GENE EXPRESSION IN THE GENUS *DEINOCOCCUS*

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Isolation and characterisation of DraI, a type II restriction endonuclease recognising a sequence containing only A:T basepairs, and inhibition of its activity by uv irradiation of substrate DNA

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ABSTRACT

A type II restriction endonuclease, DraI, isolated from *Deinococcus radiophilus* ATCC 27603 recognises the palindromic hexanucleotide sequence

5'-T-T-T↓A-A-A-3'
3'-A-A-A↑T-T-T-5'

and cleaves it, as indicated by the arrows, to produce blunt-ended fragments. The yield of enzyme is 100 to 1000 times that of the only other known type II restriction endonuclease that recognises a sequence composed solely of A:T basepairs, the isoschizomer AhaIII (1). Ultraviolet irradiation of the DNA substrate at relatively low doses inhibits the activity of DraI by "protecting" the recognition sequence and this may be exploited to give control of partial digestion of DNA by DraI.

INTRODUCTION

D. radiophilus belongs to the Family Deinococcaceae, the major characteristic of members of this group being their extreme resistance to the lethal effects of both ionising and ultraviolet radiations (2). The type species of the group *D. radiodurans* ATCC 13939, has been shown to possess a type II restriction endonuclease (3), MraI, recognising the sequence

5'-C-C-G-C-G-G-3'
3'-G-G-C-G-C-C-5'

We report here a description of the isolation and characterisation of the site-specific endonuclease DraI, and a method by which partial digestion of DNA molecules by DraI may be controlled.

METHODS

Assay for Restriction

Enzyme activity was estimated by incubating a sample (usually 1 µl) with either 1 µg λ-DNA or ColE1:Tn5 ccc DNA. One unit of enzyme represents that activity which completely digests 1 µg of DNA during 1 hour's incubation at 37°C in restriction buffer (10 mM Mg, 10 mM Tris-HCl, pH 8.0).

Protein estimation

Protein concentration was measured both by comparative absorption at 260 nm and 280 nm wavelength light and using a Bio-Rad protein assay kit.

Isolation of DraI

Three one-litre cultures of *D. radiophilus* in nutrient broth No 2 (Oxoid) were grown to early stationary phase by shaking at 37°C. It should be noted that protease activity increases greatly in late stationary and decline phase. About 15 g wet weight of cells was harvested by centrifugation at 10,000 g for 10 min. The bacteria were resuspended in 30 ml 10 mM Tris-HCl, 2 mM 2-mercaptoethanol (MSH), 0.1 mM phenylmethylsulphonylfluoride (PMSF), pH 7.5 and broken open in a French pressure cell at 3,000 psi. Following centrifugation at 10,000 g for 10 minutes to remove undamaged cells and large fragments, the lysate was centrifuged at 100,000 g for 2 hours. Most of the DNA present in the supernate was removed using polyethylene glycol 6,000 (PEG): dextran T500 phase partition (4). This procedure also removed significant amounts of non-specific exonuclease activity. The enzyme preparation was dialysed (10-20 hours) against column buffer 1 (CB1 - 10 mM Tris-HCl, 2 mM MSH, 0.1 mM PMSF and 0.075 M NaCl, pH 8.0) and applied to a 20 x 2.6 cm DEAE-sephacel (Bio-Rad) column, previously equilibrated with CB1. After washing, the column was developed with a linear

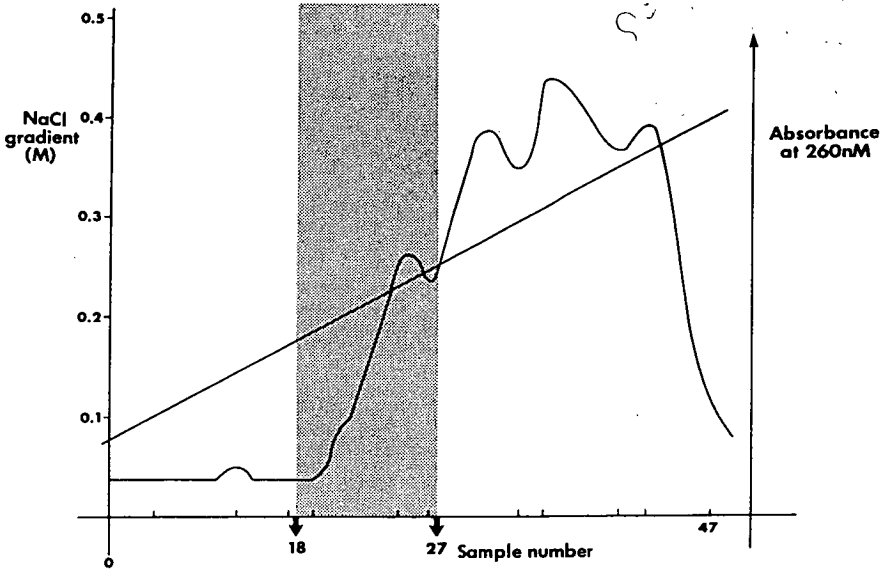

Fig. 1. Protein Elution profile of D.radiophilus cell extraction DEAE-Sephacel

0.075-0.4 M NaCl gradient, DraI activity eluting in the 0.18-0.24 NaCl region (Fig 1, shaded area represents region of 'unit' enzyme activity).

Active fractions were pooled, dialysed against column buffer 2 (CB2 - 0.01 M phosphate buffer, 2 mM MSH, 0.05 M NaCl, pH 7.5) and then applied to a 10 x 2.6 cm hydroxylapatite (Bio-Rad) column previously equilibrated with CB2. The column was washed with CB2 and then eluted with a linear gradient of 0.01 to 0.4 M phosphate buffer. DraI activity eluted between 0.20 and 0.32 M phosphate. Each collecting tube contained enough restriction enzyme-grade bovine serum albumin (BRL) to ensure that the total protein concentration did not fall below 750 $\mu g\ ml^{-1}$. Fractions showing greater than 1,000 units ml^{-1} of enzyme activity were pooled and dialysed against storage buffer (50% glycerol, 10 mM Tris-HCl, 2 mM MSH, 0.05 M NaCl, pH 8.0). DraI is stable in this buffer for > 12 months at - 20°C.

RESULTS

The yield and specific activity of DraI during various stages of isolation are shown in Table 1. The final preparation was considered to be free of contaminating 5' and 3' exonucleases since there was no alteration of cleavage patterns upon extensive incubation and because of the success of sequencing techniques which are highly sensitive to such contamination.

Optimal Conditions for DraI Activity

DraI has an absolute requirement for Mg^{2+}, a feature not uncommon in type II restriction endonucleases (5). Maximum DraI cleavage was obtained at 37°-39°C in a buffer containing 10 mM Mg^{2+} and 10 mM NaCl at pH 8.0. Concentrations of > 100 mM NaCl, > 30 mM Mg^{2+}, > 1 mM Mn^{2+} and > 0.1 mM Ca^{2+} were each inhibitory to enzyme activity. In general, conditions for maximum activity were similar to those found for MraI (3).

Table 1. Stages of DraI Purification

Stage	Total protein (mg)	Total enzyme (units)	Specific activity (units mg^{-1} protein)	Yield (%)
High speed centrifugation	1.86×10^3	8.0×10^5	4.32×10^2	100
PEG:dextran phase partition	1.22×10^3	7.5×10^5	6.15×10^2	93.75
DEAE-sephacel	3.15×10^1	4.1×10^5	1.30×10^4	51.25
Hydroxylapatite	0.57	1.4×10^5	2.46×10^5	17.50

Table 2. Specificities of Type II Restriction Enzymes DraI and AhaIII

Enzyme	Number of cleavage sites				
	pBR322	Ade 2	λ	SV40	ØX174
AhaIII	3	16	13	12	2
DraI	2 (3[a])	> 10	13	> 10	2

[a]From sequence data, see text.

Mapping of DraI cleavage sites

Cleavage of a variety of different DNA species, ie λ; Ade-2; ØX174 Rf; SV40 and pBR322 (6-9) produced a pattern of bands, after polyacrilamide gel electrophoresis (7-12% gradient), only previously seen in the case of AhaIII (1) digestions (Table 2).

Double digestions of pBR322 using each of the commercially available enzymes EcoRI, BamHI, PstI, HindIII (all from Miles Laboratories) and SalI (NBL Enzymes Ltd) with DraI indicated the presence of two DraI recognition sites in this plasmid at the map positions 3250 and 3900 respectively. These data suggested that a DNA sequencing procedure closely based upon that described for AhaIII(1) should be used. The Sau3AI fragment 9 of pBR322 was cloned into the M13mp7Rf1 BamHI site (1, 10-12), sequenced using the chain-terminator method (13) and the DraI cleavage sites determined (1 and 14). As was the case with AhaIII, during the course of sequencing a second DraI site was identified within the cloned fragment (3 in the whole pBR322 molecule) producing upon cleavage a 19bp fragment.

From the above data it was concluded that *D. radiophilus* contains a type II restriction endonuclease recognising the palindromic hexanucleotide sequence

5'-T-T-T-A-A-A-3'
3'-A-A-A-T-T-T-5'.

Inhibition of DraI activity by uv irradiation of the DNA substrate

Cleaver *et al* (15) and Hall and Larcom (16) have shown that the activity of type II sequence specific endonucleases is inhibited by thymine-thymine dimer, and possibly cytosine-thymine dimer, production within or adjacent to the enzyme recognition sequence. In those studies the type II restriction enzyme most sensitive to such inhibition was HindIII, the recognition sequence of which is

5'-A-A-G-C-T-T-3'
3'-T-T-C-G-A-A-5'

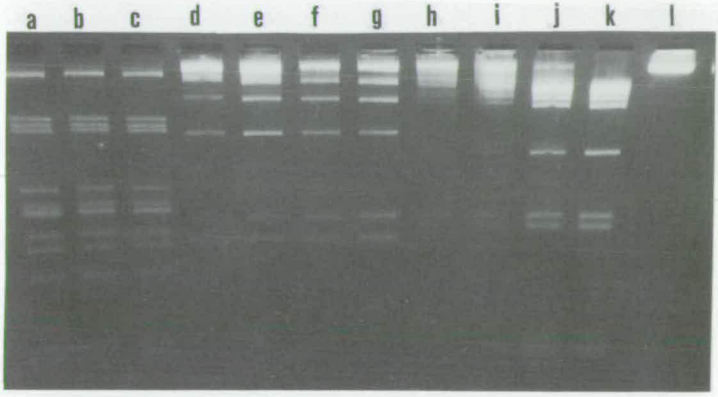

Fig 4. Cleavage of uv irradiated λ-DNA by DraI, HindIII or PstI.
Lanes *a-c* show digestion of 1 μg λ-DNA by PstI after uv radiation doses of 3600; 1800 and 0 Jm^{-2} respectively. Lanes *d-g* show cleavage of 1 μg λ-DNA by HindIII after uv doses of 3600; 1800; 300 and 0 Jm^{-2} respectively. Lanes *h-l* show digestion of 1 μg λ-DNA by DraI after uv doses of 3600; 1800; 300 and 0 Jm^{-2}. Fragment separation was by agarose gel electrophoresis (1%) and visualisation by EtBr staining.

distinctive separation of DNA molecules differing in size by only 19 basepairs. Initial alteration in the digestion pattern occurs at a uv dose of ~ 60 Jm^{-2} while above a dose of 180 Jm^{-2} a fragment of 3567 basepairs appears due to the inhibition of enzyme action at both the DraI recognition sequences at positions 3250 and 3231 on the pBR322 molecule. Finally at doses > 300 Jm^{-2} complete linear molecules of pBR322 (4362 basepairs) can be seen due to the inactivation of all DraI recognition sites.

Comparative studies using HindIII and DraI showed that inhibition of DraI activity at individual recognition sites was three to four times more sensitive to uv inhibition than that of HindIII. This is illustrated by comparative digestions of uv-damaged λ-DNA with DraI, HindIII and PstI (Fig 4). In spite of differing numbers of restriction sites in the λ-DNA, (13 for DraI, 7 for HindIII and 18 for PstI), it is still clear that DraI activity is inhibited to a greater degree than that of HindIII, whilst irradiation of the DNA has little effect upon the action of PstI, the recognition sequence of which contains no adjacent thymine residues.

DISCUSSION

In contrast to the blue green alga *Aphanothece halophytica* from which AhaIII is isolated, *Deinococcus radiophilus* is easily grown and gives a

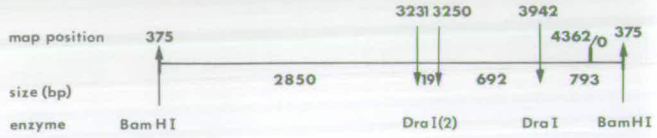

Fig 2. Schematic representation of the pBR322 molecule showing cleavage sites of DraI and BamHI.

However DraI activity is much more sensitive to inhibition following uv irradiation of the DNA substrate than HindIII. By linearising cccpBR322 (1 μg) with the type II restriction endonuclease BamHI, (map position 375 on the pBR322 molecule), followed by uv irradiation at an incident dose rate of 1.05 Jm^{-2} in a uv transparent buffer (10 mM Tris-HCl pH 8.0), the effect of different radiation doses upon DraI activity was observed (Figs 2 and 3). Unfortunately the presence in pBR322 of two DraI sites only 19 base-pairs apart does complicate interpretation of the results. The appearance of a 1489 basepair fragment allied to the simultaneous removal of both 692 and 793 basepair fragments indicates the inhibition of activity at the DraI recognition site positioned at 3942 on the pBR322 molecule. Inhibition of activity at either of the other two sites singly will not produce any new bands as the fragment size resolution of a 1% agarose gel will not allow

Fig 3. DraI digestion of uv treated, BamHI linearised pBR322.
Lanes *a-k* illustrate 1 μg pBR322 linearised with BamHI and given uv radiation doses of 4200; 3600; 3000; 2400; 1800; 1200; 600; 300; 180; 60 and 0 Jm^{-2} respectively before 1 hr DraI digestion. Lane *l* shows BamHI linearised pBR322 plasmid DNA. Fragment separation was by agarose gel electrophoresis (1%) and visualisation by EtBr staining.

relatively high yield of the AhaIII isoschizomer, DraI. *D. radiophilus* is also extremely radiation resistant and this property provides an excellent screen against contamination. These properties make it likely that DraI will become a commercially important restriction endonuclease.

Since DraI recognises a sequence containing only A:T basepairs it may be possible to expand studies on DNA-DNA and DNA-protein interactions which were being delayed by a dearth of suitable, easily available enzymes. DraI will also be useful in the study of eukaryotic DNA where, due to the marked asymmetry of A:T distribution, large regions of G:C rich DNA may be produced.

Difficulties in controlling partial digestion of DNA molecules during restriction may, to some extent, be overcome by utilising the inhibition of DraI activity by uv irradiation of the substrate DNA. Although DraI digestion produces blunt-ended fragments, this disadvantage can be removed by poly (dA) "tailing" of the restricted DNA to be cloned, followed by insertion into a poly (dT) "tailed" vector regenerating DraI sites either side of the cloned fragment. To allow gene expression within the irradiated insert, multiplication of the recombinant molecule in a uv repair-proficient strain of bacterium, yeast or tissue culture line must occur. However this process will 'reactivate' any DraI recognition sequences present within the DNA insert.

ACKNOWLEDGEMENTS

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DECLARATION

I declare that this thesis is composed of my own work and has been compiled by myself.

Ian James Purvis.

SUMMARY

The genus Deinococcus consists of four species, D. radiodurans, D. radiophilus, D. radiopugnans and D. proteolyticus. There are two strains of D. radiodurans, R1 (Anderson) and Sark. All produce red-pigmented colonies on agar and are characterised by their extreme resistance to, and non-mutability by, both ionizing and ultraviolet irradiation.

Attempts were made to phenotypically express, within members of the above genus, genes from a variety (> 10) of common or constructed plasmid vectors introduced via transformation or conjugation. All these plasmids failed to functionally express their selectable traits and this led to an investigation of possible parameters controlling such expression.

The presence of type II site-specific endonucleases and associated DNA modification systems was confirmed in D. radiodurans R1 and demonstrated in D. radiophilus. Two novel restriction enzymes, DraI and DraII, were isolated from D. radiophilus, DraI recognising the DNA sequence 5'TTTAAA 3' and now being available commercially. The extreme sensitivity of this enzyme to inhibition by pre-irradiation of the substrate DNA was demonstrated.

Gene banks of chromosomal and plasmid DNA derived from D. radiophilus were created in the Escherichia coli plasmid vector pAT153. Although the DraI restriction/modification system did not express in E. coli, it was found, on testing a wide range of mutants, that the leuB

mutation of E. coli HB101 could be complemented by a 10.24 kilobase (kb) insert of D. radiophilus DNA in pAT153. The size of the insert was subsequently reduced to a functional unit of only 800-900 basepairs which appeared to code for a protein of approximately 18 000 daltons. About two-thirds of this DNA region was sequenced and showed many open-reading frames but only one preceded by an E. coli-like promoter and without a terminator codon, in frame, close to the translation initiation site. In conjunction with further analysis of other D. radiodurans R1 cloned genes this should give valuable information on the regulation and organisation of coding regions within this odd group of bacteria.

CONTENTS

CHAPTER 1. INTRODUCTION

1 THE GENUS DEINOCOCCUS

1.1 TAXONOMY AND GENERAL BIOLOGY

Interest in the small group of non-sporing, red-pigmented bacteria that comprise the genus Deinococcus has been aroused largely because of the extreme resistance to both the lethal and mutagenic effects of ionizing and ultraviolet (UV) radiation which characterises the group. The genus consists of four species; Deinococcus radiodurans (strains R1 and Sark), D. radiophilus, D. proteolyticus and D. radiopugnans. The initial isolation of D. radiodurans (strain R1) was made in 1956 while studying a sample from a meat processing factory in Oregon, U.S.A. (Anderson et al., 1956). This organism has become the type species of the genus. The other species were found subsequently in a range of irradiated materials, i.e. Bombay duck, llama faeces and haddock. Further details can be found in the review article of Moseley (1983). Originally these species were classified in the genus Micrococcus but subsequent investigation of cell wall composition (Sleytr et al., 1973), fatty acid and phospholipid content (Thompson et al., 1980; Jantzen et al., 1974) and cell ultrastructure (Lancy and Murray, 1978) all detracted from this view. A comparative study of 16s ribosomal RNA sequences (Brooks et al., 1980) finally showed that a separate genus for the red-pigmented radiation-resistant micrococci was required, the genus Deinococcus. The genus is so distinct, in fact, that it forms by itself one of the eight recognised groups of the Eubacteria (Fox et al., 1980).

Growth of Deinococcus spp. is normally achieved in

tryptone-glucose-yeast extract (TGY) medium incubated at 30°C. Although D. radiophilus grows slowly under such conditions there is a marked increase in its growth rate if nutrient broth (Oxoid No. 2) is used. Generally, doubling times of Deinococcus spp. in liquid culture are approximately 80 to 100 minutes while cells on agar require two to three days incubation to become colonies visible to the naked eye. The organisms are rarely seen as single cells, diplo- and tetracoccal forms being prevalent. The sizes of cells varies depending upon the species. Very little is known about the detailed metabolic/biochemical infrastructure. The cells grow only in the presence of oxygen and they stain as Gram-variable. Chemically-defined media have been described (Little and Hanawalt, 1973; Shapiro et al., 1977) which allow for the isolation of a variety of auxotrophic mutants.

As previously mentioned, interest has really been focused upon the ability of members of the genus to not only withstand very high levels of radiation without loss of viability, 500 k rad or 500 Jm^{-2} (Tempest, 1978), but also to resist mutation induced by ultraviolet and ionizing rays. Although all the species are pigmented, this colouration would not appear to have any major role in radiation tolerance (Moseley, 1967). The characteristic resistance appears to reside in rapid and efficient DNA repair. In D. radiodurans R1 enzymes involved in excision repair (Moseley and Evans, 1983) have been shown to be of central importance in ultraviolet light-induced lesion repair as well as in the repair of bulky adducts

caused by chemical agents such as mitomycin c. The presence of a recombination repair pathway system, but not an error-prone mechanism, has also been inferred (Moseley and Copland, 1975). Enzymatic photoreactivation is absent.

1.2 GENETICS AND DNA CONTENT

The only known technique for inter- and intraspecies gene transfer so far available within the Deinococcaceae is via transformation (i.e. the uptake and integration of DNA from the matrix surrounding the cell)(Moseley and Setlow, 1968; Tirgari and Moseley, 1980). Despite intensive efforts, no bacteriophages capable of plaquing on Deinococcus spp. have been found (I. Masters, personal communication). No conjugal transfer of DNA either within or between the species has been observed (Tirgari, 1977). Although protoplasting and protoplast regeneration techniques have been successfully devised, no genetic evidence was obtained for protoplast fusion (G. Al Bakri, personal communication). It has been shown that at least three site-specific endonucleases exist within members of the genus, MraI in D. radiodurans R1 (formerly Micrococcus radiodurans, Wani et al., 1982) and DraI and DraII in D. radiophilus (this thesis; Purvis and Moseley, 1983). However, close examination of the chromosomal DNA of these two species indicates a lack of any methylation modification normally associated with restriction/modification systems (Mackay, 1983; Schein et al., 1972).

Genetic study of the type species D. radiodurans R1 has been complicated by the apparent existence of multiple

independently-segregating genome equivalents per viable unit (Hansen, 1978; Tirgari and Moseley, 1980; Moseley and Evans, 1981). This type of genome organisation is thought to occur throughout the genus (Purvis and Duncan, unpublished results) as well as in other bacterial species. In Azotobacter vinelandii actively dividing cells may contain as many as 40 genome equivalents (Sadoff et al., 1979). There is no positive evidence that this anomalous chromosomal organisation aids the repair to DNA damage. In fact, vegetative cells of A. vinelandii are very sensitive to ultra-violet light and D. radiodurans R1 shows no direct correlation between genome copy number and increased radiation resistance (Harsojo et al., 1981).

Further information on these characteristics can be seen in Table 1.1. Of particular interest is the recent discovery of plasmid molecules in all species of the genus apart from D. radiodurans R1 (Mackay, 1984). Molecule sizes ranged from 2.5kb (pUE 30) to 139.1kb (pUE 21). It was impossible to ascribe any characteristics of the bacteria to the possession of the plasmids but the failure to cure either D. radiophilus or D. radiodurans SARK of any plasmid type may indicate the presence of vital information on these extrachromosomal replicons.

1.3 AIMS OF THE PROJECT

The original aim was to develop vector systems capable of transferring genetic material both intra and intergenetically. The failure to do so led to an analysis of the reasons for the failure of foreign genes to express

TABLE 1.1 DNA PARAMETERS OF SPECIES WITHIN THE GENUS DEINOCOCCUS

Species	% G+C[a]	Genome size ($x10^9$ d)[b]	Plasmid content[c]	Transformation[d]
D. radiodurans R1	68	1.8	-	Yes
D. radiodurans Sark	N.T.	N.T.	pUE 10 pUE 11	Yes
D. radiophilus	65	1.5	pUE 1 pUE 2 pUE 3	No
D. proteolyticus	67	1.8	pUE 20 pUE 21	No
D. radiopugnans	68	2.9	pUE 30 pUE 31	No

(a) C.M. Duncan, personal communication
(b) I. Purvis and B.E.B. Moseley, unpublished results
(c) M. Mackay (1984).
(d) P. Tempest (1978).

N.T. - not attempted.

in D. radiodurans R1. Of particular interest was the molecular structure of the Deinococcus genotype with specific reference to the control of gene expression as determined by the nucleotide sequence of the gene itself. Nothing was known previously of the molecular mechanisms of transcription and translation within the Deinococcaceae and it was hoped that the use of E. coli, as a medium for the investigation of gene structure in the genus Deinococcus, would provide some insight.

2 REGULATION OF GENE EXPRESSION AT TRANSCRIPTION

2.1 INTRODUCTION

The functional expression of the information contained within a gene is controlled by a myriad of interacting mechanisms, many associated with the vital functions of transcription and translation. Other control parameters do come into play, particularly in the eukaryotic matrix. This section, however, will be restricted mainly to the relevant details of prokaryotic gene expression although certain similarities do exist in the realm of eukaryotic gene regulation.

The chromosome of prokaryotic organisms is organised into transcriptional units, operons, containing either single or multiple coding regions, cistrons. The operons contain regulatory sequences of DNA that determine if, when and at what level a particular gene is expressed. The action of the regulatory sequences is via complicated DNA/DNA, DNA/RNA, DNA/protein and RNA/protein interactions

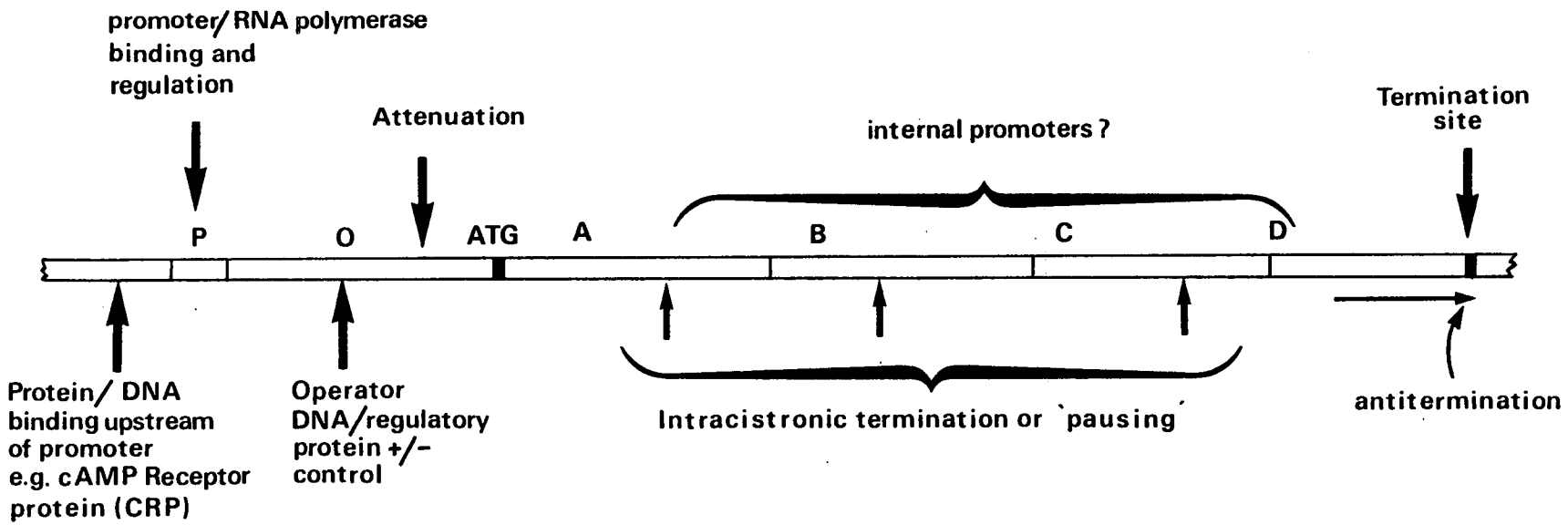
and is dependent, to a large extent, upon the primary nucleotide sequence.

2.2 CONTROL AT TRANSCRIPTION

Transcription is the first stage of gene expression and is of prime importance in regulation, particularly in the prokaryotic organism. Common prokaryotic regulatory mechanisms involved, preceding, during and directly after transcription, are shown in Figure 1.1 and excellent reviews on promoters (Rosenberg and Court, 1979), attenuation (Yanofsky, 1981) and termination (Adhya and Gottesman, 1978) are available.

Initially RNA polymerase must recognize the promoter site of a gene before translocating 'downstream' to the site of RNA strand synthesis. A promoter can be defined as a segment of DNA containing signals within the nucleotide sequence for the correct binding and subsequent activation of the RNA polymerase holoenzyme. Such interaction between DNA sequence and RNA polymerase assembly is strongly controlled by other proteins such as the σ factors and cAMP binding protein (CRP). Analysis of a large number of mainly E. coli promoters has shown the presence, within the 40 to 50 basepair region comprising the promoter, of conserved structural domains, illustrated in Figure 1.2. This consensus sequence has been derived from averaged observations (Rosenberg and Court, 1979) in a study of E. coli promoters. Other sequence organisations can exist as seen in the multiple σ modifying factors present in Bacillus subtilis (Losick and Pero, 1981). Although the major σ

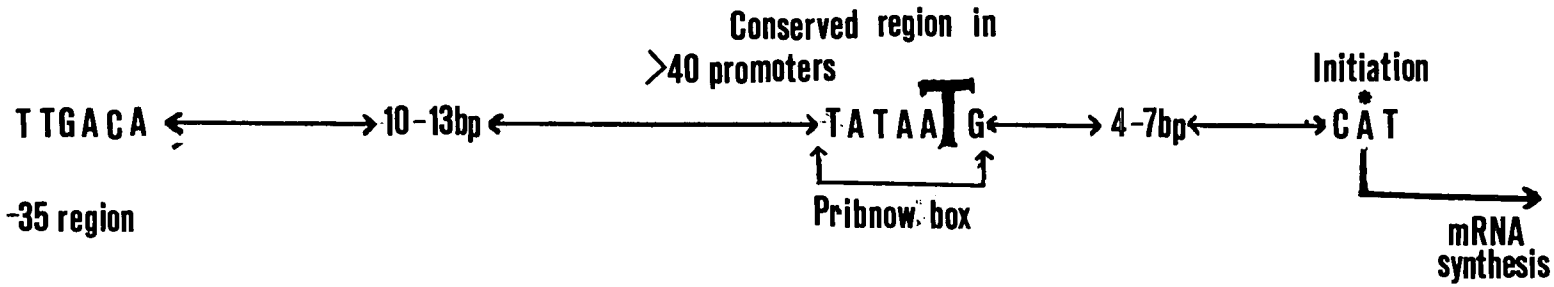
FIG. 1.1. POSSIBLE CONTROL MECHANISMS FOR PROKARYOTIC TRANSCRIPTION (Stylised operon)



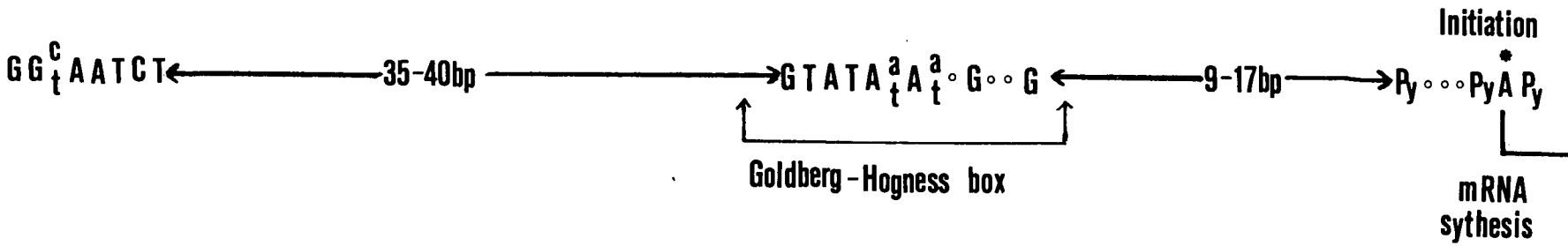
The combined action of these possible regulatory mechanisms enable the transcriptional process to respond directly to external and internal events relevant to expression of the operon.

Fig.1.2 Promoter organisation (highlighted T appears to be invariable)

(a) Prokaryotic promoter (E.coli)



(b) Eukaryotic promoter (RNA polymerase II)



factor of the vegetative cell causes initiation at the consensus sequence (seen in Fig. 1.2a), other σ factors exist within the cell that direct RNA polymerase binding to other types of promoter site. This multiple promoter organisation, modulated by different σ factors, is thought to play a major role in the temporal regulation of gene expression required for the more complicated developmental responses to fluctuating environmental conditions, e.g. sporulation. Despite qualitative variations in nucleotide sequence, a general pattern does emerge about the spatial relationships between promoter regions and RNA polymerase. In general, at least for bacterial promoters, the nucleotide sequences of regions -35 and -10, upstream of actual mRNA synthesis initiation, play a vital role.

After initiation of mRNA biosynthesis, transcription is modulated by two processes; induction/repression via protein binding at the operator site(s) or transcript termination determined either purely by nucleotide sequence or in association with a protein/ribosome. The classic model of Jacob and Monod (1961) for induction and repression of operon transcription has shown the clear effect that protein binding can have at a particular regulatory region of DNA, the operator. The relationship of regulatory proteins with operator regions of DNA is comprehensively reviewed in various articles (Miller and Reznickoff, 1978).

Recently, it has become obvious that termination of transcription plays a central role in gene regulation. Not only does termination occur at the end of an operon

but in many cases there is the possibility for pre-emptive termination or 'pausing' within both control and 'structural' regions of the operon. This intrinsic ability, largely residing in the primary nucleotide sequence, leads to the observed processes of translational polarity and attenuation. The signals for termination, like those for promoters, reside in the secondary structure of both DNA and primary transcript. In certain cases the ρ-protein is essential for transcript termination although pausing still occurs in the absence of the regulatory protein. The most striking feature common to all DNA sequences at which RNA elongation can be stopped is a region of hyphenated dyad symmetry just proximal to the termination point. For ρ independent termination in prokaryotes the dyad symmetry is surrounded by a G/C rich region allowing more stable loop-stem structures to be formed than those produced by the A/T rich regions preceding the ρ-dependent and eukaryotic termination sites. The actual point of termination would appear to occur within a run of uridine residues on the transcript. The identification and analysis of attenuator sites preceding gene clusters involved in the biosynthesis of amino-acids has demonstrated how the degree of gene transcription can be controlled by the interplay between the transcript secondary structure and translation mechanisms (Yanofsky, 1981; Gemmill et al., 1979), nucleotide sequence and regulatory molecules.

Obviously the nucleotide sequence of DNA molecules not only codes for the structural proteins and RNA molecules

of the cell but also plays a primary and varied role in the degree of gene expression. This ranges from transcription initiation through various termination controls to the ancillary sequences present upon the mRNA necessary for successful translation (Kozak, 1984). These controls combined with the plethora of regulatory mechanisms not directly influenced by the DNA nucleotide sequence e.g. pools of metabolites, protein stability, etc. enable the bacterium to recognize and respond to the principal external and internal events relevant to the expression of the operon. With the aid of modern computer analytical techniques it is hoped the sequencing of genes from members of the genus Deinococcus will give vital information on the organisation and regulation of gene expression.

3 GENE CLONING AND DNA SEQUENCING

3.1 GENERAL INTRODUCTION

Genetic manipulation can be described as the formation of novel combinations of heritable material by the insertion of nucleic acid molecules into any virus, bacterial plasmid or other vector system so as to enable their incorporation into the genetic background of a host in which they are capable of continued propagation. General aspects of recombinant DNA technology are described by Old and Primrose (1982). The essential requirements of DNA manipulation are:-

i) A DNA vehicle or vector that can replicate in living cells after foreign DNA has been incorporated into it.

ii) A DNA molecule to be cloned.

iii) A means of joining vector to target DNA.

iv) A means of introducing the recombinant DNA into a host organism which will allow stable inheritance.

v) A direct or indirect method of screening for host cells which now contain the recombinant DNA molecule.

Only recently have all these elements been available and some are still being modified. The important advances have been in the isolation of type II restriction endonucleases (Section 1.4), DNA-ligating enzymes (Higgins and Cozzarelli, 1979), bacterial transformation (Polsinelli and Mazza, 1980) and the introduction of agarose and polyacrylamide gel electrophoresis (Rickwood and Hames, 1982).

Four types of vector are generally available, i.e. bacterial plasmid, single-strand DNA bacteriophage, λ bacteriophage and cosmid, the choice being determined by the experimental aim. Only plasmid and M13 bacteriophage vectors are of direct relevance to this thesis and the reader is referred to review articles by Hendrix et al. (1983) and Collins and Hohn (1979) for information on λ vectors and cosmids respectively.

DNA sequencing has become one of the most important tools for the analysis of DNA regions isolated by the various DNA cloning techniques. As reviewed in section 2.2 the nucleotide sequence within and immediately surrounding a gene plays a major role in the regulation of expression. The development of two fast and reliable

DNA sequencing systems by Maxam and Gilbert (1977) and Sanger et al. (1977) has allowed accurate sequence determination of DNA molecules of up to 400 nucleotides in length. Longer regions may be sequenced by analysis of overlapping short stretches.

