GENETIC STUDIES ON *DEINOCOCCUS* spp. USING SPHEROPLASTS AND GENE CLONING

GHALIB H. AL-BAKRI

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SUMMARY

A protocol has been developed for making spheroplasts from Deinococcus radiodurans and D.radiophilus and conditions optimised for their regeneration. Regeneration frequencies for D.radiodurans spheroplasts ranged from 10 to 20%, while those for D.radiophilus ranged from 2 to 3%. Experiments to demonstrate fusion between spheroplasts of different mutants of D.radiodurans, attempts to transform spheroplasts of either D.radiodurans or D.radiophilus with homologous chromosomal or foreign plasmid DNAs, and efforts to cure D.radiophilus of any of its cryptic plasmids using the spheroplast formation and regeneration technique were all unsuccessful.

In an attempt to develop a cloning and/or shuttle vector for the genus Deinococcus, plasmid pUE1 (10.72kb) of D.radiophilus was cloned into the vector pAT153 and propagated in Escherichia coli HB101. A restriction map of this hybrid plasmid, pUE109, was constructed. The resistance of E.coli HB101 to ultraviolet (UV) radiation was not increased when pUE109 was introduced into it. Attempts to transform Deinococcus spp. with pUE109 were unsuccessful.

Genes were identified and selected from a gene library of D.radiodurans constructed in the cosmid pJBFH, by complementing suitable mutations in E.coli or D.radiodurans. A 51.5kb hybrid cosmid, pUE40 that transduced E.coli from leucine dependence to independence was selected, and a 3.5kb fragment which carried the leuB gene was subcloned into pAT153. Using the maxi-cell technique, the product of the cloned leuB gene was identified as a protein of approximate molecular weight 45500. An asp gene and a trp gene were identified by transforming appropriate auxotrophic mutants of D.radiodurans. The trp gene was subcloned as a 5.5kb fragment into

pAT153, but did not complement the defect in any of five different trp mutants of E.coli.

The DNA repair genes mtcA, mtcB, uvsC, uvsD, uvsE, required for the activity of two D.radiodurans UV endonucleases, were identified by transforming appropriate repair-deficient mutants of D.radiodurans to repair proficiency with DNA derived from the gene library. Hybrid cosmid pUE50 (37.9kb) which carried mtcA and mtcB was selected and 5.6 and 2.7kb DNA fragments carrying mtcA and mtcB respectively were subcloned into pAT153. The size of the insert carrying both genes was reduced to 22.0kb. The three genes, uvsC, uvsD and uvsE, were all present in the 46.0kb hybrid cosmid pUE60. The uvsE gene in a 12.2kb fragment was subcloned into pAT153 and further reduced to 6.1kb. The uvsC, uvsD, uvsE and mtcA genes were found in the 52.5kb hybrid cosmid pUE70. Thus the five DNA repair genes must all be located within a 71.0kb fragment of the D.radiodurans genome. None of the uvs genes introduced into the UV-sensitive E.coli CSR603 (uvrA) was able to complement its repair defect. No translational products of mtcA or mtcB were detected in maxi-cells. The 6.1kb fragment carrying the uvsE gene coded for two proteins of approximate molecular weight 45000 and 47000.

