ON THE MOLECULAR GENETICS OF MEMBERS OF THE GENUS DEINOCOCCUS

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SUMMARY

The genus Deinococcus comprises four species, viz. D. radiodurans, D. radiopugnans, D. radiophilus and D. proteolyticus, D. radiodurans being represented by two strains, R1 and Sark. All are characterized by their red pigmentation and extreme resistance to both the lethal and mutagenic effects of ionizing and ultraviolet (u.v.) radiation.

Plasmids were isolated from all four species; two from D. radiodurans Sark (sizes in kilobase pairs, 37.0 and 44.9); three from D. radiophilus (10.8, 27.9 and 92.2); two from D. radiopugnans (2.5 and 28.6) and two from D. proteolyticus (99.4 and 138.8). A third plasmid, 74.2, was isolated from D. radiodurans Sark and this was shown to be a dimer of the 37.0kb molecule. No plasmids were isolated from D. radiodurans R1, in spite of an extended search using a variety of methods. It seems likely that the native plasmids do not play a significant role in the radiation-resistance of the genus. Treatment of D. radiodurans Sark and D. radiophilus with ethidium bromide, sodium dodecyl sulphate, rifampicin, novobiocin, elevated temperature and u.v. irradiation failed to yield any clones with missing plasmids. Thus, phenotypic properties of these strains could not be ascribed to their plasmids.

Attempts were made to develop a gene-cloning system for Deinococcus spp. Six common plasmid vectors, viz. pML2, pBR322 and pAT153 from Escherichia coli, pC194 and pUB110 from Bacillus subtilis and pHV33 an E. coli-B. subtilis hybrid plasmid did not transform D. radiodurans R1. Recombinant plasmids derived from pAT153 and D. radiodurans chromosomal deoxyribonucleic acid (DNA), similarly failed to express a foreign marker in D. radiodurans R1 and Sark strains. An attempt to develop a gene-cloning system from a novel vector, made by insertion of a D. radiodurans Sark chromosomal marker for rifampicin resistance into a D. radiodurans Sark plasmid was unsuccessful.

Genomic libraries of *D. radiodurans* DNA were constructed; two in pAT153, each representing over 90% of the genome and two in the *E. coli* cosmid vector pJBFH, representing 97% and more than 99% of the genome. Three *D. radiodurans* genes, encoding leucine and proline independence and rifampicin resistance did not complement the respective mutations in *E. coli* HB101.

A restriction analysis of *D. radiodurans* and *D. radiophilus* genomic DNA and the same DNA cloned into *E. coli* showed that the sequence 5'-GATC-3' was under-represented, in both *Deinococcus* species, the sequence 5'-CCGCGG-3' was modified in *D. radiodurans* and the sequence 5'-TTTAAA-3' in *D. radiophilus*. Attempts were made to discover the nature of DNA modification in *D. radiodurans* by searching for the presence of methylated or unusual bases. 5 methylcytosine, 6 methyladenine and 7 methylguanine did not appear to be present. However, other unusually modified bases may be present.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

GENERAL INTRODUCTION

1. THE GENUS *DEINOCOCCUS*

1.1 BIOLOGY OF THE GENUS *DEINOCOCCUS*

1.1.1 Isolation and Classification

Prior to 1956, it was thought that highly radiation-resistant forms of bacteria existed only as endospores. However, in 1956 a Gram-positive, non-sporing bacterium was isolated in Oregon from cans of meat that had ostensibly received a sterilising dose of gamma radiation (Anderson *et al.*, 1956). On storage there was evidence of spoilage and an organism that formed red colonies on agar was found to be present in pure culture. Massive doses in excess of 6 Mrad of gamma radiation were required to sterilize stock cultures on agar and this led to the bacterium being called *Micrococcus radiodurans* (Anderson *et al.*, 1961) and its description by McWhirter and McWhirter (1969) as 'The toughest bacterium in the world'.

M. radiodurans has been isolated three times since then. It was subsequently isolated near the canning factory in Oregon and ecological studies showed it to be present in creek water upstream from the meat plant, on the hair and hide of live cattle and in samples of ground meat from the packing plant (Krabbenhoft *et al.*, 1965). It was also isolated in Ontario as an aerial contaminant in a hospital (Murray and Robinow, 1958) and was later designated *M. radiodurans* Sark. More recently, *M. radiodurans* was isolated in Japan from irradiated sawdust (Ito, 1977). In addition to these confirmed isolations, organisms resembling *M. radiodurans* have been found in several irradiated materials e.g. soil (Erikson and Emborg, 1978), chicken (Welch and Maxcy, 1979), ground peat (Parker and Vincent, 1981), air (Christensen and Kristensen, 1981) and textiles (Kristensen and Christensen, 1981) although little research has been published on these *M. radiodurans* - like bacteria and their true identity remains in doubt.

The existence of such extraordinarily radiation-resistant organisms was found not to be unique. Several other red-pigmented bacteria have been isolated from various gamma-irradiated materials and were shown to have properties similar to those of M. radiodurans, viz. M. roseus ATCC 19172, isolated from irradiated haddock tissue (Davis et al., 1963), M. radiophilus from irradiated Bombay duck (Harpodon neherus) (Lewis, 1971) and M. radioproteolyticus from the irradiated faeces of llama (Kobatake et al., 1973).

The taxonomy of these four species of radiation-resistant, red-pigmented, non-sporing bacteria has been subject to debate for a number of years. They were originally aligned with the genus Micrococcus on the basis of morphological, cultural and physiological characteristics. However, subsequent taxonomic studies by Baird-Parker (1965, 1970) demonstrated distinct differences between the cell wall composition of M. radiodurans R1 and other, radiation-sensitive, micrococci. Further studies, on cell wall composition (Work and Griffiths, 1968; Schleifer and Kandler, 1972; Sleytr et al., 1973), fatty acid components of the cell (Knivett et al., 1965; Work, 1970; Girard, 1971; Jantzen et al., 1974) cell structure (Sleytr et al., 1976; Lancy and Murray, 1978), menaquinone systems (Yamada et al., 1977) and phospholipid complement (Rebeyrotte et al., 1979, Thompson et al., 1980) confirmed that there was little or no relationship between the radiation-resistant and typical micrococci. The most striking evidence that these radiation-resistant organisms were not related to the genus Micrococcus was that the oligonucleotide catalogues derived from hydrolysis of their 16S ribosomal RNA were quite different (Brooks et al., 1980).

These data led Brooks and Murray (1981) to propose a new nomenclature for this group of radiation-resistant bacteria. They proposed Deinococcus (meaning strange or unusual berry) as the single genus of the family Deinococcaceae and assigned four species to the genus:- D. radiodurans (type species), D. radiopugnans (formerly M. roseus ATCC 19172), D. proteolyticus (formerly M. radioproteolyticus) and D. radiophilus (formerly M. radiophilus). Originally the genus contained a fifth

species, D. erythromyxa (formerly M. roseus ATCC 187) but a recent investigation involving peptidoglycan and phospholipid typing showed this organism to be unrelated to the other members of the genus Deinococcus (R.G.E. Murray, personal communication). This reclassification is now generally accepted and will be used in this thesis.

1.1.2 Cultural Characteristics

Strains of Deinococcus are normally grown in a tryptone-glucose-yeast extract broth, or in the case of D. radiophilus, in nutrient broth, at 30°C with shaking. Under these conditions they have a doubling time of about 80 min. On the relevant agar, colonies can be counted after incubation at 30°C for 2 days. Although chemically-defined media have been described (Raj et al., 1960; Little and Hanawalt, 1973; Shapiro et al., 1977) they have not been refined to the point of providing a minimal medium and growth in them is often slow and erratic.

Cells occur singly, in pairs and in tetrads and they divide alternately in two planes. In liquid culture, this soon forms a diplococcus which then proceeds to the tetracoccus stage. The majority of D. radiodurans R1 cells are in the diplococcus form with less than 10% in tetracocci (Hansen, 1978). On agar, since the bacteria divide only in two planes a sheet of cells is formed from a diplococcus and loses this regular arrangement only when friction between the agar surface and the sheet of cells causes the latter to fracture (Murray and Robinow, 1958; Driedger, 1970; Moseley and Copland, 1975a). Some mutant strains divide with such synchrony that they form tablets of cells which are clearly visible at the margins of colonies (B.E.B. Moseley, personal communication).

1.1.3 Cell Envelope

The cell walls of these organisms are structurally complex and layered and this is reflected in their chemical complexity (Work and Griffiths, 1968; Brooks et al., 1980). In contrast, cell wall profiles of other

Gram-positive bacteria such as Staphylococcus aureus and Bacillus licheniformis (Millward and Reaveley, 1974) show a single homogenous, thick component external to the plasma membrane. D. radiodurans strains have the most complex cell wall profile (Thornley et al., 1965; Schleifer and Kandler, 1972; Sleytr et al., 1973; Lancy and Murray, 1978, Thompson and Murray, 1981) and although Deinococcus species other than D. radiodurans have not been subjected to the same degree of analysis, their cell wall profiles suggest a similar level of chemical complexity. The possibility exists that the Gram-positive reaction of the genus conceals an underlying relationship to Gram-negative bacteria. This reaction is most likely due to the thickness of the peptidoglycan component which is of the type L-ornithine-glycine$_2$ and is the same for all four species (Brooks et al., 1980).

The fatty acid content of the cell wall, which is predominantly palmitoleate, suggests some alliance of Deinococcus spp. to Gram-negative bacteria (Brooks et al., 1980). However, in contrast to nearly all Gram-negative bacteria, D. radiodurans contains little or no oleic acid (Girard, 1971).

An unusual feature of membrane composition of D. radiodurans is the absence of phosphatidyl glycerol and the derivative phospholipids e.g. phosphatidyl ethanolamine (Rebeyrotte et al., 1979; Thompson et al., 1980). The phospholipids that are present give an unusual pattern on thin layer chromatography, a result that is obtained for all the deinococci (T. Counsell, R. Anderson and R.G.E. Murray, unpublished data).

1.1.4 Radiation-Resistance

In addition to their extreme resistance to the lethal effects of ionising radiation, the deinococci also exhibit a remarkable resistance to the lethal effects of ultra-violet (u.v.) radiation and to the mutagenic effects of both these forms of radiation (Sweet and Moseley, 1974, 1976; Kerszman, 1975; Tempest and Moseley, 1982). Not surprisingly, this resistance to both the lethal and mutagenic effects of ionising and u.v. radiations has been the primary reason for studying this group of bacteria.

Since 1956, many reasons have been proposed to account for this resistance. For example, the fact that cells were pigmented was suggested as one explanation (Kilburn et al., 1958). The presence of intracellular radioprotective material e.g. high sulphydryl content (Bruce, 1964) and the association of the chromosome with manganese ions (Leibowitz et al., 1976) were also offered as explanations. Although these factors may contribute to the extreme resistance of the group, they would seem to be of minor importance since efficient DNA repair systems appear to be the major reason for tolerance to radiation.

Of the four major bacterial DNA repair systems for u.v. damage viz. excision repair, post-replication recombination repair, error-prone repair and photoreactivation repair, two have been shown to exist in D. radiodurans. Boling and Setlow (1966) described an excision repair system in which u.v. induced pyrimidine dimers were removed from the DNA of D. radiodurans and excreted as part of small oligonucleotides into the surrounding medium. Recently, Moseley and Evans (1983) extended this work and showed that excision repair of pyrimidine dimers in D. radiodurans can proceed through two distinct, equally efficient pathways, the removal of either by mutation not markedly affecting u.v. resistance. The presence of a recombination repair system has also been inferred (Moseley et al., 1972a; Moseley and Copland, 1975b). However, the error-prone repair of u.v. induced damage of the type observed in Escherichia coli is absent from D. radiodurans (Sweet and Moseley, 1974; Kerszman 1975; Tempest, 1978) and the other Deinococcus spp. (Tempest and Moseley, 1982). In fact the absence of error-prone repair systems in the deinococci has been proposed as a prerequisite for extreme radiation resistance (Tempest and Moseley, 1982). Photoreactivation repair of pyrimidine dimers is also absent from D. radiodurans (Hanawalt, 1975).

1.2 GENETICS OF THE GENUS *DEINOCOCCUS*

1.2.1 *Transformation*

Transformation was first demonstrated in *D. radiodurans* by Moseley and Setlow (1968) and provided a useful tool for studying the mechanisms that confer resistance to lethal and mutagenic damage in *D. radiodurans* (Moseley *et al.*, 1972b; Moseley and Copland, 1975b). Recently, a protocol was developed that considerably enhanced the efficiency of transformation of *D. radiodurans* (Tirgari and Moseley, 1980), frequencies for single markers of 0.1-1.0% being obtained. This compares favourably with the frequencies obtained in the classical transformable species *B. subtilis* (Anagnostopoulos and Spizizen, 1961), *Diplococcus pneumoniae* (Hotchkiss, 1954) and *Haemophilus influenzae* (Spencer and Herriot, 1965). However, although transformation has proved useful, in strain construction for example, it has severe limitations when used as a method for mapping chromosomes. Transformation involves the uptake and integration of only small pieces of DNA and, therefore, cannot be used directly as a method of mapping genes that are not very closely linked.

1.2.2 *Transduction, Conjugation and Protoplast Fusion*

Despite a long and exhaustive search, no bacteriophages have been isolated which plaque on *D. radiodurans* (B.E.B. Moseley, unpublished data) and hence no transduction system is available. Attempts to develop a conjugation system in *D. radiodurans*, using *E. coli* and *Streptococcus faecalis* plasmids, have likewise failed (Tirgari, 1977; I.J. Purvis, unpublished data). Recently, an attempt was made to develop a protoplast fusion technique for *D. radiodurans* (G. Al Bakri, personal communication). Preliminary results were promising and cells were successfully protoplasted and regenerated. However, all attempts to fuse *D. radiodurans* protoplasts were unsuccessful (G. Al Bakri, personal communication).

1.2.3 *Restriction and Modification*

Restriction and modification systems in bacteria are responsible for degrading foreign DNA which enters the host cell and for protecting host

DNA from degradation by specific endonucleases (for a review see Szalay et al., 1979). Such systems exist in both Gram-positive and Gram-negative bacteria and restriction endonucleases have been isolated from many strains (Roberts, 1983). Three basic types of restriction endonuclease have been characterised but only type II enzymes have been isolated from members of the genus Deinococcus so only this type will be described.

A type II restriction endonuclease is characterised by its simple co-factor requirements and cleavage specificity. One such enzyme has been purified from D. radiodurans R1 and called MraI (Wani et al., 1982) (named when the organism was still M. radiodurans - see Smith and Nathans, 1973, for restriction endonuclease nomenclature). It was shown to be an isoschizomer of SacII and SstII which both recognize the DNA sequence CCGCGG (see Szalay et al., 1979, for sequence notation). Recently a type II enzyme was isolated from D. radiophilus (Purvis and Moseley, 1983). This enzyme recognizes the sequence TTTAAA and is an isoschizomer of the only other known type II restriction endonuclease that recognizes a sequence composed solely of AT base pairs, AhaIII (Whitehead and Brown, 1982). A second enzyme, DraII, has been isolated from D. radiophilus, the recognition sequence of which is still unknown (I.J. Purvis, personal communication).

The restriction enzymes of D. radiodurans R1 and D. radiophilus have not formally been shown to correspond to any genetically identified restriction and modification system. However, it is generally assumed that any site-specific endonuclease that is inactive upon host DNA and active against exogenous DNA is a restriction endonuclease. The discovery of restriction endonucleases in this genus and its possible implications will be discussed more fully in a later section.

1.3 THE DNA OF DEINOCOCCUS SPP

1.3.1 Base Composition and DNA Homology

The G + C content of members of the genus Deinococcus ranges between 62-68% (Brooks et al., 1980). Moseley and Schein (1964) and Schein (1966)

calculated a G + C content of 66-68% for D. radiodurans. Other workers had reported lower values (Setlow and Duggan, 1964; Alexander et al., 1965) but it is now generally accepted to be 67% (Brooks and Murray, 1981).

Chromatographic procedures (Schein, 1966) and radioactive tracer methods (Schein et al., 1972) failed to detect any unusual or modified bases in the DNA of D. radiodurans. Störl et al. (1979), using immunochemical techniques, confirmed the findings that D. radiodurans contains no 6-methyladenine. These results led to the proposal that MraI represents the first restriction endonuclease isolated from a bacterium known to lack modified bases in its DNA (Wani et al., 1982).

The DNA/DNA homology between Deinococcus spp. is not significant and that between D. radiodurans R1 and Sark strains is somewhat low at 33%.

1.3.2 DNA Complexity

The complexity of the D. radiodurans genome, measured by DNA renaturation kinetics, was calculated to be $2.0 \pm 0.3 \times 10^9$ daltons (Hansen, 1978). The number of genome equivalents per cell was then calculated from the complexity and the amounts of DNA per cell. Hansen (1978) concluded that there were four genome equivalents per resting cell and up to ten in exponentially growing cells. These conclusions were confirmed and extended by Moseley and Evans (1981), who measured the amount of DNA per stainable nucleoid and derived a value of 4.7 ± 0.9 genome complexes per non-replicating stainable nucleoid. Similarly, other members of the genus have been shown to possess multiple genome copies (I.J. Purvis and C.A.M. Duncan, unpublished data). However, the presence of multiple genome copies is not unique to the radiation resistant bacteria, as species of Micrococcus e.g. M. luteus and M. sodonenis, contain multiple genome equivalents (I.J. Purvis and B.E.B. Moseley, unpublished data) and Azotobacter vinelandii, which is radiation-sensitive, (B.E.B. Moseley, personal communication) contains forty chromosomes per cell (Sadoff et al., 1979). It follows that mere possession of such a genome arrangement is not sufficient in itself to cause radiation resistance. Harsojo et al.

(1981) provided further evidence of this. They varied the number of genome equivalents of DNA per D. radiodurans cell from 5.4 to 9.4 by varying the growth medium, and noted that cultures showed differences in their resistance to u.v. and gamma radiation but that differences could not be correlated with the differences in genome equivalents per cell.

1.3.3 Plasmids in Deinococcus spp.

Prior to 1981, little research was undertaken in this area. Tirgari (1977) failed to isolate plasmids from D. radiodurans R1. There are no other reports of attempted plasmid isolation from other members of the genus Deinococcus.

1.3.4 Restriction Analysis of Chromosomal DNA from Deinococcus spp.

There are no reports of studies involving the restriction analysis of chromosomal DNA from Deinococcus spp. This is not surprising as little work has been reported on cleavage patterns of bacterial chromosomal DNA in general.

1.4 SCOPE OF THE THESIS

The aim of this study was primarily to extend our knowledge of the molecular genetics of Deinococcus spp. and to achieve this, several lines were followed.

The first was an examination of members of the genus Deinococcus for the presence of native plasmids and the determination of the role these plasmids play. This involved a number of studies, viz. the screening of all four Deinococcus spp. for plasmids and attempts to cure D. radiodurans Sark and D. radiophilus of their plasmids in order to assign them a phenotypic trait.

The second investigation involved using gene manipulation techniques to develop tools for the study of gene expression in Deinococcus spp.

Foreign plasmid vectors, native plasmids and combinations of foreign DNA and deinococcal DNA were used in attempts to establish a gene cloning system applicable to members of the genus Deinococcus. In addition, genomic libraries of D. radiodurans were constructed as a basis for studying the expression of D. radiodurans genes in the foreign host E. coli.

The final study arose from three independent discoveries: Firstly, Deinococcus spp. were refractory to transformation by foreign plasmids. Secondly, D. radiodurans DNA showed a marked resistance to cleavage by a number of restriction endonucleases. Thirdly, restriction endonucleases were isolated from D. radiodurans R1 and D. radiophilus. Experiments were aimed at determining a common base for these discoveries that would offer explanations for the former two and elucidate the nature of restriction and modification systems in D. radiodurans R1 and D. radiophilus.

2. PLASMIDS

2.1 INTRODUCTION

2.1.1 History

The history of research conducted on plasmids since the early 1950s has been reviewed on several occasions (Meynell, 1972; Falkow, 1975; Novick, 1980). Only a brief outline will be given here.

The term plasmid was introduced by Lederberg (1952), during an analysis of genetic recombination in *E. coli*, to refer to all extra-chromosomal hereditary determinants such as the F-factor. Jacob and Wollman (1958) noted similarities between the F-factor and other elements such as bacteriophage lambda and plasmid ColEI and introduced the term episome for a genetic element that could be replicated either in a chromosomal or extrachromosomal location. These episomes were of great academic interest. However, work in Japan (Ochiai *et al.*, 1959; Akiba *et al.*, 1960; Watanabe 1963) and in Europe (Datta 1962) showed that elements similar to the F-factor were of clinical significance. These elements, R plasmids, were discovered as a result of epidemiologic and genetic studies on the rapid emergence of multiple drug resistance in the *Enterobacteriaceae*. At this time, workers reverted to the more general term plasmid for elements such as F-factor and R plasmids. In 1973, the plasmid pSC101 was successfully used as a cloning vector (Cohen *et al.*, 1973) since when a large number of plasmids have been developed for such a purpose.

The existence of plasmids has, therefore, been known for over three decades. However, the extraordinary prevalence and diversity of these extrachromosomal elements has been appreciated only recently. This realisation has depended on both the application of improved plasmid isolation techniques and the development of transformation methods for particular systems. Both aspects will be discussed later.

2.1.2 Basic Properties

Plasmids are replicons that are stably inherited (i.e. readily maintained without specific selection) in an extrachromosomal state (Novick et al., 1976). The basic properties of plasmids, such as structure, replication and function, have been described in detail (Broda, 1979; Hardy, 1981) and only a brief account will be given here.

The determination of plasmid structure has been greatly aided by the development of physical techniques such as restriction endonuclease mapping, agarose gel electrophoresis and heteroduplex methods. Plasmids have been shown to be covalently-closed, circular (CCC) molecules displaying typical DNA secondary and tertiary structure. Many plasmids have been mapped using restriction endonucleases (e.g. Archer et al., 1981) and the sizing of plasmid molecules is relatively easy (see 2.2.2).

Certain generalisations can be made about plasmid replication. Small plasmids (<30Md) e.g. ColEI (Bazaral and Helinski, 1970) are multicopy and replicate in a relaxed fashion, i.e. independent of protein synthesis for the initiation of replication. On the other hand, large plasmids (>30Md) are generally single copy and replicate under stringent control, i.e. require protein synthesis for initiation of replication and replicate only during chromosome replication. The stable inheritance of plasmids implies that there is an efficient mechanism to ensure that each daughter cell receives at least one copy of the plasmid at cell division. Many plasmid-less cells would arise during bacterial growth if low copy number plasmids were simply distributed at random at cell division. The need for an efficient segregation mechanism is presumably less critical for multicopy plasmids although each daughter cell must receive at least one copy.

Several functions have been associated with plasmids and these traits include antibiotic resistance, antibiotic production, toxin and bacteriocin synthesis, virulence factors, restriction and modification systems and an ever increasing number of metabolic categories. The current view is that plasmids carry optional functions and this is partly held because many plasmids are dispensable (see 2.3) and partly because

of the intuitive feeling that essential functions are best carried by the chromosome. However, this viewpoint may require revision as more plasmids are studied.

2.1.3 Classification

With so many plasmids being isolated it was essential that workable schemes be proposed for their classification. At present, the principal criterion for classifying plasmids is microbiological i.e. if closely related plasmids are introduced into the same cell one or other is eliminated during growth. This is termed 'Incompatibility' (Datta, 1975) and is the basis for incompatibility grouping, e.g. plasmids found in enterobacteria have been classified into over twenty incompatibility groups (Inc. groups), Staphylococcus plasmids into around seven Inc. groups and Pseudomonas plasmids into at least eleven Inc. groups.

A more direct criterion of the relatedness of two plasmids is the similarity of their base sequences. This is tested using molecular hybridisation techniques e.g. hydroxylapatite affinity methods (Brenner et al., 1969), S1 endonuclease digestion procedure (Barth and Grinter, 1975), hybridisation on nitrocellulose filters (Roussel and Chabbert, 1978) and restriction endonuclease methods (Heinaru et al., 1978). Despite the fact that there are instances of incompatible plasmids with insignificant homology (e.g. Willshaw et al., 1978) most reports confirm that incompatible plasmids do have molecular relatedness (Grindlay et al., 1973). Simple hybridisation procedures do not show which parts of two related fragments are homologous. However, electron microscopy of heteroduplexes (Davis et al., 1971) can overcome this problem and indeed has been used to determine the regions of homology of the F-factor and some R plasmids and also areas of homology between the F-factor and the bacterial chromosome (Sharp et al., 1972; Davidson et al., 1975).