3.2 PLASMIDS AS CLONING VEHICLES

Plasmids are DNA replicons that are stably inherited in an extrachromosomal state (Novick et al., 1976). This implies genetic homogeniety, constant monomeric size and ability to replicate independently of the chromosome. Many bacterial plasmids have been used as cloning vectors and all show some of the following advantages:-

a) Bacterial plasmids, especially from E. coli, may be easily isolated and purified in large quantitites. A wide range of plasmid isolation techniques are available many of which are capable of modification, depending on whether preparative quantities of DNA are required or for screening for recombinant molecules (Birnboim and Doly, 1979; Gryczan et al., 1978; Kado and Liu, 1981).

b) If not ubiquitous, plasmids do exist in a wide range of bacterial species thus allowing for the possibility of 'shuttle vector' manufacture. The ability of such a plasmid to replicate and express selectable markers in two different host systems is of great importance in the study of both chromosomal and plasmid regulatory controls (Primrose and Ehrlich, 1981).

c) Many plasmids used as vectors are relatively small in comparison with λ or cosmids and therefore are resistant to damage and have few restriction endonuclease sites.

d) Plasmids can be found which replicate either in a relaxed (multicopy) or stringent (single copy) manner. These are particularly useful if large quantities of cloned gene products are required or multicopies of the gene would lead to cell death, respectively (Hecker et al., 1983).

e) Advances are allowing the construction of positive selection vectors for many bacterial species. Transformants containing recombinant molecules can then be directly selected rather than having to rely on the classical insertional inactivation method (Hennecke et al., 1982; Gryczan and Dubnau, 1983).

f) In certain cases the number of plasmids per cell can be greatly increased (amplified) by the inhibition of protein synthesis by chloramphenicol or even by raising the temperature.

g) Expression vectors, for those genes incapable of normal function in the usual plasmid vehicles, and promoter/terminator screening vectors are also available (Rosenberg et al., 1983; Yanofsky, 1984).

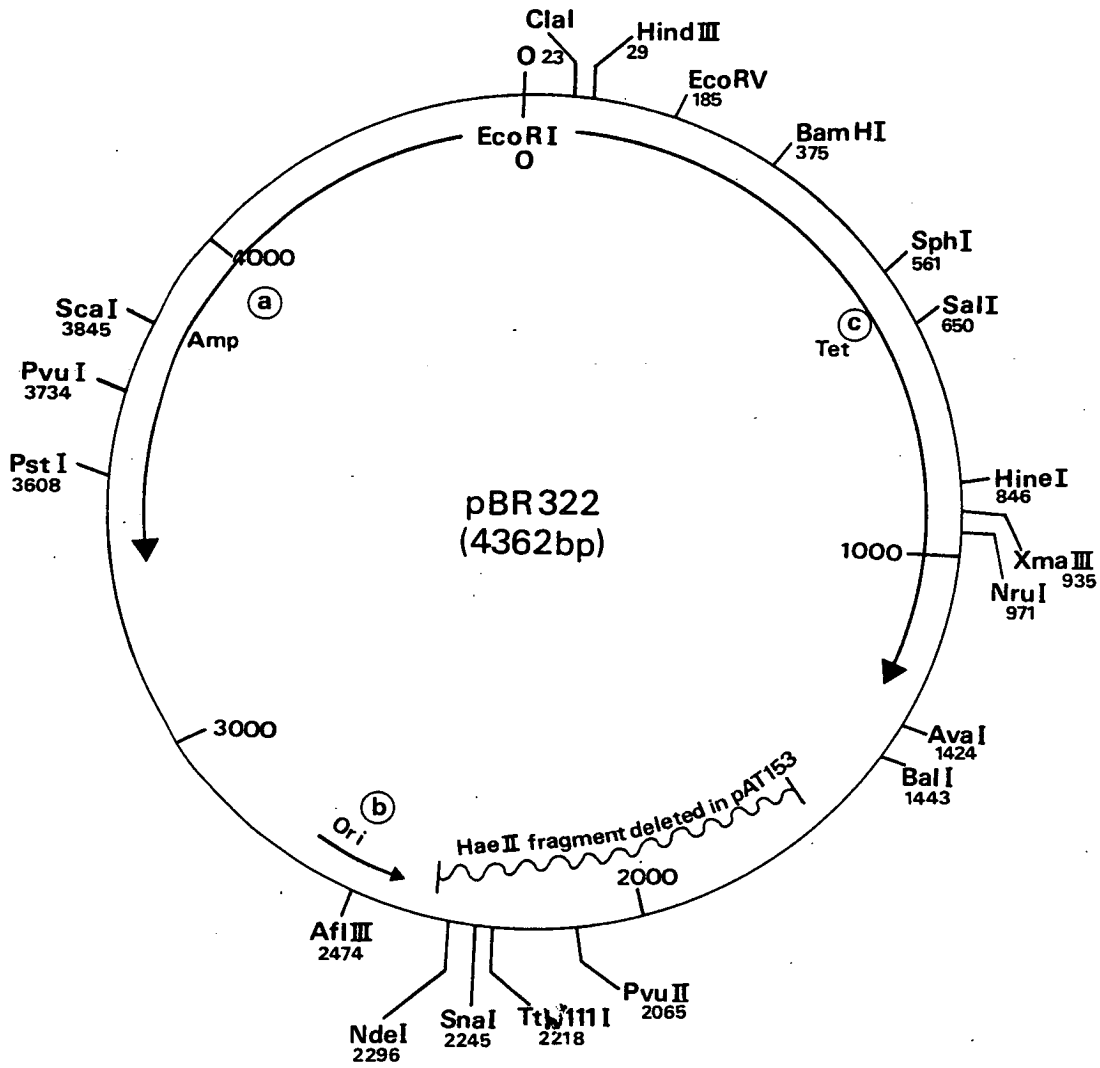
E. coli has displayed a pleasing adaptability in terms of genetic manipulation and this has led to the development of a wide range of versatile host-vector systems for this

bacterium (Bolivar and Backman, 1979). Perhaps the most commonly used plasmid vectors are pBR322 (Fig. 1.3.) and its derivatives (Twigg and Sherratt, 1980). Developed from pMB1, a colicin determining plasmid, they illustrate all the requirements of ideal plasmid cloning vehicles (Sherratt, 1979; Peden, 1983). The most important pBR322 derivative in relation to this thesis is pAT153, isolated by removing a HaeII-generated 755 basepair fragment from pBR322. This plasmid has the advantage over pBR322 of non-cotransmissibility (important for biological containment) and a slightly increased intracellular copy number.

Rapid progress is being made in the construction of cloning vectors for a wide variety of organisms, both prokaryotic and eukaryotic (Hofschneider and Goebel, 1982; Bagdasarian et al., 1983). Of particular interest are those plasmids capable of marker expression and maintenance in both B. subtilis and Staphylococcus aureus (Gryczan and Dubnau, 1978) and the complex plasmid/transposon organisation of Streptococcus faecalis (Clewell, 1981; Jacob and Hobbs, 1974) var. zymogenes.

Possibly the major disadvantage of using plasmids as cloning vehicles is the size limitation of inserted DNA. An average insert of around 5 kilobases (kb) is normal and greater than 20kb unusual. As a gene library consists of a collection of cloned DNA fragments which statistically comprise the entire genome of the organism, success obviously depends upon the average size of insert and size of the donor genome (Dahl et al., 1981). It would be impractical, in terms of individual clones required, to

FIG.1.3. THE PLASMID VECTOR pBR 322 (Showing unique restriction sites) (Sutcliffe ,1979)



a. Derived from Tn3 carried on plasmid pRSF2124
b. Origin of replication isolated from pMB1
c. Derived from the plasmid pSC101

construct gene libraries of organisms with large genome complements (eukaryotes) in plasmid vectors. Cosmid and λ vectors are candidates for this kind of work allied to subsequent sub-cloning into plasmid vehicles if necessary.

3.3 M13 - ITS ROLE IN CLONING AND SEQUENCING

The development of the single-stranded DNA bacteriophage (M13, Ff, etc.) has allowed the elegant combination of gene cloning and nucleotide sequencing in the same vector molecule. Messing and his colleagues (Messing, 1983) have constructed a range of modified M13 bacteriophages that has found wide use in recombinant DNA technology. The unique life cycle of filamentous bacteriophages, such as M13, provides large amounts of packaged (+) single-stranded DNA as well as double-stranded intracellular replicating-form plasmid (RF). M13 infects *E. coli* via the F-pilus resulting in chronic infection, the host releasing infective virions through the cell wall. M13mp bacteriophages (Messing's modified M13 range) carry part of the lac operon of *E. coli* inserted into a non-essential region of the M13 genome (Fig. 1.4). The lac DNA codes for the N-terminus of β-galactosidase which complements a host *lacZ* mutation (α-complementation) permitting the formation of blue coloured plaques on medium containing the histochemical stain, 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X gal). The bacteriophage *lacZ* DNA contains multiple unique restriction sites and insertion of foreign DNA into this region nullifies the α-complementation thus producing clear rather than blue plaques upon growth and multiplication

of the recombinant. Use of the M13mp constructs is especially attractive for the rapid association of single-stranded template necessary for DNA sequencing and hybridisation. Frequently M13mp systems are closely associated with the Sanger dideoxy terminator method of sequencing using a primer which anneals to a homologous region of M13 DNA just to the right of the cloning sites (Fig. 1.4). This permits 5' → 3' labelled extensions which randomly terminate at the insertion of a dideoxy molecule. The separate use of ddATP, ddGTP, ddTTP and ddCTP allows the production of a 'ladder' when subjected to electrophoresis and autoradiography. Such a ladder allows the determination of a nucleotide sequence of over 400 basepairs. This length can be increased by sequencing shorter, overlapping sequences either derived by random or directed subcloning techniques. The data from such experiments are assimilated by computer to produce longer DNA sequences. Directed sub-cloning routines allow for this nucleotide compilation whilst avoiding the asymptotic nature of data accumulation associated with random 'shotgun' methods of fragment production, e.g. sonication (Deininger, 1984; Hong, 1982; Guo et al., 1983). A general view of cloning routines in M13mp bacteriophages is shown in Figure 1.5.

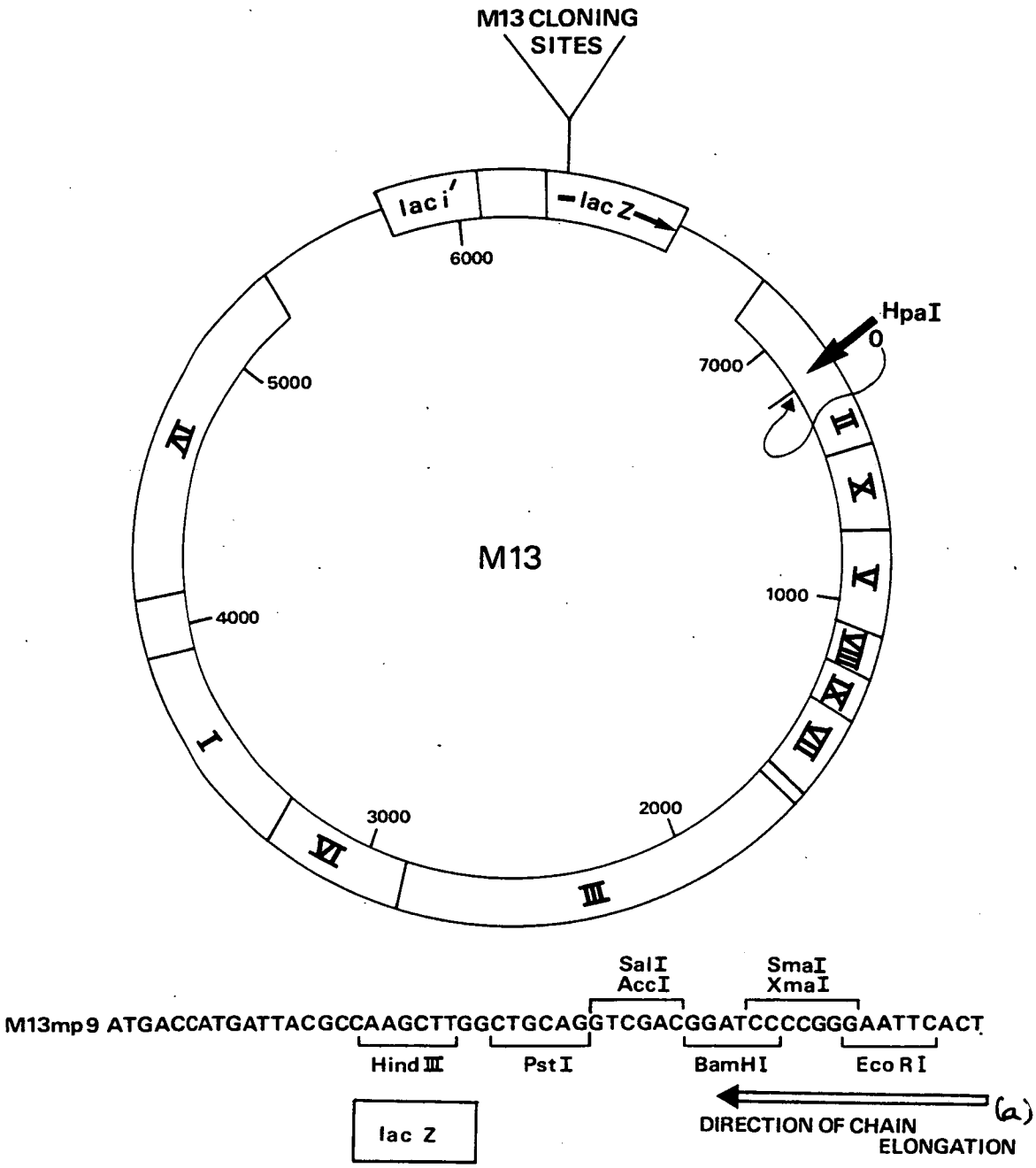


FIG. 1.4. MAP AND CLONING SITE NUCLEOTIDE SEQUENCE OF M13mp9.
(Messing & Vieira 1982)

(a) DNA polymerisation occurs in a 5'→3' direction from a 15bp primer annealled proximal to the EcoRI site of the polylinker.

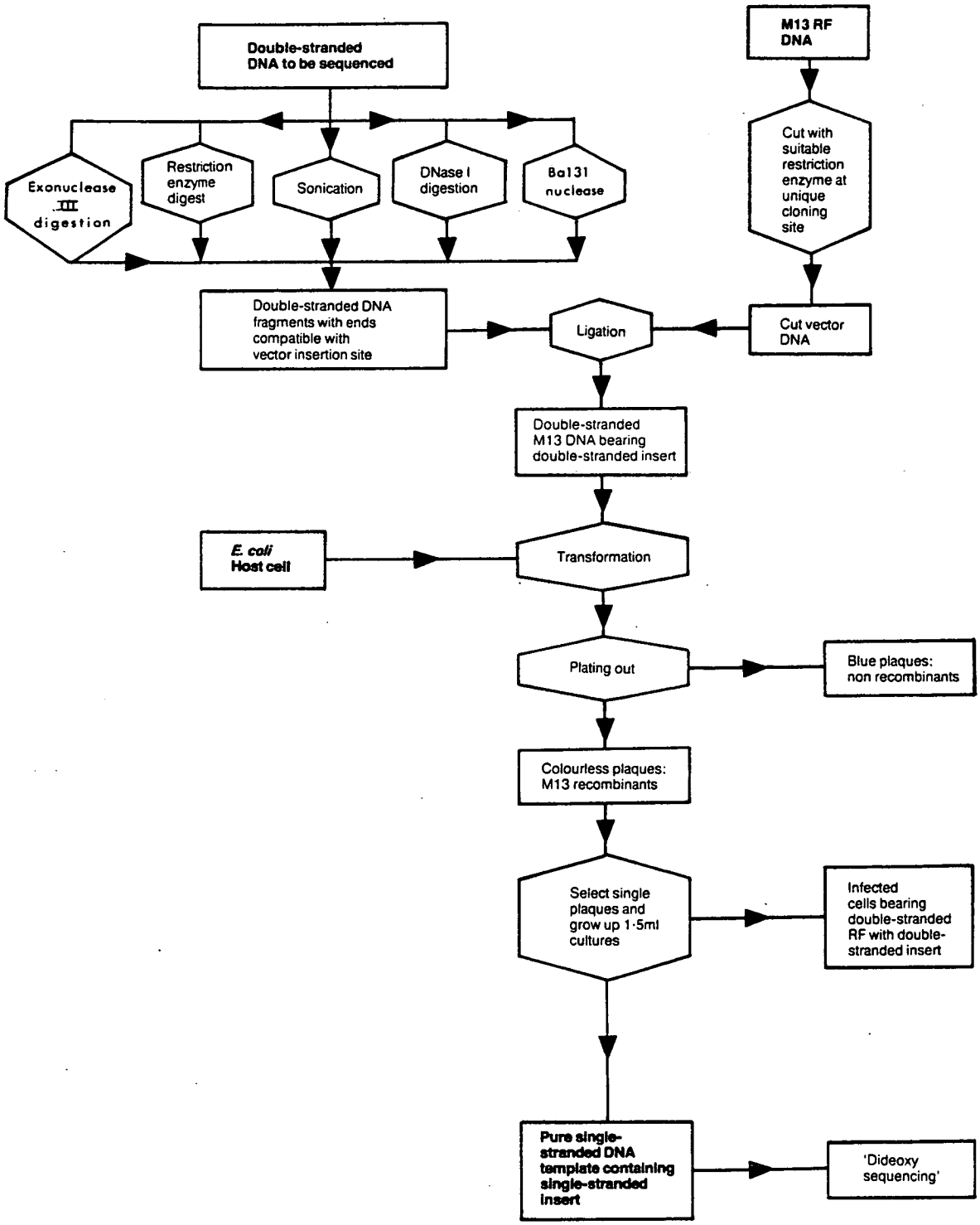


FIG. 1.5 CLONING WITH M13 TO GIVE SINGLE-STRANDED DNA TEMPLATE FOR SEQUENCING

4 SITE-SPECIFIC ENDONUCLEASES AND METHYLASES

4.1 GENERAL PROPERTIES

Sequence-specific endonucleases and their associated methylase activities are largely responsible for the host delineated barriers that regulate interstrain DNA transfer in prokaryotic cells. It was the observation of such restriction and modification which eventually led to the identification and utilization of the enzymes involved (Luria, 1953). The investigation of enzyme properties, activities and cofactor requirements has produced a classification scheme consisting of three groups; types I, II and III restriction endonucleases. Although of value to the cell, types I and III are not generally used as tools for genetic engineering and further information can be gained from the review of Yuan (1981).

4.2 TYPE II RESTRICTION ENDONUCLEASES

Type II restriction endonucleases are DNases that recognised specific oligonucleotide sequences, make double strand breaks and generate unique, equimolar fragments of a DNA molecule substrate. Their use as analytical and engineering tools resides in this predictable, controllable activity. Various review articles deal directly with their sources (Roberts, 1983), sequence specificity (Modrich, 1982) and purification (Pirrotta and Bickle, 1980). Type II classification covers a wide range of enzymes showing the general characteristics listed in Table 1.2. However, terminology such as restriction endonuclease may be misleading in so far as few of these

enzymes have been shown to be involved in restriction/modification systems *in vivo* and there may well be diverse modes of DNA-protein interactions at work.

4.3 MODIFICATION TRANSMETHYLASES (TYPE II)

Such enzymes recognise a specific nucleotide sequence and methylate bases within this sequence in the presence of S-adenosyl-L-methionine (SAM). The activity can be seen in conjunction with the complementary restriction endonucleases but often species can be found having methylase but no associated endonuclease activity. As in the case of type II site-specific endonucleases, the methylases tend to be simple proteins acting in monomeric or dimeric form.

4.4 CLONING OF RESTRICTION/MODIFICATION SYSTEMS

It would be of obvious benefit to remove the genes coding for enzymes of interest from their normal background and place them in plasmid vectors capable of allowing expression in different hosts, normally including the ubiquitous *E. coli*. There are two basic conditions that must be fulfilled in order for a restriction-modification system to be cloned and transferred successfully to a new host. It is essential that the genes for the restriction enzyme and methylase be linked upon the chromosome or plasmid of the original organism. Also the methylase gene must be functionally expressed in advance of any endonuclease production so as to protect both host and recombinant plasmid from digestion. In contrast, the only major barriers to the

TABLE 1.2. GENERALISED PROPERTIES OF TYPE II RESTRICTION ENDONUCLEASES

1. Recognition of distinct oligonucleotide sequences, frequently palindromic in nature.
2. Nuclease activity closely linked to the recognition site.
3. Simple cofactor requirements, normally Mg^{2+}.
4. Nuclease and associated methylase activities reside in separate proteins, not usually multifunctional.
5. Variable dependence upon ionic strength, salt concentration and sulphydryl compounds for activity.

-o-

TABLE 1.3. SENSITIVITY OF RESTRICTION ENZYMES TO ULTRAVIOLET INDUCED DAMAGE TO THEIR SUBSTRATES

Enzyme	Recognition Sequence and incision sites
Hind III	5' A↓AGCTT 3' TTCGA↑A
Eco RI	5' G↓AATTC 3' CTTAA↑G
Bam HI	5' G↓GATCC 3' CCTAG↑G
Sal I	5' G↓TCGAC 3' CAGCT↑C
Hae III	5' GG↓CC 3' GG↑CC
Hha I	5' GC↓GC 3' CG↑CG

decreasing sensitivity

cloning of the methylase gene activity alone is the compatibility of gene expression pathways and suitability of internal metabolic functions (Walder et al., 1983; Janulaitis et al., 1982; Schoner et al., 1983; Walder et al., 1981).

4.5 ULTRAVIOLET LIGHT DAMAGE

It has been shown that ultraviolet light can induce damage in DNA which leads to serious disruption of activity of certain type II restriction endonucleases (Hall and Larcom, 1982; Cleaver et al., 1982). Inhibition seems closely related to the presence of thymine-thymine cyclobutane dimers either within the specified enzyme recognition site or directly adjacent to it. Therefore, the thymine (and to a lesser extent cytosine) content of a recognition sequence determines the sensitivity of the associated restriction enzyme to inhibition of activity by ultraviolet light induced lesions (Table 1.3).

4.6 BIOLOGICAL ROLE OF SITE SPECIFIC NUCLEASES AND METHYLASES

There is no direct evidence for any single, common function for either restriction endonucleases or methylases. Sequence analysis of both genes and structural proteins have shown no obvious commonality. Similar enzymatic function seems to stem from convergent evolution rather than shared ancestry. Restriction endonuclease/methylase systems can function purely as barriers to foreign DNA penetration (Szyf et al., 1982). However double-stranded nucleases are intimately linked with DNA recombination in both prokaryotes and eukaryotes (Kostriken et al., 1983).

DNA methylation has been implicated in the regulation of biological processes including initiation of replication, mutagenesis and gene expression (Ehrlich and Wang, 1981). Therefore, any generalisations upon the use of these enzyme systems must be carefully considered, particularly where no extracellular/intracellular viral interlopers are seen as in the case of the members of the genus Deinococcus.

5 IDENTIFICATION OF PLASMID ENCODED PROTEINS

As a result of the widespread study of cloned genes and the biology of plasmids it has been necessary to devise methods for identifying plasmid-coded proteins not amenable to direct localisation (i.e. by sensitive radioimmunoassays). Various convenient methods of labelling such proteins with ^{35}S-methionine have been developed including maxi-cells (Sancar et al., 1979), mini-cells (Meagher et al., 1977) and in vitro translation (Yang and Zubay, 1978).

5.1 MAXI-CELLS

It was observed that heavily irradiated E. coli cells infected with λ bacteriophage DNA preferentially incorporated added label into bacteriophage-coded proteins (Ptashne, 1967). From this was developed an alternative method of labelling plasmid-coded proteins avoiding the somewhat time consuming routines of other procedures. If E. coli recA uvrA cells are irradiated with 254nm light chromosomal DNA synthesis stops and after several hours the DNA is extensively degraded leaving only traces of

chromosome remaining. However, if such a cell contains a Col E1-type multicopy plasmid, e.g. pAT153, which has a much lower chance of receiving an ultraviolet light induced lesion due to its relatively smaller size, the plasmid DNA will be amplified whilst the chromosome is being degraded. When an E. coli CSR 603 strain, phenotype recA , uvrA and phr (photoreactivating enzyme inactive) is used and irradiated then the resultant 'maxicells' contain mostly plasmid DNA directing the synthesis, almost exclusively, of plasmid proteins which could thus be labelled with ^{35}S-methionine. Obviously such detection of proteins is largely dependent upon the degree of gene expression. Therefore, products of those genes that are expressed at very low levels due to the presence of specific repressors, inefficient promoters, attenuation sites or poorly translated mRNA's are not seen.

CHAPTER 2. MATERIALS AND METHODS

BACTERIAL STRAINS

All the strains used are listed in Tables 2.1 - 2.4. Plasmids used are listed in Table 2.2 although they may have been transferred to hosts other than those in which they were received.

MAINTENANCE OF CULTURES

All the strains of the genus *Deinococcus* with the exception of *D. radiophilus* were grown in TGY broth or on TGY agar. *D. radiophilus* was grown in nutrient broth No. 2 (Oxoid). *E. coli* and *B. subtilis* strains were grown on L-broth or agar but chemically defined and supplemented media were required for phenotypic characterisation. *S. faecalis* strains were grown in Todd-Hewitt medium with a supplement of 4% horse-blood when necessary. All *Deinococcus* spp. were grown at 30°C whilst *E. coli*, *B. subtilis* and *S. faecalis* were grown at 37°C.

MEDIA

The following media were used: (All up to the litre with distilled water).

(1) TGY medium (Anderson *et al.*, 1956).

	$g\ l^{-1}$
Bactotryptone	5
D-Glucose	1
Yeast extract	3

(2) Nutrient broth No. 2

	$g\ l^{-1}$
Nutrient broth No. 2 (Oxoid)	25

TABLE 2.1 Strains of *Deinococcus* used

STRAIN	SOURCE	SELECTION MARKER
D.radiodurans R1	Dr. B.E.B. Moseley[a]	-
D.radiodurans Rif^R	"	rifampicin resistance 100 $\mu g\ ml^{-1}$
D.radiodurans Tet^R	"	tetracycline resistance 30 $\mu g\ ml^{-1}$
D.radiodurans Rec30	"	recombination deficient
D.radiodurans 302	"	mitomycin C sensitive
D.radiodurans Nuc^-	Dr. D.M. Evans[b]	extracellular nuclease deficient
D.radiodurans Sark	Prof.R.G.E.Murray[c]	-
D.radiophilus	Dr. B.E.B. Moseley	-
D.radiopugnans	"	-
D.proteolyticus	"	-

a) Department of Microbiology, University of Edinburgh

b) Department of Microbiology, University of Edinburgh

c) Department of Microbiology and Immunology, University of Western Ontario, Canada.

TABLE 2.2 Plasmids used

PLASMID	HOST	SELECTABLE[b] MARKER	SOURCE
pML2	*E.coli* HB101	Kn	Dr. M. Mackay[a]
pBR322	*E.coli* HB101	Tc,Ap	"
pAT153	*E.coli* HB101	Tc,Ap	"
pLV21	*E.coli* HB101	Kn(Su)	"
R68.45	*E.coli* J53-1	Nal,Tc,Ap,Kn	Dr. B.E.B. Moseley
RP4	*E.coli* J53-1	Nal,Tc,Ap,Kn	"
pUB110	*B.subtilis* HVS89	Kn	Dr. M. Mackay
pC194	*B.subtilis* HVS62	Cm	"
pHV33	*E.coli* HVC181	Tc,Ap,Cm	"

a) Department of Molecular Biology, University of Edinburgh

b) Abbreviations and selection concentration:

Kn - Kanamycin, 20 μg ml^{-1}

Ap - Ampicillin, 50 μg ml^{-1}

Tc - Tetracycline, 20 μg ml^{-1}

Cm - Chloramphenicol, 10 μg ml^{-1}

Su - Sulphonamide (not used)

Nal - Naladixic acid, 50 μg ml^{-1}

TABLE 2.3 Strains of S.faecalis used

STRAIN	PLASMID CONTENT	GENETIC MARKERS[b]	SOURCE
S.faecalis DS5	pAMα1	Tc^R	Dr.D.B.Clewell[c]
	pAMβ1[a]	Em^R	
	pAMγ1[a]	Hyl-Bac, UV^R, PR	
S.faecalis DS16	pAD1[a]	Hyl-Bac, UV^R, PR	"
	pAD2	Em^R(Tn917), Sm^R, Kn^R + Tn916 Tet^R[a]	
S.faecalis JH2-1	plasmid free	Fus^R	Dr.A.Jacob[d]
S.faecalis JH2-16	plasmid free	Rif^R	"

(a) Conjugative plasmids

(b) Abbreviations and selective levels

Tc - Tetracycline, 10 μg ml^{-1} (Tet^R represents Tn 916 in chromosomal situation)

Em - Erythromycin, 50 μg ml^{-1}

Sm - Streptomycin, 1000 μg ml^{-1}

Km - Kanamycin, 20 μg ml^{-1}

Fus - Fusidic acid, 40 μg ml^{-1}

Rif - Rifampicin, 50 μg ml^{-1}

Hyl-Bac - Haemolysin-Bacteriocin (see plate 2.1)

UV^R - Ultraviolet light resistance

PR - Pheromone response

(c) Department of Medicine and Dentistry, University of Michigan, USA.

(d) Department of Medicine, University of Manchester

TABLE 2.4 Other bacterial strains

STRAIN	PHENOTYPE	SOURCE
E.coli HB101	R^-M^-, *recA*, *SupE*, *lacZ*, *leuB*, *proA*, *thi*, Sm^R	Dr. B.E.B.Moseley
E.coli NM522	*hsd* $R^-M^-S^-$, *lac pro*, *SupE*, *thi*, F' *proAB laci lacZ*, *M15*	Dr. N. Murray[a]
E.coli CSH42	*thr*, *leu*, *lac*, *thyA*, *mal*, *ilv*, *thi*	Dr.I.Dawes[b]
E.coli CSH58	*ara*, *thr*, *leu*, *proA*, *lac*, *gal* *trp*, *his*, *recA*, *thyA*, Sm^R, *xyl* *mtl*, *argE*, *thy*, sup^+	"
E.coli 107	*trpD* 9778	Dr. B.E.B.Moseley
E.coli 128	*trpB* 9700	"
E.coli 311	*trpA* 88	"
E.coli 312	*trpC* am	"
B.subtilis 168	plasmid free	"

(a) Department of Molecular Biology, University of Edinburgh

(b) Department of Microbiology, University of Edinburgh

(3) Luria broth (L-broth)

	$g\ l^{-1}$
Bactotryptone	10
Yeast extract	5
NaCl	5
D-Glucose	1

(4) M9 salts (x 10 concentrate)

	$g\ l^{-1}$
$Na_2HPO_4.H_2O$	60
KH_2PO_4	30
NaCl	5
NH_4Cl	10

(dissolved in order indicated before autoclaving)

(5) M9 minimal medium

M9 salts (x 10)	100ml
20% w/v D-Glucose	20ml
0.1M $MgSO_4$	10ml
0.01M $CaCl_2$	10ml
Sterile distilled water	860ml

(each solution sterile before mixing)

(6) Penassay broth

	$g\ l^{-1}$
Bacto-Antibiotic Medium No.3 (Difco)	17.5

(7) SMMP medium

4 x strength Penassay broth

2 x strength SMM buffer

(autoclaved separately and equal volumes added)

(8) DM3 Regeneration Medium

Pre-sterilized solutions l^{-1}	
4% Agar	200ml
1M Sodium succinate	500ml
5% Casamino acids (Difco)	100ml
10% Yeast extract	50ml
3.5% K_2HPO_4/1.5% KH_2PO_4	100ml
25% D-Glucose	25ml
1M $MgCl_2$	20ml
Bovine Serum Albumin	5ml (2.5mg ml^{-1})

(9) N2GT Broth

	$g\ l^{-1}$
Oxoid nutrient broth No.2	25g
D-Glucose	2g
Tris-hydrochloride	15.8g
(pH to 7.7 using Tris-base)	

(10) Todd-Hewitt broth

	$g\ l^{-1}$
Oxoid Todd-Hewitt broth	36.4g
(optional) Horse blood (Oxoid)	40ml

(11) BGTT medium

	$g\ l^{-1}$
Calf brain infusion solids	12.5
Beef heart infusion solids	5
Proteose peptone	10
NaCl	5
Dextrose	2
$Na_2HPO_4.12H_2O$	2.5
D-Glucose	3.5
DL-Threonine	3.8

(12) 2 x YT broth

	$g\ l^{-1}$
Bacto-tryptone	16
Yeast extract	10
NaCl	5

(13) B broth

	$g\ l^{-1}$
Bacto-tryptone	10
NaCl	8

(supplemented with 1% vit B_{12} soln.)

(14) K medium

M9 glucose minimal medium + 1% casamino acids + 0.1µg ml^{-1} thiamine hydrochloride

(15) Hershey salts

	$g\ l^{-1}$
NaCl	5.4
KCl	3.0
NH_4Cl	1.1
$CaCl_2.2H_2O$	0.015
$MgCl_2.6H_2O$	0.20
$FeCl_3.6H_2O$	0.0002
KH_2PO_4	0.087
Tris base	12.1

(16) Hershey medium

Hershey salts	100ml
20% (w/v) glucose	2ml
2% (w/v) threonine	0.5ml
± 1% (w/v) leucine	1ml
2% (w/v) proline	1ml
2% (w/v) arginine	1ml
0.1% (w/v) thiamine hydrochloride	1ml

All broths were converted to plating agars by the addition of 15g agar l^{-1} (Oxoid No. 1) before autoclaving at 15 p.s.i. for 20 mins. Sloppy agars were produced by using 0.5% agar rather than 1.5%.

BUFFERS

(1) Phosphate buffer (0.067M) pH 7.0

	g l^{-1} (distilled water)
KH_2PO_4	4.56
$Na_2HPO_4.2H_2O$	4.73

(2) Butanol-saturated phosphate/EDTA buffer

	g l^{-1}
KH_2PO_4	4.56
$Na_2HPO_4.2H_2O$	4.73
EDTA	0.34
n-butanol	6% (v/v)

(3) Standard saline citrate (SSC) pH 7.0

	g l^{-1}
NaCl	8.7
Sodium citrate(dihydrate)	4.46

(4) TE buffer, pH 7.4

	g l^{-1}
Tris base	1.21
EDTA (disodium)	0.4

(5) SMM buffer, pH 6.5

	g l^{-1}
Sucrose	171
Sodium maleate	2.3
$MgCl_2$	4.0

(6) Acetate electrophoresis gel buffer pH 8.2 (x 10)

	$g\ l^{-1}$
Tris base	48.4
Sodium acetate (trihydrate)	27.2
EDTA	3.72

(7) Maxicell sample buffer pH 6.8

	$g\ 100ml^{-1}$
Sodium dodecyl sulphate(SDS)	2.3
2-mercaptoethanol (MSH)	5ml
Glycerol	10ml
Bromophenol blue (BPB)	0.05
Tris-HCl	6.25

(8) TM pH 8.0

100mM Tris HCl

100mM $MgCl_2$

(9) 10 x TBE (Tris-borate buffer) pH 8.0

	$g\ l^{-1}$
Tris base	109
Boric acid	55
EDTA	9.3

(10) Formamide dye mix

	$g\ 100ml^{-1}$
Formamide	100ml
Xylene Cyanol FF	0.1
BPB	0.1
EDTA	0.26

(11) Deoxynucleoside triphosphate (dNTP) chase mix

Deoxythymidine 5'triphosphate (dTTP)	0.25mM
Deoxyadenosine 5'triphosphate (dATP)	0.25mM
Deoxycytosine 5'triphosphate (dCTP)	0.25mM
Deoxyguanosine 5'triphosphate (dTTP)	0.25mM

all in TE buffer

(12) 40% Acrylamide stock solution

Acrylamide (electrophoretic grade)	38% (w/v)
Bisacrylamide	2% (w/v)

Solution made up in deionised water followed by the addition of 20g l^{-1} of Amberlite MB1 resin (Hopkin and Williams). Filtration removed the resin from the acrylamide stock solution.

(13) dNTP stock solutions

dTTP)
dCTP) all 50mM in TE buffer (diluted 100
dGTP) fold for use).
dATP)

(14) Dideoxynucleoside phosphate stock solutions (ddNTP)

ddTTP)
ddCTP)
ddGTP) all 10mM in TE buffer
ddATP)

(15) NTP° mixes (ratio)

		T°	C°	G°	A°
0.5mM	dTTP	25	500	500	500
"	dCTP	500	25	500	500
"	dGTP	500	500	25	500
10mM	ddTTP	50			
	ddCTP		8		
	ddGTP			16	
	ddATP				1 3 if [^{32}P] ATP is used
TE buffer		1000	1000	1000	500

ANTIBIOTICS AND NUTRITIONAL SUPPLEMENTS

All the antibiotics and nutritional requirements used in this study were obtained from Sigma Chemical Company, London.

