ASPECTS OF SHUTTLE VECTOR CONSTRUCTION IN *DEINOCOCCUS RADIODURANS* AND PURIFICATION OF ITS AP ENDONUCLEASE

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DECLARATION

I hereby declare that this thesis has been composed by myself and that all the work reported is my own.

Ian Masters

ABSTRACT

An attempt was made to develop a shuttle cloning vector for Deinococcus radiodurans and Escherichia coli using a chimaeric plasmid, pUE109, constructed from a cryptic plasmid from Deinococcus radiophilus and pAT153 from E. coli. However, the ampicillin and tetracycline resistance genes on pAT153 were not expressed in D. radiodurans, whilst a chromosomal gene for mitomycin C resistance (mtcA) from D. radiodurans and incorporated into the chimaeric plasmid, although capable of expression in a sensitive mutant strain, integrated into the chromosome. The rest of the plasmid, having no homology with the D. radiodurans chromosome, was not integrated.

New strains of Deinococcus were isolated from wet soils, providing more evidence that soil is the likely natural habitat of these organisms. Four of the new strains have provisionally been identified as D. radiodurans by other workers, but they were not transformable to rifampicin resistance using DNA from a rifampicin-resistant D. radiodurans R1. During this study, no bacteriophage active against any species of Deinococcus was found.

All the new strains showed the high resistance to ultraviolet light and production of catalase typical of the genus. Plasmids were found in a number of strains and a rapid screening method was

developed to demonstrate the presence of type II restriction endonucleases in crude extracts. Isoschizomers of the restriction endonucleases PvuI, XhoII and BstEII were isolated from strains 5/3, 4/9 and 6/1 respectively.

DNA-DNA hybridization studies suggested that the D. radiodurans R1 gene mtcA and the enzyme UV endonuclease α are highly conserved throughout the genus, but the uvsC, D and E genes, and therefore the enzyme UV endonuclease β, have diverged greatly, or possibly been lost by some species, assuming it was present in the first place. The mtcA mutation could not be transferred from D. radiodurans R1 to D. radiodurans SARK even though they are closely related and the mtcA probe hybridized to a DNA fragment of SARK.

The major AP endonuclease of D. radiodurans was purified free from exonuclease and DNA glycosylase activities and characterized. It has an approximate molecular weight of 34,500 which falls into the size range of the AP endonucleases so far described, but does not require divalent cations for activity. Evidence for base excision repair pathways in D. radiodurans was obtained from studies of enzymatic activities towards methylated DNA and DNA containing uracil. This suggests the presence of a methyl-DNA glycosylase and a uracil-DNA glycosylase.

CONTENTS

CONTENTS

CHAPTER 1

INTRODUCTION

1. The Genus Deinococcus

1.1 Occurrence and taxonomy

In 1956 Anderson et al. isolated a red pigmented, non-sporing Gram positive (Gram +ve) bacterium from meat, at a canning factory in Oregon, that had been irradiated with 2 to 3 Mrad gamma rays (Anderson et al., 1956). Doses of 6 Mrad were required to sterilize a pure culture. The bacterium was named Micrococcus radiodurans but has since been renamed Deinococcus radiodurans R1, meaning a strange or unusual (deinos) berry (coccus) that is radiation (radiatio) enduring (durans).

D. radiodurans has been isolated on four other occasions. It was shown to be present on the hides of the live cattle, grass, and in the creek water near the canning factory (Krabbenhoft et al., 1965). It was isolated as an aerial contaminant in a hospital (Murray and Robinow, 1958) and this strain was later designated Deinococcus radiodurans SARK after it was shown to differ significantly from D. radiodurans R1. D. radiodurans was also isolated in Japan from gamma-irradiated sawdust (Ito, 1977) and from irradiated suture material in Sweden (Osterberg, 1974). Other isolations, thought to be D. radiodurans have been made from soil (Erikson and Emborg, 1978), chicken (Welch and Maxcy, 1979), ground peat (Parker and Vincent, 1981), household dust and clothing (Christensen and Kristensen, 1981; Kristensen and Christensen, 1981).

Three other species of the Deinococcus genus have been isolated which show the same general characteristics of D. radiodurans. D. radiopugnans (formerly M. roseus ATCC 19172) was isolated from

irradiated haddock tissue (Davis et al., 1963) and from weathered granite (Counsell and Murray, 1986). D. radiophilus (formerly M. radiophilus) was isolated from irradiated Bombay duck (Lewis, 1971) while D. proteolyticus (formerly M. radioproteolyticus) was isolated from irradiated Llama faeces (Kobatake et al., 1973) and from animal feed and sewage (Ito et al., 1983). A fifth member was proposed for the genus viz D. erythromyxa, but has recently been excluded after studies on its phospholipids and peptidoglycan layer (Counsell and Murray, 1986).

Deinococcus spp. do not seem to occupy a natural niche. The areas of isolation do not explain the high resistance to radiation. It may be that the resistance is a redundant quality developed for an environment millions of years ago when the atmosphere differed from today and higher levels of radiation reached Earth. They are resistant to desiccation over long periods (R.G.E. Murray, pers. comm.) and may have lived in an area prone to long dry spells before spreading. It has even been suggested that they have arrived from space where they would have to survive both desiccation and high levels of radiation for very long periods of time (Hoyle and Wickramasinghe, 1981). The most likely habitat seems to be soil with other sources being soil-contaminated.

Classification of the genus proved difficult as it has few positive characters. It was aligned with the genus Micrococcus because of strong phenotypic resemblance to M. roseus, ie Gram +ve cocci which are oxidase and catalase positive aerobes. Reclassification was shown to be required after further taxonomic studies (Baird-Parker, 1965) and this was eventually implemented (Brooks and Murray, 1981).

The Deinococci are Gram +ve but resemble Gram -ve bacteria in biochemical and ultrastructural characters (Brooks et al., 1980) with the cell envelope shown to be complex in detail unlike a classical Gram +ve envelope. Studies on the cell envelope of D. radiodurans showed that it also varied from Gram -ve bacteria (Work and Griffiths, 1968; Thornley et al., 1965; Schleifer and Kandler, 1972, Sleytr et al., 1973; Lancy and Murray, 1978; Baumeister and Kubler, 1978; Thompson and Murray, 1981; Baumeister et al., 1986). Differences were also seen with studies on D. radiophilus and D. proteolyticus (Sleytr et al., 1976). The peptidoglycan layer of all the species contains the unusual amino acid composition L-ornithine-glycine$_2$ (Work, 1964). L-ornithine occurs rarely in Gram -ve cell walls with lysine or pimelic acid being more usual. The genus does not possess conventional bacterial phosphatides and shows many unidentified phospholipids (Rebeyrotte et al., 1979; Thompson et al., 1980; Anderson, 1983; Counsell and Murray, 1986). The first instance of a phosphoglycolipid isolated in nature from D. radiodurans has been reported (Anderson and Hansen, 1985). The fatty acid profile of D. radiodurans is similar to Gram -ve bacteria possessing mainly palmitoleate (Knivett et al., 1965) but no oleic acid (Girard, 1971). The Deinococcus species appear to be unusual Gram -ve bacteria concealed under a Gram +ve staining layer.

The most significant data for isolating the Deinococcus genus into a novel taxon came from 16S rRNA oligonucleotide cataloguing (Fox et al., 1977). Between species in the genus, association coefficients of 0.51 to 0.63 were found as compared to 0.29 or less for those with other Gram +ve and Gram -ve bacteria (Brooks et al., 1980). This implies that the group, Deinococcus, diverged from the

mainstream of prokaryotic phylogeny early on. Speciation was also a relatively early event though obviously occurring at a later date. The early speciation within the genus is apparent from the divergence seen in the DNA homology between Deinococcus species where there is less than 20% homology except for that between the R1 and SARK strains of D. radiodurans which have 33% (Brooks et al., 1980).

Recently, some relatives of Deinococcus have been isolated. Thermus aquaticus and T. ruber have the same peptidoglycan type but show little relationship from the 16S rRNA oligonucleotide data (Hensel et al., 1986). Deinobacter grandis shows an especially close relationship with Deinococcus radiodurans, being more closely related to the latter than are other species of Deinococcus, through 16S rRNA oligonucleotides, ornithine-glycine$_2$ peptidoglycan, fatty acids and resistance to gamma irradiation (Oyaizu et al., 1987), even though it is rod shaped and Gram -ve.

Deinococcus species have a doubling time in broth of approximately 80 minutes and it takes two days before colonies are visible on agar plates. In liquid culture the majority of D. radiodurans cells occur as diplococcus forms (Hansen, 1978). On agar, cells divide in two planes to produce sheets of cells which fracture due to friction with the agar surface (Driedger, 1970; Moseley and Copland, 1975a). A chemically-defined medium has been described (Little and Hanawalt, 1973; Shapiro et al., 1977; Tirgari, 1977) which allows for isolation of auxotrophic mutants, but growth is quite slow.

1.2 DNA of Deinococcus

Table 1.1 shows the DNA composition of Deinococcus species. The genome size was calculated from DNA renaturation kinetics (Hansen, 1978; Purvis and Moseley, umpublished results) and the occurrence of multiple genome equivalents per cell was inferred from these data. There are four genome equivalents in resting phase cells and 8 to 10 in cells growing exponentially with a doubling time of 80 min. Transformation studies with D. radiodurans showed that the genome equivalents were discrete and not confined to one large chromosome (Tirgari and Moseley, 1980), and that all the genomes were resident in a single nucleus (Moseley and Evans, 1981).

The base composition of D. radiodurans and other species was shown to be high for guanine plus cytosine (Moseley and Schein, 1964; Brooks et al., 1980). Alternating d(GC) sequences favour the formation of Z-DNA, and a Z-DNA-binding protein, which converts Z-DNA to B-DNA, has been isolated from D. radiodurans (Kitayama et al., 1985). The conformation of the DNA may be important in gene expression and DNA repair, eg the imidazole ring-opened form of 7-methylguanine is not excised from Z-DNA in E. coli (Lagravere et al., 1984). The protein does not act on DNA, with 5-methylcytosine present. This is significant because no methylated bases in D. radiodurans have been detected (Schein et al., 1972; Störl et al., 1979).

Two species contain type II restriction endonucleases. D. radiodurans has MraI (recognition sequence 5'-CCGCGG-3') (Wani et al., 1982) and D. radiophilus has DraI (5'-TTTAAA-3') (Purvis and Moseley, 1983), DraII (5'-PuGGNCCPy-3') and DraIII (5'-CACNNNGTG-3')

Table 1.1 DNA content of Deinococcus species

Species	Genome size ($x10^9$ d)[a]	% G+C[b]	Plasmid and size (kb)[c]	Restriction endonucleases
D. radiodurans RI	1.8-2.0[d]	67	pS16 (60.0)[e]	MraI[f]
D. radiodurans SARK	ND	67	pUE10 (37.0) pUE11 (44.9)	-
D. radiophilus	1.5	62	pUE1 (10.8) pUE2 (27.9) pUE3 (92.2)	DraI[g] DraII[h] DraIII[h]
D. proteolyticus	1.8	65	pUE20 (99.4) pUE21 (138.8)	-
D. radiopugnans	2.9	68	pUE30 (2.5) pUE31 (28.6)	-

(a) I. Purvis and B.E.B. Moseley, unpublished results

(b) Brooks et al., 1980

(c) Mackay, 1983

(d) Hansen, 1978

(e) Smith, personal communication

(f) Wani et al., 1982

(g) Purvis and Moseley, 1983

(h) de Wit et al., 1985

ND - not determined

(de Wit et al., 1985; Grosskopf et al., 1985). Wani proposed that MraI was the first restriction endonuclease from a bacterium which lacks methylated bases in its DNA. However, DNA in D. radiodurans and D. radiophilus must be modified in some way as DNA of each species is only cleaved by its respective enzyme after passage through E. coli (Mackay, 1983; Purvis, 1984). Mackay found unusual modified bases in D. radiodurans using thin layer chromatography but they were not characterized.

Cryptic plasmids have been found in all species of Deinococcus (Table 1.1). Unsuccessful attempts to cure plasmids suggest they may carry essential genes (Mackay et al., 1985). It is hoped that these plasmids will form the basis of a plasmid vector system.

1.3 Genetics of Deinococcus

Transformation, first demonstrated by Moseley and Setlow (1968) and improved by Tirgari and Moseley (1980), provides the only method of introducing DNA into D. radiodurans. Other members of the genus are non-transformable which has meant research has concentrated on D. radiodurans.

An extensive search for bacteriophage has proved fruitless, negating the possibility of a transduction system. The introduction of a vector system into D. radiodurans by conjugative plasmids of E. coli and Streptococcus faecalis (Tirgari, 1977; Purvis, 1984) and non conjugative plasmids from E. coli and B. subtilis (Purvis, 1984) by transformation have been unsuccessful. Al-Bakri (1985) developed a procedure for protoplasting and regenerating cells of D. radiodurans but could not demonstrate the fusion of protoplasts

which might have been useful as a means of mapping the chromosome. Transformation has been very useful for strain construction and as a method for distinguishing similar mutant phenotypes to one or more genes (Moseley and Copland, 1975b and 1978). It has also been useful in cloning wild type genes by showing complementation of mutants (Al-Bakri et al., 1985) but it has limitations in its flexibility for gene cloning and chromosome mapping.

Two genes from D. radiodurans and one from D. radiophilus have been cloned and expressed in E. coli. Peters and Baumeister (1986) cloned the HP1 protein of D. radiodurans SARK and showed that its expression was under the control of a weakly recognized Deinococcus promoter. The leuB gene from D. radiodurans R1 (Al-Bakri et al., 1985) and the leu gene from D. radiophilus (Purvis, 1984) have been cloned via complementation of the leuB mutation in E. coli HB101. These results give encouragement for further studies of gene expression. Recently, the cloning of antibiotic markers into the chromosome of D. radiodurans under heavy selection has been achieved (M. Smith, pers.comm.).

1.4 Radiation resistance and DNA repair

Most isolations of Deinococcus species has relied on their resistance to UV (254 nm) and/or gamma irradiation. It is not surprising that much research to elucidate the mechanisms of repair in this group has been carried out. They are also highly resistant to the lethal effects of other DNA damaging agents such as nitrous acid, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), methyl methanesulphonate (MMS), mitomycin C and hydroxylamine (Sweet and Moseley, 1976;

Tempest and Moseley, 1980). The resistance to such agents is not at the cost of a high mutation rate (Sweet and Moseley, 1974; Sweet and Moseley, 1976; Tempest and Moseley, 1982) and has led to the conclusion that an error prone repair pathway is absent from the species. Fortunately, D. radiodurans is mutagenically sensitive to MNNG (Sweet and Moseley, 1976; Rebeyrotte, 1983) and a strain 302, mutant in the mtcA gene is very sensitive to MNNG and MMS mutagenesis (Tempest and Moseley, 1980).

The resistance to UV and gamma irradiation has been attributed to a number of mechanisms. Originally it was thought the red pigment conferred protection but white mutants show equal resistance to wild type (Moseley, 1963; Lewis et al., 1974). The presence of a high sulphydryl content (radioprotective material) was thought to be responsible for resistance (Bruce, 1964) but was later shown to have no relevance (Serianni and Bruce, 1968). The high concentration of manganese around the DNA of D. radiodurans was thought to reduce the number of lesions on the DNA (Leibowitz et al., 1976) but the bacteria are capable of repairing 70,000 pyrimidine dimers per genome (Moseley and Copland, 1978) which represents over 1% of thymine in the DNA. Photoreactivation repair of pyrimidine dimers is absent (Moseley, 1983). It was thought that the multiple genomes present within a single nucleus (Moseley and Evans, 1981) would give greater scope for DNA repair via a recombinational pathway but Harsojo et al. (1981) showed, by varying the number of genome equivalents in a nucleus, that this did not correlate well with UV resistance.

Indirect evidence has implied that recombination plays a role in the DNA repair. The "large shoulder" of D. radiodurans survival

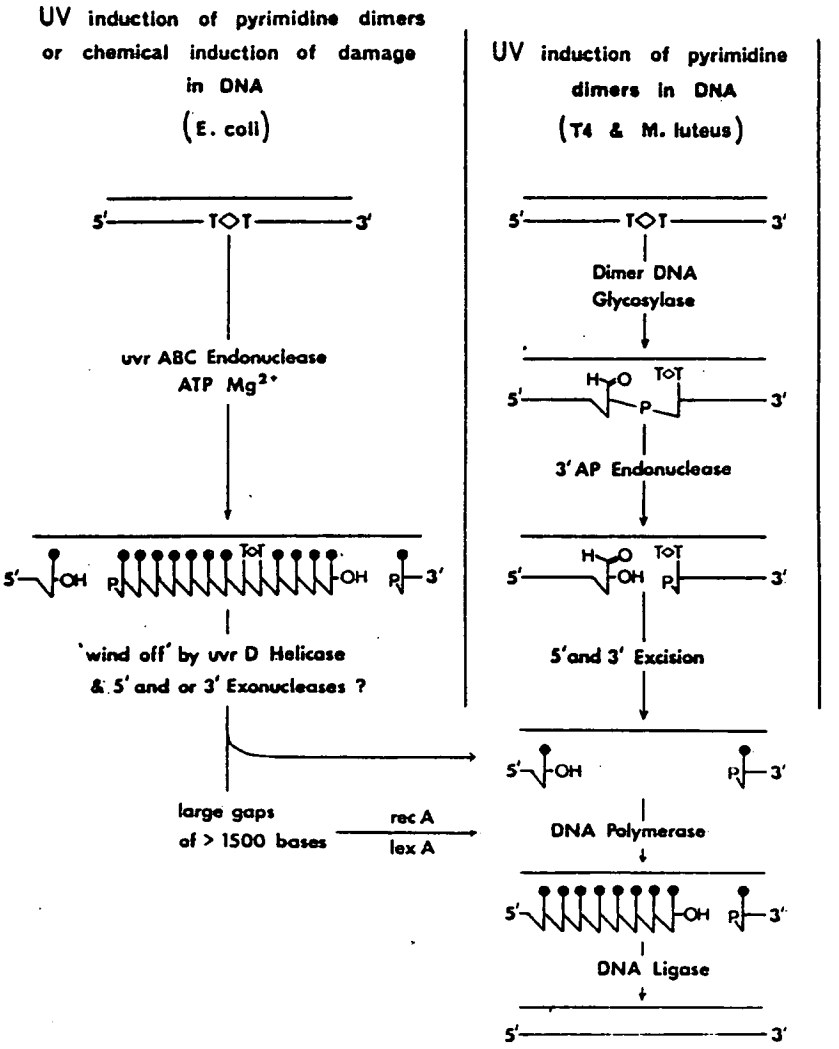
curves is thought partly to be due to recombinational repair as it disappears with transformability and increased UV sensitivity when a temperature sensitive mutant is moved to the restrictive temperature (Moseley et al., 1972). A recombination deficient mutant, rec30, has been isolated (Moseley and Copland, 1975b) which is sensitive to gamma and UV irradiation and to mitomycin C. The mutant lacks transformability and is extremely sensitive to cross-linking agents which are repaired by recombinational pathways.

Direct physical evidence of a DNA repair function is seen for excision repair. Excision repair of UV-induced pyrimidine dimers and excretion of the photoproduct as a small oligonucleotide into the surrounding medium was shown to occur in D. radiodurans as early as 1966 (Boling and Setlow). The same study showed that the repair process operated in the absence of protein synthesis and the repair mechanism therefore works constitutively. Excision repair was later shown to proceed via two pathways (Moseley and Evans, 1983) when double mutations blocking repair via both pathways resulted in a UV sensitive phenotype. The loss of a single pathway did not affect UV sensitivity to any degree with both being equally efficient.

The two excision pathways are thought to work in a similar manner to the UVRABC enzyme complex of E. coli or the M. luteus enzyme (Figure 1.1).

Endonuclease α requires the mtcA and mtcB gene products and an inducible terminator to prevent exonuclease degradation from the endonuclease incision site (Evans and Moseley, 1983). Endonuclease β requires the uvsC, uvsD and uvsE gene products and does not need an inducible terminator (Evans and Moseley, 1983). Endonuclease β has been isolated and characterised from D. radiodurans and requires

Figure 1·1 Summary of DNA excision repair pathways



manganese ions for its activity which distinguishes it from other DNA repair enzymes (Evans and Moseley, 1985). Endonuclease β specifically acts on pyrimidine dimers but endonuclease α recognizes other lesions produced by mitomycin C and alkylating agents.

Three polymerases have been identified in D. radiodurans with one showing exonuclease activity which could be involved in DNA repair and error-proof reading during DNA replication (Kitayama and Matsyma, 1977; Kitayama et al., 1978).

The two excision repair pathways operate constitutively, but there is evidence of an adaptive response in D. radiodurans. Adaptive repair of mitomycin C cross-links has been reported where cells could be preinduced for repair capacity by treatment with low levels of mitomycin C (Kitayama, 1982). Protein synthesis is required for repair of X-ray damage (Driedger, 1971), gamma ray-induced damage (Kitayama et al., 1981), and four proteins were seen to be induced after DNA damage by UV light, ionizing radiation and mitomycin C (Hansen, 1980). Recently it was shown that D. radiodurans is highly sensitive to near UV (300-400 nm) and that repair is inducible by a sublethal dose of near UV (Caimi and Eisenstark, 1986). No inducible protein has yet been ascribed to a particular repair pathway.

Excision and recombination appear to be the main methods of repair for irradiation damage, but the membrane of the cell with its unusual phospolipids may help survival at high irradiation doses. It does not protect the DNA but at high doses D. radiodurans resists radiation-induced membrane damage to a greater degree than do radiation sensitive bacteria (Merrick and Bruce, 1965).

Most DNA repair research in D. radiodurans has centred on

recombinational and nucleotide excision repair with little work done on base excision repair.

2. Cloning Vectors

2.1 Cloning vectors

A cloning vector is an autonomously replicating unit of DNA (a replicon) which can be manipulated in such a way as to insert "foreign" DNA into the unit without preventing its continued propagation in an appropriate host cell. For the DNA to be replicated an origin of replication is required. Most small pieces of DNA do not carry an origin of replication and so have to be cloned into a vector which does carry one. This allows the cloned DNA to be replicated and facilitates the analysis of its organisation and expression. Most cloning vectors are derived from replicons that are stably inherited in an extra-chromosomal state and this includes both plasmids and bacteriophages. The four main types of cloning vector are, plasmids (Bolivar and Backman, 1979), λ bacteriophage (Murray, 1983), plasmid and phage combinations, eg cosmids (Collins and Hohn, 1979) and phasmids (Brenner et al., 1982), and single-stranded DNA bacteriophages (Messing, 1983). In this thesis, only plasmids are of relevance in the construction of a cloning vector in D. radiodurans as there are no known bacteriophages for the species.

2.2 Plasmids

Plasmids have been the most widely used vectors for the cloning of DNA with most work achieved with plasmids which replicate in E. coli. The techniques applicable to plasmids have been developed mainly in E. coli which has proved to be extremely amenable to

manipulation. These techniques are now being applied with great success to other prokaryotes and to eukaryotes.

The structure, function and replication of bacterial plasmids are described in detail by Hardy (1981). There are two main categories of plasmid, conjugative and non-conjugative. Conjugative plasmids can facilitate transfer of a copy of their DNA to a recipient from the host. They are large and are not used for cloning experiments in general. Non-conjugative plasmids vary in size with small plasmids (<40 kb) present as multiple copies (relaxed plasmid) and large plasmids (>40 kb) occurring at 1 to 3 copies per chromosome (stringent plasmid). The replication of stringent plasmids is tightly controlled with replication of the chromosome (Scott, 1984; Nordström, 1984), and they are stably inherited through specific mechanisms (Sherratt, 1986).

Relaxed plasmids are not so tightly controlled in replication and are not coupled with the chromosomes replication (Clewell, 1972), but do have controls to their replication and segregation to daughter cells (Sherratt, 1986).

Plasmids carry a wide variety of phenotypic traits of which the most widely used for cloning purposes are antibiotic resistances. Other phenotypic traits include, antibiotic production, heavy-metal resistance, restriction and modification systems, sugar fermentation, toxin production and virulence factors. Some of these traits have been used to select for plasmid-transformed cells. Some plasmids appear to have no function and are described as cryptic. Related plasmids can be incompatible in a single host cell, leading to elimination of one from the cell. It is useful to cure a host cell of its natural plasmid if a cloning vector is derived from it. This

also allows easier purification of the cloning vector.

2.2.1 The use of plasmids as cloning vectors

The basic cloning vector should possess the following properties:

1. It should be small, so that it is easy to isolate from cells, and less prone to mechanical damage such as shearing.

2. It should preferably replicate in a relaxed fashion to produce multiple copies of the cloned DNA. This helps facilitate analysis of its physical organization, expression of the gene through a dosage effect and allows overproduction of the gene product, although occasionally makes the cell sick.

3. It should carry at least one, and preferably more, selectable marker genes to allow identification of transformants and to help maintain the plasmid in the bacterial population. A doubly-marked plasmid allows for selection of recombinant vectors by insertional inactivation of one.

4. It should contain as many single restriction endonuclease sites as possible in areas not essential for replication or selection.

One of the first cloning vectors used was pSC101 (Cohen, 1973; Chang and Cohen, 1974) but it does not have a high copy number. Plasmid ColE1 and its derivatives were of greater use as they replicate in a relaxed fashion (Clewell, 1972) and can be amplified even further by chloramphenicol treatment (Hershfield et al., 1974).

The most widely used plasmid has been pBR322 (Bolivar et al., 1977) and its derivatives. A summary of these plasmids is given by Balbas et al. (1986). The vector pAT153 used in this thesis was

derived from pBR322 by removal of an HaeII fragment, and has a copy number 1.5 to 3 times higher than pBR322 (Twigg and Sherratt, 1980).

Improvements made to plasmids have allowed easier selection of recombinant plasmids from cloning experiments. Direct selection cloning vectors only produce a viable host cell under appropriate selection when a fragment of DNA is cloned into a specific site (Schumann, 1979; Dean, 1981). Improvements to the low copy plasmid pSC101 (Stoker et al., 1982) has meant that cloning of genes deleterious to the host cell when present in high copy number, is possible. A second method to overcome this problem uses runaway plasmid vectors. Under certain conditions, usually low temperature, these vectors are at low copy but when the conditions are changed to high temperature, control of replication is lost and the plasmid replicates in a relaxed fashion. This produces high gene product yields before the cell ceases to grow or dies (Uhlin et al., 1979). Cloning vectors have also been used to select for promoters, with the expression of a gene on the vector dependent on a promoter sequence being isolated upstream of it (Widera et al., 1978).

Genes from Staphylococcus aureus (Chang and Cohen, 1974), Sacchoromyces cerevisiae (Struhl et al., 1976) and Neurospora crassa (Vapnek et al., 1977) have all been expressed in E. coli and it was thought that transcription and translation mechanisms were universal. As more genes were cloned, this proved not to be the case. The lack of expression was mainly due to non-recognition of promoter sites for transcription and initiation sites for translation. Other minor factors involved codon choice, secondary structure of mRNA, transcription termination and instability of some proteins in the foreign host E. coli.

Many of the problems in *E. coli* have been solved by the construction of special expression vectors. They can carry *E. coli* promoter and terminator sequences which give expression of the gene or the foreign gene can be fused to part of an *E. coli* structural gene, thus stabilizing the resultant gene product (Old and Primrose, 1985).

Plasmid cloning vectors have been developed for other organisms. *B. subtilis* has a number of cryptic plasmids (Le Hegarat and Anagnostopoulos, 1977) but having no phenotypic markers they were useless for cloning. Ehrlich (1977) showed that plasmids from *S. aureus* could be transformed into *B. subtilis* and the antibiotic markers expressed. These were used to construct cloning vectors for *B. subtilis*. Cloning in some of the important antibiotic producing *Streptomyces* spp has been made possible by the isolation of cloning vectors (Hopwood and Chater, 1982). There are also plasmid cloning vectors for microbial, plant and animal eukaryotes. Cloning in mammalian cells though, relies on the SV40 virus to provide an origin of replication.

The majority of technical progress has been achieved with *E. coli* where there are methods and tools to clone almost any DNA fragment. For this reason cloning of genes to be expressed in other organisms is performed in *E. coli* using a shuttle vector (see 2.3). All the manipulation can be done in *E. coli* with the final recombinant plasmid being purified and introduced into the second host species.

2.3 Shuttle vectors

Shuttle vectors are cloning vectors that can be stably replicated in an autonomous manner in more than one host (usually two) belonging to different genera. The criteria for a good shuttle vector are the same as those for a normal plasmid-cloning vector. It requires, in addition, replication origins that work in both hosts, marker genes that allow selection in both hosts and methods of transformation for both hosts. Usually two origins of replication are needed for viable replication in each host, eg see Ishwa and Tsuchida (1984). This is also the case for marker genes, eg see Dao and Ferretti (1985).

Most shuttle vectors use *E. coli* as one of the hosts. This is mainly for two reasons. Firstly, the transcription and translation machinery in *E. coli* can recognize signals from a wide range of organisms, eg *S. cerevisiae* (Struhl *et al.*, 1976), *B. subtilis* (Ehrlich, 1978), *D. radiodurans* (Peters and Baumeister, 1986), *Streptococcus pneumoniae* (Garcia *et al.*, 1986), and *Triticum* (Gatenby *et al.*, 1986). Special promoter sequences can be introduced into the shuttle vector to allow expression of other foreign genes in *E. coli*. This can give high levels of gene product for analysis. Secondly, and more importantly, the methods for cloning and DNA manipulation have reached a high degree of sophistication in *E. coli*. This allows for construction and analysis of recombinant vectors in *E. coli*. Large amounts of cloned foreign DNA can be made which can easily be transformed into the second host.

The use of shuttle vectors is now widely used for bacteria, eg *B. subtilis* and *E. coli* (Ishiwa and Tsuchida, 1984),

Streptococcus mutans and E. coli (Dao and Ferretti, 1985), Brevibacterium lactofermentum and E. coli (Yeh et al., 1986), Anacystis nidulans and E. coli (Gendel et al., 1983) and also for eukaryotes, eg Dictyostelium discoideum and E. coli (Firtel et al., 1985), S. cerevisiae and E. coli (Beggs, 1978) mammalian cells and E. coli (Cartier et al., 1987).

The shuttle vectors have been used extensively to form gene libraries in E. coli. Genes that complement mutations in a second host can then easily be screened for and this facilitates the study of gene regulation and gene expression in the second host, eg see Rapoport et al. (1979). Shuttle vectors have been used to look at expression of a particular gene in both hosts, eg see Hadfield et al. (1987), and to screen for promoters and terminators, eg see Van de Vossen et al. (1985). The mechanism of DNA repair and mutation has been analyzed using shuttle vectors. DNA-damaged vectors are transformed into a host cell and after repair recovered into E. coli where point mutations and other changes can be analyzed (Seidman et al., 1985; 1987). This method would probably be of use in understanding the cause of mutation in D. radiodurans from such agents as MNNG. Shuttle vectors have been put to various other specific uses to solve a particular problem.

2.4 Methodology of cloning

The manipulation of genetic material in vitro has revolutionized the study of gene structure and expression. The techniques required to achieve such manipulation have only been available since the early 1970s. Genetic manipulation involves the construction of new

combinations of genetic material using a vector system which allows propagation and amplification of DNAs within a host. It allows species barriers to be crossed, placing "foreign" genes in an unrelated organism.

Cloning of a DNA fragment requires a vector molecule (see 2.1), a method of cutting and joining DNA (see 2.4.1), a means of introducing the recombinant DNA into a host cell (see 2.4.2), its stable inheritance and a method of screening for transformed cells (see 2.4.2), and a method to monitor and analyze the new recombinant DNA molecules.

2.4.1 Type II specific restriction endonucleases and T_4 DNA ligase

Type II specific restriction endonucleases are enzymes that cut DNA reproducibly into discrete fragments while T_4 DNA ligase covalently joins the ends of DNA fragments together.

Type II restriction endonucleases were discovered in 1970 (Kelly and Smith, 1970) and recognize a specific oligonucleotide sequence which has an axis of rotational symmetry. Different enzymes which recognize the same sequence are termed isoschizomers though cutting within the site of recognition may vary. At the recognition site a double strand cut is made to leave 5' single-strand extensions, eg EcoRI, 3' single-strand extensions, eg PstI or blunt ends, eg NruI. The extensions are also called sticky ends because DNA molecules with the same complementary protruding ends will readily anneal. To date, there have been 603 isolations of type II endonucleases and their recognition sites and their sources are given in the review by Kessler and Holtke (1986).

Host cell DNA is protected from auto digestion by its own restriction endonuclease, by methylation of bases within its recognition site. This means foreign DNA entering the cell is cut if not protected in the recognition sequence. For this reason, cloning in E. coli and other species is performed if possible in restrictionless strains.

It should be mentioned that non-specific cutting of DNA by mechanical shearing (Wensink et al., 1974) has also been used to clone DNA molecules. Removal of protruding ends or filling-in of the recessed strand by S1 nuclease or klenow fragment of E. coli polymerase I respectively allows joining of DNA fragments with blunt ends (Maniatis, 1982).

The formation of stable recombinant DNA molecules by joining can be achieved by either of two methods. The first relies on T_4 DNA ligase, from phage T_4-infected E. coli, to catalyze the formation of phosphodiester bands at a single strand nick between adjacent nucleotides (Olivera et al., 1968). While the ligation of sticky ends is very efficient, blunt ends are ligated much less efficiently, requiring higher concentrations of enzyme and DNA. Joining of DNA cut with the same restriction enzyme reproduces flanking recognition sites of the restriction endonuclease. This means that the cloned fragment of DNA can easily be cut out from the vector with the same restriction endonuclease.

Bacterial alkaline phosphatase or calf intestinal phosphatase can be used to remove 5' phosphate groups from both ends of a linearized vector molecule. This prevents the vector molecule religating (Ullrich et al., 1977) and allows the formation of recombinant molecules only with two nicks being repaired in the host

cell.

A second method of joining DNA is by homopolymer tailing (Michelson and Orkin, 1982). This requires the addition of nucleotides to the 3'OH termini of a DNA fragment. A homopolymer of oligo (dA) on one DNA molecule will complement a homopolymer of oligo (dT) on another. A stable hydrogen-bonded structure forms which can be transformed into a host cell where gaps and nicks are repaired.

Agarose and polyacrylamide gel electrophoresis monitor the cutting and joining steps (Rickwood and Hames, 1982).

2.4.2 Transformation and selection of recombinants

The completion of a cloning experiment requires the DNA to be introduced into a host cell. It can be introduced like viral DNA via transduction (Holn, 1975) but usually transformation is the method used. Because *E. coli* is used extensively as the cloning host, much work has been done to make *E. coli* cells transform efficiently (Hanahan, 1983). Other organisms have proved naturally transformable or methods to render cells transformable, such as protoplasting, have been developed.

Once in the host cell, the presence of foreign DNA is selected for by growing the transformed population of cells on a selective medium. This is usually achieved by conferring antibiotic resistance or complementation of a mutation in the host cell. Indirect selection can be used with nucleic acid by hybridization and immunological techniques (Dahl *et al.*, 1981).

2.5 Problems associated with cloning in Deinococcus

Previous attempts to transform D. radiodurans with a variety of common conjugative and non-conjugative plasmid vectors from Gram +ve and Gram -ve bacteria were unsuccessful (Mackay, 1983; Purvis, 1984) as was the transformation of spheroplasts formed from D. radiodurans and D. radiophilus (Al-Bakri, 1985).

Only the two strains of D. radiodurans have proved transformable (Tirgari and Moseley, 1980) in the Deinococcus genus, and so development of a cloning system has concentrated on this species. The lack of transformation by conventional plasmids may be due to non-recognition of the origin of replication, an inability to recognize the DNA sequences required to initiate transcription and translation of the marker genes, or degradation of foreign DNA by host restriction endonucleases. Wani et al. (1981) showed that MraI in D. radiodurans R1 degrades foreign DNA but not host DNA. Some plasmids used to transform D. radiodurans R1 did not have an MraI site, eg pBR322, suggesting that the host cell restriction endonuclease while probably having an effect is not the major problem. Purvis (1984) tentatively recognized a putative Pribnow box for the D. radiophilus leu gene that complements a leuB mutation in E. coli. A leuB gene (Al-Bakri et al., 1985) and surface (HPI)-layer protein gene (Peters and Baumeister, 1986) of D. radiodurans express in E. coli from their own promoters. It therefore seems that D. radiodurans recognizes signals following the general prokaryote pattern although it may show a higher stringency of recognition of these sequences, as is seen with B. subtilis (Moran et al., 1982; Band and Henner, 1984).