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CHAPTER 1

GENERAL INTRODUCTION

1. THE GENUS DEINOCOCCUS

1.1 Isolation and classification

Deinococcus radiodurans (formerly Micrococcus radiodurans), the type species of the genus Deinococcus, was originally isolated in Oregon from canned meat which had received 2 to 3 Mrad of gamma radiation (Anderson et al., 1956). The very high resistance to ionizing radiation of this Gram-positive (G +ve), non-sporing, red-pigmented, spherical bacterium was later confirmed with pure cultures on agar which required doses of 5 to 6 Mrad for sterilisation and this led to the bacterium being designated Micrococcus radiodurans (Anderson et al., 1961). The extreme resistance of this organism to ultraviolet (UV) light was demonstrated later (Duggan et al., 1959). Another strain of M.radiodurans was isolated in Ontario, Canada, as an aerial contaminant in a hospital (Murray and Robinow, 1958) and was later designated M.radiodurans Sark. A third isolate was obtained from irradiated sawdust in Japan (Ito, 1977). Several other red-pigmented micrococci have been isolated from various gamma irradiated materials. M.roseus (ATCC 19172) was isolated from irradiated haddock tissue (Davis et al., 1963), M.radiophilus from Bombay duck (Lewis, 1971) and M.radioproteolyticus from the irradiated faeces of a llama (Kobatake et al., 1973). All were shown to have properties similar to those of M.radiodurans. Originally, these species were classified under the genus Micrococcus but subsequent studies on the structure and composition of the cell envelope (Thornley et al., 1965; Work and Griffiths, 1968; Sleytr et al., 1973; Lancy and Murray, 1978; Baumeister and Kubler, 1978; Thompson and Murray,

1981), fatty acid components (Knivett et al., 1965; Work, 1970; Girard, 1971) and phospholipid components (Thompson et al., 1980) showed that M.radiodurans is a highly unusual organism in that its cell wall is not that of a typical G +ve coccus and that it has some features in common with the walls of Gram-negative (G -ve) bacteria. These and other unique features led to the suggestion that M.radiodurans should be reclassified into a new taxon (Baird-Parker, 1965; Thornley et al., 1965; Sleytr et al., 1973). A comparative study of 16S ribosomal RNA sequences (Brooks et al., 1980) finally showed that a separate genus for the red-pigmented, radiation-resistant micrococci was required. Brooks and Murray (1981) proposed Deinococcus as a generic name and assigned four species to the genus: D.radiodurans (type species), D.radiopugnans (formerly M.roseus ATCC 19172), D.proteolyticus (formerly M.radio-proteolyticus) and D.radiophilus (formerly M.radiophilus).

1.2 Cultural characteristics

Tryptone-glucose-yeast extract (TGY) broth is used to grow strains of Deinococcus, apart from D.radiophilus which is grown in nutrient broth, at 30°C with aeration. Under such conditions, D.radiodurans has a doubling time of about 80 min. On TGY agar, colonies can be counted after incubation at 30°C for two days. In liquid cultures, D.radiodurans is rarely observed as a single cell. The majority of cells are in a diplococcus form with less that 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

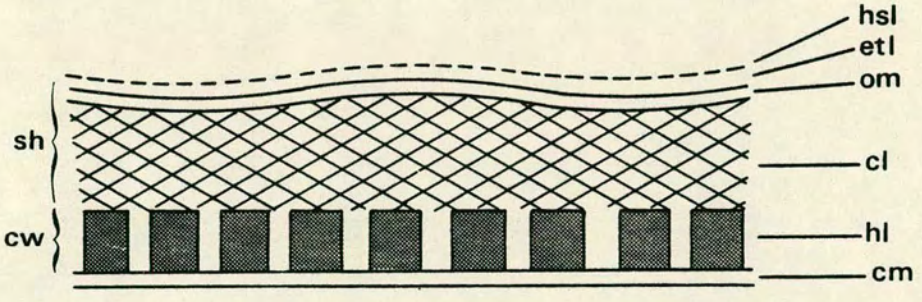
(Murray and Robinow, 1958; Dreidger, 1970; Moseley and Copland, 1975a).