2.2 ISOLATION AND SIZING

2.2.1 Isolation

Initially the demonstration of plasmids e.g. F-factor, depended on genetic experiments (Hayes, 1953). However, cryptic and non-conjugative plasmids could not be identified in this way and methods had to be developed for their physical isolation and characterisation. Plasmids were first isolated as linear molecules (Marmur et al., 1961). However, gentler methods for releasing DNA from cells were developed and several plasmids were shown, by electron microscopy, to be circular molecules (Roth and Helinski, 1967).

The numerous methods now in use to isolate plasmids exploit the difference in size between the chromosome and plasmids. Thus, on lysis of cells the plasmids retain their CCC form while the chromosome is fragmented into linear pieces of DNA. The initial concentration of plasmids can be significantly increased in some cases e.g. ColEI, where their replication does not require protein synthesis. Thus, if protein synthesis inhibitors e.g. chloramphenicol, spectinomycin, are added to the logarithmic phase of the host's growth, then chromosome replication is selectively inhibited allowing amplification of the plasmid DNA (Clewell, 1972).

Most plasmid purification procedures have a differential precipitation step, in which the long strands of chromosomal DNA, entangled in the remnants of lysed cells are preferentially removed e.g. by selective precipitation in high salt-SDS (Hirt, 1967) or by selective precipitation in acid-phenol (Zasloff et al., 1978). Many procedures also take advantage of the fact that each strand of the plasmid helix is in the CCC form and the strands cannot be separated by conditions that break most of the hydrogen bonds in the chromosome e.g. exposure to alkali (Currier and Nester, 1976), boiling (Holmes and Quigley, 1981).

Plasmids also behave differently from the chromosome when the two are centrifuged to equilibrium in caesium chloride gradients containing saturating concentrations of a DNA intercalating dye such as ethidium bromide. CCC DNA binds much less dye than linear DNA and therefore

bands at a higher density (Radloff et al., 1967). However, this procedure is expensive in both centrifuge time and materials and as a result other techniques are now being employed e.g. chromatographic procedures (Pakroppa et al., 1975; Colman et al., 1978; Vincent and Goldstein, 1980; Himmel et al., 1982).

2.2.2 Sizing

Two methods were originally used to determine the molecular weights of plasmids:- according to sedimentation velocity in sucrose density gradients (Clowes, 1972) and measurement of the contour length of plasmid molecules, in comparison to standard molecules, in the electron microscope (Lang and Mitani, 1970). However, due to inherent problems with sucrose gradients, i.e. plasmids have to be radioactively labelled and their position can only be detected by fractionating the gradient and measuring the radioactivity in each fraction, and the obvious limitations of electron microscopy, agarose gel electrophoresis is now the favoured method for sizing DNA molecules and fragments. Differences in both the molecular configuration (Thorne, 1966) and the molecular weight (Aaij and Borst, 1972) of DNA molecules can be resolved using agarose gel electrophoresis. Furthermore, the location of DNA within the gel can be determined directly by staining with low concentrations of the fluorescent intercalating dye, ethidium bromide, and detected by examination of the gel in u.v. light (Sharp et al., 1973). The various factors that affect the electrophoretic migration rate of DNA through agarose gels have been discussed by Johnson and Grossman (1977).

Graphical methods can be used for relating DNA size to mobility in agarose gels. However, the relationship between size and mobility can also be represented by a simple equation and the calculation is more accurate and often more convenient than the graphical methods (Southern, 1979).

The sizes of many plasmids have been determined by the methods described, from a 1.5Md plasmid of E. coli (Cozzarelli et al., 1968)

to plasmids of 200Md (Duggleby et al., 1977) and improved plasmid isolation methods have led to even larger plasmids being identified (e.g. Hansen and Olsen, 1978).

2.3 PLASMID LOSS AND CURING

It is often advantageous to be able to eliminate plasmids from a cell; for example, to assign a phenotypic trait to the presence of a plasmid. Probably all bacterial strains carrying non-essential plasmids generate plasmid-negative variants as a result of occasional errors in plasmid replication or segregation. The frequency of such variants in a population can often be increased by certain agents. Novick (1969) defined 'curing' agents as agents which selectively inhibit or inactivate plasmids in replication as opposed to agents which merely select for plasmid-less variants. The result of both processes is the production of plasmid-free cells. Despite the fact that numerous strategies have been employed in the curing of bacterial strains of their plasmids, the phenomenon is poorly reviewed and in many cases the mode of action is unknown. A brief description of curing agents used in this study will be given with occasional references to related agents.

2.3.1 Intercalating Agents

Acridine dyes were shown to be highly effective in curing E. coli of the F-factor (Hirota, 1960). Its elimination appeared to be due to selective interference in the replication of the plasmid (Hohn and Korn, 1969). However, acridines have little, if any, affect on the replication of R plasmids (Watanabe and Ogata, 1966).

Ethidium bromide, another intercalating agent can cure a wide range of bacteria e.g. E. coli and S. aureus, of plasmids other than F-factors i.e. R plasmids (Bouanchaud et al., 1968). The antitumour antibiotic, adriamycin is an intercalating agent that was used to cure E. coli of its F-factor (Pritchard et al., 1978). This antibiotic is similar

to the acridines in that it affects both DNA and RNA synthesis (Gale et al., 1972).

2.3.2 Other Chemical Agents

Rifampicin, an antibiotic that specifically inhibits RNA polymerase has been shown to cure E. coli of the F-factor (Bazzicalupo and Tocchini-Valentini, 1972). Mutants with an altered RNA polymerase, that were resistant to rifampicin were not cured and the authors concluded that curing was caused by an interaction of the drug with the β-subunit of RNA polymerase.

The antibiotic coumermycin inhibits the β-subunit of DNA gyrase and has been used to cure E. coli K12 strains of a number of plasmids (Danilevskaya and Gragerov, 1980). The loss of plasmids was related to the need for DNA gyrase in plasmid replication. The loss of plasmid is not the result of inhibition of plasmid replication per se, but is thought to be due to the accumulation of replication intermediates which then undergo digestion by nucleases (Tomizawa, 1978). Other gyrase β-subunit inhibitors are able to cure bacterial strains of their plasmids e.g. novobiocin cured several bacterial species of their plasmids (McHugh and Swartz, 1977; Taylor and Levine, 1979) and clorobiocin, which closely resembles novobiocin (Glasby, 1979), cured E. coli of several R plasmids (Cějka et al., 1982).

Anionic surface agents, such as deoxycholate and SDS are also known to give rise to plasmid-free bacteria (Sonstein and Baldwin, 1972; Salisbury et al., 1972). Curing E. coli of the F-factor with SDS has been reported (Inuzuka et al., 1969) but the authors were unsure whether SDS acted as a true curing agent. Further research indicated that SDS acted only by selection of spontaneous variants that did not contain the F-factor (Salisbury et al., 1972).

2.3.3 Physical Agents

Growth of plasmid-harbouring organisms at elevated temperatures also eliminates certain plasmids. For example, by growing S. aureus marginally below its maximum temperature for growth, May et al. (1964) showed the elimination of an R. plasmid. This technique has also found

application in curing the genus Rhizobium e.g. R. trifolii (Zurkowski and Lorkiewicz, 1978), R. leguminosarum (Casse et al., 1979). It is thought that the rate of plasmid replication at elevated temperatures is lower than the rate of cell division so that the number of daughter cells not inheriting the plasmid increases.

Gram-positive bacteria may be cured of their plasmids by the technique of protoplast formation and regeneration (Novick et al., 1980; Edger et al., 1981). The loss of plasmids is thought to be due to a partition defect during the several protoplasts divisions that occur prior to completion of cell wall synthesis. Divisions which occur in the absence of a cell wall may result in the disproportioning of the cytoplasmic contents so that some daughter cells inherit a chromosome but not the plasmid.

u.v. radiation has also been used as a curing agent (e.g. Schoenlein and Ely, 1983) but little has been reported on its mode of action.

3. GENE CLONING

3.1 RECOMBINANT DNA

3.1.1 Basic Concept

Recombination, *in vivo*, between DNA molecules plays a central role in the generation of genetic diversity. However, since the early 1970s it has become possible to produce recombinant DNA molecules *in vitro*, and to manipulate them in an increasingly precise fashion. The central theme of recombinant DNA technology is the process of gene cloning in which a defined fragment of DNA is produced and then propagated and amplified in a suitable host cell. This facilitates analysis of its physical organisation, enables expression of a particular gene in its normal host or foreign cell to be studied and allows an analysis of the polypeptide encoded by a particular gene. This technology, although only recently developed, provides a versatile and immensely powerful tool for the analysis of both the organisation of genetic material and control of its expression.

A gene-cloning experiment generally follows a well established sequence of events. First, discrete fragments of DNA are produced and introduced into a host cell via a replicon (e.g. phage or plasmid vector) to ensure its propagation and selective amplification. The next stage is the identification of the particular cell lines that carry the cloned fragment and then the structural and functional characterisation of the cloned DNA. These steps will be considered in more detail.

3.1.2 Restriction and ligation of DNA molecules *in vitro*

Most DNA fragments that are to be cloned are generated by the cleavage of chromosomes or other long DNA molecules. Moreover, these DNA fragments must be inserted at a precise location in the vector so as

not to interfere with the vector's ability to replicate and express a selective function after introduction into a host cell system.

Until fairly recently there was a dearth of enzymes for cleaving DNA specifically. However, within the last decade more than 300 restriction endonucleases have been discovered (Roberts, 1983). DNA fragments produced by Type II restriction endonucleases may have termini with 5' single-stranded extensions, 3' single-stranded extensions or blunt ends. The 5'-or 3'- single-stranded ends are termed cohesive or sticky ends and are complementary to, and can anneal with, any other DNA fragment end that is generated by the same enzyme.

In addition to digesting DNA with enzymes, there are a variety of treatments which result in non-specific breakage e.g. mechanical shearing of DNA by high speed stirring (Wensink et al., 1974). The termini of fragments produced by these methods, however, are either blunt ended or have short, single-stranded extensions, are not mutually cohesive and are not so readily ligated as those produced by Type II restriction enzymes.

There are currently three methods of joining DNA in vitro. The two major methods rely on the ability of DNA ligase to form phosphodiester linkages between adjacent nucleotides in a duplex DNA chain (Olivera et al., 1968). Both E. coli and phage T4 encode the enzyme and although the reactions catalysed by the respective enzymes are similar, they differ in their co-factor requirements. DNA fragments and vector molecules that have identical cohesive termini are readily ligated (Mertz and Davies, 1972) whereas the ligation of DNA fragments lacking single-stranded termini is inefficient, requires high concentrations of DNA ends and enzyme, and can only be joined with phage T4 ligase (Sgaramella et al., 1970). However, a variation of blunt-end ligation has been devised which increases its efficiency and involves ligating synthetic DNA linkers to the vector and/or donor (Rothstein et al., 1979), the linker being a short DNA fragment that contains the recognition sequence for one or more restriction endonucleases. Insertion by means of the linker creates restriction enzyme target

sites at each end of the foreign DNA, and so enables the foreign DNA to be excised and recovered after cloning and amplification in the host bacterium.

The alternative method used to join DNA molecules is homopolymer tailing (Jackson et al., 1972), which involves adding a sufficiently long homopolymer extension to the vector DNA and a complementary extension to the passenger DNA such that they are able to form a stable hydrogen bonded structure. One particular advantage is that circularisation of individual fragments is precluded because the tails of both ends of any particular fragments are identical. Another technique which is often used to prevent intramolecular joining is to remove the 5'- terminal phosphates from the DNA with E. coli or calf intestine phosphatase (Chaconas and Van de Sande, 1980), thereby making it impossible for the DNA ligase to close a nick in the DNA strand (Ullrich et al., 1977).

3.1.3 Vector systems

Four types of vector are generally used, viz. plasmids, bacteriophage lambda, cosmids and single-stranded DNA bacteriophages. Only plasmid and cosmid vectors were used in this study and only their properties will be discussed. For information relating to lambda vectors, see Hendrix et al. (1983) and for single-stranded bacteriophages, Messing et al. (1981).

The desirable properties of plasmid-cloning vectors have been reviewed many times (e.g. Sherratt, 1979) and only a brief summary will be given here. An ideal plasmid vector has low molecular weight, an ability to confer readily-selectable phenotypic traits on host cells and single sites for a large number of restriction endonucleases in regions of the plasmid that are not essential for replication. Cohen et al. (1973) were the first to construct a plasmid vector, pSC101, which was fairly small, 8.7kb, had a selectable gene conferring tetracycline resistance and a single site for EcoRI that was outside regions determining the replication of the vector and tetracycline resistance.

However, pSC101 replicates in a stringent fashion (Timmis *et al.*, 1974), whereas the plasmid ColEI (Hershfield *et al.*, 1974) was found to replicate in a relaxed fashion (Clewell, 1972) and therefore could be amplified to give higher yields of both plasmid DNA and any foreign DNA inserted into it.

Probably the most widely used plasmid vector is pBR322 (Bolivar *et al.*, 1977). A derivative of ColEI, pBR322 fulfils all the requirements of an ideal vector and its physical organisation is well characterized; indeed its complete base sequence has been determined (Sutcliffe, 1979; revised Peden, 1983). A derivative of pBR322, extensively used as a cloning vector, is pAT153, which has a copy number about 1.5 to 3 times that of pBR322 (Twigg and Sherratt, 1980).

To date, most plasmid vectors have been developed for *E. coli*. However, other systems are now being used and have been reviewed recently (Hofschneider and Goebel, 1982). The development of *B. subtilis* vectors resulted from observations that certain small, high copy-number plasmids detected in *S. aureus* could be transformed into *B. subtilis* where the plasmids were stably maintained and expressed the appropriate antibiotic resistance trait. For a review see Ehrlich *et al.* (1982). Hybrid plasmids of *E. coli* and *B. subtilis* vectors have been constructed which can be used in either host. For example, by ligating the plasmid pC194 to pBR322, Ehrlich (1978) constructed the vector pHV14 which can transform both *E. coli* and *B. subtilis*. Plasmid pHV33, a derivative of pHV14, which also encodes tetracycline resistance (Primrose and Ehrlich, 1981), is now widely used as a cloning vector.

Expression vectors, which contain promoter sequences of DNA that are required for the transcription of cloned copies of genes and the translation of their mRNAs in *E. coli* have also been developed e.g. the *trp* promoter (Kleid *et al.*, 1981), the *lac* promotor (Heiland and Gething, 1981) and *trp/lac* (*tac*) promoters (de Boer *et al.*, 1983). These vectors have been used to increase the amount of certain gene products and to facilitate expression of some foreign genes in *E. coli*.

Cosmids are vectors derived from plasmids containing the lambda cohesive (cos) ends (Collins and Brüning, 1978; Collins and Hohn, 1979). Only a small region near the cos site is recognised by the lambda packaging system, both in vivo and in vitro. Thus a length of DNA containing two cos sites in the same orientation separated by 35-53kb can be packaged in vitro and can be transduced into E. coli at high efficiency. Once inside a bacterium, the cosmid circularises, but, because it does not contain all the essential lambda genes, it is not able to go through a lytic cycle and therefore resembles a plasmid vector. Since cosmids need only contain a replication origin, selective markers and cos sites they can be very small, frequently less than 6kb e.g. pJB8 (Ish-Horowicz and Burke, 1981).

3.1.4 Introduction of Recombinant DNA into Host Cells

In most of this work restrictionless mutants of E. coli K12 have been used as recipients since they do not degrade introduced foreign DNA. For the transfer of recombinant DNA into E. coli there are basically two methods. Procedures for the first, transformation-transfection, most commonly used are all derived from the method of Mandel and Higa (1970) in which E. coli was treated with calcium ions to enhance the efficiency of transfection. Procedures for the second, transduction, are based on an in vitro system to package either lambda or cosmid recombinant DNA into infectious lambda phage particles and are derived from observations of Becker and Gold (1975) and Hohn (1975). They involve the preparation of complementary extracts from two E. coli lysogens, each of which is blocked in one step of lambda particle morphogenesis; either nonsense mutations in lambda capsid genes A and E (e.g. strains NS1128 and 433) or mutations in lambda genes D and E (e.g. strains BHB2690 and 2688). An extract from the mutant E strains lacks the major capsid protein E but contains all other head protein. This extract can therefore complement the second extract which contains empty precursor particles to complete the lambda morphogenesis.

3.2 GENOMIC LIBRARIES

3.2.1 Cloning of Gene Libraries

The construction of gene libraries involves the generation of a collection of cloned DNA fragments which comprise the entire genome of the organism and the strategy for this construction is merely an extension of the protocols described in 3.1. The number of clones required to contain all the genome depends on both the size of the donor genome and the average size of the DNA fragments cloned (Clarke and Carbon, 1976). The DNA fragments can be created in a number of ways. However, there is no guarantee that every sequence in a genome will be represented in a library and strategies have been devised to increase the probability that any sequence will be represented (for a review see Dahl *et al.*, 1981).

Plasmids have been used successfully for constructing gene libraries from both prokaryotes e.g. *E. coli* (Clarke and Carbon, 1976) and eukaryotes e.g. *Xenopus laevis* (Smith *et al.*, 1979). However, plasmid vectors are limited in that the average insert size is only 5kb which means that the number of clones required to comprise the genome can be impractically high. On the other hand, cosmid vectors have the advantage that much larger DNA inserts, 30-45kb, can be cloned. Cosmids have been used to construct gene libraries from *E. coli* (Collins and Brüning, 1978) and humans (Grosveld *et al.*, 1982).

3.2.2 Screening Clones

Procedures for screening a population of recombinant bacterial clones for the ones containing a foreign DNA sequence of interest fall into two classes: direct selection of plasmids or cosmids carrying fragments that give a selectable phenotype to the host, and indirect selection by detecting which recombinant recipient contains nucleotide sequences or gene products of the fragments of interest.

The direct selection by phenotype method is based on the suppression or complementation of a mutation, *in vivo*, by transformation with foreign DNA. The requirements for this are that the cloned gene can be transcribed and expressed in the host, e.g. Nagahari *et al.* (1980).

A range of screening procedures has been developed to detect the recombinant of interest by indirect selection e.g. nucleic acid hybridisation, hybrid-arrested and hybrid-selected translation and immunochemical techniques (for a review see Dahl et al., 1981). These approaches do not require that a gene be expressed only that the particular DNA sequence of interest is present. The detection and expression of cloned genes when the function of the gene is not known presents additional problems. Two approaches have been devised, the mini-cell (see Christen et al., 1983) and maxi-cell techniques (Sancar et al., 1979) which allow the preferential radioactive labelling of plasmid encoded proteins, therefore enabling the identification of protein produced by recombinant molecules.

4. RESTRICTION ANALYSIS OF CHROMOSOMAL DNA

4.1 PREVIOUS STUDIES

When bacterial DNA is cleaved by a type II restriction endonuclease and run on an agarose gel, a distinct banding pattern is obtained. These patterns have been used for several years to characterize closely related organisms e.g. strains of Trypanosoma cruzi (Mattei et al., 1977) Rhizobium spp. (Mielenz et al., 1979) and Lactobacillus spp. (Manachini and Parini, 1983). Recently, the analysis of restriction patterns has found application in the investigation of DNA modification profiles and although only a few reports have been published, they give an insight into the relationship between modification of bacterial DNA and the production of restriction endonucleases. For example, Norlander et al. (1981) observed that both chromosomes and plasmids from different gonococcal strains were poorly cleaved by the restriction enzymes HaeII, HaeIII and SacII which are isoschizomers of restriction enzymes produced by Neisseria gonorrhoeae, NgoI, NgoII and NgoIII respectively (Clanton et al., 1979). They concluded that resistance to cleavage was due to the hosts' restriction and modification systems. In addition, it was thought that the poor cleavage of gonococcal DNA by BamHI also reflected modification and the presence of a BamHI isoschizomer in some gonococcal strains was postulated.

Van den Hondel et al. (1983) observed that the cyanobacterium Fremyella diplosiphon exhibited a peculiar resistance to a number of restriction endonucleases. In an attempt to elucidate the underlying modification pattern of F. diplosiphon DNA they isolated two endogenous restriction endonucleases, FdiI (GGA/TCC) and FdiII (TGCGCA). The modification profile of the chromosomal DNA was thought to account for its resistance to cleavage by some restriction enzymes e.g. AosI and AvaII. In addition, they showed that the GATC sequence of the DNA was

methylated at the adenine base and the inactivity of some other enzymes e.g. MboI was attributed to this. However, a clear picture of the nature of F. diplosiphon DNA modification did not emerge from the studies as the reasons for lack of cleavage by other enzymes e.g. SstI, PvuII and SalI, could not be explained from the data available.

4.2 DETECTION OF METHYLATED BASES IN DNA

4.2.1 Restriction Endonucleases

Site specific nucleases that are unable to cleave DNA at specific methylated sequences are useful tools for studying the methylation pattern of DNA (Bird and Southern, 1978) while isoschizomers that differ in their sensitivity to methylation are particularly useful. One such pair, HpaII and MspI (Waalwijk and Flavell, 1978), have been widely used to probe the methylation status of the sequence CCGG (e.g. Sneider, 1980). The rationale is that HpaII will cleave the site when the external cytosine residue, but not the internal one, is methylated and vice versa for MspI.

Other isoschizomers that cleave at identical sequences but differ in their sensitivity to methylation are DpnI, DpnII and MboI which recognize the sequence GATC. DpnI will cleave only DNA that is methylated at the adenine residue (Lacks and Greenberg, 1975), whereas, cleavage at this sequence by DpnII and MboI is inhibited by such methylation (Lacks and Greenberg, 1977). The first isoschizomer found to be insensitive to adenine methylation was Sau3A (Sussenbach et al., 1976) and a similar property was found for FnuEI (Lui et al., 1979) and PfaI (Roberts, 1980).

A pair of isoschizomers recognizing a hexanucleotide sequence but differing in their cleavage patterns depending on specific methylation has also been reported (Youssoufian and Mulder, 1981). XmaI cleaves DNA whether or not the 3'-cytosine residue of its recognition sequence, CCCGGG, is methylated. This is in contrast to SmaI which does not cleave DNA if this cytosine residue is methylated.

4.2.2 Chromatographic Methods

Gruenbaum *et al.* (1981) developed a thin layer chromatography method which was a modification of the standard nearest-neighbour analysis technique (Josse and Swartz, 1963), to measure the extent of cytosine methylation in eukaryotic DNA. Other workers have extended the use of this technique to measure methylated adenine and guanine (Deobagkar *et al.*, 1982). High performance liquid chromatography (HPLC) has also been used to detect methylated bases in DNA (Deobagkar *et al.*, 1982).

CHAPTER 2

MATERIALS AND METHODS

BACTERIAL STRAINS

Strains of Deinococcus spp. used are listed in Table 2.1. Other bacteria used are listed in Table 2.2.

PLASMIDS AND COSMIDS

Plasmids and cosmids used during the course of this study are listed in Table 2.3. Some of them were transferred to hosts other than those in which they were received.

MAINTENANCE OF CULTURES

Strains of Deinococcus other than D. radiophilus were maintained on TGY agar. D. radiophilus, E. coli strains and B. subtilis strains were stored on nutrient agar. Genetically marked strains and those carrying plasmids were routinely subcultured onto supplemented media for characterization of the phenotype. Liquid cultures were grown in 2ml volumes in 5ml bottles, 20ml volumes in 250ml Erlenmeyer flasks or 1l volumes in 2l flasks on an orbital shaker incubator. Deinococcus strains were incubated at 30°C, E. coli and B. subtilis strains at 37°C.