The indigenous cryptic plasmid population of Deinococcus spp could provide an origin of replication but requires a marker gene. Attempts to introduce chromosomal host markers from D. radiodurans strain KRASE into SARK plasmids were unsuccessful. However, the cloning procedure may have disrupted an essential region of the plasmid, though this is unlikely.

Work on species other than D. radiodurans R1 is also hampered by the lack of mutants available.

A plasmid, pUE109, was constructed from pAT153, and the D. radiophilus plasmid, pUE1, which coded for tetracycline resistance (Mackay et al., 1985). This plasmid failed to transform D. radiodurans R1 to tetracycline resistance. Plasmid pUE1 was chosen because of its relatively small size (10.8 kb) and pUE109 gave four unique restriction endonuclease sites, PstI, MluI, SstII (MraI) and DraI.

Failure to transform D. radiodurans may have been due to one or more of the following:

i) degradation of pUE109 by restriction endonuclease cleavage at the SstII site by the indigenous restriction endonuclease MraI followed by exonucleic attack.

ii) failure to express the Tc^R gene at the transcription or translation level.

iii) disruption of the origin of replication site of pUE1 by the insertion of pAT153 at the PstI site of pUE1.

iv) non-recognition of the origin of replication of the D. radiophilus plasmid, pUE1 in D. radiodurans.

v) the gene product of the Tc^R gene does not have the correct site or conditions to function correctly.

pUE109 is still a promising starting point for the construction of a shuttle vector.

Cloning in E. coli is performed in recombination-deficient and restriction endonuclease-deficient strains, eg HB101. The isolation of such mutants in D. radiodurans would be of great use in developing a cloning strategy involving autonomously-replicating vectors. Recently, the successful cloning of antibiotic resistance genes by insertion into the chromosome under selective pressure was attained for D. radiodurans (M. Smith and K. Minton, pers.comm.). These markers will be of great help in developing an autonomously-replicating cloning vector.

3. AP endonucleases

Apurinic or apyrimidinic (AP) endonucleases recognise sites of base loss in DNA where the phosphodiester backbone is intact. They are involved in the repair of these AP sites which would otherwise be promutagenic and are a major component of the base excision repair pathway (Lindahl, 1979).

3.1 Production of AP sites

An AP site is produced by the cleavage of the N-glycosidic bond that connects the base to the deoxyribose sugar and is termed either depurination or depyrimidination. AP sites are mainly produced in three ways: spontaneously, by chemical treatment of the cells or enzymatically.

3.1.1 Spontaneous formation of AP sites

Under physiological conditions, depurination is the most frequent spontaneous DNA alteration. The rate at 37°C has been estimated as 3×10^{-11} depurinations/nucleotide residue sec^{-1} (Lindahl and Nyberg, 1972). This is equivalent to 0.5 depurinations per *E. coli* cell per generation. Depurination occurs at a higher rate at higher temperatures and so a thermophilic bacterium such as *Thermus thermophilus* lose approximately 300 purines per generation at 85°C. At pH 7 the rate of depurination is at least 20 times greater than depyrimidination (Lindahl and Karlstrom, 1973).

3.1.2 Chemical induction of AP sites

The modification of bases, leading to the formation of AP sites, can be caused by a variety of chemicals (see Loeb and Preston, 1986) with the N-7 position of guanine providing the main site of attack. This methylation at the N-3 and N-7 positions of purines and O-2 position of pyrimidines, positively charges the base, weakens the N-glycosyl bond and increases the rate of base release (Zoltewicz et al., 1970; Singer et al., 1978). Oxidizing agents increase depyrimidation by weakening the N-glycosyl bond when thymine has saturated 5, 6 bonds (Demple and Linn, 1982). The half lives of base release can vary from 3 hours for 7-methyladenine to 1.3×10^8 hours for unmodified deoxypyrimidines. Alkylation can increase the rate of depurination and depyrimidinations of DNA by as much as six orders of magnitude. A small number of AP sites can also be produced by X-irradiation and UV-irradiation (Ljungquist et al., 1974).

3.1.3 Enzymatic formation of AP sites

AP sites are produced enzymatically by the action of DNA glycosylases, small single-chain proteins with molecular weights ranging from 20,000 to 35,000. These enzymes have narrow substrate specificities and remove bases that have been modified spontaneously, chemically or by UV and ionizing irradiation. They catalyse the cleavage of the N-glycosyl bond by a hydrolysis reaction and this is the first step in base excision repair (Lindahl, 1982). DNA glycosylases are ubiquitously distributed, probably evolving early in cellular development with uracil DNA-glycosylase and 3-

methyladenine DNA-glycosylase appearing in virtually all organisms examined.

There are two classes of glycosylase; those associated with AP endonucleases and those that are not. The three known DNA-glycosylases associated with an AP endonuclease are active towards thymine glycols in E. coli (Radman, 1976) and towards pyrimidine dimers in both Micrococcus luteus (Haseltine et al., 1980) and bacteriophage T4 (Demple and Linn, 1980). Glycosylases without an associated AP endonuclease have been found to act on the modified bases uracil, hypoxanthine, 3-methyladenine, 7-methylguanine, formamido-pyrimidine and urea (Lindahl, 1982).

Lindahl argues that these enzymes evolved to deal with naturally occurring base damage. The cellular S-adenosylmethionine found in some cells has been shown to be a weak DNA alkylating agent (Lindahl, 1982). Uracil and hypoxanthine arise in DNA spontaneously or from oxidative deamination of cytosine and adenine respectively. They are potentially mutagenic since uracil can base pair with thymine and hypoxanthine with cytosine. They arise at a rate 500 times less than the rate of spontaneous depurination.

3.2 AP sites as targets for mutagenesis

The persistence of AP sites in DNA can lead to mutagenesis via misincorporation of a base opposite the AP site during DNA replication. Accumulating evidence suggests a role for AP sites in spontaneous mutagenesis and as a common intermediate in chemically-induced mutation after removal of bulky adducts in DNA. Both apurinic and apyrimidinic sites are implicated in this mutagenic

process (Schaaper and Loeb, 1981; Boiteux et al., 1985).

In vitro studies have shown that apurinic sites inhibit replication but it does proceed at a slower rate, with the frequency of base misincorporation directly proportional to the number of AP sites (Shearman and Loeb, 1979). The rate of misincorporation is substantially reduced by treating the DNA templates with alkali that hydrolyzes the phosphodiester backbone at AP sites. This demonstrates that the AP sites are responsible for the errors.

DNA polymerases from different sources were assessed for their ability to copy past AP sites (Kunkel et al., 1983). Prokaryotic DNA polymerases with their proof reading ability have high fidelity and copy past AP sites only with difficulty. Eukaryotic DNA polymerases and reverse transcriptase from RNA viruses lack the proof-reading activity and do not show high fidelity in replication. These polymerases copy past AP sites readily. Different in vitro experimental systems showed that deoxyadenosine was preferentially incorporated opposite the AP site (Kunkel et al., 1983; Sagher and Strauss, 1983; Boiteux and Laval, 1982).

Evidence now suggests that a proportion of spontaneous mutations result from replication past AP sites (Miller and Low, 1984). The transversions G:C to T:A and A:T to T:A should predominate, reflecting the fact that the rate of depurination is much higher than depyrimidination and deoxyadenosine is preferentially inserted opposite AP sites. Miller and Low (1984) showed that mutagenesis is targeted and results mainly in transversions rather than transitions produced by tautomeric or deaminated bases. In vivo studies also showed that deoxyadenosine is preferentially inserted opposite AP sites during replication (Kunkel, 1984). As stated later,

this requires the SOS system to be induced in E. coli. Why adenine is preferred over other bases is not known. The nucleotide pools have been shown to change on exposure to DNA damaging agents with increase in the dATP levels (Das and Loeb, 1984). This would seem a significant factor except that in vitro studies with equal concentrations of deoxynucleotides still show preferential incorporation of deoxyadenosine (Kunkel et al., 1983). The preferential binding of dATP in the absence of a template was also proposed but binding measurements with E. coli DNA polymerase I showed the order of preference as G > A > T > C (Englund et al., 1969). Probably a combination of stacking interactions and van der Waal's forces are responsible for deoxyadenosine incorporation at an AP site (Walker, 1984).

In vivo studies of mutagenesis in E. coli at AP sites showed the reversion of an amber mutant of ØX174 was 2 to 3 fold greater with DNA containing AP sites than with untreated DNA when transfecting E. coli spheroplasts. However, the reversion was enhanced 20 to 30 fold when the E. coli cells were exposed to UV irradiation prior to transfection (Schaaper and Loeb, 1981). Further studies implicated the SOS system in this enhanced reversion. Mutations in recA, recF or umuC, required for the induction of the SOS system. lacked mutagenic expression on apurinic DNA (Schaaper et al., 1982). The DNA polymerase in an SOS-induced system allows the bypass of AP sites more easily than in a non-induced system. The increased mutation rate in SOS-induced cells has led to the hypothesis that AP sites are cryptic lesions where the majority of mutations would only result from the SOS-induced replication complex being operative. AP sites in low numbers would not induce the SOS response and would

therefore usually remain cryptic. Mutagenesis in eukaryotic cells does not require the induction of an error-prone repair pathway with the poor fidelity of DNA polymerase accounting for mutation (Gentil, 1984).

The 2 to 3 fold enhanced revision of the depurinated ØX174 amber mutation without induction of the SOS system shows that spontaneous mutation does not require a fully-induced error-prone pathway. Spontaneous mutations could be produced from a low error frequency of the normal replicating complex or from leaky expression of the SOS system.

The SOS response is absent from D. radiodurans (Sweet and Moseley, 1976) and a range of chemicals and radiation have only small mutagenic effects on the species (Tempest and Moseley, 1980; Sweet and Moseley, 1976). Spontaneous mutation in D. radiodurans is comparable with other bacteria and it can be postulated that it occurs via misincorporation opposite AP sites as well as from misincorporation opposite tautomers or deaminated bases. High fidelity DNA replication by the DNA polymerase would, in most cases, probably stop the enzyme at an AP site until it is repaired but with the occasional copy past leading to mutation. Highly-induced levels of mutagenesis seen in SOS-induced E. coli cells would be absent from D. radiodurans. The slight sensitivity to mutation by chemical agents in D. radiodurans could be attributed to an increase in the number of AP sites produced by DNA glycosylases or increased spontaneous hydrolysis of modified bases. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) is the only potent mutagen so far found for D. radiodurans. It has been demonstrated that MNNG is mutagenic via AP site intermediates (Foster and Davis, 1987) but in the case

of D. radiodurans it must produce other types of mutagenic lesions. Perhaps it occurs at the replication forks where MNNG has been shown to affect the DNA replication complex (Cerda-Olmedo et al., 1968).

To summarise, AP sites are induced by most chemical mutagens and some irradiations. These sites can therefore act as a common intermediate in induced mutagenesis, with an error-prone replication system in operation. Usually transversions result from depurination (Foster et al., 1983) and transitions from depyrimidinations (Povirk and Goldberg, 1986). As the rate of depurination is greater than depyrimidination, transversions predominate over transitions from AP sites. The proposed pathway of chemically-induced mutagenesis would occur when DNA polymerase is stopped at an AP site or bulky DNA adduct. The bulky adduct has to be removed to allow continued replication. The resultant AP site is probably protected from AP endonucleases by the DNA replicating complex which copies past the site efficiently in eukaryote cells but with difficulty in prokaryotic cells. In prokaryotic cells which exhibit the SOS mechanism, the copy past AP sites is greatly facilitated with the consequence of increased mutation (Loeb, 1985).

3.3 AP endonucleases and their role in repair of AP sites

The mutagenic potential of an AP site necessitates its repair. AP endonucleases catalyze the cleavage of phosphodiester bonds 3' or 5' to the AP site and so prepare the DNA for subsequent excision, repair synthesis and DNA ligation. AP endonucleases are monomeric proteins of molecular weight ranging from 20,000 to 50,000. They

act on apurinic and apyrimidinic sites leaving 5'-phosphoryl ends. They are specific for double-stranded DNA where they produce single strand breaks except for the single-strand-specific endonuclease VII of E. coli (Bonura et al., 1982). Some AP endonucleases require Mg^{2+} or Mn^{2+} ions for optimal activity, whereas others work effectively in the presence of EDTA.

There are two classes of AP endonuclease: those that cleave 3' to the AP site (class I) and those that cleave 5' to the AP site (class II). In a single case, the AP endonuclease from human placenta was seen to cleave 3' or 5' to the AP site but not both (Grafstrom et al., 1982). It should also be mentioned that AP sites have labile phosphodiester bonds, particularly in the 3' bond and at neutral or alkaline pH the bond is hydrolyzed in a β-elimination reaction (Weiss, 1987). This results in a break 3' to the AP site and leaves a 2', 3'-unsaturated deoxyribose 5-phosphate which is not a suitable substrate for the E. coli DNA polymerase I 3'-5' exonuclease activity.

3.3.1 Class I AP endonuclease

All but one of the four 3' nicking AP endonucleases have an associated glycosylase. The human fibroblast enzyme which is absent from the human DNA repair deficient disease xeroderma pigmentosum lacks a known glycosylase activity (Mosbaugh and Linn, 1980). The other three are the glycosylases active against thymine glycols and pyrimidine dimers mentioned in section 3.1. It was originally thought that these enzymes leave 3'-OH base-free deoxribose ends which act as poor primer templates for DNA synthesis by E. coli

polymerase I (Warner et al., 1980). In vitro it was shown that the base-free deoxyribose had to be removed by a 5' acting AP endonuclease to provide a good primer template. This suggested that the base-free deoxyribose was not a substrate for the 3'-5' exonuclease activity of DNA polymerase I. Bailly and Verly (1984) demonstrated that this activity could remove a 3' terminal AP site with a 3'-OH end. This suggested that 3' AP endonucleases do not leave 3'-OH ends. Further work by them showed that endonuclease III of E. coli is a catalyst for a β-elimination reaction, leaving 2', 3'-unsaturated deoxyribose phosphate which is not a substrate for DNA polymerase I and its 3'-5' exonuclease activity (Bailly and Verly, 1987) (see Figure 1.2). They suggest endonuclease III should not be termed an AP endonuclease.

This has led to discussion over the definition of a true AP endonuclease especially as oligopeptides containing aromatic amino acids such as lys-trp-lys can promote hydrolysis at AP sites via a β-elimination reaction (Pierre and Laval, 1981). This is probably the mechanism by which endonuclease III and the AP endonuclease activities of the pyrimidine dimer DNA-glycosylases of M. leutus and bacteriophage T4 operate. In vitro reactions may vary from those in vivo so that a protein can spuriously act as a 3' AP endonuclease in vitro but not in vivo. This problem requires much further study.

3.3.2 Class II AP endonucleases

The 5' nicking AP endonucleases all leave 3'-OH ends which are good template primers for E. coli DNA polymerase I (see Figure

1.2). They are ubiquitous, being found in prokaryotes, microbial eukaryotes, plants and animals (Friedberg, 1985). Some have associated properties such as exonuclease III of E. coli which functions as a 5'-AP endonuclease, 3'-phosphatase, 3'-5' exonuclease and ribonuclease H (Weiss, 1981). The enzyme is thought to have one domain which acts as an active site for all activities associated with the enzyme. The majority of the AP endonucleases do not show any extra specific function.

3.3.3 The biological role of AP endonucleases

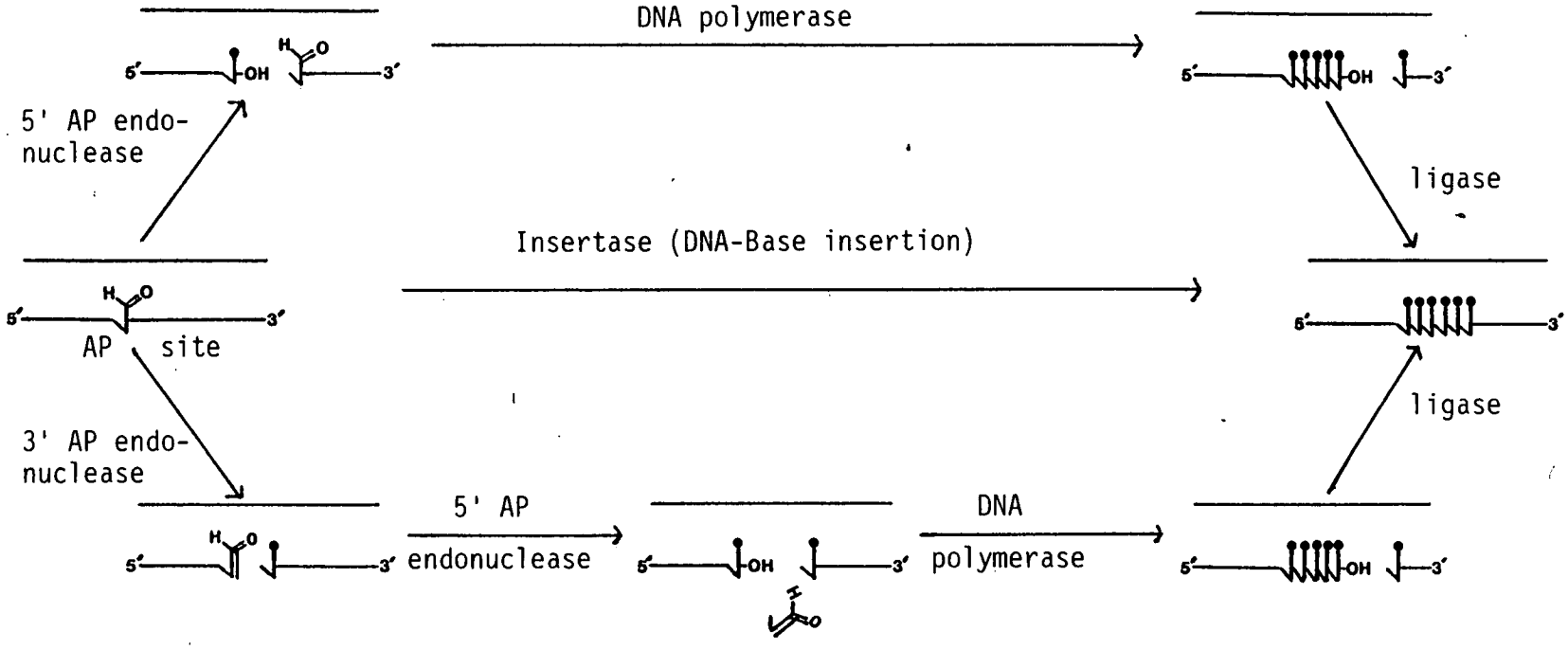
The biological role of AP endonucleases has been difficult to study due to the lack of mutants available. Most organisms appear to have more than one type of AP endonuclease making it difficult to select for mutants. E. coli shows redundancy in having at least four AP endonucleases that act on double stranded DNA (Friedberg, 1985). Exonuclease III mutants (gene xth) were obtained by mass screening, looking for the loss of this major AP endonuclease in E. coli (Yajko and Weiss, 1975). Mutants of endonuclease III(nth) in E. coli were isolated by first selecting cloned DNAs which over-produced the enzyme and using the cloned DNA to produce mutants (Cunningham and Weiss, 1985). Recently, a mutant for endonuclease IV (nfo) was obtained (Cunningham and Weiss, 1987). It was shown that nfo mutations increase the sensitivity of xth mutants to various DNA damaging agents such as alkylating agents and hydrogen peroxide. This is not surprising as the two enzymes from these genes account for over 95% of the 5' acting AP endonuclease activity in E. coli. Endonuclease III mutations did not enhance

sensitivity to most DNA damaging agents and its role as an AP endonuclease was seen not to be vital. It is only useful against DNA containing thymine glycols or urea.

It is thought that the 5' AP endonucleases are the primary AP site repair enzymes. The 3' AP endonucleases probably help the repair of AP sites not readily recognized by 5' endonucleases. This could be the case because there may be different classes of AP sites. The nfo mutant of E. coli is more sensitive to some types of DNA damage than xth mutants even though it is the minor 5' AP endonuclease in the cell (Cunningham and Weiss, 1987).

The three AP endonuclease mutations in E. coli should help establish the role of AP endonucleases in the cell. The characterisation of substrate specificity of the enzymes will also help. The diagrammatic scheme in figure 1.2 shows the repair pathway for AP sites. The purine insertase pathway shown in figure 1.2 is included for completeness but this repair route is controversial (Deutsch and Linn, 1979; Luineh et al., 1979).

Figure 1.2 Repair pathways for AP sites



4. Aims of the project

Genetic manipulation within the Deinococcus genus is limited to transformation of homologous DNA into the species D. radiodurans. To facilitate a greater range of genetic analysis, an attempt was made to construct a cloning shuttle vector with E. coli which would allow the study of gene expression in D. radiodurans.

New strains of Deinococcus were isolated to provide a further insight into their growth environment. These strains were screened for plasmids to provide possible material for vector constructions and for high-yielding restriction endonucleases. The new strains were also used to ascertain the extent of interrelatedness of Deinococcus species with respect to genes involved in the repair of ultraviolet light induced damage (mtcA and B; uvsC, D and E).

The final study involved the purification and characterisation of the major AP endonuclease of D. radiodurans. This enzyme is thought to be important in the repair of intermediate DNA lesions (AP sites) resulting from bulky DNA damage. A search for uracil DNA glycosylase activity and a methylated DNA glycosylase activity was undertaken during the purification of the AP endonuclease.

CHAPTER 2

MATERIALS AND METHODS

Bacterial strains

All *Deinococcus* strains used are listed in Table 2.1, and *Escherichia coli* strains in Table 2.2.

Plasmids

All plasmids used and constructed are listed in Table 2.3.

Maintenance and growth of cultures

Strains of *Deinococcus* were maintained on TGY agar while those of *E. coli* were maintained on LB agar with antibiotic present in the case of resistant strains. Genetically-marked strains were always checked by subculturing onto appropriate selective medium before use.

All *Deinococcus radiodurans* strains were grown in TGY broth or a defined medium. All other strains of *Deinococcus*, with the exception of *D. radiophilus*, were grown in TGY broth. *D. radiophilus* was grown in nutrient broth No. 2. *E. coli* was grown in LB broth with relevant antibiotic selection when required.

Deinococcus strains were grown at 30°C and *E. coli* strains at 37°C.

Table 2.1 Strains of *Deinococcus* used

Strain	Genotype	Reference
D. radiodurans R1	Wild Type	Anderson *et al.*, 1956
" SARK	Wild Type	Murray and Robinow, 1958
" KRASE*	kan^R, rif^R, acr^R, str^R, ery^r	Tirgari and Moseley, 1980
" 78	*uvsE*, *mtcA*	Evans and Moseley, 1983
" 262	*mtcB*	Evans and Moseley, 1983
" 302	*mtcA*	Moseley and Copland, 1978
" 18-1	*mtcA*, *trp1*	Al-Bakri, 1985
" 112	*mtcA*, *rec1*	Evans, 1984
" rec30	*rec30*	Moseley and Copland, 1975b
D. radiopugnans	Wild Type	Davis *et al.*, 1963
D. radiophilus	Wild Type	Lewis, 1971

* A mutant strain of *D. radiodurans* RI, resistant to 30 µg kanamycin ml^{-1}, >100 µg rifampicin ml^{-1}, 5 µg acriflavin ml^{-1}, 200 µg streptomycin ml^{-1}, 25 µg erythromycin ml^{-1}.

Table 2.2 Strains of *E. coli* used

Strain	Genotype	Reference
HB101	*pro leuB thi recA* r^-_k m^-_k	Boyer and Roullard-Dussoix (1969)
B/CT/LT*	*thy*	Evans (1984)

* B/CT/LT required 3 µg thymine ml^{-1} for growth.

Table 2.3 Plasmids used

Plasmid (a)	Selectable Marker (b)	*Deinococcus* genes carried on plasmid	Reference
pAT153	Ap, Tc		Twigg and Sherratt, 1980
pUE109	Tc	*D. radiophilus* plasmid pUE1	Mackay *et al.*, 1985
pUE58	Ap, Tc	*mtcA*	Al-Bakri *et al.*, 1985
pUE59	Ap, Tc	*mtcB*	Al-Bakri *et al.*, 1985
pUE502	Ap	*mtcA*, *mtcB*	Al-Bakri *et al.*, 1985
pUE81	Ap, Tc	*trp*	Al-Bakri, 1985
pUE200	Ap	*uvsC*, *D*, *E*	Lab plasmid

(a) All plasmids contain the pAT153 origin of replication and use *E. coli* as the host system for replication.

(b) Abbreviations and selectable levels:

Ap = Ampicillin 50 $\mu g\ ml^{-1}$

Tc = Tetracycline 20 $\mu g\ ml^{-1}$

Media

The following media were used - all were made up in distilled water:

1. TGY broth

	gl^{-1}
Bactotryptone (Oxoid)	10
Yeast Extract (Oxoid)	5
D-Glucose	1

2. Nutrient broth No. 2

	gl^{-1}
Nutrient broth No. 2 (Oxoid)	25

3. Luria-Burrows broth (LB broth)

	gl^{-1}
Bactotryptone (Oxoid)	10
Yeast Extract (Oxoid)	5
NaCl	3
D-Glucose	1

4. Deinococcus minimal media (cited in Tigari, 1977)

i) Basal medium

	gl^{-1}
D-Glucose	2 g
Monosodium Glutamate	2 g
Tris Base	2 g
Ammonium Acetate	1 g

ii) Solution A	gl^{-1}
Potassium acetate	200 g
Magnesium acetate $4H_2O$	100 g
Sodium acetate	5 g
Calcium chloride $2H_2O$	5 g

iii) Solution B	gl^{-1}
Ammonium phosphate	87 g
Ammonium sulphate	20 g

iv) Solution C	mg/100 ml
Nicotinic acid	50
Vitamin B (aneurin HCl)	50
Biotin	1
Vitamin B12 (cyanocobalamin)	0.1

v) Solution D	mg/100 ml
Boric acid	50
Manganese acetate $4H_2O$	50
Zinc acetate $2H_2O$	40
Ferric chloride	20
Ammonium molybdate	10
Potassium iodide	10
Copper acetate H_2O	4
Citric acid	100

vi) Solution E

	g/100 ml
L-Cysteine	1.2
L-Methionine	0.8
L-Lysine monohydrochloride	0.92
L-Histidine	0.78

To 1 l of basal medium, 1 ml of solutions A, B and C, 0.1 ml of solution D and 2 ml of solution E were added. The pH of the medium was adjusted to 7.8 using glacial acetic acid. 0.1% casamino acids could be used in the medium in place of solution E.

5. M9 salts x10 (concentrate)

	gl^{-1}
$Na_2HPO_4 . H_2O$	60
KH_2PO_4	30
NaCl	5
NH_4Cl	1

Dissolved in order listed before autoclaving.

6. M9 minimal medium

M9 salts (x10)	100 ml
20% w/v D-glucose	10 ml
0.1M $MgSO_4$	10 ml
0.01M $CaCl_2$	10 ml
H_2O	870 ml

(Each solution was sterilized separately before mixing.)

7. K medium

M9 Minimal media	196 ml
15% w/v Casamino acids	4 ml
Thiamine hydrochloride (1 mg ml^{-1})	20 µl

Suitable plating agar was made by the addition of 15 g Agar No. 3 (Oxoid) to 1 l of broth. In the case of minimal media, 15 gl^{-1} of Agar No. 1 (Oxoid) was used. Sterilization of all media was by autoclaving at 15 psi for 20 min.

Buffers

1. Phosphate buffer (0.067 M), pH 7.0

	gl^{-1}
Na_2HPO_4	4.73
KH_2PO_4	4.56

2. Phosphate EDTA buffer (PEB), pH 7.0

	gl^{-1}
KH_2PO_4	0.52
K_2HPO_4	2.82
EDTA	3.36

3. Butanol-saturated PEB (BSPEB)

PEB	94 ml
Butan-1-ol	6 ml

4. Standard saline sodium citrate (x1) (SSC), pH 7.0

	gl^{-1}	
NaCl	9.0	(0.15 M)
Na_3 citrate $2.H_2O$	4.0	(0.015 M)

5. TE Buffer, pH 7.5

	gl^{-1}	
Tris base	1.21	(0.01 M)
EDTA	0.38	(0.001 M)

6. TES buffer, pH 8.0

	gl^{-1}	
Tris base	6.05	(0.05 M)
EDTA	1.86	(0.005 M)
NaCl	2.92	(0.05 M)

7. Acetate electrophoresis gel buffer (x10), pH 8.2

	gl^{-1}	
Tris base	48.4	(0.4 M)
Sodium acetate. $3H_2O$	27.2	(0.2 M)
EDTA	3.72	(0.01 M)

The pH was adjusted with acetic acid.

8. Denhardt's solution (x100)

	gl^{-1}
Ficoll	20
Polyvinylpyrrolidone	20
Bovine Serum Albumin	20

The solution was stored at -20°C.

9. Prehybridisation solution

SSC buffer (x10)	6.0 ml
Denhardt's solution (x100)	0.5 ml
10% (w/v) SDS	0.25 ml
0.1M EDTA	1.0 ml
Denatured salmon or calf sperm DNA	1.0 mg
Distilled water	to 10 ml

10. STOP buffer

SDS	0.4 g
EDTA	0.2 g
Ficoll	4.0 g
Bromophenol Blue	0.2 g
Tris Base	0.1 g
Distilled water	to 20 ml

1 µl of STOP buffer was added per 20 µl of reaction mixture.

11. Lysis buffer, pH 8.0

	gl^{-1}	
Tris base	3.03	(25 mM)
EDTA	3.72	(10 mM)
D-Glucose	9.01	(50 mM)

The pH was adjusted with hydrochloric acid.

12. STET buffer, pH 8.0 (Do not autoclave - store at 4°C)

Sucrose	16 g	(8% w/v)
Triton X-100	1 ml	(0.5% v/v)
EDTA	3.72 g	(50 mM)
Tris base	1.21 g	(50 mM)
Distilled water	to 200 ml	

13. Polyacrylamide gel electrophoresis buffer (x10), pH 8.8

Glycine	42.0 g
Tris base	9.0 g

SDS	6.0 g	
Distilled water	to 500 ml	

14. Polyacrylamide gel separating buffer (x4), pH 8.8

Tris base	90.75 g	(1.5 M)
EDTA	1.50 g	(8 mM)
SDS	2.00 g	(0.4% w/v)
Distilled water	to 500 ml	

15. Polyacrylamide gel stacking buffer (x4), pH 6.8

Tris base	30.25 g	(0.5 M)
EDTA	1.50 g	(8 mM)
SDS	2.00 g	(0.4% w/v)
Distilled water	to 500 ml	

The pH was adjusted with hydrochloric acid.

Chemicals

Sodium dodecyl sulphate (SDS), ethylene diamine tetra acetic acid: sodium salt (EDTA), Tris(hydroxymethyl)aminomethane (Tris base), Tris-hydrochloride (Tris-HCl), caesium chloride, polyethylene glycol 6000 (PEG), ethidium bromide, 3-(N-Morpholino) propanesulphonic acid (MOPS), acrylamide and bisacrylamide (electro-phoretic grade) and routinely-used salts were purchased from BDH Chemicals Ltd., England. Ficoll, agarose (type 1-low EEO), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), mitomycin C (MTC), deoxyadenosine 5-triphosphate (ATP), thymine, bovine serum albumin (BSA) and phenylmethylsulphonylfluoride (PMSF) were purchased from Sigma Chemical Co., London. Dithiothreitol (DTT), ultrapure phenol, tetramethylethylenediamine (Temed) were purchased from BRL Bethesda Research Laboratories (UK) Ltd., Cambridge. λ-DNA and øX174 DNA were purchased from Boehringer Mannheim, Lewes, England and New England Biolabs, Bishop Stortford, England respectively. Methyl methanesulphonate (MMS) was purchased from Eastman-Kodak and Co., Rochester, New York.

Enzymes

T_4 DNA ligase (E.C. 6.5.1.1), calf intestinal alkaline phosphatase (E.C. 3.1.3.1), S1 nuclease (E.C. 3.1.30.1), Bal31 and a number of restriction endonucleases were purchased from Boehringer Mannheim, Lewes. Exonuclease III, nick translation kit (containing DNase I and E.coli polymerase I) and a few restriction endonucleases were purchased from Bethesda Research Laboratories. Pancreatic RNase (E.C. 3.1.4.22) and lysozyme were purchased from Sigma. Mung bean nuclease and a number of restiction endonucleases were purchased

from Pharmacia (GB) Ltd., Milton Keynes, England. DraI restriction endonuclease was a gift from I.J. Purvis, University of Edinburgh.

Radioactively-labelled Compounds

[6-^{3}H]deoxythymidine (dThd) 27 Ci mmol^{-1}, 1 μ Ci ml^{-1} in water and [α-^{32}P] deoxycytosine 5'-triphosphate were purchased from Amersham International, Bucks, England.

Antibiotics and nutritional supplements

All the Antibiotics and nutritional supplements required were purchased from Sigma.

Storage of DNA-damaging agents

MMS was stored at room temperature in the dark. MNNG was dissolved in 67 mM Phosphate buffer at 2 mg ml^{-1}, filter sterilized and stored at -20°C in 0.5 ml aliquots until required. MTC was dissolved in 50% methanol: 50% distilled water at 500 μg ml^{-1}, and stored in the dark at 4°C.

Room temperature

In this thesis, room temperature indicates a temperature of between 18°C-24°C.

Measurement of bacterial growth

The rate and stage of culture growth was determined using a nephelometer (Evans Electroselenium Ltd., Halstead) with an orange filter.

Isolation of chromosomal DNA

Chromosomal DNA was prepared from members of the genus Deinococcus, E. coli and isolates of Deinococcus-like bacteria. A modification of the Marmur method (1961) was used for all cultures except for E. coli where the initial butanol step was omitted.

One litre of stationary-phase culture was centrifuged at 16,000 g for 10 min and the pellet of cells drained well. The cells were suspended in 50 ml BSPEB and the suspension left for 1 h at room temperature. This treatment leaves the cell walls of Deinococcus spp. sensitive to lysozyme (Dreidger and Grayston, 1970). The cells were pelleted by centrifugation, washed in PEB and re-suspended in 25 ml PEB. Lysozyme (0.25 g) was added and mixed with swirling until fully dissolved and the mixture incubated at 37°C for 90 min. The cells were then lysed by the addition of 2.5 ml 11% SDS (in PEB), and gentle swirling of the mixture to produce a sticky lysate. Addition of 6.5 ml sodium perchlorate solution (70.25 g NaCl O_4 H_2O, 4.4 g NaCl in 100 ml H_2O), helped stabilize the DNA, and 35 ml of chloroform/isoamyl alcohol (24:1 v/v) were added and mixed carefully with the sticky lysate. The initial two phases of the mixture became one continuous milky phase after 10 minutes mixing, and this was left overnight at 4°C. The mixture was centrifuged at 30,000 g for 30 min in 25 ml Corex tubes. Three layers resulted in a bottom chloroform/isoamyl alcohol layer, a middle solid layer of denatured protein and a top aqueous layer containing the nucleic acids. The top layer was removed carefully with a 10 ml pipette, to help prevent shearing of the chromosomal DNA, and added to two volumes of cold ethanol. Precipitated DNA formed at the aqueous/ethanol interface and was wound onto a glass

rod, dried in the air and dissolved in TE buffer. RNase was added (preboiled at 100°C for 15 min to inactivate contaminating DNase) to a concentration of 1 mg ml^{-1} and incubated at 37°C for 1 h. An equal volume of chloroform/isoamyl alcohol (24/1) was added and mixed as before for 10 min, to give a milky emulsion. This was centrifuged at 30,000 g for 30 min and the clear top aqueous layer removed. The chromosomal DNA was collected as before via precipitation in cold ethanol and after drying was resuspended in TE buffer. The concentration of DNA was estimated by measuring absorption at 260 nm and 280 nm light, followed by use of a nomograph.