1.3 Cell envelope

The cell envelope of both strains of D.radiodurans, R1 (Anderson strain) and Sark, is most unusual in terms of both its structure and composition. It has a multilayered structure which differs from those of any bacteria described. The cell envelope consists of at least four layers exterior to the cytoplasmic membrane (Fig. 1.1). The innermost layer contains peptidoglycan and appears to be perforated (the holey layer). The next layer appears to be divided into compartments (the compartmentalised layer). The third layer is the outer membrane, while the outermost layer consists of regularly arranged ring-shaped subunits linked in a hexagonal array by fine fibrils (the hexagonal subunit layer). The last two layers are separated by an electron-transparent zone (Thornley et al., 1965; Work and Griffiths, 1968; Sleytr et al., 1973; Lancy and Murray, 1978; Thompson and Murray, 1981). Only the holey layer is involved in septum formation during cell division. The other layers are regarded as a sheath, since they surround groups of cells and form on the surface of daughter cells as they separate (Thornley et al., 1965; Work and Griffiths, 1968; Sleytr et al., 1973). The sheath has a more elaborate structure than normal capsules, although it has some features in common with the sheath of the blue-green algae (Ris and Singh, 1961). Such a cell wall structure is completely different from that of a typical G +ve bacterium, e.g. Staphylococcus aureus or Bacillus licheniformis (Millward and Reaveley, 1974), which has a single homogeneous

Fig.1.1

Diagram of the cell envelope layers of Deinococcus radiodurans based on electron micrographs of the cell envelope of D.radiodurans Sark (Lancy and Murray, 1978). (cm) cytoplasmic membrane, (hl) holey layer (peptidoglycan layer), (cl) compartmentalised layer, (om) outer membrane, (etl) electron transparent layer, (hsl) hexagonal subunit layer, (sh) sheath, (cw) cell wall.



thick component external to the plasma membrane, and also different from that of a typical G -ve bacterium, e.g. Escherichia coli which usually consists of two layers external to the plasma membrane, the peptidoglycan layer and the outer membrane (Burdett and Murray, 1974).

In terms of chemical composition, D.radiodurans is an atypical G +ve, showing some features corresponding to those of G -ve bacteria, but also having some unique features. The peptidoglycan layer which is responsible for the rigidity of the cells contains L-ornithine, which occurs very rarely in bacterial cell walls, as the principle diamino acid, instead of the more usual diamino pimelic acid or lysine (Work, 1964; Work and Griffiths, 1968; Schleifer and Kandler, 1972). Like G -ve bacteria, the cell envelope of D.radiodurans contains lipopolysaccharides, but they are not those characteristic of G -ve bacteria since heptose is not present (Work and Griffiths, 1968; Brooks et al., 1980). The fatty acid content of the cell wall which is predominantly palmitoleate, suggests some alliance of Deinococcus spp. to G -ve bacteria (Brooks et al., 1980). However, in contrast to nearly all G -ve bacteria, D.radiodurans contains little or no oleic acid (Girard, 1971). Also, D.radiodurans has none of the conventional phospholipids found in bacteria (Thompson et al., 1980).

1.4 Radiation resistance

In addition to their extreme resistance to the lethal effects of ionizing radiation, the deinococci can withstand very high levels of UV radiation without loss of viability (Tempest, 1978). They also show resistance to the mutagenic effects of both these

forms of radiation and other damaging agents (Sweet and Moseley, 1974, 1976; Kerszman, 1975; Tempest and Moseley, 1982). This resistance to both the lethal and mutagenic effects of ionising and UV radiations has been the main reason for studying this group of bacteria.

Early explanations for this resistance centred upon possible intracellular radioprotective compounds such as the carotenoid pigments (Kilburn et al., 1958) or the high sulphydryl content (Bruce, 1964). The high concentration of manganese around the DNA of D.radiodurans was another possible source of radioprotection (Leibowitz et al., 1976). Although these factors may contribute to the extreme resistance of this group, they would seem to be of minor importance since efficient DNA repair systems appear to be the major reason for tolerance of radiation. It has been shown that D.radiodurans possesses only two mechanisms for the repair of UV-irradiated DNA: excision repair (Boling and Setlow, 1966) and recombination repair (Moseley et al., 1972; Moseley and Copland, 1975b). The excision repair process, in which UV induced pyrimidine dimers are removed from the DNA of D.radiodurans and excreted in the form of small oligonucleotides into the suspending medium, can proceed through two distinct, equally efficient pathways (Moseley and Evans, 1983). One pathway requires functional mtcA and mtcB genes, while the other pathway requires functional uvsC, uvsD and uvsE genes. Only when both pathways are blocked by mutations are the bacteria incapable of excising pyrimidine dimers, although strains blocked only in mtcA or mtcB are mitomycin C sensitive (Moseley and Copland, 1978). The error-prone repair of UV-induced damaged DNA of the type observed in E.coli (Witkin, 1976), 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 (Moseley and Laser, 1965).