RADIOACTIVELY-LABELLED COMPOUNDS

Labelled [^{32}P] deoxyadenosine 5'-triphosphate, [^{35}S] deoxyadenosine 5'-triphosphate and [^{35}S] L-methionine were gifts from either Dr N. Brown (University of Bristol) or Dr M. Mackay (University of Edinburgh).

ENZYMES

T_4 DNA ligase (EC.6.5.1.1.), calf intestinal alkaline phosphatase (EC.3.1.3.1.), S1 nuclease (EC.3.1.30.1), Bal 31 nuclease and a variety of restriction endonucleases were purchased from Boehringer Mannheim, Lewes. *E. coli* DNA polymerase I (Klenow fragment) was supplied by both NBL enzymes Ltd., Cramlington and PL Laboratories, Milton Keynes. Pancreatic RNase (EC.3.1.4.22) and lysozyme came from Sigma. Various restriction endonucleases were also obtained from New England Biolabs, Bishops Stortford, NBL enzymes, Miles Laboratories, Stoke Poges, and Bethesda Research Laboratories (BRL), Cambridge.

CHEMICALS

Sodium dodecyl sulphate (SDS), ethylene diamine tetra acetic acid: disodium salt (EDTA), hydroxymethyl methylamine (Tris base), Tris-hydrochloride (Tris-HCl), caesium chloride (AnalaR), polyethyleneglycol (AnalaR) 6000,

bromophenol blue (BPB), ethidium bromide, xylene cyanol FF, 3-(N-Morpholino) propanesulphonic acid (MOPS), acrylamide and bisacrylamide (electrophoretic grade), S-adenosyl L-methionine and spermidine were all obtained from BDH Chemicals Ltd., England. Ficoll, agarose (type II low EEO), deoxyadenosine 5-triphosphate (dATP), deoxyguanosine 5' triphosphate (dGTP), deoxythymidine 5' triphosphate (dTTP), deoxycytosine 5' triphosphate (dCTP), dideoxyadenosine 5' triphosphate (ddATP), dideoxy guanosine 5' triphosphate (ddGTP), dideoxythymidine 5' triphosphate (ddTTP) dideoxycytosine 5' triphosphate (ddCTP), and phenylmethylsulfonylfluoride (PMSF) were all supplied by Sigma. Dithiothreitol (DTT), ultrapure phenol, isopropylthio-β-galactoside (IPTG), 5-bromo-4-chloro-3 indolyl-β-D-galactoside (Xgal) and (N,N,N'N')-tetramethylethylenediamine (Temed) were products of BRL. Hydroxylapatite-HTP and dextran T-500 were from Pharmacia (GB) Ltd., Milton Keynes. Triton X-100 was obtained from Koch-light Laboratories, Colnbrook.

DYE-BUOYANT DENSITY GRADIENT CENTRIFUGATION

DNA was prepared from a variety of sources for purification on $CsCl_2$ gradients. These gradients were formed by adding 11g of $CsCl_2$ and 1ml of ethidium bromide (10mg ml^{-1} in TE buffer) to 10ml of cleared lysate. The refractive index was adjusted to 1.3925 (density 1.625g cm^{-3}), using an Abbe 60 Refractometer (Bellingham & Stanley Ltd., England), by adding further $CsCl_2$ crystals.

The solution was then transferred to two 10ml polypropylene tubes (MSE) and centrifuged at 130 000g for 60hr at 18°C in a 10 x 10ml fixed head rotor. When both chromosomal and plasmid DNA were present, two bands formed in the middle of the gradients. The lower covalently closed circular (ccc) band was intact plasmid DNA, the upper band being chromosomal and 'nicked' plasmid DNA. When required, the bands were extracted by carefully puncturing the side of the tube with a 19 gauge needle and drawing off the solution using a 1ml sterile syringe. The ethidium bromide was extracted with salt saturated isopropanol (5M NaCl, 10mM Tris base, 1mM EDTA pH 7.5). The DNA was collected by precipitation with 2vol. H_2O and 6vol. ethanol at -20°C for 2 - 5 h. After centrifugation the pellet was resuspended in an appropriate volume of TE buffer.

DNA ISOLATION

(a) CHROMOSOMAL DNA

Chromosomal DNA was prepared from *D. radiophilus* by a modification of Marmur's method (1961). Two l of cell culture, grown to stationary phase (approx. two days), were spun at 8000g for 10 min. The pelleted cells were resuspended and washed in 50ml of SSC buffer followed by recentrifugation. The cells were rendered sensitive to lysozyme activity by resuspending the pellet in 40ml butanol-saturated phosphate/EDTA buffer and leaving the suspension at room temperature for 45 mins. (Driedger and Grayston,

1970). After collecting the cells and washing them again in SSC, the total volume of the suspension was brought to 40ml and lysozyme added to a final concentration of 2mg ml^{-1}, the mixture being incubated at 37°C for 30 - 60 mins. Complete cell lysis was achieved by the addition of 0.1 vol of 20% SDS. After swirling to ensure adequate mixing, 13ml of sodium perchlorate solution (70.25g $NaClO_4.H_2O$ and 4.4g NaCl per 100ml water) was added, followed immediately by an equal volume of a 24:1 chloroform:iso-amyl alcohol mixture. Deproteinisation occurred during 30 min. of agitation of the solution followed by centrifugation at 30 000g for 20 mins. in 'Corex' 25ml pyrex tubes. DNA and RNA were present in the upper aqueous layer and were carefully removed without disturbing the protein pellet present and at the solvent interface. Careful pouring of the aqueous component into 2 vol. of cold ethanol allowed the nucleic acids to be wound out of solution on glass rods, dried and then resuspended in a small volume (approx. 5ml) of SSC buffer. Chromosomal DNA of very high purity was collected after dye-buoyant density gradient centrifugation (Radloff et al., 1967).

(b) PLASMID DNA

(i) E. coli

For large scale extraction of the majority of E. coli plasmids used in this study (excluding the large R factors) the basic alkaline extraction procedure of Birnboim and Doly (1979) was used. E. coli cells were grown in 1l amounts

of L-broth at 37°C usually in the presence of a relevant selective antibiotic. Chloramphenicol amplification was possible for all ColE1 derived plasmids but was not employed for the preparation of any recombinant plasmids. For pBR322 and pAT153 extractions, the host cells were grown to a turbidity of 60 - 100 (measured in a nephelometer) before chloramphenicol was added to a final concentration of 150μg ml^{-1}. The culture was then shaken at 37°C for 16 h. One l of culture of plasmid-bearing cells were spun down by centrifugation at 7000g for 10 min. allowed to drain, then resuspended in 30ml of lysis mix (50mM glucose, 25mM Tris-HCl, 10mM EDTA, pH 8.0). To this, 10ml of lysis mix containing 8mg ml^{-1} of lysozyme was added and the mixture incubated for 10 mins on ice. Cell lysis was achieved by the addition of 80ml of 0.2M NaOH; 1% SDS solution w/v (fresh) and incubation on ice for a further 5 mins. Sixty ml of high salt solution (3M sodium acetate adjusted to pH 4.8 using CH_3COOH) was then added and the mixture kept on ice, with occasional inversion, for 60 mins. Centrifugation at 12000g for 20 mins allowed a 'clear lysate' to be separated from the insoluble mass of cell debris/chromosomal material. Filtration through a tea strainer removed any remaining lumps. To this supernatant, 2 vol. of cold ethanol was added and after complete mixing the solution was placed at -20°C for at least 30 mins. The precipitate was collected by centrifugation at 5000g for 5 mins and resuspended in 50ml of

low salt solution (0.1M sodium acetate at pH 6.0). Ethanol precipitation was repeated and the pellet resuspended in <10ml of TE buffer. Plasmid DNA was then separated from contaminating chromosomal DNA, proteins and RNA by dye-buoyant density centrifugation.

For rapid screening of colonies for the presence of plasmid DNA the boiling method of Holmes and Quigley (1981) was used. 1.5ml cultures of E. coli in L-broth were grown overnight, poured into 1.5ml Eppendorf tubes and spun for 15 s in a microfuge (Hettich Mikroliter). The pellet was resuspended in 350µl of STET buffer (0.8% sucrose, 0.5% Triton X-100, 50mM Tris, 1mM EDTA pH 8.0) and 25µl of lysozyme solution (10mg ml^{-1}) added. Immediately upon mixing, the tube was placed in a boiling-water bath for 40s and then centrifuged at 4°C for 15 mins. The gelatinous pellet was removed with a toothpick and the supernatant precipitated by adding 40µl of 4M sodium acetate and 400ul of cold isoproponol. After 5 mins at ambient temperature the precipitate was collected by centrifugation and the pellet washed with 70% ethanol before being vacuum dried. The DNA was then resuspended in 40µl of distilled water. This method was used for the isolation of both recombinant plasmid and M13 RF DNA molecules, the DNA being suitable for direct restriction enzyme digestion and also for bacterial transformation.

(ii) B. subtilis

Plasmid isolation from B. subtilis was achieved using Niaudet and Ehrlich's (1979) modification of the Gryczan

et al. (1978) method.

250ml of an overnight culture (L-broth) was centrifuged at 7000g for 10mins and the cells washed and resuspended in 50 of buffer consisting of 0.1M NaCl, 0.05M Tris base and 1mM EDTA at pH 7.4. Lysozyme (0.25ml, 20mg ml^{-1}, freshly made) was added and the mixture incubated at 37°C for 20 mins. 2.4ml of 5M NaCl, 0.6ml of 0.5M EDTA, pH 8.0 and 12.5ml of freshly prepared 2% SDS (w/v) - 0.7M NaCl were added in that order and left overnight at 4°C. The lysed cells were spun at 38000g for 30mins, the supernatant collected and the salt concentration adjusted to 1M by adding 5M NaCl. One third volume of 40% polyethyleneglycol 6000 (PEG) was added and the DNA precipitated out during a 1h incubation at 0°C. The precipitate was collected by centrifugation at 7000g for 5mins, and the pellet resuspended in 5ml TE buffer. Further purification was achieved by density gradient centrifugation.

(iii) S. faecalis

Essentially the plasmid preparation procedure for S. faecalis was that described by Le Blanc and Lee (1979) but with modifications in the lysis stages (Kado and Liu, 1981) and by Jacob (personal communication).

Cultures of S. faecalis were grown up in 20ml of BGTT broth at 37°C with aeration and harvested by centrifugation at 8000g for 15mins at 4°C. The pellet was resuspended in 0.5ml of 0.01M Tris-HCl, 25% sucrose pH 8.0, followed by the addition of 0.5ml of lysozyme solution (40mg ml^{-1} in

0.25M - Tris HCl pH 8.0). These solutions were well mixed and incubated at 37°C for 30mins. The cells were lysed by the addition of 0.25ml of 0.25M EDTA pH 8.0 and 2.5ml of lysis mix (50mM Tris-HCl, 3% SDS (w/v) adjusted to pH 12.6 with 2M NaOH). The lysate was gently mixed and incubated at 65°C for 30mins. Proteins and cell debris were separated by extraction with 2 vol of a 1:1 phenol:chloroform mixture (previously buffered by 1M Tris base, pH 8.0). The layers were emulsified by gentle shaking before phase generation during a 15mins spin at 3000g. The upper aqueous layer was carefully removed and nucleic acids precipitated by the addition of 0.54vol cold isopropanol. The DNA was recovered by centrifugation and resuspended in about 100µl of TE buffer. The plasmid isolated in this manner was used directly for transformation or as a check for plasmid presence by agarose gel electrophoresis.

BACTERIAL TRANSFORMATION

(a) TRANSFORMATION OF *E. coli* HB101 WITH PLASMID DNA

E. coli HB101 was transformed with plasmid DNA using the technique developed by Humphreys *et al*. (1978). An overnight culture in L-broth (37°C) was diluted 100 fold and grown at 37°C with vigorous aeration until the optical density reached approximately 30 (2 - 3 h). For each transformation 4x1.5ml Eppendorf tubes were filled with the culture and spun for 20s in a microfuge. The pellets were washed in 0.5vol of 10mM $CaCl_2$ and once again pelleted

by spinning for 20s in the microfuge. The cells were resuspended in 50µl of 75mM $CaCl_2$, 10mM MOPS, 0.5% glucose, pH 6.5 and all 4 tube contents pooled, i.e. from 6ml culture 200µl of competent cell culture is produced. To the 200µl cell suspension the transforming DNA was added (normally dissolved in 100µl of 0.1M Tris buffer pH 7.0) followed by 200µl of 75mM $CaCl_2$, 10mM MOPS, 0.5% glucose, pH 6.5. The mixture (0.5ml) was kept on ice for 45mins before a heat-shock treatment of 10mins at 42°C. The transformed culture was then transferred to 1.5ml of L-broth and shaken for 30 to 60mins at 37°C to allow for phenotypic lag.

(b) TRANSFORMATION OF *E. coli* NM522 WITH M13RF DNA

The re-introduction of manipulated M13 mp9 DNA into the indicator strain *E. coli* NM522 was achieved using the method of Messing (1983).

E. coli NM522 was grown up overnight in M9 minimal medium + thiamine. The culture was diluted 100 fold into 2 x YT medium and grown at 37°C with shaking until the turbidity reached approximately 60. The cells were collected by centrifuging 1.5ml amounts in Eppendorf tubes for 20s (2 x 1.5ml tubes per transformation). The pellets were resuspended in ice-cold 50mM $CaCl_2$ and left on ice for 20mins. The cells were again pelleted by centrifugation for 20s and resuspended in 150µl of cold $CaCl_2$, 2 tubes being combined to give 300µl of competent cell mix. To this the M13 DNA was added and after gentle mixing the

tube was kept on ice for 40mins and then transferred to 37°C for 15mins to induce DNA uptake. The transformed culture (0.3ml or appropriate dilutions) was added to 3ml of B sloppy agar containing 20ul IPTG (25mg ml^{-1}), 20µl X-Gal (25mg ml^{-1}), 200µl fresh indicator strain (NM522) and 20µl thiamine (1mg ml^{-1}). After vortexing for 10s the mixture was poured onto dry B-agar plates and left to set before incubating overnight at 37°C.

(c) TRANSFORMATION OF B. subtilis BY PLASMID DNA

B. subtilis 168 was transformed with plasmid DNA using the polyethylene glycol (PEG) induced DNA uptake by protoplasted cells, a method developed by Chang and Cohen (1979). Twenty ml of a mid-exponential culture of B. subtilis 168 growing in Penassay broth at 37°C was harvested and resuspended in 2ml of SMMP solution followed by the addition of lysozyme, to give a final concentration of 2mg ml^{-1}. The suspension was incubated for 2 h at 37°C with gentle aeration. The cells were centrifuged at 2500g for 15mins and washed once in SMMP before being pelleted. The resultant pellet of protoplasts was resuspended in 2ml SMMP and 0.5ml samples were mixed with 0.1ml of 2 x SMM, 5µg plasmid DNA, 1.5ml 40% w/v PEG (40g PEG 6000, 50ml 2 x SMM buffer in 100ml) in order. After 2 mins, 5ml of SMMP was added and the mixture centrifuged at 2500g for 10mins. The pellet was resuspended in 1ml SMMP and incubated at 30°C for 1.5h with gentle shaking to allow for phenotypic

lag. The protoplasts were plated directly onto DM3 regeneration medium plus relevant antibiotics and incubated at 37°C for 2-3 days.

(d) TRANSFORMATION OF D. radiodurans STRAINS

Transformation, with either chromosomal or plasmid DNA, of D. radiodurans strains R1, 302, Nuc 1, Rec 30 and Sark followed the procedure developed by Tirgari and Moseley (1980). An overnight culture of the required strain was diluted 20 fold into fresh, warmed TGY broth and incubated at 30°C with shaking until an optical density of about 30 was reached (∿3 h). Ten ml of the culture was harvested by centrifugation at 12000g for 5mins, resuspended in 5ml of prewarmed TGY broth and 2ml of 0.1M $CaCl_2$ solution added. One ml samples of this culture were used for transformation by adding 1-10µg of DNA and by chilling on ice for 10mins. These samples were then transferred to a 30°C water bath for 90mins before 9ml of TGY broth was added and the transformed cells allowed to express the integrated DNA over a period of 4 to 10 h. The relevant selection pressure was applied by plating onto selective TGY agar and incubating for 3 - 4 days at 30°C.

CONJUGATION

E. coli

The transfer of E. coli conjugative R factors RP4 and R68.45 was followed by using a filter-mating technique. Donor and recipient cells (Table 2.5) were grown separately

Table 2.5. Strains of *E. coli* and *D. radiodurans* used in Conjugation Analysis.

	Chromosomal Markers	Plasmid markers
Donor Strains		
E. coli J53-1 (RP4)	Nal^R	Tc^R Ap^R Kn^R
E. coli J53-1 (R68-45)	Nal^R	" " "
Recipient Strains		
E. coli HB101	Sm^R	-
D. radiodurans Rif^R	Rif^R	-

(abbreviations: Nal = naladixic acid, 50μg ml^{-1}; Sm = streptomycin, 200μg ml^{-1}; Rif = rifampicin, 20μg ml^{-1}; Tc = tetracycline, 10μg ml^{-1}; Ap = ampicillin, 20μg ml^{-1}; Kn = kanamycin, 20μg ml^{-1}).

overnight at 37°C without shaking in L-broth (or for D. radiodurans, TGY broth). In all cases both donor and recipient strains had chromosomal markers allowing for differential selection after mating. 0.1ml of donor culture was added to 0.9ml of recipient cells and 9ml of fresh broth, either L-broth or TGY broth, was added. After thorough mixing, the culture was put through a 0.45µm Millipore filter. The filter was taken out of the support assembly, placed on a dry L or TGY agar plate and incubated overnight at 37°C or 30°C. The filter was then removed, the cells washed into fresh broth and donor, recipient and transconjugant cells enumerated by the plating of dilutions onto the appropriate selective medium. The frequency of conjugation was calculated by dividing the number of transconjugants by the number of donors.

S. faecalis

(a) FILTER MATING

Filter matings between streptococcal species were carried out using the method outlined by Franke and Clewell (1981). 0.05ml of an overnight donor culture, grown at 37°C with shaking in N2GT broth, was mixed with 0.5ml of overnight recipient culture in a total volume of 5ml N2GT broth. The cells were collected on a 0.45um Millipore filter which was then removed from its housing and placed on horse-blood agar and incubated overnight at 37°C. The cells were washed off in 1ml of N2GT broth and appropriate dilutions spread onto selective plates. Total numbers of

donors, recipients and transconjugants were determined, the conjugation frequency being calculated by dividing numbers of transconjugants (minus spontaneous mutants) by the number of donors. If *D. radiodurans* Tet^R was used as the recipient, the method remained the same except that TGY broth and agar replaced the media noted above (this seemed to have no effect on *S. faecalis* viability and little effect on streptococcal conjugation frequency).

As well as the filter-mating protocol, a technique developed by Smith and Guild (1980) was used. In this case the procedure was essentially identical except that the filter was overlayed with TGY agar (only used in *S. faecalis* x *D. radiodurans* crosses) before incubating overnight. This embedding in agar has been shown to increase the frequency of conjugation in *Streptococcus pneumoniae* by between 10 and 100 fold.

(b) BROTH MATING

S. faecalis x *S. faecalis* and *S. faecalis* x *D. radiodurans* broth matings were undertaken using the procedure described by Dunny and Clewell (1975). Overnight cultures of the donor and recipient strains of *S. faecalis* (or *D. radiodurans*) were grown at 37°C in either N2GT or TGY broth. Then, to 4.5ml of fresh broth, 0.5ml of recipient, and 0.05ml of donor culture, were added. The mixture was incubated at 37°C for 4h before vortexing followed by serial dilution and plating on the appropriate selective medium for enumeration of donors, recipients and transconjugants.

MEASUREMENT OF BACTERIAL GROWTH

The phase of growth of a bacterial culture was monitored by following changes in the optical density of the medium using a nephelometer (Evans Electroselenium Ltd., Halstead) with an orange filter.

MEASUREMENT OF DNA AND PROTEIN CONCENTRATIONS

The concentrations of DNA preparations were determined spectrophotometrically with a Pye Unicam 5P6-500 uv spectrophotometer. The assumption was made that an absorbance reading, at 260nm, of 1.0 corresponded to a DNA concentration of 50μg ml^{-1}. The comparative absorbances of a solution at 260nm and 280nm not only determined DNA content more accurately, but also allowed calculation of the solutions protein content by using a nomograph. Protein concentration was also measured using a Bio-Rad protein assay kit.

AGAROSE GEL ELECTROPHORESIS

Horizontal agarose gel electrophoresis allowed the identification of a wide range of intact and endonuclease restricted DNA molecules. Concentrations of agarose, from 0.8 to 1.5% were used and in all cases gels were run in a Tris-acetate buffer. Before loading into the wells of the gel each sample had 0.1 vol. of STOP buffer added (0.05% Bromophenol blue; 20% w/v Ficoll; 30mM SDS, 0.5M EDTA) and was heated to 65°C for 10 mins. Electrophoresis conditions were dependent upon the separation of DNA bands required but were rarely above 10V cm^{-1} or below 2.5V cm^{-1}. Band visualization was by staining with the fluorescent dye

ethidium bromide either by post-electrophoresis staining in a 0.5µg ml^{-1} solution or pre-electrophoresis incorporation of the agent into the buffered agarose at 0.5µg ml^{-1}. Upon illumination with 260 - 300nm light, DNA bands could be seen down to a concentration of about 50ng. Photographs were taken using a Polaroid MP-4 camera with Polaroid type 55 band film, and a Kodak 22A Wrattan filter.

Elution of particular restriction fragments (Yang, Lis and Wu, 1979) was achieved by cutting a 'u' shaped well directly in front of the required band (visualized in a pre-stained agarose gel with incident ultraviolet light). This well was then lined with dialysis tubing and filled up with fresh electrophoresis buffer. The current was then continued until all the relevant stained material in the gel had moved into the well. A short period of reversal of electrical polarity released any closely bound DNA from dialysis membrane and the well contents were collected. The DNA was then phenol extracted twice (using 1M Tris base pH 8.0 buffered phenol), with phenol:chloroform (1:1 twice and ether (water saturated) washed once, before being ethanol precipitated by the addition of 0.1vol 4M sodium acetate and 2vol cold ethanol. After 2 - 3 h at -20°C the DNA precipitate was collected by centrifugation, dried in a vacuum and resuspended in an appropriate volume of TE buffer, usually about 50µl. Fragments of agarose gel were removed, when necessary, from the preparation, before the phenol extractions, by filtering the well contents through siliconised glass wool.

The DNA fragments isolated in the above manner were suitable for both restriction analysis and sub-cloning using T_4 DNA ligase.

POLYACRYLAMIDE GEL ELECTROPHORESIS FOR RESTRICTION MAPPING

Analysis of linear DNA fragments of <400bp is virtually impossible on agarose gels, even of high concentration. In order to accurately size such fragments, gradient, protein denaturing, vertical polyacrylamide gels were used. Gel dimensions were 23cm x 17cm x 0.05cm and the gradient was produced by the controlled mixing of the solutions shown in Table 2.6. When the 5 - 15% resolving gel was set, a 4.5% polyacrylamide stacking gel, 3cm deep, was made above it into which the comb was set.

Before application, the DNA samples (50µl) had 0.1vol loading dye added (2% SDS w/v, 10% Glycine w/v, 0.05% bromophenol blue w/v, pH 8.0) and were heated to 65°C for 15mins. Electrophoresis proceeded at a voltage gradient of 10V cm^{-1} using an electrophoresis buffer consisting of 1.5% w/v glycine 0.6% w/v Tris base and 0.1% w/v SDS at pH 8.4. DNA bands were visualized by staining for 15mins in a 0.5µg ml^{-1} solution of ethidium bromide followed by illumination with 300nm light. Photography was the same as for agarose gels.

SIZING OF DNA FRAGMENTS FROM AGAROSE AND POLYACRYLAMIDE GEL ELECTROPHORESIS

Southern (1979) has described a simple equation representing the relationship between the molecular weight of a DNA molecule or fragment and that fragment's mobility in a

Table 2.6. Relative Composition of Polyacrylamide Mixing Solutions

	Solution A (15%) ml	Solution B (5%) ml	Stacking gel ml
Tris 1.5M, pH 8.8	3.25	3.25	-
Tris 0.5M, pH 6.8	-	-	2.5
Acrylamide solution (29.2% acrylamide, 0.8% bisacrylamide)	6.5	2.2	1.5
1% SDS	1.3	1.3	1
H_2O	0.5	6.2	5
Glycerol	1.3	-	-
Temed	15µl	15µl	25µl
10% Ammonium Sulphate	20µl	20µl	20µl

gel during electrophoresis. This equation is more convenient than graphical methods, accurate over a wide range of sizes and is outlined in Figure 2.1.

ENDONUCLEASE AND METHYLASE ASSAYS

Assay conditions for all commercially supplied restriction endonucleases were as stated in the manufacturers instructions. The standard assay condition used for DraI was the incubation of 1µg DNA sample with 2 units of DraI for 60 mins at 37°C in DraI restriction buffer (10mM Mg Cl_2, 1mM MSH, 25mM NaCl, 20mM Tris base pH 7.5).

The activity of DraI methylase was tested by incubating 5µl of protein sample, 2µg DNA (plasmid pPL3 p86), 1µl of S-adenosyl-L-methionine (0.25mM) and 92ul 0.2M Tris-HCl pH 8.0/8mM EDTA for 1h at 37°C. The mixture was then phenol extracted twice, phenol-chloroform extracted twice, ether washed and finally ethanol precipitated. The DNA was collected by centrifugation and resuspended in 40µl of DraI restriction buffer. One unit of DraI was added, incubated at 37°C and the activity of DraI methylase indicated by the absence of DraI digest products upon analysis using agarose gel electrophoresis.

ENZYME PURIFICATION

(a) RESTRICTION ENZYME SCREENING

A variety of methods were used in attempts to identify the presence of site-specific endonucleases within deinococcal species. Small volumes of culture (about 100ml) were spun down at 7000g for 10mins and the pellet resuspended in 10ml

Fig. 2.1. Calculation of size of DNA molecules and fragments from mobility in agarose gels

Three points were chosen corresponding to size standards L_1, L_2 and L_3 with mobilities m_1, m_2 and m_3 respectively. The value m_0 that determines that these three points are joined by a straight line is given by,

$$m_0 = \frac{m_3 - m_1\left(\left(\frac{L_1 - L_2}{L_2 - L_3}\right)x\left(\frac{m_3 - m_2}{m_2 - m_1}\right)\right)}{1 - \left(\left(\frac{L_1 - L_2}{L_2 - L_3}\right)x\left(\frac{m_3 - m_2}{m_2 - m_1}\right)\right)}$$

The values k_1 and k_2 were then calculated from the following equations,

$$k_1 = \frac{L_1 - L_2}{\left(\frac{1}{m_1 - m_0}\right) - \left(\frac{1}{m_2 - m_0}\right)}$$

$$k_2 = \frac{L_1 - k_1}{m_1 - m_0}$$

The equation given below was then used to calcuiate the size of a molecule or fragment L with a mobility m

$$L = \frac{k_1}{m - m_0 + k_2}$$

10mM Tris-HCl, 0.1mM phenylmethylsulphonylfluoride (PMSF) pH 7.5 before the cells were broken open in a French pressure cell at 3000p.s.i. (most species were refractory to lysis by sonication). The presence of restriction enzymes was investigated in this 'crude' extract after cell debris had been removed by slow speed centrifugation (10000g for 10mins). The crude extract was concentrated and fractionated by using either ammonium sulphate precipitation or polyethyleneglycol 6000:dextran T500 phase partition (Schleif, 1980). In the former case AnalaR (BDH) ammonium sulphate (90% w/v solution) was added slowly to the crude extract to final concentrations of 35, 45, 55, 65 and >75% sequentially. At each concentration stage the mixture was left for 1h at 0°C before the precipitate was collected by centrifugation at 7000g for 15mins. Each pellet was resuspended in 10mM Tris-HCl, 0.1mM PMSF, pH 7.5 buffer and extensively dialysed, with multiple buffer changes for 5h, before being tested for enzyme activity. In the Schleif (1980) method, to each 1ml of crude extract the following solutions were added: 0.5ml H_2O, 0.6g polymer concentrate (64g dextran T500, 256g polyethyleneglycol 6000, 900ml H_2O) and one of the following volumes of 4M NaCl: 0.002, 0.005, 0.01, 0.02, 0.04, 0.08, 0.17, 0.32, 0.64 and 1.2ml. After the additions, the mixtures were vortexed in 3ml Eppendorf tubes and centrifuged for 5mins in an Eppendorf microcentrifuge model 5413. The resulting clear supernatants were then assayed for endonuclease activity.

(b) PREPARATIVE ENZYME PURIFICATION OF DraI and DraII

Three 1l cultures of D. radiophilus were grown in nutrient broth No. 2 (Oxoid) to stationary phase at 30°C-37°C with shaking. About 15g wet weight cells were harvested from this volume by centrifugation at 10000g for 10mins. The cells were resuspended in 30ml 10mM Tris-HCl, 2mM MSH and 0.1mM PMSF, pH 7.5, and broken open in a French pressure cell at 3000-4000psi. The cell debris was removed by a low speed spin at 10000g for 10mins at 0°C, the supernatant then being subjected to a high speed spin at 100000g for 1 to 2h at 0°C in a MSE Prepspin 65 ultracentrifuge. Polyethyleneglycol-dextran phase partition was carried out on the separated supernatant by adding per ml of extract 0.5ml H_2O, 0.5g polymer concentrate and 0.32ml 4M $NaCl_2$. After mixing, the phases were separated by centrifugation at 6000g for 10mins, the upper solution being kept and dialysed overnight against column buffer 1 (CB1 - 10mM Tris-HCl, 2mM MSH, 0.1mM PMSF, 0.075M NaCl, pH 8.0). The dialysed extract was applied to a 20 x 2.6cm DEAE-sephacel (Bio-Rad) column previously equilibrated with CB1. The sample was applied at a rate of 10ml h^{-1}, washed by 2x the column volume of CB1 at a rate of 10cm h^{-1} and then the column was developed at 5ml h^{-1} with a linear 0.075-0.4M NaCl gradient of approximately 2x column volume. Two ml samples of eluate were collected in acid-washed test tubes and DraI/DraII activity tested. Fractions showing greater than unit activity (complete digestion of 1µg DNA in 1h at 37°C) were pooled and dialysed overnight against

column buffer 2 (CB2 - 0.01M phosphate buffer, 2mM MSH, 0.05M NaCl, pH 7.5). After dialysis, the sample was applied at a rate of 50ml h^{-1} to a 10 x 2.6cm column of Bio-Gel HTP grade hydroxylapatite (Bio-Rad) previously equilibrated with CB2. The column was washed with 2x column volume of CB2 at 50ml h^{-1} and then developed with a linear 0.01M - 0.4M phosphate buffer gradient, of approximately 2x column volume, at 40ml h^{-1}. Two ml fractions were collected in acid-washed tubes each containing 100µl, 10mg ml^{-1} of bovine serum albumin (BSA, restriction enzyme grade, BCL) and tested for restriction enzyme activity. All samples showing greater than 'unit' activity were pooled and dialysed against CB3 (20mM Tris-HCl, pH 8.0) overnight. This sample was applied to a 5 x 2.5cm heparin-agarose column (BCL) at a rate of 5ml h^{-1}, washed with 20ml of CB3 at the same rate and the column developed using a 20ml 0 - 0.8M NaCl linear gradient. 0.7ml fractions were collected in tubes containing 10µl, 50mg ml^{-1}, BSA, tested for the activity of restriction endonucleases, and those fractions showing DraI activity (at least 1 unit μl^{-1}) were pooled and dialysed against storage buffer (50% v/v glycerol, 2mM MSH, 50mM NaCl, pH 8.0) overnight. The enzyme preparation was stored in this form, at -20°C.

ULTRA-VIOLET IRRADIATION

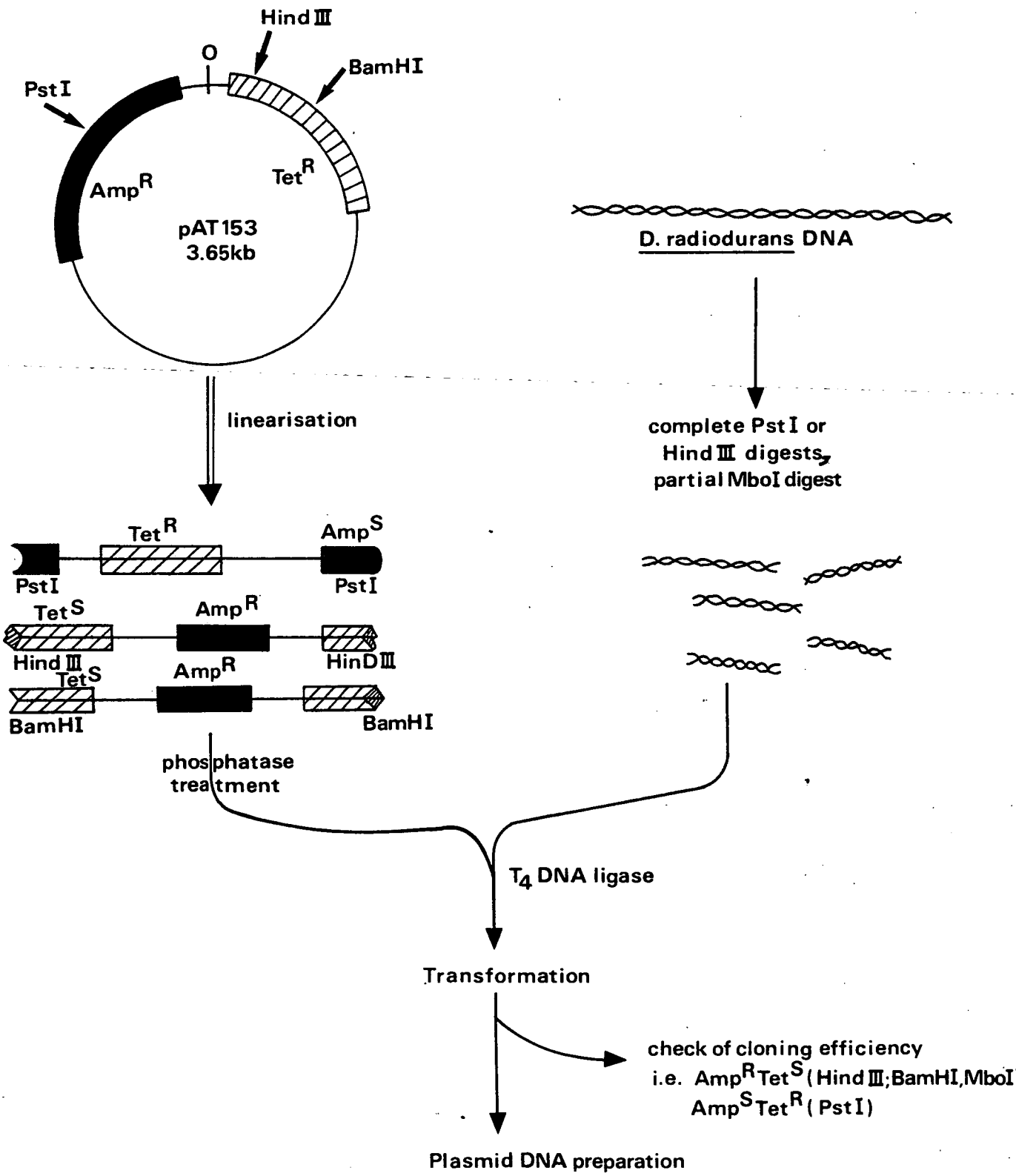
DNA with desired levels of ultra-violet light damage was produced by resuspending the nucleic acid in an ultra-violet transparent buffer (10mM Tris-HCl, pH 7.4) and

irradiating 10ul droplets for specified times at a dose rate of 1.05 J m^{-2} s^{-1}.