Isolation of plasmid DNA

a. Deinococcus spp.

The Mackay (1983) modification of the Birnboim-Doly (1979) plasmid isolation method was employed. This method selectively denatures high molecular weight chromosomal DNA at an alkaline pH, but covalently-closed-circular (ccc) DNA remains double stranded. On neutralization the chromosomal DNA renatures into an insoluble clot, leaving ccc plasmid DNA in the aqueous phase.

A rapid screening method for the detection of plasmids was developed using 10 ml of culture. Cells from a 10 ml overnight culture were centrifuged at room temperature and resuspended in 1 ml BSPEB for 45 min. The cell suspension was transferred to a microcentrifuge tube and the cells pelleted in a microcentrifuge. After a single wash in 5 ml lysis buffer, the pellet was resuspended in 200 µl lysis buffer containing lysozyme at a concentration of 2.5 mg ml^{-1} and left at room temperature for 30 min. 400 µl of 0.2M NaOH,

1% SDS, was added and well mixed, to give good lysis. After 5 min, 300 µl of 3M sodium acetate (pH 4.8) was added and well mixed before leaving on ice for 1 h. The precipitate formed was removed by centrifugation at 4°C for 15 min. The supernatant was divided between two microcentrifuge tubes and the DNA precipitated by the addition of 900 µl cold ethanol to each. After exposure to a temperature of -20°C for 30 min, the precipitated DNA was collected by centrifugation at 4°C for 15 min. The two pellets were air dried and pooled after resuspending in 0.1 M sodium acetate (pH 6.0) to a final volume of 300 µl. The DNA was precipitated by the addition of 600 µl of cold ethanol and collected by centrifugation as before. The pellet was vacuum dried and resuspended in 20 µl TE buffer. STOP buffer was added to the preparation and it was electrophoresed through a 0.8% agarose gel for analysis, as described later.

In preparation of plasmid DNA from some of the strains, considerable protein and RNA contamination was carried over. Most of this was removed using the phenol/chloroform extraction technique described later. This step was used at the second DNA precipitation stage, the DNA being resuspended in 100 µl TE buffer instead of 20 µl.

Large scale preparation of plasmid from Deinococcus spp. was done by scaling up the previous procedure. Cells from two litre cultures in stationary phase were centrifuged and resuspended in 100 ml BSPEB for 45 min at room temperature. The cells were centrifuged, well drained and resuspended in 30 ml lysis buffer. 10 ml of 8 mg ml^{-1} lysozyme in lysis buffer was added, well mixed, and then left at room temperature for 30 min. Lysis was achieved by the addition of 80 ml 0.2 M NaOH, 1% SDS and mixing thoroughly by

inversion. The lysate was kept at 0°C for 5 mins and then 60 ml of 3 M sodium acetate (pH 4.8) was added and gently mixed by inversion. The mixture was kept at 0°C for 60 mins and then centrifuged at 16,000 g for 15 mins resulting in a clear lysate over a pellet of cell debris and chromosomal DNA. The lysate was filtered through a tea strainer to remove any floating insoluble lumps. To the supernatant two volumes of cold ethanol were added and mixed, followed by exposure to -20°C for 45 min. The precipitate was collected by centrifugation at 4,000 g for 15 mins and resuspended in 25 ml of 0.1 M sodium acetate (ph 6.0) before a second precipitation of DNA with 50 ml cold ethanol. After 30 mins at -20°C the DNA precipitate was collected by centrifugation, vacuum dried and resuspended in TE buffer. Plasmid DNA was further purified using the dye-buoyant density gradient centrifugation technique (Radloff et al., 1967) which will be described below.

b. E. coli

Small scale isolation of plasmid from E. coli for rapid screening, employed the boiling method of Holmes and Quigley (1981). Cells from 1.5 ml of an overnight culture of E. coli were collected by microcentrifugation for 30 s. The cells were resuspended in 350 µl of STET buffer and 25 µl of lysozyme solution (10 mg ml^{-1} in STET buffer). The microcentrifuge tube containing this mixture was immediately placed in a boiling water bath for 1 min and then centrifuged for 15 mins at 4°C. The sticky pellet which formed was removed using a toothpick and 40 µl of 4 M sodium acetate added to the supernatant. Precipitation of plasmid DNA was achieved by the addition of 400 µl of cold propan-2-ol (iso propanol) with mixing by

inversion. The precipitate was collected by centrifugation for 6 mins at 4°C and washed with 70% ethanol to remove propan-2-ol before being dried by vacuum. The pellet was dissolved in 20 µl TE buffer and STOP buffer added before agarose gel electrophoresis. If the plasmid was to be digested with a restriction endonuclease, the pellet was resuspended in 36 µl of distilled water and 4 µl of the appropriate x10 restriction endonuclease buffer. To remove protein and small molecular weight RNA, the dissolved pellet was cleaned using the phenol/chloroform extraction procedure to be described later.

Large scale production of plasmid from E. coli cultures was done using the method of Birnboim and Doly (1979). The procedure followed was the same as that employed for Deinococcus spp. but with the initial butanol treatment omitted and using only 1 litre of overnight culture. Plasmid DNA was further purified using the dye-buoyant density gradient centrifugation technique.

Dye-buoyant density gradient centrifugation

To purify DNA from any source, the dye-buoyant density gradient centrifugation technique was used (Radloff et al., 1967). The DNA was always dissolved in TE buffer before the addition of 1.15 g of caesium chloride and 0.1 ml of ethidium bromide solution (10 mg ml^{-1}) per 1 ml of clear lysate. The solution should have a density of 1.60-1.62 g ml^{-1}. If the density fell outside this range, it was changed by the addition of distilled water or caesium chloride to decrease or increase density respectively. The solution was then transferred to a 10 ml polypropylene tube (MSE) and centrifuged at 130,000 g for 60 h at 18°C in a Prepspin 65 MSE ultracentrifuge.

Two intense red bands formed of which the lower contained the ccc plasmid DNA. This band was drawn from the gradient into a syringe by inserting a 19 guage needle through the side of the tube, just below the band. The ethidium bromide was removed from the DNA by several extractions with NaCl-saturated isopropanol (5 M NaCl). The DNA was then precipitated by adding two volumes of distilled water and six volumes of cold ethanol. The mixed solution was then held at -20°C for 2 h before the precipitate was collected by centrifugation. The pellet formed was dried by vacuum and then dissolved in TE buffer. This DNA solution was stored at 4°C, if being used continuously, or at -70°C when not in use.

Phenol and chloroform extraction technique

This technique was used to rid mini-preparations of plasmid DNA of contaminating protein and low molecular weight RNA, and so allow clearer visualization of DNA on agarose gels and to prevent inhibition of restriction digests.

An equal volume of phenol/chloroform mixture (1:1) saturated with TE buffer was added to the DNA solution. Two phases formed which were well mixed before microcentrifugation for 30 s. Denatured protein collected at the aqueous-phenol/chloroform interphase. The top, aqueous phase was carefully removed and the procedure repeated in a fresh microcentrifuge tube. A final extraction using chloroform only, to remove the last traces of phenol in the aqueous phase, was followed by precipitation of the plasmid DNA on addition of two volumes cold ethanol to the aqueous phase.

Volumes of 100 to 200 µl were used in general for extraction.

Preparation of radiolabelled DNA

A thy$^-$ strain of E. coli B (Evans, 1984) was grown overnight in M9 medium containing 3 µg thymine ml^{-1}. It was then diluted 800 fold into M9 medium containing 3 µg thymine ml^{-1}, 250 µg deoxyadenosine ml^{-1} (Boyce and Setlow, 1962) and 1 mCi ^{3}Hd Thd, before growing for 12 h. The cells were harvested and the chromosomal DNA extracted using the normal procedure. The specific activity of the DNA was 10,000 cpm µg^{-1}.

Alkylation and depurination of DNA

The labelled chromosomal DNA was alkylated and depurinated using the procedure of Verly (1981). One volume of DNA in SSC buffer was mixed with two volumes of 1 M sodium phosphate pH 7.0. Methyl methanesulphonate was added to a molarity of 0.3 M and the mixture incubated at 37°C for 1 h. After cooling on ice, the solution was dialysed against two changes of 1 litre SSC buffer over 16 hours at 4°C. This treatment results in approximately 1 alkylated nucleotide moiety per 7 nucleotides. To depurinate the DNA, the alkylated DNA was incubated at 50°C for 6 h and cooled to 4°C before dialysing against 2x 1 litre changes of SSC buffer. The DNA contained approximately one AP site per 20 nucleotides.

Plasmid DNA was depurinated by the procedure of Teebor and Brent (1981). 10 µl of DNA in TE buffer (1 mg ml^{-1}) was mixed with 1.6 µl 0.1 M Na acetate/0.1 M NaCl pH 4.4 and incubated at 70°C for 20 min. The solution was then rapidly cooled to room temperature and neutralized with 1.6 µl of 0.2 M Tris-HCl, pH 8.0 (at 25°C)/ 1 mMEDTA. This method produces about one depurination per 3,000 nucleotides.

The following procedure was employed to methylate plasmid DNA. 10 µl of a 1% MMS solution (in 10 mM Tris; pH 7.5) was added to 100 µl of plasmid DNA (~ 500 µg ml^{-1}) and left at 37°C for 20 min. Then 300 µl of 10 mM Tris pH 7.5 and 40 µl of 4 M sodium acetate were added and mixed. The DNA was precipitated by the addition of 900 µl cold ethanol and microcentrifugation for 15 min. The resulting DNA pellet was dissolved in 400 µl of 0.1 M sodium acetate pH 6.0 and 800 µl of cold ethanol added. Precipitated DNA was again collected by centrifugation, drained and dried by vacuum before being resuspended in 10 mM Tris pH 7.5.

Deamination of cytosine to uracil in plasmid DNA

A solution of 2 M sodium bisulphite ($NaHSO_3$) pH 5.2 was made by adding 2 M sodium sulphite (Na_2SO_3) to 10 ml of 1 M sodium metabisulphite ($Na_2S_2O_5$) until a pH of 5.2 was reached. The solutions should be made up with deoxygenated distilled water. To 50 µl of DNA in TE (2 µg $µl^{-1}$), 400 µl of the 2 M $NaHSO_3$ solution was added in a microcentrifuge tube and nitrogen gas used to displace any remaining air above the solution. The microcentrifuge tube was sealed and incubated at 37°C for 15-16 hours before the addition of 45 µl of 4 M Na acetate and 300 µl of cold isopropanol. This mixture was placed at -20°C for 10 min and centrifuged for 5 min. The supernatant was discarded and the pellet resuspended in 500 µl 0.1 M Na acetate pH 6.0. The DNA was then collected by precipitation with 300 µl cold isopropanol and centrifugation. The pellet of DNA was resuspended in 450 µl of NaOH pH 10.6 and left for 5 min at room temperature. The DNA was then precipitated by the addition of 45 µl 4 M Na acetate and 300 µl of cold isopropanol. The precipitate

was collected by centrifugation, vacuum dried and resuspended in SSC buffer.

Cloning in plasmid DNA

Vector and target DNA were digested with appropriate restriction endonucleases to produce compatible ends. The vector DNA was resuspended in 40 µl of alkaline phosphatase buffer (50 mM Tris-HCl 0.1 M EDTA, pH 8.0) and 1 unit of alkaline phosphatase added for incubation at 37°C for 30 min. This removed the terminal phosphate groups from the 5' ends of the linearized vector DNA and prevented recircularization of vector DNA. Vector and target DNAs were purified by phenol/chloroform extraction, and were resuspended together in the ratio of 3:1 respectively in ligase buffer (20 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 0.6 mM ATP, pH 7.6). For ligation of sticky ends, the DNA was resuspended at a concentration of 50 μg^{-1} and 0.5 units of T4 DNA ligase added. The mixture was incubated at 12°C for 16 h before the ligation mixture was ethanol-precipitated and then used to transform E. coli HB101. For ligating blunt ends, the DNA was resuspended at a concentration of 200 µg ml^{-1} and 200 units of T4 ligase added for 16 h at 23°C.

Removal of a restriction endonuclease site with Bal31 nuclease

The equation in Figure 2.1 was used to calculate the time required to remove nucleotides from DNA. The plasmid DNA was linearized with the restriction endonuclease whose site was to be removed. The DNA (10 µg) was precipitated and dissolved in a BSA solution (500 µg ml^{-1}) to a concentration of 50 µg ml^{-1}. An equal volume of Bal31 buffer (24 mM $CaCl_2$, 24 mM $MgCl_2$, 0.4 M NaCl, 40 mM

Figure 2.1 Rate of removal of nucleotides from both termini of duplex DNA using Bal31 nuclease

Bal31 nuclease degrades both 3' and 5' strands of linear DNA in a controlled progressive manner under the correct conditions (Gray et al., 1975). The rate at which Bal31 nuclease removes nucleotides from duplex DNA is given by the formula $\frac{dMt}{dt} = -2\ Vm\ Mn/[Km + (S)_o]$

where:

M_t - molecular weight of linear duplex DNA after t minutes of digestion

V_m - maximum reaction velocity (moles of nucleotide removed l^{-1} min^{-1})

M_n - average molecular weight of sodium mononucleotide (330)

K_m - Michaelis-Menton constant (moles of duplex termini l^{-1})

(S_o) - moles of duplex DNA ends l^{-1} at t = o min.

At an enzyme concentration of 40 units ml^{-1}:

$V_m = 2.4 \times 10^{-5}$ $K_m = 4.9 \times 10^{-9}$

It is assumed that the value of V_m alters in proportion to enzyme dilution.

Tris HCl, 2 mM EDTA, pH 8.0) and the appropriate amount of Bal31 enzyme added before incubation at 30°C. At specific times, samples from the reaction mixture were removed and added to 0.2 M EGTA (pH 8.0) to give a final concentration of 20 mM EGTA. Samples were kept at 0°C. Phenol/chloroform extraction was used to purify the DNA which was then precipitated with ethanol. The DNA was resuspended in ligase buffer and the ligation procedure for blunt ends followed.

Agarose Gel Electrophoresis

Agarose gels were always run horizontally using a Tris-acetate buffer. Concentrations of gel from 0.6% to 1.0% were used. 1 µl of STOP buffer was added to every 20 µl of sample before loading into the gel wells. Gels were run at voltage gradients between 2 Vcm^{-1} and 8 Vcm^{-1} depending on the size and resolution required of the gel. Gels were stained with the fluorescent dye ethidium bromide by either incorporating it into the gel and the buffer (0.5 µg ml^{-1}) before running or by diffusion of ethidium bromide into the gel from solution (0.5 µg ml^{-1}) after running for 20 min. DNA bands could then be visualized by transmitted UV light (302 nm). Photographs were taken by using a Polaroid MP-4 camera with Polaroid Type 55 film, and a Kodak 22A Wrattan filter.

SDS Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gels were cast in home made cassettes of dimensions 110 x 150 x 1 mm or 150 x 200 x 1 mm, using 4 mm glass and perspex spaces sealed with 1% agarose. The polyacrylamide gel was poured in two parts. The lower separating gel and the upper

stacking gel. The ratio of acrylamide to bisacrylamide in the gels was 36.5:1. The separating gel was made down to the required acrylamide concentration from a stock of 30% with 4x separating buffer and distilled water. 30 ml per gel was made and to initiate polymerization of the gel 1 µl of TEMED per 1 ml of gel and 150 µl of 10% ammonium persulphate was added and well mixed. The separating gel, when poured, was overlaid with water saturated-n-butyl alcohol to help form a smooth gel surface. Shortly before use, the n-butyl alcohol was totally removed by washing and the gel overlaid with a 4.5% acrylamide stacking gel. Again, polymerization was initiated with 1 µl TEMED per 1 ml of gel (8 ml) and 150 µl of 10% ammonium persulphate. A comb was immediately inserted with either 10 or 20 wells. Samples were run on the gel at a voltage of 8 to 10 Vcm^{-1}.

Visualization of DNA on polyacrylamide gels

Analysis of DNA fragments of size 200 base pairs (bp) to 1,000 bp were done on acrylamide gels. The gels were formed as described previously with the separating gel being 5% acrylamide. 40 µl samples had 20 µl of sample buffer added (0.15 M Tris, 1 mM EDTA, 6% (w/v) SDS, 30% (v/v) glycerol, 0.03% (w/v) bromophenol blue, pH 6.8) before loading onto the gel. In the case of λ DNA, the sample was heated to 65°C for 10 min before rapid cooling on ice and loading onto the gel. This prevented the fragments containing the cos sites from annealing and running as a single band of DNA instead of two lanes. Gels were stained in water containing 0.5 µg ml^{-1} of ethidium bromide for 10 min before visualization of the DNA bands with transmitted UV light (302 nm).

Sizing of DNA fragments from gel electrophoresis

The equation described by Southern (1979) were used to calculate the size of DNA fragments. The equation utilizes the fact that the molecular weight of a fragment has a proportional effect on the mobility of the fragment through a gel. The relationship is accurate in the 0.5 kilobases (kb) to 20 kb range. The equations are shown in Figure 2.2.

Elution of DNA fragments from an agarose gel

Restricted DNA was eluted from 0.8% agarose gels, run with 0.5 $\mu g\ ml^{-1}$ ethidium bromide present. DNA bands were visualized, using transmitted UV light (302 nm) and the elution procedure followed once the band of interest was well separated from the other bands of DNA (Yang et al., 1979). A trough of 2 mm width was cut in front of the band and EDTA cleaned dialysis tubing inserted into the trough so that it lay against the far side of the agarose gel from the DNA. The trough was filled with electrophoresis buffer and the gel run until the DNA fragment had run into the trough and attached to the dialysis membrane. A two second reversal of current detaches the DNA from the membrane, so that it can be easily taken up with the electrophoresis buffer present in the trough. The DNA was then cleaned using phenol, phenol/chloroform and chloroform extraction procedure or by using an Elutip-d minicolumn purchased from Schleicher and Schüll.

Figure 2.2 Calculation of size of DNA fragments from mobility in agarose or polyacrylamide 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_o that determines that these three points are joined by a straight line is given by:

$$m_o = \frac{m_3-m_1\ [((L_1-L_2)/(L_2-L_3))\ x\ ((m_3-m_2)/(m_2-m_1))]}{1\ -\ [((L_1-L_2)/(L_2-L_3))\ x\ ((m_3-m_2)/(m_2-m_1))]}$$

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

$$k_1 = \frac{L_1-L_2}{1/(m_1-m_o)-1/(m_2-m_o)}$$

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

The equation below was then used to calculate the size of a fragment L with mobility m:

$$L = \frac{k_1}{(m-m_o)+k_2}$$

Transformation of D. radiodurans with chromosomal and plasmid DNA

In the genus Deinococcus only the species D. radiodurans has been found to be transformable. The method used was that of Tirgari and Moseley (1980). An overnight culture was diluted with fresh TGY to give a nephelometer reading of 10 and grown at 30°C until a reading of 30-40 was reached. 10 ml of cells were harvested and resuspended in 5 ml TGY and 2 ml 0.1 M $CaCl_2$. The suspension was held at 0°C for 10 minutes and then 0.3 ml aliquots were added to 50 µl of DNA (0.1-1 mg ml^{-1}), and mixed gently. It was then incubated at 30°C with gentle shaking for 90 min, followed by the addition of 2.7 ml TGY broth. Incubation at 30°C with vigorous shaking for 5 hours allowed expression of phenotypes. In certain cases phenotypic expression was allowed overnight before selection. After marker expression, serial dilutions of the transformed culture were made and plated onto relevant selection plates and also onto TGY plates for a viable count. Viable counts could be scored after 2 days incubation at 30°C but counts from selective plates were possible only after 3-4 days.

To test for possible Rec^- phenotypes (ie lack of transformability) a simplified version of the above procedure was used. 10 ml of cell culture were harvested at nephelometer readings of between 20 and 100. These were resuspended in 5 ml TGY and 2 ml 0.1 M $CaCl_2$ and then kept at 0°C for 10 min. 0.3 ml was gently mixed with 50 µl of chromosomal DNA containing the rif^R marker gene and incubated at 30°C for 90 min before the addition of 5 ml TGY and further incubation at 30°C overnight. Only a tenfold dilution was used to plate

onto TGY plates containing 30 μg rifampicin ml^{-1}, to score for transformability, a possible Rec^- phenotype being shown by non-transformability.

Transformation of E. coli HB101 with plasmid DNA

The procedure developed by Humphrey et al. (1979) was used to transform plasmid DNA into E. coli HB101. An overnight culture of E. coli HB101 was diluted 30-fold into fresh prewarmed (37°C) LB broth and incubated at 37°C with shaking until a nephelometer reading of 30 was reached. 20 ml of culture was centrifuged in a bench-top centrifuge and the cells washed in 10 ml of cold 10 mM $CaCl_2$. The cells were recentrifuged and resuspended in 1 ml of 75 mM $CaCl_2$, 10 mM MOPS, 0.5% (w/v) glucose, pH 6.5. 200 μl of the cell suspension was then gently mixed with 50 μl plasmid DNA (10-20 μg ml^{-1}) in 0.1 M Tris pH 7.2. A further 250 μl of fresh 75 mM $CaCl_2$, 10 mM MOPS, 0.5% (w/v) glucose pH 6.5 was added and the mixture incubated at 0°C for 30 min. The transformation mixture was then transferred to 40°C for 5 min and followed by the addition of 1 ml warm LB broth. The culture was then incubated with shaking at 37°C for 2 h for phenotypic expression. Appropriate dilutions were plated onto relevant antibiotic selective plates for incubation at 37°C for 16 hours.

A second method was employed whereby competent cells were stored at -70°C. A 100 ml culture of E. coli HB101 was grown to an OD of 30 and cooled rapidly on ice for 10 min. The cells were harvested at 4°C and resuspended in 6 ml of cold 0.1 M $CaCl_2$. The suspension was kept at 0°C for 30 min and then distributed between 4 microcentrifuge tubes. The suspensions were centrifuged and each pellet was resuspended in 250 μl of 0.1 M $CaCl_2$. To store the cells,

0.35 ml of sterile glycerol was added to the 1 ml of suspended cells, distributed into 50 µl amounts and frozen at -70°C. To transform the cells, 10 µl of DNA (10-100 µg ml^{-1}) was mixed with 50 µl of the thawed E. coli suspension. The transformation mixture was put on ice for 30 min and then transferred to 40°C for 5 min, followed by the addition of 1 ml prewarmed LB broth. The culture was then incubated at 37°C for 2 h to allow phenotypic expression and appropriate dilutions plated out onto relevant selective media.

Isolation of new Deinococcus species

Solid (eg soil) and water samples collected from various natural habitats into sterile bottles were screened for possible new Deinococcus species. Solid samples (1-2 g) had 15 ml of sterile 67 mM phosphate buffer mixed vigorously with them. Debris and soil were allowed to settle out and the supernatant decanted into a second sterile 20 ml bottle. Any supernatant with a large amount of colloidal material present was centrifuged in a bench top centrifuge for 30 s, to remove most of it. The supernatant was placed in a sterile glass petri dish to form a shallow layer, no deeper than 1 mm, and irradiated with 254 nm UV light, from a Hanovia germicidal lamp, at a dose rate of 1.05 Jm^{-2} sec^{-1} for 20 min. A sterile magnetic bar was used to stir the supernatant during the irradiation and samples were taken at 10, 15 and 20 min. 100 µl samples were plated out on NB2 and TGY plates, and then incubated at 30°C for 2-4 days. Any red/pink/orange colony was picked and streaked on NB2 or TGY plates to get a pure single colony. Isolates were then screened for Deinococcus-like properties. All the procedures up to and including plating, with the exception of the UV irradiation, were

conducted in a laminar air flow cabinet to prevent reisolation of a lab strain from the air.

Measurement of UV survival

An overnight culture was diluted to give an OD reading of 10 and allowed to grow to an OD of 30. Cells from 10 ml of culture were harvested and resuspended in 10 ml 67 mM phosphate buffer. 5 ml was placed in a sterile glass petri dish and stirred well with a sterile magnetic bar during UV irradiation. The suspension was irradiated with 254 nm UV light, from a Hanovia germicidal lamp (Hanovia Lamps Ltd, Slough, Bucks) at a dose rate of 1.05 Jm^{-2} s^{-1}. 100 µl samples were taken at appropriate times and diluted in TGY broth. 100 µl sample of appropriate dilutions were spread on TGY plates and incubated at 30°C for 3 days before recording the number of colonies. Survival curves were constructed by working out the relative survival at a given time to that at time zero.

A rough guide as to the UV survival of a culture was obtained by exposing a streak of the culture on a TGY plate to increasing doses of UV. One side was not exposed to UV and the other to 12 min in 2 min steps. This gave a quick visual idea as to the resistance of the strain to 254 nm light.

Rapid screening method for restriction endonucleases

This method was developed to screen for restriction endonucleases from strains in which large quantities of enzyme were produced that would work in a NaCl salt concentration of 50 mM. Dense cultures of the bacterial strains were grown in 100 ml TGY broth over 1-2 days. The cells were harvested, washed with TE

buffer and centrifuged. The cells were resuspended in 10 ml of 10 mM Tris, 1 mM EDTA, 0.1 mM PMSF, pH 7.5, and then broken by passing them through a French pressure cell at 40,000 psi at 0° to 4°C. Unbroken cells and debris were collected as a hard pellet by centrifugation at 27,000 g for 10 min. 17 µl of supernatant was added to 1 µl DNA (λ, pBR322 or øX174), 2 µl of x10 medium salt buffer (0.5 M NaCl, 0.1 M $MgCl_2$, 0.1 M Tris base, 10 mM DTT, pH 7.8) and then incubated at 37°C for 2 h. 1 µl of STOP buffer was added and the samples loaded onto 0.8% or 0.6% agarose gels. Fragment sizes were calculated for λ and pBR322 and compared with the restriction patterns produced by known restriction endonucleases.

Southern blotting

A modified method of Southern (1975) was used. 5 µg samples of different chromosomal DNAs were digested with 50 units of EcoRI for 2 hours and run on a 0.8% agarose gel containing ethidium bromide. λ DNA cut with HindIII and specific plasmids (of which one would contain the DNA fragment to be used as the probe) cut with EcoRI were also run on the same gel, as controls. Following electrophoresis, the gel was illuminated with 302 nm UV light and photographed. Excess gel was trimmed away and the DNA denatured by placing the gel in two volumes of 0.5 M NaOH, 1.5 M NaCl solution for 30 min at room temperature. The gel was washed in distilled water and neutralized with two volumes of 1 M NH_4 acetate, 0.02 M NaOH for 30 to 60 min at room temperature. A piece of nitrocellulose filter and 10 sheets of blotting paper (Whatman 3 MM paper) were cut to the size of the gel. The gel was placed on a glass plate and then the nitrocellulose filter (Schleicher and Schuell BA85) saturated

in 1 M NH_4 acetate, 0.02 M NaOH was placed on top of the gel. Then, in ascending order, 4 sheets of saturated blotting paper, 6 dry blotting paper sheets, a stock of paper towels (about 6 cm high), and another glass plate were placed on the nitrocellulose filter. Care was taken not to trap air bubbles between soaked layers of filter or blotting paper. A weight was put on the top glass plate to distribute contact evenly over the gel. Transfer of DNA to the nitrocellulose filter was achieved in approximately 1 h at room temperature. Bidirectional transfer was also done where a second nitrocellulose filter is placed beneath the gel. The transfer then proceeded as with unidirectional transfer. After transfer, one corner of the filter was cut for orientation and then washed in 500 ml of 2 x SSC for 2 min at room temperature. The filter was dried at 37°C and baked at 80°C for 2 h in a vacuum.

The baked filter was soaked in prehybridization buffer for 3 h at 37°C, in a plastic bag. The denatured probe DNA (heated at 100°C for 15 min) was then added and air bubbles removed from the bag before resealing. The filter was incubated at 37°C overnight and washed to remove unbound label. In a stringent wash, 0.1 x SSC, 0.1% SDS was used to wash the filter in 200 ml changes for 1 min, 5 min, 15 min, 15 min and 20 min in succession at 37°C. This was followed by a wash in 0.1 x SSC for four 15 min periods (250 ml each). A less stringent wash used 2 x SSC, 0.1% SDS in 200 ml changes for 1 min, 5 min, 10 min, 20 min and 30 min in succession at 37°C. This was followed by a wash in 1 x SSC for four 25 min periods (250 ml each). The washed filters were then dried at 37°C and placed on blotting paper. Saran wrap was placed over the filter and applied to X-ray film for 1-4 days at -70°C to obtain an autoradiograph image.

Preparation of probe DNA by nick-translation

Probe DNA was prepared by using the nick translation method of Rigby et al. (1977), using the BRL nick translation reagent kit. DNA from Deinococcus radiodurans and Deinococcus radiophilus was used to probe filters. 10 µCi of [α-^{32}P] labelled dCTP was used to label 1 µg of plasmid DNA. After nick translation, the probe was cleaned of unincorporated [α-^{32}P] nucleotide by passing through a Sephadex G-50 fine column (Pharmacia) in a 1 ml syringe. TES buffer was used to wash the probe DNA through the column whilst leaving the unincorporated nucleotides behind in the sephadex matrix. The reaction mixture (55 µl) was loaded onto a washed G-50 column and centrifuged at 1,500 rpm for 4 min to collect the labelled probe in a microcentrifuge tube. A second wash of the column with 50 µl TES collected labelled probe DNA-which did not pass through the first time. The radioactivity of the probe was measured and the probe was boiled for 15 min before addition to prehybridisation buffer. The probe is denatured by the boiling to allow renaturation to homologous DNA on the filter.

Preparation of enzyme extracts for AP endonuclease and glycosylase activity from Deinococcus radiodurans

Cells (20 g wet weight) were washed in 50 mM Tris 10 mM EDTA. 0.1 M NaCl, pH 7.6. The cells were resuspended in 30 ml of the same buffer containing 0.15 mM PMSF (serine protease inhibitor) and broken using a French pressure cell at 40,000 psi at 4°C. Extracts were always kept at 0-4°C from this point. Cell debris and unbroken cells were removed by centrifugation at 25,000 g for 10 min to leave a clear red supernatant. To remove DNA, streptomycin sulphate was

added to 1% (w/v) and mixed for 5 min. The white precipitate formed was removed by centrifugation at 25,000 g for 10 min. To the supernatant, solid ammonium sulphate ($(NH_4)_2SO_4$) was added to give different saturation levels of $(NH_4)_2SO_4$. A magnetic bar was used to stir the supernatant, dissolving the $(NH_4)_2SO_4$ and mixing the solution. Precipitates produced at each saturation level were collected by centrifugation at 12,000 g for 10 min. The precipitates were resuspended in 20 mM Tris 2 mM EDTA pH 7.6, and dialyzed against 1 litre of the same buffer overnight. The different $(NH_4)_2SO_4$ cuts of protein were assayed for enzyme activity, to show the range over which the particular enzyme precipitated with $(NH_4)_2SO_4$.

Further purification of AP endonuclease was undertaken using three different chromatography matrices.

Preparation of chromatography materials

1. DEAE Sephacel (Pharmacia)

DEAE Sephacel is an anion exchanger and binds negatively charged ions. The DEAE Sephacel was washed in 50 mM Tris 5 mM EDTA pH 7.6 and then poured into a column for packing at a rate of 10 ml h^{-1}. The column size was 20 cm x 2.6 cm. The column was washed with 6 times its volume of 20 mM Tris 2 mM EDTA, pH 7.6. Elution of proteins bound to the matrix was done using step or linear gradients of NaCl in 20 mM Tris 2 mM EDTA, pH 7.6.

The pH and salt concentrations required to bind and elute AP endonuclease from the DEAE Sephacel column were calculated from small pilot tests. 0.5 ml of DEAE Sephacel was washed 5 times in a 0.5 M Tris-HCl buffer. A pH range of 5 to 9 was used with 0.5 pH

unit steps. The Sephacel in each tube was then equilibriated at its particular pH with a 20 mM Tris HCl buffer by washing 5 times. One µl of a dialyzed ammonium sulphate fraction containing AP endonuclease was then added to each tube at 0°C and mixed. After 30 min the enzyme was assayed from the supernatant. AP endonuclease was shown to be bound at pH 7.0 but not 6.5. A pH of 7.6 was used to give good binding of AP endonuclease to DEAE Sephacel while allowing easy elution with a salt gradient.

2. Hydroxylapatite (HT grade, Bio-rad)

Hydroxylapatite was prepared in a similar way to DEAE Sephacel with the buffer 10 mM phosphate, 1 mM EDTA, 50 mM NaCl, 5% (v/v) glycerol, pH 7.0 being used to pack and wash the column (10 cm x 2.6 cm). The column was packed at 50 ml h^{-1}. Elution of bound protein was done using a linear phosphate buffer gradient.

3. S-200 (Pharmacia)

S-200 is a gel filtration matrix. It was washed with 20 mM Tris, 2 mM EDTA, 5% (v/v) glycerol, 0.2 M NaCl pH 7.6 and packed in a column 70 cm x 1.6 cm at 150 ml h^{-1} with the same buffer. Samples were loaded and developed at approximately 5 ml h^{-1} in the above buffer.

All chromatographic procedures were done at 4°C and all solutions (except samples) were degassed before use. Fractions were collected in acid washed test tubes.

Assay of protein concentration

Protein concentrations were assayed using a Bio-Rad (Watford, Herts) protein assay kit.

Estimation of the molecular weight of AP endonuclease

The molecular weight of a protein can be estimated using a gel filtration matrix such as Sephacryl-200 (Pharmacia).

The volume of buffer required to elute a protein and its K_{AV} is linearly related to the $\log_{10}$ molecular weight of the protein. This means that a column can be calibrated using known molecular weight standards. Standards used on the S-200 column were Dextran 2000 (Pharmacia mw >1,000,000), alcohol dehydrogenase (Sigma mw 150,000), bovine serum albumen (Sigma mw 66,000), ovalbumen (Sigma mw 45,000), carbonic anhydrase (Sigma mw 29,200), ribonuclease A (Sigma mw 13,700) and potassium ferrocyanide (BDH mw 330). A Pharmacia UV-1 monitor using a 280 nm filter was used to monitor peaks of protein as they were eluted off the column. The 70x1.6 cm sephacryl-200 column was calibrated to give the curve in Figure 2.3 using the following formula:

$$K_{AV} = \frac{V_e - V_o}{V_t - V_o}$$

K_{AV} - is the fraction of the stationary gel volume which is available for diffusion of a given solute species.