1.5 Genetics and DNA content

A thorough genetic analysis of radiation resistance in this group of bacteria is limited by the fact that transformation using homologous chromosomal DNA is, so far, the only method available for genetic exchange in the two strains of D.radiodurans (Moseley and Setlow, 1968; Tirgari and Moseley, 1980) while none is available for the other three species. Although transformation has been useful for investigating the mechanisms that confer resistance to lethal and mutagenic damage in D.radiodurans, e.g. Moseley and Copland (1978), it has extreme limitations when used as a means of mapping chromosomal genes. In spite of a long search, no phages have been isolated that plaque on Deinococcus spp., and hence no transduction system is available, while all attempts to develop a conjugation system has likewise been unsuccessful (Moseley, 1983; I. Masters, personal communication). Attempts to mobilise the chromosome by introducing plasmids from other species of bacteria have been unsuccessful (Tirgari, 1977; Mackay, 1983; Purvis, 1984).

Four type II restriction endonucleases have been isolated from members of the genus Deinococcus. MraI has been isolated from D.radiodurans R1 (named when the organism was known as M.radio-

durans) (Wani et al., 1982) and was shown to be an isoschizomer of SacII and SstII which both recognise the DNA sequence 5'-CCGC↓GG-3'. DraI isolated from D.radiophilus (Purvis and Moseley, 1983) recognise the sequence 5'-TTT↓AAA-3' and is an isoschizomer of AhaIII (Whitehead and Brown, 1982). Another two enzymes DraII and DraIII have been isolated from D.radiophilus and their recognition sequences are 5'-PuG↓GNCCPy-3' and CACNNN↓GTG respectively (Grosskopf et al., 1985). However, close examination of the chromosomal DNA of these two species indicated a lack of any methylation modification normally associated with restriction/modification systems (Schein et al., 1972; Störl et al., 1979; Mackay, 1983).

The presence of multiple, independently-segregating genome equivalents per viable unit of D.radiodurans has been demonstrated and the genome size was calculated to be $3x10^6$ base pairs (Hansen, 1978; Tirgari and Moseley, 1980; Moseley and Evans, 1981). Similarly, other members of the genus have been shown to possess multiple genome copies (I.J. Purvis and C.A.M. Duncan, personal communication). There is no evidence that such a genome arrangement aids the repair to DNA damage and, in fact, some UV-sensitive bacterial species, e.g. Micrococcus luteus, Micrococcus sodonensis (I.J. Purvis and B.E.B. Moseley, personal communication) and Azotobacter vinelandii (Sadoff et al., 1979) also contain multiple genome copies per cell. Harsojo et al. (1981) found that D.radiodurans shows no direct correlation between a genome copy number and increased radiation resistance.

Plasmid molecules are present in all strains of the genus Deinococcus apart from D.radiodurans R1 (Mackay et al., 1985). There are three size-classes of plasmid in D.radiophilus, while in D.radiodurans Sark, D.proteolyticus and D.radiopugnans there are

two. Their sizes range from 2.5 kb (kilobase pairs) (pUE30 of D.radiopugnans) to 139 kb (pUE21 of D.proteolyticus). It was not possible to ascribe any characteristics of the bacteria to the possession of plasmids but the failure to cure either D.radiophilus or D.radiodurans Sark of any of their plasmids may indicate that these plasmids carry genes for essential functions, the loss of which results in cell death (Mackay et al., 1985).