MEDIA

The following media formulations were used:

(1) TGY medium (Anderson et al., 1956) for the growth of D. radiodurans R1, D. radiodurans Sark, D. proteolyticus and D. radiopugnans.

	gl^{-1}
Bactotryptone (Difco)	5
D-Glucose	1
Yeast Extract	3

TABLE 2.1

STRAINS OF *DEINOCOCCUS* USED

STRAIN	SOURCE	REFERENCE
D. radiodurans R1	Dr. B.E.B. Moseley(3)	Anderson *et al.*, 1956
D. radiodurans Krase(1)	"	Tirgari and Moseley, 1980
D. radiodurans Sark	Prof. R.G.E. Murray(4)	Murray and Robinow, 1958
D. radiodurans (Rf^r)(2)	Ms. S. Whyte(3)	
D. radiopugnans	Dr. B.E.B. Moseley	Davis *et al.*, 1963
D. radiophilus	"	Lewis, 1971
D. proteolyticus	"	Kobatake *et al.*, 1973

(1) Mutant strain of *D. radiodurans* R1 resistant to 30μgml^{-1} kanamycin, >100μgml^{-1} rifampicin, 5μgml^{-1} acriflavine, 200μgml^{-1} streptomycin and 25μgml^{-1} erythromycin.

(2) Mutant strain of *D. radiodurans* Sark resistant to 100μgml^{-1} rifampicin.

(3) Dept. of Microbiology, University of Edinburgh.

(4) Dept. of Microbiology and Immunology, University of Western Ontario.

TABLE 2.2

OTHER SPECIES OF BACTERIA USED

STRAIN	SOURCE
E. coli HB101	Dr. B.E.B. Moseley
E. coli BHB 2688	Dr. P. Whittaker (1)
E. coli BHB 2690	Dr. P. Whittaker
B. subtilis 168	Dr. I.W. Dawes (2)

(1) M.R.C. Mammalian Genome Unit, Kings Buildings, Edinburgh

(2) Dept. of Microbiology, University of Edinburgh.

TABLE 2.3

PLASMIDS AND COSMID USED

PLASMID	HOST	SELECTABLE MARKER (1)	SOURCE
pML2	*E. coli* HB101	Kn	Mr. G. Coupland (2)
pBR322	*E. coli* HB101	Ap, Tc	"
pAT153	*E. coli* HB101	Ap, Tc	"
pC194	*B. subtilis* HVS62	Cm	Dr. S.D. Ehrlich (3)
pUB110	*B. subtilis* HVS89	Kn	"
pHV33	*E. coli* HVC181	Ap, Cm, Tc	"
Cosmid pJBFH	*E. coli* HB101	Ap	Dr. P Whittaker

(1) Abbreviations: Kn - Kanamycin; Ap - Ampicillin;
Tc - Tetracycline; Cm - Chloramphenicol

(2) Dept. of Molecular Biology, University of Edinburgh.

(3) Institut de Recherche en Biologie Moleculaire, Paris.

(2) Nutrient broth for the growth of D. radiophilus.

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

(3) Luria broth (L-broth) (Lennox, 1955) for the growth of B. subtilis and E. coli.

	gl^{-1}
Bactotryptone	10
Yeast Extract	5
NaCl	5
D-Glucose	1

For in vitro packaging, E. coli was grown up in L-broth +0.4% (w/v) maltose.

(4) Nutrient broth for the growth of E. coli.

	gl^{-1}
Nutrient Broth (Oxoid)	13

(5) M9 salts (x10 concentrate) for E. coli minimal media.

	gl^{-1}
$Na_2H\ PO_4.H_2O$	6
$KH_2\ PO_4$	3
NaCl	0.5
NH_4Cl	1

Dissolved in order indicated and autoclaved.

(6) M9 minimal media.

M9 salts	100ml
20%w/v D-Glucose	20ml
0.1M $MgSO_4$	10ml
0.01M $CaCl_2$	10ml
Sterile distilled water	to 1000ml

Each solution autoclaved separately and components mixed aseptically before use.

(7) Penassay broth.

	gl^{-1}
Bacto-Antibiotic Medium No. 3 (Difco)	17.5

(8) SMMP medium.

4x strength Penassay broth

2x strength SMM buffer

Autoclaved separately and equal volumes added.

(9) DM3 Regeneration media.

Sterile solutions l^{-1}

200ml	4% Agar
500ml	1M Sodium succinate
100ml	5% casamino acids (Difco)
50ml	10% Yeast extract
100ml	3.5% $K_2H\ PO_4$/1.5% KH_2PO_4
25ml	25% D-Glucose
20ml	1M $MgCl_2$
5ml	Bovine Serum Albumin

Media were solidified as required with $15gl^{-1}$ Agar No. 1 (Oxoid). Sterilisation of media was by autoclaving at 15lb in^{-2} for 15 minutes.

BUFFERS

(1) Phosphate buffer, 0.067M, pH 7.0

	gl^{-1}
KH_2PO_4	4.56
$Na_2HPO_4 2H_2O$	4.73

(2) Butanol saturated phosphate/EDTA buffer

	gl^{-1}
KH_2PO_4	4.56
$Na_2HPO_4 2H_2O$	4.73
EDTA	0.34
n-butanol	6%

(3) Standard Saline Citrate (SSC), pH 7.0

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

(4) TE buffer, pH 7.4

	gl^{-1}
0.01M Tris	1.2
0.001M EDTA	0.4

(5) TES buffer, pH 8.0

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

(6) SMM buffer, pH 6.5

	gl^{-1}
0.5M Sucrose	171.0
0.02M Maleate	2.3
0.02M $MgCl_2$	4.0

(7) Nick Translation buffer (NT), pH 7.4
made up as 10x solution;

	gl^{-1}
0.5M Potassium phosphate	114.1
10mM 2-mercaptoethanol	0.78
67mM $MgCl_2$	13.6

(8) Nearest Neighbour Analysis buffer (NNA) pH 8.3
made up as 10x solution;

	gl^{-1}
1M Tris	121.1
0.1M $CaCl_2$	11.1

CHEMICALS

Sodium dodecyl sulphate (SDS), ethylene diamine tetra-acetic acid-disodium salt (EDTA), hydroxymethyl methylamine (Tris), Tris-hydrochloride, caesium chloride, polyethylene glycol-6000 (PEG), bromophenol blue and ethidium bromide were obtained from BDH Chemicals Ltd., England; Ficoll, agarose Type 1 (low EEO), adenosine 5'-triphosphate, 2'-deoxyadenosine 5'-monophosphate, 2'-deoxycytidine 5'-monophosphate, 2' deoxyguanosine 5'-monophosphate and 2' deoxythymidine 5'-monophosphate from Sigma Chemical Co. Ltd., London; dithiothreitol (DTT) and ultra pure phenol from BRL Bethesda Research Laboratories (UK) Ltd., Cambridge and Sephadex G-75 from Pharmacia (GB) Ltd., Middlesex.

ANTIBIOTICS AND NUTRITIONAL SUPPLEMENTS

The antibiotics ampicillin, chloramphenicol kanamycin, rifampicin, tetracycline and the nutritional supplements leucine, proline and thiamine were obtained from Sigma.

RADIOACTIVELY-LABELLED COMPOUNDS

[α-^{32}P] labelled deoxyadenosine 5'-triphosphate, deoxycytosine 5'-triphosphate, deoxyguanosine 5'-triphosphate and deoxythymidine 5'-triphosphate were either obtained from Amersham International p.l.c., England or were gifts from Dr. Edwin Southern, MRC Mammalian Genome Unit, Edinburgh.

ENZYMES

T_4 DNA ligase (E.C. 6.5.1.1.) and calf intestine alkaline phosphatase (lyophilised) (E.C. 3.1.3.1.) were purchased from Boehringer Mannheim, Lewes, England. Pancreatic RNase (E.C. 3.1.4.22), pancreatic DNase (E.C. 3.1.4.5.), bovine spleen phosphodiesterase (3.1.4.18), micrococcal nuclease (E.C. 3.1.31.1) and lysozyme from Sigma. E. coli DNA polymerase I (E.C. 2.7.7.7) from Cambridge Biotechnology, England. Restriction endonucleases were bought from several sources:- New England Biolabs, Bishops Stortford, England; Boehringer Mannheim, Miles Laboratories, Stoke Poges, England; NBL enzymes Ltd., Cramlington, England; Bethesda Research Laboratories or were gifts from Dr. Edwin Southern, MRC Mammalian Genome Unit, Edinburgh. Restriction endonucleases were used in accordance with the manufacturers' instructions.

MEASUREMENT OF BACTERIAL GROWTH

Growth rates were determined by following changes in the turbidities of cultures, measured in a nephelometer (Evans Electroselenium Ltd., Halstead) with an orange filter.

GENETIC TRANSFORMATION

(a) Transformation of D.radiodurans with chromosomal DNA.

The transformation procedure used was essentially that of Tirgari and Moseley, (1980). One ml of an overnight culture of D. radiodurans in TGY medium was diluted into 20ml of prewarmed TGY broth in a 250ml conical flask and incubated with aeration at 30°C to give an exponential culture with a turbidity reading of 30 (~1 x 10^8 viable units ml^{-1}). Ten ml of the culture was centrifuged at 12,000g for 5 min, resuspended in 5ml of prewarmed TGY broth and 2ml of 0.1M $CaCl_2$ solution added. Samples of this culture (1ml) were then added to 10µg of transforming DNA and placed on ice for 10 min. The mixtures were shaken gently in a water bath at 30°C for 90 min, 9ml volumes of TGY broth added to the mixtures and the cultures shaken at 30°C for 4 hr. to allow for phenotypic expression of the transformed marker. Appropriate dilutions (0.1ml) were inoculated onto TGY plates and incubated at 30°C for 3-4 days.

(b) Transformation of D. radiodurans by plasmid DNA.
A similar method to the chromosomal transformation procedure was employed. In general, 5µg of plasmid DNA was used in the transformation mixture and phenotypic expression was allowed over 24 hr. Plates were incubated at 30°C for 7-10 days. In addition, the transformation procedure of Humphreys et al., (1978) which is used to transform E. coli with plasmid DNA was tested in a limited number of experiments.

(c) Transformation of B. subtilis by plasmid DNA.
The 'Polyethyleneglycol-Induced Transformation of B. subtilis Protoplasts' method (PIP transformation) as developed by Chang and Cohen (1979) was used. Twenty ml of a mid exponential-phase cell culture of B. subtilis 168 freshly grown in Penassay broth at 37°C to 1-2 x 10^8 viable units ml^{-1} was harvested and resuspended in 2ml of SMMP solution, lysozyme was added to $2mgml^{-1}$ final concentration and the suspension incubated at 37°C for 2 hr. with gentle shaking. Cells were centrifuged at 2,600g for 15 min. and washed once by resuspending them gently in 2ml SMMP and centrifuged a second time. The pellet was resuspended in 2ml

SMMP. A sample of the protoplast suspension (0.5ml) and 0.1ml of 2 x SMM were added to 5µg of plasmid preparation followed immediately by the addition of 1.5ml 40%~~W~~PEG (40g PEG 6000, 50ml 2 x SMM buffer in 100ml). After 2 min., 5ml of SMMP was added and centrifuged at 2,600g for 10 min. The pellet was resuspended in 1ml SMMP and incubated at 30°C for 1.5 hr. with gentle shaking to allow phenotypic expression, before plating directly onto DM3 regeneration media plus the relevant antibiotic. Scoring for transformants was done after incubation of protoplasts on regeneration medium plates at 37°C for 2 days.

(d) Transformation of E. coli by plasmid DNA

E. coli HB101 (hsd, recA, thi, pro, leu) was used as a recipient in transformation procedures. Four methods of transformation were tested. The first technique was developed by Humphreys et al. (1978). E. coli HB101 was grown without shaking in L-broth overnight at 37°C and the culture diluted 1:20 with fresh pre-warmed broth. It was grown for 95 min. (turbidity reading 30) at 37°C with shaking. The recipient cells from 12ml of culture were harvested by centrifugation in a microcentrifuge (Hettich Mikroliter). The cells were resuspended and washed once in 6ml of 10mM $CaCl_2$ at 0°C. The cells were then resuspended in 0.6ml 75mM $CaCl_2$, 10mM MOPS, 0.5% glucose, pH6.5 and 0.2ml were transformed in 75mM $CaCl_2$, 10mM MOPS, 0.5% glucose, pH6.5 in a final volume of 0.5ml, with a DNA concentration of 2µgml^{-1}, for 45 min. at 0°C. The transformation mixture was then transferred to 42°C for 10 min. and 0.5ml L-broth added before incubating at 37°C for 2 hr. with shaking.

The method of Davis et al. (1980) involved diluting an overnight L-broth culture of E. coli HB101 1:100 into fresh L-broth. The cells were collected at a turbidity reading of 60. Then, 6ml of the recipient culture were harvested in a microcentrifuge and resuspended in 3ml of 50mM $CaCl_2$ at 0°C for 45 min. The cells were sedimented and resuspended in 0.3ml of 50mM $CaCl_2$ at 0°C for 45 min. Plasmid DNA (1µg in 0.1ml, 0.1MTris, pH7.2) was added to 0.1ml cells and

placed at 0°C for 10 min. The transformation mixture was then heat treated at 37°C for 2 min. before adding 1ml L-broth and incubating at 37°C for 2 hr with shaking. A modification of this method was tested using 50mM $CaCl_2$, 10mM Tris pH8.0 instead of 50mM $CaCl_2$.

The method of Maniatis et al. (1982) also involved diluting an overnight culture of E. coli HB101 1:100 into fresh L-broth and collecting the cells at a turbidity reading of 60. The culture was chilled on ice for 10 min. before harvesting 9ml by centrifugation. The cells were then resuspended in 4.5ml of 50mM $CaCl_2$, 10mM Tris, pH8.0 and the suspension placed on ice for 15 min. After collecting the cells by centrifugation they were resuspended in 0.6ml of 50mM $CaCl_2$, 10mM Tris, pH8.0 and 0.2ml aliquots were dispensed into pre-chilled tubes. The cells were stored at 4°C for 20 hr. Plasmid DNA (1µg in 0.1ml, 0.1M Tris, pH7.2) was added to the 0.2ml aliquot and placed in a waterbath at 42°C for 2 min. After this heat treatment 1ml of L-broth was added and incubated at 37°C for 2 hr. with shaking. In all four cases 0.1ml of an appropriate dilution of the transformation mixture was placed onto L-plates plus antibiotic. Plates were incubated at 37°C for 20-40 hr.

ISOLATION OF CHROMOSOMAL DNA

Chromosomal DNA was isolated from members of the genus Deinococcus by a modification of Marmur's method (1961). Two litres of a 40 hr. cell culture were centrifuged, the pellet washed in 50ml SSC and resuspended in 40ml butanol saturated phosphate/EDTA buffer. This treatment renders the cell wall sensitive to lysozyme degradation (Driedger and Grayston, 1970). This suspension was left at ambient temperature for 45 min. before collecting the cells and resuspending them in 40ml SSC. Lysozyme was added to 2.0mgml^{-1} and the mixture was incubated at 37°C until a 0.5ml aliquot lysed on the addition of 0.05ml of 20% SDS in water. The bacteria were lysed by the addition of 20% SDS to a final concentration of 2%. After swirling the flask to ensure complete lysis, 13ml of a sodium perchlorate solution

(70.25g $NaClO_4.H_2O$ and 4.4g NaCl in 100ml distilled water) and an equal volume of chloroform: isoamyl alcohol mixture (24:1) were added for deproteinisation. The mixture was shaken by hand for 30 min. before centrifuging at 30,000g for 20 min. The mixture separated into two layers with an interface protein layer. The aqueous top layer contained DNA and RNA. The top layer was removed carefully to avoid shearing and the nucleic acids were precipitated by pouring the aqueous component carefully into two volumes of absolute alcohol. The nucleic acids were wound onto clean glass rods to dry at ambient temperature before resuspending them in a minimum volume of SSC. Further purification entailed adding preboiled pancreatic RNase to a final concentration of $1mgml^{-1}$ and incubating at $37^{o}C$ for 1hr. An equal volume of chloroform: isoamyl alcohol (24:1) was added and the mixture shaken for 10 min. It was then centrifuged at 30,000g for 30 min., the aqueous layer removed and the DNA precipitated as before. The DNA was dissolved in 2 to 4ml of TE buffer and the concentration measured spectrophotometrically. After a final ethanol precipitation the DNA was dissolved in TE buffer to give a concentration of $1mgml^{-1}$. The preparation was stored at $4^{o}C$ for use.

ISOLATION OF PLASMID DNA

(a) From members of the genus Deinococcus

The protocol employed initially in this study was that of Hansen and Olsen (1978). This method, which in part is a synthesis of three extant plasmid isolation procedures (Guerry et al., 1973; Humphreys et al., 1975; Currier and Nester, 1976), was designed for the isolation of large bacterial plasmids.

Cells were grown in one litre of culture and harvested at ambient temperature. The cells were resuspended in 50ml butanol-saturated phosphate/EDTA buffer, incubated for 30 min. at ambient temperature, collected and washed once in phosphate buffer. The washed pellet was resuspended in 33.5ml 25% sucrose/0.05M Tris, pH8.0 and 2.5ml of a fresh lysozyme solution ($10mgml^{-1}$ in 0.25M Tris, pH8.0) added. The

suspension was mixed gently and placed on ice for 5 min. before adding 12.5ml 20% SDS (in TE buffer). This was followed by 8 cycles of heat pulse which each comprised of 15 sec at 55°C then 5 inversions at ambient temperature during a further 15 sec. After this heat pulse, 12.5ml of freshly prepared 3N NaOH was added which raised the pH to 12.1-12.3. The pH was lowered to 8.5-9.0 by adding drops of Tris-saturated concentrated hydrochloric acid. Then 16.25ml of 20% SDS and 31.25ml 5M NaCl were added simultaneously. After mixing gently the suspension was centrifuged at 18,000g for 40 min. at 4°C. The volume of supernatant was measured and precipitated by adding 0.25 volumes 50% PEG 6000. This mixture was incubated overnight at 4°C and centrifuged at 18,000g for 5 min. The precipitate was dissolved in 6ml TES buffer and inverted gently. Proteins were precipitated by adding 6ml Tris-saturated phenol and mixed before centrifugation at 18,000g for 10 min. Twice the volume of cold 95% ethanol was added to precipitate the DNA. After overnight incubation at -20°C the precipitate was collected by centrifugation and dissolved in 10ml TES buffer. Plasmid DNA was purified by the dye-buoyant density gradient centrifugation technique (Radloff *et al.*, 1967).

Rapid screening of members of the genus *Deinococcus* for the presence of plasmid DNA was performed using a modification of the Birnboim and Doly (1979) technique. The principle of this method is selective alkaline denaturation of high molecular weight chromosomal DNA while covalently closed, circular DNA remains double stranded. Upon neutralisation, chromosomal DNA renatures to form an insoluble clot, leaving plasmid DNA in the supernatant.

Liquid cultures were incubated for 24 hr. at 30°C with shaking and 1.5ml of culture transferred to a microcentrifuge tube for plasmid extraction. The tube was centrifuged in a microcentrifuge for 10 sec. The supernatant was decanted and the tube allowed to drain inverted for 1 min. The pellet was resuspended in 300µl butanol saturated phosphate/EDTA buffer and left at ambient temperature for 30 min.

This suspension was centrifuged and the supernatant throroughly drained before resuspending the pellet in 100µl lysozyme solution (lysozyme 2mgml^{-1}, 50mM glucose, 10mM EDTA, 25mM Tris-HCl, pH8.0). The cell pellet was thoroughly suspended and after a 30 min. period of incubation at 0^{o}C, 200µl of 0.2N NaOH, 1% SDS was added. The tube was inverted gently and maintained at 0^{o}C for a further 5 min. The suspension became almost clear and slightly viscous at this stage. After 5 min. 150µl of 3M sodium acetate (pH4.8) was added. The contents of the tube were gently mixed by inversion for a few sec. during which time a clot formed. The tube was maintained at 0^{o}C for 60 min. which allowed most of the protein, high molecular weight RNA and chromosomal DNA to precipitate. Centrifugation for 5 min. at 4^{o}C yielded a slightly clouded supernatant. The DNA was precipitated by adding 1ml of cold ethanol to the supernatant and holding at -20^{o}C for 30 min. The precipitate was collected by centrifugation at 4^{o}C for 3 min. and the pellet allowed to dry before resuspending in 100µl of 0.1M sodium acetate (pH6.0). The DNA was precipitated by adding 200µl cold ethanol and holding at -20^{o}C for 10 min. The precipitate was again collected by centrifugation and the pellet dissolved in 40µl TE buffer. Loading dye was added to the preparation before applying to an agarose gel for electrophoretic analysis.

Large scale preparations of plasmid DNA from members of this genus were performed using a scale-up modification of the previous procedure.

Two litres of an overnight broth culture were harvested and resuspended in 25ml butanol-saturated phosphate/EDTA buffer. After incubation at ambient temperature for 30 min. the suspension was centrifuged and the pellet left to drain for 2 min. The cells were resuspended in 30ml lysis solution. Then 10ml of 8mgml^{-1} lysozyme in lysis solution was added and mixed gently. After 30 min, at 0^{o}C, 80ml of 2N NaOH, 1% SDS was added and mixed gently. After 5 min. incubation at 0^{o}C, 60ml of 3M sodium acetate (pH4.8) was added, mixed by inversion and held at 0^{o}C for 60 min. Centrifugation for 10 min. at 16,000g in a Sorvall GSA rotor yielded a cloudy supernatant which when decanted through a plastic

tea-strainer became almost clear. The DNA was ethanol precipitated and the precipitate collected by centrifugation at 4,000g for 15 min. The pellet was dissolved in 25ml 0.1M sodium acetate pH6.0 and once again ethanol precipitated. After 20 min. at -20°C the precipitate was again collected by centrifugation before dissolving in 10ml TE buffer. Plasmid DNA was purified by the dye-buoyant density gradient centrifugation technique (Radloff et al., 1967), which will be described later.

(b) From B. subtilis

The S. aureus plasmids pC194 (Ehrlich, 1977) and pUB110 (Gryczan et al., 1978), which were used as cloning vectors in B. subtilis, were isolated from B. subtilis by Niaudet and Ehrlich's (1979), modification of the Gryczan et al. (1978) procedure.

Cells were collected from 250ml of an overnight culture in L-broth washed with 100ml of a buffer which contained 0.1M NaCl, 0.05M Tris pH7.4 and 0.001M EDTA and resuspended in 10ml of 25% sucrose, 0.1M NaCl and 0.05M Tris pH7.4. Fresh lysozyme (0.25ml of a $20mgml^{-1}$ solution in water) was added and the mixture incubated at 37°C for 20 min. The following solutions were then added in order:- 2.4ml of 5M NaCl, 0.6ml of 0.5M EDTA pH8.0 and 12.5ml of freshly prepared 2% SDS-0.7M NaCl. This suspension was gently but thoroughly mixed and left overnight at 4°C. The lysate was centrifuged for 30 min. at 38,000g in a Sorvall SS-34 rotor, the supernate collected and adjusted to 1M by adding 5M NaCl. One third volume of 40% polyethylene glycol 6000 (PEG) was then added to precipitate the DNA, and the suspension incubated for 1 hr. at 0°C. The precipitate was collected by centrifugation and dissolved in 5ml TE buffer. Plasmid DNA was purified by density gradient centrifugation.

(c) From E. coli

The Ish-Horowicz and Burke (1981) procedure for preparative extractions of E. coli plasmid DNA was employed. Cells were grown in 1l of L-broth

plus the relevant selective antibiotic. Chloramphenicol amplification was optional and if used, the cells were grown to a turbidity of 100 then chloramphenicol, 150μgml^{-1} final concentration, added and the culture shaken at 37^{o}C overnight. Cells were harvested by centrifugation and allowed to drain upside down for 2 min. before suspending the pellet in 36ml of 50mM glucose, 25mM Tris pH8.0, 10mM EDTA. Fresh lysozyme was added, 4ml of a 5mgml^{-1} solution in 50mM glucose, 25mM Tris pH8.0, 10mM EDTA or 20mgml^{-1} lysozyme solution if chloramphenicol amplification was used. This suspension was incubated for 10 min. at ambient temperature before adding 80ml 0.2N NaOH, 1% SDS (fresh) and placing at 0^{o}C for 5 min. Addition of 5M potassium acetate pH4.8 caused an insoluble clump to form and this mixture was incubated at 0^{o}C for 30 min. Distilled water, 10ml, was added and the lysate centrifuged at 10,000g in a Sorvall GSA rotor. The supernatant was filtered through a plastic tea-strainer to remove floating lumps and 0.6 volumes of cold propan-2-ol added. After incubation at -20^{o}C for 30 min. the precipitate was collected by centrifugation, the supernatant drained off and the pellet dried in an evacuated dessicator for 1 min. The pellet was resuspended in 9ml TE buffer plus 0.6ml 0.2M EDTA and neutralised with 2M Tris base. Plasmid DNA was purified by density gradient centrifugation.