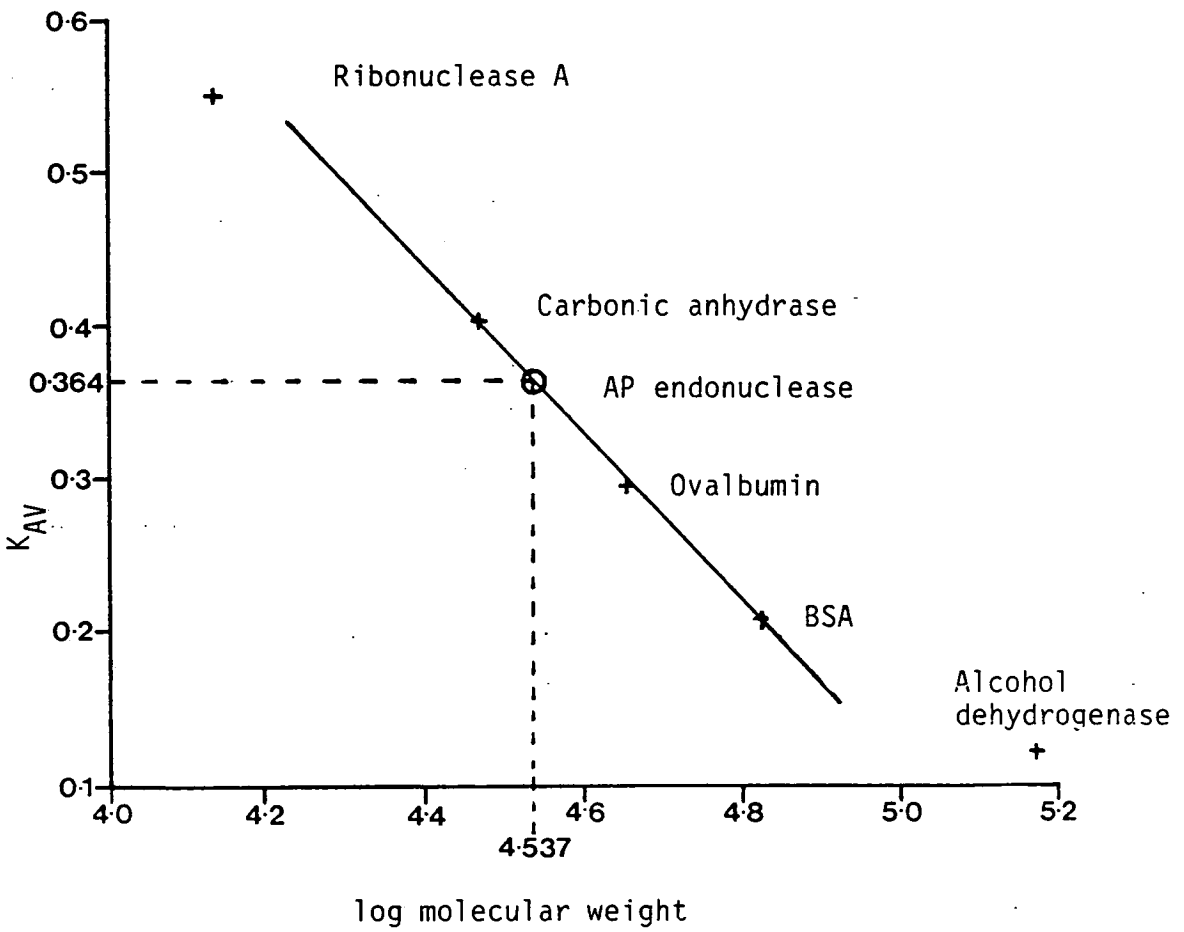
V_e - is the elution volume of a protein

V_o - is the void volume (measured using Dextran 2000)

V_t - is the total volume of the column (measured using a small molecule which distributes freely in the mobile and matrix phases, eg potassium ferrocyanide).

Knowing the K_{AV} of the studied protein allows the calculation of its molecular weight from the curve in Figure 2.3.

Figure 2.3 Calibration curve for the 70 cm x 1.6 cm Sephacryl-200 column for the estimation of molecular weights



Visualization of proteins on SDS polyacrylamide gels

Separating gels of 12% polyacrylamide were made and overlaid with a stacking gel of 4.5% polyacrylamide. To 40 µl of sample, 20 µl of sample buffer (0.15 M Tris base, 6 mM EDTA, 6% (w/v) SDS, 30% (v/v) glycerol, 0.03% (w/v) bromophenol blue, pH 6.8) and mercaptoethanol to 2 mM was added and mixed. The samples were then boiled for 2 min before cooling and loading into the wells. The gel was run at 10 V cm^{-1} for 5-6 h. Gels were stained using kenacid blue R250 (BDH) or silver stain.

Kenacid blue staining

The gel was incubated overnight in 0.25% (w/v) kenacid blue, 45% (v/v) methanol, 8% (v/v) glacial acetic acid and was destained with 5% (v/v) methanol, 7.5% (v/v) glacial acetic acid, with numerous changes.

Silver staining

The Bio-rad silver staining technique was used. Three solutions were prepared just prior to use:

a. Oxidizer - 1 g potassium chromate and 0.2 ml concentrated nitric acid in 1 l H_2O
b. Silver reagent - 2 g silver nitrate in 1 l H_2O
c. Developer - 30 g sodium carbonate and 0.5 ml formaldehyde in 1 l H_2O.

The above solutions and others shown in Table 2.4 were used to silver stain proteins in the gel.

Table 2.4 Silver staining table

Reagent	Volume (ml)	Time (0.5 mm-1.0 mm gel)
1. Fixative - 40% methanol/10% acetic acid v/v	400	30 min
2. " - 10% methanol/5% acetic acid v/v	400	15 min
3. " - 10% methanol/5% acetic acid v/v	400	15 min
4. Oxidizer	200	5 min
5. Distilled H_2O	400	5 min
6. " "	400	5 min
7. " "	400	5 min
8. Silver reagent	200	20 min
9. Distilled H_2O	400	1 min
10. Developer	200	30 s
11. "	200	~ 5 min
12. "	200	~ 5 min
13. STOP - 5% acetic acid v/v	400	5 min

Steps 10-12 are the most important.

The reactions were undertaken at room temperature.

Assays for AP endonuclease

Two methods were used; a qualitative method using a plasmid and a quantitative method using radiolabelled chromosomal DNA. The plasmid assay system used depurinated plasmid DNA in conditions of pH 7.0 to 7.6 with ionic strength of 10-100 mM. The presence of an AP endonucleolytic activity was detected by the conversion of depurinated covalently closed circular (ccc) plasmid DNA to the open circular (oc) form, as detected via agarose gel electrophoresis. This method was extremely sensitive, requiring only one nick in the plasmid, to show the presence of an AP endonuclease.

The second method relied on the release of radiolabelled oligonucleotides which are perchloric acid soluble, and which can be counted in a scintillation counter. After incubation of 20 µl depurinated radiolabelled DNA (50 µg ml^{-1}) with 20 µl of enzyme preparation for 15 min, the reaction was quickly cooled to 0°C. 100 µl of 2 mg ml^{-1} calf thymus DNA in 0.1 x SSC was added and well mixed by inversion. 500 µl of 6.4% (v/v) perchloric acid was added immediately and well mixed by inversion. This mixture was left on ice for 15 min, and then centrifuged for 15 min in a microcentrifuge. 500 µl of supernatant was added to 3 ml of a dioxan-based scintillant (NE250-Nuclear Enterprises, Edinburgh). The radioactivity in the vial was determined in a scintillator counter (Packard Instruments Ltd, Caversham, Bucks).

For both methods, the enzyme Exonuclease III was used as a control. With calcium ions present as the only divalent cation, Exonuclease III has only an AP endonuclease activity. Reactions were performed in 50 mM Tris, 5 mM Ca^{2+}, 1 mM mercaptoethanol, pH 8.0 for both methods, with 0.05 x SSC also present in the radio-

labelled DNA method. In the radiolabelled DNA method, 20 µl of 0.4 M NaOH was added to 20 µl of the depurinated DNA in a control reaction at 37°C for 15 min. This procedure nicks the DNA at every AP site to give a maximal reading on the scintillation counter.

Assays for methylated DNA glycosylase

Both methods used to assay for an AP endonuclease were employed to assay for a glycosylase which removed methylated nucleotides from DNA. A linked assay was developed using the sequential reactions of glycosylase and AP endonuclease to identify the presence of a glycosylase specific for methylated nucleotides. AP sites produced by the action of the glycosylase were nicked by the AP endonuclease. This was seen either by the conversion of ccc methylated plasmid DNA to the oc form, as detected by agarose gel electorphoresis, or by the liberation of perchloric acid-soluble radiolabelled oligonucleotides from methylated radiolabelled DNA.

Assay for uracil glycosylase

Only the plasmid method was used, showing conversion of uracil-containing ccc plasmid DNA to the oc form as visualised by agarose gel electrophoresis. Again the presence of an AP endonuclease was required for the assay to work.

CHAPTER 3

RESULTS

1. Attempts to construct a shuttle vector between *D. radiodurans* and *E. coli*

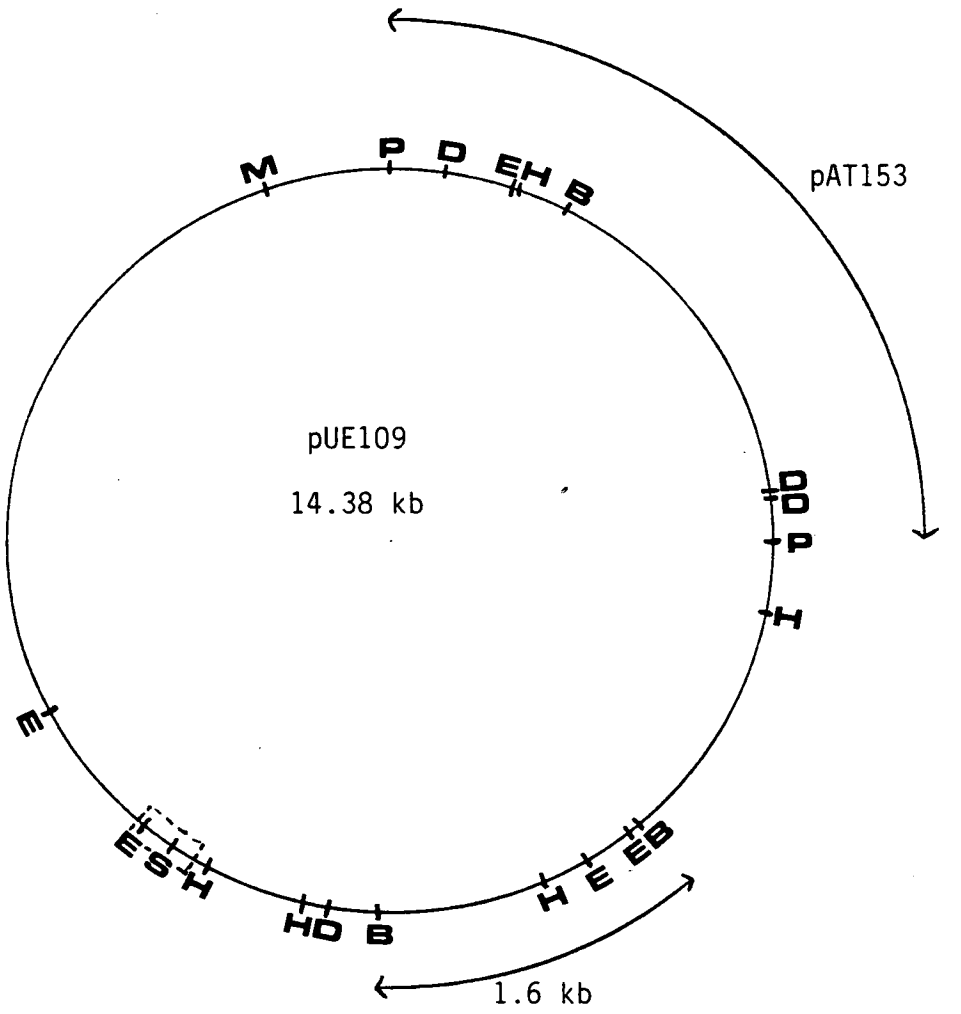
It has been shown previously that plasmids derived from Gram +ve and Gram -ve strains of bacteria, could not transform any Deinococcus species (Mackay, 1983; Purvis, 1984). Attempted transformation of protoplasts of *D. radiodurans* and *D. radiophilus* with a wide range of plasmids and antibiotic markers was also unsuccessful (Al-Bakri, 1985). Al-Bakri developed a chimeric plasmid composed of the *D. radiophilus* cryptic plasmid, pUE1, and the *E. coli* plasmid, pAT153 (Al-Bakri *et al.*, 1985). This plasmid, pUE109 (Figure 3.1) has a functional tetracycline resistance (Tc^R) gene in *E. coli* HB101 but it did not transform *D. radiodurans* to Tc^R.

1.1 Removal of the *Sst*II site of pUE109

The sticky ends produced by cutting with *Sst*II (recognition sequence 5'-CCGC↓GC-3') could not be filled in by using the Klenow fragment of DNA polymerase, because the recessed site was on the 5' end of the DNA. S1 nuclease is a single strand specific nuclease which should remove the protruding ends of the *Sst*II cut site. After numerous attempts with changes of enzyme concentration, buffer constituents and selection procedure, only pUE109 was reisolated with an intact *Sst*II site. Mung Bean nuclease works in a similar manner to S1 nuclease but it is not usually as effective. The use of Mung Bean nuclease did not remove the *Sst*II site either.

*Bal*31 nuclease, a highly specific single strand exonuclease that catalyzes the removal of mononucleotides and oligonucleotides

Figure 3.1 Map of pUE109 showing the region of DNA removed to produce pUE119



⬚ - fragment removed by Bal31 digestion to give pUE119

E- EcoRI, **H**- HindIII, **B**- BamHI, **P**- PstI,
M- MluI, **S**- SstII, **D**- DraI

from both 5' and 3' termini of double stranded DNA, was a more powerful tool to remove the SstII site. Degradation of both strands occurs at approximately the same rate, which is dependent on the concentration of both enzyme and duplex termini. An enzyme concentration was used which gave a rate of degradation of approximately 60 bp min^{-1} DNA $strand^{-1}$. Samples were taken at 15 s, 30 s, 60 s, 2 min and 4 min. After purifying the DNA by phenol-chloroform extractions and ligation in 20 µl ligation buffer (10 mM $MgCl_2$, 20 mM Tris, 10 mM DTT, 0.6 mM ATP, pH 7.6) with one unit T4 DNA ligase, it was transformed into HB101. Tc^R clones were screened for pUE109 plasmids missing the SstII site, using the boiling method to prepare plasmid and then digesting it with SstII restriction enzyme. Three plasmids from the sample removed after 60 s were found not to be cut by SstII. A large scale preparation of one plasmid was made. Lane 3 of Figure 3.2 shows that the plasmid was not cut by SstII whilst pUE109 was (lane 2). The plasmid was called pUE119 and was shown to have one MluI site (lane 6) and two PstI sites, to liberate pAT153 (lane 9).

pUE119 was sized and compared with pUE109 using EcoRI and HindIII digests (Figure 3.3). The fragment sizes are shown in Figure 3.3. The HindIII digests show a loss of 0.49 kb, the EcoRI digests a loss of 0.52 kb and the double digest shows a loss of 0.42 kb. The Bal31 digest removed between 0.42 and 0.52 kb of DNA and this area is shown in Figure 3.1. A greater stretch of DNA was removed from one side of the SstII site than the other. This could be due to the map positions of the restriction sites being inaccurate or the DNA to the EcoRI side of the SstII site having a greater proportion of A-T base pairs. Bal31 degrades A-T rich DNA at a greater rate then G-C rich DNA.

Figure 3.2 0.8% agarose gel to show the loss of the SstII site in plasmid pUE119 derived from pUE109

Lanes: 1, Undigested pUE109; 2, pUE109 + SstII; 3, pUE119 + SstII; 4, undigested pUE119; 5, pUE109 + MluI; 6, pUE119 + MluI; 7, λ + HindIII; 8, pUE109 + PstI; 9, pUE119 + PstI; 10, pAT153 + PstI; 11, pUE109 + SstII + PstI; 12, pUE119 + SstII + PstI.

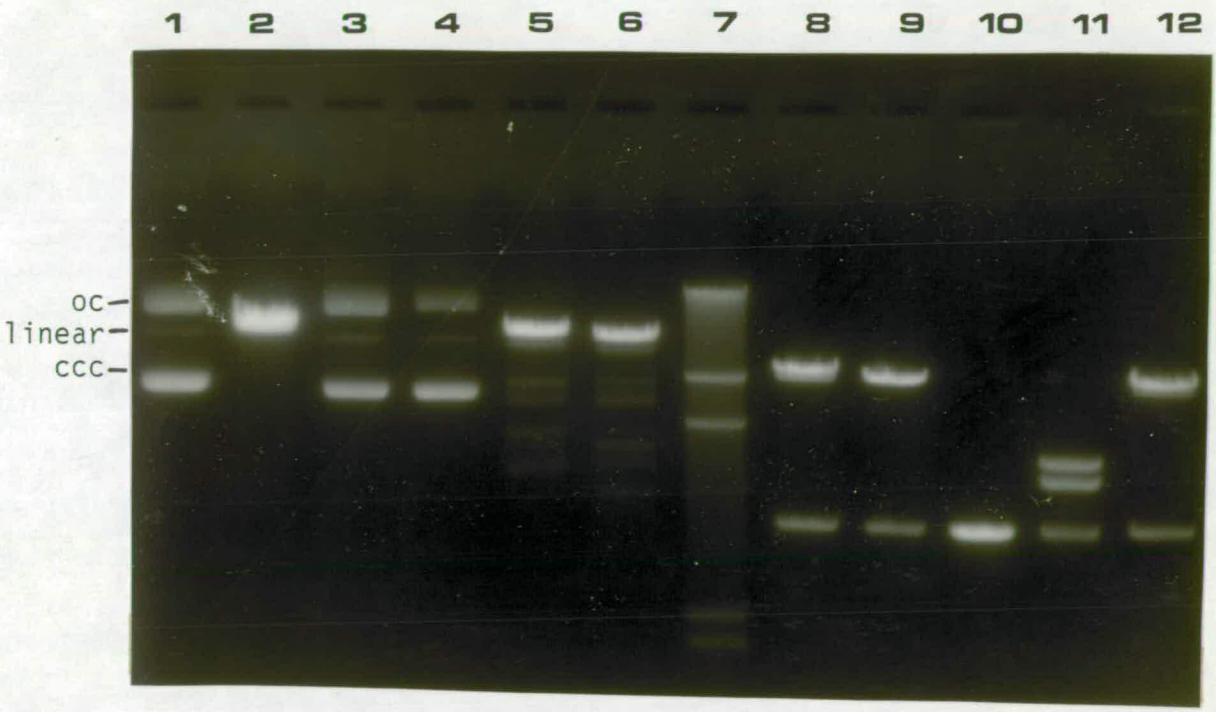
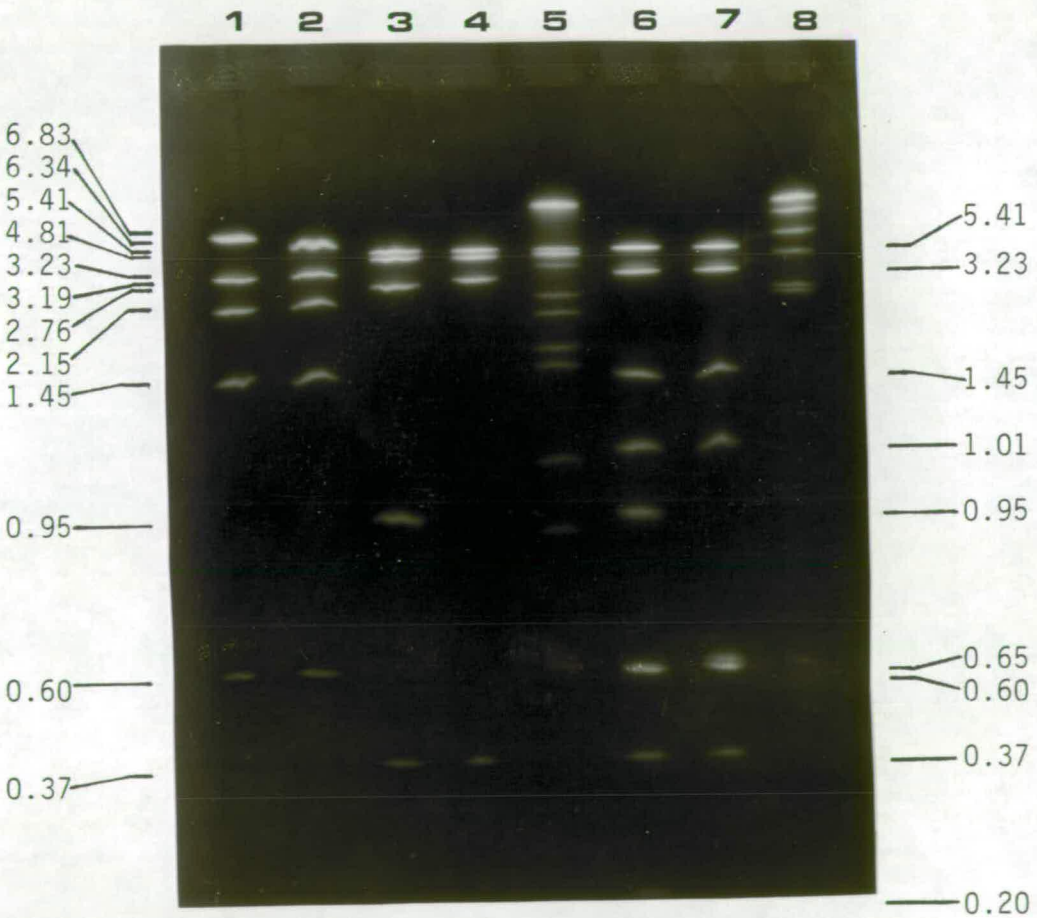


Figure 3.3 5% polyacrylamide gel showing the fragment sizes of digested plasmids pUE109 and pUE119

Lanes: 1, pUE109 + HindIII; 2, pUE119 + HindIII; 3, pUE109 + EcoRI; 4, pUE119 + EcoRI; 5, λ + HindIII + EcoRI; 6, pUE109 + HindIII + EcoRI; 7, pUE119 + HindIII + EcoRI; 8, λ + HindIII.

Fragment sizes in kilobases



Transforming pUE119 into D. radiodurans produced no increase in Tc^R colonies over the spontaneous mutation frequency on TGY plates containing tetracycline at a concentration of 0.5 μg ml^{-1}. The lack of transformability of D. radiodurans to Tc^R may not therefore be due to the presence of the SstII site in pUE109. The site may still be a problem but there is a second problem preventing the expression of the plasmid in D. radiodurans. The removal of the SstII site may have prevented the plasmid from being restricted but its removal may have taken out an important sequence involved with replication. An MraI$^-$ strain of D. radiodurans would be more useful than removing SstII sites from plasmids. Attempts at producing such a strain have not been successful (B.E.B. Moseley, pers.comm.). The removal of approximately 500 bp is not satisfactory when the loss of 6 bp would have been sufficient. But the use of SI nuclease, Mung Bean nuclease and low levels of Bal31 did not produce a plasmid with a few bps removed, as might have been expected.

1.2 Rearrangement of pUE119 to produce a plasmid with an active Ap^R gene

pUE119 was cut with BamHI and the DNA put in a ligation mixture. Tc^R transformants of E. coli HB101 were screened for plasmids which had lost the 1.6 kb BamHI fragment (Figure 3.1). Such a plasmid, pUE129, was identified and purified. PstI was used to cut pUE129 and the pUE1-derived DNA was separated and purified from the pAT153-derived DNA by electroelution and phenol-chloroform extraction. The pUE1-derived DNA was religated and then cut with BamHI. The plasmid was religated to pAT153 cut at its BamHI site. $Ap^R Tc^S$ transformants

of E. coli HB101 were screened and pUE120 found (Figure 3.4).

Both pUE129 and pUE120 were used in an attempt to transform D. radiodurans to Tc^R and Ap^R respectively on TGY plates containing ampicillin at 0.2 μg ml^{-1} or tetracycline at 0.5 μg ml^{-1}. No transformants were found. The Ap gene which produces β-lactamase is probably not expressed at either the transcriptional or translational level. A third possibility is that the β-lactamase enzyme is not transported across the cell envelope, to act on the ampicillin.

A similar series of plasmids from pUE109 also gave negative results. The two markers Ap^R and Tc^R do not work in a D. radiodurans environment so it was decided to try to use a D. radiodurans chromosomal marker.

1.3 Construction of plasmids containing the mtcA and mtcB genes of D. radiodurans

The two genes mtcA and mtcB, involved in the resistance to mitomycin C of D. radiodurans, are present in a 26.0 kb fragment cloned into pAT153, pUE502 (Al-Bakri, 1985). The plasmid contains two StuI sites (Figure 3.5) and from transformation experiments the smaller 7.3 kb fragment was shown not to contain either the mtcA or mtcB genes. This fragment was removed from the plasmid by digestion of the plasmid with StuI and religation of the plasmid. Screening Ap^R transformants of E. coli HB101 yielded pUE512 (Figure 3.5). Restriction endonuclease analysis of pUE512 showed that EcoRV cut at three sites to produce fragment sizes of 10.21, 5.21 and 4.78 kb. pUE512 was digested with EcoRV and ligated with alkaline phosphatase treated pAT153 cut at its EcoRV site. The middle sized fragment

Figure 3.4 Construction of pUE129 and pUE120 from pUE119

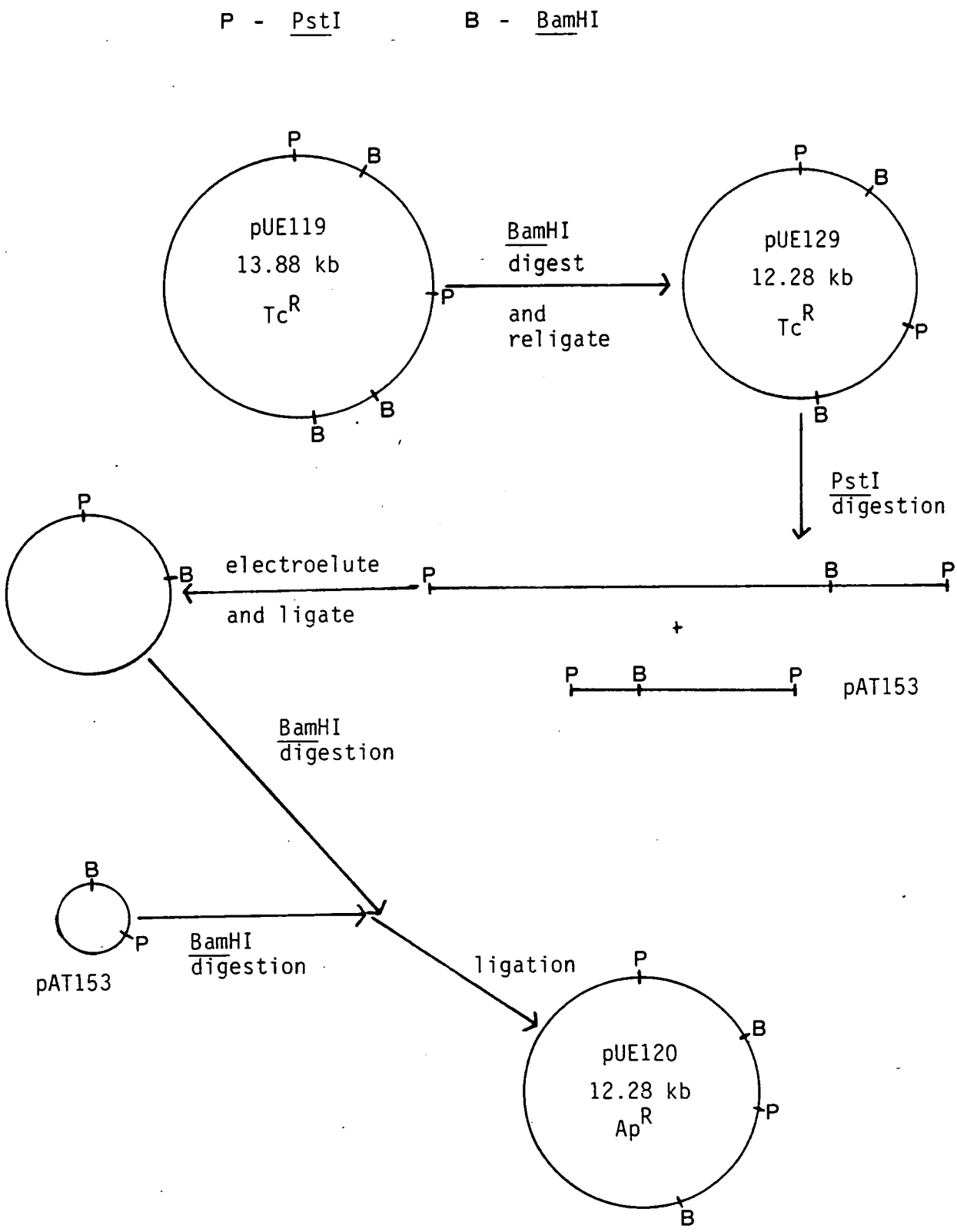
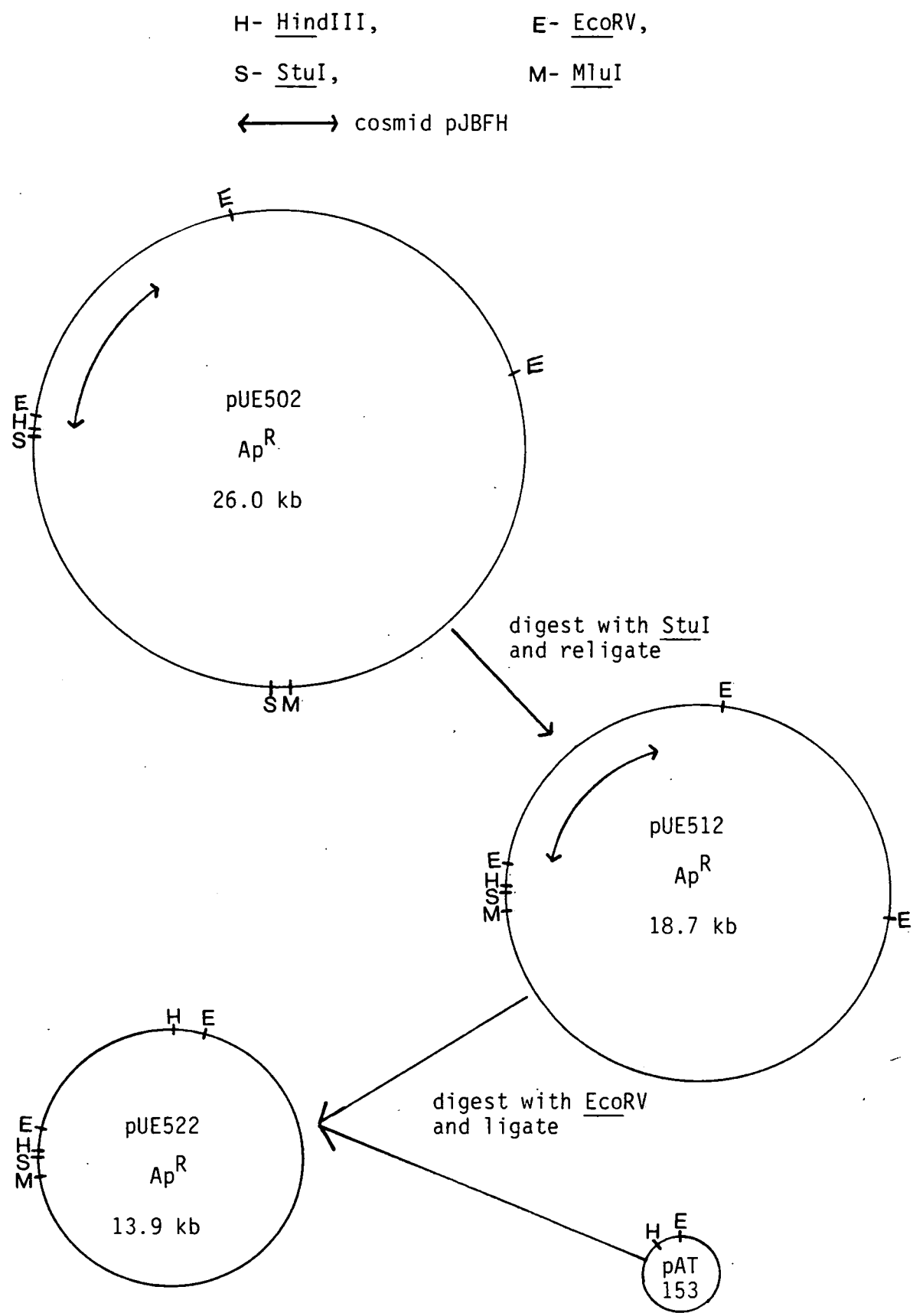


Figure 3.5 Construction of plasmids pUE512 and pUE522 from pUE502



religated to itself to form pUE523, containing the origin of replication and the Ap^R gene from pUE512. The large and small fragments were ligated to pAT153 to form pUE522 and pUE524 respectively. These three plasmids with pUE512 and pUE502 were used to transform the mtcA and mtcB strains of D. radiodurans, 78 and 262 respectively. The frequency of transformation to Mtc^R phenotype is shown in Table 3.1. The plasmid, pUE522, was shown to carry both the mtcA and mtcB genes of D. radiodurans. pUE522 has one MluI site (Figure 3.5). The site was shown not to be in the structural or control region of either mtcA or B by digesting pUE522 with MluI and transforming 78 and 262 to mitomycin C resistance. This MluI site was therefore used to clone into the single MluI site of pUE119 to form pUE641. This plasmid was easily selected for in E. coli HB101 on LB agar containing ampicillin and tetracycline as it received the Ap^R gene from pUE522 and the Tc^R gene from pUE119. This plasmid was used to transform strains 78 and 262 to Mtc^R phenotype. The transformation frequency was $6x10^{-3}$ for the mtcA and $1.3x10^{-5}$ for the mtcB gene. These frequencies did not vary substantially from those obtained using the plasmids shown in Table 3.1. This suggested that both mtcA and mtcB were transforming the mutant strains to wild type via a recombinational event between the plasmid and the chromosome, rather than the plasmid remaining an autonomous replicating unit. Preparing mini-plasmid preparations of 48 transformants each of 78 and 262 yielded no visible plasmids on a 0.8% agarose gel. pUE641 is a large plasmid (13.88 + 13.90 = 27.78 kb) with two pAT153 derived sections. This perhaps makes the plasmid unstable in the Rec^+ environment of D. radiodurans. This instability in D. radiodurans would ensure that only recombinational events into the

Table 3.1 Transformation of D. radiodurans mutants 78 and 262 with plasmids derived from pUE502 containing the mtcA and mtcB genes

Plasmid	Plasmid size (kb)	Transformation frequency to Mtc resistance	
		in 78	in 262
pUE502	27.80	6.8×10^{-3}	1.5×10^{-5}
pUE512	20.50	2.4×10^{-3}	1.8×10^{-5}
pUE522	14.10	7.8×10^{-3}	1.5×10^{-5}
pUE523	5.12	C[(a)]	C
pUE524	8.45	C	C
Control (no DNA)	-	$< 1 \times 10^{-9}$	$< 1 \times 10^{-9}$

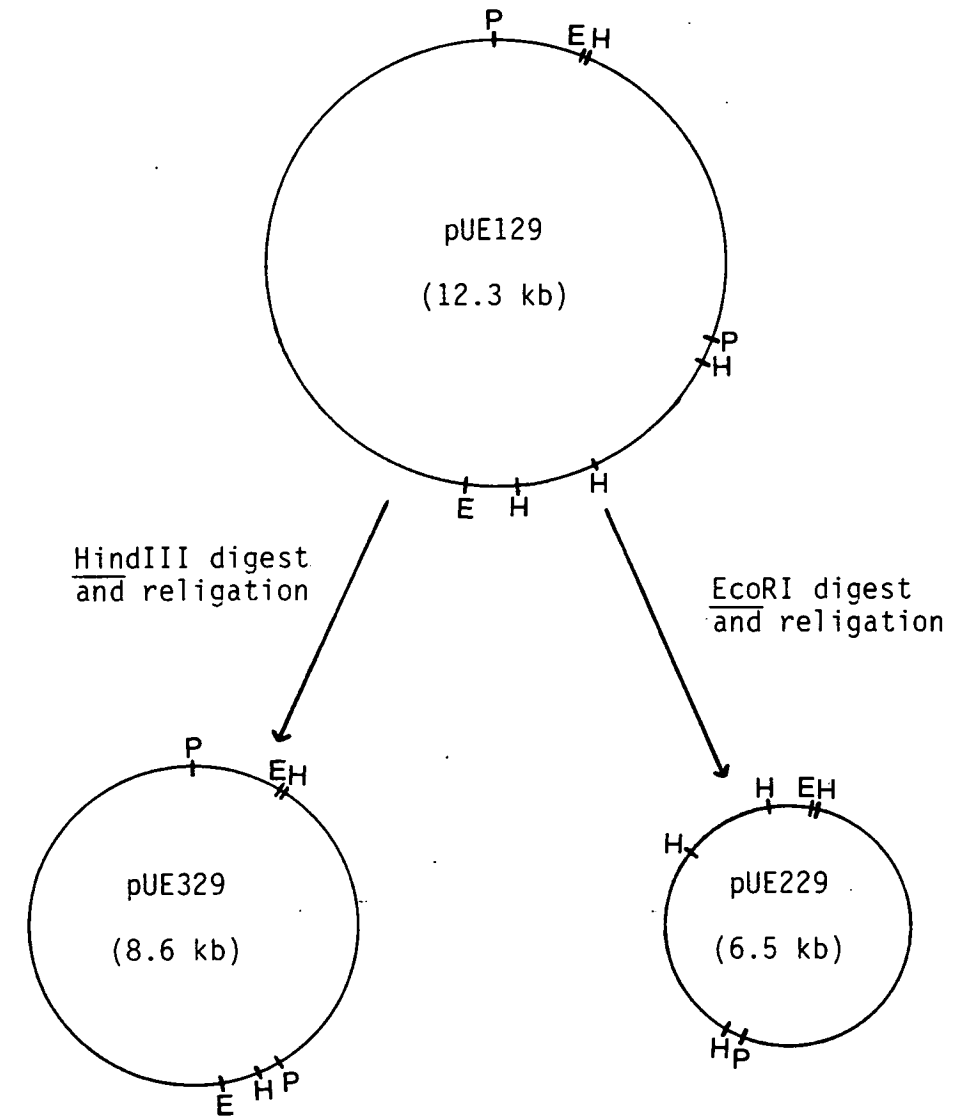
(a) C - control level

chromosome would transform the cell to Mtc^R.