1.6 Aims of the project

The first aim was to start protoplast genetic studies in the genus Deinococcus by developing suitable procedures for protoplast formation and regeneration of cell walls in order to

i) Develop a protoplast fusion system for D.radiodurans

ii) Transform protoplasts with both plasmid and chromosomal DNA

iii) Cure plasmids by regenerating protoplasts.

The second aim was to use one of the native plasmids in order to develop a cloning vehicle for this genus, which would be helpful in cloning genes of interest.

A third aim was to select various genes, especially those involved in DNA repair, as large DNA fragments, from a gene library constructed from D.radiodurans in a cosmid vector (Mackay, 1983), and to determine their expression in E.coli.

2. BACTERIAL PROTOPLAST GENETICS

2.1 Introduction

Although the first observation on bacterial protoplasts (cells completely deprived of their wall) and their fusion, goes back to 1925 (Mellon, 1925), meaningful genetic studies employing them are quite recent. Fodor and Alföldi (1976) and Schaeffer et al. (1976) reported the first complementation of bacterial auxotrophic mutants by controlled protoplast fusion and since that time the field of bacterial protoplast genetics has expanded rapidly, and many data have been accumulated in both basic and applied areas of research. Results on protoplast fusion and plasmid transfer by protoplast fusion, transformation of protoplasts with plasmid and chromosomal DNA, loss of plasmid by regeneration of protoplasts and protoplast transfection, obtained in the past few years have indicated clearly the possibilities and importance of this new field of genetics.

2.2 Protoplast formation and regeneration

For any microbial protoplast genetics experiment suitable procedures need to be developed both for generating protoplasts and for creating the conditions required for converting protoplasts back to wall-bearing vegetative cells.

Bacterial protoplast genetic studies have been applied more successfully to G +ve species than to G -ve ones, since it is relatively easy to prepare protoplasts from the former. Most G +ve bacteria, especially in the genus Bacillus can be converted to protoplasts by a simple lysozyme treatment (Fodor and Alföldi, 1976; Schaeffer et al., 1976). Staphylococcus can be converted

into protoplasts using lysostaphin (Novick et al., 1980). In the case of Clostridium (Allcock et al., 1982) and Streptomyces (Sagra et al., 1971; Okanishi et al., 1974; Hopwood et al., 1979) preconditioning the cells by growing them in medium containing glycine renders them more sensitive to lysozyme. The presence of the outer membrane in G -ve bacteria makes their conversion into true protoplasts very difficult, and usually a part of the cell wall may not be completely dissolved by lysozyme and the additive compounds, so that the membrane, at least partially, will be covered with wall material. For this reason the term "spheroplast" is used when complete removal of the cell wall is not demonstrated. Weiss (1976) has described a procedure for converting E.coli to true protoplasts using a combination of lysozyme and ethylenediaminetetra-acetic acid (EDTA). Spheroplasts were prepared from Providence alcalifaciens using glycine-lysozyme-EDTA treatment which resulted in exposing areas of the cytoplasmic membrane through breaks in the outer membrane (Coetzee et al., 1979).

Successful regeneration of the protoplasts into viable vegetative cells is essential in order to give progeny capable of being characterised genetically and used for further studies. Although some useful generalisations can be discerned, different nutritional and other conditions are needed for optimal regeneration even in quite closely-related species. Materials such as gelatin, serum bovine albumin and casamino acids are often used to enhance regeneration (Landman et al., 1968; De Castro-costa and Landman, 1977; Gabor and Hotchkiss, 1979; Akamatsu and Sekiguchi, 1981; Allcock et al., 1982). Apart from optimization of the medium components, the choice of physical conditions can be important for

regeneration. The incubation temperature, the osmolarity of the medium, the way of plating protoplasts on the regeneration medium, i.e. embedding them in soft agar overlays on hypertonic medium or direct spreading, have all been shown to be important (Baltz and Matsushima, 1981; Gabor and Hotchkiss, 1979). The physiological state of the microorganism, e.g. growth phase, also has an important role in protoplast formation and regeneration (Baltz, 1978). Optimizing conditions for regeneration of B.subtilis (Gabor and Hotchkiss, 1979) and some Streptomyces spp. (Baltz and Matsushima, 1981) resulted in nearly 100% regeneration.