CONSTRUCTION OF GENE LIBRARIES OF D. radiophilus

Banks of D. radiophilus genomic DNA were constructed in the E. coli vector plasmid pAT153 using three different restriction endonuclease systems, i.e. cloning at the PstI site (amp^R inactivation), at the HindIII site (tet^R inactivation) or using a MboI partial genomic digest inserted at the BamHI site of pAT153 (tet^R inactivation) (Fig. 2.2). The genomic fragments for cloning were generated by digestion of 2µg D. radiophilus chromosomal DNA and 0.2µg D. radiophilus plasmid DNA (a gift from G. Al-Bakri) with 5 units of PstI or HindIII for 2h. In the case of MboI partial digestion, the same DNA concentrations were divided into three and separately digested with 0.04, 0.08 and 0.15 units of MboI for 2h and the digestion products pooled (M. Mackay, 1983). All restriction reactions were terminated by heating to 70°C for 10mins. In parallel, the vector plasmid DNA was prepared by digesting 2µg pAT153 for 1 hour with 5 units of the relevant restriction endonuclease, PstI, HindIII or BamHI. The linearised plasmid was then ethanol precipitated and resuspended in 40ul H_2O and 2µl of 20x alkaline phosphatase buffer added (1M Tris pH 9.5, 20mM EDTA; spermidine to 1mM). The plasmid DNA had the 5' terminal phosphate groups removed by the addition of 1 unit of calf intestinal alkaline phosphatase (BCL) and incubation for 30 mins at 37°C. This treatment prevented the recircularisation of plasmid

FIG. 2.2. CONSTRUCTION OF D.radiophilus GENE BANK USING INSERTIONAL INACTIVATION OF pAT153



vector molecules unless D. radiophilus DNA was inserted. The reaction was terminated by heating to 65°C for 20mins. All DNA molecules were deproteinised by two phenol and two phenol:chloroform extractions followed by washing with water saturated ether and ethanol precipitation at -20°C. De-phosphorylated vector molecules were then mixed with the appropriate DNA fragments from D. radiophilus (i.e. common PstI ends, HindIII ends or BamHI/MboI ends) in the ratio of 4:1 so that the final DNA concentration in 20µl of ligation buffer (10mM Tris-HCl, pH 7.8, 10mM $MgCl_2$, 20mM DTT, 0.6mM ATP) was no greater than 50µg ml^{-1}. Ligation was carried out by adding 0.1 units of T_4 DNA ligase (BCL) to the 20µl of DNA in ligase buffer and allowing incubation for 16h at 14°C. The DNA was then ethanol precipitated, resuspended in 100µl 0.1M Tris, pH 7.2 and used to transform E. coli HB101. After transformation, a sample of cells was taken to test the level of insertional inactivation and cloning efficiency whilst the rest of the culture was used to prepare plasmid DNA by the Birnboim-Doly (1979) method using the still-active antibiotic resistance marker for pre-selection. The gene banks were stored as DNA solutions at -4°C rather than individual colonies.

DETERMINATION OF DNA SEQUENCE REPRESENTATION IN A GENOMIC LIBRARY

The exact probability of having any DNA sequence represented in the genomic library was calculated from the formula:

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

where P was the desired probability, f the fractional proportion of the genome in a single recombinant and N was the necessary number of recombinants (Clarke and Carbon, 1976). For easy use the equation can be rearranged as:-

$$P = 1 - (1 - f)^N \quad (2)$$

In addition an assumption could be made that the length (x) of a required DNA segment was small in comparison with the length (L) of the inserts thus reducing the effects of random breaks occurring within length (x). The equation can thus be modified to read

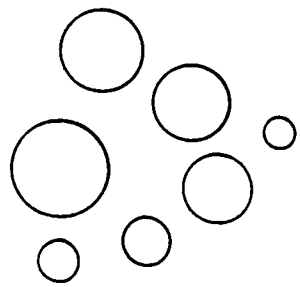
$$f^* = (1 - \frac{x}{L})f \quad (3)$$

with the f^* value including the effect of random breaks, these being particularly important if the size of the average cloned fragment is small.

SCREENING OF THE GENOMIC LIBRARIES

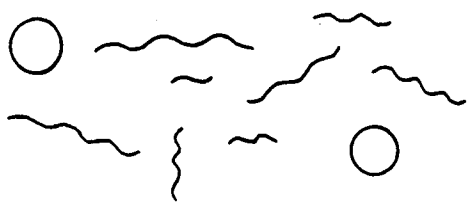
As D.radiophilus is refractory to DNA uptake and therefore cannot be transformed even by homologous DNA, all screening for genomic markers was carried out in E.coli mutants. This procedure of course, has its own problems which are covered in the Discussion. A wide variety of

FIG. 2.3 SCREENING OF RECOMBINANT GENE BANKS FOR PRESENCE OF MODIFICATION METHYLASE ACTIVITY



Recombinant gene bank in plasmid pAT153

Digestion with DraI



Linearization of all plasmids not protected by DraI methylase modification (plasmid borne)

Transformation of E.coli HB101 r^-m^- (only ccc form transforms effectively)

Selection for Amp^R (Hind III or Bam HI/Mbo I) or Tet^R (Pst I)

Small scale rapid screening for presence of recombinant plasmids carrying DraI methylase gene

auxotrophic, nutritional and other selectable phenotypic traits were screened by attempted transformation of appropriate E.coli mutants with 2 μg of each of the three gene bank DNAs. Auxotroph to prototroph conversion required plating onto defined medium lacking the appropriate growth factor. Sugar fermentation complementation i.e. xylose$^-$ to xylose$^+$ depended upon growth with that compound as the sole carbon source. Other lesions could be checked by complementation restoring at least partial resistance to normally bacteriocidal doses of certain agents e.g. ultra-violet light.

Of principal importance were the attempts to clone the modification/restriction genes of D.radiophilus. Screening for the expression of these genes in E.coli HB101 was attempted in two ways. Firstly E.coli HB101 was transformed with 2 μl of each gene bank and allowed 2h for phenotypic expression. To one sample, T_4 bacteriophage was added in L-broth to a multiplicity of infection of 10 (i.e. 10 phage per bacterium). Infection was allowed 60 minsto complete and then the cells were spun down in Eppendorf microcentrifuge tubes and washed three times with a 25 mg ml^{-1} pronase solution. The cell pellet was finally resuspended in a buffered 1% sucrose solution (10mM Tris-HCl, 1mM EDTA, pH 7.4) and plated out onto either ampicillin (40 μg ml^{-1}) or tetracycline (20 μg ml^{-1}) selective medium. Thus, selection was for T_4^R amp^R or T_4^R tet^R cells. Secondly, the procedure for methylase expression described by Walder et al. (1983) was followed

(Fig. 2.3). Fifty μg DNA from each gene bank was digested for 3 h with 100 units of DraI, the DNA being collected by phenol extraction followed by ethanol precipitation. The DNA was resuspended in 100 ul 0.1M Tris pH 7.2 and used for the transformation of E.coli HB101. After phenotypic lag, the transformed cells were plated onto either ampicillin (40 μg ml^{-1}) or tetracycline (20 μg ml^{-1}) L-agar depending on which gene bank was originally used.

In both cases all colonies growing on antibiotic selective plates were screened for the presence of recombinant plasmids (see earlier).

RECOMBINANT MOLECULE LIGATION

The religation of matching, 'sticky' ended DNA fragments has already been described in the section on the construction of the gene libraries. 'Blunt-ended' ligation required modification of the reaction conditions as this reaction exhibits bimolecular reaction kinetics at low substrate concentration and is not linear with respect to the DNA ligase concentration (Modrich and Lehman, 1970). Thus for ligation involving blunt ends higher concentrations of DNA were used, >200 μg ml^{-1}, with 1 unit of DNA ligase per 20 μl of ligase buffer (0.6mM ATP, 10mM DTT, 20mM Tris, 10mM $MgCl_2$, pH 7.8).

M13mp8 and mp9 BACTERIOPHAGE ASSAY

A single colony of indicator strain, E.coli NM522, was taken from M9 minimal medium agar + thiamine and inoculated into 2 ml of 2 x TY broth and grown at 37°C for 7 h with vigorous shaking. 0.2 ml of these host cells were added

to 0.01 ml of bacteriophage dilution, 0.01 ml of 100mM IPTG, 0.05 ml of 2% Xgal and 3 ml of B sloppy agar and thoroughly mixed by vortexing for 10 s before the agar was poured onto dried B broth agar plates. The sloppy agar was allowed to set and the plates incubated overnight at 37°C. Blue plaques represent non-recombinant M13mp phage whilst clear plaquesindicate the removal of α-complementation by insertion of DNA into the M13mp phage constructed cloning sites.

SIZE ASSAY

As stated previously all sizing of M13mpRF molecules was done using the Holmes and Quigley boiling technique (1981).

C-TESTING

Insert orientation and complementarity was checked using the renaturation technique employed by Messing (1983). Indicator cells were produced as above and when the optical density reached around 10, 2 ml samples were removed and fresh plaques resuspended in them. The cultures were vigorously rotated at 37°C for 6-7 h before being transferred to 1.5 ml Eppendorf tubes. The tubes were centrifuged for 5 mins at room temperature in a microfuge, the supernatant being carefully removed into a different tube. Twenty µl of each supernatant was mixed with 20 µl of a control supernatant of a M13mp clone definitely carrying inserted DNA of interest in the opposite orientation and 1 µl of 2% SDS with 3 µl loading buffer added. The solutions were well mixed and overlayed with light mineral

oil before being heated at 65°C for 1 h. The sample was then electrophoresed through a 0.8% agarose gel. DNA isolates with opposite orientations of the same insert reannealed together, altering the position of the bands within the agarose gel.

GROWTH AND ISOLATION OF M13mp9 AND M13mp8 RF DNA

A blue plaque was picked from a fresh overlay plate, inoculated into 1 ml of 2 x TY broth and the culture grown overnight with vigorous aeration at 37°C. One litre of 2 x TY broth was inoculated with this starter culture and 10 ml of a fresh NM522 culture. The flask was shaken for a further 4 h before the RF DNA of the M13 'phage was isolated by the Birnboim and Doly method (1979) as described earlier. The purified M13mp9 and 8 DNA was taken from the $CsCl_2$ gradient and tested for its suitability to act as a substrate for restriction and ligation. One ug of DNA was restricted with 4 units SmaI for 1 h at 30°C in the relevant enzyme buffer. This sample was divided into two and both had the DNA recollected by ethanol precipitation. Both samples were resuspended in ligation buffer but only one had the T_4 ligase enzyme added. The DNAs were then used to transform competent E.coli NM522 and suitable dilutions of the transformed culture made to enable single plaque identification. Comparison of plaque numbers and morphology between a control of 0.5 µg uncut DNA, 0.5 µg SmaI cut DNA and 0.5 µg SmaI cut then religated DNA gave a strong indication of the susceptibility of the DNA to restriction enzyme digestion and to the level of chromosomal

DNA contamination of the RF DNA preparation.

PREPARATION OF TEMPLATE DNA

E.coli NM522 was inoculated into fresh 2 x TY broth, from an overnight culture (40 x dilution) and grown to an optical density of 30 by shaking at 37°C. 1 ml samples of this culture were placed in bijoux bottles and freshly plated plaques were inoculated into each sample. The bottles were shaken vigorously for 4½ h at 37°C and the cultures poured into 1.5 ml Eppendorf tubes and centrifuged in a microfuge for 5 mins at room temperature. The supernatants were transferred carefully to other Eppendorf tubes (approx. 0.8 ml) and 200 µl of a 2.5M NaCl, 20% w/v polyethylene glycol solution added. The tubes were incubated for 30 mins at room temperature before being spun for another 5 mins period at room temperature. The supernatants were discarded and all traces of liquid around the pellet removed using a drawn out capillary. The pellets were resuspended in TE buffer and 50 µl phenol added to each. The tubes were vortexed for 10 s, left standing for 10 mins, vortexed again and finally the phases separated by centrifugation for one min. The upper aqueous layer was removed and to it was added 10 µl 4M sodium acetate and 250 µl ethanol. After incubation at -70°C for 5 mins the tubes were spun for 10 mins at 4°C and the pellets washed with 1 ml of ethanol. The procedure of resuspension followed, ethanol precipitation and washing was repeated, then the pellets vacuum dried and resuspended in 50 ul TE buffer. The M13mp templates were ready for DNA sequencing and were stored at -20°C. Each template was checked by agarose gel electrophoresis (5 µl of 50 µl) before sequencing was begun.

GENE PRODUCT ANALYSIS - THE MAXI-CELL TECHNIQUE

The E.coli strain CSR603 was transformed with the recombinant plasmid as previously described in this chapter. Individual clones were tested for the appropriate antibiotic resistance marker expression and for the presence of plasmid DNA of the correct size using the Holmes and Quigley boiling method (1981). Three ml of K medium was inoculated with an E.coli CSR603 isolate containing the recombinant plasmid and the sample was incubated, with shaking, at 37°C overnight. 0.3 ml of the culture was then diluted into fresh K medium (10 ml) and allowed to reach an optical density of around 30. From this exponential phase culture 2.5 ml was removed and placed in an open petri dish where a $\overset{uv}{\lambda}$ dose of 6 J m^{-2} was administered (with continuous stirring). After irradiation, 2 ml of the sample was transferred to a sterile bottle and incubated for 1 h at 37°C. This was followed by the addition of 15 ul of 15 mg ml^{-1} cycloserine and incubation for 16-20 hours at 37°C in a shaking water-bath. At the end of the incubation period, a 1.5 ml sample was removed and the cells harvested by centrifugation in a microfuge for 40-50 s at room temperature. The cell pellet was washed twice with 2 x 1 ml of Hershey salts before it was resuspended in 0.8 ml of Hershey medium. After incubation for 1 h at 37°C, 0.2 ml of Hershey medium containing 5 μCi of [^{35}S]-methionine was added with further incubation for 2 h at 37°C. The labelled cells were pelleted and washed

twice with 2 x 1 ml 100mM NaCl. The cell pellet was resuspended in 50 µl of sample buffer, vortexed thoroughly and heated to 100°C for 3-5 mins. The sample was loaded onto a 7-15% gradient SDS-polyacrylamide gel, electrophoresed at 10V cm^{-1} for 5 h and then the gel fixed by immersion in 40% methanol/10% acetic acid (v/v) for 15 mins. The gel was then given 2 washes with 10% ethanol/5% acetic acid (v/v) before soaking overnight in 30% methanol/3% glycerol (v/v). This prevented cracking of the gel during the subsequent vacuum drying. Radioactive labelling was detected by autoradiography, non-labelled size-control proteins were run on the gel concurrently and visualized by kenacid blue staining. These size controls consisted of thyroglobulin (330000 daltons), bovine serum albumin (68000 daltons), egg albumin (43000 daltons) and trypsin inhibitor (20000 daltons).

DNA SEQUENCING (Sanger et al., 1977)

The single stranded templates were purified in the manner previously described. Annealing of the template to the synthetic primer (15-mer, New England Biolabs) was accomplished by the incubation of 5 ul template (1 µg DNA), 1 µl TM buffer, 5 µl primer (0.2 p mol ul^{-1}) and 3 µl H_2O at 60°C for 1 h. The Eppendorf tube was centrifuged briefly to collect any condensation. All subsequent sequencing reactions were carried out in 1.5 ml uncapped Sarstedt tubes capable of centrifugation in an Eppendorf Model 5413 centrifuge. Into 4 tubes (per clone) 2 µl of one of the respective 'NTP' mixes was added (G^o, A^o, T^o and C^o) using a Hamilton repetitive dispenser. This was followed

by the addition of 2 µl of the template/primer annealed mix. Finally, to each tube 2 µl of a 'Klenow' mix was added. This solution consisted of 3.2 µl 100mM Tris pH 7.5, 3.2 µl 100 mM DTT, 25 µl H_2O with 5 units of Klenow enzyme and 16 µCi of [^{35}S]ATP (note: these volumes are sufficient for 4 sets of clones). These solutions were mixed during a short centrifugation and the tubes left at room temperature for 25 mins. Then 2 µl of the chase solution was mixed into each reaction tube and they were left for a further 20 mins at room temperature. Prior to loading, 2 µl of formamide dye mix was added and the tubes placed in a boiling water bath for 3 mins. 15s. Approximately half of each sample was loaded onto the sequencing gel, a wattage of around 40W (1500 volts) was applied and the gel run for 2-2½ h. The denatured sequenced strands separate by virtue of differing chain length by polyacrylamide gel electrophoresis. The gel itself was 0.3 mm thick, 20 cm wide and 40 cm long, and was produced by mixing 35 ml of 0.5 TBE gel mix with 70 µl of ammonium persulphate (0.25% w/v) and 70 µl Temed. For greater resolution a buffer-gradient gel was used, requiring a second solution of 7 ml 2.5 TBE gel mix, 14 µl ammonium persulphate and 14 µl TEMED to be made. In this case 4 ml of the first solution was taken up in a 10 ml pipette along with 6 ml of the latter. This mixture was dispensed into the taped-up gel plates (notched plate being siliconised) followed quickly by the rest of the 0.5 TBE gel mix solution. The comb was then inserted and the gel allowed to set.

After electrophoresis was completed the gel was fixed in 10% methanol, 10% acetic acid and 80% H_2O for 15 mins. The gel itself was then transferred to blotting paper, covered in cellophane wrapping (clingfilm) and dried on a Biorad Model SE1125B gel drier. The gel was then placed under X-ray film, the developing of the autoradiogram depending upon the strength of banding required.

CHAPTER 3. RESULTS

1. FOREIGN GENE EXPRESSION IN D. radiodurans

1.1 Construction and preparation of a range of S.faecalis plasmids

S.faecalis DS-16 normally contains two plasmids, pAD1 and pAD2, and two transposons, Tn916 and Tn917 (Clewell, 1981). Table 2.3 shows the selectable markers carried by the plasmids and transposons, Tn916 being inserted in the chromosome and Tn917 in pAD2. From the original DS-16 strain it was possible to isolate novel cointegrate plasmid molecules produced during the process of conjugation. During broth mating experiments between DS-16 and the rifampicin-resistant recipient strain JH2-16, the conjugative plasmid pAD1 not only transferred a copy of itself but also in rare cases stimulated the transfer of modified plasmid molecules. By testing for transfer of either of the transposable elements, tetracycline or erythromycin resistance, as well as a zone of haemolysis around the colonies grown on Todd-Hewitt agar + 4% horse blood, a variety of modified S.faecalis plasmid molecules were isolated (Fig. 3.1 and Table 3.1). Alteration of the degree of haemolysin production is indicative of the insertion of DNA either into the control region of the gene (super-haemolytic or non-haemolytic) or structural region of the gene (non-haemolytic). The differences in the zone of haemolysin production is illustrated in plate 1. The three presumptive plasmid types GS-6, GS-28 and GS-32 were screened for the presence of plasmid DNA using the modified Kado and Liu technique (1981). All three isolates showed the presence

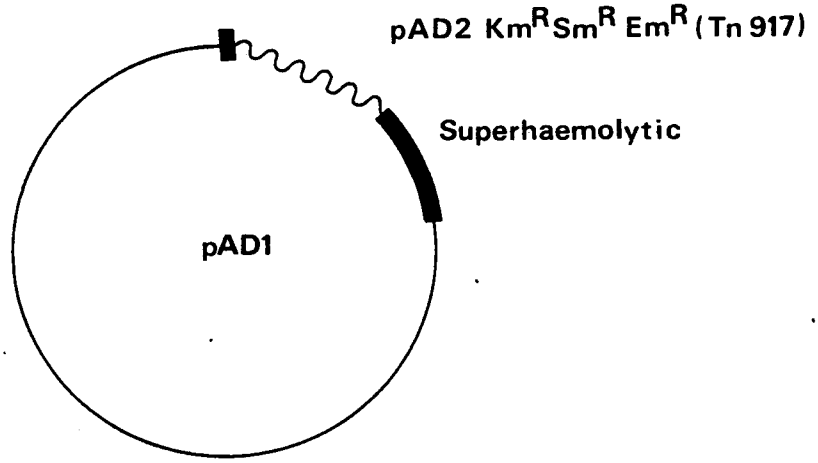
TABLE 3.1 S.faecalis plasmids constructed for transformation/conjugation into Deinococcus

Plasmid/ Strain	Original host	Phenotype[a] (plasmid) Em	Tc	Kn	Sm	rif/ fus	Haemo-lytic	Plasmid type per cell		Conjugate transfer
GS-6	DS-16	R	S	R	R	S	S.Hyl	plasmid	20kb	All markers
GS-28	DS-16	R	S	S/R	S	S	S.Hyl	plasmid	20kb	All markers
GS-32	DS-16	R	R	R	R	S	Non-Hyl	plasmid	20kb	All markers
AMβ	DS-5	R	S	S	S	S	Non-Hyl	plasmid	10-20kb	Em^R

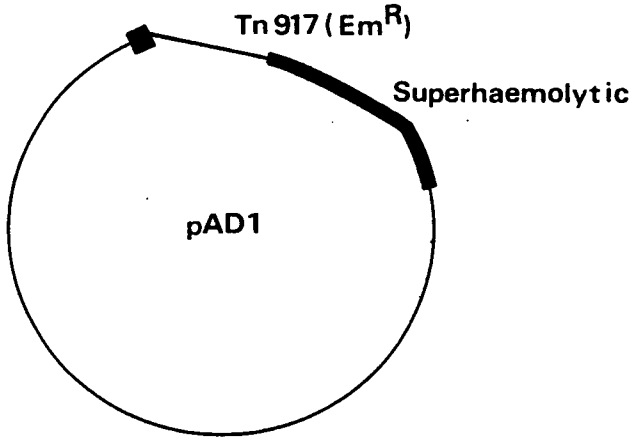
[a]Abbreviations:
Em - Erythromycin 50μg ml^{-1}
Tc - Tetracycline 10μg ml^{-1}
Kn - Kanamycin 20μg ml^{-1}
Sm - Streptomycin 1000μg ml^{-1}
rif - rifampicin 50μg ml^{-1}
fus - fusidic acid 40μg ml^{-1}
S.Hyl - super-haemolytic
Non-Hyl - non-haemolytic
R - cells grow in indicated level of antibiotic
S - cells do not grow in indicated level of antibiotic.

FIG. 3.1. PLASMID CONTENT OF MODIFIED S. faecalis STRAINS

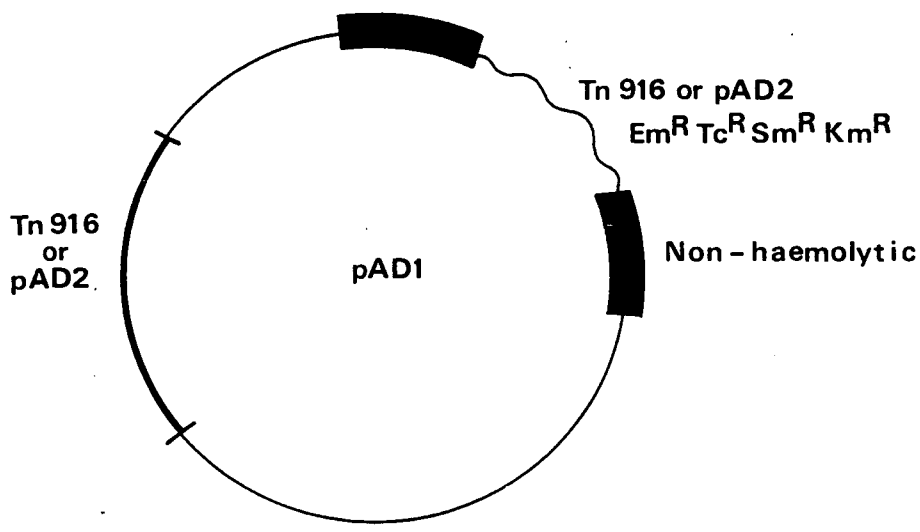
a) GS-6 ; pAD1/pAD2 cointigrate molecule



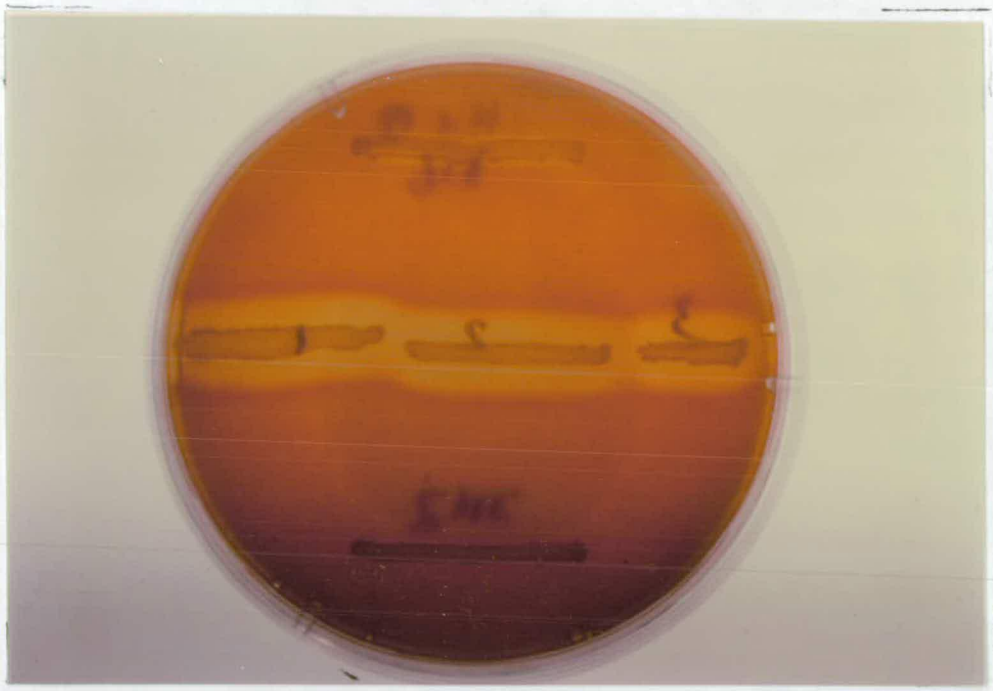
b) GS-28 ; Tn 917 insertion into pAD1



c) GS-32 ; Tn916/pAD2/pAD1 cointegrate molecule



Zones of haemolysis around streaks of Streptococcus faecalis grown on Todd-Hewitt agar + 4% horse blood



1 - S.faecalis + haemolysin plasmid pAD1

2 - S.faecalis + derepressed haemolysin gene due to tn917 transposition

3 - S.faecalis with no haemolytic plasmid present

of plasmid DNA of approximately the correct size to reinforce the proposed structure, although accurate molecule sizing was very difficult. If further broth mating experiments were done using the S.faecalis strain JH2-1 (fusidic acid resistant) as the recipient and JH2-16/pAD1 cointegrate plasmids, as the donor then all the plasmid markers were transferred as a linked group at high frequency (10^{-3}-10^{-4} per donor). All available evidence would therefore suggest the novel plasmid structures proposed in Figure 3.1 are correct.

In addition to the construction of novel plasmid/transposon conjugative vectors from strain DS-16, the multiple conjugative resistance factor pAMβ1 was investigated. The S.faecalis strain DS-5, which carries the pAMβ1 plasmid, harbours two other plasmids pAMα1 and pAMγ1 (Clewell, 1981). The latter plasmid is capable of inducing conjugation but its presence is inhibitory to conjugation stimulated by pAMβ. To remove the inhibitory effect of the pAMγ1 molecule DS-5 was grown in Todd-Hewitt broth containing 0.5 ug ethidium bromide at elevated temperature (42^{o}C). Such treatment produced erythromycin resistant/non-haemolytic colonies at a frequency of 3.2×10^{-3}. The screening of 20 of these colonies showed that 75% appeared to have lost the pAMγ1 molecule. When one of the 'cured' strains was tested for the ability to conjugate the pAMβ1 molecule into strain JH2-16 it showed a 300 fold increase in erythromycin resistance transfer over the original DS-5 strain.

One of the recipient JH2-16 colonies ($Em^{R}Rif^{R}$) was used as the basis for further experiments as now both

original DS-5 plasmids, pAMγ1 and pAMα1, were absent leaving pAMβ1 alone. The plasmid pAMβ is a multiple resistance factor (MLS-macrolide, lincomycin and spectinomycin) capable of conjugal transfer not only into the original host species but also into unrelated genera, e.g. Lactobacillus casei (Clewell, 1981).

1.2 Transformation of D.radiodurans strains with non-endogenous plasmid DNAs

A variety of plasmid DNAs from E.coli, B.subtilis and S.faecalis were purified using techniques described in Chapter 2. In the case of plasmids isolated from E.coli and B.subtilis the biological activity of the preparations was tested by transformation and subsequent plasmid marker selection within the appropriate plasmid-free recipient (E.coli HB101 or B.subtilis 168). The results of these experiments are in Table 3.2. All the plasmid isolates, except for the very large R68.45 plasmid, showed significant transforming activity. Unfortunately, no transformation technique has been developed for S.faecalis and therefore, plasmids derived from this organism were tested purely by electrophoresis in a 0.6% agarose gel.

A variety of D.radiodurans R1 mutants (302, Rec30 and Nuc^-) as well as the wild type D.radiodurans R1 and Sark strains were then transformed with approximately 5 ug of each of the 11 different plasmid DNAs shown in Table 3.3. After the normal D.radiodurans transformation procedure had been completed (Chapter 2) the cells were left growing in TGY broth for 10 h before plating out serial dilutions onto antibiotic media. Although a wide variety of

TABLE 3.2 Biological activity of plasmids assayed by transformation

Plasmid	Host	Antibiotic[a] selection concentration ug ml^{-1}	Frequency of Host Transformation ug^{-1} DNA
pML2	E.coli	Kn25	7.0×10^{-3}
pBR322	E.coli	Tc20 or Ap50	7.0×10^{-3}
pAT153	E.coli	Tc20 or Ap50	2.4×10^{-3}
pLV21	E.coli	Kn25	8.0×10^{-4}
R68.45	E.coli	Ap40 or Tc20	2.0×10^{-7}
pUB110	B.subtilis	Kn25	1.2×10^{-4}
pC194	B.subtilis	Cm15	3.2×10^{-5}
pHV33	E.coli	Cm15 or Tc20	3.1×10^{-4}

[a] Abbreviations: Kn - Kanamycin

Cm - Chloramphenicol

Tc - Tetracycline

Ap - Ampicillin

BLE 3.3 Attempted transformation of D.radiodurans strains using foreign plasmid DNA.

rain/ asmid	D.radiodurans RI	D.radiodurans SARK	D.radiodurans 302	Nuc⁻	Rec30
L2 n 12.5 ug ml^{-1}	-	-	-	-	-
R322 p 1 ug ml^{-1} c 1 ug ml^{-1}	-	-	-	-	-
T153 p 1 ug ml^{-1} c 1 ug ml^{-1}	-	-	-	-	-
V21 n 12.5 ug ml^{-1}	-	-	-	-	-
845 c 1 ug ml^{-1}	-	-	-	-	-
V33 p 1 ug ml^{-1} c 1 ug ml^{-1} m 10 ug ml^{-1}	-	-	-	-	-
B110 n 12.5 ug ml^{-1}	-	-	-	-	-
194 m 10 ug ml^{-1}	-	-	-	-	-
-6 m 20 ug ml^{-1} m 10 ug ml^{-1} n 125 ug ml^{-1}	-	-	-	-	-
-28 m 20 ug ml^{-1}	-	-	-	-	-
-32 m 20 ug ml^{-1} c 1 ug ml^{-1} m 10 ug ml^{-1} m 12.5 ug ml^{-1}	-	-	-	-	-

different antibiotic markers from different organisms were used as well as S.faecalis transposable elements there was no evidence for stable expression of any of these 'foreign' genes. At no time were the spontaneous mutation frequencies for the various antibiotic resistance markers (Table 3.4) surpassed by the relevant transformation frequencies (Table 3.3).

1.3 Conjugal transfer of foreign plasmids into D.radiodurans R1.

A number of common or constructed resistance transfer factors were tested for biological activity in their normal donor-host systems. Conjugations between E.coli strains and intra-species transfer in S.faecalis strains were achieved as described in Chapter 2 and the results are shown in Table 3.5. All the conjugative plasmids were capable of transferring their selectable markers at high frequency. However, when D.radiodurans R1 (tetracycline or rifampicin resistant) was substituted as the recipient cell there was no evidence for the subsequent conjugal transfer of any plasmid as indicated by the expression of the antibiotic resistance markers. Attempted conjugation using E.coli HB101 carrying R68.45 or RP4 was by normal filter mating techniques. When the constructed S.faecalis resistance factors GS-6, GS-28 and GS-32, were used both broth and filter matings were attempted, the former with and without the presence of the recipient S.faecalis strain JH2-1. The D.radiodurans R1 recipient cells did appear to be inhibited slightly by the bacteriocin produced from pAD1 expression in strains GS-6 and GS-28 but not GS-32 probably indicating a linkage between haemolysin

TABLE 3.4 Spontaneous mutation frequencies

Strain	Marker & concentration ($\mu g\ ml^{-1}$)	Spontaneous mutation frequency (sensitive to resistant)
E.coli HB101	Ampicillin 50	$< 1.0 \times 10^{-9}$
	Tetracycline 20	4.5×10^{-8}
	Kanamycin 12.5	6.9×10^{-7}
	Chloramphenicol 10	$< 1.0 \times 10^{-9}$
	Rifampicin 50	4.5×10^{-8}
S.faecalis JH2-16	Tetracycline 10	$< 1.0 \times 10^{-9}$
	Erythromycin 50	1.6×10^{-8}
	Streptomycin 500	4.5×10^{-7}
	Kanamycin 20	7.2×10^{-8}
	Rifampicin 25	8.3×10^{-7}
D.radiodurans R1	Ampicillin 1	$< 6.2 \times 10^{-8}$
	Tetracycline 1	$< 5.8 \times 10^{-8}$
	Kanamycin 12.5	2.0×10^{-6}
	Chloramphenicol 10	5.8×10^{-8}
	Erythromycin 20	8.4×10^{-8}
	Streptomycin 50	7.4×10^{-8}

TABLE 3.5 Biological activity of plasmids assayed by conjugation

Plasmid	Host	Antibiotic[a] Selection Concentration ug ml^{-1}	Conjugation[b] frequency
R68.45	E.coli	Ap40; Sm100	2.8×10^{-3}
RP4	E.coli	Ap40; Sm100	1.9×10^{-4}
GS-6	S.faecalis	Em50; Rif50	1.9×10^{-3}
GS-28	S.faecalis	Em50; Rif50	1.7×10^{-3}
GS-32	S.faecalis	Em50; Rif50	9.7×10^{-4}
AMβ	S.faecalis	Em50; Rif50	1.61×10^{-4}

[a] Ap - Ampicillin
Sm - Streptomycin
Rif - Rifampicin
Em - Erythromycin

[b] Filter mating method

and bacteriocin expression. A modified technique for filter mating with S.faecalis strains containing the broad host range plasmid pAMβ, developed by Smith and Guild (1980), was used in an attempt to enhance any possible transfer of the pAMβ plasmid into D.radiodurans but this had no obvious affect.

2. NUCLEASE ACTIVITY IN THE DEINOCOCCACEAE

2.1 Extracellular nucleases

The production of active extracellular endo/exonucleases could be a major influence on the ability of ccc (covalently closed circular) DNA to transform competent cells. In order to ascertain whether species of the genus Deinococcus produced such enzymes, nutrient agar plates (TGY for D.radiodurans R1 wt, Nuc$^-$, Sark, D.proteolyticus, D.radiopugnans and E.coli HB101 but NB2 agar for D.radiophilus) were covered with a thin layer of electrophoretic grade agarose (1% w/v) containing either cccDNA (pML2) or salmon sperm DNA. A light streak of each bacterial species was placed upon the set agarose and the plates incubated at 35^{o}C until the streak had fully grown. The organisms were carefully wiped off the agarose which was then separated from the supporting agar and stained for 10 mins in a 0.5 μg ml^{-1} solution of ethidium bromide. Zones of DNA degradation were viewed by illuminating the thin agarose sheet with 300 nm wavelength light. In comparison with the control organism E.coli HB101, it was obvious that D.radiodurans R1 and Sark produced large amounts of an extracellular nuclease capable of diffusing through the agarose layer

and digesting completely both ccc and linear DNA. The mutated D.radiodurans strain designated Nuc$^-$ completely lacked this nuclease activity. D.radiophilus and D.radiopugnans produced only a slightly more detectable zone of clearing than the non-nuclease producing E.coli strains, whilst D.proteolyticus seemed to be intermediate between the two extremes. Alteration of the agarose concentration in the overlay (0.6% w/v) appeared to have no discernable effect upon the relative zones of DNA degradation.

DNA being incubated with D.radiodurans R1 cells under transforming conditions was reisolated by removing the cells, and ethanol precipitation. The DNA, whether originally homologous linear, heterologous linear or heterologous plasmid, did not seem to show any degree of degradation in comparison with control DNA upon agarose gel electrophoresis (1% w/v gel).