The rapid boiling method (Holmes and Quigley, 1981) was used to screen small cultures for recombinant DNA inserts and prepare recombinant plasmids from E. coli for restriction analysis. Cells were collected from 1.5ml of an overnight culture in L-broth by centrifugation for 15 sec. in a microcentrifuge. The pellet was resuspended in 350μl of 0.8% sucrose, 5% Triton-X 100, 50mM Tris pH8.0 (STET buffer) and 25μl of a 10mgml^{-1} solution of freshly prepared lysozyme was added. The solution was placed in a boiling water bath for 40 sec. and centrifuged immediately at 4^{o}C for 15 min. in a micro-centrifuge. The gelatinous pellet was removed with a toothpick and the supernatant precipitated by adding 40μl of 4M sodium acetate and 400μl of cold propan-2-ol. After 5 min. at ambient temperature the

precipitate was collected by centrifugation and the pellet washed with 70% ethanol to remove residual propan-2-ol. The pellet was dried *in vacuo* and resuspended in 40µl distilled water. A sample of this preparation was used directly for electrophoretic analysis or for restriction enzyme digestion. At the end of the restriction enzyme digestion preboiled pancreatic RNase was added to a final concentration of $20\mu g ml^{-1}$ and the reaction continued for a further 2 min. at $37^{o}C$. Loading dye was added to the preparation before applying to an agarose gel for electrophoretic analysis.

DYE-BUOYANT DENSITY CENTRIFUGATION

Ethanol or propan-2-ol precipitated lysates from the genus *Deinococcus*, *B. subtilis* and *E. coli* were purified for covalently closed circular plasmid DNA by the dye-buoyant density gradient centrifugation technique (Radloff *et al.*, 1967). Gradients were formed by adding 11g of caesium chloride and 1ml of an ethidium bromide solution ($10mg ml^{-1}$ in TE buffer) to 10ml of cleared lysate. The refractive index was adjusted, using an Abbe 60 Refractometer (Bellingham and Stanley Ltd., England), to 1.3925 (density $1.625 g cm^{-3}$) by adding caesium chloride. The mixture was then transferred to two 10ml polypropylene tubes (MSE) and centrifuged at 130,000g for 60 hr. at $18^{o}C$ in a Prepspin 65 MSE ultracentrifuge.

Two bands formed in the middle of the gradients. The lower CCC plasmid DNA band was removed carefully using a 1ml syringe with a 19 gauge needle inserted through the side of the tube and extracted with caesium chloride saturated propan-2-ol to remove ethidium bromide. The preparation was then dialysed against four changes of cold TE buffer. The DNA was finally ethanol precipitated and resuspended in TE buffer before storing at $4^{o}C$.

DETERMINATION OF DNA CONCENTRATION

The concentrations of DNA solutions were determined spectrophotometrically with a Pye Unicam 5P6-500 u.v. spectrophotometer. A 50µl sample of

DNA preparation was diluted in TE buffer to 4ml and absorbance readings taken at 260nm and 280nm against a TE buffer blank. The equation that an absorbance reading, taken at 260nm, of 1 corresponded to a DNA concentration of 50μgml^{-1} was assumed. The reading at 280nm gave an indication of protein contamination.

AGAROSE GEL ELECTROPHORESIS

Both horizontal and vertical types of apparatus were constructed in this laboratory using perspex or glass. Agarose gels (0.25-2.0%) were run in either Tris-acetate buffer (0.04M Tris; 0.02M sodium acetate; 0.001M EDTA, pH8.2) or Tris-phosphate buffer (0.08M Tris-phosphate; 0.008M EDTA, pH8.0). Samples were mixed with 1/10 volume of loading buffer which contained 0.25% Orange G, 30% Ficoll, 0.5M EDTA made up in 10x electrophoresis buffer. Running conditions depended on the nature of the material to be electrophoresed.

In general, mini gels were run for 2-4 hr. at a voltage gradient of 10Vcm^{-1}, preparative gels for 16-24 hr. at 1-2.5Vcm^{-1} and high resolution gels for 24-48 hr. at 0.25-1.0Vcm^{-1}.

Gels were stained with the fluorescent dye ethidium bromide (Sharp et al., 1973) either by incorporation into the gel and running buffer (0.5μgml^{-1}) or by immersing the gel in water containing ethidium bromide at 0.5μgml^{-1} for 45 min. after electrophoresis was complete. The DNA bands were either visualised by u.v. illumination at 302nm on a u.v. transilluminator (u.v. Products Inc., Cambridge) or by incident u.v. light. Photographs were taken using a Polaroid MP-4 camera with either Polaroid Type 55 film or Ilford fast film and Kodak 22A Wrattan filter.

SIZING OF DNA MOLECULES AND FRAGMENTS FROM AGAROSE GEL ELECTROPHORESIS

The method of Southern (1979) was adopted. The relationship between the molecular weight of a DNA molecule or fragment was represented by a simple equation which was accurate and more convenient than graphical methods. The relationship was accurate over a relatively wide range provided the separation was carried out with a low voltage gradient. The details of the calculation are outlined in Fig. 2.1.

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

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

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

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

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

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

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

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

ELECTRON MICROSCOPY

Plasmids isolated from members of the genus Deinococcus were examined in an electron microscope (Siemens Elmiskop 101) using the protein film technique (Kleinschmidt, 1968) as modified by Davis et al. (1971). Purified plasmid DNA was dissolved in 0.1M Tris-HCl buffer containing 0.01M EDTA, 50% formamide and 0.02% cytochrome C, pH8.5. The contour lengths of the molecules were measured from photographic enlargements with a Ferranti digitiser and Olivetti P6040 minicomputer. Plasmid pAT153, 3.657kb, was used as an internal standard.

CURING OF PLASMIDS

A 0.01ml inoculum of an overnight culture was added to 10ml broth with various concentrations of ethidium bromide, SDS, rifampicin or novobiocin. The culture containing the highest concentration of these agents that did not prevent growth of the test organisms was diluted and spread onto the appropriate agar. After 3 days growth at $30^{o}C$ several single colonies were sub-cultured into broth, grown up overnight and examined for plasmids by the rapid screening technique described.

Alternatively, cultures of test organisms were grown just below their restrictive temperature for 24 hr. before spreading onto the appropriate agar. Cultures were also u.v. irradiated ($1.1 \times 10^{3} Jm^{-2}$) before growing up and plating onto agar. In both cases, single colonies were subcultured into fresh broth and screened for plasmid DNA.

INHIBITORY CONCENTRATIONS OF ANTIBIOTICS

The minimum inhibitory concentrations (MIC) of antibiotics against Deinococcus spp. were determined by inoculating 0.005ml of an overnight broth culture onto solid media containing serial dilutions of antibiotic. The MIC was judged to be the lowest concentration of antibiotic which inhibited the growth of >95% of organisms after 48 hr. incubation at $30^{o}C$.

ANALYSES OF ANTIBIOTIC RESISTANCE AND HEAVY METAL RESISTANCE

Cultures of the strains to be tested were grown up in the relevant broth overnight and used to seed agar plates. When dry, antibiotic

discs (Oxoid Multodiscs or Difco Mastrings) or home made discs impregnated with heavy metals (20µl of a 10mM heavy metal solution allowed to dry onto sterile filter discs) were placed on the seeded plates and incubated overnight at 30°C.

CONSTRUCTION OF HYBRID MOLECULES WITH D. RADIODURANS SARK PLASMIDS AND D. RADIODURANS (Rf^r) CHROMOSOMAL DNA

Plasmid DNA (2µg), isolated from D. radiodurans Sark and chromosomal DNA (2µg), isolated from D. radiodurans (Rf^r) were digested separately with either 10u EcoRI or 8u BamHI for 1 hr. at 37°C. Pairs of samples from individual digestions, ie both EcoRI digests or both BamHI digests, were pooled and ethanol precipitated before resuspending in 20µl ligation buffer. T4 DNA ligase (1u) was added and the mixture incubated at 7°C overnight. The samples were then ethanol precipitated before resuspending in 100µl 0.1M Tris buffer (pH7.0). The pH was raised to 12.4 by the addition of 1M NaOH, then neutralised by the addition of 3M sodium acetate (pH4.8). The samples were once again ethanol precipitated before resuspending in 0.1M Tris buffer (pH7.0) prior to use in transformation experiments. The described deinococcal transformation procedure was employed with D. radiodurans R1 and Sark strains. After the transformation protocol, 0.1ml samples were plated on TGY plates containing 20µgml^{-1} rifampicin and incubated for 2.5 days.

CONSTRUCTION OF D. RADIODURANS KRASE GENOMIC LIBRARIES

1. Plasmid Vector System

Plasmid pAT153 was used as a vector and the technique of insertional inactivation employed to detect recombinant molecules. Two methods were used to create libraries of D. radiodurans Krase genomic DNA. The first procedure (Fig. 2.2) involved incubating 2µg of purified pAT153 with 5u of the restriction endonuclease BamHI at 37°C for 2 hr. The reaction was stopped by heating the mixture at 65°C for 10 min. In parallel to this, three individual 1µg samples of D. radiodurans genomic DNA were restricted with different amounts of the enzyme MboI which gave

various degrees of partial digestion. Conditions for partial digestion of chromosomal DNA were ascertained as follows:- Aliquots of DNA (1µg) were incubated with doubling dilutions of enzyme 5u - 0.04u. The reaction mixtures were then incubated at 37°C for 1 hr. and digestion halted by heating to 65°C for 10 min. Products of the digestion were visualised by gel electrophoresis and the size range of fragments obtained was determined by comparing them to standards produced by a HinDIII digestion of phage lambda DNA.

After linearisation of plasmid pAT153 and partial digestion of D. radiodurans DNA, the preparations were ethanol precipitated, pooled and resuspended in 20µl ligation buffer, (10mM Tris HCl pH7.8, 10mM $MgCl_2$; 20mM DTT, 1mM ATP). Ligation was carried out by adding 1u of T4 DNA ligase and incubating at 7°C. After 48 hr. the DNA was ethanol precipitated and resuspended in 100µl 0.01M Tris pH7.2. This preparation was used to transform E. coli HB101 to ampicillin resistance according to the method of Humphreys et al. (1978).

The level of insertional inactivation of the tetracycline gene was determined by replica plating ampicillin-resistant colonies onto L-plates containing $20\mu g ml^{-1}$ tetracycline. To determine the size of the recombinant plasmid, and thus the insert fragment size, at least 30 ampicillin-resistant, tetracycline-sensitive colonies were selected and screened for plasmids using the Holmes and Quigley (1981) rapid boiling method. Three plasmid standards were run, pBR322 (4.3kb), pHV33 (6.6kb) and pML2 (10.8kb). The size of the recombinant plasmids were calculated using the numerical technique of Southern (1979).

The second method involved a modification of the Directional Cloning technique (Maniatis et al., 1982) (Fig. 2.3). Plasmid pAT153 (2µg) and D. radiodurans DNA were digested with 10u EcoRI by incubating at 37°C for 1 hr. The DNAs were ethanol precipitated and resuspended in HinDIII restriction buffer. Digestion with HinDIII at 37°C for 1 hr. followed. The mixtures were then heated to 65°C for 10 min. to halt the reaction, ethanol precipitated and resuspended in ligation buffer. Ligation was carried out as before by adding 1u T4 DNA ligase to the pooled samples.

Transformation of E. coli HB101, determination of insertional inactivation, and calculation of insert size were identical to the method previously described.

2. Cosmid vector system

Preparation of the cosmid vectors

Cosmid pJBFH, a derivative of pJB8 (Ish-Horowicz and Burke, 1980) was used as a vector. Two procedures were used to prepare the vector. The first (Fig. 2.4) simply involved the restriction of 10µg pJBFH by 50u BamHI. The second method (Fig. 2.5) involved using cosmid vector arms according to the technique of Ish-Horowicz and Burke, (1980). Instead of digesting the vector with BamHI the vector was divided into two aliquots, each of which was cleaved with a different restriction enzyme. The restriction enzymes HinDIII and SalI were used and the resultant linearised DNAs were dephosphorylated with alkaline phosphate by adding 20x phosphatase buffer (1M Tris pH9.5, 20mM EDTA; spermidine to 1mM) to the restriction digest and incubating it at 37°C for 1 hr. with 1u calf intestine alkaline phosphatase. The dephosphorylated DNAs were then cleaved with BamHI and mixed to create cohesive ends which could be ligated to genomic DNA. Both linearised DNAs were phenol extracted twice (phenol: chloroform: isoamylalcohol, 25:24:1) and chloroform extracted twice (chloroform: isoamylalcohol, 24:1) before ethanol precipitation and resuspension in TE buffer.

Preparation of target DNA

D. radiodurans Krase DNA was prepared for cloning by partially digesting with MboI. The procedure used was described in the section on plasmid vector systems. After choosing mixtures suitably digested the DNA was dephosphorylated as before. After phosphatase treatment, the DNA was phenol extracted and chloroform extracted. To ensure minimal loss of material the phenol and chloroform interfaces were re-extracted with TE buffer. This was followed by ethanol precipitation and resuspension TE buffer.

Fig. 2.2. Construction of *D. radiodurans* Krase genomic library using the technique of insertional inactivation.

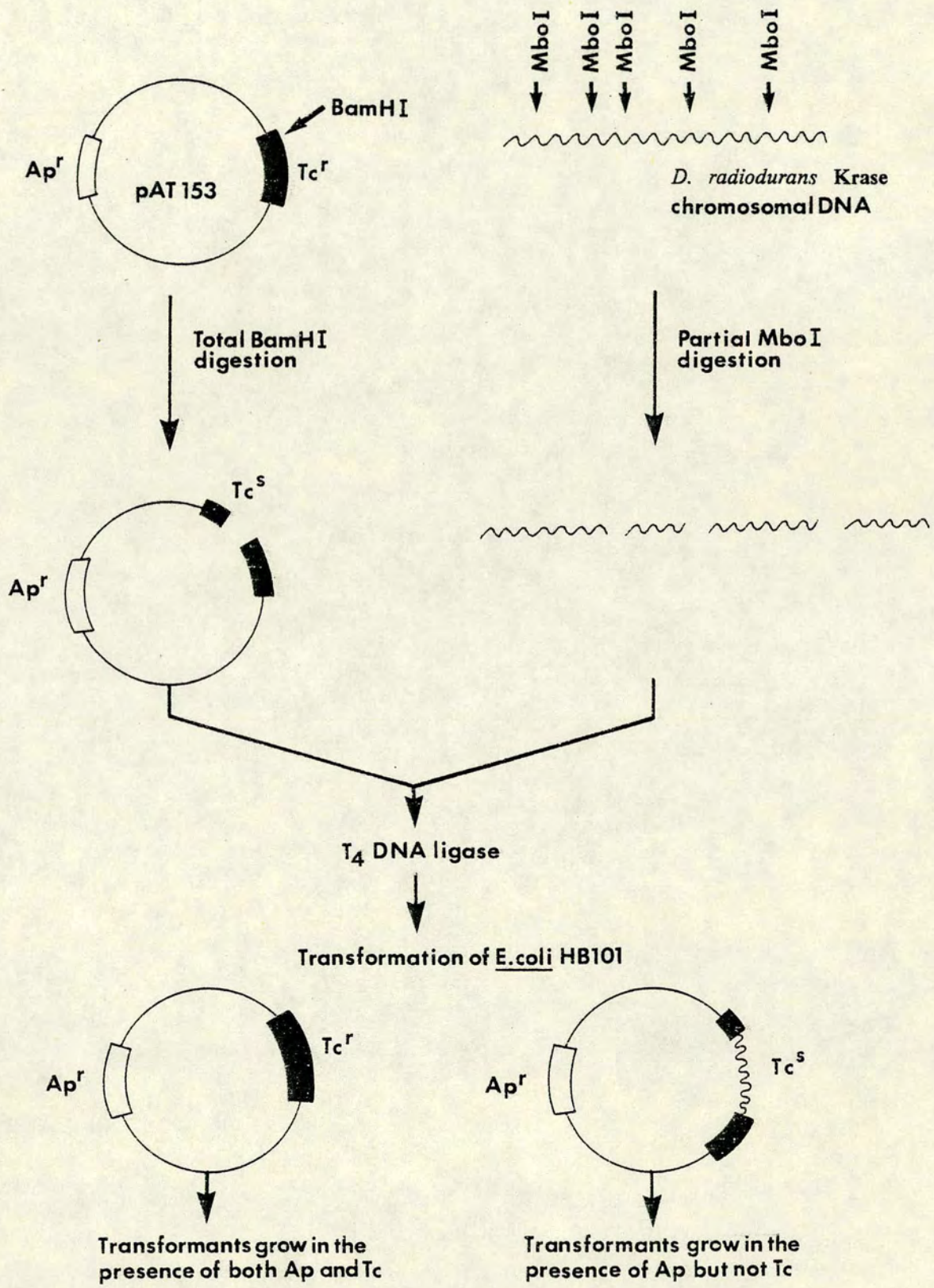


Fig. 2.3. Construction of *D. radiodurans* Krase genomic library using a modification of the directional cloning technique.

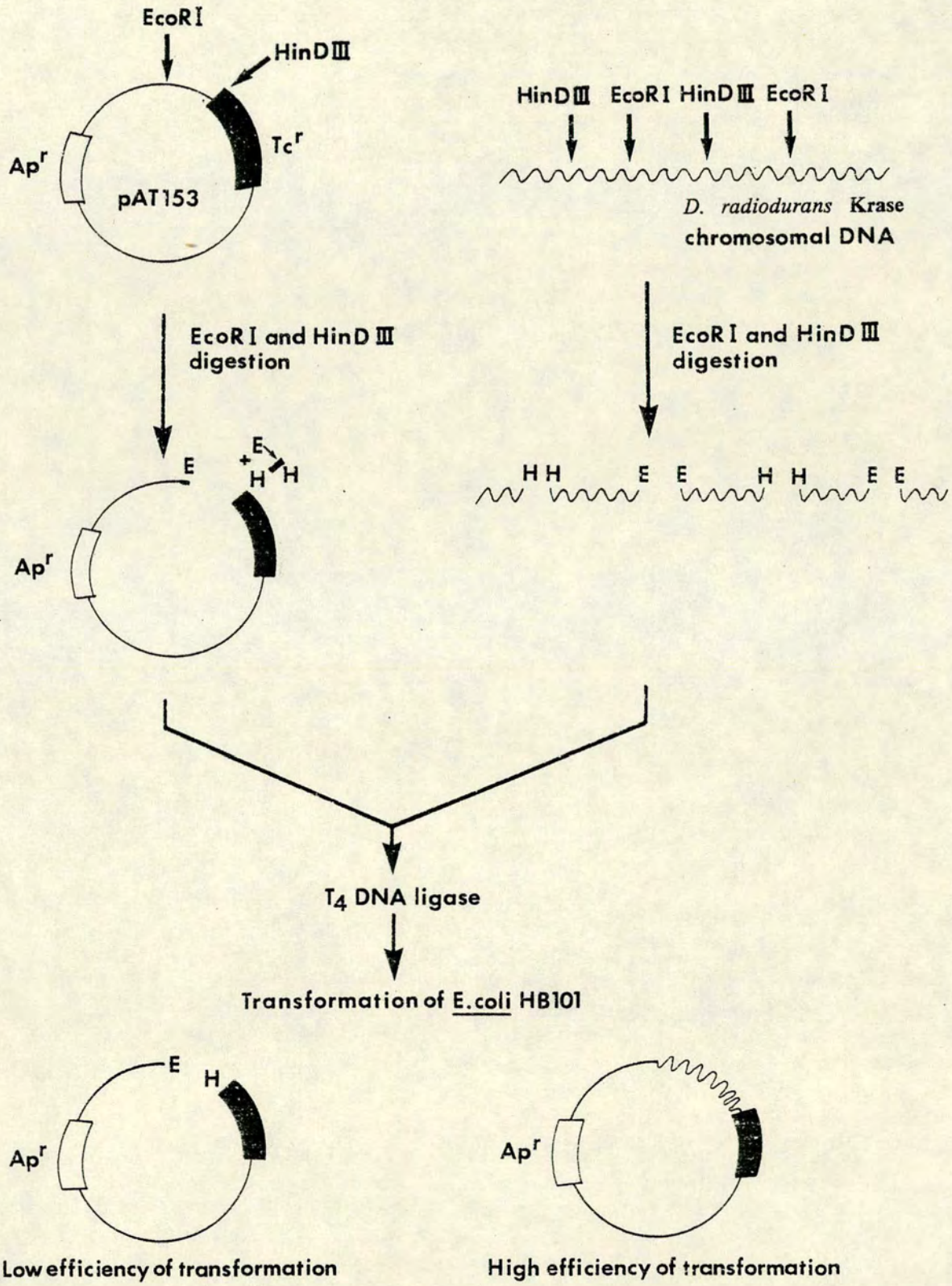


Fig. 2.4. Construction of *D. radiodurans* Krase genomic library using cosmid pJBFH