2.3 Protoplast fusion

The first controlled microbial protoplast fusion and complementation of genetic markers was achieved with the yeast-like filamentous fungus Geotrichum candidum (Ferenczy et al., 1972). This was followed by the successful, controlled intraspecific bacterial protoplast (Fodor and Alföldi, 1976; Schaeffer et al., 1976) and yeast protoplast (Sipiczki and Ferenczy, 1976) fusion as well as successful fusion of Aspergillus (Ferenczy, 1976), Penicillium (Anne et al., 1976), Streptomyces (Hopwood et al., 1977) and algal (Matagne et al., 1979) protoplasts.

In early fusion experiments, a centrifugal force (Ferenczy et al., 1972, 1974) was applied to induce the fusion. Later, cold potassium chloride (Ferenczy et al., 1975a) or calcium phosphate (Fodor and Alföldi, 1976) was used. After the discovery that polyethylene glycol (PEG) acts as a fusogenic agent of plant protoplasts (Kao and Michayluck, 1974) it was soon introduced into microbial protoplast fusion (Ferenczy et al., 1975b; Anne and Peberdy, 1975)

and is nowadays used almost exclusively. Recently, electric fields have been used to stimulate protoplast fusion (e.g. Zimmerman, 1982).

Different polymers and concentrations of PEG were used to induce fusion in different microorganisms. In bacterial protoplast fusion experiments, 40% PEG6000 (now known as PEG8000) is usually used (Fodor and Alföldi, 1976; Coetzee et al., 1979; Rastogi et al., 1983).

The fusion of protoplasts derived from different mutants is usually assessed by the detection of complementation. This can be done either by plating the fused protoplasts directly on selective regeneration medium (Baltz, 1978; Fodor and Alföldi, 1979) or by allowing protoplast regeneration to take place on non-selective regeneration medium and recombinants within the regenerated population detected by subsequent replication onto diagnostic medium (Schaeffer et al., 1976; Coetzee et al., 1979). Many reports of successful fusion and complementation in G +ve bacteria have been published (e.g. Dancer, 1981; Baltz and Matsushima, 1981; Stahl and Pattee, 1983). Recombinants were also obtained after fusion of Mycobacterium aurum spheroplasts (Rastogi et al., 1983). This new technique should prove to be very useful with bacteria for which no genetic transfer mechanism exists, e.g. B.megaterium, and also to improve the industrially important microorganisms like Streptomyces (for more information see Ferenczy, 1981; Hopwood, 1981). Only a few reports have been published on successful protoplast or spheroplast fusion of G -ve bacteria, e.g. Tsenin et al. (1978) on E.coli and Coetzee et al. (1979) on Providence alcalifaciens.

The transfer of plasmids during cell fusion have been demonstrated in Bacillus spp. (Dancer, 1980), E.coli (Vorobjeva and

Khmel, 1979) and between different species of Staphylococcus (Gotz et al., 1981). The transfer process can proceed in the presence of DNase which indicates that it does not involve PEG-induced transformation after lysis of protoplasts (Dancer, 1980).