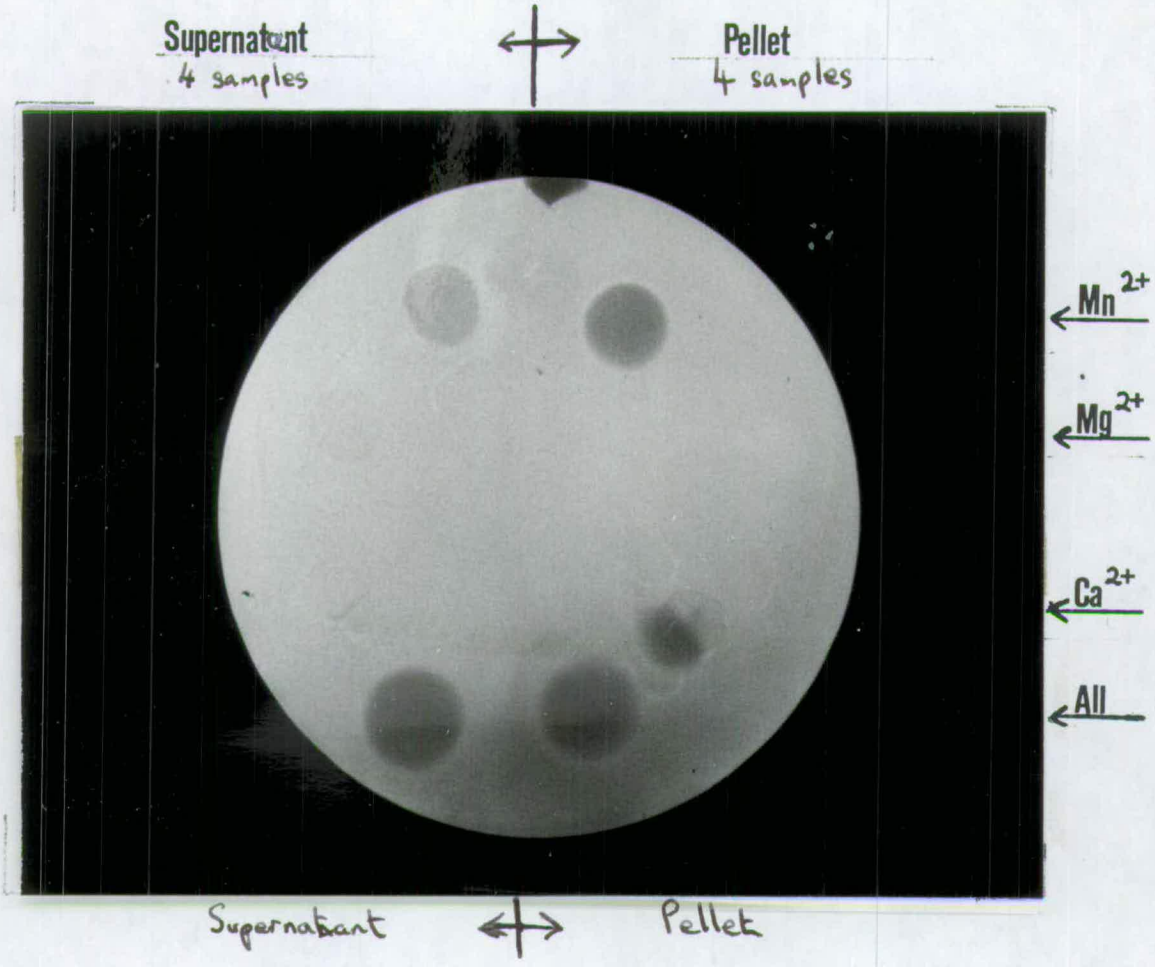
2.2 Non-specific nuclease activity associated with the cell

A range of non-specific DNA degrading enzymes probably exist, to varying degrees, in most bacterial cells. To examine the extent of such enzyme activity in deinococcal cells, 100 ml of each strain and E.coli HB101 was grown to late exponential phase in the appropriate nutrient broth. The cells were harvested at 10000 g and washed once in TE buffer. The pellet was resuspended in 10 ml 50mM Tris HCl, 2mM EDTA, 0.2mM PMSF, pH 8.0 buffer and the cells broken open in the French pressure cell (3000 p.s.i.). The lysed cells were centrifuged at 24000 g for 15 mins at 0^{o}C and the supernatant separated from the pellet. The latter was washed three times

in resuspension buffer to remove any traces of supernatant and 20 µl of the resuspended mixture mixed with 20 µl of 20 mM Mg^{2+}, 20mM Mn^{2+}, 20mM Ca^{2+} or distilled water. The same procedure of recentrifugation (three times) in a 1.5 ml Eppendorf tube followed by mixing with various ions was used with the cell-extract supernatant. The 20 µl samples were placed as discrete drops onto a thin layer of 1% w/v agarose containing about 1 mg ml^{-1} of purified plasmid pML2 DNA. The drops were allowed to dry by evaporation before the agarose sheets were incubated at 30°C overnight. The sheet was then stained with a 0.5 µg ml^{-1} solution of ethidium bromide and zones of DNA degradation viewed by ultra-violet light illumination. In parallel, 10 µl of each of the extracts + ion mixtures were incubated with 1 µg of plasmid pML2 DNA or 1 µg λDNA for an hour at 30°C and then STOP buffer was added (0.1 vol.) and the effect upon the DNA observed by agarose gel electrophoresis and ethidium bromide staining.

In all cases a certain degree of DNA digestion was seen both in the stained agarose layers and after gel electrophoresis. However, D.radiodurans R1 and D.radiodurans Sark both displayed by far the greatest amount of degradation. This intracellular activity appeared to be largely located in the cell supernate rather than membrane preparation and was stimulated by the presence of Mn^{2+} ions. Also, there was a Ca^{2+} stimulated nuclease activity present in the membrane preparations of these two strains only (Fig.3.2). All other genus members displayed levels of intracellular non-specific degradation of DNA intermediate between the

Fig.3.2 Test for the ion requirements of non-specific nucleases present in the D.radiodurans R1 cell.



10ul of each extract preparation + the different ions shown above, spotted onto an 0.8% agarose sheet containing DNA and incubated for 24h at 30C. Dark areas represent zones of DNA degradation.

aforementioned strains and the E.coli control organism. The other major point of note was the presence in D.radiophilus of an enzymatic activity, stimulated by Mg^{2+} only, which altered banding patterns of both the λ and pML2 plasmid DNAs. This observation stimulated the screening of all members of the group for the presence of such enzymes tentatively identified as a restriction endonuclease.

2.3 Sequence-specific endonuclease activity in the Deinococcaccae

All members of the genus Deinococcus were screened for the presence of type II restriction endonuclease activity as described in chapter 2. Not only were crude cell extracts tested for enzyme activity but also protein concentrates obtained by ammonium sulphate precipitation and polyethylene-glycol-dextran T500 phase partition. For each method both linear (λ DNA) and ccc (pAT153) DNAs were used under an extensive variety of restriction buffer conditions. These were; high (100mM), medium (50mM) or low (0mM) salt concentration; ± 2-mercaptoethanol (MSH); pH 7.0 or pH 8.0 medium salt Tris buffers; 30°C or 37°C incubation for one hour and finally, the substitution of KCl for NaCl in the medium salt buffer. All the buffers had constant magnesium ion (10mM) and Tris (20mM) concentrations. A resumé of the screening results is shown in Table 3.6. Two species of the genus were shown to contain site-specific endonucleases. D.radiodurans R1 contains the enzyme MraI, this enzyme being discovered by Wani et al (1982) and described in a paper published just after my own initial observation.

TABLE 3.6 Presence of site specific endonuclease activity in the genus Deinococcus

Species	Crude extract[a]	Ammonium sulphate precipitation	Polyethylene glycol dextran phase separation
D.radiodurans R1	-	+	+/-
D.radiodurans Sark	-	-	-
D.radiophilus	+	+	+
D.proteolyticus	-	-	-
D.radiopugnans	-	-	-

[a]All the techniques used both λ and pAT153 DNA, high, medium and low salt buffers, + or - 2-mercaptoethanol and also a KCl restriction buffer rather than NaCl.

Their characterisation of the enzyme reinforced my initial observations that:-

a) the enzyme is essentially undetectable in crude cell lysates due to the swamping activity of the non-specific nucleases previously described in section 2.2:

b) The enzyme has no site of action in the pAT153 molecule but 2 or more sites in λ DNA.

In fact Wani et al. (1982) showed that the enzyme is an isoschizomer of SstII and SacII, the recognition sequence being:-

5' C C G C G G 3'

3' G G C G C C 5'

The other member of the genus definitely shown to produce sequence-specific endonucleases was D.radiophilus. Alteration of DNA banding patterns after supernatant incubation and agarose gel electrophoresis was seen under all the various conditions used for screening. The enzymes, for it was later proven that there were in fact at least two, are from now on called DraI and DraII. Magnus Hansen (Novo Industries) has given Roberts (1983) personal communication confirming my own findings on DraII but also proposing the existence of a third enzyme DraIII. This enzyme appears to cut λDNA 13 times but in none of my experiments was there any indication of this third enzyme.

From the results it would seem that D.radiodurans Sark, D.proteolyticus and D.radiopugnans do not contain active restriction endonucleases, at least under the conditions used. For the first of these organisms the result is confirmed by the observation that Sark DNA carrying selectable

markers will transform R1 at a much lower frequency (at least 100 fold) than *vice versa*. Of course the clear-cut absence of restriction enzymes from the remaining three *Deinococcus* strains could not be totally confirmed due to the possible deficiencies in isolation and assay procedure. Also there appeared transient band alteration of ccc DNA on occasions, in all cases tested. The production of multiple faint bands intermediary between ccc and open circular (oc) forms may be due to topoisomerase activity or even inefficient DNA digestion by a restriction endonuclease. This latter explanation is unlikely as the linear DNA run concurrently showed no pattern alteration.

2.4 Purification of site-specific endonucleases from *D.radiophilus*

It was obvious from the screening procedures that *D.radiophilus* produced sufficient quantities of restriction endonuclease activity to make attempts at purification a feasible proposition. The procedures outlined in Chapter 2 were therefore employed.

During the different phases of purification various important observations were made. The growth state of the *D.radiophilus* cells influenced dramatically the final yield of enzyme. If the cells were left in a stationary phase of growth for too long (one day) subsequent lysis appeared to free a very active protease which rapidly removed most of the restriction enzyme activity present. This destruction was not inhibited by the presence of PMSF and seemed to be absent from cultures in late exponential or early stationary phase. High-speed centrifugation did

remove large amounts of cell debris and ribosomes but the addition of magnesium ions to aid this operation stimulated non-specific nuclease activity at this stage. At the poly ethylene glycol:dextran T500 phase separation, I had already shown that restriction enzyme activity remained bound to the dextran phase at salt concentrations of $<$0.8 M NaCl. It was possible to release this bound activity by washing the dextran phase, after centrifugation, with high salt buffer. Although the enzyme preparation lost 99% of the protein content it also lost 70% of the original enzyme activity. This method could be of use to rapidly prepare relatively small amounts of enzyme (without nuclease contamination). For larger scale purification of both enzymes it was thought preferable to resort to the standard preparatory use of the phase partition technique. That was to use a high salt concentration, the restriction enzyme separating into the poly ethyleneglycol supernatant. Very little enzyme activity was lost and almost all non-specific nuclease contamination was removed. Also, a large proportion of the DNA and RNA was excluded from the preparation, this being of vital importance for successful column chromatography later. During the overnight dialysis against column buffer 1 the presence of PMSF was essential to prevent proteolytic degradation of the restriction enzymes by contaminating serine proteases. The cell extract was then fractionated during chromatography through a series of protein purification columns as described in Chapter 2. In each case a small scale experiment was performed to ensure that enzyme activity was not adversely influenced by the column

material and that binding/elution at differing buffer concentrations could occur. DraI activity eluted from the DEAE-sephacel column in the 0.18-0.24M NaCl buffer although considerable 'tailing' of enzyme activity did occur in later fractions (Fig. 3.3). The vastly lower concentrations of DraII enzyme eluted only slightly after the first DraI fraction thus preventing any reasonable separation of the two enzymes at this stage. However, a hydroxylapatite column allowed the removal from the DraI enzyme preparations of contaminating DraII activity as well as any remaining non specific exo/endonuclease. DraI eluted from this column within the 0.20-0.32M phosphate buffer fractions whilst no DraII activity could be detected (Fig.3.4). At this stage it was shown to be essential that the protein concentration of the collected fractions did not fall below 500 $\mu g\ ml^{-1}$. If this occurred then rapid and irreversible loss of enzyme activity occurred even with material stored at $-20^{o}C$. Therefore, it was necessary to add 500 $\mu g\ ml^{-1}$ restriction grade Bovine Serum Albumin (BSA) to each collecting tube with the unfortunate effect of preventing accurate protein and specific enzyme activity determination at the last purification step. Although protracted enzymatic digestion indicated that the DraI preparation was free of non-specific exonuclease activity, the inability of T4 DNA ligase to work on fragments produced by this preparation implied the presence of phosphatase contamination. This barrier was overcome during the final phase of purification, heparin-agarose column chromatography. The fractions containing

3.3 Protein elution profile of *D.radiophilus* cell extract on DEAE-sephacel

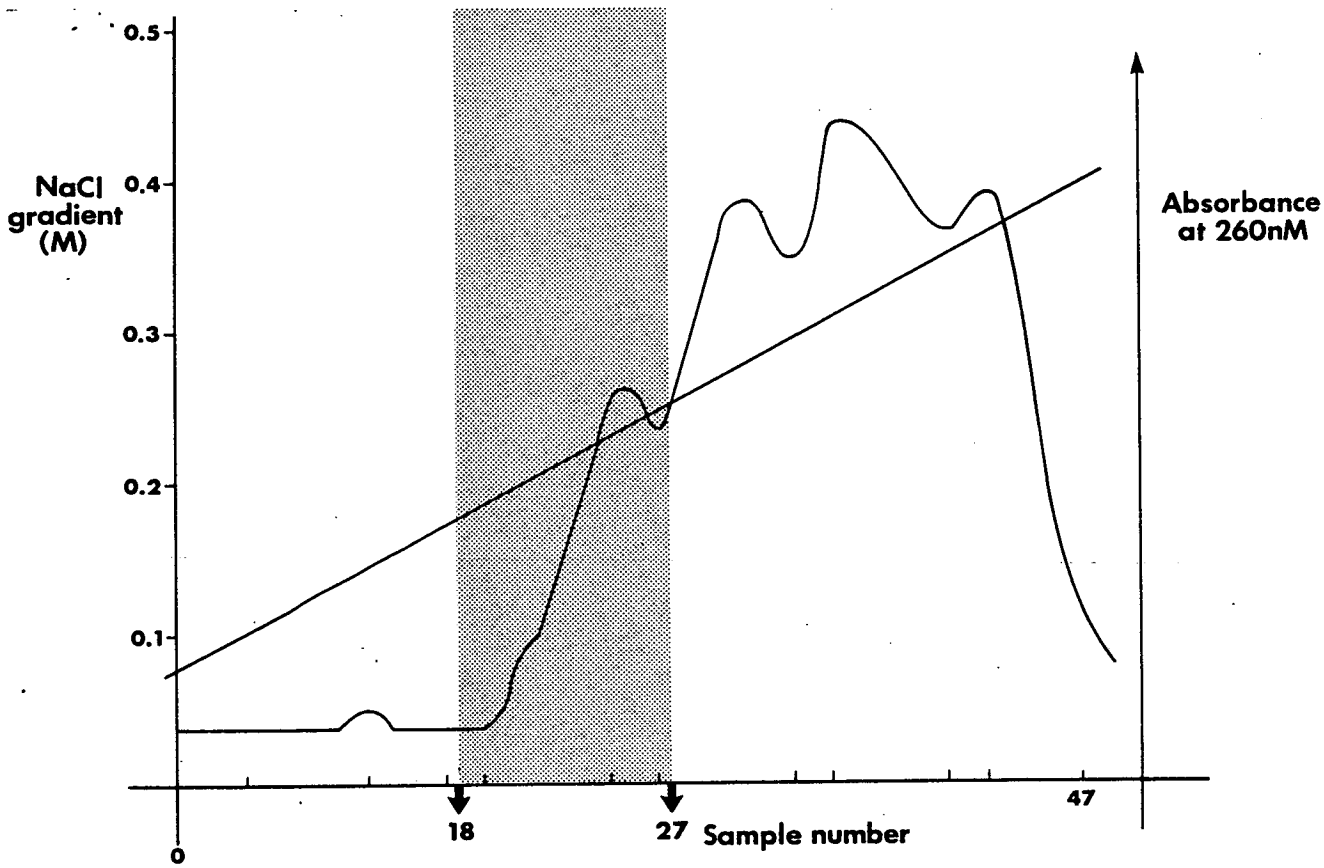
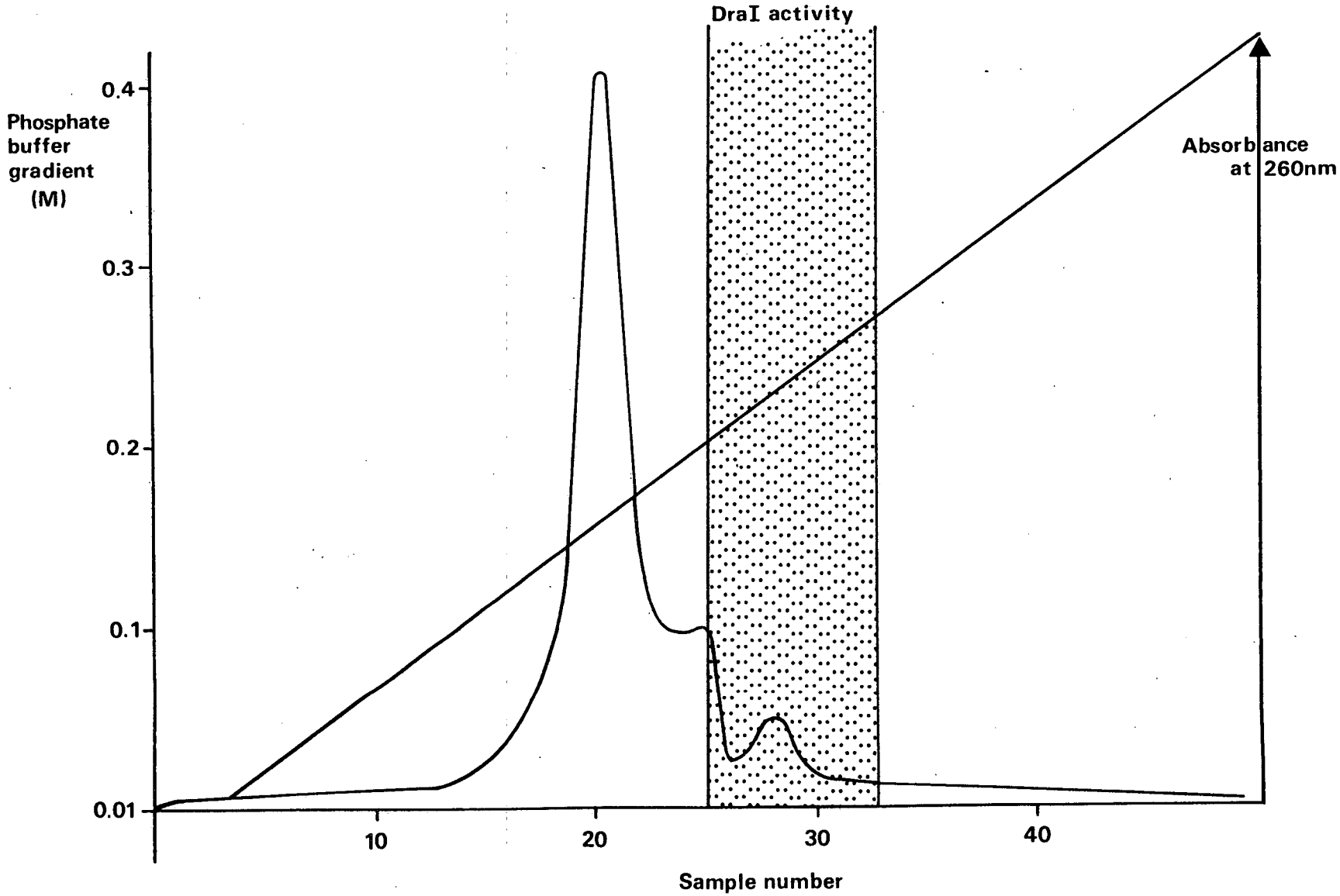


FIG. 3.4 PROTEIN ELUTION PROFILE OF D. radiophilus CELL EXTRACT ON HYDROXYAPATITE



peak DraI activity appeared in the 0.60-0.70 M NaCl eluate, the fractions being pooled, dialysed against storage buffer and kept at -20°C. This preparation was free of non-specific nuclease and phosphatase activities, as shown by the success of subsequent sequencing techniques which are extremely sensitive to such contaminants. The DraI-cut DNA did not religate efficiently, as indicated after agarose gel electrophoresis, under the standard conditions used for 'sticky-ended' joining, i.e. $<50\ \mu g\ ml^{-1}$ DNA concentration and 0.1 units of T4 DNA ligase at 14°C for 24 h. However, by increasing the ligase concentration to one unit, the DNA concentration to 100 μg DNA ml^{-1} and increasing the incubation temperature to 20°C a much higher level of religation was observed. This result strongly implies that DraI action produces blunt-ended fragments of DNA. The results of each stage of DraI purification are shown in Table 3.7, the final preparation showing the presence of four clear protein bands upon polyacrylamide gel electrophoresis (same as for DNA mapping) followed by a silver staining protocol.

If the DEAE-sephacel eluate containing peak DraII activity was loaded directly onto the heparin-agarose column after dialysis then partial separation from DraI could be achieved. The DraII activity eluted in the 0.55-0.65M NaCl range and although the pooled fractions contained elevated DraII levels they still possessed a large amount of contaminating DraI. Thus, from an approximate ratio of DraI:DraII of $\sim$10000:1 units this preparation was in the 5:1 region. Unfortunately, the enriched DraII preparation still had enough contaminating

TABLE 3.7 Stages of DraI Purification

Stage	Total protein (mg)	Total enzyme (units)	Specific activity (units mg^{-1} protein)	Yield (%)
High speed centrifugation	1.86×10^3	8.0×10^5	4.32×10^2	100
PEG-dextran phase precipitation	1.22×10^3	7.5×10^5	6.15×10^2	93.75
DEAE-sephacel	3.15×10^1	4.1×10^5	1.30×10^4	51.25
Hydroxylapatite	0.57	1.4×10^5	2.46×10^5	17.50
Heparin-agarose	N.D.	3.0×10^4	-	3.75

nuclease activity to prevent the subsequent analysis of its active site using DNA sequencing.

2.5 Characteristics of DraI and DraII

Before detailed analysis of the recognition sequence of either of these enzymes was made it was necessary to investigate the parameters affecting enzymatic digestion of substrate DNA. From section 1.4 of the Introduction it is obvious that the major influences upon other type II restriction endonucleases are those of divalent cation concentration, pH, temperature and salt concentration. By holding all factors except one constant and varying this other parameter its effect upon DNA digestion by DraI was shown. By doing this it was possible to produce reaction conditions which give a reproducible, maximised DraI digestion of λ DNA (Table 3.8). Of these factors the magnesium concentration seemed to allow the least variation before seriously affecting the DraI digestion rate, DraI activity declining rapidly outside 5-25mM Mg^{2+} limits. A wide variation in salt (NaCl) concentration of between 0-75mM was compatible with full activity. The enzyme had a slight peak in activity in different pH buffers at pH 8.0 and it lost activity rapidly when the temperature was raised above 44°C. Table 3.9 shows the influence of various other treatments upon the activity of DraI. As well as being inhibitory in the presence of Mg^{2+} other divalent cations did not successfully replace Mg^{2+} in the reaction buffer. High ionic concentrations were inhibitory, i.e. ≥100mM NaCl or ≥100mM Tris-HCl. KCl could replace NaCl in the reaction buffer with no obvious detrimental effect.

TABLE 3.8 Optimum conditions for DraI activity

Mg^{2+} ion Concentration	NaCl[a] Concentration	pH	Temperature
10mM	20mM	8.0	37°C

[a]Tris buffer concentration 20mM throughout

TABLE 3.9 Inhibition of DraI activity[a]

Compound	Concentration
Ammonium Sulphate	5% w/v
SDS	0.1% w/v
Cu^{2+} ions	1mM
Zn^{2+} ions	0.1mM
Ca^{2+} ions	0.1mM
Mn^{2+} ions	1mM
Tris-HCl	100mM
NaCl	100mM

[a]All reactions carried out in 10mM Mg^{2+}, 20mM Tris-HCl, pH 8.0 buffer with 1 unit of DraI and 1 µg plasmid (pML2) DNA incubated for 1 h at 37°C. Concentration of compound shown produces complete inhibition of DraI activity (except for Tris-HCl and NaCl where it indicates the level of inhibition initiation).

The addition of adenosine triphosphate (ATP), dithiothreitol (DTT) or 2-mercaptoethanol (MSH) (all 10mM) had neither a stimulatory or inhibitory influence. It may be possible that the addition of weak reducing agents such as DTT or MSH increases protein stability during long term storage. The restriction enzyme reaction could be terminated by addition of STOP buffer containing dye + 0.1% SDS or by heating at 65°C for 5 mins. The enzyme activity was stable in storage buffer at 4°C for >10 weeks and at -20°C for >2 years without major loss of activity. (50% v/v glycerol, 10mM Tris-HCl, 50mM KCl, pH8.0)

The inability to purify DraII free of DraI unfortunately clouds the ionic requirements of the former enzyme. No conditions were found that inhibited DraI activity whilst leaving DraII unaffected. However, in very low salt buffers the DraII activity began to wane. It would seem that the enzymatic requirements of DraII closely match those of DraI and both bear close similarity to MraI (Wani et al., 1982).

2.6 Mapping of DraI and DraII cleavage sites

The number and position of restriction enzyme sites in a variety of different DNA species was investigated by agarose and polyacrylamide gel electrophoresis. These results are shown in Table 3.10 and it should be noted that in all cases of DraII digestion the DNA was simultaneously digested with contaminating DraI activity. The two observed DraI sites in pBR322 were then mapped to within ±50 bp by using double enzyme digestions of the DNA (Table 3.11). By using DraI in association with 5 single-cutting enzymes, the DraI sites were shown to be around map

FIG. 3.5 APPROXIMATE POSITIONS OF DraI & DraII INCISION IN THE pBR322 MOLECULE (GEL ELECTROPHORESIS)

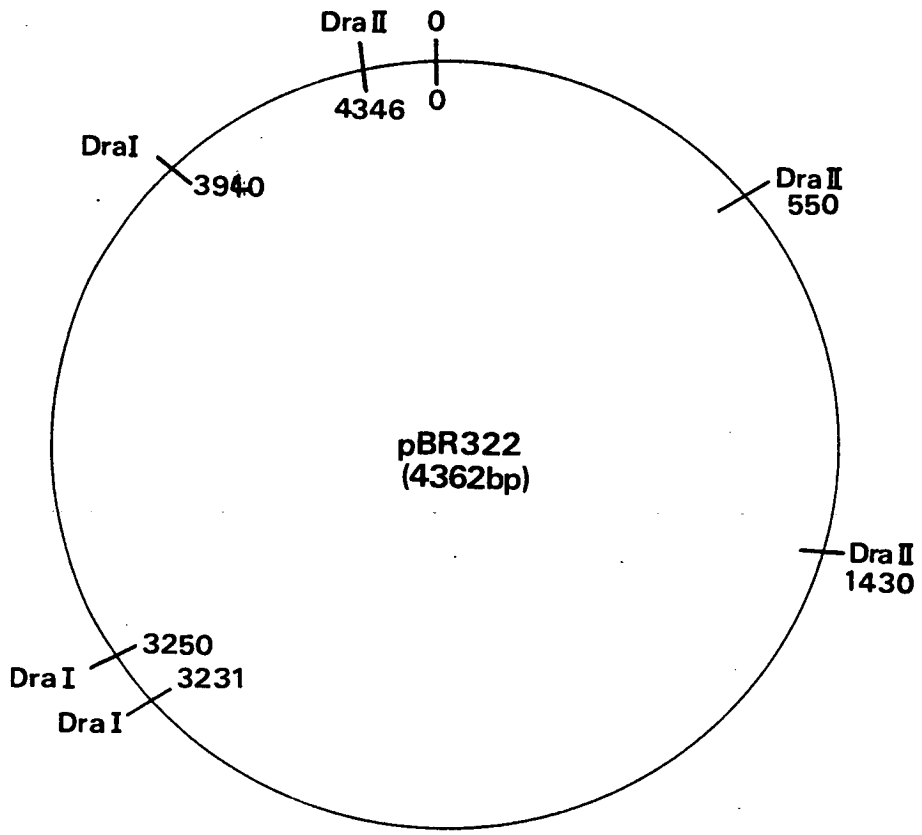


Table 3.10 DNA SPECIFICITIES OF TYPE II RESTRICTIONS ENZYMES DraI & DraII

ENZYME	NUMBER OF CLEAVAGE SITES				
	pBR322	λ	Ade2	SV40	øx 174
DRA I	3(2)[a]	13	>10	>10	2
DRA II	3	1	?	?	?

(a) Only two fragments visible using agarose gel electrophoresis or non-radioactive polyacrylamide gel techniques

TABLE 3.11 Mapping of DraI sites on pBR322 using double enzyme digestions

(composite agarose and polyacrylamide gel electrophoresis results)

	Enzymes					
	DraI	Dra+EcoRI	Dra+BamHI	DraI+HindIII	DraI+PstI	DraI+SalI
~ Fragment Sizes (bp)	3435	3075	2625	3445	3300	2249
	655	655	828	681	335	1046
		452	655	505	305	655

positions 3250 and 3940 (Fig.3.5). All double digestions were done in the restriction buffer of the second enzyme as DraI showed unit activity in all these varying buffers. The positioning of the DraI sites on the pBR322 molecule, the number and approximate position of the sites in ØX174 (DraI/PstI double digest) and SV40 prompted a search within the data produced by Fuchs et al, (1980) for a palindromic sequence matching these findings. In fact, the only palindrome which appeared to fit was the hexanucleotide,

5' T T T A A A 3'

3' A A A T T T 5'

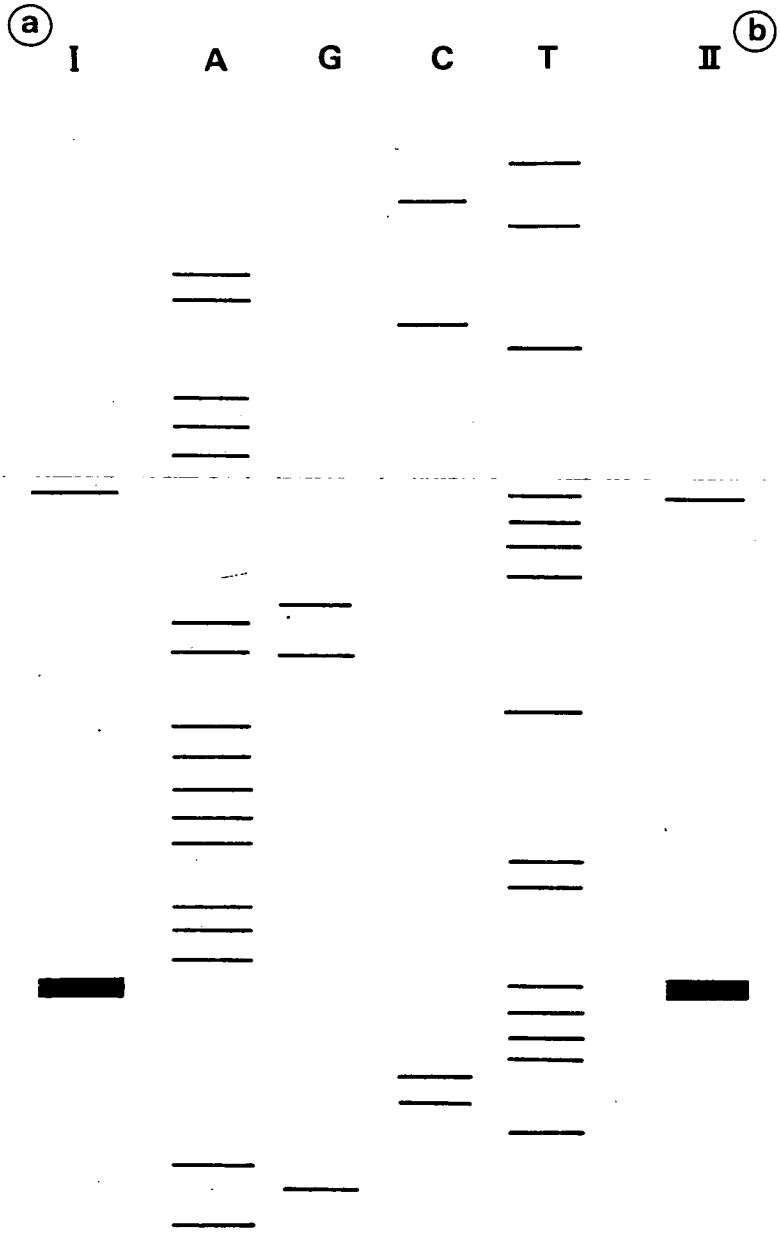
Thus, DraI seemed to be an isoschizomer of the recently isolated enzyme AhaIII (Whitehead and Brown, 1982), a product of the blue-green alga Apanothece halophytica. This made confirmation of the recognition sequence relatively simple in that the enzymes DraI and AhaIII should produce exactly the same fragement patterns from DNA digestions, which they did, and the sequencing protocol recently perfected for AhaIII could now be applied to DraI. The Sau3AI fragment 9 of pBR322 was subcloned into M13mp7 in the manner described by Whitehead and Brown (1982). Briefly, the fragment was eluted from an excised agarose block by soaking in 4 gel volumes of 500 mM ammonium acetate, 1mM EDTA, 0.1% w/v SDS at 38°C for five hours. Gel fragments were removed by filtration through glass wool (siliconized) and the DNA precipitated with 2 vol. ethanol + 0.1 vol 4M sodium acetate. The purified fragment was subcloned into the BamHI site of M13mp7. Clear plaques were isolated after transformation into E.coli NM522 and the molecular size of the recombinant

checked by the boiling isolation method of Holmes and Quigley (1981). The cloned pBR322 fragment was sequenced by the chain terminator method (Sanger et al., 1977) in essentially the same manner as described in Chapter 2 except for the following variations:-

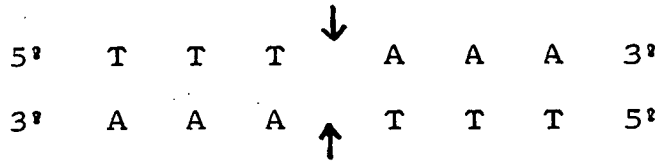
a) [^{32}P]ATP was used to label the DNA rather than [^{35}S]ATP, so that the ddATP in the reaction mixture for the 'A' lane was increased fourfold as [^{32}P]ATP incorporates more efficiently than [^{35}S]ATP.

b) Two lanes, denoted I and II were run simultaneously either side of the GATC sequencing lanes. Both contained the labelled Sau3AI fragment cut with DraI under conditions which inhibit complete digestion. The difference between the two lanes was the addition of the Klenow fragment of DNA polymerase in lane II. This means that 3 possibilities exist. The 3'-5' exonuclease function of this enzyme removed a protruding 3' sticky-end (resulting in the band in lane II being a number of basepairs shorter than lane I), the enzyme had no effect (lane I and II showed bands of the same size) or the 5' - 3' polymerase activity filled in a 3' recessed terminus (lane II showed a band larger than lane I). Using partial digestion conditions, it was possible to locate the third DraI site in pBR322 only 18 basepairs away from the primer proximal site of the Sau3AI fragment 9 molecule. A diagrammatic representation of the resulting autoradiograph is shown in Figure 3.6, clearly reaffirming the recognition site of the enzyme DraI as,

FIG. 3.6 DIAGRAMATIC REPRESENTATION OF AN AUTORADIOGRAM OF THE SEQUENCE OF THE pBR322Sau3AI FRAGMENT WHICH CONTAINS DraI CLEAVAGE SITES



(a) LOCATION OF DraI CLEAVAGE IN SEQUENCED STRAND
(b) LOCATION OF DraI CLEAVAGE IN TEMPLATE STRAND



cleavage producing blunt-ended fragments.