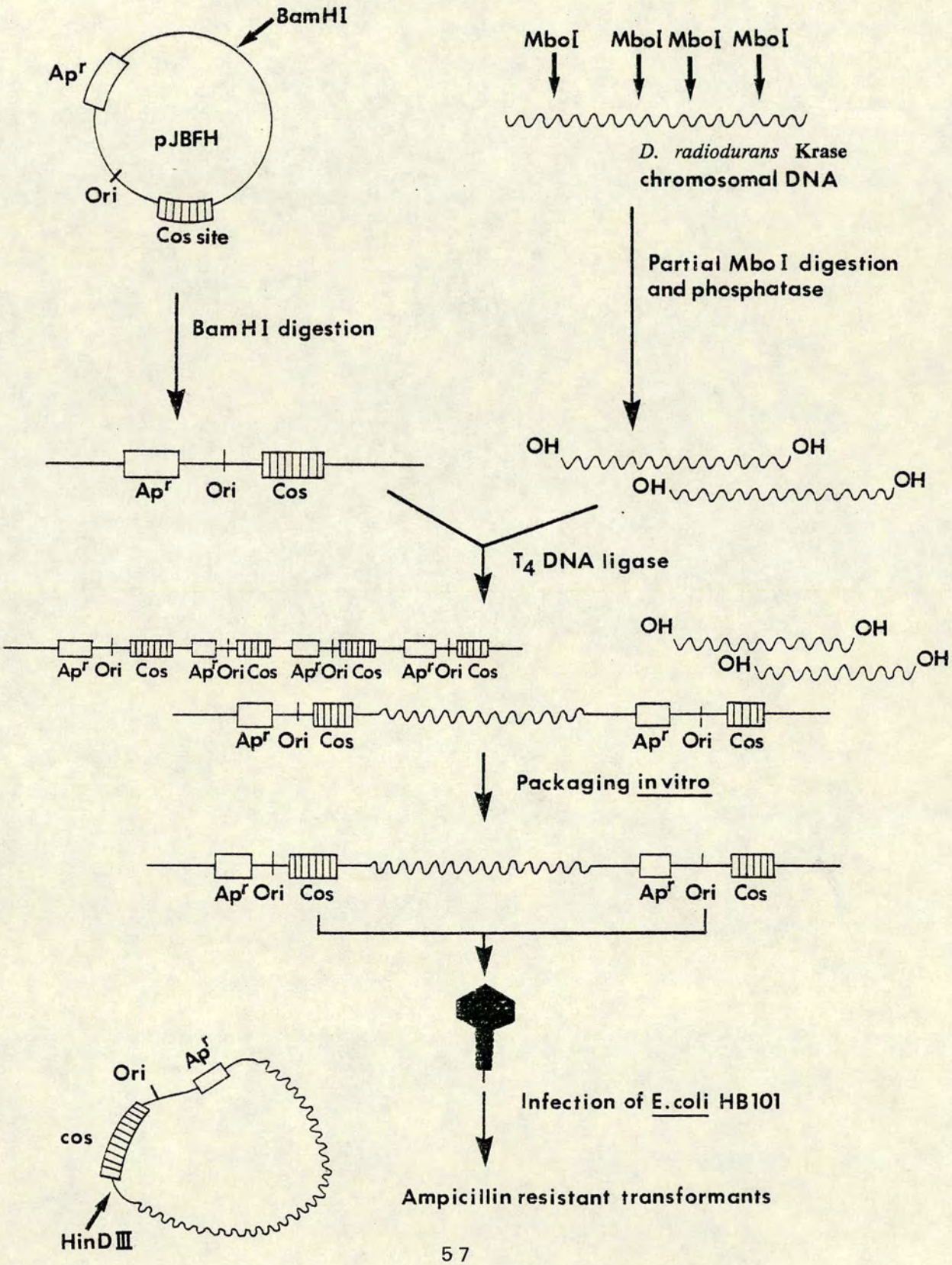
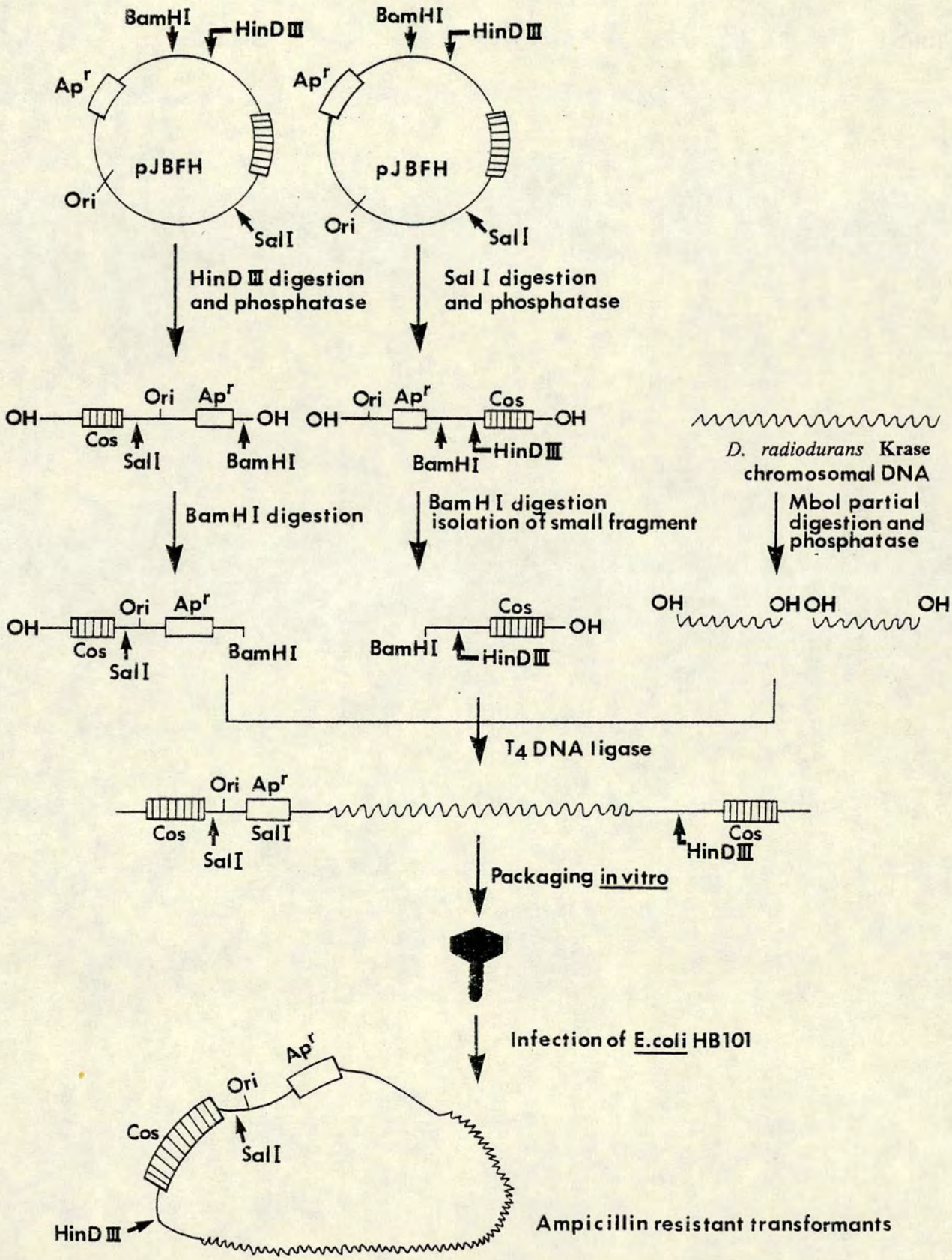


Fig. 2.5. Construction of *D. radiodurans* Krase genomic library using the Ish-Horowicz and Burke (1981) efficient cloning in cosmids procedure.



Ligation

Ligation was carried out under the following conditions:- Ligation buffer (20mM Tris, 10mM $MgCl_2$) was added to 1µg genomic DNA and 2µg vector DNA. This was made up to 1mM ATP and 10mM DTT in a total volume of 9µl. One unit of T4 DNA ligase was added to the mixture and incubated at 14°C for 16 hr.

Preparation of packaging extracts

The protocol used for the preparation of packaging extracts (Maniatis et al., 1982) involved two different extracts, a sonicated extract (SE) from induced E. coli BHB2690 (prehead donor) and a freeze/thaw lysate (FTL) from induced E. coli BHB2688 (packaging protein donor). Stocks of E. coli BHB2690 and BHB2688 were stored in glycerol at -70°C and checked before use for temperature sensitivity (each strain carries an mutation that renders the cI-gene product temperature sensitive).

The sonicated extract was prepared by growing E. coli BHB2690 in L-broth at 30°C for 16 hr. An aliquot of this culture was used to inoculate 500ml L-broth and grown at 30°C until it recorded a turbidity of 30. The prophage was induced by incubation at 45°C for 15 min. without shaking then transferred to 37°C and incubated for 1 hr. with vigorous aeration. Successful induction was checked by adding a drop of chloroform to a small sample of culture and observing clearing after a few minutes. The cells were recovered by centrifugation at 4,000g for 10 min. at 4°C. The supernatant was completely drained and the pellet was resuspended in 3.5ml of buffer A (20mM TrisHCl, pH8.0; 3mM $MgCl_2$, $6H_20$: 0.05% 2-mercaptoethanol, v/v; 1mM EDTA). The suspension was transferred to a small plastic tube and was sonicated, ensuring no foaming, for 5 sec. bursts until no longer viscous. The sonicated sample was then transferred to a centrifuge tube and centrifuged at 12,000g for 6 min. to remove debris. Aliquots of the supernatant (5-10µl) were distributed into pre-cooled microcentrifuge tubes and frozen quickly in liquid nitrogen. The tubes were stored at -70°C.

The freeze/thaw lysate was prepared in a similar fashion. E. coli BHB2688 was grown, harvested and induced in an identical manner to the sonicated extract procedures. However, instead of sonication the cells were resuspended in 3ml cold 10% sucrose, 50mM Tris - HCl pH7.5 in an ultracentrifuge tube. Fresh lysozyme solution (75µl of a $2mgml^{-1}$ in 0.2M Tris HCl pH7.5 solution) was added and mixed gently. The mixture was quickly frozen in dry ice and placed in liquid nitrogen. The extract was thawed in ice and 75µl of buffer M1 (6mM Tris, HCl pH8.0; 50mM spermidine, 50mM putrescine, 20mM $MgCl_2$; 30mM ATP; 30mM 2-mercaptoethanol) added and mixed gently. The thawed extract was centrifuged at 48,000g for 35 min. at $4^{o}C$. The supernatant was distributed in 50-100µl aliquots in microcentrifuge tubes, frozen in dry ice and stored at $-70^{o}C$. Each batch of sonicated extract and freeze/thaw lysate was tested with a standard preparation of intact phage lambda.

Packaging in vitro

The packaging reaction involved adding 7µl buffer A, 2µl buffer M1, 6µl SE and 10µl FTL to 1µl of ligated DNA. The mixture was incubated at $25^{o}C$ for 1 hr. before diluting with 0.5ml phage buffer (1M Tris: 0.1M NaCl; 1% gelatin, 100mM MgSo4; 10mM $CaCl_2$, pH7.4). Addition of 10µl chloroform to the packaging mix ensured sterility. After packaging was complete, 50µl of the mixture was added to 50µl E. coli HB101 which was grown in the presence of 0.4% maltose. The phage particles were allowed to adsorb by incubating at $37^{o}C$. After 20 min., 1ml L-broth was added and the incubation continued for a further 45 min. The culture was spread onto L-plates containing $100\mu gml^{-1}$ ampicillin and incubated overnight at $37^{o}C$. After counting the number of bacterial colonies, a number of individual colonies were subcultured into 5ml L-broth and a boiling preparation performed on them. The plasmid DNA was digested with restriction enzymes prior to electrophoretic analysis allowing the size of the resultant fragments to be determined.

DETERMINATION OF DNA SEQUENCE REPRESENTATION IN A GENOMIC LIBRARY

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

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

Where P was the desired probability, f the fractional proportion of the genome in a single recombinant and N was the necessary number of recombinants (Clarke and Carbon, 1976). This equation was rearranged to the following form to ease calculation:-

$$P = 1 - (1-f)^N$$

In addition, an assumption was made that the length (x) of a required DNA segment was small in comparison with the length (L) of the inserts, reducing the effects of random breaks occurring within length (x). To include the effect of these random breaks an f* value obtained from the formula,

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

was substituted into the probability equation when the size of the average cloned fragment was small.

SCREENING GENOMIC LIBRARIES

The screening procedure used was based on the suppression or complementation of E. coli HB101 mutations in vivo by transformation with the foreign DNA. D. radiodurans Krase is $Thi^+Leu^+Pro^+$ whereas E. coli HB101 is $Thi^-Leu^-Pro^-$. Leucine and proline markers were used by direct selection of the phenotype. Genomic libraries were replica plated onto M9 minimal media which contained either thiamine and proline or thiamine and leucine. In addition, D. radiodurans Krase is resistant to $100\mu g ml^{-1}$ rifampicin whereas E. coli HB101 is sensitive to rifampicin.

Therefore, this marker was similarly screened by replica plating onto nutrient agar plates containing 20μgml^{-1} rifampicin. Putative transformants were screened for the presence of plasmids.

RESTRICTION ANALYSIS OF TOTAL CHROMOSOMAL DNA

Chromosomal DNA was isolated from D. radiodurans Krase and D. radiophilus by the method described. Restriction endonuclease analysis was performed on 2.5μg aliquots of DNA using enzymes recognizing tetranucleotide or hexanucleotide sequences. The DNA was incubated at 37^{o}C with enzyme in the appropriate restriction buffer. The time of incubation was generally 3 hr. although longer incubations were necessary when only small amounts of enzyme were available. After incubation, the DNA was ethanol precipitated, collected by centrifugation and redissolved in 9μl water and 1μl loading buffer. The sample was then applied to an agarose gel. DNA restricted with tetranucleotide recognizing enzymes was run for 16 hr. at 2.5Vcm^{-1} on 1% gels or 24 hr. at 2Vcm^{-1} on 2% gels. On the other hand, DNA restricted with hexanucleotide recognizing enzymes was run for 16 hr. at 2.5Vcm^{-1} on 1% gels or 48 hr. at 0.5Vcm^{-1} on 0.25% gels. Gels were stained and photographed as previously described.

RESTRICTION ANALYSIS OF CLONED CHROMOSOMAL DNA

Chromosomal DNA isolated from D. radiodurans Krase was cloned into the cosmid vector pJBFH essentially by the procedure described to obtain the cosmid clone gene bank. Modifications of that procedure implemented for this investigation involved restricting pJBFH with BamH1 and treating the linear molecule with alkaline phosphatase. As regards passenger DNA, a total BamH1 restriction was also performed before ligating to the vector in a vector to passenger ratio of 2:1. In vitro packaging was performed using the same extracts of E. coli BHB2690 and E. coli BHB2688 as previously prepared for the cosmid clone gene bank.

A number of ampicillin resistant clones were chosen for further analysis. They were grown up in L-broth and cosmids were isolated by the boiling method (Holmes and Quigley, 1981). Restriction analysis

was performed on small aliquots derived from the isolation procedure and run on 0.8% agarose gels. Gels were stained and photographed as previously described.

ANALYSIS OF METHYLATED BASES IN D. RADIODURANS KRASE DNA

A modification of the technique developed by Gruenbaum et al. (1981) was used to determine the degree of base methylation in D. radiodurans Krase DNA.

Nick translation

D. radiodurans Krase chromosomal DNA (1-10µg) was incubated with 5µl NT buffer, 1µl of nucleotide labelled with ^{32}P at the position (e.g. d[α-^{32}P]GTP), 5u of E. coli DNA polymerase I and 0.002µgml^{-1} pancreatic DNase I. This mixture was made up to 50µl in water and incubated at 15^{o}C for 15 min. The labelled DNA was purified by phenol:chloroform (1:1) extraction and the unincorporated nucleotides removed by Sephadex G-75 chromatography.

Nearest Neighbour Analysis

After chromatography the labelled DNA was ethanol precipitated and redissolved in 18µl H_2O plus 2µl NNA buffer. The DNA was then digested to deoxynucleoside 3'-monophosphates in two stages. Firstly by incubating with 1µl micrococcal nuclease (140µgml^{-1}) at 37^{o}C for 45 min. The pH was then lowered to 6.5 by the addition of 1M HCl and 2µl bovine spleen phosphodiesterase (7u ml^{-1}) added. Incubation was continued for a further 30 min. An aliquot of the digest was applied directly to a cellulose thin layer chromatography (TLC) sheet (Eastman-Kodak). In addition, 1µl of each 5' mononucleotide marker (dATP, dCTP, dGTP, dTTP - 10mgml^{-1}) was applied and chromatographed in two dimensions (Cedar et al., 1979). The first solvent contained isobutyric acid: H_2O: ammonia (66:20:1) and was run until the solvent reached 1-2cm before the top of the sheet (approx. 3 hr.). The sheet was dried before cutting off the top 1-2cm which would otherwise affect

chromatography in the second dimension. The second solvent contained saturated ammonium sulphate: propan-2-ol: 1M sodium acetate (40:1:9) and was run until the solvent reached the top of the sheet. Once again the sheet was dried. A hand held u.v. light source (UVS-54, u.v. Products Inc. Cambridge) was used to detect mononucleotide standards. Chromatograms were exposed to RX (safety) film (Fuji) for 12-24 hr. at -70^{o}C and autoradiagrams were developed. This procedure was performed for each labelled base.

CHAPTER 3

RESULTS

1. PLASMIDS IN MEMBERS OF THE GENUS *DEINOCOCCUS*

1.1 ISOLATION OF PLASMIDS

On the basis of the extraordinary prevalence and diversity of plasmids in prokaryotes it seemed reasonable to assume that members of the genus *Deinococcus* would harbour extrachromosomal elements.

1.1.1 Methods of Isolation

The Hansen and Olsen (1978) method was chosen quite arbitrarily as the first method for detecting and isolating plasmids from *Deinococcus spp*. It was found to be a protracted and unwieldy method but, nevertheless, after centrifugation of cleared lysates in caesium chloride gradients, two bands corresponding to chromosomal and plasmid DNA were observed with *D. radiophilus* and *D. proteolyticus*. These bands were very faint and DNA yields were assumed to be low. No plasmid bands were obtained with cleared lysates of *D. radiodurans* R1 and *D. radiopugnans*. Various attempts were made to improve the yield of *D. radiophilus* and *D. proteolyticus* plasmids and to detect plasmids in *D. radiodurans* R1 and *D. radiopugnans*. These included; variations in lysozyme and SDS concentrations, the heat pulse was increased or omitted, the alkaline denaturation step was omitted, the time of PEG 6000 precipitation was varied and the phenol step was excluded. These permutations neither enhanced the yield of plasmids from *D. radiophilus* and *D. proteolyticus* nor detected plasmids in *D. radiodurans* R1 and *D. radiopugnans*.

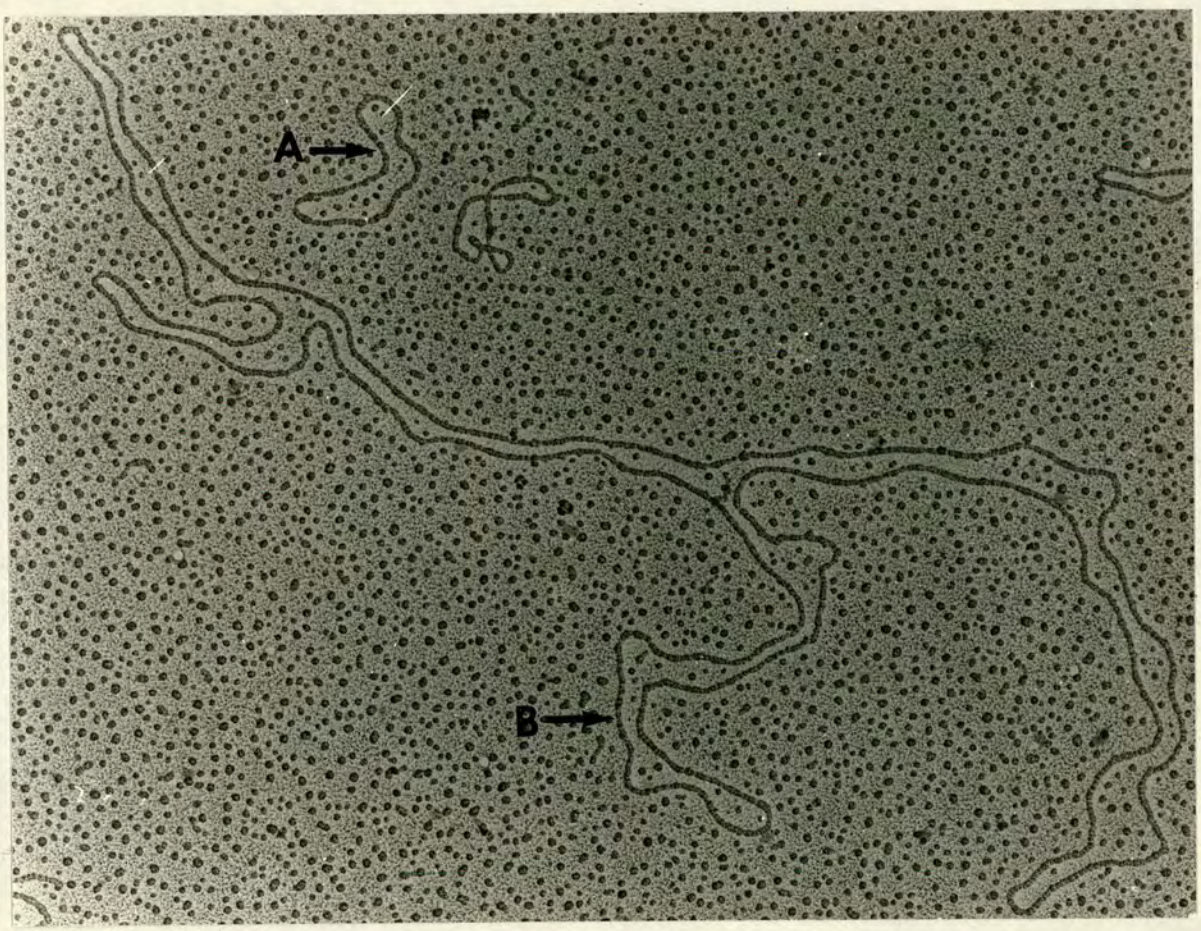
The Birnboim and Doly (1979) technique, used next, was expeditious, simple to perform and gave generally reproducible results. However, a butanol-pretreatment step was essential to render the cell wall of *Deinococcus spp*. sensitive to lysozyme degradation (Driedger and Grayston, 1970). Thereafter, the standard procedure was employed. Initially, small volumes were used for the rapid screening of

D. radiodurans R1, D. radiodurans Sark, D. radiophilus, D. proteolyticus and D. radiopugnans for the presence of plasmids. Cleared lysates produced by this technique were ethanol-precipitated and examined on agarose gels. Plasmid bands were observed in tracks loaded with DNA from D. radiodurans Sark and D. radiophilus but not from D. radiodurans R1, D. proteolyticus and D. radiopugnans. At this stage it was difficult to determine the number of plasmid class-sizes harboured by D. radiodurans Sark and D. radiophilus as relatively complex banding patterns were obtained. Variations in the small-scale technique e.g. lysozyme and SDS concentrations and times and temperatures of incubation of certain steps, failed to produce a rapid method of plasmid isolation for D. radiodurans R1, D. proteolyticus and D. radiopugnans. However, when cleared lysates, produced from 21 cultures were ethanol-precipitated and the redissolved DNA run on caesium chloride/ethidium bromide gradients, a plasmid band was present in the gradients of all four species, only D. radiodurans R1 failing to show a plasmid band.

1.1.2 Sizing of Plasmids

The purified plasmids were visualised by electron microscopy e.g. Fig. 3.1, and their measured sizes are shown in Table 3.1. D. radiophilus harboured 3 plasmid class-sizes, whilst D. radiodurans Sark, D. proteolyticus and D. radiopugnans each contained 2 plasmid class-sizes. From Table 3.1 it can be seen that there is a wide diversity of plasmid sizes within the group, ranging from the 2.5kb molecule of D. radiopugnans to the 138.8kb molecule of D. proteolyticus. However, only the 27.9kb molecule of D. radiophilus and the 28.6kb molecule of D. radiopugnans shared similar sizes. In general, the plasmids isolated tended towards the large size and as agarose gel electrophoresis sizing techniques are less accurate in the large size range (Southern, 1979), electron microscopy was adopted as the method for measuring the plasmid molecules. Agarose gel electrophoresis was used, however, in two cases to measure the plasmids.

Fig. 3.1. An electron micrograph of the 37.0kb molecule of *D. radiodurans* Sark



A — pAT153

B — pUE10

TABLE 3.1

Sizes of plasmids, as determined by electron microscopy, in members of the genus Deinococcus.

Bacteria Species and Strain	Plasmid Designation	Plasmid Size ±SD (kb)	No. of Molecules Measured
D. radiophilus	pUE1	10.8 ± 0.2	24
	PUE2	27.9 ± 1.1	18
	pUE3	92.2 ± 2.0	17
D. radiodurans Sark	pUE10	37.0 ± 0.4	18
	pUE11	44.9 ± 0.4	21
		(74.2 ± 1.2)	(11)
D. proteolyticus	pUE20	99.4 ± 1.0	15
	pUE21	138.8 ± 1.7	21
D. radiopugnans	pUE30	2.5 ± 0.1	40
	pUE31	28.6 ± 0.3	6

The 10.8kb molecule of D. radiophilus, pUE1, was sized by agarose gel electrophoresis to confirm the data obtained from electron microscopy. This plasmid was chosen because it can be cleaved at a unique site by the restriction endonuclease PstI (G. Al Bakri, personal communication) and it was therefore possible to measure it both as a linear molecule and as a covalently-closed circle (Table 3.2). The method of Southern (1979) was used for the calculations. The standards used were a HinDIII digest of lambda DNA for linear molecules and plasmids pAT153, pHV33 and pML2 for CCC molecules. The electron microscopy measurements and the two sizes obtained for pUE1 from agarose gel electrophoresis were almost identical.

Agarose gel electrophoresis was also used to resolve a problem pertaining to the 74.2kb plasmid of D. radiodurans Sark. Being twice the size of plasmid pUE10 (37.0kb) the possibility arose that this molecule was simply a dimer of pUE10 rather than an independent plasmid. To resolve this, restriction analyses with BamHI, EcoRI, HinDIII, PstI, SalI and SstII were performed on a purified plasmid preparation for D. radiodurans Sark with a HinDIII digest of lambda DNA for standards. The rationale for this procedure was that if the 74.2kb molecule was an independent plasmid, having unique DNA sequences, fragments from the restriction digests would summate to approx. 156kb i.e. 37.0 plus 44.9 plus 74.2kb. On the other hand, if the 74.2kb molecule was a dimer of pUE10 then the sum of the fragment sizes from the restriction digest would only total 82kb. The results of the digests are compiled in Table 3.3 and show that the sum of fragment sizes from the restriction digests all totalled approx. 82kb, providing unequivocal evidence that the 74.2kb molecule was a dimer of pUE10. It is interesting to note that this molecule was observed in the electron microscope on numerous occasions, whereas, no such dimer of pUE11 was seen. Whether this is due to an artefact of the isolation procedure or whether the plasmid pUE10 has a propensity to dimerize remains unclear.