2.4 Plasmid curing during the regeneration of protoplasts

The possibility that the bacterial cell envelope is involved in plasmid maintenance was discussed by Jacob et al. (1963). Kawakami and Landman (1965) reported that some or all of the genetic markers carried by some plasmids in Salmonella typhimurium were lost when spheroplasts were formed and allowed to regenerate, indicating that certain plasmids might be cured in this way. Novick et al. (1980) found that some plasmids are, indeed, eliminated following the regeneration of Staphylococcus aureus protoplasts, but that others are not. They concluded that curing does not appear to involve physical expulsion of plasmid DNA during the formation of protoplasts, but is probably due to an aberration of plasmid replication or partition during the regeneration process. Also, it was found that neither the size of the plasmid, nor the copy number is correlated with curing. Curing of plasmids by protoplast formation and regeneration has been demonstrated in many microorganisms, e.g. Bacillus subtilis (Edger et al., 1981), Streptomyces kasugaensis (Furumai et al., 1982), Thermus thermophilus (Vasquez et al., 1983), Streptococcus lactis (Gasson, 1983) and Lactobacillus reuteri (Vescovo et al., 1984).

2.5 Transformation of protoplasts by plasmid and chromosomal DNA

Protoplasts proved to be a useful tool not only for fusion but

also for transformation by plasmid DNA. When Streptomyces protoplasts are exposed to plasmid DNA in the presence of PEG and subsequently regenerated, a very high proportion of the resulting colonies (up to 80%) are transformed (Bibb et al., 1978). Chang and Cohen (1979) found that exposing B.subtilis protoplasts to plasmid DNA in the presence of PEG is a highly efficient method of transformation, 80% of the regenerated colonies being transformants (with an efficiency of 4.0×10^7 transformants per μg of supercoiled DNA). In both cases, the special physiological state of competence was not required for the introduction of plasmid DNA into protoplasts. This technique of transformation has been successful in other bacteria, e.g. B.megaterium (Brown and Carlton, 1980), B.thuringiensis (Miteva et al., 1981), Streptococcus lactis (Kondo and McKay, 1982), Staphylococcus carnosus (Götz et al., 1983) and Clostridium acetobutylicum (Lin and Blaschek, 1984).

B.subtilis protoplasts are also transformable with chromosomal DNA in the presence of PEG, but with a much lower frequency than that with plasmid DNA (Levi-Meyrueis et al., 1980).

3. GENE CLONING

3.1 Introduction

In the past 15 years, the field of molecular genetics has been revolutionised. This revolution has not been the result of a single new development in instrumentation or a theoretical breakthrough, but is rather due to the application of a variety of techniques collectively referred to as recombinant DNA technology, also known as gene cloning or gene manipulation. Gene manipulation is defined as the formation of new combinations of heritable material by the insertion of nucleic acid molecules, produced by whatever means outside the cell, into any virus, bacterial plasmid or other vector system so as to allow their incorporation into a host organism in which they do not naturally occur but in which they are capable of continued propagation (Old and Primrose, 1985). A typical cloning experiment requires

i) A DNA of interest, sometimes called foreign, passenger or target DNA

ii) A cloning vector

iii) A means for cutting and joining the DNA

iv) A means for monitoring the cutting and joining reactions

v) A means for introducing the recombinant DNA into a host organism which will allow stable inheritance

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

This methodology is now available so that it is possible to produce recombinant DNA molecules in vitro and to manipulate them in an increasingly precise fashion. Some aspects of this methodology are discussed below in more detail.

3.2 Cloning vectors

Since most DNA fragments are incapable of self-replication, an autonomously replicated segment of DNA (cloning vector) must be used to accomplish replication of the desired DNA fragment. Most cloning vectors have been derived from extrachromosomal replicons such as bacteriophages and plasmids. Four types of vectors are generally used, i.e. plasmids, single-stranded DNA bacteriophages, λ bacteriophage and cosmids. The choice of the cloning vector is determined by the experimental aim. Only plasmid and cosmid vectors are of direct relevance to this thesis and their properties will be discussed. For information relating to λ vector, see Hendrix et al. (1983) and for single-stranded bacteriophages, Messing et al. (1981) and Norrander et al. (1983).