By employing the double-digestion techniques used initially in the case of DraI preliminary site localization, it was possible to locate regions of pBR322 where DraII cleaved the molecule. However, due to contamination of the enriched DraII preparation with DraI enzyme these digestions tended to involve three and not two restriction endonucleases, Table 3.12. Further information was gathered from digestion of the pBR322 molecule with a tetranucleotide sequence-recognising enzyme, Sau3AI(GATC), FnuDII(CGCG) or HaeIII(GGCC) before incubation with either DraI or the DraII/DraI preparation. Analysis of the fragment sizes produced by polyacrylamide gel electrophoresis (resolution of around 60 basepair fractions after ethidium bromide staining) allowed the DraII sites in pBR322 to be located with an accuracy of $\pm$ 25 basepairs. These sites are illustrated in Fig.3.5 and occur at positions 550, 1476 and 4346. However when this information was fed into a computer to analyse possible common recognition sites, no known palindromic sequence capable of producing the observed fragment configurations of DraII digestion of pBR322 or λ could be discovered. The definitive DNA sequencing protocol utilized for DraI site confirmation could not be used for DraII as the very small quantities of the enzyme could not be separated from a contaminating nuclease. This latter enzyme was removed from DraI

TABLE 3.12 Mapping of DraII sites using triple enzyme digestions of pBR322

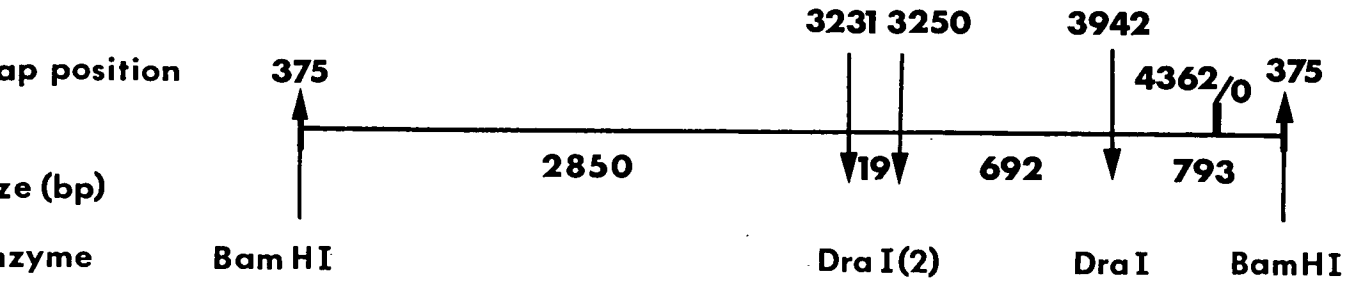
	Enzymes				
	DraI+DraII	DraI+DraII+ PstI	DraI+DraII+ HindIII	DraI+DraII+ BamHI	DraI+DraII+ SalI
Fragment Sizes (bp)	1832	1832	1832	1832	1832
	968	968	968	968	811
	710	575	710	710	710
	575	445	518	445	575
	445	376	445	407	445
		334	(57?)	(168?)	(157)

during hydroxylapatite chromatography but as already noted DraII was completely and irreversibly inactivated (or bound) by this material, and also by phosphocellulose.

2.7 Inhibition of DraI activity by ultraviolet irradiation of the substrate

As previously reported in section 4.5 of the Introduction it has been shown that the activity of type II sequence-specific endonucleases is inhibited by thymine-thymine, or to a lesser degree cytosine-thymine, dimer damage within the substrate DNA molecule. This interference with enzyme action only takes place however if the lesions occur within, or directly adjacent to, the recognition sequence. Obviously a recognition sequence containing a high proportion of thymine residues ought to be rendered particularly sensitive to ultraviolet light-induced lesions in the DNA. To investigate whether this trend holds for the enzyme DraI, the site of which contains a total of 6 thymine residues in comparison with 4 for HindIII (Chapter 2), the dose-dependent inhibition of DraI activity by ultraviolet light irradiation of the pBR322 molecule was studied. A total of 6 μg of pBR322 was linearised with the restriction endonuclease BamHI, Figure 3.7, (6 units for 1 h at 37^{o}C), the DNA precipitated with ethanol and resuspended in an ultraviolet transparent buffer (Chapter 2). The linear pBR322 was then irradiated for a series of time periods corresponding to doses ranging from 0 to 3800Jm^{-2}, samples being removed at set time periods and digested for 1 hour at 37^{o}C with 2 units of DraI.

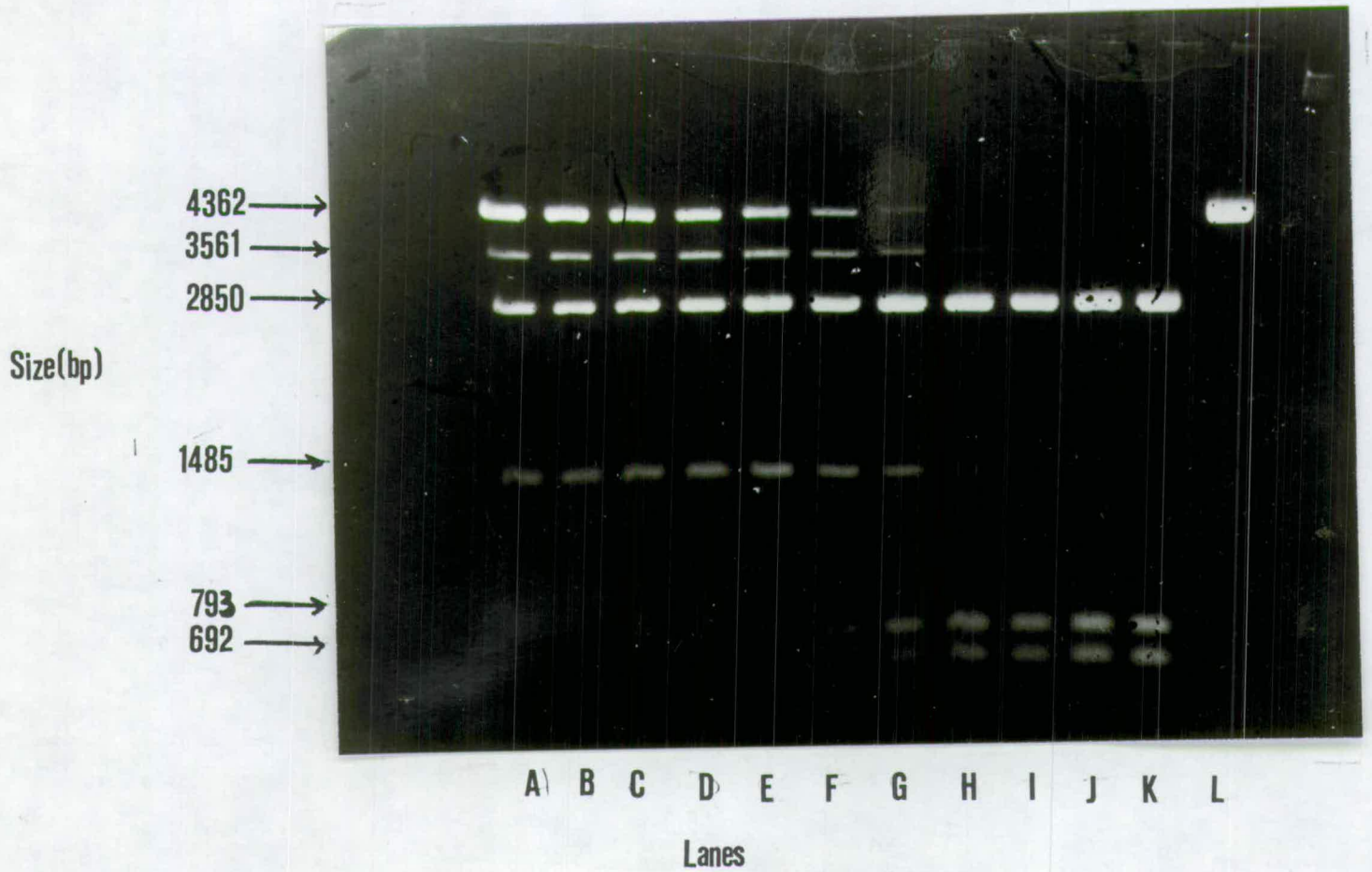
3.1 Sites of action for the various restriction enzymes used to study the inhibition of DraI activity on pBR322 after ultraviolet irradiation (see text).



The reactions were terminated with STOP buffer and the DNA fragments analysed using agarose gel electrophoresis. In this manner the affect of increasing doses of irradiation upon the action of DraI can be displayed (Fig. 3.8). Unfortunately, the presence of two DraI sites only 18 basepairs apart on the pBR322 molecule does complicate interpretation of the results. The appearance of a 1485 basepair fragment allied to the simultaneous removal of both 692 and 793 basepair bands as the ultra-violet dose increases does indicate the inhibition of DraI enzyme action at position 3942 on the pBR322 molecule. Inhibition of either of the two other DraI sites (3230 or 3250) would not result in band alteration as fragment size resolution on the 1% agarose gel used can not allow the distinctive separation of bands differing in size by only 20 basepairs. The initial alteration in banding pattern was seen after a dose of around 60 Jm^{-2}. Above a dose of 180 Jm^{-2} a fragment of 3567 basepairs appears due to the inhibition of both DraI sites at positions 3250 and 3230. Finally, at doses of >300 Jm^{-2}, complete linear molecules of pBR322 (4362 basepairs) were seen due to the complete inactivation of all DraI recognition sites by ultra-violet induced lesions. This enzymatic inactivity could not be overcome by increasing either the length of incubation or the amount of enzyme. If HindIII (recognition sequences) was used as a comparative control for this experiment (this being previously the most sensitive enzyme to ultra-violet lesion inhibition tested), band alterations were

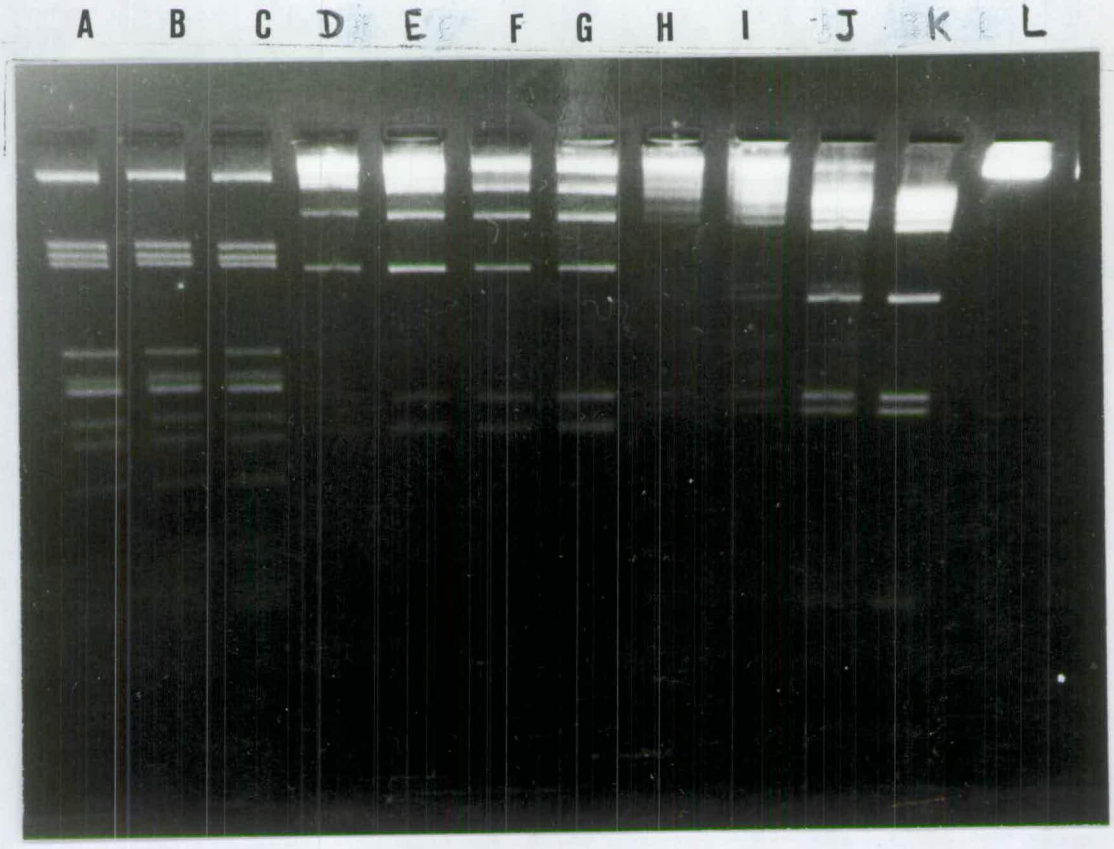
Fig.3.8

Dra1 digestion of uv treated, BamHI linearised pBR322.
Lanes A-K illustrate 1 ug pBR322 linearised with BamHI and given uv doses of 4200, 3600, 3000, 2400, 1800, 1200, 600, 300, 180, 60 and 0 Jm^{-2} respectively before 1h DraI digestion. Lane L shows BamHI linearised pBR322 plasmid DNA. Fragment separation was by 1% agarose gel electrophresis.



3.9

Cleavage of uv irradiated λ-DNA by Dra1, HindIII or PstI. Lanes A-C show digestion of 1 ug λ-DNA by PstI after uv radiation doses of 3600, 1800 and 0 Jm^{-2} respectiely. Lanes D-G show cleavage of 1 ug λ-DNA by Hind III after doses of 3600, 1800, 300 and 0 Jm^{-2} respectively. Lanes H-K show digestion of 1 ug of λ-DNA by DraI after doses of 3600, 1800, 300 and 0 Jm^{-2} respectively. Lane L is 1 ug of undigested λ-DNA. Fragment separation was by agarose gel (1%) electrophoresis.



first observed after doses of between 180 and 300 Jm^{-2}. In this case NruI was used to linearise the pBR322 molecule (position 971) as a HindIII/BamHI double digest would normally produce a 327 basepair fragment which is on the limit of resolution in a 1% agarose gel. The inhibition of DraI activity resulting from ultraviolet irradiation of the substrate DNA was therefore 2-4 times greater than that of HindIII. A direct comparison of the differing sensitivities of restriction endonucleases to this kind of damage is clearly illustrated in Figure 3.9. In this case λDNA was irradiated with increasing doses of ultraviolet light and then digested for 1 hour with 4 units of either DraI, HindIII or PstI at 37°C in the appropriate restriction buffer. Fragment visualization was by agarose gel electrophoresis (1% w/v), ethidium bromide staining and ultraviolet light transillumination. There was a striking difference in the banding pattern of DraI digested DNA with and without irradiation. After a dose of 3600 Jm^{-2} almost all the DraI sites present in the λDNA had been inactivated (13 altogether). To a lesser degree the inhibition of HindIII action is also obvious but PstI digestion was completely unaffected by the radiation damage even at the highest dose.

3. MODIFICATION METHYLASE ACTIVITY

Evidence for some manner of DNA modification associated with DraI restriction enzyme activity is clear cut. Both chromosomal and plasmid DNA from D.radiophilus were isolated (Chapter 2) but proved to be completely resistant to digestion by both purified DraI and enriched DraII enzyme preparations. The plasmid pUE1 from D.radiophilus has been purified free of other plasmid types and cloned into pAT153 (G. al Bakri, personal communication). The plasmid itself, when isolated directly from D.radiophilus, is not linearized by DraI but the composite molecule pUE109 does show that a DraI site exists within the pUE1 region of DNA, this site only becoming active after multiplication in E.coli. Chromosomal DNA from A.halophytica (gift of N. Brown) was resistant not only to AhaIII degradation but also DraI action. The former enzyme also showed no activity upon incubation with D.radiophilus DNA. Thus modification seemingly occurred in such a manner as to render the sequence 5'-T T T A A A-3' resistant to both enzymes, although not necessarily via the same alteration.

D.radiophilus late exponential phase cells were harvested, washed in T.E. buffer (pH 7.4) and then lysed in the French pressure cell as previously described for restriction enzyme isolation. An assay for type II modification methylase activity was undertaken as described in Chapter 2. When the assay conditions included the

presence of the methyl group donor S-adenosyl-L-methionine then incubation of the DNA with crude cell extract in the absence of divalent cations resulted in the protection of the DNA present from subsequent degradation by DraI. However, if S-adenosyl-L-methionine was excluded from the reaction mix then no protection from DraI degradation accrued to the DNA. These results strongly suggest the existence of a 'classical' type II modification methylase activity. A previous investigation into DNA modification in Deinococcus spp. (Mackay, 1984) indicated the absence of any of the standard methylated bases in D.radiodurans (N^6-methyl adenine and 5-methyl cytosine) even though MraI exists within the cell and the chromosomal DNA is modified to resistance against the enzyme. Preliminary work did indicate the absence of methylated bases in D.radiophilus also and perhaps some novel modification system is common to both of these organisms.

4. DEVELOPMENT OF THE CLONING AND EXPRESSION OF D.radiophilus GENES

4.1 Construction of D.radiophilus gene libraries

The experimental details of the construction of D.radiophilus genomic libraries in the E.coli plasmid vector pAT153 have been described in Chapter 2. In the case of partial MboI digestion,with insertion into the BamHI site of pAT153, the degree of MboI action was controlled by using different dilutions of the enzyme ranging from 1 to 0.01 units per ug D.radiophilus chromosomal DNA. The fragment patterns were visualized

after agarose gel electrophoresis (Fig. 3.10). It was decided to pool the three partial digestions resulting from using 0.04, 0.08 and 0.15 units for use in the recombination experiments.

The results from the three different plasmid cloning experiments are shown in Table 3.13. The probability of a DNA sequence being represented by the gene banks was calculated from equation 3 in Chapter 2. The maximum insert size observed in 25 randomly selected clones from each gene bank was 20.6 kb, produced from a HindIII digest. The major drawback with using the plasmid vector system was the relatively small insert size, particularly in the case of the MboI/BamHI method.

4.2 Screening of recombinant DNA banks

Plasmid DNA was extracted from the cells of each gene bank, using the Birnboim-Doly method (1979) and this was used as the basis for testing, via transformation, for the expression of *D.radiophilus* genes in *E.coli* mutant strains. Each of the mutant *E.coli* strains (Table 3.14) was challenged with DNA from all 3 gene banks and successful complementation of the chromosomal lesion detected as described in Chapter 2. In the case of the attempted cloning of the restriction/modification system of DraI, incubation of 50 μg of gene bank DNA with 100 units of DraI for 2 h at 37^{o}C caused a 10^{5} fold decrease in the number of antibiotic resistant transformants. In fact, plasmid containing transformants were recovered only from the PstI gene bank. Eight out of 20 of these showed a pAT153 molecule completely lacking the region containing all three DraI sites.

Fig.3.10

Partial digestion of D.radiophilus DNA using the restriction endonuclease MboI.Each lane consists of 2 ug chromosomal and 0.2 ug plasmid DNA digested for 2h at 37 C with the appropriate amount of MboI. Band separation was by agarose (1%) gel electrophoresis.

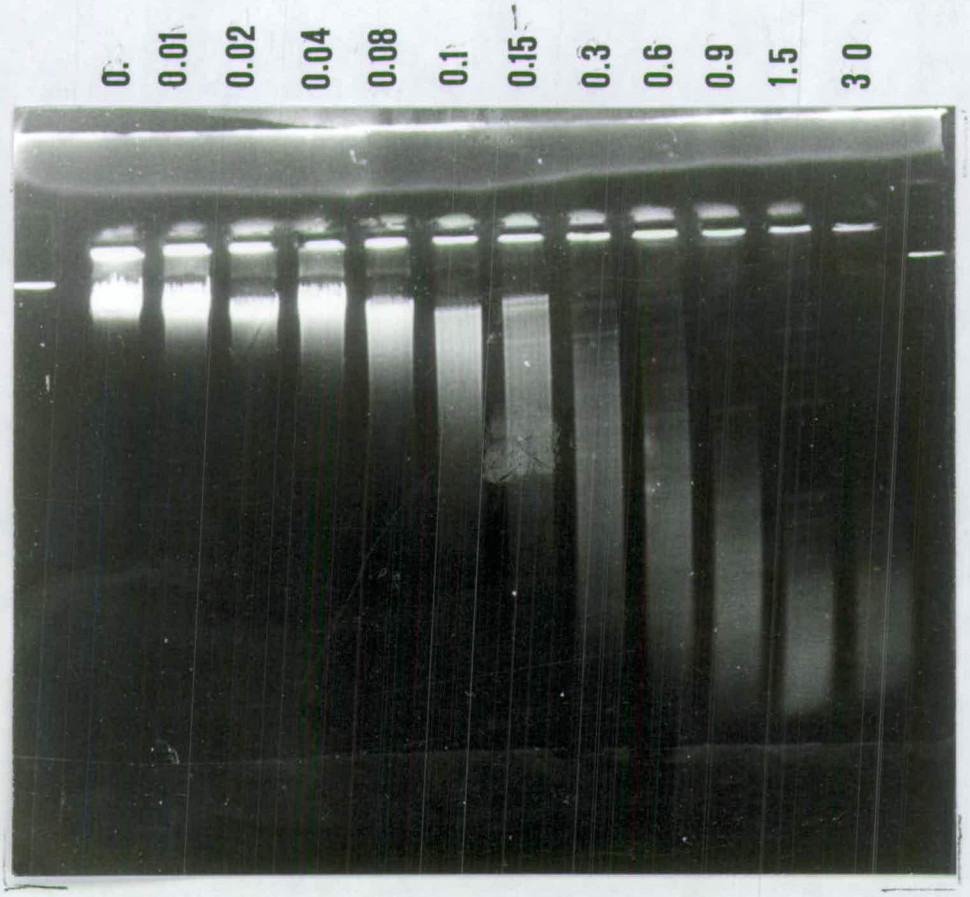


TABLE 3.13 Construction of genome libraries of D.radiophilus DNA using plasmid pAT153

Enzymes	Number of transformants	% Insertional Inactivation	Average Insert size (kb)	[a]Probability of any DNA sequence being represented
HindIII	1250	76	3.2	0.75
PstI	1849	92	2.9	0.89
BamHI/ MboI	5300	96	1.2	0.82

[a]Using equation 3 from chapter 2.

TABLE 3.14 Selection of recombinant plasmids capable of complementing *E.coli* chromosomal mutations.

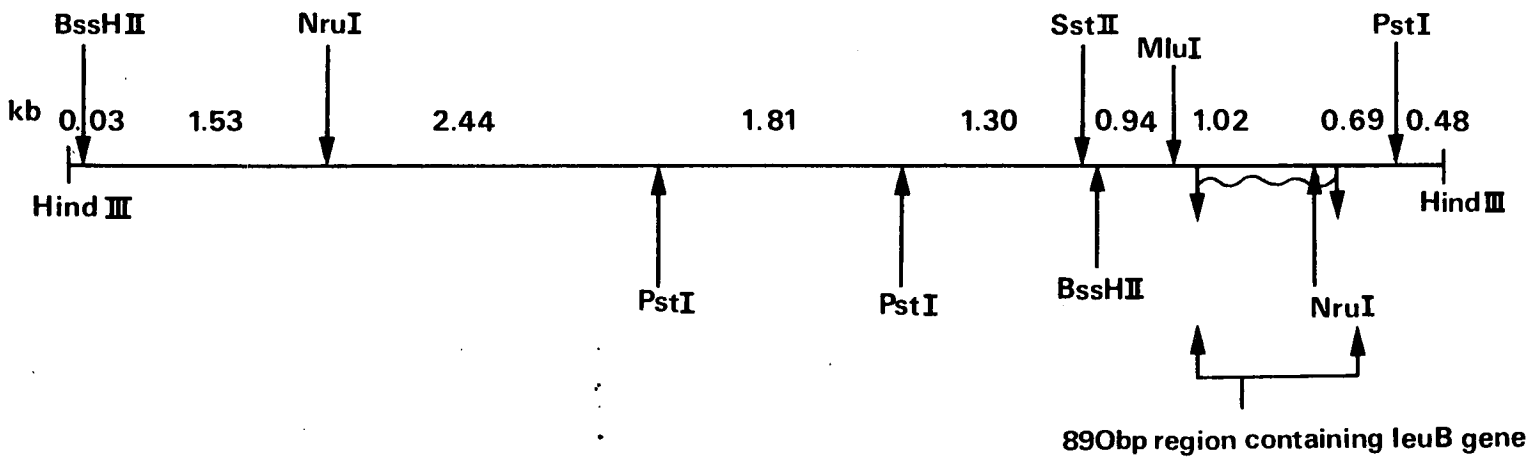
Phenotypic trait	*E.coli* Strain	No. transformants ug^{-1} DNA carrying complementary marker + appropriate antibiotic resistance
R^-M^-(DraI)	HB101	0
M^-(DraI)	HB101	0
uvrA	159	0
proA	HB101	0
leuB	HB101	11
thr$^-$	CSH42	0
mal$^-$	CSH42	0
ilv$^-$	CSH42	0
trp$^-$	CSH58	0
his$^-$	CSH58	0
arg$^-$(E)	CSH58	0
trpD	107	0
trpB	128	0
trpA	311	0
trpC	312	0

Of the remaining clones, none were resistant to DraI cleavage. Thus, attempts to clone and express the DraI methylase gene in E.coli were unsuccessful. From all the transformations attempted with the derived gene banks, successful complementation of the leucine B mutation was the only one observed (Table 3.14).

4.3 Mapping and subcloning of the leuB-complementing DNA from D.radiophilus.

In the original experiment 11 E.coli HB101 colonies were isolated with the $leu^{+}amp^{R}$ phenotype and these were denoted as pleu 1 to 11. Of these only 8 showed stable inheritance of the leucine prototrophic marker, although all contained plasmid DNA. The recombinants pleu1, 7 and 10 all appeared to have a high level of internal recombination resulting in the production of $leu^{-}amp^{+}$ daughter cells containing deleted plasmid DNA. In the most severe case, pleu1, 47 out of 50 plasmid-containing cells had lost the ability to synthesize leucine. The plasmid DNA from all leu^{+} clones was extracted using the Holmes and Quigley boiling technique (1981) and restricted with HindIII, PstI or EcoRI and the fragments analysed by agarose gel electrophoresis. This showed that there were no EcoRI sites in any of the inserts and that in all cases the insert DNA was of the same size and orientation, except in leu1, 7 and 10 where complicated patterns due to deletion products were seen. Further double digestions with the restriction enzymes SstII/HindIII or PstI/SstII appeared to confirm that in all cases the insert DNA was identical and inserted at the

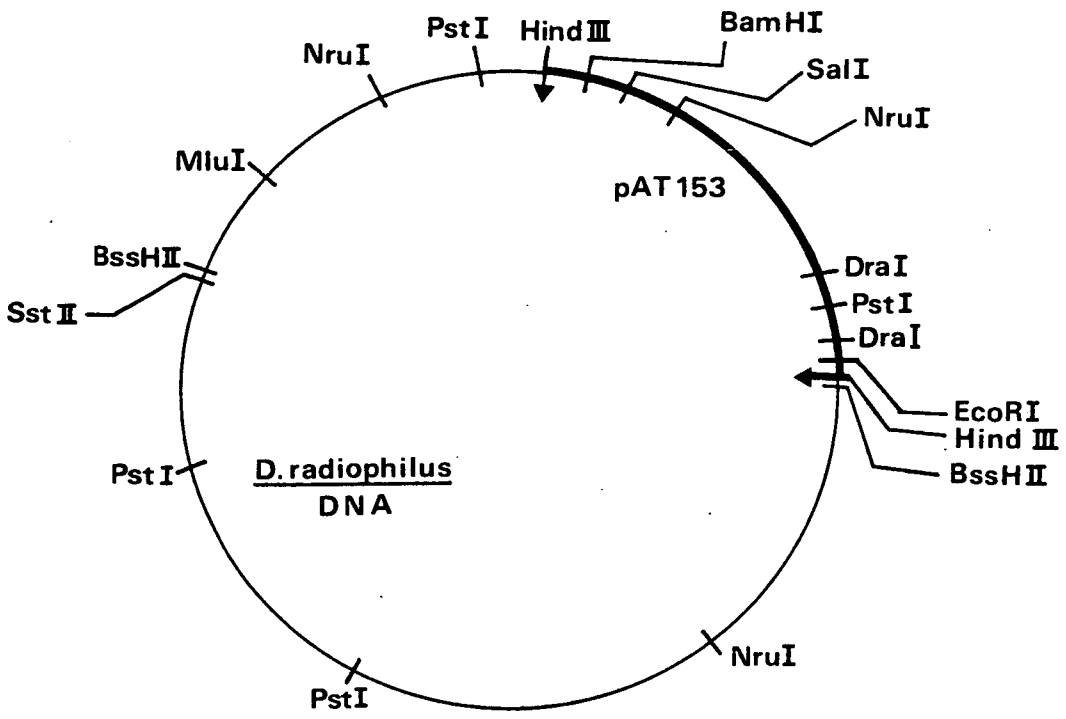
FIG. 3. 11 (a) RESTRICTION ENZYME MAP OF PLASMID pleu5 INSERT



TOTAL SIZE = 10.24 kb

FIG. 3.11 (b) PLASMID pLEU 5 RESTRICTION MAP

SIZE = 13.89kb



Plasmid pleu 5

HindIII site of pAT153. Large-scale plasmid preparations were then made of pleu5 and pleu8 DNA in order for accurate restriction mapping. Various single, double and triple enzymatic digestions were attempted with a wide range of 6 basepair sequence-specific endonucleases. The fragment patterns produced were analysed and sized using agarose (fragments $>$500 basepairs) and polyacrylamide (fragments $<$ 500-60 basepairs) gel electrophoresis. The results were collated to produce the insert restriction map shown in figure 3.11(a). The insert contained no EcoRI, BamHI, SalI, SmaI or DraI recognition sites. The total length of the insert was 10.24 kilobases and the orientation of insertion into the HindIII site of pAT153 is shown in Figure 3.11 (b), the total plasmid pleu5 being 13.89 kilobases in length.

The three enzymes which had multiple sites within the insert DNA, PstI(3), NruI(2) and BssHII(2) were then used in attempts to subclone the DNA region coding for the leuB-complementing activity. Both PstI and NruI digestions of pleu5 (1µg DNA digested with 4 units of enzyme for 1 hour at 37^{o}C) were ligated into the appropriately digested pAT153 molecule. The conditions for ligation differed for the two treatments as PstI produces sticky-ends and NruI blunt-ends. After transformation of the ligation mixtures into E.coli HB101 tet^{R} leu^{+} or amp^{R} leu^{+} transformants were selected respectively. No amp^{R} leu^{+} clones were isolated, presumably indicating that one of the NruI sites is within or very close to the region of DNA linked to the relief of leucine auxotrophy. However, about 10% of

tet^R transformants were also leu^+ and plasmid screening of these clones showed the presence of the 4.01 kilobase fragment inserted into the PstI site of pAT153. One of these $tet^R leu^+$ clones was purified and used for large scale plasmid preparation by the Birnboim-Doly method (1979) and caesium chloride density equilibrium centrifugation. This plasmid was termed pPL3. Attempts to use the enzyme BssHII in such cloning experiments were unsuccessful. This may have been due to enzyme impurities or to the BamHI site used (as BssHII has no recognition sequence present in the pAT153 molecule) and the BssHII produced fragments requiring S1 nuclease treatment to remove any extensions before blunt-ended ligation could be applied.

4.4 Reorientation and stability of the leuB-complementing insert

A total of 24 $tet^R leu^+$ transformants produced by the PstI digestion of pleu5 and re-insertion into the pAT153 were tested for insert orientation. This was done by isolating the plasmid DNA by the Holmes and Quigley boiling method (1981) followed by digestion with HindIII and SstII. Band analysis was by agarose gel electrophoresis. In all cases the NruI site was proximal to the 2 β-lactamase promoter sites (Stüber and Bujard, 1981). To confirm this result pPL3 was digested with PstI, treated with 1 unit of alkaline phosphatase at 37°C for 15 mins and then 56°C for 15 mins. (both steps repeated again) and finally, after phenol extraction, ligated into the PstI site of pAT153 again. Analysis of 10 subsequent transformants, once

again, showed only the one type of insert orientation, that with the NruI site not the SstII site close to the HindIII site of pAT153.

The stability of the recombinant plasmid molecules pleu5 and pPL3 was compared with that of the vector molecule pAT153 by growth of the E.coli strain harbouring the respective plasmids in L-broth lacking any antibiotic selection. The cultures were kept at 37°C in exponential phase for >100 generations and samples removed at different times. Individual clones were isolated from these by dilution plating on L-agar and the presence of plasmid-borne phenotypic traits tested by replica plating. The results are shown in Table 3.15. It is obvious that for the period of investigation the recombinant plasmids were as stable as the progenitor pAT153 molecule.

Insertion of DNA may also influence the ability of the DNA to transform the bacterial indicator strain. In fact pAT153 gave an average of 3×10^5, pPL3 an average of 1.4×10^5 and pleu5 an average of 0.8×10^5 transformants μg^{-1} DNA. It would seem likely that this variation was due to size differences rather than any inherent instability caused by the insertion of foreign DNA.

4.5 Subcloning using nuclease Bal31

From the results above it is reasonable to assume that the leuB-complementing activity produced by expression of the insert DNA is localised in a region near to or surrounding the NruI site within the 4.01 kilobase PstI fragment. Further subcloning routines such as partial digestion with

TABLE 3.15 Plasmid stability in the absence of antibiotic selection

No. of generations	pAT153 % colonies[a] amp^R tet^R	pleu5 % colonies amp^R leu^+	pPL3 % colonies tet^R leu^+
0	100	100	100
5	99	88	96
15	99	100	100
30	100	100	100
50	96	100	100
68	100	100	90
80	100	99	100
104	100	100	99

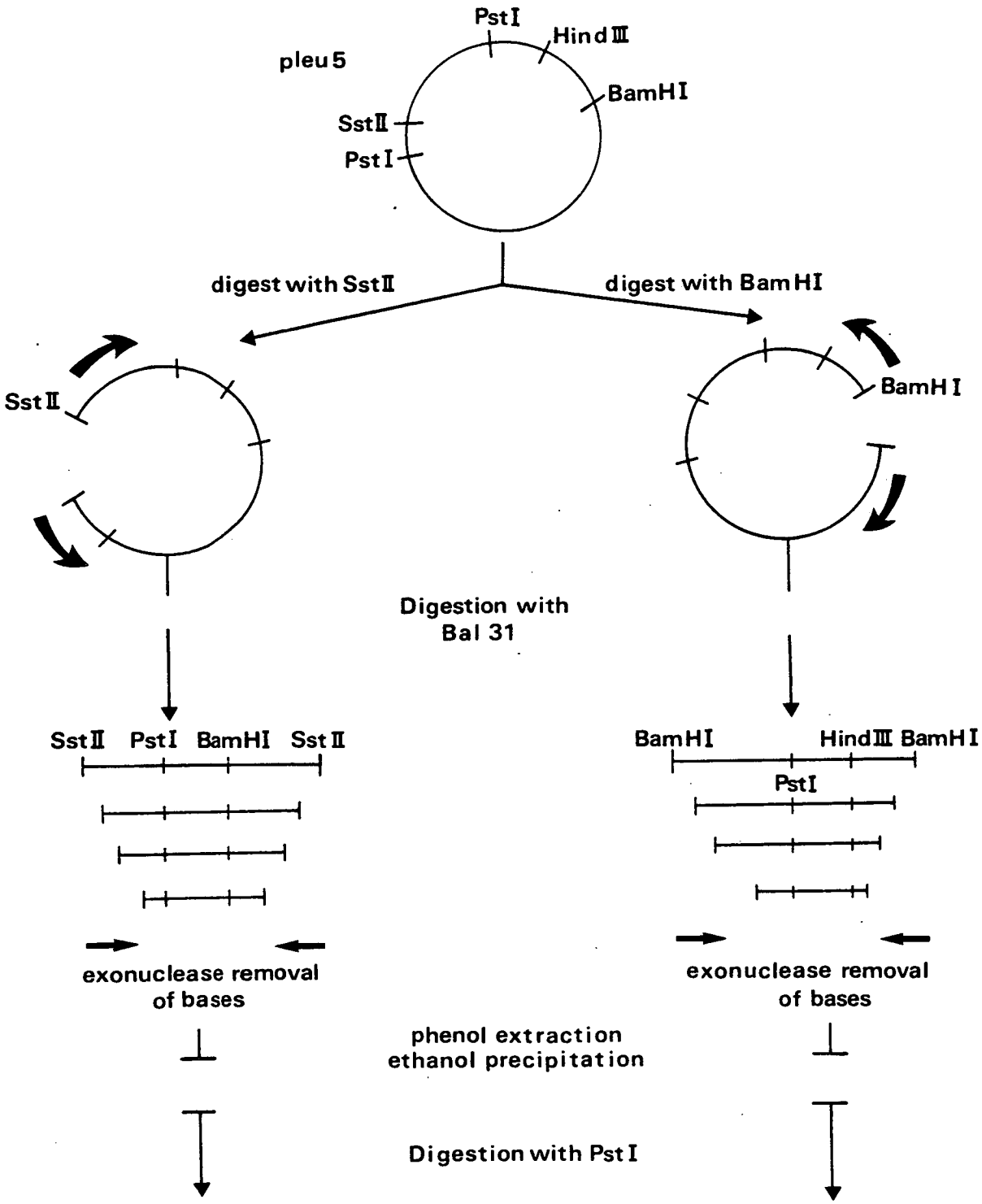
[a]In each case 100 colonies were tested for amp^R (30 $\mu g\ ml^{-1}$); tet^R (20 $\mu g\ ml^{-1}$) and leu^+ (min.med + proline + thiamine)

a restriction enzyme with a 4 basepair recognition site eg. HaeIII, Sau3AI, might have been used. However the presence of unique SstII and BamHI sites near the region of interest prompted the use of Bal31 nuclease. As mentioned in Chapter 2 Bal 31 nuclease has the ability to sequentially digest DNA from both ends of a linear fragment simultaneously. The rate of digestion is dependent upon the relative concentrations of enzyme to DNA and also the G:C ratio of the region being degraded. A major advantage of Bal31 nuclease is that it requires Ca^{2+} ions for activity and therefore its activity can be inhibited by the addition of ethylene glycol tetra acetic acid (EGTA) which chelates Ca^{2+} but not Mg^{2+} ions in any restriction buffer added. If this Ca^{2+} chelation is followed by the dilution of the 200mM NaCl concentration present in the Bal31 nuclease buffer, by the addition of a salt-free restriction buffer, digestion of the Bal31 treated DNA with restriction enzymes can be accomplished quickly. Before the sub-cloning regimen was initiated a rough estimate of the rate of Bal31 nuclease digestion was determined on plasmid pleu5 after digestion with either SstII or BamHI. Using this information 2 µg of pleu5 was digested with 4 units of either BamHI or SstII for 1 h at 4°C. After ethanol precipitation, the linear DNA was digested with Bal31 nuclease and samples removed after periods during which approximately 300-500 basepairs were removed. In each sample the reaction was terminated with EGTA (4 x concentration of Ca^{2+} ions) and buffer added to allow PstI digestion of the resultant deletions (Fig.3.12a). Such treatment resulted in the

formation of a series of PstI/blunt-ended fragments which were then cloned into a modified pAT153 molecule (Fig.3.12 b). This molecule had the PstI-EcoRI 755 basepair fragment removed and was therefore deficient in the normal β-lactamase promoter region at position 4150. After ligation with T4 DNA ligase and transformation into E.coli HB101, selection was for tet^R leu^+ colonies. The insert sizes of 24 recombinant plasmids of each type (SstII or BamHI digestion) were screened by using the rapid boiling method.