It was decided to make smaller and more stable plasmids from pUE129 using again the mtcA and B genes of D. radiodurans. pUE129 was digested separately with either EcoRI or HindIII. They were religated separately to produce the plasmids pUE229 and pUE329 from the EcoRI and HindIII digests respectively (Figures 3.6 and 3.7), both being Tc^R in E. coli HB101. From Figure 3.7, it can be seen that pUE229 has single cloning sites at PstI and EcoRI. pUE329 has single cloning sites for MluI and BamHI. The two plasmids represent 76% of the D. radiophilus pUE1, and it was hoped that one could produce a plasmid that would replicate in D. radiodurans,

Previously, the mtcA and mtcB genes had been subcloned into pAT153 (Al-Bakri, 1985), a 5.6 kb EcoRI fragment containing the mtcA gene (pUE58) and a 2.7 kb EcoRI fragment containing the mtcB gene (pUE59). pUE58 and pUE59 were digested with EcoRI and the D. radiodurans DNA fragments electroeluted from an agarose gel. These were ligated separately into pUE229 at its single EcoRI site. Tc^R transformants were screened. Plasmids pUE158 and pUE159 were produced containing the mtcA, 5.6 kb fragment, and the mtcB, 2.7 kb fragment in pUE229 respectively (Figure 3.8). The two plasmids and others containing the mtcA or mtcB gene were used to transform strains 78 and 262 to Mtc^R. The transformation frequencies are shown in Table 3.2. Transformants were screened for plasmids using the mini-preparation method for D. radiodurans. No plasmids were detected. The transformation frequency for all the plasmids with the mtcA gene are of the same order as are the frequencies of transfer for the mtcB gene. Using chromosomal marker genes in the plasmids is a problem, as the recombination system in D. radiodurans appears to be

Figure 3.6 Construction of plasmids pUE229 and pUE329



H- HindIII E- EcoRI P- PstI

Figure 3.7 0.8% agarose gel showing restriction analysis of plasmids pUE229 and pUE329

Lanes: 1, pUE229 + DraI; 2, pUE229 + BamHI; 3, pUE229 + MluI; 4, pUE229 + PstI; 5, pUE229 + HindIII; 6, pUE229 + EcoRI; 7, pUE129 + EcoRI; 8, λ DNA + HindIII; 9, pUE129 + HindIII; 10, pUE329 + HindIII; 11, pUE329 + EcoRI; 12, pUE329 + PstI; 13, pUE329 + MluI; 14, pUE329 + BamHI; 15, pUE329 + DraI.

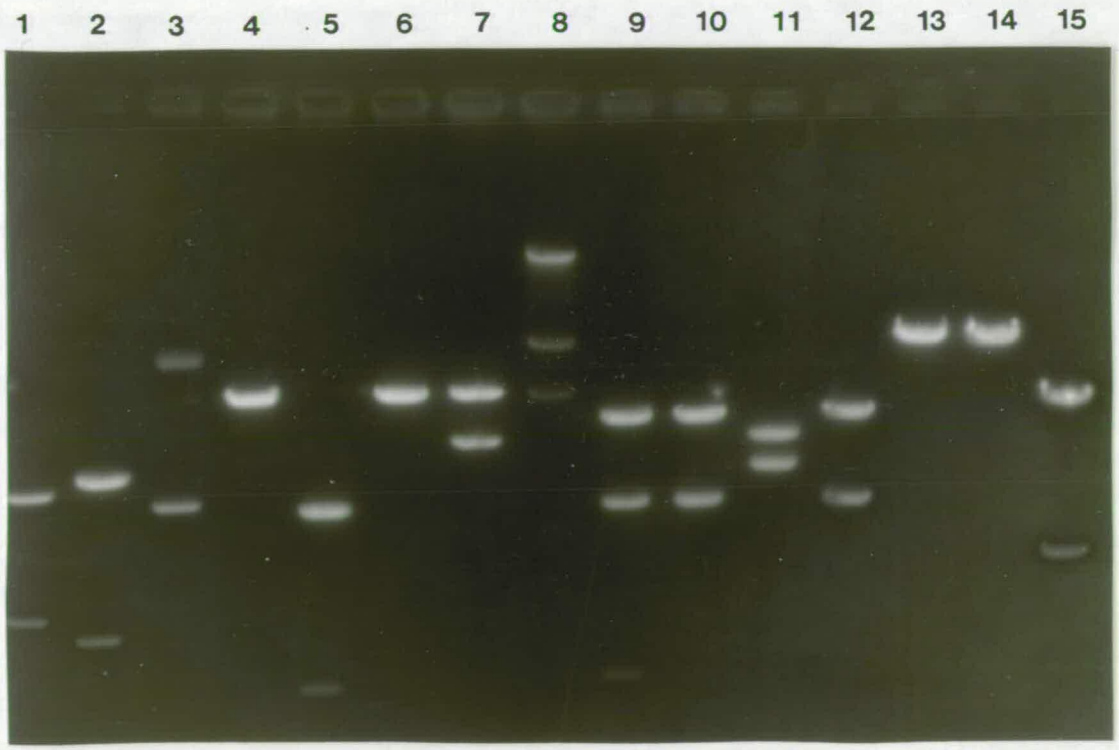


Figure 3.8 Construction of pUE158 and pUE159

0.8% agarose gel. Lanes: 1, λ + HindIII; 2, pUE59 + EcoRI; 3, pUE158 + EcoRI; 4, pUE229 + EcoRI; 5, pUE159 + EcoRI; 6, pUE58 + EcoRI.

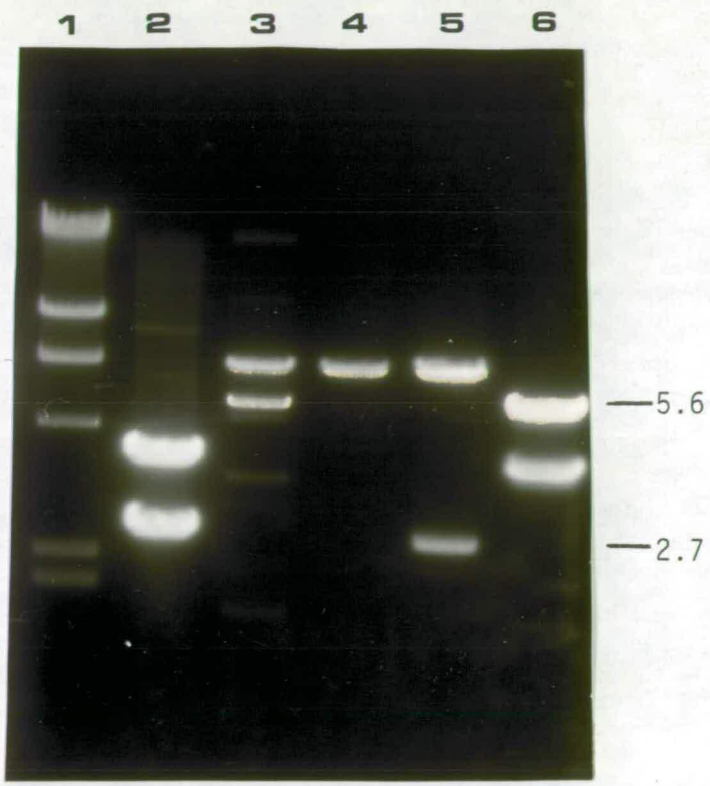


Table 3.2 Transformation of D. radiodurans 78 and 262 with plasmids containing the mtcA gene or mtcB gene, or both

Plasmid	Transformation frequencies to Mtc resistance	
	in 78 (b)	in 262 (b)
pUE522	3.2×10^{-2}	5.8×10^{-5}
pUE641	9.6×10^{-3}	4.9×10^{-5}
pUE58	7.2×10^{-3}	C
pUE158	7.7×10^{-3}	C
pUE59	C (a)	5.6×10^{-5}
pUE159	C	4.3×10^{-5}
Control	$< 1 \times 10^{-9}$	$< 1 \times 10^{-9}$

(a) C - Control level

(b) 78 is defective in the mtcA gene and 262 in the mtcB gene.

extremely efficient. A plasmid containing a chromosomal marker would not exist autonomously for long in D. radiodurans if this applied. The process of recombination into the chromosome may still be useful if there is one recombinational event which takes the whole plasmid into the chromosome and so introduces genes in this fashion (Figure 3.9). This process would not allow the production of multiple copies of the gene as produced from a relaxed plasmid (multiple copies per cell). If the whole plasmid is not taken into the chromosome then transformation into a recombination-deficient strain of D. radiodurans or a strain with a deletion of the marker gene may be useful. Whether the whole plasmid is taken into the chromosome can easily be shown using the technique of Southern blotting.

1.4 Southern blotting of Mtc^R transformants of strains 78 and 262

The DNA-DNA hybridisation technique of Southern (1975) was used to show whether the whole plasmid or just the chromosomally-derived marker gene was integrated into the chromosome of D. radiodurans after transformation. Strains 78 and 262 were transformed to Mtc^R by pUE158 and pUE159 respectively. Six transformants from each strain were grown and chromosomal DNA extracted by the Marmur (1961) method. 5 µg of each DNA preparation was digested with EcoRI and run on a 0.8% agarose gel. The DNA was blotted onto nitrocellulose paper and probed with labelled pUE229 or pUE158 or pUE159. The results of the probe with pUE159 is shown in Figure 3.10. The pUE159 probe lights up a band on each lane containing the chromosomal DNA digests corresponding with the 2.7 kb fragment of pUE159. The probe

Figure 3.9 Integration of a plasmid into a chromosome

Through a single recombinational event at the region of homology, eg, the mtcA gene on pUE158 with that on the D. radiodurans chromosome, it is possible to integrate the plasmid and therefore a gene of interest into the chromosome.

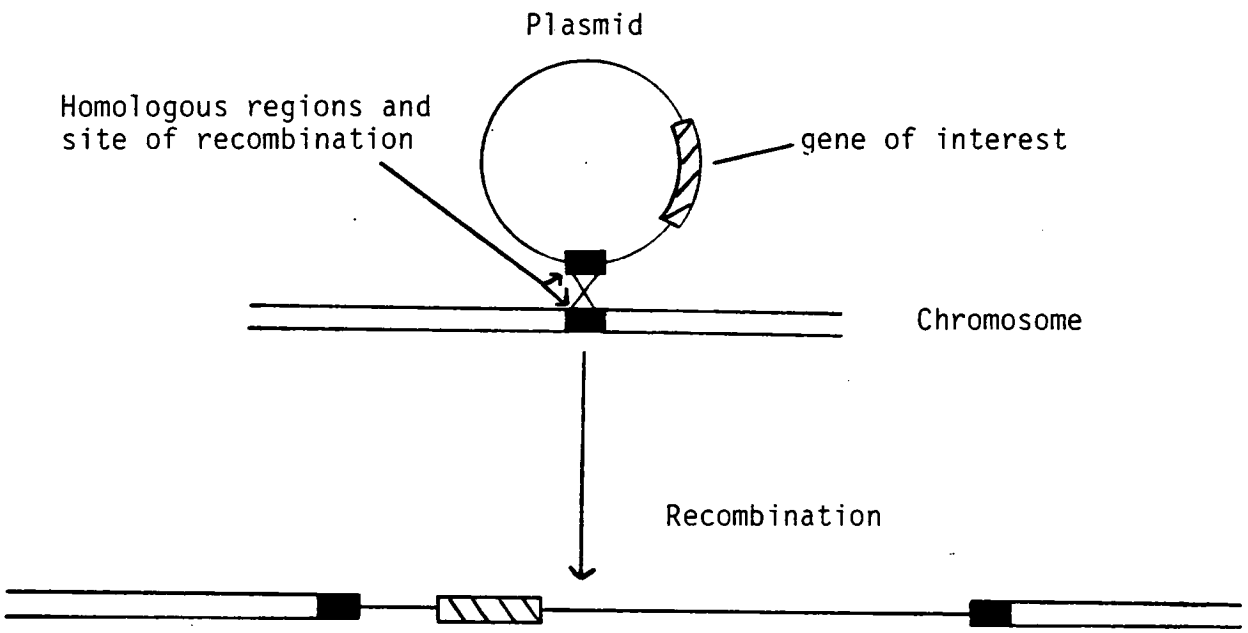
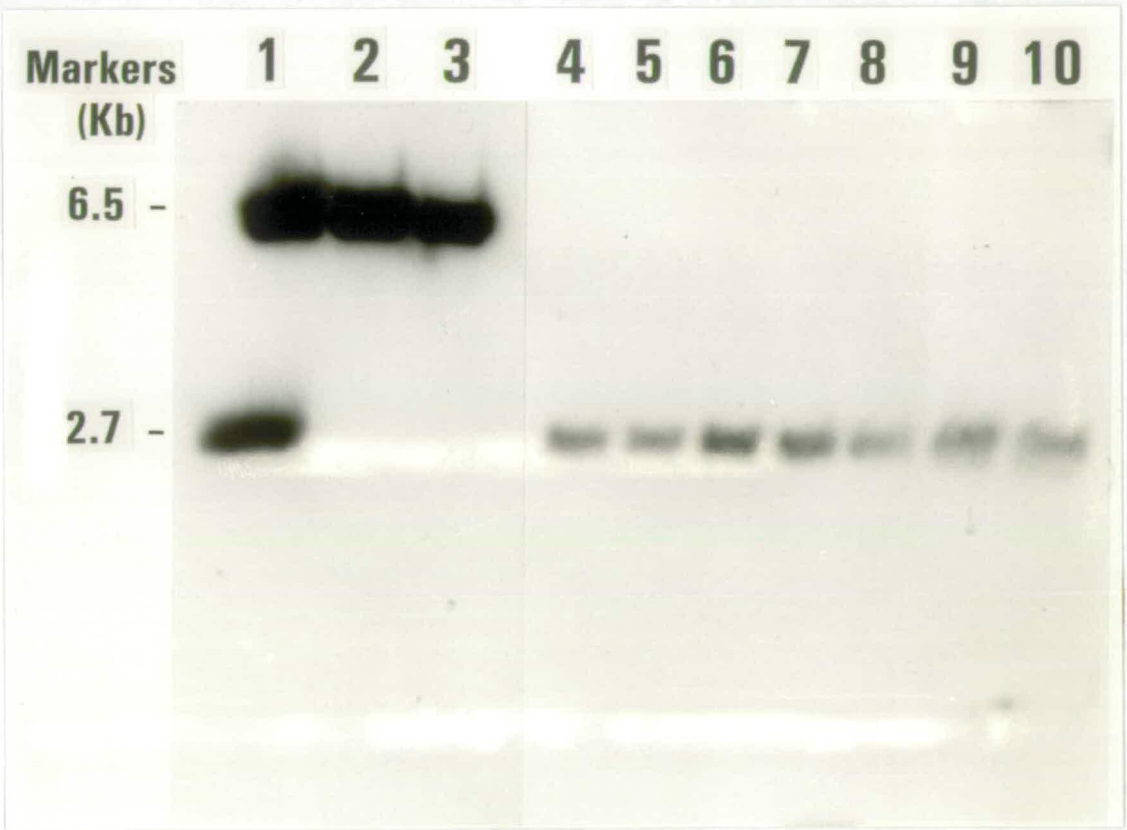


Figure 3.10 Southern blot hybridization analysis of MTC^R transformants of D. radiodurans 262 to see if the plasmid vector part of pUE159 (pUE229) has been integrated into the chromosome of D. radiodurans

The DNA was probed with pUE159.

All DNAs were digested with EcoRI.

Lanes: 1, pUE159; 2, pUE158; 3, pUE229; 4, Chromosomal DNA (C DNA) of transformant (T)1; 5, C DNA of T2; 6, C DNA of T3; 7, C DNA of T4; 8, C DNA of T5; 9, C DNA of T6; 10, C DNA of wt D. radiodurans



also lights up all the DNA fragments in the plasmid lanes except for the 5.7 kb EcoRI fragment from pUE158 carrying the mtcA gene. The probe does not light up any other fragment other than the 2.7 kb one, showing that the non-chromosomally-derived DNA of pUE159 was not incorporated into the chromosome. This was also shown with the pUE158 probe where only a 5.7 kb fragment containing the mtcA gene lights up on the chromosomal lanes. Probing with pUE229 shows clearly that it is not integrated into the chromosome when no bands light up in the chromosomal lanes (data not shown). These results would indicate that either double cross-over events occur much more frequently than single cross-overs or that foreign DNA sequences are not tolerated in the genome. It appears that introducing genes into D. radiodurans cannot be done via an integrative step as seen in yeast (Hinnen et al., 1978) and B. subtilis (Rubin et al., 1980).

A recombination deficient strain of D. radiodurans is required which contains a relevant marker to screen for its transformation by a plasmid. The construction of such a strain was undertaken.

1.5 Construction of a recombination-deficient strain of D. radiodurans with a mutant marker gene

Two recombination-deficient strains have been produced, 112 (Evans, 1984) and rec30 (Moseley and Copland, 1975b) both being sensitive to mitomycin C. Strain 112 is a double mutant, rec1 mtcA formed by mutating strain 302 with MNNG. It is three times more sensitive to mitomycin C than strain 302 and was totally recombination deficient. Strain rec30 was produced by MNNG-induced mutation of wild type D. radiodurans and is 10-fold more sensitive to

mitomycin C than strain 302. It was originally found to be totally recombination-deficient but during this study was found to be transformed by the rifampicin-resistance marker at a frequency one tenth that of wild type. This change is probably due to improvements in the transformation technique. Transforming the plasmids pUE158, pUE159, pUE512 and pUE641 into rec30 gave no increase in resistance to mitomycin C.

Al-Bakri (1983) showed that a leu gene of D. radiodurans complemented the leuB mutation in E. coli HB101. A leucine-requiring mutant of D. radiodurans would therefore have been very useful. After an extensive search, no leu mutants could be made by mutating either 302 or wild type D. radiodurans.

The trp gene of D. radiodurans was chosen because minimal medium is easily produced by using casamino acids where the tryptophan is destroyed by autoclaving. A trp mutant strain of D. radiodurans already existed, viz 18-1, which grew on minimal medium supplemented with 20 μg ml^{-1} of tryptophan. The trp gene of D. radiodurans that complements the mutation in 18-1 had already been cloned in the plasmid pUE81 (Al-Bakri, 1985). Because 112 and rec30 are transformation deficient, the recombination deficient genes had to be transferred to the trp^- strain. The Rec^- phenotype confers mitomycin C sensitivity and so could be identified on transfer. First, the mtcA mutation in strain 18-1 had to be removed as it confers mitomycin C sensitivity to the strain. This was done by transforming the strain with pUE158 which carries the $mtcA^+$ gene. Selection of transformants was on TGY plates containing 0.04 μg ml^{-1} of mitomycin C. Several resistant colonies were picked and checked for mitomycin C resistance and tryptophan dependence. One of these transformants

was picked, 18-B, and shown to be recombination-proficient after being transformed to rif^R at a frequency of 3.3×10^{-3} with DNA from the KRASE strain of D. radiodurans. Strain 18-B was then transformed with DNA from strain 112 (rec1). After overnight growth (to segregate recombined DNA), 10^{-5} dilutions were plated on TGY plates and grown for 36 hours. The colonies were replica plated onto TGY plates containing 0.04 μg ml^{-1} mitomycin C. 28 colonies from 10,000 screened showed sensitivity to mitomycin C. These were tested for recombination proficiency by transforming them with KRASE DNA containing the rif^R gene. All 28 were transformed to rifampicin resistance at a high frequency (data not shown). It appeared that all had transformed the mtcA gene from 112. Several were transformed back to mitomycin C resistance by plasmids containing the mtcA gene. It was originally expected that 50% of the sensitive transformants would carry the rec1 mutation. The effect on mitomycin C by the rec1 gene may only apply when in the background of a mtcA mutation which prevents endonuclease α from working. Transforming 112 with pUE158 did not give a rare recombination event to produce a transformant resistant to more than 0.04 μg ml^{-1} mitomycin C. Transformation of 18-1 with rec1 DNA did not produce a strain more sensitive to mitomycin C.

Strain rec30 differs from 112 in having a low level of recombinational activity. DNA from rec30 was transformed into 18-B and mitomycin C-sensitive transformants identified using the replica plating method. Twenty-six colonies from 10,000 screened showed sensitivity to 0.04 μg ml^{-1}. All were transformed to rifampicin-resistance at a high frequency. The growth of 4 transformants was compared with rec30 and 18-B. They were sensitive to 0.04 μg ml^{-1}

of mitomycin C but not 0.005 μg ml^{-1} as shown by rec30. This shows that the mitomycin C sensitivity seen in rec30 involves at least two mutations. The results strongly suggest that mtcA or mtcB mutations have been transferred from rec30 to 18-B. pUE522 was used to transform the 26 strains. All 26 strains were transformed to mitomycin C-resistance at high frequency showing that it was probably the mtcA or B gene that transferred to 18-B. It must be the second mutation in rec30 which makes it sensitive to 0.005 μg ml^{-1} mitomycin C and also recombination deficient. Strain rec30 is partially recombination proficient so pUE522 was transformed into the strain. There was no increase in the resistance to 0.005 μg ml^{-1} mitomycin C in TGY agar. The control with DNA containing the rif^R gene transformed at a low frequency. This could mean that the second mutation in rec30 is responsible for a sensitivity of 0.005 μg ml^{-1} mitomycin C as a single mutation in the cell. A second possibility is that for some reason the mtcA, B mutation is not complemented due to an effect by the second mystery mutation. This second mutation was not transferred to 18-B and there is obviously some block to its transformation.

Five of the 18-B transformants to mitomycin C sensitivity were grown up and transformed with rec30 DNA for a second time and replica plating was done on 0.01 μg ml^{-1} mitomycin C. Again, no transformants were found.

2. Screening of new Deinococcus isolates

An attempt to isolate bacteriophages of Deinococcus species was undertaken. Samples from varying habitats were analyzed in different ways to search for a bacteriophage. After an extensive search no virus was found for any of the four Deinococcus species. Many bacteriophages exist as prophages within lysogenic bacterial cells. An attempt was made, therefore, to isolate new Deinococcus species in the hope of finding a bacteriophage in a lysogenic bacterium. The new isolates were also tested with regard to survival properties including UV sensitivity, presence of restriction endonucleases and presence of plasmids. No lysogenic bacteria were found.

2.1 Screening for new isolates

Samples from various sites were spread on TGY agar and irradiated for 10, 15 and 20 mins as shown in Materials and Methods. Any red/pink pigmented colonies were picked off and spread on a fresh TGY agar plate to get colonies derived from single cells. Most samples were taken from Nottingham, a good distance from Edinburgh, so that the risk of reisolation of strains originating from the laboratory was minimized. The presence, or absence, of Deinococcus-like bacterial colonies from these sites is shown in Table 3.3. All sites producing resistant, red-pigmented cocci were from soil samples from damp or wet habitats and not dry areas. This was an unexpected finding, as the Deinococcus species are highly dessication resistant (Murray, pers.comm.). All the isolates are shown in Table 3.4. These produced smooth, convex and regularly edged colonies while

Table 3.3 Samples screened for Deinococcus-like bacteria and the occurrence of such bacteria

Sample		Presence or absence of red-pigmented colonies
Raw sewage	(E)[a]	-
Activated sewage	"	-
Grass pellets	"	-
Silage	"	-
Cow manure	"	-
Sheep manure	"	-
Soil next to burn	"	+
Soil next to brook	(N)[a]	+
Manure and soil	"	-
Soil and sand	"	-
Submerged soil	"	+
Soil by lake	"	+
Bird excreta	"	-
Putrefied leaf by brook	"	+
Lake water	"	-
Soil and weed on bank of brook	"	-
Garden soil	"	-

(a) E - samples taken in Edinburgh

N - samples taken in Nottingham

Table 3.4 Preliminary UV survival data, Gram staining and size of coccus for the isolates and Deinococcus spp

Strain	UV resistance min[a]	Gram stain	Size of coccus[b]
D. radiodurans R1	12	+	A
D. radiodurans SARK	10	+	A
D. radiophilus	10	+	B
D. radiopugnans	12	+	B
D. radiodurans 78	2	+	A
1/10 (E)[c]	10	+	B
1/1 "	12	+	A
1/3 "	12	+	A
1/4 "	12	+	A
4/1 (N)[c]	6	+	B
4/5 "	4	+	B
4/8 "	8	+	B
4/9 "	9	+	B
4/11 "	8	+	B
4/12 "	10	+	B
4/13 "	8	+	B
4/16 "	6	+ -	B
5/1 "	10	+	B
5/3 "	8	+	B
5/5 "	10	+	B
5/6 "	10	+	B
5/7 "	10	+	B
5/9 "	9	+	B
5/10 "	9	+ -	B
5/13 "	8	+ -	B
5/14 "	6	+	B
6/1 "	9	+	B
8/1 "	10	+	A
E. coli	2	-	-

(a) UV resistance estimated using the streak method

(b) A - 1.5-3.0 μm
B - 1.0-2.0 μm

(c) E - isolate from Edinburgh
N - isolate from Nottingham

cells seen under the microscope were all single or diplo-cocci, characteristic of Deinococcus. All isolates produced catalase as evidenced by the production of bubbles from hydrogen peroxide solution placed on the colonies, again a characteristic of Deinococcus spp. The colour of the colonies ranged from orange-red through red to purple red with the exception of isolate 4/5 which was a pale pink colour. Prolonged incubation on TGY agar plates caused many isolates to produce "watery" colonies. Table 3.4 shows the results of the streak method of estimating UV sensitivity. All isolates showed greater resistance to UV than E. coli or the UV-sensitive strain of D. radiodurans, 78. The Gram test stains showed up positive in most cases, as seen with the Deinococcus species. Three isolates gave ambiguous results and may be due to the nature of its cell wall. Members of the Deinococcaceae family stain Gram +ve but the fatty acid profile of the species differ from other Gram +ve organisms (Thompson et al., 1980). The cell wall of Deinococcus species appears to be unique and complex (Thompson et al ., 1981; Thompson et al., 1982) hiding an underlying Gram -ve nature. It would not be surprising, therefore, to find strains of Deinococcus which have a variable or negative Gram stain. Isolates have been sent to Professor R.G.E. Murray at the University of Western Ontario, Canada for cell wall and membrane studies. The size of the cocci fell into two categories. Most had cocci of a size similar to D. radiophilus and D. radiopugnans (1.0-2.0 μm), with a few similar to D. radiodurans (1.5-3.0 μm). So all the isolates have large-sized cocci, also a characteristic of the Deinococcaceae.

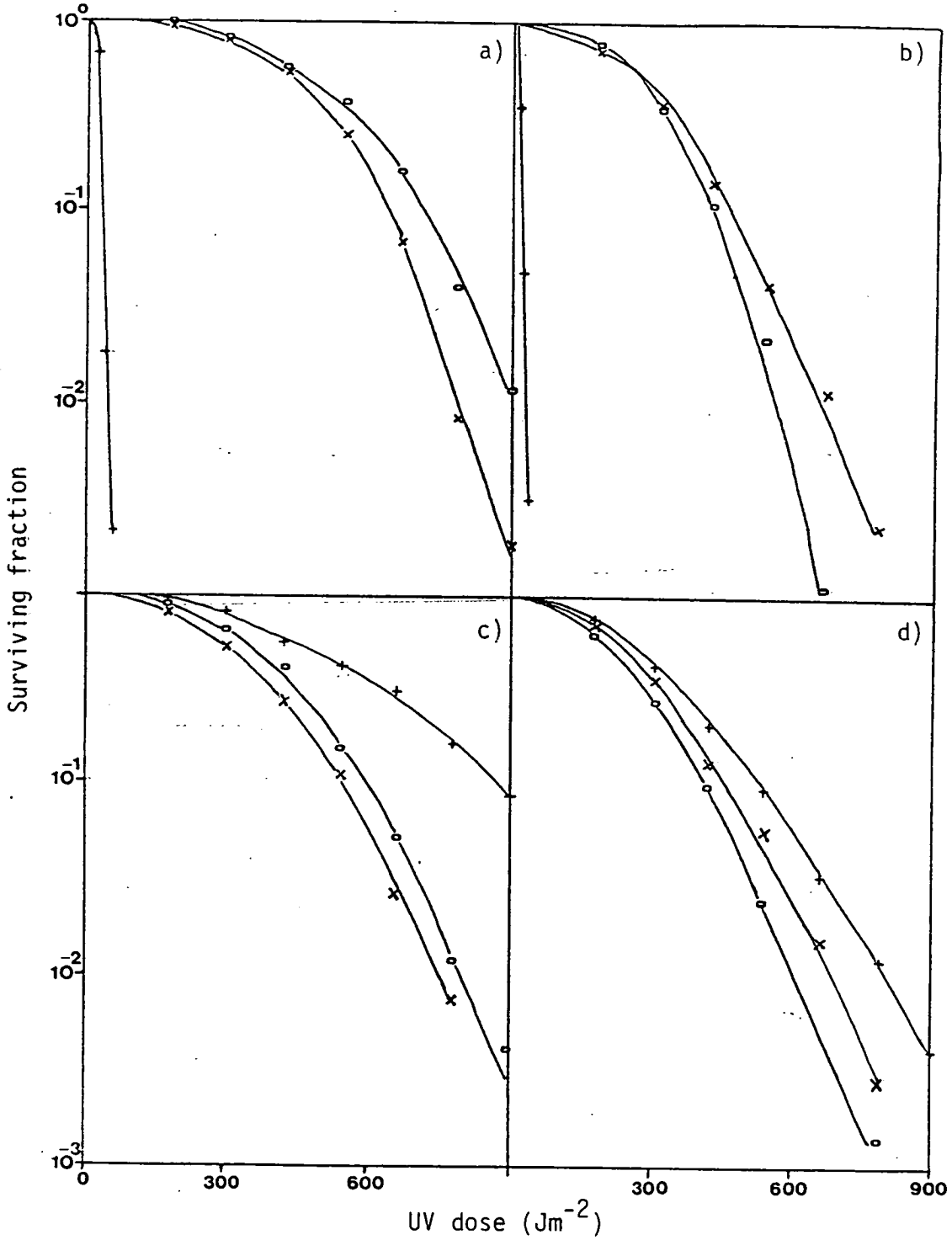
2.2 UV survival curves of the isolate

UV survival curves were made for all the isolates and most are shown in Figures 3.11 and 3.12. Isolates 4/1, 4/13 and 5/14 showed the least resistance to UV but, compared with E. coli and UV sensitive strain of D. radiodurans, 78, even these had a high resistance to UV. Isolate 4/5 showed high survival to UV by the plating method as opposed to the streak method (Table 3.4). It probably requires a greater time to mend the DNA damage and start growth again than the other isolates. Thus, in the streak method it would appear to show no growth at particular UV doses due to this "resting phase" of the cells. In the plate method colonies took 1-2 days longer than other isolates to grow after UV irradiation supporting this idea.

Isolates 1/1, 1/3 and 1/4 showed survival curves similar to that of D. radiodurans. They all have the same size cocci as D. radiodurans and the colour of colonies grown on TGY medium were indistinguishable. These isolates were probably reisolations of the laboratory strain. They all transformed to rifampicin resistance with DNA from the KRASE strain at the same order of magnitude as the frequency of transfer to D. radiodurans wild type (2 to 8×10^{-3}). Isolate 1/1 was transformed with strain 112 chromosomal DNA carrying the mtcA mutation. Five colonies out of 1,000 were found to be mitomycin C sensitive. Study on these three isolates was taken no further.

The high UV resistance, colony morphology, Gram staining, catalase production and coccus-shaped vegetative cells are all properties of Deinococcus spp. All the isolates showed these properties and this is good evidence that the isolates are members of

Figure 3.11 Survival of new Deinococcus isolates after UV irradiation



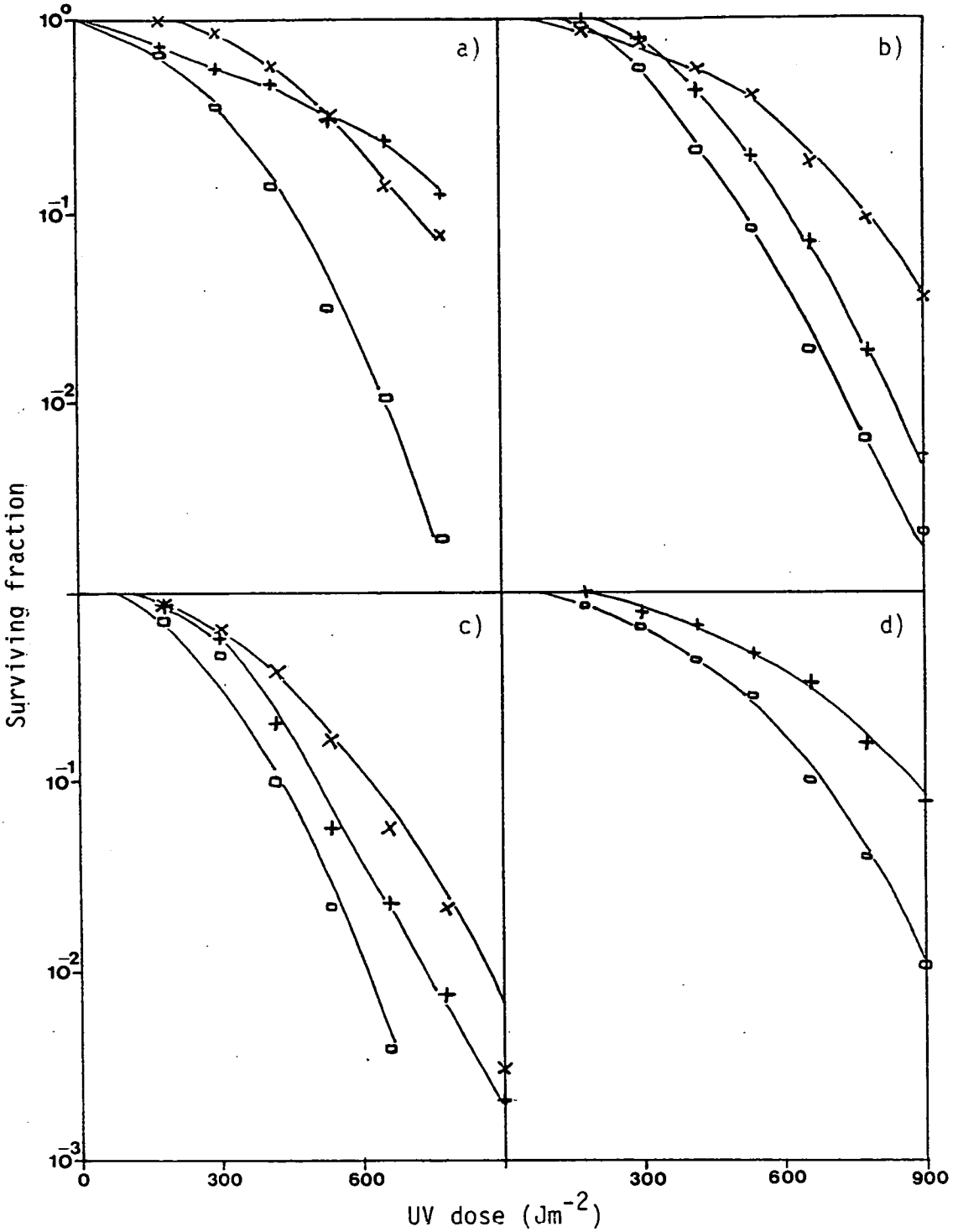
a) + - 78; ● - D. radiodurans; × - 1/10

b) + - E. coli; ● - 4/1; × - 4/5

c) ● - 4/8; + - 4/9; × - 4/11

d) + - 4/12; ● - 4/13; × - 4/16

Figure 3.12 Survival of new Deinococcus isolates after UV irradiation



a) + - 5/1; o - 5/3; x - 5/5

b) + - 5/6; x - 5/7; o - 5/9

c) + - 5/10; x - 5/13; o - 5/14

d) + - 6/1; o - 8/1

the Deinococcaceae. Further studies on the cell wall structure, constituent fatty acids, peptidoglycans and the 16s ribosomal RNAs would clarify if any of the isolates belong in the Deinococcacaea family.