TABLE 3.2

Comparison of electron microscopy and agarose gel electrophoresis techniques used to size the D. radiophilus plasmid pUE1.

	Electron Microscopy	Agarose Gel Electrophoresis	
		CCC form	Linear form
Size ± S.D. of pUE1 (kb)	10.8 ± 0.2	10.9 ± 0.3	10.7 ± 0.1

TABLE 3.3

Restriction analysis of D. radiodurans Sark purified plasmid preparation.

	BamHI	EcoRI	HinDIII	PstI	SalI	SstII
Fragments (kb)	19.5	14.8	16.7	16.7	17.4	17.6
	17.2	12.1	15.1	14.8	16.0	13.6
	14.7	10.7	14.0	12.1	13.6	10.0
	11.9	9.9	12.3	10.3	11.2	9.5
	11.0	8.4	8.8	9.1	9.5	9.0
	10.3	7.2	6.8	6.1	5.2	7.5
	1.4	6.1	5.6	4.6	4.1	7.1
	0.7	3.8	4.0	3.8	3.5	6.1
		3.0		3.0	3.0	4.5
		2.2		1.8	2.0	1.3
		2.1		0.8	1.1	0.7
		1.0				
Sum of fragment sizes	86.7kb	81.3kb	83.3kb	83.1kb	86.6kb	86.9kb

No experiments were performed to determine the copy-number of any of the plasmids. However, two plasmids, pUE1 and pUE30, were visualised much more frequently in the electron microscope than any of the other plasmids. Whilst not being definitive evidence that pUE1 and pUE30 were multicopy, it does give an indication that they may be. In addition, one would expect the other plasmids, because of their relatively large size, to be single copy.

1.1.3 Search for Plasmids in *D. radiodurans* R1

Concerning the apparent absence of plasmid DNA in *D. radiodurans* R1, the possibility existed that this was due to the limitations of the two isolation methods used. For this reason, a diligent search was made for plasmids in this organism employing many variations of the two original protocols plus a number of other methods, i.e. the cleared lysate techniques of Guerry *et al.* (1973) and Humphreys *et al.* (1975), the Niaudet and Ehrlich (1978) modification of Gryczan *et al.* (1978) technique, the Ish-Horowicz and Burke (1981) modification of the Birnboim and Doly (1979) method and Holmes and Quigley's (1981) boiling method. These methods all gave negative results.

In addition to experimenting with alternative methods, cells of *D. radiodurans* R1 were u.v. irradiated ($1.0Jm^{-2}$ - $315Jm^{-2}$) and subsequently grown up prior to employing a plasmid isolation technique. The rationale for this procedure was based on findings of Yeats *et al.* (1982) that plasmid production in the Archaebacterium *Sulfolobus acidocaldarius* was induced by u.v. irradiation and that without it, yields of plasmid DNA were either very low or were not recovered at all. No plasmids were detected in *D. radiodurans* R1 using this procedure.

In another approach, the Birnboim and Doly (1979) plasmid isolation technique was used on a Nuc$^-$ mutant of *D. radiodurans* R1 that has considerably reduced levels of extracellular nuclease production (D.M. Evans, personal communication). Timmis and Winkler (1973) showed that reproducible yields of plasmids were obtained from mutants of *Serratia marcescens* defective in extracellular nuclease production but

not from the corresponding wild type. However, no plasmids were detected in the Nuc⁻ mutant of D. radiodurans R1.

It was difficult to conceive that the most studied strain, D. radiodurans R1, does not harbour plasmids, although the fact that it is the most studied could be the reason for its lacking plasmids. Plasmids may have been lost from the strain during the constant subculturing of the organism and if this were the case one would imagine that cultures, freeze-dried many years previously, would harbour plasmids. To verify this reasoning, several cultures, freeze-dried in the mid-sixties, were resuscitated and investigated for the presence of plasmids. However, they too proved to be plasmidless. Although the possibility remains that D. radiodurans R1 harbours plasmids that are very difficult to isolate, the weight of evidence suggests that this laboratory strain is plasmidless. If this is the case it may prove useful as a plasmidless recipient.

1.2 CURING OF PLASMIDS

1.2.1 Methods of Curing

An efficient mechanism for eliminating plasmids from a plasmid-harbouring host is essential both for the assignation of phenotypic traits to a plasmid and for an investigation of its transfer characteristics. In this study a number of chemical and physical methods were used in an attempt to cure D. radiodurans Sark and D. radiophilus of one or all of their plasmids. These species were chosen for examination in preference to D. proteolyticus and D. radiopugnans because a rapid plasmid isolation technique had already been developed for them and this greatly promoted the screening of putatively cured bacteria.

Four chemical agents, ethidium bromide, SDS, rifampicin and novobiocin were chosen on the basis of their proven plasmid-curing capacity in other bacterial systems. The procedure for assaying the curing frequency of these agents against D. radiodurans Sark and D. radiophilus was essentially the same (see Chapter 2). The MICs of

the four agents against the two species in broth culture were determined (Table 3.4) and appropriate dilutions of the culture which contained the highest concentration of agent which allowed growth were plated onto the relevant solid media. Individual colonies were screened for the presence of plasmids. Two physical methods, elevated temperature and u.v. irradiation, were also used in an attempt to cure D. radiodurans Sark and D. radiophilus of their plasmids and the procedure used is outlined in Chapter 2.

1.2.2 Screening of Putatively Cured Clones

These procedures although rather laborious, were relatively simple to perform as only one experiment per treatment was required to yield thousands of putatively cured colonies. The limiting factor was the rate at which single colonies could be screened for their plasmid content. The results of curing D. radiodurans Sark and D. radiophilus plasmids are shown in Table 3.5. Neither the chemical agents nor the physical methods tested cured D. radiodurans Sark or D. radiophilus of any of their plasmids. Of course, these results can only be considered in terms of the number of colonies screened because it could be that curing was occurring at lower frequencies than could be detected from the number of colonies tested.

1.3 PHENOTYPIC TRAITS OF NATIVE PLASMIDS

Many plasmids carry genes that specify readily-detectable functions e.g. antibiotic and heavy-metal resistance (for a review see Foster, 1983). To test whether plasmid-carrying Deinococcus spp. were specifically resistant to antibiotics, antibiotic-impregnated discs were used on lawns of the four plasmid-harbouring species, D. radiodurans Sark, D. radiophilus, D. proteolyticus and D. radiopugnans with D. radiodurans R1 being included as a plasmid-free strain. The results of these tests are shown in Table 3.6. Of 15 antibiotics tested only two gave what may have been, significant results. D. radiophilus was resistant to streptomycin whilst D. radiopugnans was resistant to rifampicin.

TABLE 3.4

The MICs of ethidium bromide, SDS, rifampicin and novobiocin against *D. radiodurans* Sark and *D. radiophilus*.

	Ethidium Bromide ($\mu g ml^{-1}$)	SDS ($\mu g ml^{-1}$)	Rifampicin ($\mu g ml^{-1}$)	Novobiocin ($\mu g ml^{-1}$)
D. radiodurans Sark	2.0	60.0	0.5	0.5
D. radiophilus	2.0	40.0	0.5	0.5

TABLE 3.5

Evaluation of four chemical and two physical curing treatments against *D. radiodurans* Sark and *D. radiophilus*.

TREATMENT			*D. radiodurans* Sark		*D. radiophilus*		
			pUE10	pUE11	pUE1	pUE2	pUE3
Ethidium Bromide $1.5\mu gml^{-1}$	No. of colonies from each species screened	100					
	Cured clones		0	0	0	0	0
	Frequency of curing		<1%	<1%	<1%	<1%	<1%
SDS $55\mu gml^{-1}$ and $35\mu gml^{-1}$	No. of colonies from each species screened	100					
	Cured clones		0	0	0	0	0
	Frequency of curing		<1%	<1%	<1%	<1%	<1%
Rifampicin $0.4\mu gml^{-1}$	No. of colonies from each species screened	50					
	Cured clones		0	0	0	0	0
	Frequency of curing		<2%	<2%	<2%	<2%	<2%
Novobiocin $0.4\mu gml^{-1}$	No. of colonies from each species screened	60					
	Cured clones		0	0	0	0	0
	Frequency of curing		<1.5%	<1.5%	<1.5%	<1.5%	<1.5%
Elevated Temperature ($38.5^{o}C$)	No. of colonies from each species screened	100					
	Cured clones		0	0	0	0	0
	Frequency of curing		<1%	<1%	<1%	<1%	<1%
u.v. Irradiation ($1.1x10^{3}Jm^{-2}$)	No. of colonies from each species screened	50					
	Cured clones		0	0	0	0	0
	Frequency of curing		<2%	<2%	<2%	<2%	<2%

TABLE 3.6

Resistance of members of the genus Deinococcus to common antibiotics

Antibiotic (conc $\mu g ml^{-1}$)		D.radio-durans Sark	D.radio-philus	D.proteo-lyticus	D.radio-pugnans	D.radio-durans R1
Ampicillin	25	S	S	S	S	S
Bacitracin	10	S	S	S	S	S
Cephaloridine	25	S	S	S	S	S
Chloramphenicol	10	S	S	S	S	S
Cotrimoxazole	25	S	S	S	S	S
Erythromycin	10	S	S	S	S	S
Kanamycin	5	S	S	S	S	S
Methicillin	5	S	S	S	S	S
Neomycin	30	S	S	S	S	S
Nitrofurantoin	50	S	S	S	S	S
Penicillin G	10	S	S	S	S	S
Polymyxin B	100	S	S	S	S	S
Rifampicin	5	S	S	S	R	S
Streptomycin	10	S	R	S	S	S
Tetracycline	10	S	S	S	S	S

Disc Sensitivity Tests,

S - sensitive, zone of inhibition around disc.

R - resistant, no zone or very small zone of inhibition around disc.

In a similar experiment, heavy metal solutions were impregnated into sterile discs and the disc diffusion method used to detect resistance of the previously tested strains to a number of heavy metals. The data (Table 3.7) show that all five strains were resistant to the concentrations of sodium iodide, sodium selenite and lithium lactate used. However, only D. radiophilus was resistant to other heavy metal solutions at the concentrations tested, namely stannous chloride and sodium arsenate.

D. radiophilus and D. radiopugnans were then treated with a plasmid curing agent and several hundred colonies screened to ascertain whether colonies of the former retained their resistance to streptomycin, stannous chloride or sodium arsenate and colonies of the latter retained resistance to rifampicin. Ethidium bromide was chosen as the curing agent because it has been used successfully to cure a large number of bacterial species. Over 1,000 ethidium bromide-treated cells of each of D. radiophilus and D. radiopugnans were plated onto the relevant media (average 100 colonies $plate^{-1}$) and the resulting colonies replica-plated onto media containing singly streptomycin, stannous chloride or sodium arsenate (D. radiophilus) and rifampicin (D. radiopugnans) at concentrations which allowed replica-plated colonies of the wild-type to grow. No colony from either species failed to grow on the supplemented media. It was concluded that either the phenotypes tested were not plasmid encoded e.g. streptomycin and rifampicin resistance are generally chromosomally mediated, or ethidium bromide did not cure these species of their plasmid complement even at very low frequencies e.g. <0.1%.

Changes in colonial morphology were also looked for. Plasmid elimination in some E. coli strains has been associated with changes in the morphology of colonies (Rosas et al., 1983). However, no morphological changes in ethidium bromide-treated cells of D. radiophilus and D. radiopugnans colonies were accompanied by plasmid loss.

TABLE 3.7

Resistance of members of the genus Deinococcus to heavy metals.

Heavy Metal	D.radio-durans Sark	D.radio-philus	D.proteo-lyticus	D.radio-pugnans	D.radio-durans R1
Antimony Potassium Tartrate	S	S	S	S	S
Cadmium Sulphate	S	S	S	S	S
Cobaltous Chloride	S	S	S	S	S
Lead Acetate	S	S	S	S	S
Lead Nitrate	S	S	S	S	S
Lithium Lactate	R	R	R	R	R
Mercuric Chloride	S	S	S	S	S
Mercuric Sulphate	S	S	S	S	S
Phenyl Mercuric Acetate	S	S	S	S	S
Sodium Arsenate	S	R	S	S	S
Sodium Arsenite	S	S	S	S	S
Sodium Iodide	R	R	R	R	R
Sodium Selenite	R	R	R	R	R
Stannous Chloride	S	R	S	S	S
Thallous Acetate	S	S	S	S	S
Zinc Acetate	S	S	S	S	S
Zinc Chloride	S	S	S	S	S

Disc Sensitivity Tests,

Each heavy metal disc contained 20µl of a 10mM heavy metal solution.

S - sensitive, zone of inhibition around disc.

R - resistant, no zone or very small zone of inhibition around disc.

2. DEVELOPMENT OF A GENE CLONING SYSTEM

2.1 PLASMID VECTORS

2.1.1 Foreign Plasmid Vectors

The first stage in the development of a gene-cloning system for Deinococcus spp. involved attempts to transform D. radiodurans R1 with a number of common plasmid vectors, viz. the ColE1 derived plasmids pML2 (Hershfield et al., 1974), pBR322 (Bolivar, 1977) and pAT153 (Twigg and Sherratt, 1980), the S. aureus plasmids used as cloning vectors in B. subtilis, pC194 (Ehrlich, 1977) and pUB110 (Gryczan et al., 1978) and the E. coli-B.subtilis hybrid plasmid, pHV33 (Primrose and Ehrlich, 1981).

Plasmid Preparations

Plasmids were prepared by the procedures detailed in Chapter 2. The purity of plasmid preparations was checked by agarose gel electrophoresis and concentrations determined by spectrophotometric analysis. Biological activity of the plasmid preparations was assayed by transformation of the host organism. It can be seen from Table 3.8 that the six plasmid preparations were biologically active and therefore suitable for the attempted transformation of D. radiodurans.

Transformation experiments

The MICs of marker antibiotics against D. radiodurans R1 were, kanamycin 2.5μgml^{-1}, ampicillin 0.01μgml^{-1}, tetracycline 0.1μgml^{-1} and chloramphenicol 2.5μgml^{-1} and these values were in general agreement with those of Hawiger and Jeljaszewicz (1967). In experiments with D. radiodurans R1, concentrations of marker antibiotics 5 to 10 times that of the MIC were used to score for transformants. Table 3.9 shows that the plasmids tested failed to yield any transformants of

D. radiodurans R1. Variations of the transformation protocol e.g. increasing plasmid concentration and lengthening the time allowed for phenotypic expression of the marker, were ineffective. In addition, experiments using alternative transformation procedures e.g. an E. coli plasmid transformation procedure (Humphreys et al., 1978) and a B. subtilis PEG-induced transformation procedure (Chang and Cohen, 1978) were also unsuccessful. Similar attempts to transform D. radiodurans R1 and other Deinococcus spp. with other E. coli plasmid vectors e.g. RP4, pLV21 and R68.45 failed to yield transformants (I.J. Purvis, personal communication). It was concluded that D. radiodurans R1 is refractory to transformation by foreign plasmid vectors and in the light of these results the search for a known plasmid vector was terminated.

2.1.2 Foreign Plasmid/D. radiodurans Krase Chromosomal DNA Hybrid Vectors

An alternative approach to establishing a gene-cloning system for Deinococcus spp. involved attempting to transform D. radiodurans R1 and Sark strains with a novel plasmid vector.

Construction of novel vectors

Hybrid plasmids were constructed by cloning fragments of D. radiodurans Krase DNA into pAT153. The technique employed was identical to that described for the construction of the D. radiodurans genomic library using the directional cloning method (see Chapter 2). Briefly, D. radiodurans DNA was cleaved, first with EcoRI, then HinDIII restriction endonucleases and cloned into pAT153 which had also been cleaved with the two enzymes. After ligation of the fragments, the material was used to transform E. coli HB101. This procedure was straightforward and no problems were experienced with ligation of D. radiodurans chromosomal DNA into E. coli plasmid DNA. The level of insertional inactivation was 55% and ten ampicillin-resistant, tetracycline-sensitive clones, harbouring recombinant plasmids with chromosomal inserts of between 2.3 and 9.6kb were chosen for further analysis.

TABLE 3.8

Biological activity of plasmids assayed by transformation.

Plasmid	Host	Antibiotic Resistance Selected and Concentration used ($\mu g ml^{-1}$) (1)	Biological Activity: Frequency of Host Transformation	Number of Transformants μg^{-1} DNA
pML2	E. coli	Kn25	6.2×10^{-3}	3.2×10^{3}
pBR322	E. coli	Tc20	5.0×10^{-3}	4.2×10^{5}
pAT153	E. coli	Tc20	3.5×10^{-3}	1.7×10^{5}
pC194	B. subtilis	Cm30	2.0×10^{-3}	1.6×10^{5}
pUB110	B. subtilis	Kn25	8.8×10^{-4}	8.0×10^{4}
pHV33	E. coli	Tc20	2.0×10^{-4}	1.8×10^{4}

(1) Abbreviations:- Kn - Kanamycin, Tc - Tetracycline,
Cm - Chloramphenicol

TABLE 3.9

Attempted transformation of D. radiodurans R1 using foreign plasmid vectors.

Plasmid	Antibiotic Resistance Selected for and Concentration Used ($\mu g ml^{-1}$) (1)	Frequency of Transformation (2)
pML2	Kn 12.5	$<7.4 \times 10^{-7}$
pBR322	Ap 0.1	$<1.0 \times 10^{-8}$
	Tc 1.0	$<1.0 \times 10^{-8}$
pAT153	Ap 0.1	$<1.3 \times 10^{-8}$
	Tc 1.0	$<1.3 \times 10^{-8}$
pC194	Cm 12.5	$<4.0 \times 10^{-7}$
pUB110	Kn 12.5	$<3.4 \times 10^{-7}$
pHV33	Ap 0.1	$<6.0 \times 10^{-7}$
	Tc 1.0	$<6.0 \times 10^{-7}$
	Cm 12.5	$<6.0 \times 10^{-7}$

(1) Abbreviations:- Kn - Kanamycin, Ap - Ampicillin, Tc - Tetracycline, Cm - Chloramphenicol

(2) No transformants were detected. If there had been one colony then the frequencies would have been those shown. No spontaneous antibiotic-resistant mutants were isolated for any marker.

Plasmid preparations

Recombinant plasmids were prepared using the Holmes and Quigley (1981) boiling method. Restriction mapping of plasmids derived from the clones was used to check the pedigree of the recombinants i.e. that each recombinant molecule possessed one EcoRI site and one HinDIII site and that a double digest with the enzymes released a fragment the size of pAT153. (In theory, this fragment should be 31 base pairs less than pAT153.) Transformation of E. coli HB101 to ampicillin resistance was tested as a guide to biological activity. The results (Table 3.10) show that the frequency of transformation using recombinant plasmids derived from the boiling preparation were not as biologically active as purified plasmid preparations used in 2.1.1. A combination of the crude nature of the boiling preparations and the fact they were recombinant plasmids was thought to account for this.

Transformation experiments

Plasmids derived from the ten clones were used in attempts to transform D. radiodurans R1 and Sark strains to ampicillin resistance. The results are shown in Table 3.10. The ampicillin marker carried on recombinant plasmids failed to express in D. radiodurans R1 and Sark strains. Another method in which, instead of transforming E. coli HB101 to derive recombinant molecules, ligated D. radiodurans chromosomal DNA and pAT153 was used directly to transform D. radiodurans R1 and Sark strains. No ampicillin-resistant colonies could be isolated. It was concluded that foreign plasmid vectors which contain D. radiodurans chromosomal inserts do not express their foreign markers in D. radiodurans.

2.1.3 Native Plasmids

The lack of foreign gene expression in D. radiodurans led to attempts to develop vector systems for Deinococcus spp. using native plasmids.

TABLE 3.10

Size, biological activity and frequency of transformation of *D. radiodurans* R1 and Sark strains of clones 1-10.

Clone	Size of chromosomal insert (kb)	Biological Activity: Frequency of *E. coli* transformation	Frequency of *D. radiodurans* Transformation (1)	
			R1 strain	Sark strain
1	2.3	1.7×10^{-4}	$<4.3 \times 10^{-7}$	$<1.3 \times 10^{-8}$
2	2.5	3.1×10^{-4}	$<4.3 \times 10^{-7}$	$<1.3 \times 10^{-8}$
3	2.8	3.2×10^{-4}	$<5.1 \times 10^{-7}$	$<5.1 \times 10^{-7}$
4	3.3	2.0×10^{-5}	$<5.1 \times 10^{-7}$	$<5.1 \times 10^{-7}$
5	3.6	1.0×10^{-4}	$<8.0 \times 10^{-7}$	$<7.0 \times 10^{-7}$
6	3.7	5.0×10^{-5}	$<8.0 \times 10^{-7}$	$<7.0 \times 10^{-7}$
7	5.1	5.0×10^{-5}	$<6.0 \times 10^{-7}$	$<5.7 \times 10^{-7}$
8	5.4	8.0×10^{-5}	$<6.0 \times 10^{-7}$	$<5.7 \times 10^{-7}$
9	6.0	6.0×10^{-5}	$<8.0 \times 10^{-7}$	$<7.0 \times 10^{-7}$
10	9.6	2.3×10^{-5}	$<8.0 \times 10^{-7}$	$<7.0 \times 10^{-7}$

(1) No transformants were detected. If there had been one colony then the frequencies would have been those shown. No ampicillin-resistant *D. radiodurans* spontaneous mutants were isolated.

Choice of plasmid

D. radiodurans Sark plasmids, pUE10 and pUE11 were chosen as possible cloning vectors for two reasons, both advantageous, viz. rapid plasmid preparations could be made from this organism and D. radiodurans Sark is a transformable strain. The disadvantage of using any of the native plasmids was that they were cryptic. In an attempt to overcome this problem, experiments were carried out to insert a rifampicin-resistance marker from D. radiodurans (Rf^r) chromosomal DNA onto a D. radiodurans Sark plasmid.

Choice of restriction enzyme

The choice of BamHI and EcoRI for use in the manipulation experiments was dictated by two results. Firstly the results in Table 3.3 showed the size of fragments obtained when D. radiodurans Sark plasmids were digested by some restriction enzymes. There was no way of knowing whether those enzymes cleaved essential regions of the native plasmids but BamHI and EcoRI cleaved both plasmids at least once and not to an excessive degree. Secondly, it was essential that these enzymes did not affect transformation of the rifampicin-resistant marker. This was shown by digesting 5µg samples of D. radiodurans (Rf^r) DNA prior to transformation of the wild type strain. Table 3.11 shows that restriction of D. radiodurans (Rf^r) DNA with the enzymes BamHI, EcoRI HinDIII and PstI had little affect on transformation to rifampicin resistance, whereas, SalI digestion resulted in a 500 fold decrease in transformation efficiency. It was concluded that the former 4 enzymes must have left significant fragments of DNA intact whilst SalI cleaved either within or close to the gene conferring rifampicin-resistance.

Table 3.11

Effect of digesting D. radiodurans (Rf^r) chromosomal DNA with restriction enzymes prior to transformation of D. radiodurans Sark to rifampicin resistance.

Enzyme	Transformation Frequency	No. of Transformants μg^{-1}
None	8.8×10^{-4}	1.1×10^{4}
BamHI	1.9×10^{-4}	4.1×10^{3}
EcoRI	2.2×10^{-3}	2.2×10^{3}
HinDIII	1.2×10^{-3}	1.1×10^{4}
PstI	1.0×10^{-4}	1.0×10^{4}
SalI	1.5×10^{-6}	8.7×10^{1}

Strategy for insertion of marker

The experimental details of this strategy has been outlined in Chapter 2. Basically, BamHI and EcoRI were used in independent experiments to cleave both D. radiodurans Sark plasmids and D. radiodurans (Rf^r) chromosomal DNA. This material was ligated to form recombinant molecules. An alkaline denaturation step was then used to inactivate linear DNA, which would otherwise have transformed the recipient cultures, leaving the plasmids intact. This DNA was used in attempts to transform D. radiodurans R1 and Sark strains to rifampicin resistance. No transformants were isolated with either recipient. It was reasoned that either the design of the experiment or problems inherent in D. radiodurans transformation e.g. failure to take up plasmids, were responsible for the lack of success of this strategy.

Due to the lack of expression of foreign genes in D. radiodurans and the difficulty encountered with the construction of native plasmid vectors, D. radiodurans genomic libraries were made with a view to examining D. radiodurans gene expression in E. coli.

