." *Escherichia coli* and *Salmonella Typhimurium*: Cellular and Molecular Biology. Ed F. C. Neidhardt. American Society for Microbiology. (Washington, D.C.) First Edition, 1555-1577.

Wada, C., Imai, M. and Yura, T. (1987). "Host control of plasmid replication: Requirement for the σ factor σ^{32} in transcription of mini F replication initiator gene." Proc. Natl. Acad. Sci. USA **84**: 8849-8853.

Walker, G.C. (1987). "The SOS response of *Escherichia coli*." *Escherichia coli* and *Salmonella Typhimurium*: Cellular and Molecular Biology. Ed F. C. Neidhardt. American Society for Microbiology. (Washington, D.C.) First Edition, 1346-1357.

Wang, J.C., Caron, P.R. and Kim, R.A. (1990). "The role of DNA topoisomerases in recombination and genome stability: A double-edged sword." Cell **62**: 403-406.

Watanabe, H., Mise, K. and Hashimoto, H. (1982). "Recombination between two IS1's flanking the r-det determinant of R100-1: involvement of *dor* and *rec*A gene functions in *Salmonella typhimurium*." J. Bact. **150**: 113-121.

Wegrzyn, G., Neubauer, P., Krueger, S., Hecker, M. and Taylor, K. (1991). "Stringent control of replication of plasmids derived from coliphage lambda." Mol. Gen. Genet. **225**: 94-98.

Weinert, T.A., Derbyshire, K.M., Hughson, F.M. and Grindley, N.D.F. (1984). "Replicative and conservative transpositional recombination of Insertion Sequences." CSHSQB **49**: 251-260.

Weinstock, G.M. (1987). "General Recombination in *Escherichia coli*." *Escherichia coli* and *Salmonella typhimurium*: Cellular and molecular Biology. Ed F. C. Neidhardt. American Society for microbiology. (Washington, D.C.) First Edition, 1034-1043.

Whittaker, P.A., Campbell, A.J.B., Southern, E.M. and Murray, N.E. (1988). "Enhanced Recovery And Restriction Mapping Of DNA Fragments Cloned In A New Lambda Vector." NAR **16**:: 6725-6728.

Wickner, S., Hoskins, J. and McKenney, K. (1991). "Function of DnaJ and DnaK as chaperones in origin-specific DNA binding by RepA." Nature **350**: 165-167.

Willetts, N. and Skurray, R. (1987). "Structure and function of the F factor and mechanism of conjugation." *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology. Ed F. C. Neidhardt. American Society for Microbiology. (Washington, D.C.) First Edition, 1110-1135.

Willetts, N. and Wilkins, B. (1984). "Processing of plasmid DNA during bacterial conjugation." Microbiol. Rev. **48**: 24-41.

Willetts, N.S. (1977). "The transcriptional control of fertility in F-like plasmids." J. Mol. Biol. **112**: 141-148.

Wu, H.-Y., Shyy, S., Wang, J.C. and Liu, L.F. (1988). "Transcription generates positively and negatively supercoiled domains in the template." Cell **53**: 433-440.

Wu, J.H. and Ippen-Ihler, K. (1989). "Nucleotide sequence of *traQ* and adjacent loci in the *Escherichia coli* K-12 F-plasmid transfer operon." J. Bact. **171**: 213-221.

Yang, Y. and Ames, G.F. (1988). "DNA gyrase binds to the family of prokaryotic repetitive extragenic sequences." Proc. Natl. Acad. Sci. USA **85**: 8850-8854.

Yanish-Perron, C., Vieira, J. and Messing, J. (1985). "Improved M13 phage cloning vectors and host strains: Nucleotide sequence of the M13mp18 and pUC19 vectors." Gene **33**: 103-119.

York, M.K. and Stodolsky, M. (1981). "Characterization of P1*arg* derivatives from *Escherichia coli* K-12 transduction." Mol. Gen. Genet. **181**: 230-240.

Yoshioka, Y., Ohtsubo, H. and Ohtsubo, E. (1987). "Repressor gene finO in plasmids R100 and F: constitutive transfer of plasmid F is caused by insertion of IS3 into finO." J. Bact. **169**: 619-623.

Young, M. and Cullum, J. (1987). "A plausible mechanism for large-scale chromosomal DNA amplification in streptomycetes." FEBS **212**: 10-14.

Zerbib, D., Gamas, P., Chandler, M., Prentki, P., Bass, S. and Galas, D. (1985). "Specificity of insertion of IS1." J. Mol. Biol. **185**: 517-524.

Zerbib, D., Jakowec, M., Prentki, P., Galas, D.J. and Chandler, M. (1987). "Expression of proteins essential for IS1 transposition: specific binding of InsA to the ends of IS1." EMBO.J **6**: 3163-3169

GLASGOW
UNIVERSITY
LIBRARY