2.3 Screening for plasmids in the isolates

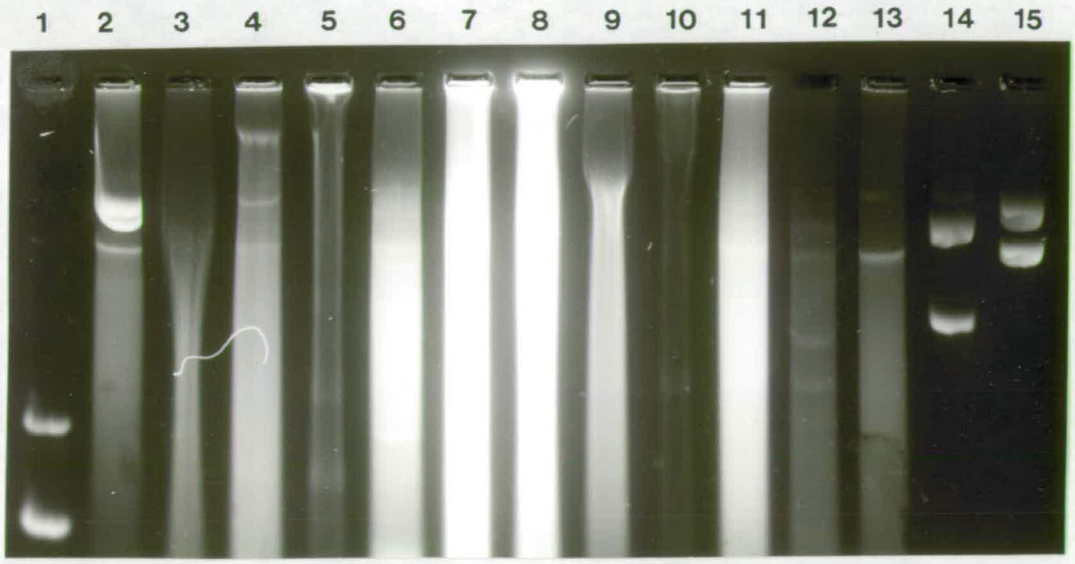
All the isolates were screened using the mini-screening method. Plasmids were shown to be present in isolates 4/1, 4/12, 5/10, 5/14 and 6/1 using this method (Figure 3.13). The lack of plasmids in other isolates does not exclude the possibility of them having plasmids. This mini-method does not show plasmids of D. radiopugnans and a large scale plasmid preparation procedure from 2 l of culture is required to show their presence. This probably applies to the isolates showing no plasmids. There appears to be a plethora of plasmids available for study and the possible construction of a plasmid vector for use in D. radiodurans.

2.4 Screening the isolates for the presence of restriction endonucleases

Four restriction endonucleases have been isolated from members of the Deinococcaceae. MraI from D. radiodurans (Wani et al., 1982), DraI (Purvis and Moseley, 1983), DraII and DraIII (Grosskopf et al., 1985) from D. radiophilus. Deinococcus species may thus be a good reservoir of restriction endonucleases and so the isolates were screened using the rapid screening method. It had previously been shown that restriction enzymes, if present in sufficient quantity,

Figure 3.13 Presence or absence of plasmids from the new isolate

Lanes: 1, pAT153 (3.7 kb); 2, strain SARK; 3, strain 4/5; 4, strain 4/1; 5, strain 4/9; 6, strain 4/12; 7, strain 4/13; 8, strain 5/3; 9, strain 5/5; 10, strain 5/9; 11, strain 5/10; 12, strain 5/14; 13, strain 6/1; 14, pDE200 (11 kb); 15, pDE16 (19 kb).



could be assayed for from crude extracts (Purvis and Moseley, 1983). From an initial study, isolates 4/8, 4/9, 5/3, 6/1 and 1/10 appeared to show endonuclease activity.

2.4.1 Isolate 5/3

Further studies of 5/3 showed that it produced an enzyme that cut λ DNA four times to give fragment sizes of 14.45, 13.00, 12.16 and 9.66 kb. These compared well with the fragment sizes produced by a PvuI digest of λ DNA (14.25, 12.70, 12.00 and 9.55 kb). Figure 3.14 compares the action of PvuI on λ DNA and pBR322 with that of the enzyme from isolate 5/3. Lanes 2 and 3 show that the two enzymes cut λ DNA to give identical fragments. The top band in lane 2 and the top two in lane 3 are present because of partial digestion of the λ DNA. pBR322 is cut with PvuI and the enzyme from 5/3 (lanes 5 and 6). For evidence that they both cut at the same site in pBR322, a double digest with NruI and each enzyme was done. Lanes 8 and 9 show that PvuI and NruI gave the same restriction pattern as NruI with the enzyme from the 5/3. This results shows that the restriction enzyme in isolate 5/3 was an isoschizomer of PvuI and therefore recognises the sequence 5' C G A T C G 3'.

2.4.2 Isolate 4/9

Further analysis of 4/9 showed that it produced an enzyme that cut pBR322 into small fragments of less than 2 kb. It also cut λ DNA into at least 16 fragments as visualized on a 5% polyacrylamide gel (Figure 3.15). Table 3.5 compares the fragment sizes, obtained

Figure 3.14 Comparison of restriction endonuclease PvuI with that found in isolate 5/3 on a 1% agarose gel.

Lanes: 1, λ DNA; 2, λ DNA + PvuI; 3, λ DNA + 5/3; 4, λ DNA + HindIII; 5, pBR322 + 5/3; 6, pBR322 + PvuI; 7, pBR322 + HindIII; 8, pBR322 + 5/3 and NruI; 9, pBR322 + PvuI and NruI; 10, pBR322.

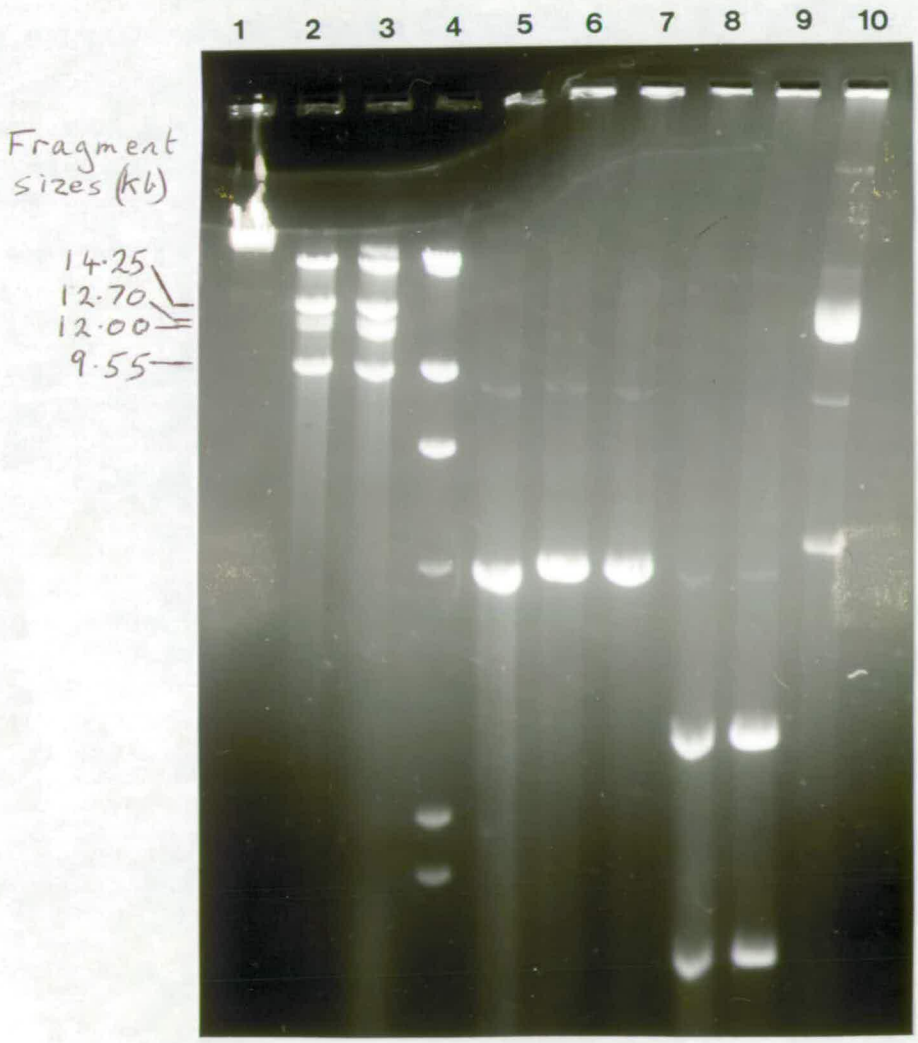


Figure 3.15 5% polyacrylamide gel showing restriction of λ DNA pBR322 and ØX174 DNA by the restriction endonucleases from isolates 4/9 and 6/1

Lanes: 1, ØX174 + 4/9; 2, pBR322 + 4/9; 3, λ + 4/9; 4, λ + HindIII + EcoRI; 5, λ + 6/1; 6, ØX174 + 6/1; 7, pBR322 + 6/1.

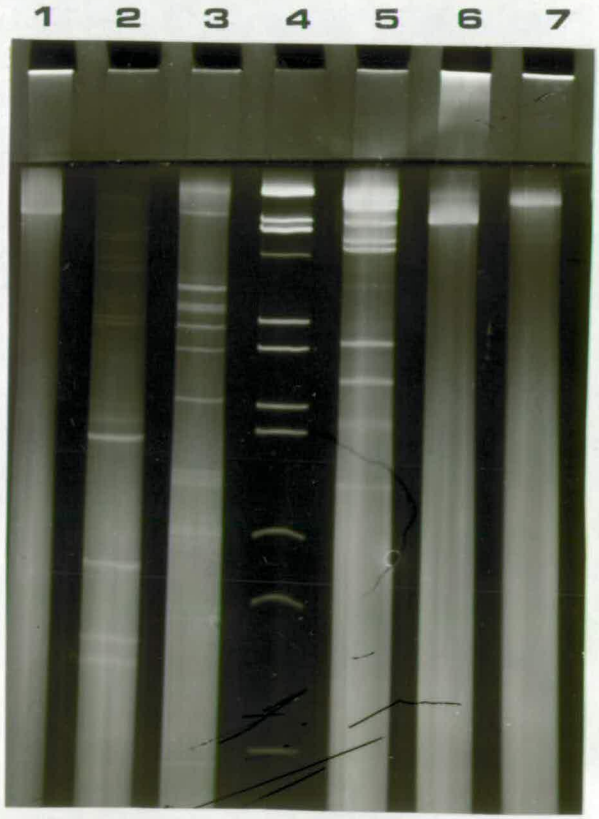


Table 3.5 Comparison of restriction fragment sizes of λ and pBR322 digested with XhoII and the enzyme from the crude extract of isolate 4/9

λ fragment sizes (kb)[c]		pBR322 fragment sizes (kb)	
XhoII digested	4/9 digested	XhoII digested	4/9 digested
15.924	14.69	1.449	1.355
6.041	5.87	1.292	0.922
4.073	3.87	0.768	0.750
2.974	2.85	0.726	0.701
2.516	2.43	0.086	NS
2.392	2.32	0.017	NS
2.156	2.16	0.012	NS
2.086	2.07	0.011	NS
1.621	1.596		
1.212	1.202		
1.191	1.180		
(1.144)[a]	1.161		
0.945	1.007		
0.925	0.995		
0.917	0.980		
0.833	0.807		
0.762	0.787		
0.729[a]			
0.561	0.579		
0.451[a]			
0.090	NS[b]		
0.079	NS		
0.060	NS		

(a) fragment sizes 0.729 and 0.451 kb contain the cohesive ends of λ and so "stick" together and run as a 1.144 kb fragment. This was seen as the 1.161 kb measured fragment of the 4/9 digested λ DNA.

(b) NS - fragments not seen on gel, having probably run off the end of the gel.

(c) λ fragment sizes were obtained from a restriction enzyme data file used by the programs MAP, MAPSORT and MAPPLOT.

from the polyacrylamide gel and a 0.8% agarose gel, with the fragment sizes produced by incubation of λ DNA or pBR322 with the restriction endonuclease XhoII. There is a good correlation between the fragment sizes produced by the enzyme in extract 4/9 and those formed by the restriction endonuclease XhoII. XhoI enzyme cuts at a single site in λ DNA, found in the 4.073 kb fragment of λ digested with XhoII. To show that the enzyme in extract 4/9 was an isoschizomer of XhoII, a double digest of extract 4/9 with XhoI was done. If the enzyme in 4/9 cuts at the same palandromic sequence as XhoII then the fragment of size 4.073 should disappear and two fragments of sizes 3.072 and 1.001 kb should appear. Figure 3.16 shows the disappearance of the 4.073 kb fragment (third band down in lane 3 disappears in lane 2). The 2.974 bp fragment appears brighter in lane 2 due to the migration of the 3.072 kb band to virtually the same spot. The 1.001 kb fragment has run off the end of the gel. The enzyme in extract 4/9 is an isoschizomer of XhoII and therefore recognises the sequence 5'-Pu G A T C Py-3' (Pu-purine, Py-pyrimidine).

2.4.3 Isolate 6/1

Further analysis of the enzyme in the lysate of isolate 6/1 showed that it did not cleave pBR322 but cut λ DNA at least 8 times as visualized on a 0.8% agarose gel. The fragments were sized from the gel and those on the 5% polyacrylamide gel are shown in Figure 3.15. Table 3.6 compares these sizes with those produced by digestion of λ DNA with restriction endonuclease BstEII, and shows good correlation. The single site in λ DNA recognised by XhoI cuts in the third largest fragment of a BstEII digest. This 6.369 kb

Figure 3.16 0.8% agarose gel showing the restriction pattern produced by digestion of λ DNA with XhoI and the extract from isolate 4/9

Lanes: 1, λ + XhoI; 2, λ + 4/9 + XhoI; 3, λ + 4/9.

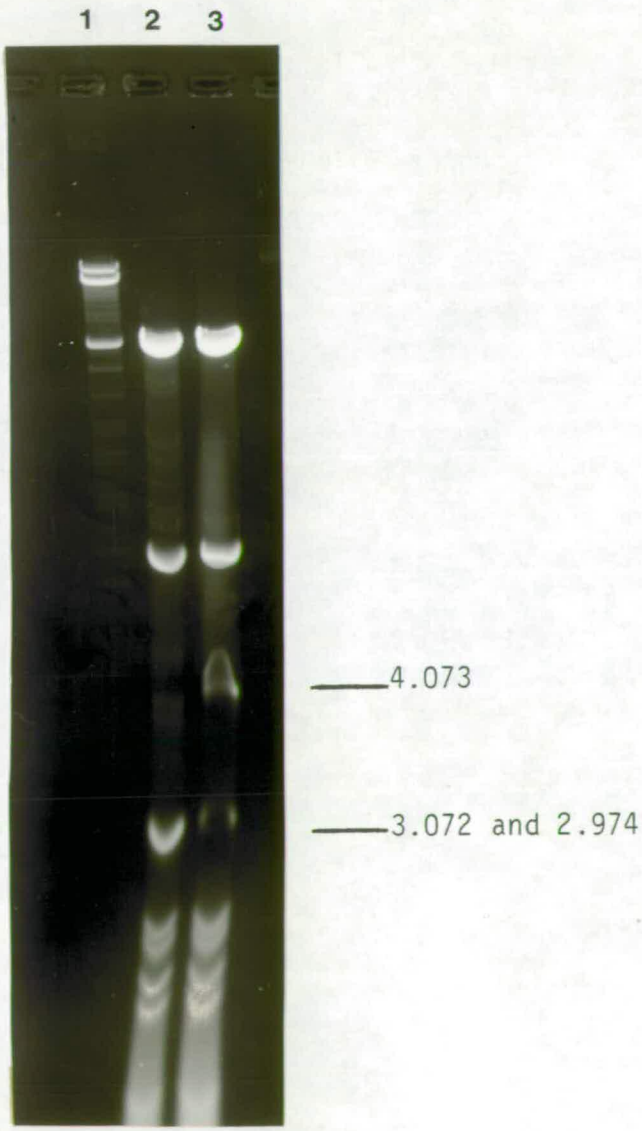


Table 3.6 Comparison of restriction fragment sizes of λ digested with BstEII and the enzyme from the crude extract of isolate 6/1

Size of λ fragments (kb)[b] digested with BstEII	Size of λ fragments (kb) digested with 6/1
8.453	8.13
7.242	6.76
6.369	5.96
5.687	5.31
4.822	4.75
4.324	3.890
3.675	3.581
2.323	2.642
1.929	1.718
1.371	1.445
1.264	1.156
0.702	NS[a]
0.224	NS
0.117	NS

(a) NS - fragments not seen on the polyacrylamide gel

(b) λ fragment sizes were obtained from the programs MAP, MAPSORT and MAPPLOT

fragment is cut into a 3.493 and a 2.876 kb fragment. Figure 3.17 shows that this happens when λ DNA is digested with XhoI and the crude extract of isolate 6/1. The reaction has not fully completed, probably because the XhoI enzyme does not work well under the conditions in the crude extract. It can be seen though that in lane 1, the third band has partially disappeared and two new bands appear between the seventh and eighth fragments which correspond with the expected 3.493 and 2.876 kb fragments. The enzyme in extract 6/1 is thus an isoschizomer of BstEII and recognises the sequence 5'- G G T N A C C -3' (N = anybase).

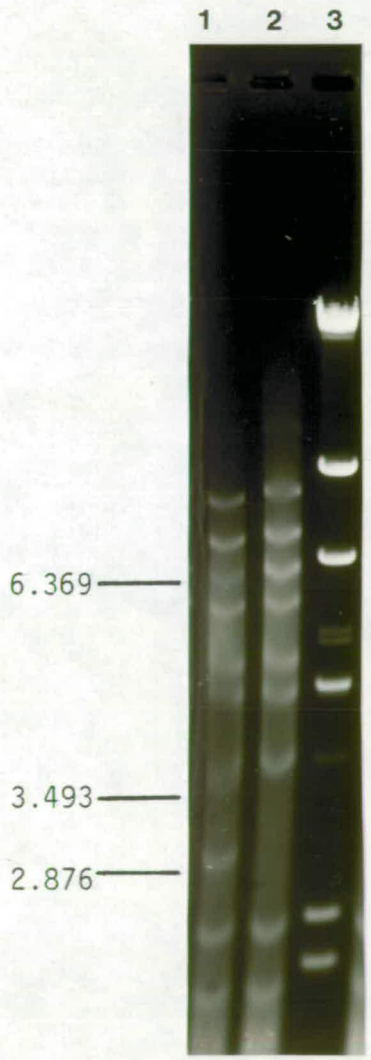
2.4.4 Isolates 1/10 and 4/8

On further analysis, isolate 4/8 gave no indication of an enzyme cutting λ and so was not studied further. It may have a restriction endonuclease at a low concentration and the first screen was fortunate to pick it up. The strain may produce a protease which degrades the enzyme when the cells are lysed and before it can be assayed. This protease would not be inhibited by PMSF or the presence of EDTA.

Isolate 1/10 gave a couple of encouraging gels but the banding was blurred and the restriction was always partial. After changing assay conditions and the length of incubation, no useful information was obtained from this strain. The blurring or absence of bands may be due to the presence of an exonuclease which operates in the assay conditions used. Again, 1/10 may produce a protease which degrades the enzyme. Purvis and Moseley (1983) reported that the growth phase of D. radiophilus influenced the final yield of DraI with

Figure 3.17 0.6% agarose gel showing the restriction pattern produced by digestion of λ DNA with XhoI and the extract from isolate 6/1

Lanes: 1, λ + 6/1 + XhoI; 2, λ + 6/1; 3, λ + HindIII.



stationary cells producing an active protease which rapidly degraded DraI, Isolate 1/10 may produce a protease at a constant level which is resistant to inhibition by PMSF or the divalent cation-chelating effect of EDTA.

2.5 Homology between species of Deinococcus and three new isolates

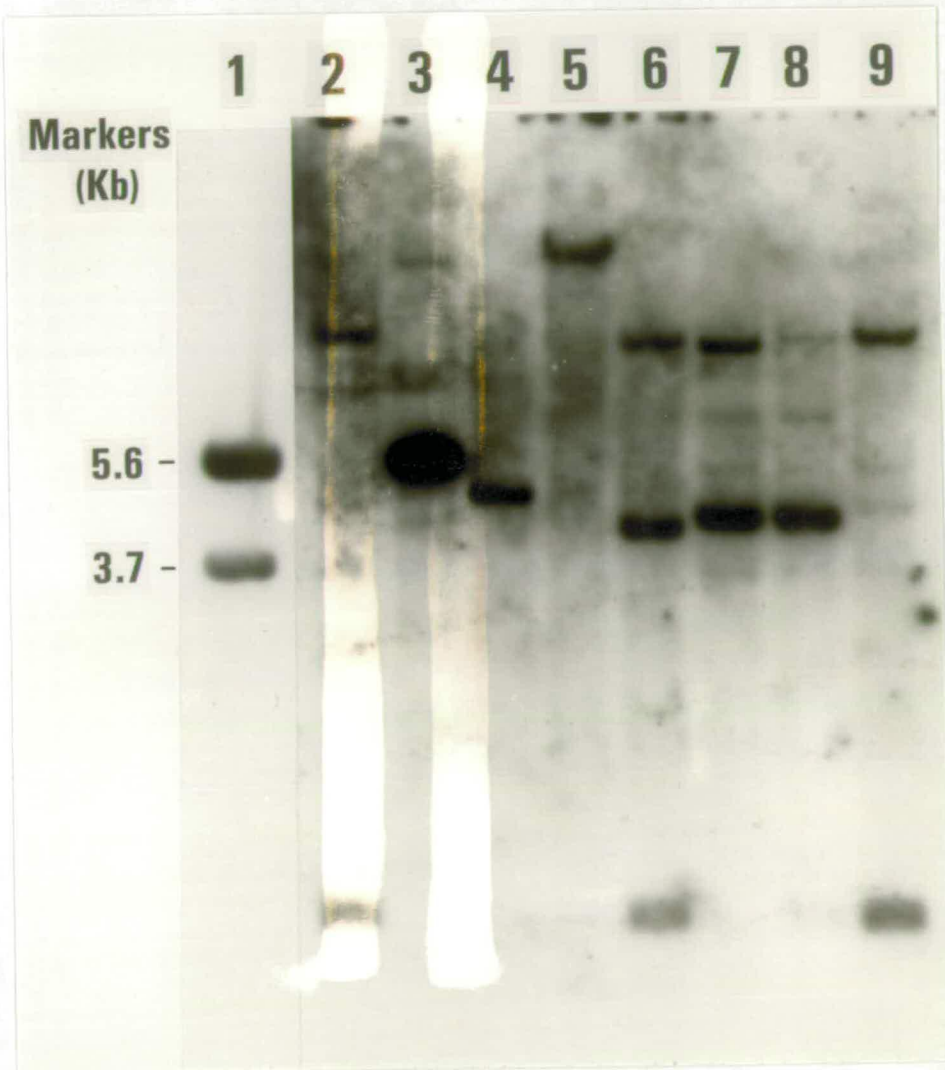
One of the criteria used to show that a species is a member of the Deinococcaceae family is its high resistance to UV and ionizing radiation. In an attempt to see if there was any homology between genes involved in DNA repair, Southern blot hybridization analysis was used. The probes used were pUE58, carrying the mtcA gene and pUE200 carrying the uvs genes C, D and E.

5 µg of chromosomal DNA of each species was digested with EcoRI and run on a 0.8% agarose gel, blotted onto nitrocellulose and probed with either pUE58 or pUE200. The filters were washed with 2xSSC, 0.1% SDS and then by 1xSSC at 37°C. The autoradiographic images are shown in Figures 3.18 and 3.19. Figure 3.18 shows the result of probing with pUE58. As expected, a band in the D. radiodurans lane migrates to the same position as the EcoRI fragment carrying mtcA in pUE58. All the other lanes have bands lighting up, showing that there is homology between the DNA fragment containing the mtcA gene of D. radiodurans RI and the other species. D. radiophilus and D. radiodurans SARK show single bands as do the new isolates 4/1, 4/12 and 4/13. Bands which light up faintly are produced by either incomplete digests or they are, perhaps, areas of DNA with less homology to the mtcA gene than that producing strong bands.

Figure 3.18 Southern blot hybridisation analysis, probing the chromosomal DNA of different species of Deinococcus for homology with the mtcA gene of D. radiodurans R1

All DNAs were digested with EcoRI.

Lanes: 1, pUE58; 2, strain 4/13; 3, D. radiodurans R1; 4, D. radiophilus; 5, D. radiodurans SARK; 6, D. radiopugnans; 7, strain 1/10; 8, strain 4/12; 9, strain 4/1.



D. radiopugnans and isolate 1/10 show two strong bands each and is most likely due to the EcoRI enzyme cutting the area of homology into two separate fragments. Another possibility is that the area of homology in these two strains was duplicated at some time in the past. The bands would migrate to different places because flanking DNA would have EcoRI sites at different points.

These results offer possible evidence of a conserved endonuclease α enzyme complex in the genus Deinococcus. This evidence is not absolute proof. Hybridisation could be due to a fortuitous, limited homology between the mtcA gene and a section of DNA in the second species. An assay method for endonuclease α would be helpful in trying to show if all strains carry this enzyme complex, but attempts to produce an assay have failed.

The autoradiogram does show that D. radiodurans RI, D. radiodurans SARK, D. radiophilus and new isolate 4/12 are separate strains. D. radiopugnans and isolate 1/10 show a similar banding pattern but the lower band has migrated slightly further in D. radiopugnans than in isolate 1/10. This could indicate that they are closely related with only slight divergence between the two species. 1/10 was isolated from a burn, 400 metres from the laboratory. There is a possibility that the isolate 1/10 was the laboratory strain of D. radiopugnans that had escaped into the surrounding area. The difference in band migration could be due to the loss of an EcoRI site in isolate 1/10 which would produce a slightly larger fragment when digested with EcoRI. Protein and plasmid profiles of 1/10 and D. radiopugnans also show slight differences (data not shown).

Isolates 4/1 and 4/13 appeared from the autoradiogram as if they might have been the same isolate, with the strong, single-bands

migrating to the same position. These two isolates and D. radiopugnans produce a small-size fragment which lights up with the probe. This is either a very small fragment from the area of homology or an RNA species. If it was an RNA species, it would have to survive the RNase treatment of the chromosomal DNA in its preparation.

Filters probed with pAT153 showed no hybridization in the lanes containing chromosomal DNA.

Figure 3.19 shows the results of probing with pUE200. Again the D. radiodurans RI lane has a strong band corresponding to the fragment of DNA containing the uvs genes, C, D and E. The band has not migrated as far as the plasmid because pUE200 does not contain the whole EcoRI fragment from D. radiodurans RI. The D. radiodurans SARK strain also gives a strong band showing an area of homology with uvsC, D and E. The other lanes show no bands or faint bands produced by either limited fortuitous homology or by a gene cluster similar to uvsC, D and E which has diverged sufficiently to produce limited homology. D. radiopugnans has a faint band at the same position as seen in D. radiodurans RI but this does not appear to be present in isolate 1/10. The single faint bands for 4/1 and 4/13 do not migrate exactly to the same point. This could be an artefact caused by the gel or the bands do represent DNA fragments of different lengths. 4/1 and 4/13 come from the same sample of submerged mud from Nottingham and it is more likely that they are the same isolate.

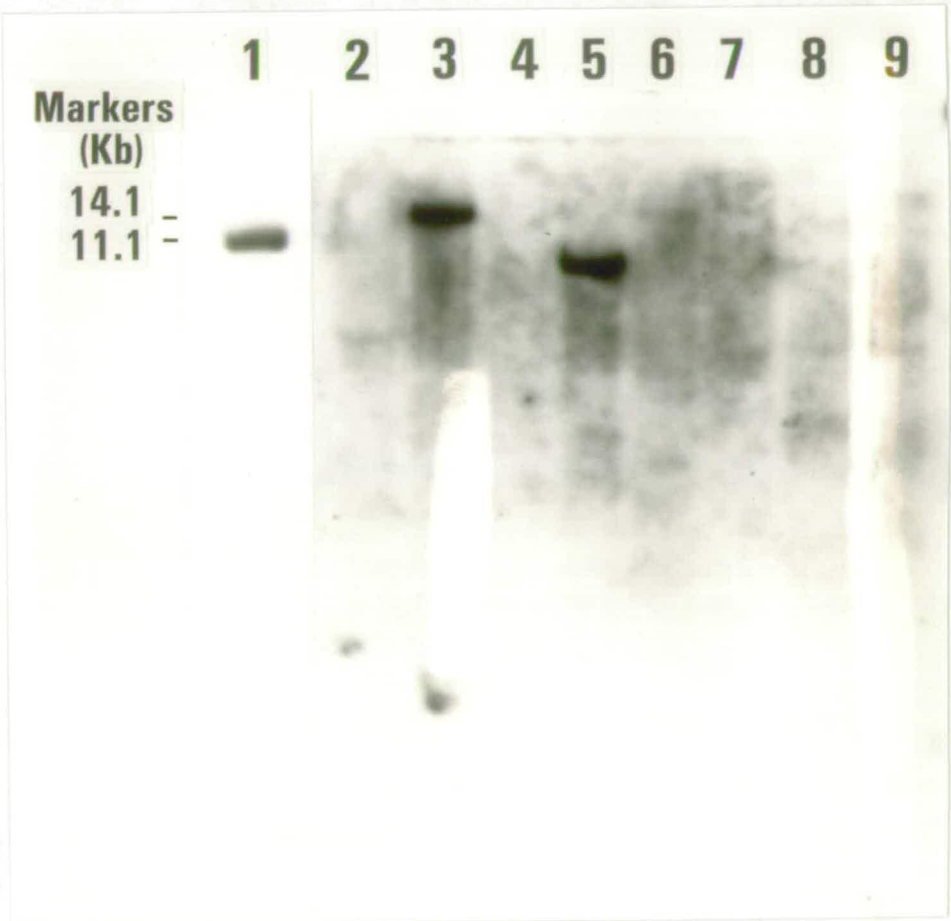
Probing E. coli genomic DNA with both probes showed no homology (data not shown).

It would be of interest to probe all the new isolates with pUE58 and pUE200, and also with different probes derived from the

Figure 3.19 Southern blot hybridization analysis, probing the chromosomal DNA of different species of Deinococcus for homology with the uvsC, uvsD and uvsE genes of D. radiodurans R1.

All DNAs were digested with EcoRI.

Lanes: 1, pUE200; 2, strain 4/13; 3, D. radiodurans R1; 4, D. radiophilus; 5, D. radiodurans SARK; 6, D. radiopugnans; 7, strain 1/10; 8, strain 4/12; 9, strain 4/1.



cosmid band of D. radiodurans RI to see the level of interrelatedness of the strains.

2.6 Transformation of the new isolates

DNA from the KRASE strain of D. radiodurans was prepared and used to transform all the new isolates. TGY plates containing rifampicin (10 μg ml^{-1}) or kanamycin (10 μg ml^{-1}) were used to screen for transformants. The control strain of D. radiodurans transformed the two antibiotic markers at high frequency. There were no transformants produced for the new isolates, only the occasional single colony on a plate arising from spontaneous mutation. The lack of transformation could be due to:

a) failure to take up DNA

b) non-specific digestion of DNA in cell

c) lack of homology between the incoming DNA and that of the host

d) the inability of transcription-translation apparatus to recognize control regions.

It would be of interest to make DNA of rifampicin-resistant clones of all the isolates and to transform each wild type isolate and D. radiodurans with these DNA preps. This would show which isolates, if any, take up DNA and allow recombination events to take place. However, experience with D. radiophilus, D. radiopugnans and D. proteolyticus does not offer much hope.

2.7 Attempted construction of a mitomycin C-sensitive strain of D. radiodurans SARK

It can be inferred from the hybridization studies shown here, and those of Brooks et al. (1980), that D. radiodurans RI and SARK are more closely related to each other than any other two members of the genus. SARK does not have a restriction endonuclease system (Purvis, 1984), it is transformable and it has its own indigenous plasmids. With an origin of replication from one of its own plasmids and with no restriction enzyme, two of the problems associated with strain RI would be overcome immediately. The major problem with SARK is the lack of available mutant strains and the unsuccessful attempts to cure it of plasmids (Mackay, 1984). If a cloning vehicle could be constructed, it may exist with the indigenous plasmids or may appear in 50% of transformed cells due to random elimination of itself or the parental plasmid, or due to segregation of the two plasmid types.

Searching for mutants is time-consuming. With D. radiodurans RI and SARK being closely related, a mutation may be transformed from RI to SARK. DNA from D. radiodurans RI strain 78rif^R, containing the mtcA$^-$ and rif^R mutations was used to transform SARK. The SARK strain was transformed to rifampicin resistance at a frequency of 5.7×10^{-3} but no Mtc$^-$ transformants of SARK were found after replica plating colonies onto TGY plates containing mitomycin C at a concentration of 0.03 μg ml^{-1}. This concentration was used because wild type cells of SARK grew poorly on TGY with mitomycin C at 0.04 μg ml^{-1}. The non-transformability of the Mtc$^-$ phenotype to SARK was not due to lack of DNA transfer as the Rif^R phenotype was

transformed. The homology between the DNAs may not have been high enough for recombination to take place. A second more unlikely explanation is that the DNA fragment of SARK which lights up when probed with pUE58 was a random region of DNA which happened to have much homology with the RI DNA in probe pUE58.

A second method to obtain Mtc^- mutants of SARK was tried. Evans (1984) developed a procedure in D. radiodurans RI for getting mutants in genes coding for the endonuclease α enzyme complex. He proposed that there was an inducible terminator to an exonuclease which acted on DNA incised by endonuclease α. When the terminator was not produced, by the use of chloramphenicol, there was uncontrolled degradation of the DNA after the cells had been UV irradiated. This hypothesis was used as the basis of a selection procedure. Mutagenized cells were spread onto a sterile filter which was placed on a TGY agar plated containing 10 μg chloramphenicol ml^{-1}. The cells were irradiated with 600 Jm^{-2} of 254 nm UV, incubated at 30°C for 16 hours, and then on fresh TGY agar plates only. Wild type cells should die from uncontrolled breakdown of the DNA. Cells mutant in genes of the endonuclease complex would survive because the DNA is not incised to produce a substrate for the exonuclease. A mutation which produces constitutive amounts of inducible terminator would also survive. This method worked well with RI to give survivors with mutations in mtcA and B (Evans, 1984).

This procedure was tried on SARK with no success. Colonies that grew up on the filters all showed the same resistance to mitomycin C as wild type. These may have survived because they were protected by other cells from the effects of the UV irradiation or possibly an inducible terminator if present in SARK is produced at

a low level constitutively which allows some cells to survive where the DNA has a minimal number of pyrimidine dimers.

The formation of a mitomycin C-sensitive mutant of SARK would have to be by classical mutation and search methods. The production of mutants of SARK may prove profitable in the long term with its own plasmids and lack of restriction system. It would though still be recombination proficient and, like RI, a well-studied recombination deficient mutant would be useful.