2.2 CONSTRUCTION OF GENOMIC LIBRARIES OF D. RADIODURANS KRASE

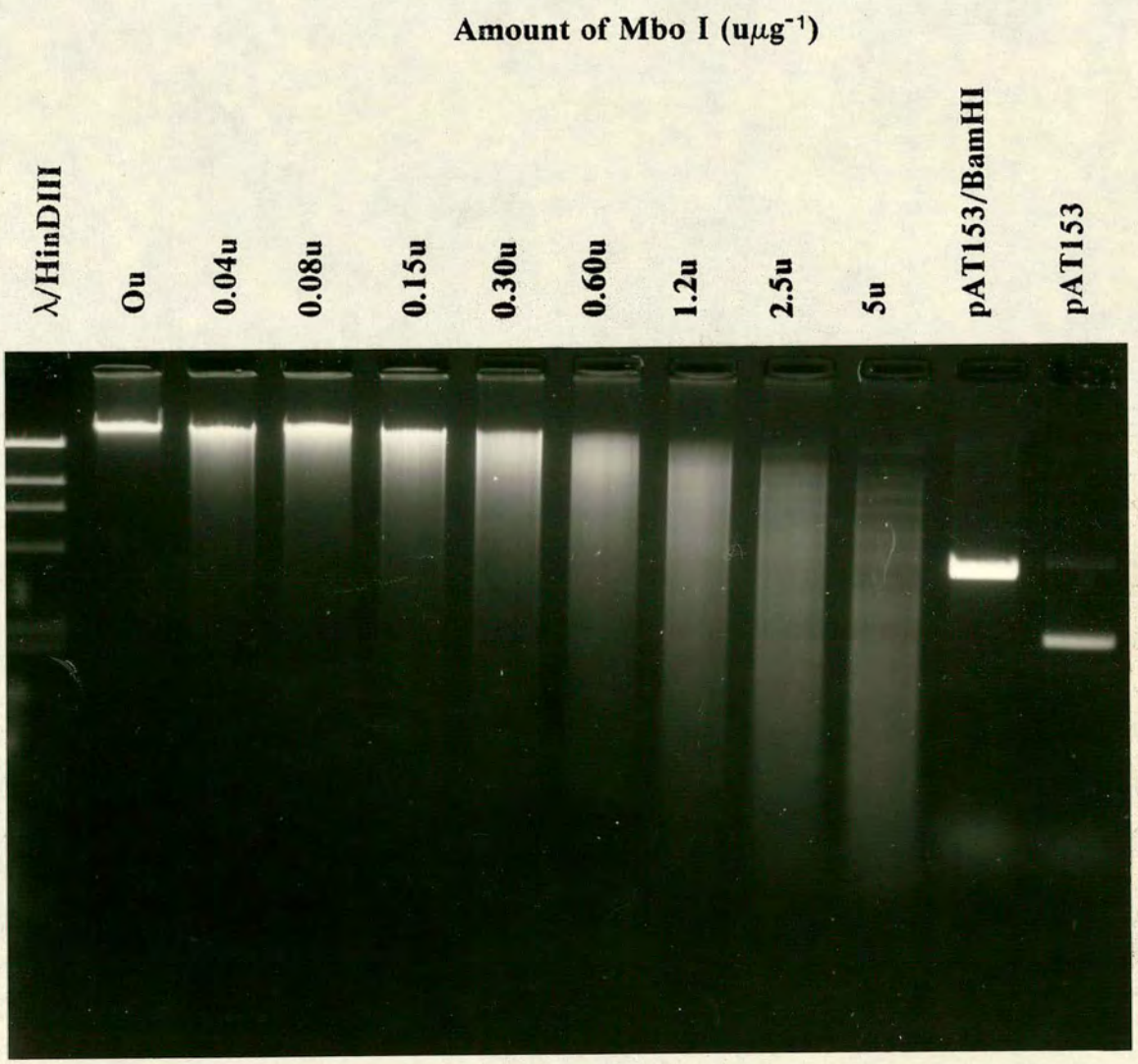
2.2.1 Plasmid Genomic Libraries

The E. coli plasmid pAT153 was used as a vector and two methods of constructing D. radiodurans genomic libraries were pursued; insertional inactivation using BamHI cleaved vector and MboI partially digested target DNA and a directional cloning technique using EcoRI and HinDIII double-digested vector and target DNA.

Conditions for MboI partial digestion

Products of MboI endonuclease digestion with various amounts of enzyme were visualised by agarose gel electrophoresis (Fig. 3.2). Three partial-digestion samples, resulting from cleavage of D. radiodurans Krase DNA by 0.04u, 0.08u and 0.15u, were pooled and used in the manipulation experiments.

Fig. 3.2. Agarose gel electrophoresis of the products of partial digestion of *D. radiodurans* Krase DNA with MboI. One μg samples of DNA were digested with doubling dilutions of MboI (5u-0.04u) and incubated at 37°C for 1 hour.



Transformation procedures

Four transformation methods were evaluated by measuring the transformation frequencies obtained under similar conditions to those used in the manipulation experiments. The protocols of Humphreys *et al.* (1978), Davis *et al.* (1980), a modification of Davis *et al.* (1980) and Maniatis *et al.* (1982) were tested as outlined previously. Plasmid pAT153 (0.5μg) was used as transforming DNA and tetracycline transformants of the recipient strain *E. coli* HB101 were scored. The results (Table 3.12) indicate that the method of Humphreys *et al.* (1978) is best suited to that particular concentration of DNA and to the recipient strain used.

Construction of libraries

Experimental details of the construction of *D. radiodurans* Krase genomic libraries has been described previously (Chapter 2). Table 3.13 illustrates typical results. The major drawback with these techniques was the small average size of the cloned *D. radiodurans* fragment. Considering plasmid vectors have the capability to carry 15kb of insert DNA it was surprising to obtain average insert sizes of only 3.5kb and 2.8kb.

Screening of libraries

Both libraries contained over 90% of the genome and both were used to ascertain whether the *D. radiodurans* Krase genes, Rf^r, Leu^+ or Pro^+ were expressed in *E. coli* HB101. A small number of Rf^r, Leu^+ or Pro^+ *E. coli* clones resulted from both methods. However, no recombinant plasmids were isolated from either Rf^r or Pro^+ *E. coli* colonies, which were assumed, therefore, to be spontaneous mutants (both these markers gave relatively high spontaneous mutant frequencies). A small number of *E. coli* Leu^+ cells were isolated, however, which did contain recombinant plasmids. Unfortunately, these cells lost their ability to grow in the absence of leucine over the course of one or two subcultures, even when selective pressure was maintained. It was concluded that, *D. radiodurans* Leu^+ genes may have been expressing in *E. coli* but that expression was extremely unstable.

TABLE 3.12

Efficiency of four E. coli-plasmid transformation procedures using pAT153 (0.5μg) as transforming DNA and E. coli HB101 as recipient.

Procedure	Number of tetracycline transformants μg^{-1}pAT153	Frequency of Transformation
Humphreys et al. (1978)	1.6×10^{5}	1.6×10^{-3}
Davis et al. (1980)	4.0×10^{2}	2.4×10^{-6}
Davis et al. (1980) Modification	1.1×10^{4}	1.1×10^{-4}
Maniatis et al. (1982)	1.6×10^{2}	1.7×10^{-5}

TABLE 3.13

Evaluation of D. radiodurans Krase genomic libraries, constructed using the techniques of insertional inactivation and directional cloning.

Technique	Number of Ampicillin Resistant Transformants	% Insertional Inactivation	Average size of Insert (kb)	Probability of any gene being represented (1)
Insertional Inactivation	8.0×10^3	37.5%	3.5kb	0.92
Directional Cloning	7.6×10^3	60.0%	2.8kb	0.93

(1) Calculated as described in Chapter 2, taking the genome size of D. radiodurans to be 3×10^6 base pairs.

2.2.2 Cosmid Genomic Libraries

The cosmid pJBFH, a derivative of pJB8 (Ish-Horowicz and Burke, 1981), was chosen as an alternative vector system.

Construction of libraries

Two methods for cloning in cosmid vectors were employed; the first involved the restriction of cosmid pJBFH with BamHI, and restriction with MboI, and phosphatase treatment of, *D. radiodurans* Krase chromosomal DNA and the second, a more complex approach, involved the use of cosmid arms (Ish-Horowicz and Burke, 1981). The protocols for these methods have been given previously. The results of cosmid cloning experiments are presented in Table 3.14. Method 1 gave excellent transduction frequencies and a representation of the genome of over 99%. On the other hand, Method 2, had some inherent technical difficulties which resulted in a very low transduction frequency and attempts to improve this frequency were time consuming and unsuccessful.

Screening of libraries

Screening experiments were performed similar to those used with the plasmid vector genomic libraries and similar results were obtained i.e. no *E. coli* HB101 colonies which were Rf^r, Leu^+ or Pro^+ were found to contain recombinant molecules. For *D. radiodurans* genes to express in *E. coli*, it was concluded that an *E. coli* promoter sequence is required. In the case of the cosmid libraries, *E. coli* promoters are likely to be too far away from the relevant *D. radiodurans* genes.

Table 3.14

Construction of genomic libraries of D. radiodurans Krase DNA using the cosmid vector pJBFH.

Technique	Number of ampicillin resistant transductants μg^{-1} DNA	Average size of insert (kb)	Percentage representation
Method 1	8.6×10^4	35.0	>99.9%
Method 2	4.0×10^2	35.0	97.0%

3. RESTRICTION STUDIES ON CHROMOSOMAL DNA

3.1 RESTRICTION ANALYSIS OF GENOMIC DNA

The discovery that two members of the genus Deinococcus produced restriction endonucleases and therefore possessed host restriction and modification systems was unexpected, especially as D. radiodurans R1 had been reported to contain no biomethylated bases and no unusual bases (Schein, 1966; Schein et al., 1972; Störl et al., 1979). A second discovery was made during this project that D. radiodurans DNA appeared to be partially resistant to cleavage by certain restriction enzymes. As a result of both findings, three investigations were started: Firstly, to see if the observation that D. radiodurans DNA was resistant to cleavage by some enzymes could be extended to encompass other enzymes, and another Deinococcus spp. viz. D. radiophilus. Secondly, to see if D. radiodurans and D. radiophilus DNA gave similar restriction patterns when cloned into E. coli HB101. Thirdly, to search for methylated or unusual bases present in D. radiodurans.

Predicted number of restriction sites

Chromosomal DNA was isolated from D. radiodurans Krase and D. radiophilus by the modification of Marmur's (1961) method as outlined in Chapter 2. Restriction digests were performed by incubating samples of DNA with a number of restriction endonucleases. A scale, necessary to measure the degree of cleavage of DNA by a particular enzyme, was devised, based on figures calculated from an equation which predicts the average spacing of restriction sites for any given restriction endonucleases (Upholt, 1977; Moore and Moore, 1982). Three assumptions were made: cleavage sites occur randomly; DNA strands are long; and the occurrence of any given base pair is an independent event. The probability of occurrence P_g or P_c of the bases G or C is given by,

$$P_g = P_c = F_{gc}/2$$

and the corresponding probability of A or T as,

$$P_a = P_t = F_{at}/2$$

where F_{gc} and F_{at} are the respective mole fractions of GC and AT in the DNA. The factor 2 takes into account the fact that DNA is double stranded, with occurrence on either strand equally likely but mutually exclusive, thus,

$$F_{gc} + F_{at} = 1$$

The assumption of independence allows calculation of probability of any given sequence of bases as the product of independent probabilities e.g. for a restriction site consisting of M_{gc} base pairs of GC and M_{at} base pairs of AT then,

$$P = (F_{gc}/2)^{M_{gc}} \times (F_{at}/2)^{M_{at}}$$

where M_{gc} and M_{at} are the total number of base pairs in both strands of the recognition site. Then,

$$\text{Average Site Spacing} = {}^{1}/P$$

In addition,

$$\text{The Most Probable Number of Fragments} = P \times \text{Genome Size.}$$

Taking the GC ratio of D. radiodurans and D. radiophilus to be approximately 66% (a compromise between their respective values) and both genome sizes to be 3×10^6 base pairs, then calculations can be made giving the probability of a sequence in the genome, the most probable number of fragments and the average spacing of sites for any given restriction endonuclease. Table 3.15 gives the calculated results, determined for restriction enzymes that recognize tetra-and hexanucleotide sequences respectively.

TABLE 3.15

Probability of restriction sites, most probable number of fragments and average spacing of sites in D. radiodurans and D. radiophilus genomes with enzymes recognizing tetranucleotide (cases 1-3) and hexanucleotide sequences (cases 4-7).

Case	Characteristic Pairs	Example	Probability of a restriction site in genome	Most probable number of fragments	Average spacing of sites (kb)
1	2AT 0GC	AATT, no known example	7.4×10^{-4}	2.2×10^{3}	1.35
2	1AT 1GC	GATC, MboI	3.0×10^{-3}	8.9×10^{3}	0.34
3	0AT 2GC	CCGG, MspI	1.2×10^{-2}	3.6×10^{4}	0.08
4	3AT 0GC	TTTAAA, DraI	2.0×10^{-5}	6.0×10^{1}	49.5
5	2AT 1GC	GAATTC, EcoRI	8.1×10^{-5}	2.4×10^{2}	12.4
6	1AT 2GC	GGATCC, BamHI	3.2×10^{-4}	9.7×10^{2}	3.1
7	0AT 3GC	CCGCGG, MraI	1.3×10^{-3}	3.9×10^{3}	0.8

G+C content taken to be 66%.

Genome size taken to be 3×10^{6} bp.

Restriction Analysis

The results from a restriction analysis of D. radiodurans and D. radiophilus DNA using enzymes recognizing tetranucleotide sequences (Table 3.16) and hexanucleotide sequences (Table 3.17) were not as predicted. The first pattern to emerge related to the restriction analysis of D. radiodurans and D. radiophilus DNA using enzymes that recognize the sequence GATC or have GATC as the internal sequence of their recognition site. Enzymes MboI and Sau3A cleaved both DNAs to the same extent, whereas, DNA digested with DpnI showed no difference in migration on an agarose gel compared with undigested DNA. These results indicated that the adenine base of sequence GATC is not methylated in either D. radiodurans or D. radiophilus. Although MboI and Sau3A cleaved the DNAs, cleavage was much less than predicted from probability calculations, e.g. the average spacing of sites was predicted to be 0.34kb whereas some DNA fragments were as large as 20kb with most fragments being greater than 1kb. This apparent resistance of D. radiodurans DNA to cleavage by MboI when compared with the restriction analysis with other enzymes recognizing tetranucleotide sequences is shown in Fig. 3.3. This partial resistance to cleavage was extended to hexanucleotide-recognizing enzymes with GATC as the internal sequence, e.g. BamHI, BclI, BglII and XorII. Fig. 3.4 shows digestion patterns of D. radiodurans DNA with BamHI, BglII, XorII and HpaI compared with the pattern given by, and predicted for, EcoRI. The resistance of D. radiodurans DNA to cleavage by HpaI is discussed later. One explanation for the resistance to enzymes with GATC as the recognition sequence or GATC as the internal sequence of both DNAs would be that the sequence GATC is being used as a restriction sequence in these organisms as it is often used as such, e.g. in Bacillus, Diplococcus, Escherichia, Moraxella and Staphylococcus (Sussenbach et al., 1976). Isoschizomers HpaII and MspI and isoschizomers SmaI and XmaI gave identical patterns with D. radiodurans and D. radiophilus DNA. It was therefore concluded that methylation of the sequences CCGG and CCCGGG does not occur in either organism.

D. radiodurans DNA was not cleaved by SstII or SacII, both isoschizomers of the organism's own restriction endonuclease MraI. This result was not unexpected as in most cases the resistance of a bacterium's DNA to its own restriction enzymes can be generalised to include resistance to all other restriction enzymes which have the same sequence specificity. As expected, the restriction endonuclease isolated from D. radiophilus, DraI, did not cleave its own DNA. In both cases, this lack of cleavage was genuine, and not simply due to inhibition of enzyme action by a component in the digestion mix and was shown by lambda DNA added to the digestion mixes, being cleaved normally.

The reasons for D. radiodurans DNA being partially resistant to cleavage by ClaI and HpaI were less clear. These enzymes have the bases -A--T- in common in their recognition site but so do the enzymes PstI, SalI and XhoI and these give a normal cleavage pattern. On the other hand, PstI, SalI and XhoI cleave sequences possessing 1AT to 2GC pairs, whereas, ClaI and HpaI cleave sequences having 2AT pairs to 1GC and this might be significant. Resistance to cleavage by HpaI is shown in Fig. 3.4 where it can be seen that the average site spacing is >20kb whereas the predicted value is about 12kb. An explanation for this resistance to HpaI and ClaI digestion could be that D. radiodurans possesses a second restriction enzyme and that the sequence -A--T- is part of its recognition site.

TABLE 3.16

Restriction analysis of D. radiodurans Krase and D. radiophilus DNA using restriction endonucleases that recognize tetranucleotide sequences.

Enzyme	Recognition Sequence	Restriction Analysis	
		D. radiodurans	D. radiophilus
AluI	AGCT	+	+
DpnI	GATC	-	-
FnuDII	CGCG	+	+
HaeIII	GGCC	+	+
HhaI	GCGC	+	+
HpaII	CCGG	+	+
MboI	GATC	±	±
MspI	CCGG	+	+
Sau3A	GATC	±	±
TaqI	TCGA	+	+

+ Normal Cleavage

± Poor Cleavage

- No Cleavage

TABLE 3.17

Restriction analysis of D. radiodurans Krase and D. radiophilus DNA using restriction endonucleases that recognize hexanucleotide sequences.

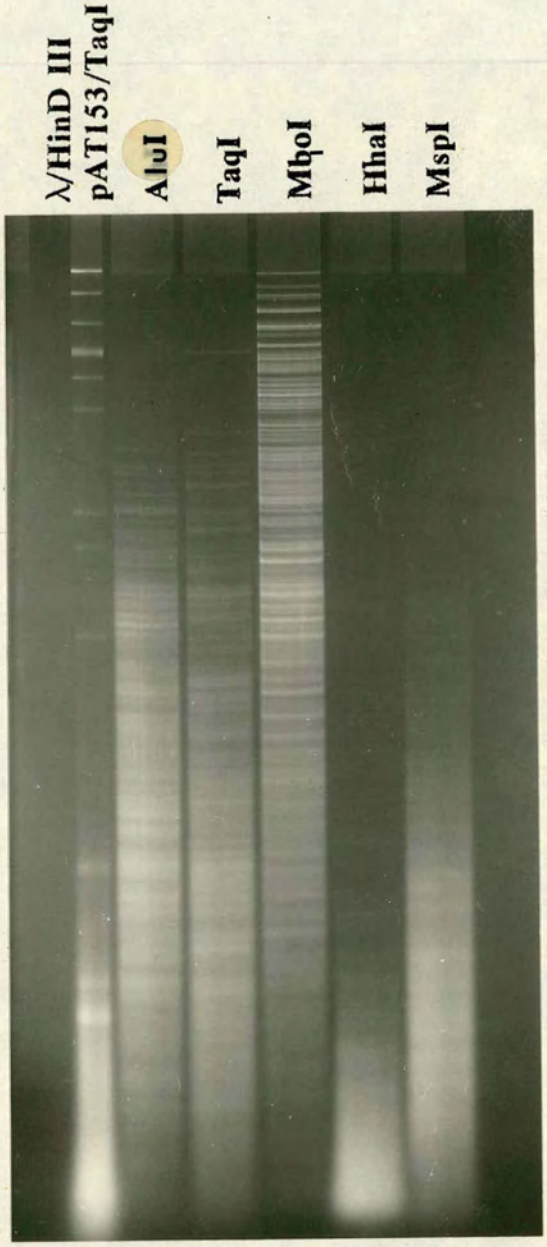
Enzyme	Recognition Sequence	Restriction Analysis	
		D. radiodurans	D. radiophilus
BamHI	GGATCC	±	±
BclI	TGATCA	±	±
BglII	AGATCT	±	±
BssHII	GCGCGC	+	+
ClaI	ATCGAT	±	+
DraI	TTTAAA	+	-
EcoR1	GAATTC	+	+
HinDIII	AAGCTT	+	+
HpaI	GTTAAC	±	±
KpnI	GGTACC	+	+
MluI	ACGCGT	+	+
NruI	TCGCGA	+	+
PstI	CTGCAG	+	+
PvuII	CAGCTG	+	+
SacII	CCGCGG	-	+
SalI	GTCGAC	+	±
SmaI	CCCGGG	+	+
SphI	GCATGC	+	+
SstI	GAGCTC	+	±
SstII	CCGCGG	-	+
XbaI	TCTAGA	+	±
XhoI	CTCGAG	+	+
XmaI	CCCGGG	+	+
XorII	CGATCG	±	±

+ Normal Cleavage

± Poor Cleavage

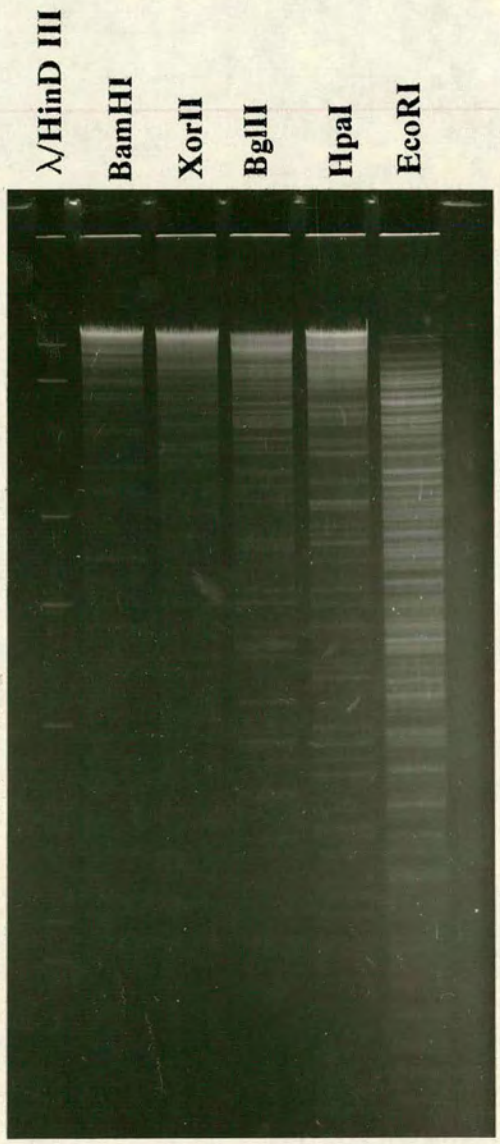
- No Cleavage

Fig. 3.3. Restriction analysis of *D. radiodurans* DNA with some enzymes recognizing tetranucleotide sequences.



2% agarose gel. 2 V cm^{-1} 17.5 hours

Fig. 3.4. Restriction analysis of *D. radiodurans* DNA with some enzymes recognizing hexanucleotide sequences.



0.8% agarose gel. 1Vcm^{-1} 48hours

D. radiophilus DNA was partially resistant to cleavage by HpaI and XbaI whose recognition sequences, GTTAAC and TCTAGA respectively, are only one pair of bases removed from the DraI recognition site, TTTAAA. Thus, rarity of DraI recognition sites could have an effect on HpaI and XbaI digestion patterns. The reasons for resistance of *D. radiophilus* DNA to SalI (GTCGAC) and SstI (GAGCTC) are less obvious, but one possibility is that the second restriction enzyme from *D. radiophilus*, DraII (I.J. Purvis, personal communication) has a similar sequence to either of these enzymes. Until the recognition sequence of DraII is resolved, this explanation will remain speculative.

3.2 RESTRICTION ANALYSIS OF CLONED GENOMIC DNA

In order to detect modified sequences in the DNA of *D. radiodurans* and *D. radiophilus*, fragments of their DNA were cloned into *E. coli* HB101, the rationale being that whatever modification of the DNA either organism possessed would be lost when cloned. Different restriction patterns would then be expected to result from digests of genomic DNA and cloned genomic DNA. Most of this work concentrated on *D. radiodurans* DNA cloned in cosmid vectors although results from *D. radiophilus* cloning experiments will also be reported.