Of all the molecules screened the smallest plasmid recombinant found still capable of producing a leu^+ phenotype upon transforming E.coli HB101 was plasmid pTL27 (Fig. 3.13). This chaemeric molecule was the product of SstII/Bal31 nuclease digestion and consisted of a 1.38 kilobase insert as measured by agarose gel electrophoresis. The molecule lacked both SstII (as expected) and MluI sites but still retained the NruI site of the insert. The smallest molecule produced upon digestion into the 4.01 kilobase region from the other direction, i.e. BamHI/Bal31 nuclease treatment was only 400 basepairs smaller than the complete region (i.e. 3550 basepair insert). The plasmid pTL27 was then purified on a large scale in the standard manner and used as a basis for further localizing the DNA region of interest. About 1 μg of pTL 27 was linearised at the remaining PstI site and digested with Bal31 nuclease, samples being taken at periods corresponding to 200 basepair deletion. These samples were then allowed to recircularise using T4 DNA ligase and were used to transform E.coli HB101. Transformants with leu^+tet^R

Fig. 3.12(a) PRODUCTION OF TARGET DNA BY SEQUENTIAL Bal 31 NUCLEASE DIGESTION



Ligation into modified pAT153 vector at Pst/ blunt-end sites. Selection for $leu^{+}tet^{R}$ transformants.

FIG. 3.12(b) SUB-CLONING ROUTINES USED FOR LOCALIZING THE leuB GENE OF D. radiophilus

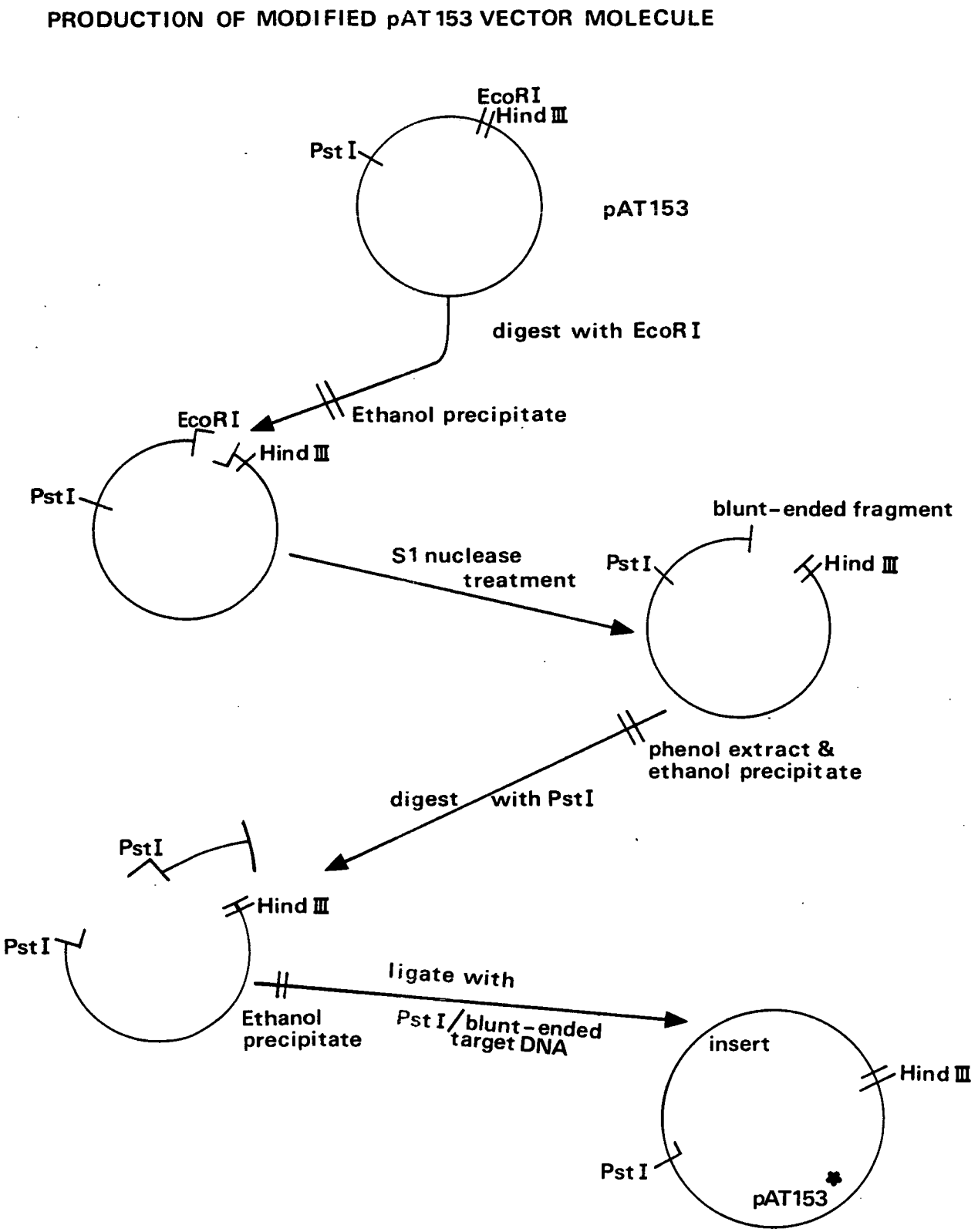
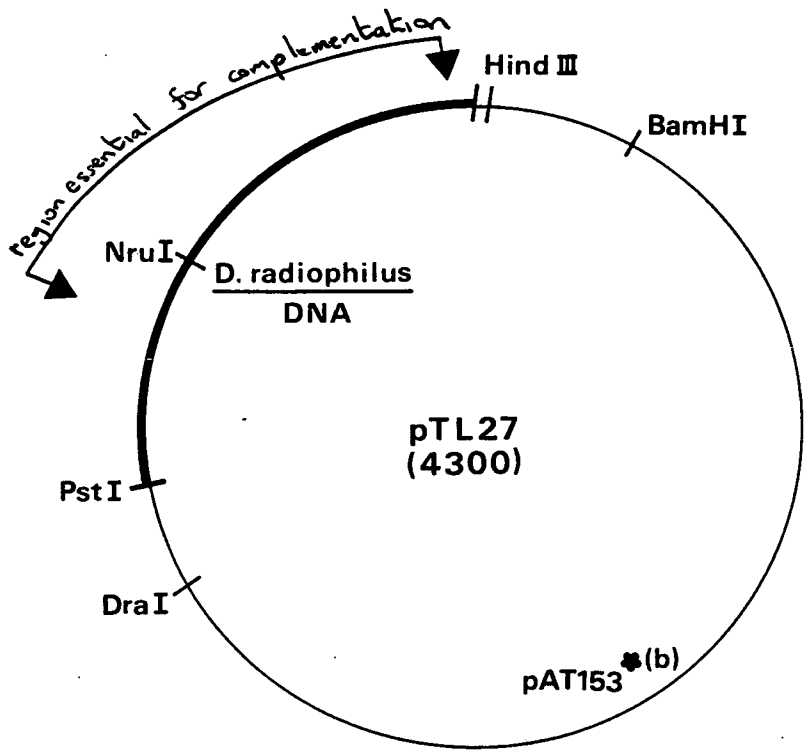


FIG. 3.13 PLASMID pTL27 (a)



(a) Derived from Sst II / Bal 31 / PstI treatment of pleu 5

(b) pAT153* = pAT153 - 755 bp region from EcoRI → PstI site (fig. 3.12/a)

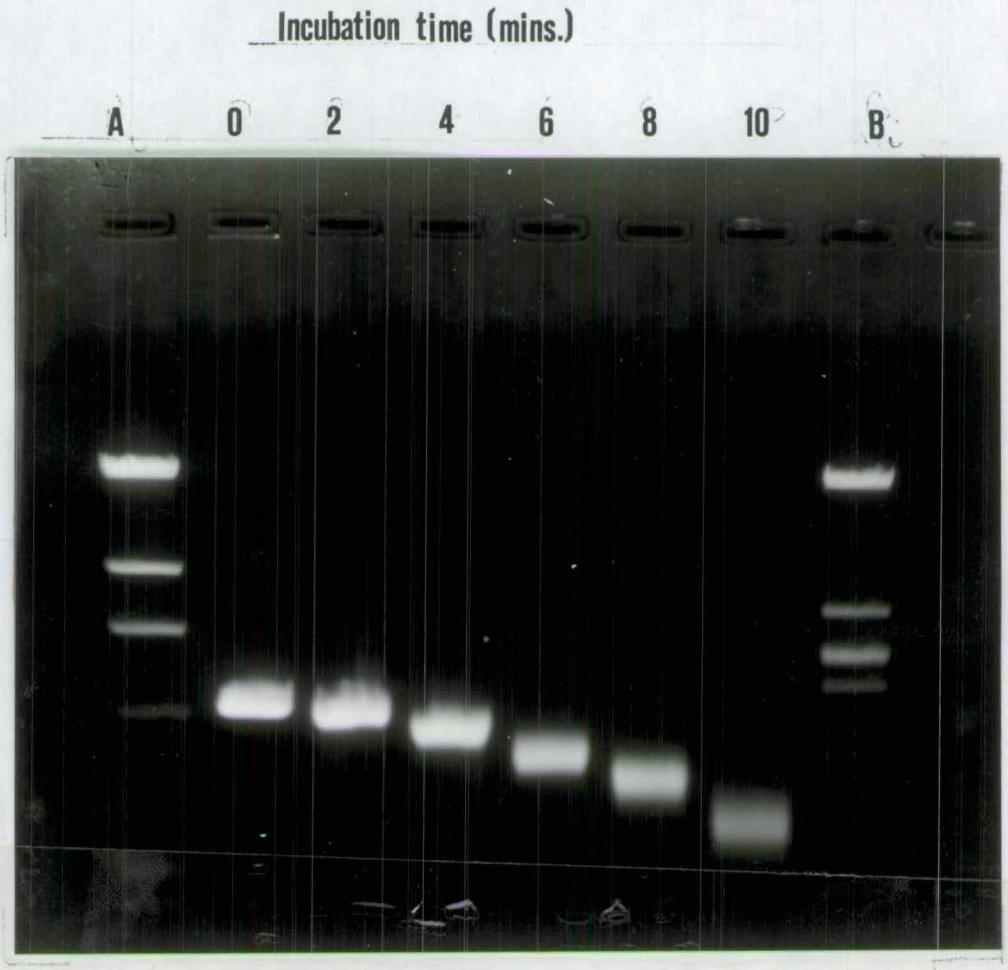
phenotype were selected and screened for plasmid DNA. It was found that the smallest recombinant molecule, pTL54, had an insert of only 850 basepairs. The molecule still retained the NruI site, the region of interest lying between NruI and MluI sites of the original insert.

4.6 Sequencing the iso-propylmalate dehydrogenase gene of D.radiophilus along with flanking DNA regions.

The advantages of using the M13 bacteriophage vector systems for sequencing were discussed in Chapter 1. The choice of the particular vector is dependent upon the restriction site orientation required. For sequencing the 900 basepair region delineated by the cloned D.radiophilus fragment in plasmid pTL54 the vector used was M13mp9 (M13mp8 for one particular clone set). Random techniques such as sonication are not particularly suited for fragmenting such small regions successfully and accentuates the asymptotic nature of information gathering produced normally. The non-random method of choice was developed from the use of Bal31 nuclease by Guo et al. (1983) to produce sequential deletions along the DNA region being sequenced. The success of this method depends to a great extent upon the presence of 2 unique restriction endonuclease sites flanking the insert. This allows the use of 'forced' cloning into the M13mp9 vector. It was for this reason that pTL27 was constructed with unique HindIII and PstI sites on either side of the D.radiophilus DNA. Although pTL54 has a smaller insert, the flanking enzyme sites, DraI and HindIII, are not as easily used since DraI produces

blunt-ended fragments no different functionally from those produced by the Bal31 nuclease treatment. Therefore, it was necessary to use pTL27 instead and sequence about 400 basepairs of flanking DNA. By digestion with either PstI or HindIII followed by controlled Bal31 nuclease digestion incremental deletions of 200 basepairs could be produced. The alternate restriction enzyme was then used to create fragments with one blunt and one sticky end. The deleted molecules were then force-cloned into SmaI and PstI or HindIII digested M13mp9. The complete inserts were cloned by HindIII/PstI double digestion followed by insertion into HindIII/PstI digested M13mp8 or M13mp9, thus giving both orientations. The experimental details are shown in Figures 3.14 and 3.15. The action of Bal31 nuclease on pTL27 was determined by digestion of 10 µg of the plasmid with 20 units of PstI for 1 hour at 37°C. After ethanol precipitation the DNA was resuspended in 45 µl H_2O and 45 ul Bal31 nuclease buffer (x2). A single unit of Bal31 nuclease was added and after mixing, the Eppendorf tube was incubated at 30°C, 15 µl samples being removed at 0, 2, 4, 6, 8 and 10 mins. The reaction in each sample was immediately terminated by the addition of 15 µl of 100mM EGTA. The samples were loaded onto a 1% (w/v) agarose gel along with size markers and electrophoresed overnight. From the results, (Fig. 3.16) it was possible to accurately gauge the length of incubation time with Bal31 nuclease under these fixed conditions which would give sequential deletions of about 200 basepairs. To improve the percentage yield of correct inserts, the

Fig.3.16 Sequential digestion of PstI linearised pTL27 DNA using the nuclease Bal31.



The two control lanes A and B consisted of 1ug λ DNA digested for 1h with 2units of HindIII and EcoRI respectively. Bal31 digestion (0.2 units) of 2 ug of linearised pTL27 DNA occurred for the time periods shown above at 30° C. Electrophoresis was through a 1% agarose gel.

FIG. 3.14 STRATEGY FOR SUB-CLONING leuB REGION FROM pTL 27 INTO M13mp9 FOR COMPLETE SEQUENCE DETERMINATION

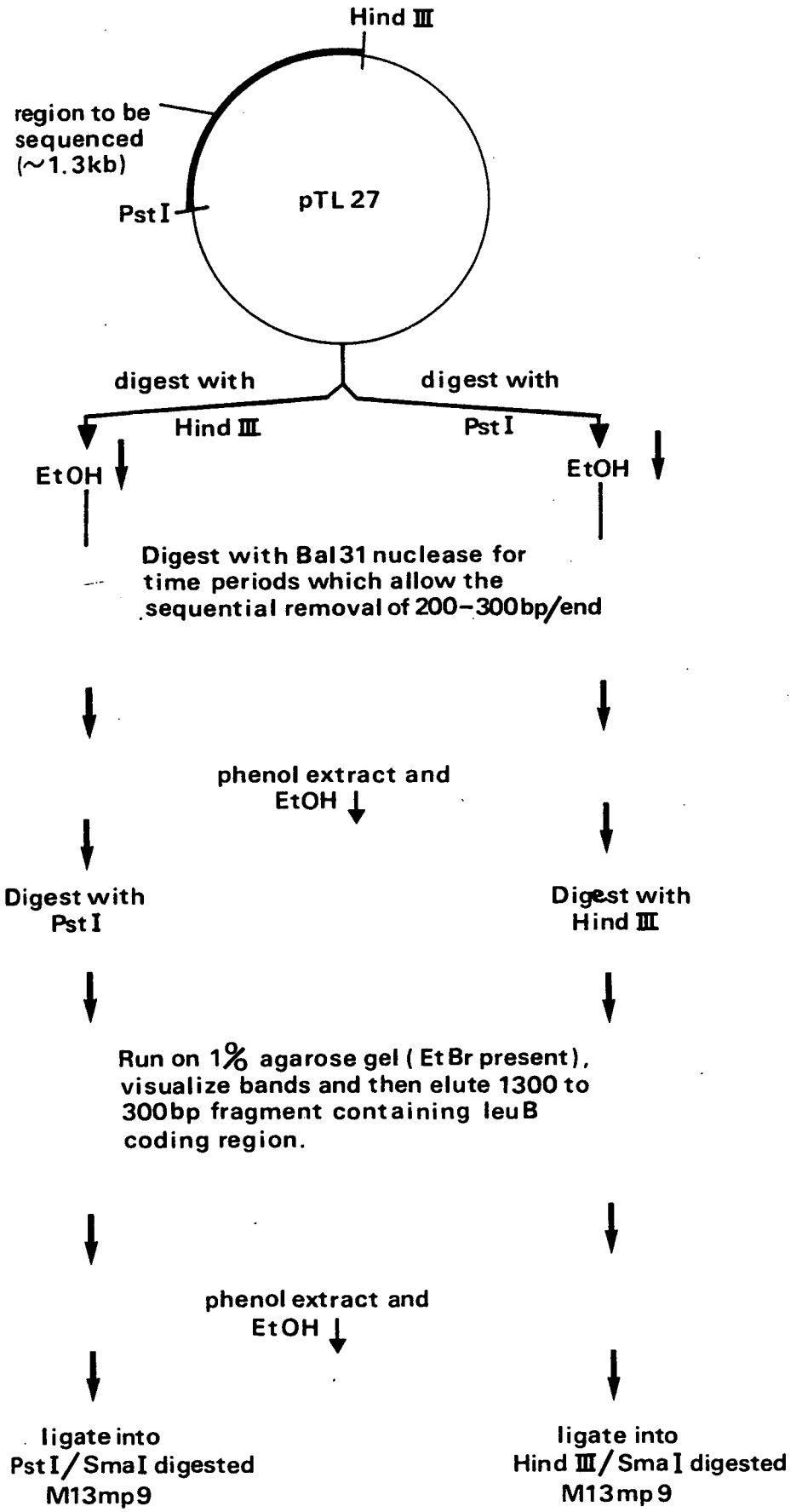
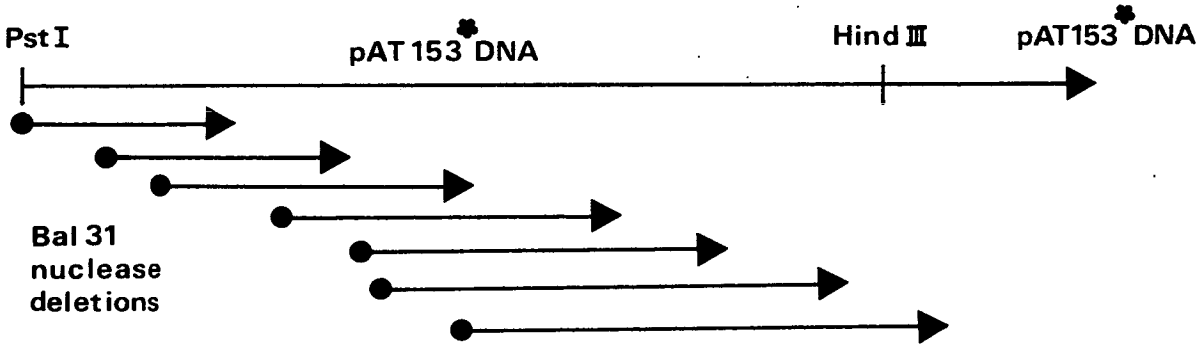
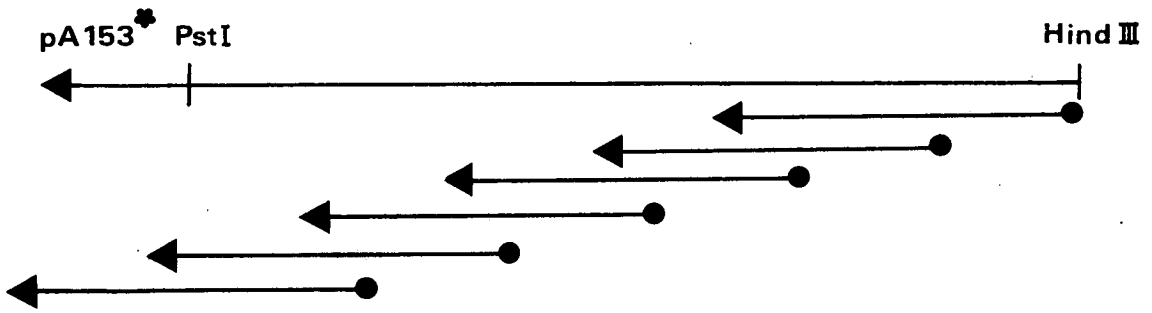


FIG. 3.15. SUB-CLONING INTO M13mp9 using Bal 31 NUCLEASE SEQUENTIAL DIGESTION ('PONCZING ALONG')

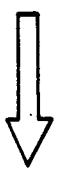
(a) DIGESTION WITH Pst/ Bal 31 NUCLEASE / Hind III



(b) DIGESTION WITH Hind III / Bal 31 NUCLEASE/ Pst I



(ARROWS INDICATE SEQUENCED REGION OF EACH DELETION)



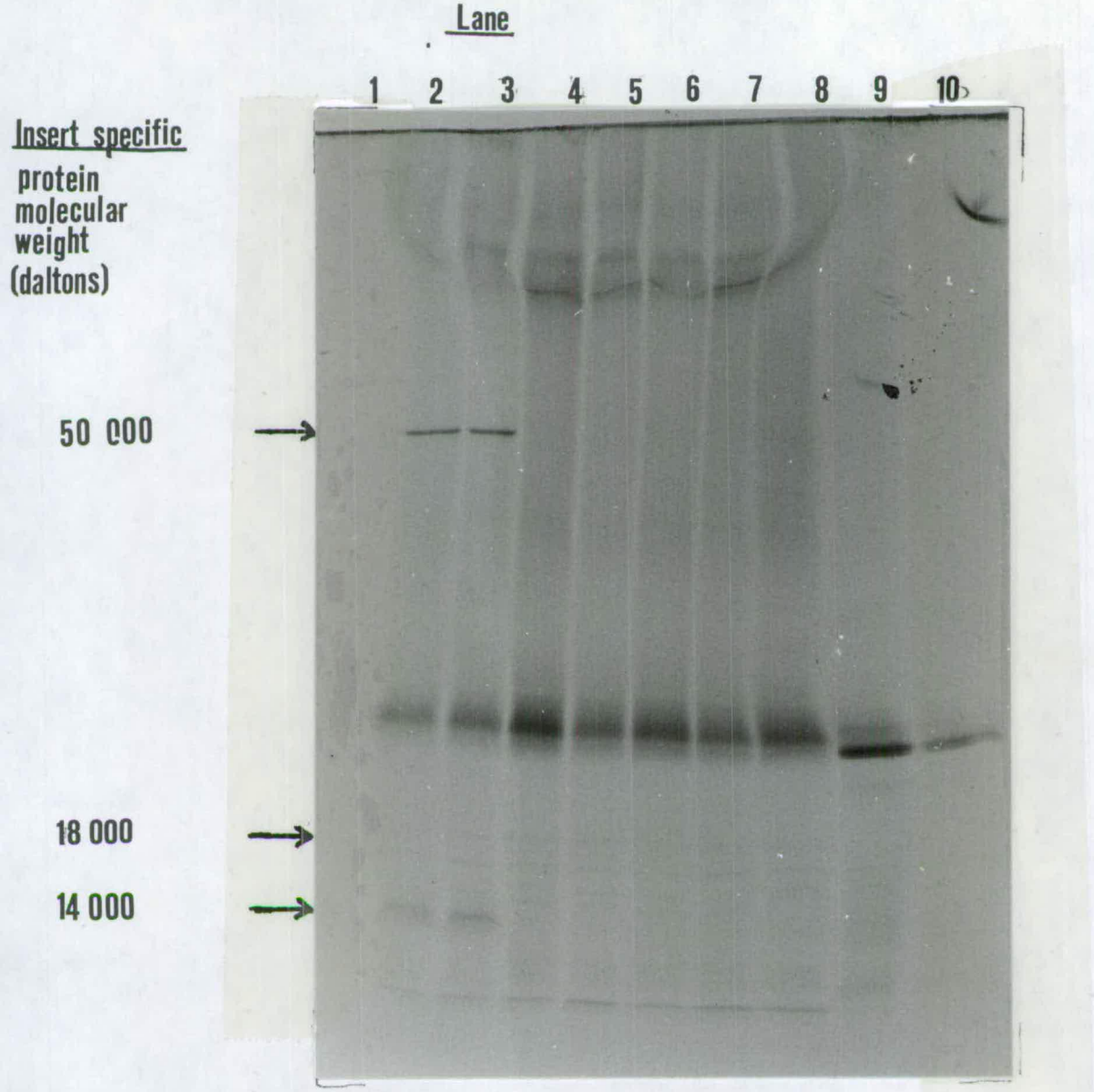
Ligation into (a) SmaI/ Hind III
or (b) SmaI/ Pst I - digested M13mp9

deleted regions of DNA of interest were purified, after digestion with the second restriction enzyme (2 units μg^{-1} DNA) and agarose gel electrophoresis. The fragments were eluted into wells cut in the agarose and then phenol extracted before being inserted into the appropriately treated M13mp9 vector using T4 DNA ligase. The DNA was transformed into the E.coli indicator strain NM522 and clear plaques picked, purified and tested for the insert size. In this manner a series of 40 deletions were produced covering the insert DNA and being in both orientations. These recombinants were then used to produce the M13mp9 templates required for sequencing (Chapter 2). The combination of forced cloning and Bal31 nuclease deletions is an elegant and rapid way of providing enough template information to cover the DNA sequence of both DNA strands. In this case, however, it was thought advisable also to sequence the extra 500 basepairs of flanking sequence in pTL27, subsequently shown not to be involved directly by the construction of pTL54.

4.7 Protein products of the leu$^+$ subclones

The protein products of sub-cloned plasmids pPL3, pTL27 and pTL54 were investigated using the maxicell technique described in Materials and Methods. When compared to the products of plasmid pAT153, or just the host strain (E.coli CSR603) without plasmid, certain insert specific proteins could be identified (Fig. 3.17). The plasmid pPL3 coded for proteins of approximate molecular

Fig.3.17 Maxi-cell analysis of the translational products of recombinant plasmid molecules with the leuB gene of D.radiophilus present.



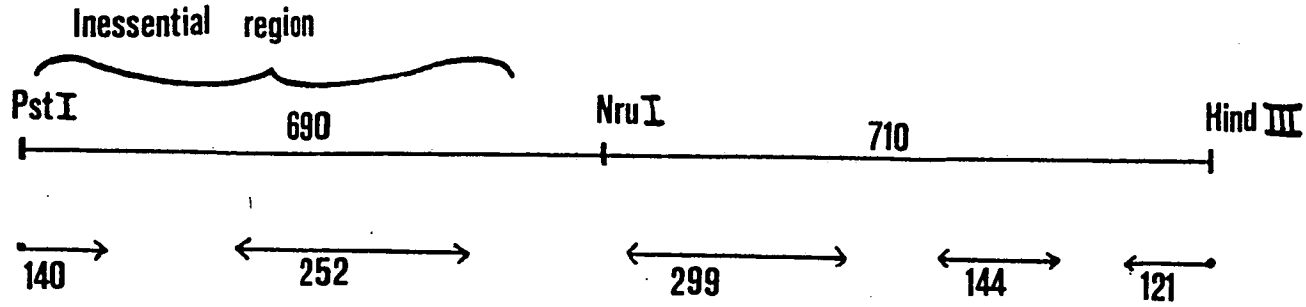
LANE	E.coli strain	Plasmid	Growth supplements
1	CSR603	-	-
2	"	pPL3	+ leucine
3	"	pPL3	-
4	"	pTL27	+ ampicillin
5	"	pTL27	+ leucine
6	"	pTL27	-
7	"	pTL54	+ leucine
8	"	pTL54	-
9	"	pAT153	+ leucine
10	"	pAT153	-

weights 50000, 18000 and 14000 daltons in addition to those bands represented in the control lanes. In the cases of pTL27 and pTL54 the 50000 and 14000 dalton proteins disappear leaving only the 18000 dalton band being insert specifically coded. If leucine is removed from the incubating medium just before labelling there appears to be no effect upon the plasmid coded proteins. This was also the case when a sub-lethal level of ampicillin (0.1 $\mu g\ ml^{-1}$) was added to one of the pTL27 preparations. It would seem that neither the absence of leucine or the presence of ampicillin have a regulatory role in the expression of any of the proteins produced from the insert DNA.

4.8 Nucleotide sequence in regions of the pTL27 insert

A total of 40 templates were tested for correct DNA insertion using the reannealing procedure of Messing (1981). Only 2 out of 40 failed this test. Thanks to the co-operation of Dr. Scaife's laboratory in the Department of Molecular Biology in Edinburgh 10 of these templates were sequenced allowing approximately 2/3 of the unique sequence of the D.radiophilus 1.4 kilobase insert to be distinguished using the Sanger chain terminator method (1977). The approximate regions covered by the nucleotide sequence and the actual sequence itself are displayed in Figs.3.18 and 3.19.

 Regions of plasmid pTL27 sequenced using the chain terminator method



Nucleotide sequence of regions within the *D. radiophilus* *leuB*$^{+}$ insert.