3. The major AP endonuclease of D. radiodurans

As stated in the Introduction, AP sites in DNA can act as sites for mutagenic change, DNA polymerase being capable of incorporating any nucleotide opposite such a site in E. coli. Depurination is the most frequent spontaneous alteration in DNA, and is probably one of the major pathways for spontaneous mutation in D. radiodurans. A study of its major AP endonuclease was undertaken to look at its activity and characteristics.

3.1.1 Purification and molecular weight determination of the major AP endonuclease

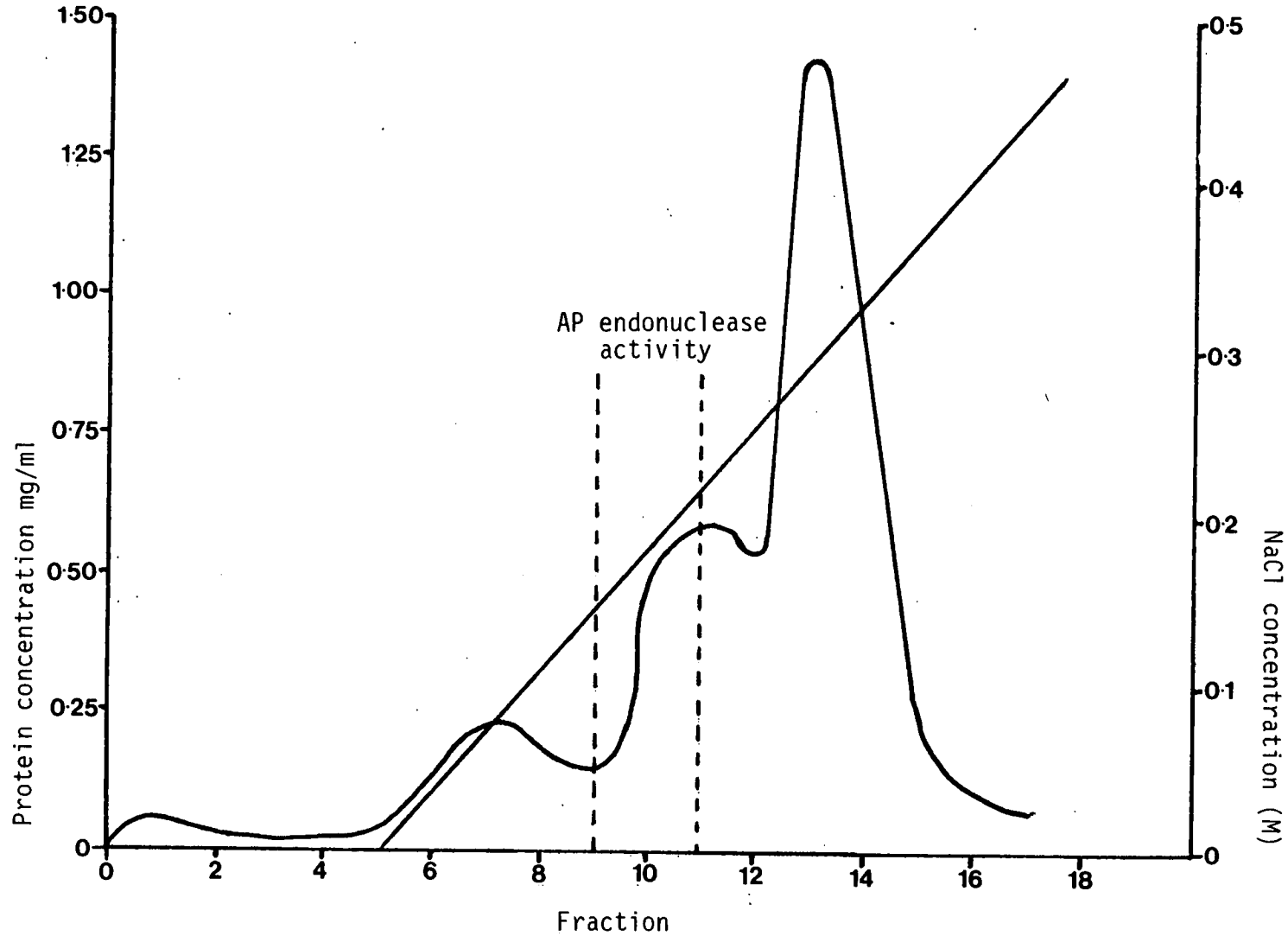
During the initial stages of the work only the plasmid assay system was used to show the presence or absence of an AP endonuclease. Exonuclease III (ExoIII) was used as a control. ExoIII has no effect on untreated ccc plasmid DNA. With acid depurinated DNA, and in the presence of Ca^{2+} ions, the endonuclease activity of ExoIII nicks the plasmid to produce oc form plasmids. In the presence of Mg^{2+} ions both the endonuclease and exonuclease functions work and cause total digestion of the acid-depurinated ccc plasmid DNA. Some of the methodology developed for the purification of AP endonuclease is included in the following results.

Ammonium sulphate is useful for performing the initial purification step after lyzing of the cells because it removes large amounts of contaminating protein before the application of column chromatography. The majority of AP endonuclease activity, ie 90%, was found at the 43% to 75% saturation ammonium sulphate cut. This

step also removed 60% of the contaminating cell protein. This cut formed fraction A which was dialized for 16 hours against two changes of 1 l 20 mM Tris, 2 mM EDTA, pH 7.6 (Buffer A) before loading onto the DEAE Sephacel column. Initial small scale studies showed that the enzyme eluted between 0.15 M and 0.21 M (Figure 3.20) in buffer A. A step gradient of 0.3 M NaCl was found to be more suitable as an initial large scale step as a linear gradient released enzyme over a large range of salt concentrations when the column was heavily loaded. This would necessitate a longer concentrating step (concentration was always achieved using 10% PEG 6000 in the dialyzing buffer) before loading on the next gel filtration column. Even though AP endonuclease was found to be quite stable at 4°C there was a slight decrease in activity from day to day, especially when in a more dilute form. The step gradient removed 80% of contaminating protein from fraction A. The concentrated eluent off the DEAE Sephacel column fraction B, containing AP endonuclease had at first been loaded onto a phosphocellulose column but results proved erratic with poor resolution or complete loss of enzyme activity.

Fraction B was dialized against buffer A containing 0.2 M NaCl and 5% (v/v) glycerol before loading on the S-200 column. The glycerol was found to stabilize the enzyme when in dilute form. The 0.2 M NaCl was required to prevent inter-protein interactions. AP endonuclease aggregated into a large complex with other proteins when the salt was not present. The column was loaded with 5.2 ml and resolved at a flow rate of 5.4 ml hr^{-1}. Fractions were collected into tubes containing bovine serum albumen to achieve a final concentration of 500 µg ml^{-1}. This helped stabilize the enzyme in a dilute form. The majority of the AP endonuclease activity fell

Figure 3.20 Protein elution profile of fraction A on DEAE-Sephacel.
Column 10 x 2.6 cm. Flow rate 10 ml hr^{-1}.



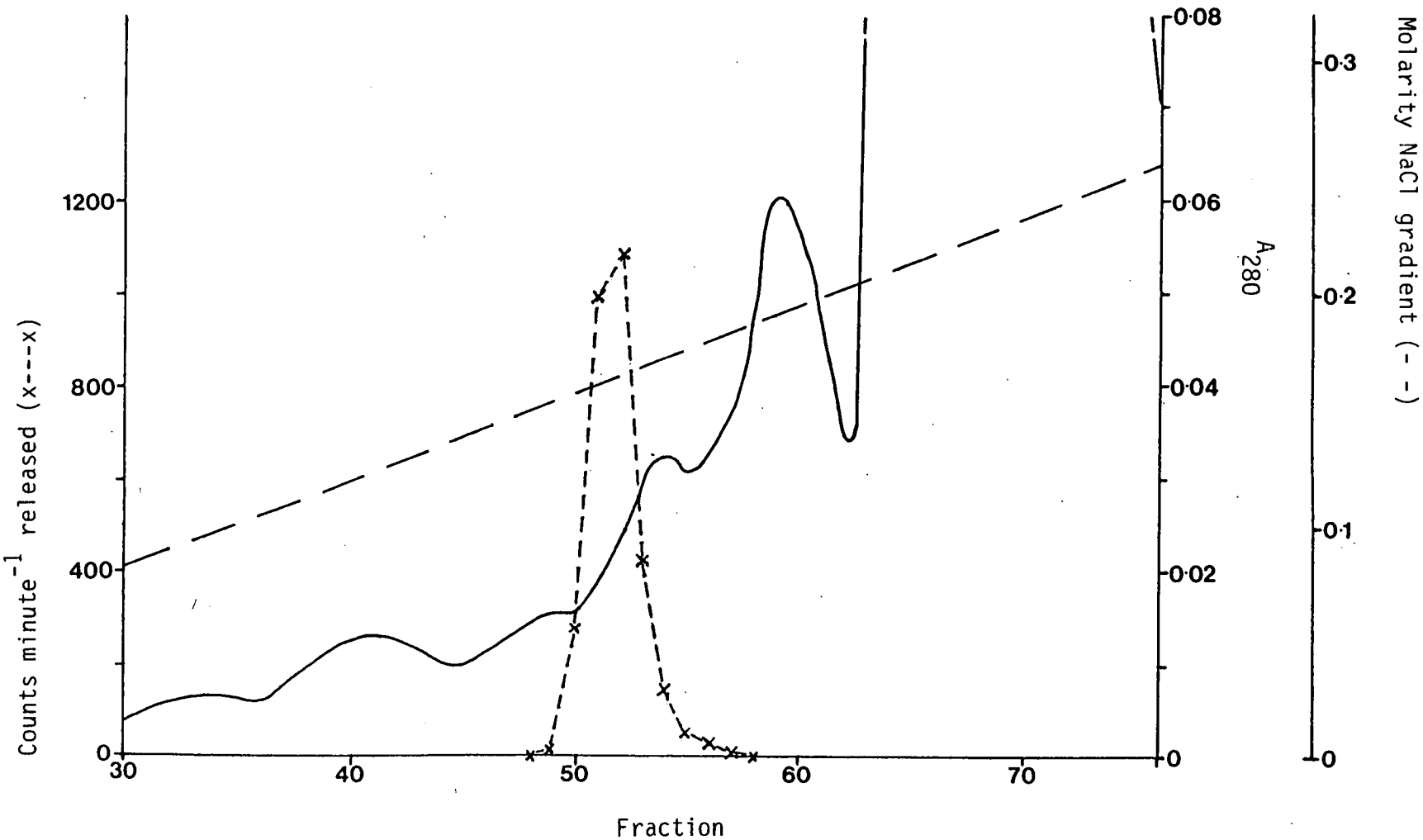
into four hourly fractions with the peak enzyme activity eluted after 86.4 ml. The K_{AV} for this elution is 0.364 and from the calibration curve in Figure 2.3 gives a molecular weight estimate of 34,500 for AP endonuclease. The four fractions contained over 95% of the AP endonuclease activity off the column and were pooled to form fraction C.

This was dialized for 16 hours against two changes of 1 l 10 mM phosphate, 1 mM EDTA, 50 mM NaCl, 5% glycerol pH 7.0 (buffer B). The concentrated fraction C was loaded onto the hydroxylapatite column where the enzyme bound and a linear 0 to 0.3 M phosphate gradient in buffer B was applied and developed at 8.4 ml hr^{-1}. The enzyme eluted over the range 0.08 M to 0.13 M with peak activity at 0.103 M phosphate. Again bovine serum albumen was present at 500 µg ml^{-1} in the collected fractions. Fractions containing 95% of the eluted AP endonuclease activity were pooled to form fraction D. Fraction D was concentrated and dialized against buffer A with 5% glycerol (v/v) present.

3.1.2 Visualization of AP endonuclease on an SDS polyacrylamide gel

Fraction D was loaded onto the DEAE Sephacel column and developed using 400 ml of a 0-0.3 M NaCl linear gradient in the running buffer (buffer A + 5% glycerol (v/v)). AP endonuclease activity appeared in fractions 50-54 (Figure 3.21), separate from the bovine serum albumen and globulins. The enzyme eluted at a salt concentration between 0.155 and 0.175 M. Fractions 48 to 61 were run on a SDS polyacrylamide gel and the appearance of bands which predominated in fractions 51 and 52, and to a lesser extent in fractions 50, 53

Figure 3.21 Elution of AP endonuclease activity in fraction D from a DEAE Sephacel column using a NaCl linear gradient at a flow rate of 10 ml hr^{-1}. Fractions of 5 ml were collected and AP endonuclease activity measured by the release of acid soluble radioactivity using the standard assay described in Materials and Methods.



and 54, were looked for (Figure 3.22). There appears to be two possibilities. One shows very faint bands in lanes from fractions 51 and 52 which migrate to approximately the same position as a 34,000 mw standard. This would agree well with the estimate of the molecular weight from the S-200 gel filtration column. The second possibility shows ... stronger bands in the lanes from fractions 51 and 52 with lighter bands in the lanes from fractions 50 and 53. Fractions 50 to 53 were pooled to form fraction E. This was dialyzed against 20 mM Tris, 2 mM EDTA, 50 mM NaCl, 50% glycerol (v/v) pH 7.6 for 20 hours. Fraction E was concentrated to 2 ml.

A silver stained SDS polyacrylamide gel with the protein profile at each purification stage is shown in Figure 3.23. When the bands attributable to the bovine serum albumen are ignored, 10 bands are left. Of these, one of the two arrowed bands is thought to be the AP endonuclease protein. Further purification would be possible with larger linear gradient volumes but would increase the risk of losing enzyme activity at low dilutions. A gel filtration column which separated over the molecular weight range of 10,000 to 70,000 (eg G-75 Sephadex-Pharmacia) would also improve purification. Gel types which interact with DNA binding proteins, such as heparin-agarose may also improve purification. Table 3.7 shows the percentage recovery at each stage of purification.

Accurate determination of the protein concentration and calculation of the enzymes activity were not possible after the first DEAE-Sephacel step because of the presence of 500 µg bovine serum albumen ml^{-1}.

Figure 3.22 Silver stained 12% SDS polyacrylamide gel showing fraction D proteins eluted in different fractions from a DEAE Sephacel column

Lanes: 1, mw marker - 34,000; 2 mw marker - 24,000; 3 to 16, fractions 61 down to 48 respectively; 17, fraction D from hydroxylapatite column.

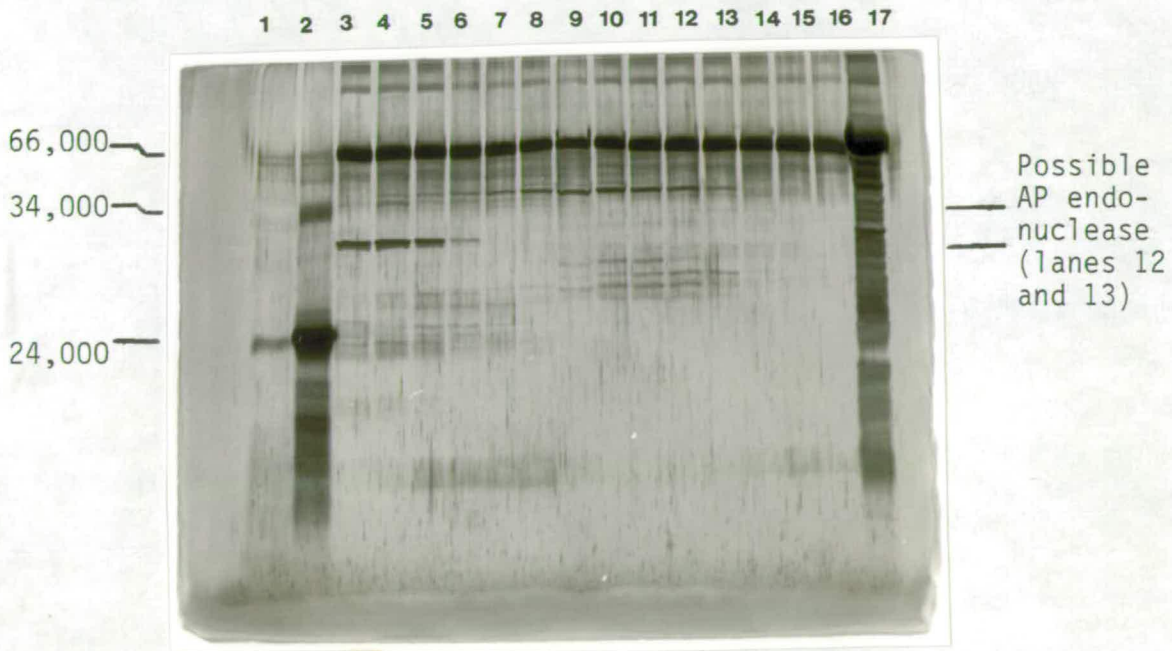


Figure 3.23 Silver stained 12% SDS polyacrylamide gel showing the protein profile at each purification stage of the AP endonuclease

Lanes: 1, mw standard 66,000; 2, mw standard 20,100; 3, Crude extract; 4, fraction A; 5, fraction B; 6, fraction C; 7, fraction D; 8, fraction E; 9, mw standard 24,000; 10, mw standard 34,000.

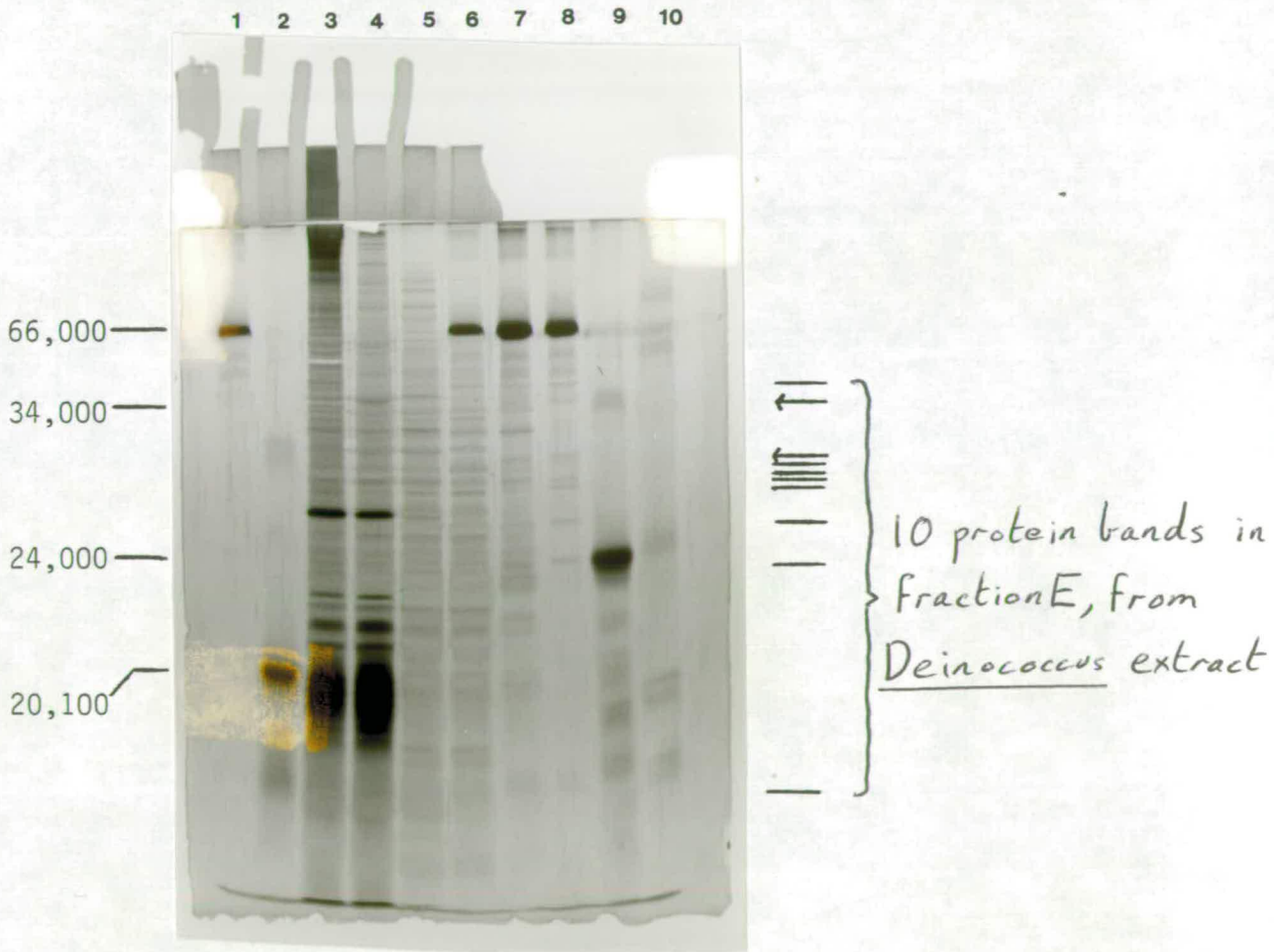


Table 3.7 Stages of AP endonuclease purification

Stage	Total protein[a] (mg)	Total enzyme (units)[b] $\times 10^3$	Specific activity[a] (units mg^{-1} protein)	Yield (%)
Crude extract	600	1,050	1,850	100
Ammonium sulphate (43-70% cut)	138.2	910	6,600	86.6
1st DEAE-Sephacel (step gradient)	33.28	520	15,700	49.5
S-200	-	312	-	29.7
Hydroxylapatite	-	201	-	19.1
2nd DEAE-Sephacel (linear gradient)	-	80	-	7.6

(a) The total protein and therefore the specific activity were not calculated for the final three stages because BSA was added to collected fractions to a concentration of 500 µg ml^{-1}.

(b) Here one unit is defined as the minimum activity required to convert 1 µg of depurinated ccc DNA to oc DNA in 30 min at 30°C.

3.2 Characterization of the major AP endonuclease of D. radiodurans

3.2.1 Purity of the AP endonuclease activity in Fraction E

From Fraction E, ten protein bands are visible on a silver-stained SDS polyacrylamide gel. Fraction E was assayed using the qualitative plasmid assay to see if any of the proteins was an exonuclease. Figure 3.24 shows that exonucleases are present in the crude extract of D. radiodurans (lanes 6 and 12). After 6 hours the plasmid DNA was almost totally digested.

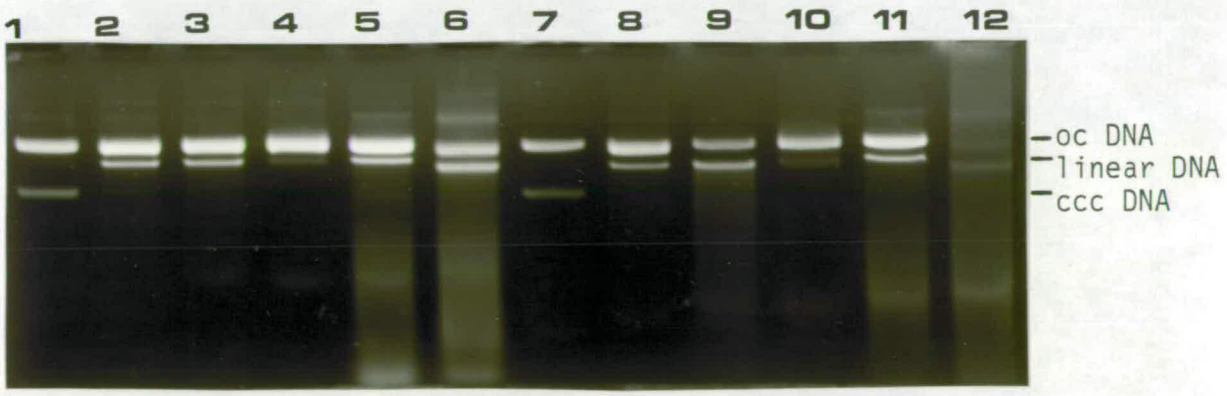
AP endonuclease works in the absence of divalent cations (Figure 3.24, lanes 3 and 9). In the presence of divalent cations Mg^{2+} and Mn^{2+} there appears to be slight exonuclease activity after 30 min (lane 4) as the linear DNA band is faint. But after 6 hours (lane 10) the linear DNA band is still present. This suggests that the faint band is not the result of the action of an exonuclease on the linear DNA. Using the quantitative radiolabelled assay, there was no difference in the liberation of acid soluble radioactivity between the assay with or without Mg^{2+} and Mn^{2+} ions. This again shows that no exonuclease is present. At different pH values between 6 and 8, and salt concentrations up to 0.2 M exonuclease activity was not shown. The Mg^{2+} and Mn^{2+} ions do appear to have an effect on the DNA with Fraction E. The cations probably have an inhibitory effect on the action of AP endonuclease, with relaxed open circular DNA.

Figure 3.24 0.8% agarose gel showing the activity of fraction E and crude extract of D. radiodurans on depurinated DNA

All lanes contain depurinated DNA. Reaction time for lanes 1 to 6 was 30 min and for lanes 7 to 12 it was 6 hours.

Enzymes and covalent ions added to each reaction were as follows:

Lanes 1 and 7 - none; lanes 2 and 8 - fraction E; lanes 3 and 9 - ExoIII; lanes 4 and 10 - fraction E + Mg^{2+} + Mn^{2+} (5 mM each); lanes 5 and 11 - crude extract; lanes 6 and 12 - crude extract + Mg^{2+} + Mn^{2+} (5 mM each).



3.2.2 Specificity of the AP endonuclease

Some AP endonucleases are associated with a glycosylase activity, eg UV endonuclease of Micrococcus luteus (Demple and Linn, 1980). The radioactive assay method was used on untreated DNA, MMS-treated DNA, depurinated MMS-treated DNA and UV-treated DNA. Table 3.8 shows that the action of NaOH on MMS-treated DNA released radioactivity five times above the control level with untreated DNA. The MMS treatment has introduced a small number of apurinic sites but depurination produces over 200 fold more NaOH labile sites in the DNA than present in untreated DNA. Fraction E had no effect on untreated DNA and released 62% of acid-soluble radioactivity from depurinated DNA, as compared with the DNA treated with NaOH over the extended 30 min assay time. Further incubation would have produced more acid-soluble radioactivity but some radioactivity released by NaOH is at alkali-labile sites other than AP sites in MMS-treated DNA (Teebor and Brent, 1981). The small amount of radiolabel released by Fraction E on MMS-treated DNA is 54% of the total release using NaOH. The difference again is due to incomplete alkaline hydrolysis of all AP sites and the presence of non apurinic alkali labile sites. The proportions of radiolabelled release from MMS-treated and depurinated DNAs by Fraction E show an 8% higher level on depurinated DNA. This means that the purified AP endonuclease contains no glycosylase activity which would act on the methylated DNA to produce more AP sites. The crude extract released more radiolabel from MMS-treated DNA than was released by alkali hydrolysis. This showed that an enzyme in the crude extract was acting on the MMS-methylated DNA. It is most likely to be a

Table 3.8 Activity of the AP endonuclease on different substrates

Substrate DNA [a]	Assay component [b]	cpm released	Corrected cpm value
UT	-	25	0
UT	NaOH	38	13
UT	Fraction E	35	10
UT	Crude extract	40	15
AP	-	38	0
AP	NaOH	2,854	2,816
AP	Fraction E	1,780	1,742
AP	Crude extract	1,301	1,263
MMS	-	26	0
MMS	NaOH	98	72
MMS	Fraction E	65	39
MMS	Crude extract	160	121
UV	-	33	0
UV	NaOH	51	18
UV	Fraction E	34	1
UV	Crude extract	170	137

(a) UT - untreated DNA

AP - depurinated methylated DNA

MMS - MMS-treated DNA

UV - UV-treated DNA (600 Jm^{-2})

(b) The assay was for 30 min.

3-methyladenine glycosylase which removes 3-methyladenine residues, a major product of MMS-treated DNA (Riazuddin and Lindahl, 1978). Fraction E had no effect on UV-treated DNA whereas the crude extract gave a small release of radiolabel. This release was due to the activity of the high UV endonuclease (Evans, 1984).

Using the plasmid assay method it was demonstrated again that Fraction E has no activity towards MMS- or UV-treated DNA. It was also evident that it did not act on DNA treated with bisulphite, which deaminates cytosine to uracil in DNA. The crude extract converted ccc plasmid DNA to the oc form; this was probably due to the combined action of an uracil-DNA glycosylase and the AP endonuclease (data not shown).

Within the range of substrates used Fraction E was specific for DNA containing AP sites.

3.2.3 The effect of cations and other small molecules on the activity of the AP endonuclease

Table 3.9 shows the effect of different cations on the activity of the AP endonuclease. At concentrations of 5 mM, Cu^{2+} and Fe^{2+} ions totally inhibited activity while Zn^{2+} and Co^{2+} ions partially inhibited activity. At 10 mM, Zn^{2+} ions completely inhibited the enzyme and Co^{2+} ions gave only 8% of control activity. Ca^{2+} ions had no effect on enzyme activity at concentrations up to 20 mM. At 5 mM the Mg^{2+} and Mn^{2+} ions had no effect on activity, but at 10 mM and above Mn^{2+} ions inhibited the activity and above 10 mM concentrations of Mg^{2+} ions also inhibited activity. Table 3.10 shows an increasing inhibitory effect of Mn^{2+} and Mg^{2+} ions above a

Table 3.9 Activity of AP endonuclease in the presence of different cations

Cation[(a)]	Concentration (mM)	% activity with respect to the control
Control	-	100
Fe^{2+}	5	0
Cu^{2+}	5	0
Zn^{2+}	5	22.0
Zn^{2+}	10	0
Co^{2+}	5	68.5
Co^{2+}	10	8.0
K^{+}	5	120 - 179
Ca^{2+}	5	97.5
Mn^{2+}	5	102.0
Mn^{2+}	10	73.0
Mn^{2+}	20	21.5
Mg^{2+}	5	107.0
Mg^{2+}	10	104.0
Mg^{2+}	20	49.5
$Mg^{2+}+Mn^{2+}$	10 (each)	27.0

(a) Control has no cations added to the normal reaction. All other mixtures contain the normal reaction constituents, plus the cation to the specified concentration.

Table 3.10 The effect of Mn^{2+} and Mg^{2+} ion concentrations on the activity of AP endonuclease

Cation and concentration	% activity
Control[a]	100
1 mM Mn	103
2 mM Mn	103
4 mM Mn	95
8 mM Mn	51
16 mM Mn	47.5
20 mM Mn	41.0
1 mM Mg	94.0
2 mM Mg	117.5
4 mM Mg	90.5
8 mM Mg	73.5
16 mM Mg	71.0
20 mM Mg	67.0

(a) Control has no Mn^{2+} or Mg^{2+} ions in the reaction.

5 mM concentration level. The results with Mn^{2+} ions are interesting as these ions are found at up to 100x higher concentrations around D. radiodurans DNA than in most other bacteria (Leibowitz et al., 1976). It may seem surprising that the enzyme is inhibited by 5 mM Mn^{2+} ions but the concentration around the DNA in D. radiodurans is only 0.3 to 3.2×10^{-18} mol/chromsome, well below 5 mM.

Initial results with K^{+} ions gave large increases in activity (Table 3.9) but later results gave only increases of 20 to 30%. The presence of K^{+} ions did increase the activity of the enzyme and this was due to an increase of the ionic concentration of the reaction mixture (see 3.2.4). Further study on the effects of Mn^{2+} and Mg^{2+} ions on the activity of the enzyme might reveal interesting results.

The provision of a reduced environment using 10 mM DTT gave a 50% increase in activity of the enzyme. ATP, caffeine, N-ethylmaleimide and Triton X-100 all had no effect on the enzymes activity.

3.2.4 The effect of ionic strength and pH on the AP endonuclease

Figure 3. 25 shows that enzyme activity drops dramatically after a concentration of 0.4 M NaCl is reached. NaCl concentration of 0.7 M or above totally destroyed enzyme activity, probably through disruption of its tertiary structure. At NaCl concentrations of 20 mM and below there was a drop in enzyme activity of up to 20%. Optimal activity was seen in NaCl concentrations of 0.1 to 0.2 M.

Figure 3.26 shows the effect of pH on enzyme activity. The optimal pH was 7.5 and the enzyme had high activity across the pH range 6.0 to 8.5. Controls where no AP endonuclease (Fraction E)

Figure 3.25 The effect of ionic strength on AP endonuclease activity as seen by the release of radioactivity as TCA soluble material

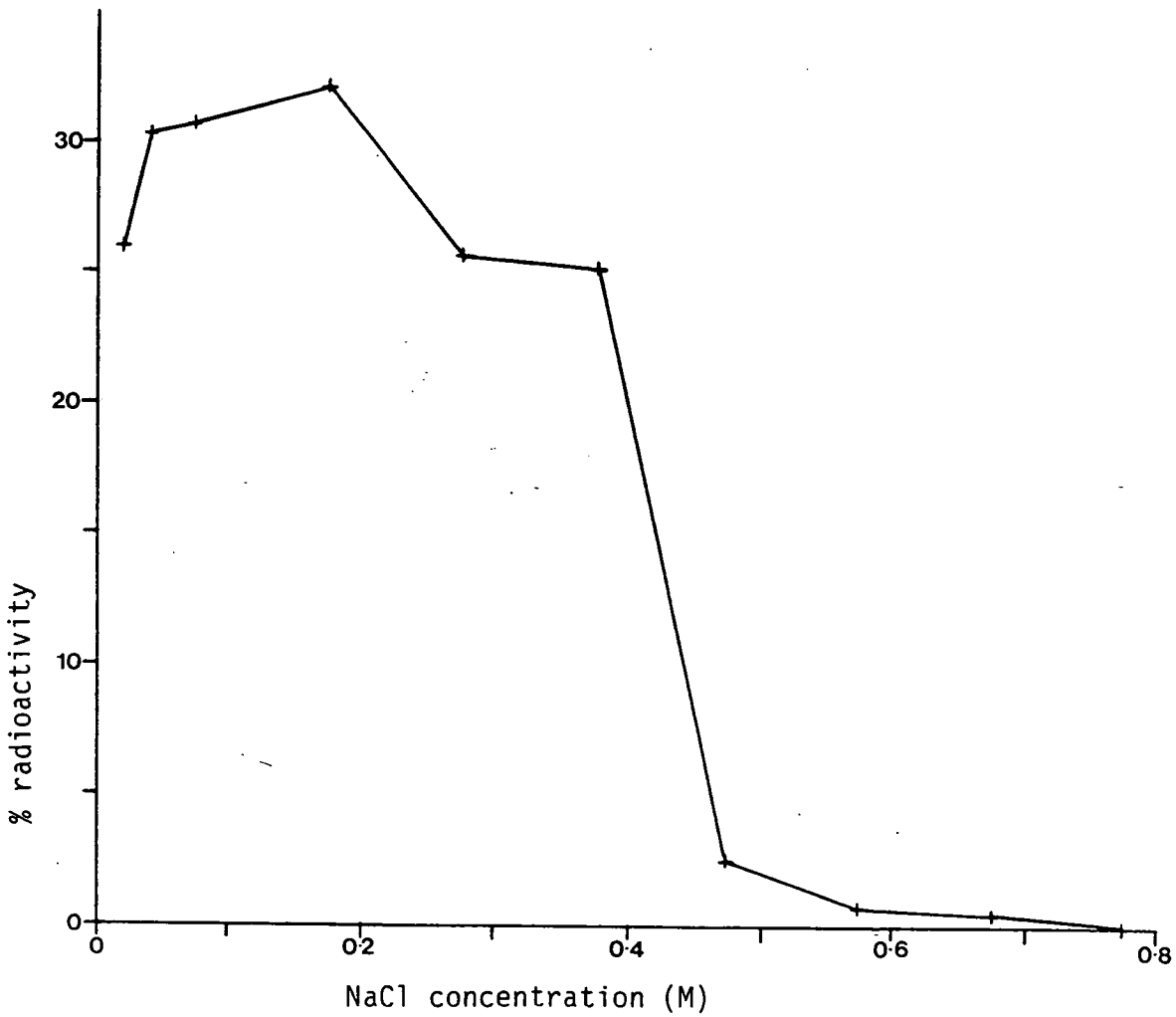
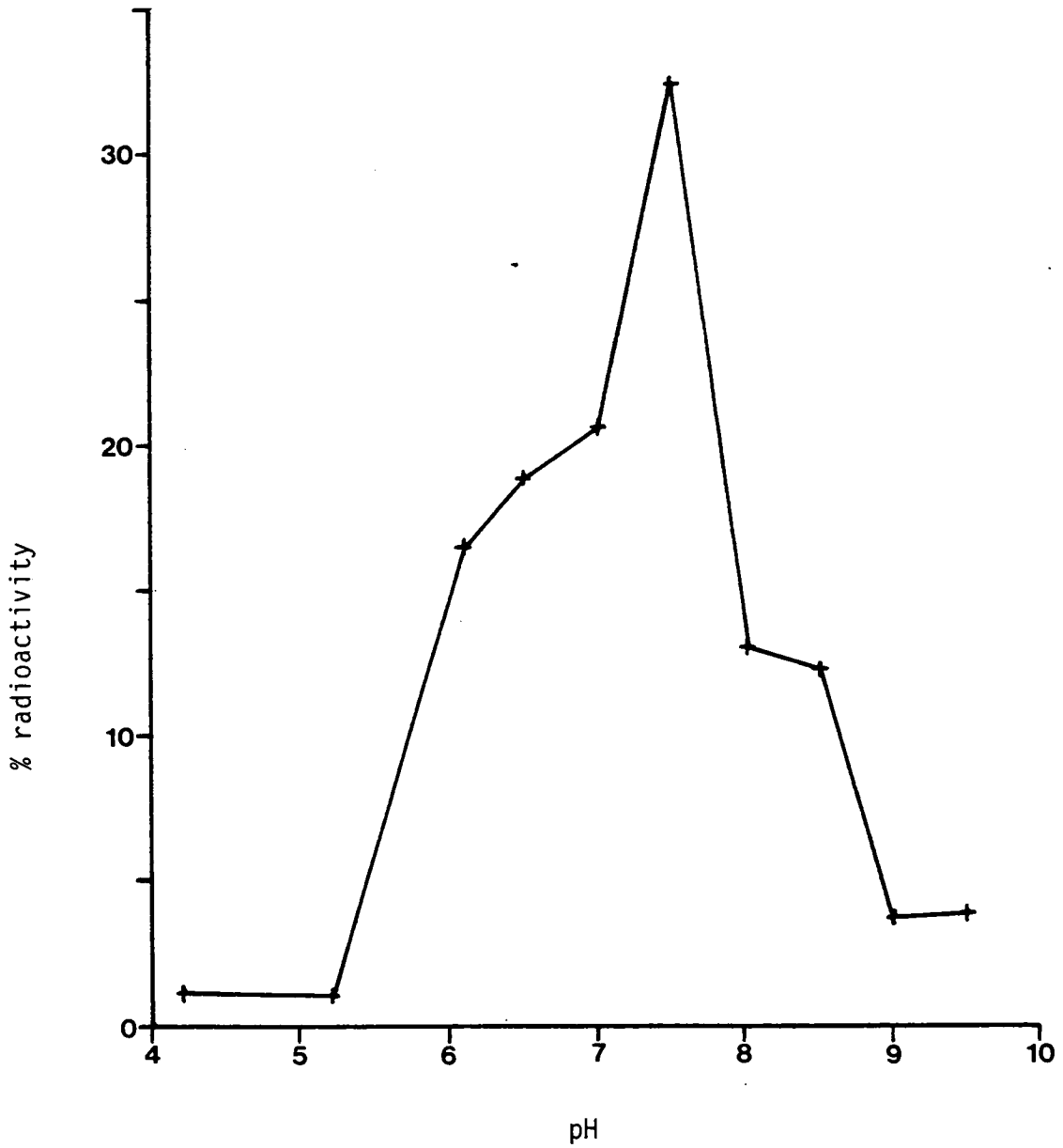


Figure 3.26 The effect of pH on AP endonuclease activity as seen by the release of radioactivity as TCA soluble material



was added to the assay tubes showed the same background level of acid-soluble radioactivity at all pHs. So the low levels of enzyme activity recorded at pHs 4.2, 5.2, 9.0 and 9.5, were due to low enzyme activity and were not a consequence of the acidic or alkaline enviroment.