Cloning *D. radiodurans* DNA into cosmid vectors

Large DNA fragments (up to approx. 40kb) from *D. radiodurans* Krase were cloned into the cosmid vector pJBFH and transduced into *E. coli* HB101. Recombinant molecules were then isolated and restricted with a number of enzymes. The cloning procedure (detailed in Chapter 2) resulted from an observation that a BamHI digest of *D. radiodurans* DNA gave a number of bands in the 30-45kb region and would be ideally suited for cosmid cloning. Highly efficient packaging of the DNA was achieved and this was reflected in 9×10^4 cosmid clones μg^{-1} DNA being obtained. Not surprisingly, many recombinant molecules from isolated clones were shown to have identical restriction patterns and were therefore formed

from the same BamHI fragment of D. radiodurans. However, ten clones were chosen which gave distinct cleavage patterns with BamHI, EcoRI and SstII.

Restriction analysis of D. radiodurans DNA

The number of fragments obtained with BamHI, EcoRI and SstII digestion of the recombinant molecules from the ten clones are summarised in Table 3.18 and the size of each D. radiodurans insert was measured and gave a combined length of 348kb. The restriction map of cosmid pJBFH possesses 1 BamHI site (the cloning site), 2 EcoRI sites, only a few base pairs either side of the BamHI cloning site (thus acting as only 1 site) and no SstII sites. These values were taken into account when calculating the number of restriction sites in the D. radiodurans inserted DNA. A second caveat related to the use of BamHI to digest vector and target DNA prior to cloning. This meant that if modification is disregarded then each recombinant should possess 2 BamHI sites. However, the BamHI digest of D. radiodurans DNA was not phosphatase treated prior to ligation and more BamHI sites could have resulted from non-contiguous fragments of target DNA joining together before ligating to the vector. A simple experiment showed that the extra sites found in clones 9 and 32 were probably due to non-contiguous fragments forming the insert and not to loss of a modified BamHI recognition sequence when cloned into E. coli. The experiment involved running BamHI restriction digests of clones 9 and 32 in parallel to a total BamHI digest of D. radiodurans DNA and matching the cloned fragments to genomic fragments. In both cases the cloned fragments had identical mobilities to genomic fragments and it was concluded that they were due to ligation of non-contiguous fragments.

From these results it appears that the low number of BamHI sites in D. radiodurans DNA was not caused by modification of the DNA but was due to under-representation of the recognition site. It was considered probable that BclI, BglII and XorII sites were also relatively rare and resulted from a lack of GATC sequences.

TABLE 3.18

Number of BamHI, EcoRI and SstII sites in ten recombinant clones constructed from ligation of a BamHI digest of D. radiodurans Krase DNA and a BamHI digest of cosmid pJBFH.

Clone	Size of insert (kb)	BamHI Sites in insert	EcoRI Sites in insert	SstII Sites in insert
7	32.4	2	5	2
9	33.6	3(1)	5	4
10	37.1	2	4	2
11	32.4	2	3	2
13	36.0	2	1	3
14	36.1	2	0	7
18	35.1	2	1	1
23	38.9	2	4	3
27	33.8	2	2	5
32	32.8	3(1)	2	5
Total number of sites in 348kb		22	27	34
Predicted number of sites in 348kb		112	28	435

(1) BamHI was used for cloning purposes, therefore extra site probably due to ligation of non-contiguous BamHI fragments. See text.

An interesting discovery was that SstII cleaved D. radiodurans DNA inserts when cloned into E. coli. In the combined length of cloned DNA (348kb) there were 34 SstII sites and this indicated that SstII recognition sequences were present in D. radiodurans genomic DNA but were modified so that they were resistant to SstII digestion. However, there were only 34 actual sites while the predicted value was over 400 (348 ÷ 0.8). It was concluded that SstII recognition sites were under-represented, in addition to being modified, in D. radiodurans DNA. The number of EcoRI sites in the cloned DNA was 27 and therefore similar to the predicted number of 28 sites.

Restriction analysis of D. radiophilus DNA

There were considerably fewer data for the restriction analysis of D. radiophilus cloned DNA than for D. radiodurans. However, one result emerged: The D. radiophilus plasmid, pUE1, was cloned into plasmid pAT153, forming the recombinant plasmid pUE109 and transformed into E. coli (G. Al Bakri, unpublished data). Plasmid pAT153 contains 3 DraI sites (Purvis and Moseley, 1983) while pUE1 isolated from D. radiophilus contains no DraI sites. However, a DraI digest of pUE109 from E. coli HB101 yielded 4 fragments, meaning that cloned pUE1 was cut once. This evidence suggested that D. radiophilus DNA, at least plasmid pUE1, contained DraI sites which were modified to protect against DraI digestion.

The two major conclusions drawn from these results were that the CCGCGG sequence of D. radiodurans DNA and the TTTAAA sequence of D. radiophilus DNA is modified in such a way as to protect the DNA from host restriction enzymes. The question remained - what is the nature of this modification in D. radiodurans and D. radiophilus?

3.3 ANALYSIS OF METHYLATED BASES IN *D. RADIODURANS* DNA

3.3.1 *Technique*

A modification of the Gruenbaum *et al.* (1981) technique for the analysis of nearest neighbour bases was used in order to determine whether any bases are methylated in *D. radiodurans* Krase DNA. Methylated bases cannot be determined by the conventional nearest neighbour analysis technique (Josse and Swartz, 1963), since in this technique only the *in vitro* synthesized nucleotides are analysed. Thus, wherever a methylated base is present in the native DNA, it will be exchanged by the unmethylated base in the newly synthesised, radioactively-labelled DNA. In order to determine methylated bases in *D. radiodurans* Krase DNA, randomly-placed nicks were inserted into the DNA with DNaseI and used as a primer for *E. coli* DNA polymerase I using one of the deoxy nucleotide triphosphates (dNTPs) labelled as α-^{32}P e.g. d[α-^{32}P]GTP. This experiment was repeated for each d[α-^{32}P]NTP. After digestion to nucleoside -3'-monophosphates only the nucleotide on the 5'-side of the nick will be labelled e.g. in the case of d[α-^{32}P]GTP, after digestion the ^{32}P label is transferred to the nearest neighbour of G on the 5'-side. This means that if the DNA contains the four normal bases, then the technique should yield four spots upon chromatography. If, however, methylated bases or unusual bases are present, a corresponding number of spots will be visualised. Fig. 3.5 shows a diagrammatic representation of observed separation of standard dNMPs following chromatography.

3.3.2 *D. radiodurans* analysis

The results of analysis of *D. radiodurans* Krase DNA are presented in Fig. 3.6. Experiments using d[α-^{32}P]ATP or d[α-^{32}P]TTP gave identical autoradiograms, both having spots, in the position of the four normal bases, upon chromatography. Whereas, experiments using d[α-^{32}P]GTP or d[α-^{32}P]CTP gave different results. Firstly no 5 methylcytosine, 6 methyladenine or 7 methylguanine spots were observed with either labelled base. However, in addition to the four expected mononucleoside

phosphates, several other spots were seen; when d[α-^{32}P]GTP was used, there were four additional spots which ran close to A, one spot just ahead of G and one spot ahead of C. When d[α-^{32}P]CTP was used, there was one additional spot ahead of C in a similar position to the spot observed when dGTP was labelled.

Considering the evidence for DNA modification in this organism it would seem reasonable that at least some of the additional spots could be due to an unusually modified base e.g. methylated at an unusual position. However, the possibility exists that the additional spots may be due to artefacts of the procedure e.g. hydrolysis products of the mononucleotides. However, the spots only occurred when labelled dGTP or dCTP was used and they were reproducible in repeat experiments.

Fig. 3.5. Separation of deoxynucleotide monophosphates on two-dimensional TLC. Diagrammatic representation of observed separation of standard d NMP's following chromatography on TLC plates using iso butyric acid:water:ammonia (66:20:1) in the first dimension and saturated ammonium sulphate:propan-2-ol:1M sodium acetate (40:1:9) in the second dimension.

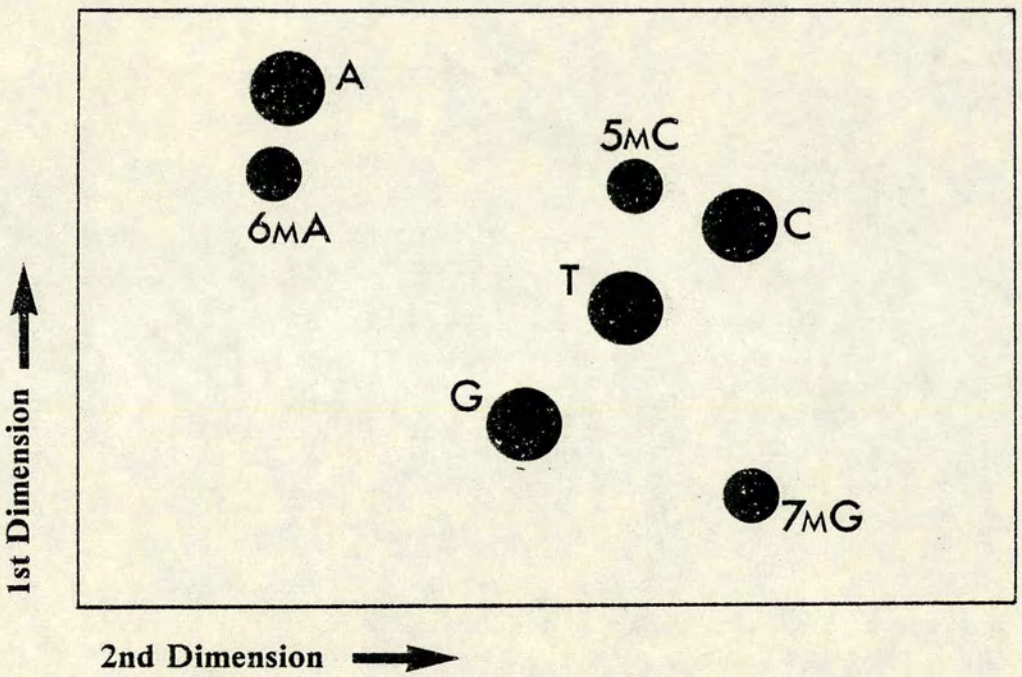
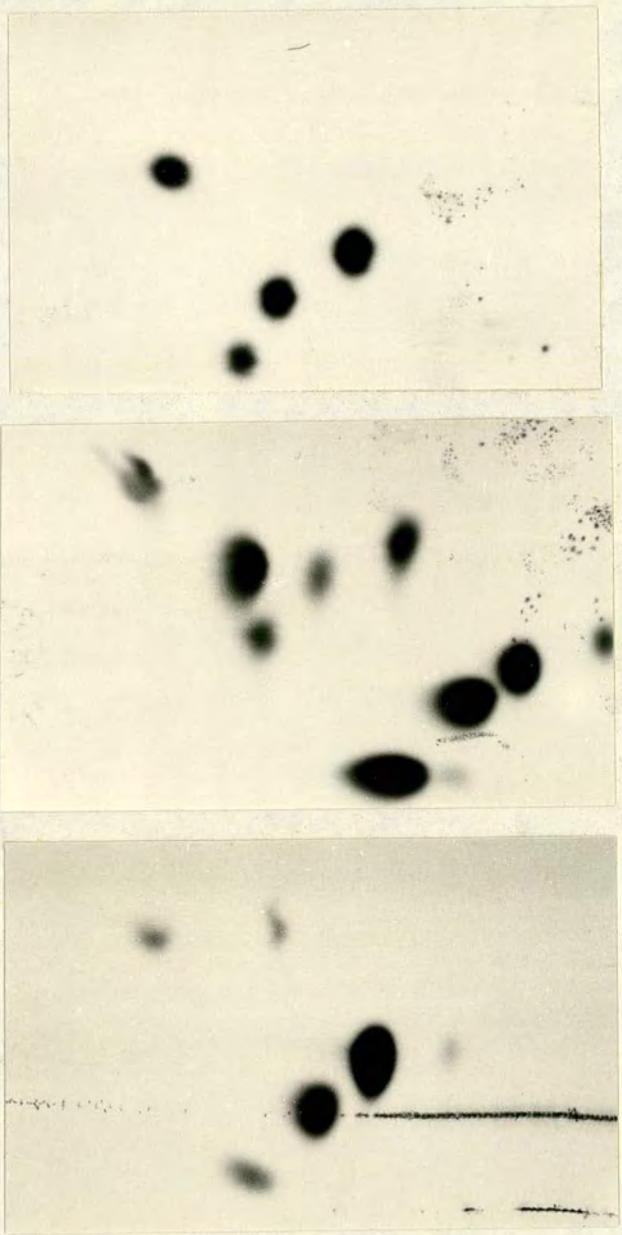


Fig. 3.6. $3^{1}\alpha$-^{32}P labelled nucleotide monophosphates were chromatographed under the same conditions as those used for the separation of standard NMP's (Fig. 3.5)

1. Autoradiogram of 3^{1}dNMP's following nick translation in the presence of d[α-^{32}P] ATP

2. Autoradiogram of 3^{1}dNMP's following nick translation in the presence of d[α-^{32}P] GTP

3. Autoradiogram of 3^{1}dNMP's following nick translation in the presence of d[α-^{32}P] CTP



CHAPTER 4

DISCUSSION

1. PLASMIDS IN DEINOCOCCUS SPP.

Plasmids have been observed in representatives of virtually every bacterial genus examined for their presence. It was not surprising, therefore, to find plasmids in all four Deinococcus spp. However, it was unexpected that one strain, D. radiodurans R1, in spite of an extensive search, did not yield plasmids. It is possible that this strain harbours very large plasmids and that the variety of techniques used were not gentle enough to allow their isolation. However, one protocol attempted in this study, the Hansen and Olsen (1978) method was specifically devised to enable the isolation of very large plasmids and has been used successfully with many bacterial genera. More likely then is that the laboratory cultures of D. radiodurans R1 used in this study were plasmidless, which suggests that the native plasmids do not play a significant role in the characteristic radiation-resistance shown by these organisms.

The occurrence and distribution of the different plasmid profiles in Deinococcus spp., apparently did not correlate with any evolutionary relationships the respective isolates may have. Only plasmids pUE2 and pUE31 from D. radiophilus and D. radiopugnans respectively, shared a similar size and even then it is not known if these plasmids are identical. However, this disparity in sizes does not preclude the possibility of significant homology between native plasmids and it may simply reflect the contrasting environmental ecology of the hosts' isolation source.

To date, the Deinococcus plasmids have remained cryptic. Whether this is a genuine result, i.e. the plasmids code only plasmid functions, or whether the inadequacies of the techniques used to ascribe a phenotypic trait are at fault remains unknown. However, the presence of plasmids in all four Deinococcus spp. allied to the relatively large size of the respective molecules, suggests that they play a role in the survival of the organisms. Of course the 2.5kb

plasmid of D. radiopugnans, pUE30, may be similar to p15A, a small minimally encumbered E. coli plasmid (Cozzarelli et al., 1968) and may represent an efficient plasmid replicon possessing functions only to ensure its own replication and perhaps to facilitate transmission to other cells. Whilst this hypothesis is appealing for small plasmids its relevance is doubted for the large plasmids of the group.

The failure to cure D. radiodurans Sark and D. radiophilus of their plasmids is not unique as many workers have encountered such difficulties, e.g. with Erwinia stewartii (Coplin et al., 1981) and Caulobacter spp. (Schoenlein and Ely, 1983). However, considering the range of potential curing agents and methods used in this study and the assiduous screening for plasmidless variants or clones with missing plasmids, it was surprising that neither species were cured of any plasmid. These results raise doubts about the widely held belief that plasmids only carry optional functions and the likelihood arises that D. radiodurans (Sark) and D. radiophilus plasmids carry essential function, the loss of which result in cell death.

In summary, three main points arose from this part of the thesis,

1. All four Deinococcus spp. harbour plasmids.
2. D. radiodurans R1 does not harbour plasmids.
3. D. radiodurans Sark and D. radiophilus were not cured of their plasmids by conventional agents.

Future research should be aimed at the physical characterisation of the plasmids e.g. restriction mapping and sequence homology studies. The D. radiophilus plasmid, pUE1, has already been cloned (G. Al Bakri, unpublished data) and a restriction map constructed.

2. DEVELOPMENT OF A GENE-CLONING SYSTEM

Gene-cloning systems developed for bacteria have been largely facilitated by the realization that the same plasmids can function in many bacterial species e.g. in different species of Gram-negative bacteria (for a review, see Reanney, 1976), and this ability represents the foundation for genetic exchange amongst diverse species. However, the foreign plasmid vectors used in this study did not express any markers in *D. radiodurans*. Three main factors could account for this situation, viz. foreign DNA may not be taken up by *D. radiodurans*, foreign DNA may be degraded on entry to the cell by a host restriction endonuclease and expression of the foreign genes is repressed because *D. radiodurans* is unable to transcribe them. Other attempts to transform *D. radiodurans* using hybrid plasmids, constructed with plasmid vector and host chromosomal DNA, similarly failed and the same explanations for lack of expression could apply in this case. The fact that an enzyme has been isolated from *D. radiodurans* R1 (Wani *et al.*, 1981), that degrades foreign DNA but does not degrade host DNA, suggests that a restriction and modification system may be of major significance when considering the failure of foreign gene expression in *D. radiodurans*.

Experiments aimed at developing a novel cloning vector, derived from *D. radiodurans* Sark plasmid and chromosomal DNA, failed, in that no host marker was expressed. Nevertheless, future attempts to develop a cloning system for *Deinococcus spp.* will almost certainly involve the use of native plasmids as cloning vectors. For example, native plasmids could be genetically marked by *in vivo* or *in vitro* insertion of a transposon, a technique used to mark cryptic plasmids of *Anacystis nidulans* (van den Hondel *et al.*, 1980). Another strategy would involve the construction of a shuttle plasmid that could transfer between *E. coli*, for example, and a *Deinococcus spp.* This method is now widely used for many bacterial species e.g. between *A. nidulans* and

E. coli (Kuhlemeir et al., 1981), between B. subtilis and E. coli (Primrose and Ehrlich, 1981) and between S. sanguis and E. coli (Macrina et al., 1980). Attempts should also be made to isolate restrictionless mutants of D. radiodurans that could allow the transformation, by foreign vectors, of D. radiodurans.

The alternative course used for studying D. radiodurans gene expression involved the construction of D. radiodurans genomic libraries in E. coli plasmid and cosmid vectors. The construction of a cosmid library was particularly successful and had the advantage, over a plasmid library, that large DNA fragments of D. radiodurans were carried on the vector. This enabled relatively easy screening, for expression of D. radiodurans genes in E. coli and allowed more manageable storage of the library. The direct screening methods were largely unsuccessful and this was surprising as many foreign genes have been shown to express in E. coli e.g. B. subtilis leucine genes (Nagahari and Sakaguchi, 1978), Streptococcus mutans aspartate-semialdehyde dehydrogenase genes (Jagusztyn-Krynicka et al., 1982), Achromobacter sp. β-lactamase genes (Levesque and Roy, 1982) and Clostridium thermocellum cellulase genes (Cornet et al., 1983). However, these results did not preclude the expression of D. radiodurans genes in E. coli as logistical problems could have accounted for the lack of expression. The major question of whether D. radiodurans genes can express in E. coli could be resolved by the use of mini-cells or maxi-cells and these techniques would have the advantage that the function of particular genes need not be known. If D. radiodurans genes are not able to express in E. coli, it may be due to E. coli being unable to transcribe D. radiodurans promoter regions and therefore requiring E. coli promoters. This problem could be circumvented with plasmid genomic libraries constructed in expression vectors e.g. in ptrpL1 (Edman et al., 1981).

Although this study did not result in the development of a gene-cloning system for D. radiodurans, several positive aspects emerged. For example, the discovery that D. radiodurans DNA is amenable to basic gene

manipulation techniques, such as restriction and ligation to foreign DNA, is a foundation for further studies. In addition, the ease with which D. radiodurans gene libraries were constructed in both plasmid and cosmid vectors of E. coli will undoubtedly facilitate further investigations.

3. RESTRICTION STUDIES ON CHROMOSOMAL DNA

An observation, made during the course of this project, that both *D. radiodurans* R1 and *D. radiophilus* DNA was resistant to, or partially resistant to, a number of restriction endonucleases, led to a restriction analysis being made of both genomic DNA and cloned genomic DNA from both organisms. From the results, attempts were made to define the nature of DNA modification in *D. radiodurans* R1. The major findings of this study were;

1. The sequence GATC is under-represented in both *D. radiodurans* and *D. radiophilus* DNA: Enzymes whose recognition sequence is, or included, GATC as the internal sequence, were shown to cleave DNA from both organisms much less than expected and this partial resistance extended to *D. radiodurans* DNA cloned into *E. coli*. In addition, digests of DNA from either organism with MboI, DpnI and Sau3A indicated that the adenine base of GATC was not methylated.

It was stated previously that the sequence GATC is often used in restriction systems and the possibility arises that this sequence plays such a role in *D. radiodurans* and *D. radiophilus*. However, neither organisms DNA was completely resistant to an enzyme with GATC in its recognition sequence (excepting DpnI, that only cleaves methylated DNA) and this casts some doubt on the restriction role.

2. The sequence CCGCGG is modified in *D. radiodurans* R1: SstII and SacII, isoschizomers of the *D. radiodurans* R1 restriction enzyme, MraI (Wani *et al.*, 1981), did not cleave *D. radiodurans* DNA but did

cleave the same DNA when cloned into E. coli. This provided major evidence that this recognition sequence is modified.

3. The sequence TTTAAA is modified in D. radiophilus: DraI, isolated from D. radiophilus (Purvis and Moseley, 1983) only cleaved D. radiophilus DNA that had been cloned into E. coli.

The last two findings both conform to the generally held views that DNA isolated from a strain producing a restriction enzyme is not cleaved by its own restriction enzyme and generally not cleaved by isoschizomers of that restriction enzyme (Brooks and Roberts, 1982). Speculation concerning the nature of DNA modification is particularly interesting for D. radiodurans because previous reports (Schein, 1966; Schein et al., 1972; Störl et al., 1979) had failed to detect any unusual or modified bases in the DNA of D. radiodurans and in one report (Schein et al., 1972), no DNA methyltransferase activity was detected. A search for 5 methylcytosine, 6 methyladenine and 7 methylguanine in D. radiodurans failed to detect any of these methylated bases. However, other bases, with unusual modifications, may have been present, as additional 'bases' were isolated on DNA analysis using TLC. The identity of these unusual 'bases' was not revealed during the course of the study and may have been due to breakdown products of normal bases, i.e. were artefacts of the technique. However, the presence of these additional 'bases' was reproducible and they were isolated only when specific radioactively-labelled nucleotide monophosphates were used i.e. d[α-^{32}P]G and d[α-^{32}P]C. The fact that the recognition sequence of MraI contains only cytosine and guanine may be significant. Further analysis of D. radiodurans DNA is necessary to determine the nature of DNA modification and may necessitate the use of more sensitive techniques such as HPLC. A similar approach could be used to study the nature of D. radiophilus DNA modification and thus compare the respective modes of modification. It is known that bacteriophages possess multiple mechanisms for avoiding DNA restriction systems of their hosts (for

a review see Krüger and Bickle, 1983) e.g. phage Mu contains acetimidated adenine which protects the DNA against several restriction endonucleases (Hattman, 1979) and it is possible that D. radiodurans and D. radiophilus possess unusual forms of DNA modification. Thus, these organisms may not adhere to the traditional concept that bacterial DNA is only modified as a result of cytosine or adenine methylation.

4. Cytosine methylation does not occur in the DNA sequences CCGG or CCCGGG of D. radiodurans and D. radiophilus: Pairs of isoschizomers MspI, HpaII and SmaI, XmaI cleaved D. radiodurans and D. radiophilus DNA as expected and to the same extent, thus showing that neither site was methylated at the cytosine residues.

5. Enzymes ClaI and HpaI cleaved D. radiodurans DNA less than expected: The possibility arises that the recognition sequences of these enzymes, viz. ATCGAT and GTTAAC respectively, may be similar to, or play a role in, another restriction enzyme from this organism.

6. Enzymes SalI and SstI cleaved D. radiophilus DNA less than expected: The same explanation, as given above, could apply here particularly as another restriction enzyme, DraII (I.J. Purvis, personal communication), has been isolated from this organism.

Resolution of the last two findings could be achieved by isolation of further enzymes from D. radiodurans and determination of the recognition sequence of DraII.

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