HindIII

TGCAGAAGGCGCCCAGGCGGCGTTTAGAAGCAGCAGAGCAGCCTGGCCCTGAGGGTAGTGGTGTGGCCTGGACGGCA
ACGTCTTCCGCGGGTCCGCCGCAAATCTTCGTCGTCTCGTCGGACCGGGACTCCCATCACCACACCGGACCTGCCGT

TGAGAATACTAAACAGATTAGGACTAGAGGTATTGGCAACCTTATGCAGACTTGCTTCTA**********
ACTCTTATGATTTGTCTAATCCTGATCTCCATAACCGTTGGAATACGTCTGAACGAAGAT

****

CGATCGCGCGTATGATAGTTTGAAGGCAGAATACGGTATCCCGATTCATCXGXGGTCCGCGATTCGCGAACGTATTA
GCTAGCGCGCATACTATCAAACTTCCGTCTTATGCCATAGGGCTAAGTAGXCXCCAGGCGCTAAGCGCTTGCATAAT

AACACCTATATAGAACCATATACAAGAAAGTAATAAAGGTCAACGGATAAGTCAATTTGGTCGGTGCGCGCCATCGA
TTGTGGATATATCTTGGTATATGTTCTTTCATTATTTCCAGTTGCCTATTCAGTTAAACCAGCCACGCGCGGTAGCT

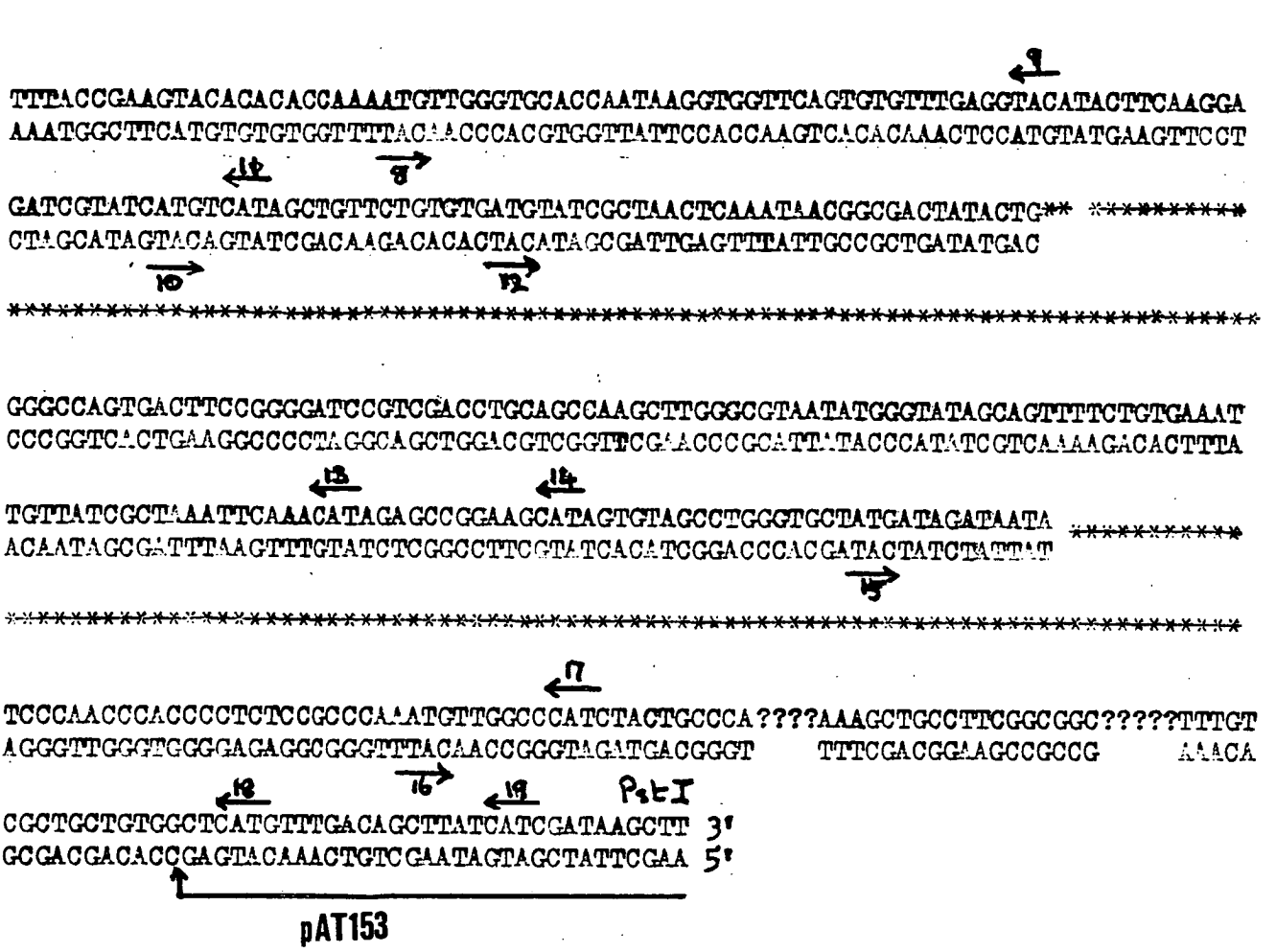
ATATCGGCAAGCGCGGTCAACAAAGTCTCTCTTATGAAGTATTCCTTTGCTTCAGATGGTGAAGCATTTCGCCACGT
TATAGCCGTTCGCGCCAGTTGTTTCAGAGAGAATACTTCATAAGGAAACGAAGTCTACCACTTCGTAAAGCGGTGCA

TTAGAGTTTGAACGTGA**********
AATCTCAAACTTGCACT

****

CCAGTGCCAAGCTTGGCTGCAGGTTTGCCCGTGGTCAACTTGGACACCAGATTAGACTACCGTGTCATTGACTTGAG
GGTCACGGGTTGAACCGACGTCCAAACGGGCACCAGTTGAACCTGTGGTCTAATCTGATGGCACAGTAACTGAACTC

ACCGTCACCAACCAAGCTATTTTAGGATTCCAAGCTGGTGTTTGTGAGCTGTTCAGAGAATACTTGGCCACAAAGAAA
TGGCAGTGGTTGGTTCGATAAAATCCTAAGTTCGACCACAAACACTCGACAAGTCTCTTATGAACCGGTGTTTCTTT



tosine A=adenine X=unknown base

anosine T=thymine ?=secondary structure interference

possible AUG open reading frames are underlined and numbered

CHAPTER 4. DISCUSSION

1. FUNCTIONAL EXPRESSION BY GENES OF FOREIGN ORIGIN IN BACTERIA

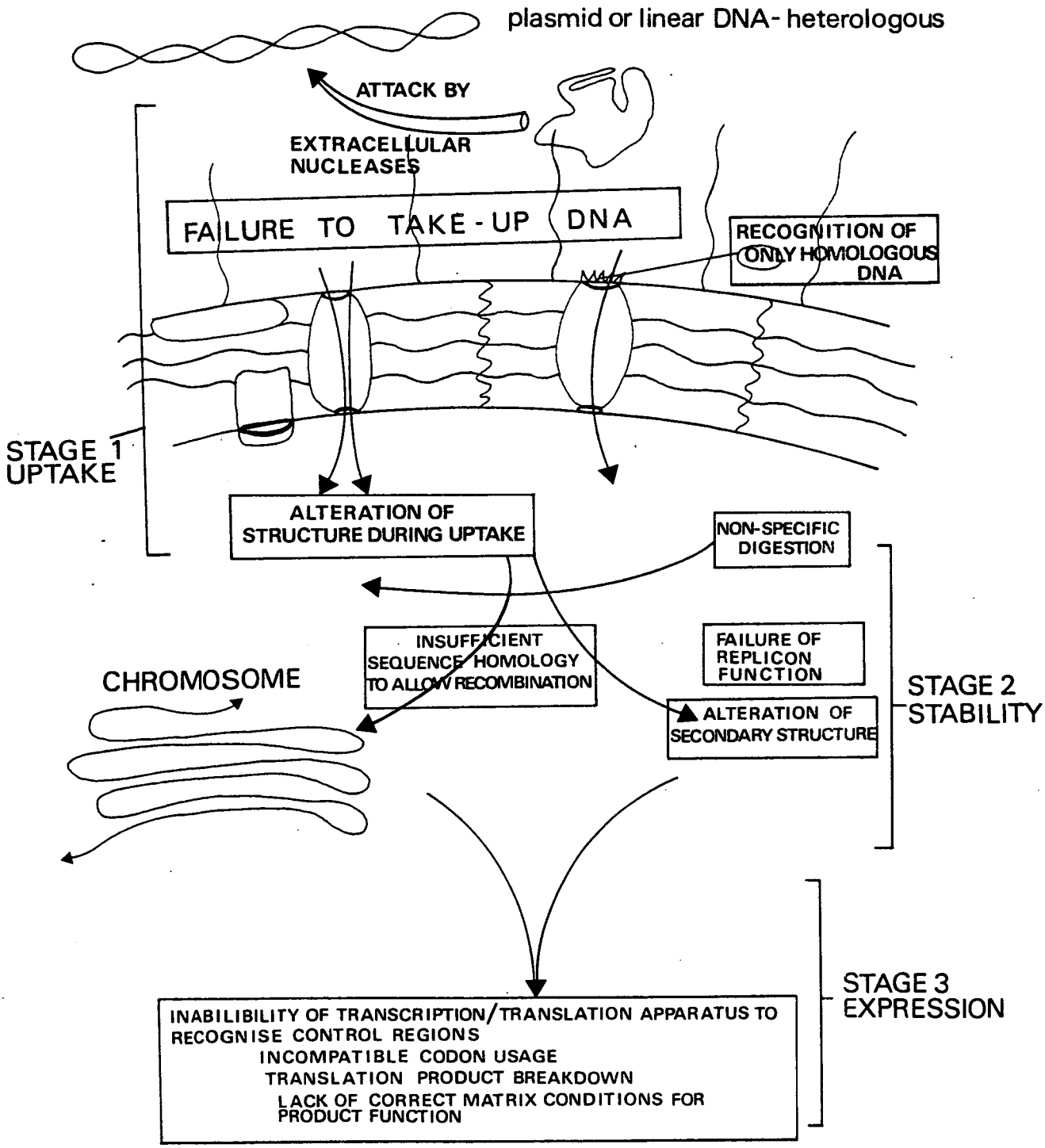
1.1 Barriers to Gene Transfer

The information embodied in the DNA primary sequence of any organism basically determines the latter's developmental and phenotypic traits. Due to the universal usage of the DNA molecule to encode these data, it should theoretically be possible for any gene to function within foreign hosts of widely different phylogenetic backgrounds. That this is not the case indicates the existence of factors which modulate this simplistic view of DNA expression. The studies of gene structure and function in bacteria have largely been dependent upon the use of interspecies 'vectors' capable of function in many different bacterial species (Reanney, 1976). The basic barriers to the successful incorporation of foreign DNA into a bacterial model system are shown in Figure 4.1. Superimposed on these may be a myriad of developmental processes particularly relevant when considering eukaryotic/prokaryotic gene transfers, although as mentioned previously (section 2.2) even the 'simple' bacterium may display a variety of differentiational controls.

1.2 The *Deinococcus* gene organisation - an enigma?

The organisation of the genetic code and the levels at which gene expression may be regulated in the genus *Deinococcus* has never previously been studied. As trans-

FIG. 4.1 FACTORS ANTAGONISTIC TO SUCCESSFUL FUNCTIONAL EXPRESSION OF FOREIGN GENES INTO A MODEL BACTERIAL CELL.



formation of D. radiodurans R1 and Sark cells with genetically 'marked' homologous DNA had already been demonstrated at relatively high frequency (Tirgari and Moseley, 1980) and endogenous plasmid molecules shown to exist in all Deinococcus species except D. radiodurans R1, it was hoped that the development of plasmid vector systems for genetic manipulation studies would be possible. It is obvious from my results that conventional vector systems, whether via conjugation or transformation, were ineffective, even though a wide range of selectable markers and replicons derived from both Gram-positive and Gram-negative bacterial sources were used.

It is perhaps not surprising that conjugation between such unlikely bedfellows as E. coli or S. faecalis and D. radiodurans did not work. This process is a highly specific mechanism, involving cell to cell contact, for the introduction into the recipient of genetic information. Genetically-distinct conjugation systems exist within Gram-negative bacteria whilst transfer mechanisms in Gram-positive types are distinct biochemically (Willetts and Wilkins, 1984; Clewell, 1981). The failure of both E. coli and S. faecalis conjugative systems to function with D. radiodurans as a recipient could have been due to a breakdown in the complicated DNA processing necessary, involving initiation of DNA transfer, successful cell/cell contact and binding, separation of the two plasmid strands, strand transfer, conjugative DNA synthesis in both donor and recipient cells and recircularisation of the transferred

plasmid. This situation would be further complicated by the presence in D. radiodurans of an elaborate cell envelope (Thornley et al., 1965) as well as to the enzymatic and expressional barriers against the addition of foreign genes to the cell phenotype. The obvious avenue of information transfer into a member of the genus Deinococcus, i.e. transformation, appeared refractory to all the standard or specially constructed plasmid vectors used. The failure of such a wide variety of plasmids to express their selectable markers could have been due to any one of the inhibitory factors described in Figure 4.1. Attempts to transform D. radiodurans R1 with hybrid recombinant plasmids consisting of D. radiodurans marked chromosomal DNA allied to either E. coli or D. radiophilus replicons have also failed (Mackay, 1984; G. Al Bakri, personal communication). Also, the plasmid pUE1, isolated from D. radiophilus, has been successfully cloned into pAT153 and mapped using restriction enzymes (Al Bakri, Mackay and Moseley, personal communication) yet did not successfully transform.

D. radiodurans R1

Homologous DNA is taken up by D. radiodurans R1 and Sark, recombination producing highly variable levels of transformation depending upon the marker selected. 'Dominant' genes such as rifampicin resistance transform at a high frequency, in the region of 10^{-2}, and express the marker relatively quickly ($\sim$ 2 hours) whilst 'recessive' markers such as streptomycin resistance may only reach a

frequency of 10 000 fold less after many more hours of phenotypic lag. Whether such behaviour, thought to be associated with the multigenomic nature of the Deinococcus cell (Tirgari and Moseley, 1980), would have an adverse effect upon heterologous DNA suppression associated to an independent replicon is debatable. It is quite reasonable to assume, however, that a recessive marker inserted into a plasmid would never reach the homozygous state required for phenotypic selection. The cell always carrying one or more copies of the 'sensitive' dominant allele upon the chromosome. Such a recessive marker could only succeed in altering the cell phenotype by integration into the chromosomal allelic site followed by segregation until the homozygous state was achieved.

Previous DNA labelling experiments (Tirgari, 1977) have shown that E. coli plasmid DNA is taken into D. radiodurans from the surrounding matrix. Thus, there is no selection of homologous DNA for uptake as is seen in Haemophilus influenzae (Danner et al., 1980). Although it has been shown that both transformable strains of D. radiodurans secrete highly active non-specific nucleases into the growth medium, such behaviour has little effect upon the transformation process. No alteration to heterologous plasmid structure could be demonstrated during the short time period prior to DNA uptake, using the standard transformation protocol, presumably because most enzyme activity would be removed during resuspension of the bacteria

into fresh medium. Nuc^- mutants lacking the extracellular nuclease activity showed no difference in response to transformation compared with the Nuc^+ wild type.

The structural conformation of the transforming DNA in the extracellular matrix is unimportant. Chimaeric DNA constructed from *E. coli* plasmid and *D. radiodurans* R1 chromosomal DNA will transform the cell at the same frequency whether originally in covalently-closed-circular or linear form (G. Al Bakri, personal communication).

The binding and uptake of foreign DNA itself appears not to be a problem. However, for successful plasmid marker expression in a host having little DNA homology with the plasmid, and therefore, no recombination, stable independently-replicating molecules would have to be produced. Although any DNA can be taken up by the cell, major structural alterations to plasmid integrity may occur during the uptake process, perhaps in a manner analogous to the *B. subtilis* system (Piechowska and Fox, 1971). Both transformable strains of *D. radiodurans* were shown to have a Ca^{2+} dependent nuclease associated with the cell membrane fraction. As transformation in these organisms is vastly stimulated by the presence of this divalent ion, but no other, in the medium, it is not impossible that this enzyme is closely linked to DNA uptake and processing. Thus, the transformation frequency obtained using homologous DNA of size <2kb containing a selectable marker can be greatly increased by ligating the transforming DNA into a non-homologous

E. coli plasmid DNA which is then linearised (G. Al Bakri, personal communication).

Another major problem facing molecules attempting to retain circular integrity during uptake by D. radiodurans R1 is the presence, in the latter, of the restriction endonuclease MraI (Wani et al., 1982). The host chromosomal DNA is modified in such a manner that it is protected from the enzyme. However, chromosomal DNA cloned into E. coli plasmid molecules and replicated within E. coli is not modified and so no longer retains this intrinsic resistance. Such a restriction/modification system may play a fringe role in the inability of foreign plasmids to transform D. radiodurans R1 successfully. The apparent absence of such enzymes in D. radiodurans Sark and the lack of MraI sites in some ineffective plasmid vectors, e.g. pBR322 suggests that other factors are of more importance.

It is clear that at least two members of the Deinococcaceae, D. radiodurans R1 and D. radiophilus contain type II site-specific endonucleases. The various theories for the biological role for such enzymes in vivo have been aired in the Introduction, section 4.6. Perhaps no simple empirical raison d'être exists. No bacteriophages have been found capable of parasitising any member of this genus. Conjugation does not seem to be a viable proposition and transformation unlikely to be of biological importance in the natural environment. Therefore, the persistence of such enzymes purely as a defense mechanism against incoming DNA

appears slightly perverse, particularly in the case of D. radiophilus where at least two and possibly three separate systems occur. The balance of methylation modification and gene expression has clearly been demonstrated in eukaryotes and the novel method of DNA modifications seen in these two organisms may have a vital role to play in gene control. Also, the association of site-specific enzymes SceI and SceII in yeast DNA recombination may be mirrored in the highly complex genomic organisation found in the Deinococcaceae. That comparable enzymes have not been found in the other genus members may be a function of experimental ineptitude rather than non-existence.

Unlike D. radiophilus the blue-gree alga Apanothece halophytica, the source of the DraI isoschizomer AhaIII, is very difficult to grow and the yield of purified AhaIII is very low (Whitehead and Brown, 1982). Therefore, although AhaIII was commercially produced it was rapidly superseded by DraI since D. radiophilus is easily grown in liquid culture and produces large amounts ($\sim$ 200 000 units g^{-1} wet weight cells) of enzyme which can rapidly be purified into stable preparations. The enzyme recognises a sequence containing only A:T bases and this property in itself boosts the importance of its discovery. Very few enzymes behave in this manner; previously only AhaIII, modified EcoRI (Woodbury et al., 1980) and the tnpR gene product of the transposon Tn3 did so (Reed and Grindley, 1981). The dearth of available enzymes showing this property has hindered the

expansion of DNA/protein studies particularly into the highly A:T-rich eukaryotic DNAs. Here, due to a marked degree of asymmetry in A:T distribution large regions of G:C rich DNA may be produced.

DraI, unlike the vast majority of restriction endonucleases does show a high level of activity against bacteriophage T_4 DNA. The DNA of T-even bacteriophages is characterized by the presence of 5-hydroxymethylcytosine glucosylated at either the α or β position. This glucosylation renders the DNA resistant to most restriction endonucleases but has no effect, theoretically, upon such enzymes acting only at T:A DNA. Digestion of T_4 DNA by DraI does occur but although the same pattern is produced upon semi-glucosylated DNA (β position only) my inability to obtain T_4 DNA free of glycosyl adducts prevented clear proof of complete digestion of the original T_4 DNA by DraI. It was shown that increasing the DraI enzyme concentration from 1 to 5 units or increasing the incubation period to 5 hours had no effect upon the T_4 DNA digestion pattern.

The interesting behaviour of DraI upon ultraviolet irradiation of the substrate DNA has been used to accurately control the partial digestion of DNAs required for cloning. Assuming random lesion production within the DNA, varying the ultraviolet dose can reduce the number of DraI sites in a population of DNA molecules. Previously, partial digestions had been achieved by lowering the amount of enzyme

per μg DNA to well below one unit. However, the easily-controllable technique of ultraviolet lesion inhibition affords some obvious advantages but previously the lengthy period of irradiation necessary was a problem. This is no longer true in the case of DraI. The partially digested DNA can be directly cloned into a vector molecule at any blunt-ended restriction site, directly or by using poly(dA) and poly(dT) tailing. This latter method has the advantage of recreating DraI sites at either end of the original insert if correctly done, i.e. poly(dT) tailing of the vector and poly(dA) for the target DNA. To allow gene expression within the insert DNA, the recombinant molecule must be allowed to replicate in an ultraviolet damage repair-proficient strain of bacterium, yeast or cell line. This process would remove any translational/transcriptional or replication block caused by the ultraviolet light-induced lesion but would also reactivate any internal DraI sites.

Also of significance is that the three cleavage sites in pBR322 are centred closely around the single PstI cloning site. This enzyme is frequently used for cloning restriction fragments or cDNA by the G/C tailing technique. Thus, such fragments may be recovered from vector DNA with little flanking pBR322 sequence via DraI digest even though the original PstI insertion sites are no longer operative.

The mode of plasmid replication and partition within the genus Deinococcus is unknown. Attempts to cure both D. radiodurans Sark and D. radiophilus of any of their endogenous plasmids have failed (Mackay, 1984). Spatial

compartmentation to protect plasmid and chromosomal DNA from the many non-specific nuclease activities present within the cells may occur. The absence of such separation might explain the absence of plasmid DNA from D. radiodurans R1. Obviously, exposure of incoming plasmid molecules to non-specific endonuclease activity would be disastrous in terms of molecular integrity if not for the ability of linear DNA to transform. The significant effects of super-helicity of a DNA molecule upon its ability to be efficiently transcribed have been detailed (West and Rodriguez, 1982), but the possible secondary form of plasmid molecules successfully surviving in the Deinococcus cells has never been studied. In the case of vector molecules derived purely from foreign sources, interference with the replicating mechanisms encoded by the plasmid is not uncommon. In B. subtilis, vector molecules not only require selectable B. subtilis markers genes but also a B. subtilis replicon to be present. The lack of transposition from 'suicide' plasmids unable to replicate indicates that the breakdown of plasmid replication machinery is not the simple answer to why no transformants were found. Experiments aimed at developing novel cloning vectors derived from D. radiodurans Sark plasmid DNAs have failed, and the chimaeric pAT153/pUE1 recombinant molecule (pUE109) does not transfer effectively between E. coli and D. radiodurans. Future development of a successful shuttle vector will largely depend upon a detailed understanding of the structure and function of

Deinococcus genes and its replicational apparatus.

If a foreign gene product is successfully translated from DNA it faces extreme difficulty in functioning efficiently in a completely novel environment. Compatible biochemical pathways or modes of anti-bacterial agent resistance cannot be assumed to exist. In the extreme cases where mammalian genes such as somatostatin or inteferon are cloned into bacteria it would be folly to expect them to function normally within these cells. Careful consideration must be given, when analysing evidence for the inability of genes to function, to the type of markers in question. Furthermore, the presence of proteases capable of recognising inessential, mistranslated or foreign proteins may be a possibility.

1.3 Transcriptional Unit Organisation

Fundamental to the successful expression of genes between bacterial species is the compatibility of their transcriptional and translational mechanisms. The basis for these reside around and within the transcriptional units themselves, whether operons or single cistrons, although there may be a degree of developmental control for the purposes of differentiation. The failure to construct shuttle vectors capable of gene isolation and transfer between established bacterial systems such as E. coli or B. subtilis and D. radiodurans precluded the study of the latter's gene structure and control mechanisms directly.

The alternative course involved the construction of Deinococcus genomic libraries in E. coli vectors, maintaining and studying the recombinants mainly in this organism. E. coli is amenable to a variety of cell manipulation techniques and has the advantage that its gene expression mechanisms function successfully upon a wide range of foreign genes from such diverse organisms as B. subtilis (Nagahari and Sakaguchi, 1978), Streptococcus mutans (Jagusztyn-Krynicka et al., 1982), Achromobacter (Levesque and Roy, 1982), Clostridium thermocellum (Cornet et al., 1983) and Thermus thermophilus (Nagahari et al., 1980).

Construction of D. radiophilus (this thesis) and D. radiodurans R1 (M. Mackay, 1984) gene banks were successfully accomplished in parallel. In the case of D. radiophilus the absence of a transformation system and a lack of mutants required the search for structural genes to be limited to those which successfully complemented E. coli lesions. The success in finding a D. radiophilus gene capable of removing leucine dependence in E. coli HB101 opened up the possibility of studying the primary structure of a functional Deinococcus gene. The complementing of the E. coli leuB mutation was later paralleled using the D. radiodurans R1 gene bank (G. Al Bakri, personal communication). Two questions are of central importance:-

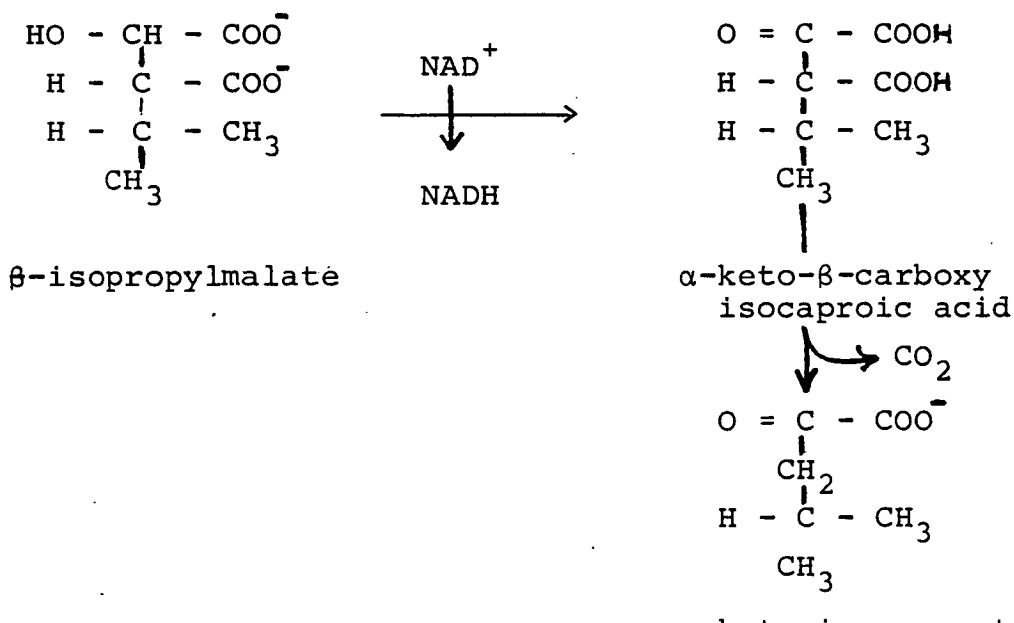
1. Is the product of the coding DNA a protein capable of complementing the β-isopropylmalate dehydrogenase activity absent in E. coli HB101 or is it

a molecule capable of suppressing the actual E. coli lesion itself?

2. Does the insert contain all or most of the regulatory sequences necessary for successful expression both in E. coli and D. radiophilus or is transcription and translation a product of inadvertant read-through from E. coli regulatory regions?

1.4 The leuB gene of D. radiophilus

In E. coli the biosynthesis of leucine is largely effected by the enzyme products of four genes, leuA, B, C, and D. These code for α-isopropylmalate synthetase, β-isopropylmalate dehydrogenase and sub-units of α-isopropylmalate isomerase respectively. The enzyme β-isopropylmalate dehydrogenase catalyses the loss of protons from β-isopropylmalic acid associated to the removal of a carboxyl group,



β-isopropylmalate

α-keto-β-carboxy isocaproic acid

α keto-isocaproate

This activity corresponds to that of the leuC gene product in B. subtilis. Regions of DNA which produce proteins complementing the leuB lesion in E. coli have been successfully cloned from an extremely wide variety of organisms including Salmonella typhimurium (Gemmill et al., 1983), Azotobacter vinelandii (Medhora et al., 1983), B.subtilis (Nagahari and Sakaguchi, 1978), Bacillus No. 221 (Honda et al., 1984) and Thermus thermophilus (Nagahari et al., 1984). Even if the operon organisation present within these various species may vary, expression controls such as attenuation regions (Wessler and Calvo, 1981; Gemmill et al., 1983) and co-ordinate regulation (Travers, 1984), common in amino-acid biosynthetic gene clusters of E. coli and S. typhimurium, could be present. Whether the D. radiophilus recombinant plasmid pleu5 contains other leucine biosynthetic genes as well as the leuB complementing gene and whether such genes would be functional in E. coli can only be shown when leuA, leuC and leuD mutants of E. coli become available (a set of such mutants has been reported (Honda et al., 1984) and a request made for them to be sent, but they have not arrived from Japan). Why the enzyme β-isopropylmalate dehydrogenase should be so amenable as to function in E. coli from such a wide range of organisms can only be speculated upon. From the sequence data from all these organisms allied to that accruing from both D. radiophilus and eventually D. radiodurans R1, the complete primary, secondary and tertiary structures of the proteins should become available. It would

seem unlikely that a protein involved in an amino acid biosynthetic pathway would show great evolutionary conservation. However, certain analogous features associated with the NAD linked dehydrogenase/carboxylase activity may occur.

The sub-cloning rationale followed has allowed the identification of the coding region to an 850 basepair region present in plasmid pTL54. All of the sequentially produced smaller clones pPL3 → pTL27 → pTL54 produce plasmid specific proteins as illustrated by the maxi-cell results (Fig. 3.17).A protein of about 18 000 daltons appears to be the only protein common to all sub-clones but absent in all control lanes. This fits in well with the sequence results. Such a protein would not allow much DNA spare for further proteins or even a tRNA molecule unless overlapping reading frames or transcription of the opposite DNA strand occurred. It would seem that *D. radiophilus* DNA, cloned originally into pleu5 and finally modified to produce an 850 basepair insert in pTL54, encodes an 18 000 dalton protein capable of complementing the leuB gene mutation of *E. coli*.

The question of whether *D. radiophilus* control signals are being recognised by the *E. coli* expression apparatus has been complicated by the sub-cloning routines used. The plasmid pAT153, a derivative of pBR322, has 5 major transcription initiation sites (Fig. 4.2)(Stüber and Bujard, 1981). The original insertion of DNA into the HindIII site should have precluded any initiation from the *tet* promoter, P_2.

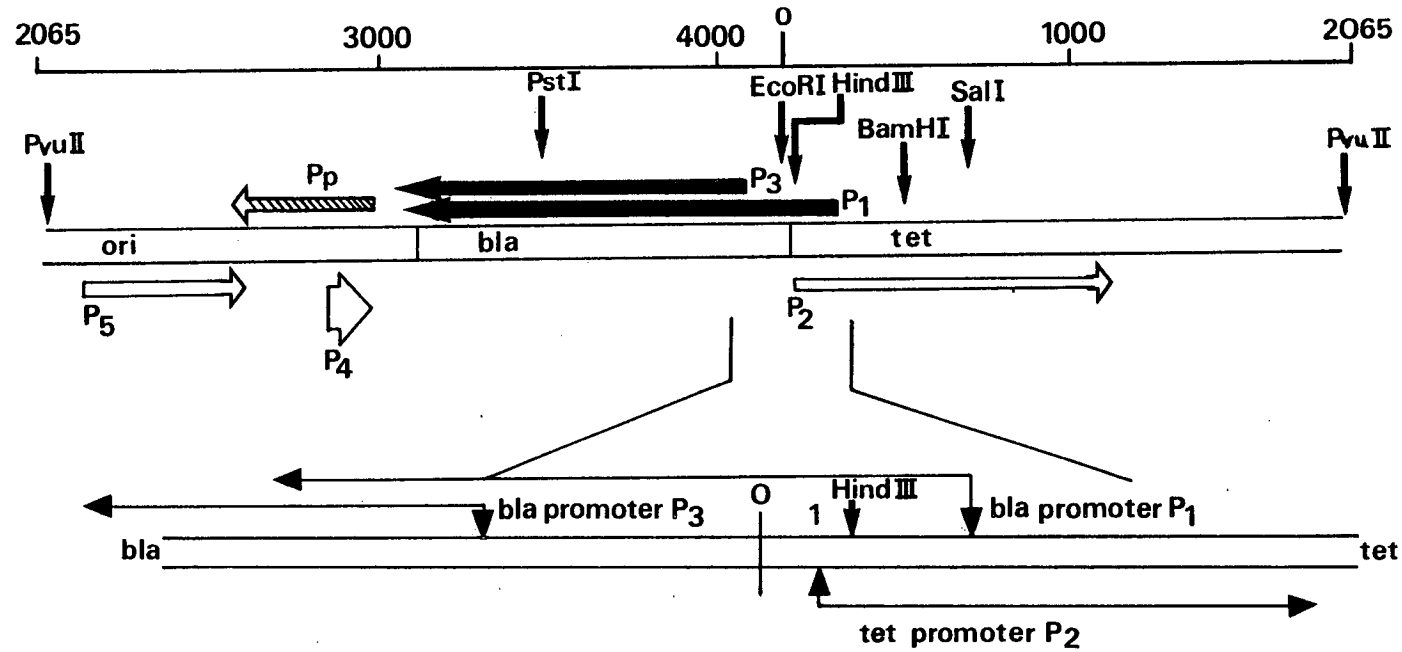


FIG. 4.2 PROMOTER SITES OF pBR322
THE TRANSCRIPTIONAL UNITS ARE DEFINED BY ARROWS, FILLED-IN, (mRNA) CLEAR (PROTEINS) OR HATCHED (transcript which primes DNA synthesis). THE PRESENCE OF P_1 ALLOWS TRANSCRIPTION OF RNA FROM DNA INSERTED INTO THE Hind III SITE. INSERTION OF DNA INTO THE 4000→0 REGION RESULTS IN SUPEREXPRESSION OF THE tet GENE INDICATING THE PRESENCE OF FURTHER CONTROL REGIONS IN THIS AREA UPSTREAM OF THE PROMOTER.

However, it is still possible that the strong bla gene promoter $\underline{P}_1$ situated to the right of $\underline{P}_2$ was operating, thus transcribing into the right side of the leu5 insertion. As the leuB region is situated only 1600 basepairs away from this promoter it is not impossible for read-through to occur. When the 4.01kb PstI/PstI fragment of pleu5 was sub-cloned into the PstI site of pAT153 it may have come under the influence of both bla promoters although one might have expected a fused protein product. The only orientation successfully achieved matches the direction of transcription that would result from $\underline{P}_1$ into the leu5 insert. However, orientation of inserts is strongly influenced by secondary structure and it is not uncommon for one orientation to be favoured many-fold over its opposite. The plasmid pPL3 directed the production of three insert-specific proteins of molecular weights 50 000, 18 000 and 14 000 daltons. Therefore, if transcription was initiating at the bla promoters at least the internal translational signals of D. radiophilus must have been directing the production of three separate proteins only one of which could have been a fusion product whilst another was a functional protein. However, during the sub-cloning which produced pTL27 the insert was forcibly re-orientated by the technique used, the truncated insert being cloned directly behind the $\underline{P}_1$ promoter. The methodology of isolation and selection would, of necessity, produce the correct relative positioning between gene and E. coli

promoter. The inability to successfully remove the HindIII/ BamHI region (23-350) without destroying the *leu*$^+$ phenotype tends to indicate that the insert was dependent upon P_1 for initiation of transcription, the Bal31 nuclease sequential removal of bases allowing the correct alignment between promotion and translation. If this is correct then a *D. radiophilus* promoter must be responsible for transcription of *leuB* genes in both pPL3 and pleu5 as in both cases *E. coli* promoters would have led to the transcription of the non-coding strand. This problem may be resolved by studying the sequence data of the 850 basepair essential region. It should be noted that in clones pTL27 and pTL54 the deletion of the PstI (2857) → EcoRI (o) fragment of pAT153 leads to hyper-expression of the tet gene indicating that this region normally has a negative-controlling role.

1.5 The sequence of the leuB gene of *D. radiophilus*

Nearly 2/3 of the DNA region of interest has been sequenced (Fig. 3.19). Even this would not have been possible without the massive assistance of Dr Scaife's group and in particular Dr M. Mackay. As DNA sequencing, particularly of such large stretches of DNA is very expensive and the MRC has decided to terminate Dr Moseley's grant, not only will sequencing of the remaining 3/4 of unused templates for the leuB clone of *D. radiophilus* (almost certainly covering the final 1/3 of sequence remaining) be prevented but also that of the clones isolated by G. Al

Bakri of the uvrC, uvrD, uvrE, leuB, trp and asp genes in D. radiodurans.

Obviously, drawing clear conclusions from partial data can be dangerous. In the sequenced regions of the leuB insert there exist 19 possible sites for protein synthesis initiation (if one assumes an AUG initiation codon). Of these, 6 are in an area deemed inessential for mutation complementation. In the remainder only one appears to have no stop codon (UAA, UAG or UGA) closely associated in phase. This start codon has been denoted 13 in Figure 3.19 and resides round 150 - 250 basepairs to the left of the HindIII insertion site. More detailed examination of this region is shown in Figure 4.3.

As can be seen 21 basepairs upstream of this open reading frame is a possible site of transcription initiation ($C\overset{*}{A}T$). Also preceding this site at -5 to -9 is the heptanucleotide TATTATC which closely resembles the consensus sequence of TATAATG for E. coli promoters at this region (5 out of 7 bases in the same position). Unfortunately, the -35 region lies in an unsequenced area of the insert. Following the AUG initiator codon is a region of 32 amino acid codons in frame without a terminator codon, however the DNA moves again into an unsequenced region. These similarities to E. coli may be coincidence, without the full sequence data it is impossible to tell as the correct initiation site may lie in an unsequenced area. However, if this is not the case then two points are obvious,

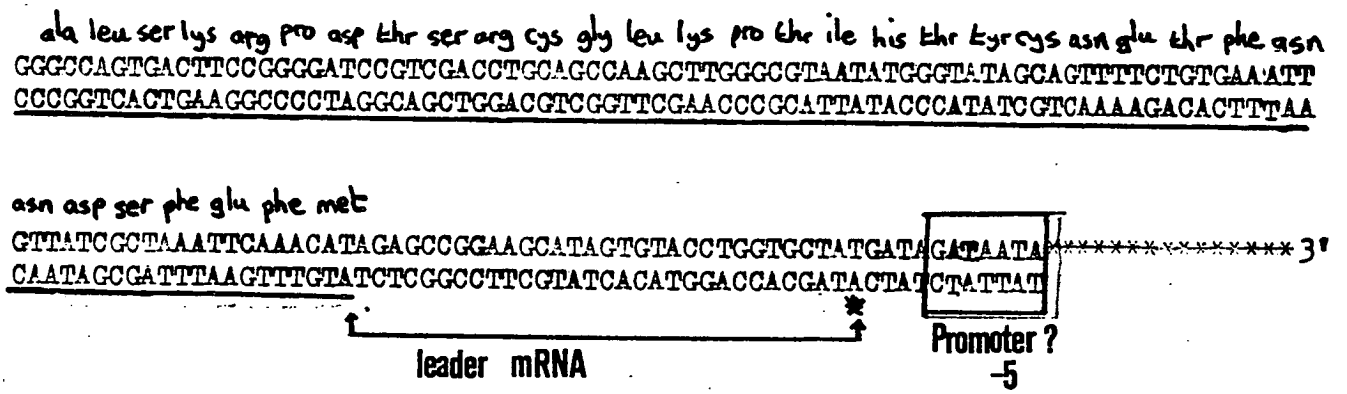


Fig.4.3 Possible open reading frame for the leuB gene of D.radiophilus .The amino-acid sequence is present above the appropriate nucleotide triplet. Initiation of mRNA synthesis is indicated by the * below the sequence.

firstly the E. coli promoter in the pAT153 molecule is not essential and secondly no attenuation site occurs in the leader mRNA sequence (reinforcing the maxi-cell information upon the constitutive protein production in the presence and absence of leucine in the medium).

1.6 Where Are We Now?

It has been shown that at least one D. radiophilus gene, that coding for β-isopropylmalate dehydrogenase, can functionally express in the novel environment of the E. coli cell. The D. radiophilus R1 gene also behaves in a similar manner. No absolute conclusions can be drawn as to the nature of any 'consensus' sequences associated with Deinococcus genes until numerous other examples have been sequenced and analysed. However, the successful transcription of these two genes does at least give a foothold into the investigation. The absence of complementation of other E. coli mutations is attributable either to the failure of foreign protein products to harmonise into an unusual biological matrix or to the existence, as in B. subtilis, of many types of promoter/translation systems for developmental considerations.

From this study we can attribute the inability to select for successful foreign gene expression in the Deinococcaceae to no single factor. Undoubtedly, the unknown aspects of DNA uptake and processing are important particularly in view of plasmid integrity. The need for a known,

functional D. radiodurans replicon capable of insertion into vectors for these transformable species is vital. Although restriction of incoming DNA should be a consideration it may only play an important role in future work on D. radiodurans R1 and D. radiophilus. Possibly the most important factors are the differences between DNA expressional apparatus and the choosing of a selectable marker capable of function.

From this, and the closely-associated studies of M. Mackay and G. Al Bakri, however, it may soon be possible to overcome most if not all these difficulties. Markers have been isolated, leuB, both from D. radiophilus and D. radiodurans capable of effective selection in both the Deinococcaceae and E. coli. A vector, pUE109 (pAT153/pUE1) has been constructed that is capable of existing in E. coli and carries a Deinococcus replicon. From the sequence data it should be possible to localise the control mechanisms associated with the leuB regions and manipulate them so that any gene of choice can receive a promoter capable of directing transcription in E. coli and D. radiodurans or D. radiophilus. The major obstacles remaining are:-

1. The isolation and use of a D. radiodurans plasmid replication origin.
2. Ensuring transformation does not affect plasmid integrity.
3. Development of transformation and mutations in D. radiophilus.
4. Purification of DraI and MraI modifying enzymes to ensure the limitation of the effect of restriction.

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