3.2.5 The effect of temperature on the AP endonuclease

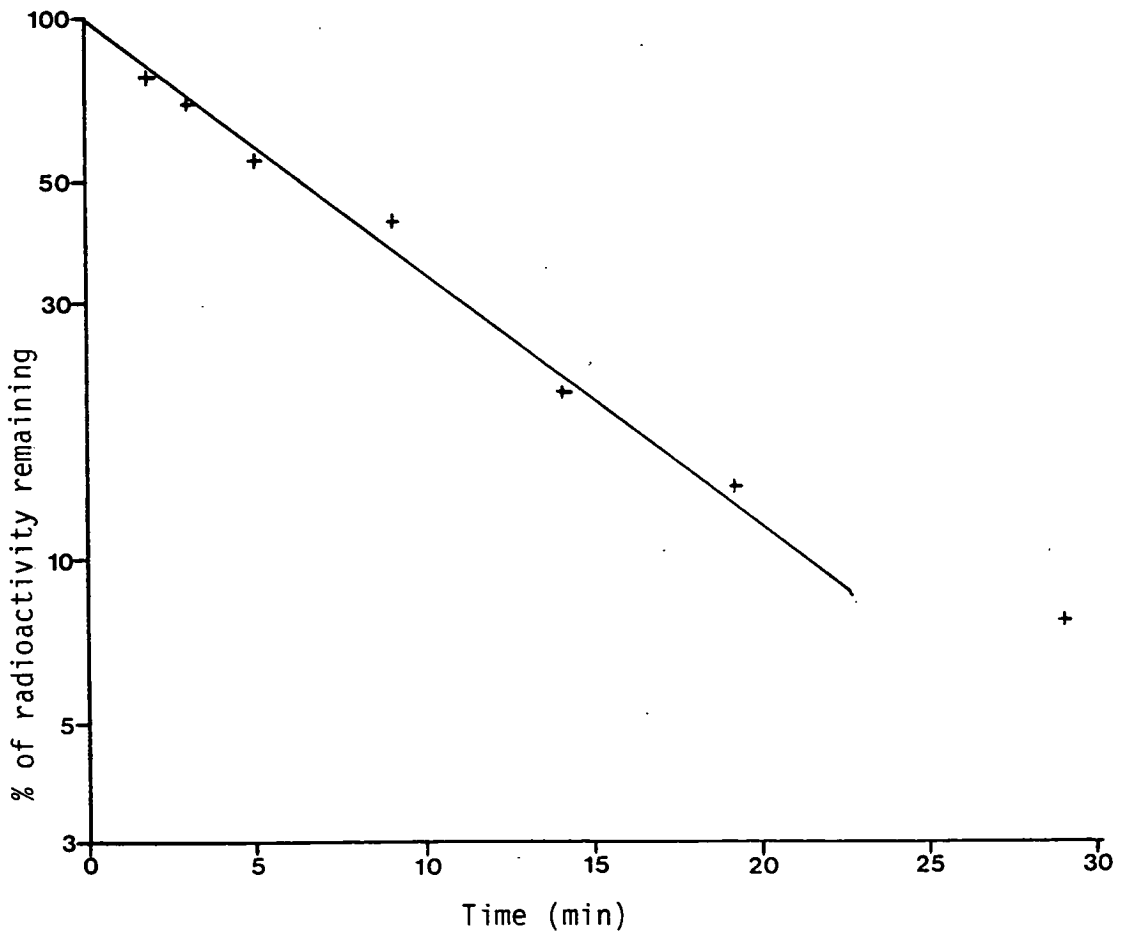
The optimal temperature for enzyme activity was 42°C, with high activity of at least 75% of the 42°C level, over the range 30 to 55°C. This was the case for the standard 20 min radiolabelled DNA assay. Activity decreased at 45°C and temperatures above.

The heat sensitivity of the AP endonuclease at 45°C was tested. Fraction E was diluted 10 fold in 1xSSC, to prevent protection of the enzyme by the 50% glycerol present in Fraction E. The diluted form was held at 45°C and 20 µl samples taken at various times and immediately cooled to 0°C. 20 µl of radiolabelled DNA (50 µg ml^{-1}) was added and the usual assay procedure followed. As can be seen in Figure 3.27, the enzyme shows a half life of 6.5 min at 45°C. When the enzyme was held at 45°C with 50% glycerol present, there was only a small decline in activity over 30 min. The 10 fold diluted form of Fraction E, when held at 60°C, showed no activity after 15 min.

3.2.6 Uracil-DNA glycosylase and methyl-DNA glycosylase of D. radiodurans

During the study of AP endonuclease, the 30 to 70% saturation

Figure 3.27 Heat sensitivity of AP endonuclease at 45°C as shown by the release of radioactivity as TCA soluble material



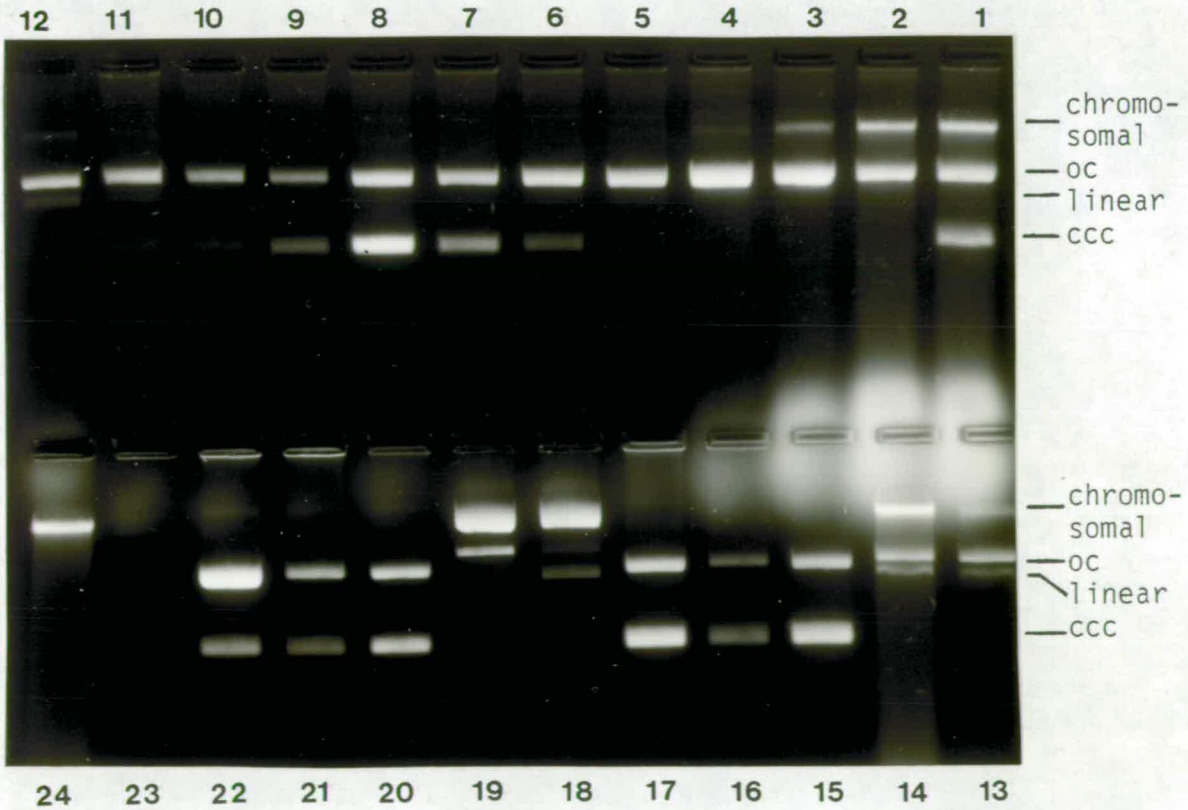
ammonium sulphate cut of the D. radiodurans crude extract was assayed for the presence of uracil-DNA glycosylase and methyl-DNA glycosylase. Plasmid DNA containing uracil or MMS methylated bases was prepared as described in Materials and Methods. Assays were made at 30°C in 10 mM Tris, 2 mM EDTA, 0.1 M NaCl pH 7.5. The presence of the AP endonuclease in the extract was essential for the assay. The AP endonuclease nicked the plasmid, at AP sites produced by the action of the glycosylase, and the conversion of ccc plasmid DNA to oc DNA was visualized on 0.8% agarose gels.

Figure 3.28 shows that enzymes which recognize DNA containing uracil and methylated bases were present in the D. radiodurans extract. Lanes 2, 3 and 4 show enzyme activity that recognizes MMS-methylated bases. The main products of MMS-treated DNA are 7-methylguanine (85%) and 3-methyladenine (12%). 7-methylguanine is tolerated in the genome as it does not lead to miscoding (Ludlum, 1970), or affect cell viability (Prakash and Strauss, 1970). Glycosylases active towards 7-methylguanine have been reported (Gallagher and Brent, 1984), and all appear to be due to the action of broad specific 3-methyladenine-DNA glycosylases. Wild type D. radiodurans has also demonstrated enzymic excision of 7-methylguanine (Tempest, 1978). Activity towards 7-methyladenine is much lower than towards 3-methyladenine and the presence of such activity probably reflects the slow rate of imidazole ring cleavage seen in 7-methylguanine at neutral pH. The cleavage results in 2, 6-diamino-4-oxy-5-N-methylformanindo-pyrimidine, which is potentially lethal as it blocks DNA synthesis (Boiteux and Laval, 1983). Spontaneous hydrolysis of the glycosidic bond to form apurinic sites occurs 300 times more rapidly that the ring cleaving reaction at neutral pH (Lindhal, 1982) and the AP sites form, potentially, more

Figure 3.28 0.8% agarose gel showing enzymatic activity towards DNA containing methylated bases or uracil

Dilution of crude extract	Type of DNA[a]			
	DNA	URA	MMS	AP
	Lane numbers			
1/1	1	14	2	
1/4		13	3	
1/16		12	4	
1/64		11	5	
1/256		10	6	
1/1024	8	9	7	
0	15	16	17	18
ExoIII	20	21	22	19

[a] DNA - unmodified DNA, URA - DNA containing uracil, MMS-methylated DNA, AP - depurinated DNA. Lanes 18 and 19 contain a different plasmid to the other lanes and the form of plasmid indicated at the side of the picture does not correspond to these two lanes. Lane 24 contains only crude extract.



of a mutagenic problem for the cell. 3-methyladenine is not tolerated by cells and is released rapidly in bacterial and mammalian cells by the action of a 3-methyladenine-DNA glycosylase (Bjellard and Seeberg, 1987; Karran et al., 1982; Gallagher and Brent, 1984). It is removed rapidly because it is highly cytotoxic and weakly mutagenic (Karran et al., 1980). The D. radiodurans enzyme is most probably a 3-methyladenine-DNA glycosylase because of the lesions high cytotoxicity and the seeming ubiquity of this type of glycosylase enzyme. Further study on the product released from the methylated DNA, will show what the glycosylase acts on, eg paper chromatography or HPLC chromatography.

Lanes 12, 13 and 14 in Figure 3.28 show the presence of an enzyme which recognises uracil in DNA. The enzyme is most probably a uracil-DNA glycosylase which catalizes the hydrolysis of the glycosyl bond between the uracil base and the deoxyribose-phosphate backbone leaving an apyrimidinic site. Uracil-DNA glycosylase is an ubiquitous enzyme found in bacteria (E. coli - Lindhal et al., 1977) mammals (Human - Krokan and Wittwer, 1981) and plants (Bensen and Warner, 1987) and its main function is to remove uracil in DNA produced by the deamination of cytosine. This forms a potentially mutagenic lesion (Duncan and Miller, 1980) as uracil base pairs with adenine and not guanine as is the case with cytosine.

Figure 3.28 has controls in lanes 1 and 15 to 22. Lane 1 shows that the D. radiodurans extract has no effect on unmodified plasmid DNA. Lanes 15-22 show that MMS-treated DNA and the bisulphite-treated ccc DNA do not contain AP sites before the addition of the extract.

The crudely prepared extract of the two glycosylases was

assayed under different ionic and pH conditions. The uracil glycosylase was active over 10 mM-300 mM NaCl with an optimum at 200 mM. The methyl glycosylase was active over the range 20 mM-300 mM with an optimum at 100 to 200 mM. Both enzymes were active at pHs between 5.5 and 8.5 with optimal activity between pH 7 to 8. The enzymes did not require divalent cations, working in the presence of EDTA, as exemplified by all known glycosylases.

D. radiodurans is shown to have enzymes active towards methylated DNA and DNA containing uracil.

CHAPTER 4

DISCUSSION

4.1 Development of a cloning shuttle vector between D. radiodurans and E. coli

Attempts by Mackay (1983), Purvis (1984) and Al-Bakri (1985) to produce a cloning vector for D. radiodurans from a variety of plasmids and the use of spheroplasts were unsuccessful. The construction of a shuttle vector using native Deinococcus plasmids was seen as a logical method of achieving a cloning system for Deinococcus. Al-Bakri constructed a chimeric plasmid pUE109 (Mackay et al., 1985) which failed to transform D. radiodurans to tetracycline resistance from the Tc^R gene functional in an E. coli host.

It has been shown previously that there is no barrier to plasmid DNA uptake in D. radiodurans (Tirgari, 1977) and that the highly active extracellular nucleases have little effect on transformation using the transformation procedure in Materials and Methods where the nucleases are washed away (Purvis, 1984). B. subtilis requires plasmid oligomers for transformation (Canosi et al., 1978) and are transformed with monomeric plasmid DNA only because of contamination with multimeric forms when purified from E. coli (Mottes et al., 1979). D. radiodurans should therefore transform at a low frequency if oligomers are required.

Failure to express may have been due to the endogenous MraI restriction endonuclease which recognizes a site in pUE109. Removal of the site did not confer Tc^R on D. radiodurans transformed with modified plasmid. The significance of MraI as a problem to plasmid integrity may be over-estimated. In other bacteria a restriction system lowers the frequency of transformation or virus infection when non-modified DNA is used (Arber, 1974), but some DNA escapes

digestion and is protected subsequently by modification mechanisms (eg methylation). Restriction enzymes only restrict double-stranded DNA unmodified in both strands to prevent digestion of DNA during replication where the new strand remains unmodified for a period of time. If D. radiodurans was transformed with single-stranded DNA, then this might also protect the DNA. It is likely that MraI does play a role in lowering transformation via foreign DNA into D. radiodurans but this barrier could be removed by isolation of a MraI mutant, defective in enzyme activity.

A major problem in constructing a cloning vector for D. radiodurans has been the lack of a marker gene. Experiments using D. radiodurans SARK plasmids and chromosomal DNA from a D. radiodurans strain resistant to rifampicin did not produce any rifampicin-resistant transformants (Mackay, 1984). Using plasmids containing the mtcA or mtcB genes gave transformants to mitomycin C resistance in strains mutant in these two genes but no plasmids were detected on agarose gels. A more sensitive method to detect plasmids would have been to transform E. coli cells with DNA preparations from the D. radiodurans transformants and to select for Ap^R or Tc^R. It seemed more likely that the transformants resulted from integration of the wild type mtcA or B genes into the chromosome via recombination at the site of homology. Indeed, this was the strategy used to clone the mtcA and B genes originally (Al-Bakri et al., 1985).

With hindsight the E. coli ampicillin and tetracycline resistance markers in pUE120 and pUE109 may not have been a good choice. Foster (1983) and Lorian (1986) have reviewed the subject of plasmid-determined resistance to antimicrobial drugs. Resistance to ampicillin requires the transportation of the Ap^R gene product,

β-lactamase, across the cell envelope to inactivate ampicillin before the latter interferes with cell wall peptidoglycan synthesis. Tetracycline inhibits proteins synthesis by binding to the ribosomal 30S subunit and preventing access of the aminoacyl-tRNA to the acceptor site of the ribosome. Tetracycline resistance is thought to occur via an efflux mechanism and decreased uptake of tetracycline, both of which require interaction with the cell membrane and wall. Thus, even if the Ap^R and Tc^R genes are transcribed and translated in D. radiodurans, the gene products are probably ineffective due to the unusual nature of the cell envelope (Thornley et al., 1965) not providing the correct sites for the transport and function of these proteins. If the problem is post-translational then it should be possible to pick out transcription and translation products of these genes in D. radiodurans by using Northern blots (RNA-DNA hybridization) (Thomas, 1980) and Western blots (Protein-antibody reaction) (Burnette, 1981) respectively.

Resistance to kanamycin (Km) and chloramphenicol (Cm) has recently been achieved in D. radiodurans by the transformation of the heterologous E. coli Km^R and Cm^R genes (M. Smith and K. Minton, pers.comm.). Resistance to these antimicrobial drugs is achieved by acetylation of the drugs in the cytoplasm by enzymes encoded by Km^R and Cm^R genes. These genes would only require to be transcribed and translated to function in the cytoplasm. These markers will be of great help in future development of cloning vectors.

The use of chromosomal marker genes indicated recombination of the genes into the chromosome. Southern blot hybridization of Mtc^R transformants of D. radiodurans showed that non D. radiodurans DNA on the plasmid was not integrated into the chromosome. This

suggested that double cross over events occur more readily than single cross overs or that foreign sequences are not tolerated in the chromosome. However, the recent work with Km^R and Cm^R markers has shown them to be integrated into the chromosome from a plasmid carrying D. radiodurans DNA sequences (M. Smith and K. Minton, pers. comm.).

Recombination in D. radiodurans is very efficient and plasmids containing homologous DNA sequences appear always to integrate into the chromosome and not to exist as an autonomous unit. To overcome this problem, deletion mutants could be constructed for genes already identified from the gene bank. These mutants could be used to test the hypothesis of integration, assuming that the origin of replication of pUE1 (in pUE109), or those from plasmids in SARK, work in D. radiodurans R1.

A second method to achieve this would be to isolate a recombination-deficient strain carrying a second mutational marker gene. The attempt to construct such a strain in this study was unsuccessful. The major problem was the lack of understanding of the Rec^- phenotypes of strains 112 and rec30. The recl mutation of strain 112 could not be transformed into the Trp^- strain of D. radiodurans and might indicate that the defective gene produces a local area of deficient recombinase which prevents the DNA from recombining into the chromosome. The recl phenotype requires further study of its complex phenotype and may prove to be a multiple mutation effect.

This study showed that the strain rec30 which is highly sensitive to mitomycin C carries at least two mutations, one being in the mtcA or mtcB gene. Again, this strain requires further study to characterize the nature of its phenotype. It may be proved that the

high mitomycin C sensitivity and the recombination deficiency are caused by different mutations or that these two phenotypic traits require the same, but more than one, mutation.

How near to an autonomously-replicating cloning plasmid in D. radiodurans are we? The major requirements for a vector are marker genes and an origin of replication. Heterologous antibiotic-resistance markers (M. Smith and K.Minton, pers.comm.) and homologous chromosomal markers (Al-Bakri, 1985) are now available and the major problem is the isolation of an origin of replication. The native plasmids of Deinococcus should provide one by digesting them with restriction endonucleases and ligating resulting fragments to markers lacking an origin of replication. At present the Km^R and Cm^R markers will be more useful until a recombination-deficient strain of D. radiodurans is isolated. Until such a mutant is found, it will be difficult to clone D. radiodurans genes on an autonomously-replicating plasmid, but the cloning of genes from other bacteria should be possible. Cloning of genes from other species of Deinococcus might be possible in a recombination proficient strain of D. radiodurans R1, provided the analogous genes have reduced or no homology. This would allow complementation tests to be performed without the high degree of DNA homology required at the present.

The present method of studying the expression and structure of D. radiodurans genes relies mostly on complementation of E. coli mutants. So far, only one has been found (Al-Bakri et al., 1985). A cloning vector in D. radiodurans would allow more detailed study of expression and structure of Deinococcus genes.

4.2 Isolation of new Deinococcus species

4.2.1 Ecology of Deinococcus

The first Deinococcus species was discovered over 30 years ago, but a common natural habitat for these highly radiation-resistant organisms has not been found. The diverse sources from which isolations have been made, eg fish (Davis et al., 1963), cattle and grass (Krabbenhoff et al., 1965), sawdust (Ito et al., 1977), dust and clothing (Christensen and Kristensen, 1981), faeces (Kobatake et al., 1973), soil (Erikson and Emborg, 1978) do not explain why these bacteria have kept such an efficient DNA repair capacity.

Deinococcus species are desiccation resistant (Sanders and Maxcy, 1974; R G E Murray, pers.comm.) and it has been suggested that this may account for their radiation resistance. The resistance to drying could explain its wide-ranging isolation where desiccated cells travel by air on dust particles, or by themselves, to reach a new habitat. The isolations during this study were from wet soils next to a lake in Nottingham and a burn in Edinburgh, which are not prone to desiccation. It can be surmised that the Deinococcus family evolved in areas of high water content that were prone to droughts. Strains which could survive drought while desiccated,would survive and form the basis of the next bacterial generation when the water returned. Desiccation is thought to produce some forms of DNA damage (Jackson, 1987) that would require an efficient repair mechanism on rehydration. Deinococcus would therefore grow in wet conditions but be able to survive a period of drought.

Deinococcus species require some amino acids for good growth and are stimulated by others (Raj et al., 1960). The lake and burn water were very murky and probably contained many nutrients. Soil micro-fauna and -flora would also break down complex food sources to release amino acids and sugars into the environment. Soil appears to be the most likely source of the bacterium with aerial contamination accounting for isolation at other sites.

In most cases, a selection procedure for the isolation of Deinococcus has had to be employed because faster growing bacteria outnumber them by up to 5 orders of magnitude. It is doubtful if Deinococcus will be found in large numbers at any site except areas with good nutritional supply prone to drying out for long periods of time, or at artificially created sites where the high radiation resistance of the organism is a vital factor (eg areas in and around nuclear power sources).

The strains isolated in this study were all highly resistant to UV radiation and Professor R.G.E. Murray has confirmed that four of them are new D. radiodurans isolates but they were non-transformable to rifampicin resistance by DNA from the KRASE strain of D. radiodurans R1.

4.2.2 Plasmids and restriction endonucleases

The new strains were not reisolates of laboratory strains as they varied with regard to UV survival curves, plasmid profiles and, in some cases, restriction endonucleases.

All four species of Deinococcus carry plasmids and so it was no surprise to find some of the new strains carrying plasmids. Not

all new strains were screened and the method used is not overly sensitive, so strains not showing visible bands may nevertheless harbour plasmids. It has, so far, proved impossible to cure any strain of Deinococcus of its plasmids and this has led to the suggestion that they may carry essential genes (Mackay et al., 1985). Whether the plasmids in the new strains are of relevance to survival was not studied.

Deinococci have proved a useful source of restriction endonucleases (Wani et al., 1982; Purvis and Moseley, 1983; De Wit et al., 1985; Grosskopf et al., 1985) and three of the new strains produced such enzymes in large quantities. Isoschizomers of PvuI (Gingeras et al., 1981), XhoII (Gingeras and Roberts, 1984) and BstEII (Lautenberger et al., 1980) were isolated from strains 5/3, 4/9 and 6/1 respectively. Each strain would have to protect its own DNA from autodigestion and it would have been of interest to see if DNA isolated from 5/3, 4/9 and 6/1 was protected from the endonucleic activity of the respective isoschizomers PvuI, XhoII and BstEII.

Further purification of the crude extracts of other strains would surely produce more enzymes. MraI enzyme activity of D. radiodurans R1 is seen in an ammonium sulphate fraction of the crude extract, but not in the crude extract itself (Purvis, 1984).

The hypothesized biological role of these enzymes is for the protection of the cell against invading foreign DNA, particularly viruses. However, in this study and others, no bacteriophage has been isolated for any Deinococcus. Does this mean the restriction endonucleases in Deinococcus species are redundant? It would be remarkable if the bacteria were not parasitized by a virus and searches for such may have been unlucky or the wrong techniques used

for their isolation. D. radiodurans is easily transformable and the enzymes could be a defence against the invading DNA. This does not explain the presence of DraI, DraII or DraIII in D. radiophilus, which is non-transformable. Perhaps the restriction endonucleases have functions in vivo other than repelling invading DNA.

4.2.3 Interrelatedness of Deinococcus strains

DNA homology studies show different species and strains of Deinococcus do not have great homology at the chromosomal level (Brooks et al., 1980). To assess possible interrelatedness at the gene level, Southern blot hybridization analysis with the DNA repair genes mtcA, uvsC, uvsD and uvsE was performed.

DNA from all the species showed homology to the mtcA gene (Figure 3.18) suggesting that the gene and its product, a subunit of UV endonuclease α are conserved throughout the genus.

Strain 1/10 was isolated from a burn 400 m from the laboratory and it resembles D. radiopugnans in colony morphology. The mtcA-probed filter shows two bands for these two bacteria with the lower bands migrating to slightly different positions. The possibility that 1/10 is an escaped strain of D. radiopugnans which has diverged slightly during its isolation must be considered. There are only a few differences in their protein profiles (data not shown). The extent to which they are related could be seen from DNA homology studies and by comparing their rRNA oligonucleotide profiles.

UV survival curves and the mtcA probe data suggest that 4/1 and 4/13 are the same organism which would not be surprising as they come from the same sample. They both have in common with D. radiopugnans

the very small oligonucleotide molecule which lights up with the mtcA probe. The larger single bands that light up correspond with the larger of the two D. radiopugnans bands. Perhaps 4/1 and 4/13 are closely related to D. radiopugnans where again further comparison studies are required. The strains 4/1 and 4/13 could not be escaped laboratory strains as they were isolated from a sample 250 miles away.

The probe containing the uvs genes showed homology only to the two D. radiodurans strains. This could be explained by the other strains not possessing the gene and therefore the enzyme UV endonuclease β, or the nucleotide sequence for this gene complex has diverged sufficiently to produce little or no homology. Extreme radiation resistance in these organisms would require only one of the two UV endonuclease activities to show wild type levels of UV resistance as mutations in both pathways are required to produce a UV sensitive strain of D. radiodurans (Moseley and Evans, 1983). If only one repair pathway is present in the other strains of Deinococcus it would be logical to have the UV endonuclease α complex as it confers resistance to mitomycin C damage as well as UV damage. The DNA hybridization studies suggest that this is the case. Whether these strains lost the second repair pathway due to its possible redundant capacity or D. radiodurans acquired the second pathway fortuitously for a specific reason, it will be hard to determine. If D. radiopugnans and D. radiophilus have only the equivalent of the UV endonuclease α repair pathway, it should be simple to isolate UV sensitive strains.

D. radiodurans R1 and SARK strains are more closely related than other Deinococci and antibiotic resistance has been transferred from

one strain to the other (B. E. B. Moseley, pers.comm.). The attempted transfer of the mtcA mutation to D. radiodurans SARK was unsuccessful and the homology may not have been sufficient for recombination to occur.

From the data, it appears that the mtcA gene has been quite highly conserved whereas the uvs complex of genes has diverged greatly, or possibly been lost by some species if it was present in the first place.

4.3 The AP endonuclease of D. radiodurans

Apurinic (AP) and apyrimidinic (AP) sites are common in DNA and are repaired with the help of AP endonucleases which incise the DNA adjacent to the AP site. D. radiodurans, like all organisms examined so far, shows AP endonuclease activity. All are specific for double stranded DNA with the exception of endonuclease VII of E. coli (Bonura et al., 1982) and they don't have a requirement for ATP. They are all monomeric proteins with molecular weights ranging from 20,000 to 50,000. There is variation with respect to EDTA resistance, cofactor requirements, associated activities, eg. glycosylase activity, and cleavage, 3' or 5', to the AP site. Most of the differences encountered are exemplified by the five known E. coli AP endonucleases which are compared with the D. radiodurans AP endonuclease in Table 4.1. AP endonucleases from other organisms are listed by Friedberg (1985) and Weiss (1987).

Fraction E of the D. radiodurans AP endonuclease was not contaminated with exonucleases, 3-methyladenine-DNA glycosylase, uracil-DNA glycosylase or UV endonuclease activities. Its estimated molecular weight of 34,500 falls into the size range for AP endonucleases and it will, therefore, most likely be a monomeric protein. It works well over a pH range of 6 to 8.5 with optimal activity at 7.5. Its preferred ionic strength of 0.1 to 0.2 M is higher than most of the other enzymes, but endonuclease IV of E. coli is more active at 0.2 M. The D. radiodurans enzyme is not excessively heat stable with a half life of 6.5 min at 45°C. Endonuclease IV of E. coli and the AP endonuclease of the thermophile Desulfotomaculum nigrificans have long half lives at 60°C (Sako et al., 1984; Ljungquist, 1977).

Table 4.1 AP endonucleases of *E. coli* and *D. radiodurans*

	Endo III[(a)]	Endo IV[(b)]	Endo V[(c)]	Endo of ExoIII[(d)]	Endo VII[(e)]	Endo of *D. radiodurans*
Molecular weight	~ 27,000	~ 33,000	~ 20,000	~ 32,000	~ 56,000	~ 34,500
Divalent cation requirement	None	None	Mg^{2+}	Ca^{2+} or Mg^{2+}	None	None
pH optimum	7.5	8.0 - 8.5	9.25	8.0 - 8.5	7.0	7.5
Preferred ionic concentration (mM)	50 - 100	200 - 300	< 50	50	50 - 100	100 - 200
Class of AP endo	I	II	ND	II	ND	ND
Associated activities	Thymine glycol-DNA glycosylase	NF	Cleaves DNA at a variety of damage	3'-5' exonuclease, 3'-phosphatase, RNase H	ND	NF

ND - not determined

NF - not found

endo - endonuclease

exo - exonuclease

(a) Radman, 1976

(b) Ljungquist, 1977

(c) Gates and Linn, 1977

(d) Weiss, 1981

(e) Bonura *et al.*, 1982

D. nigrificans requires a highly heat stable enzyme to work at the high growing temperatures it can survive. Endonuclease IV is probably the main E. coli enzyme active at high temperatures during the heat shock response.

Further purification and study of the enzyme would allow classification of its cleavage 3' or 5' to the AP site and analysis of the protein structure. Being the major AP endonuclease of D. radiodurans and seemingly not having an associated glycosylase activity, would suggest that it will prove to be a 5' acting endonuclease to provide a suitable substrate for DNA repair by a DNA polymerase and DNA ligase.

It is probable that D. radiodurans has more than one AP endonuclease as is the case with E. coli, S. cerevisiae, M. luteus and Homo sapiens (Friedberg, 1985; Pierre and Laval, 1980). A very weak AP endonuclease activity was detected separately from the major AP endonuclease off a hydroxylapatite column on one occasion. It was not reproducible and might have been a spurious activity produced by oligopeptides containing aromatic amino acids (Pierre and Laval, 1981).

It would be of interest to study the D. radiodurans gene and a mutant defective in the AP endonuclease. There has been some success in cloning D. radiodurans genes in E. coli (Purvis, 1984; Al-Bakri et al., 1985; Peters and Baumeister, 1986) and perhaps it may be possible to complement the E. coli double mutant nfo, xth defective in the 5' acting AP endonucleases IV and of exonuclease III, which is sensitive to a number of DNA damaging agents (Cunningham and Weiss, 1986). Depending on any redundancy of AP endonuclease activity occurring in D. radiodurans, a mutant might be expected to be MMS

sensitive. MMS-sensitive mutants of D. radiodurans produced in the laboratory all showed proficient AP endonuclease activity.

Enzymatic activities towards methylated DNA and DNA containing uracil, suggested the presence of a methyl-DNA glycosylase, probably active against 3-methyladenine, and a uracil-DNA glycosylase. Further purification and study of the enzymatic reaction with the use of HPLC will enable the release products of these enzymes to be recognized.

D. radiodurans has been shown to possess DNA repair enzymes involved in lowering the mutational and lethal effects of uracil-containing DNA from deamination of cytosine and methylated DNA.

D. radiodurans is highly resistant to the lethal and mutagenic effects of alkylating agents and agents which promote deamination of cytosine to uracil (Tempest and Moseley, 1980). This resistance will in part be due to a concerted effort of the DNA glycosylases, AP endonucleases, DNA polymerase and DNA ligase. But these enzymes are also present in E. coli, which is more susceptible to killing and mutation by these agents. This may be explained by the lack of an error prone repair pathway in D. radiodurans and the possession of other extremely efficient repair pathways via UV endonuclease α which recognises a wide range of DNA lesions (Evans and Moseley, 1983) and also by recombination (Sweet and Moseley, 1976).

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