3.2.1 Plasmids and cosmids as cloning vectors

Plasmids are double-stranded DNA molecules which are capable of replicating independently of the host chromosome and are stably inherited in an extrachromosomal state. Plasmids are widely used as cloning vectors and the ideal plasmid vector should have the following properties (Rodriguez and Tait, 1983).

i) It should be as small as possible with little or no extraneous genetic information. Small plasmids are much easier to handle, more efficient in transformation and there is less chance of having multiple sensitive sites for any restriction endonuclease.

ii) It should be well characterised with respect to gene location, restriction enzyme cleavage sites and, preferably, nucleotide sequence.

iii) It should be easily propagated in the desired host so that

large quantities of vector and recombinant DNA can be obtained.

iv) It should have a selectable marker that allows selection of transformants

v) It should have an additional genetic marker that can be inactivated by the insertion of a foreign DNA fragment (insertional inactivation).

vi) It should have the maximum number of unique restriction endonuclease cleavage sites. This provides maximum flexibility in terms of cloning different kinds of restriction fragments.

Among the first plasmid vectors used were the natural plasmid ColE1, which specifies the production of colicin E1, and the plasmid pSC101 (Cohen et al., 1973) which has a selectable gene conferring tetracycline resistance and a single site for EcoRI. However, pSC101 replicates in a stringent fashion (Timmis et al., 1974), whereas ColE1 and its derivatives have the advantage of replicating in relaxed fashion (i.e. have a high copy number) and can be enriched by chloramphenicol treatment (Clewell, 1972). Undoubtedly, the most widely used plasmid vector is pBR322 (Bolivar et al., 1977). It is derived from ColE1 and fulfils all the requirements of the ideal vector. It is small (4363 base pairs) and has two selectable markers, ampicillin resistance (Ap^R) and tetracycline resistance (Tc^R). It has been completely sequenced (Sutcliffe, 1979; revised Peden, 1983) and there are 20 known enzymes that cleave it at unique sites (Old and Primrose, 1985). pAT153 (Twigg and Sherratt, 1980) is a derivative of pBR322 made by removing a HaeII-generated fragment from pBR322 and is also widely used.

Early plasmid vectors were developed mainly for E.coli. However, rapid progress is being made in the construction of

cloning vectors for a wide variety of organisms, both prokaryotic and eukaryotic (Hofschneider and Goebel, 1982; Bagdasarian et al., 1983). The development of B.subtilis vectors resulted from observations that plasmids isolated from Staphylococcus aureus, e.g. pT127 and pC194 can be transformed into B.subtilis where they replicate and express antibiotic resistance normally (Ehrlich, 1977). Since E.coli plasmids do not replicate efficiently in B.subtilis nor are their markers expressed, hybrid plasmids were constructed by the ligation of an E.coli vector to a B.subtilis vector so they can be used in both hosts. Such plasmid vectors are called shuttle vectors. pHV14 (Ehrlich, 1978) which can transform E.coli and B.subtilis has been constructed by the ligation of pC194 to pBR322. Its derivative, pHV33, which encodes Tc^R (Primrose and Ehrlich, 1981) is now widely used as a cloning vector. Cloning vectors that shuttle between E.coli and other microorganisms e.g. Cyanobacterium Anacystis nidulans (Gendel et al., 1983) and Neurospora crassa (Hughes et al., 1983) have also been constructed.

After the successful expression in E.coli of genes originating from S.aureus (Chang and Cohen, 1974), Saccharomyces cerevisiae (Struhl et al., 1976) and N.crassa (Vapenek et al., 1977), it was assumed that genes from bacteria and higher organisms would express in any bacterium. Later it was found that many cloned genes were not expressed in their new genetic background, and that the first requirement for expression of a foreign structural gene in E.coli, inserted in vitro in a vector, is that the gene be placed under the control of an E.coli promoter. In order to do that many expression vectors were developed that contain promoter sequences required for the transcription of the inserted gene. Different promoters have

been used in the construction of these vectors e.g. trp promoter (Tacon et al., 1980), λ P_L promoter (Remaut et al., 1981), lac promoter (Heiland and Gething, 1981), trp/lac (tac) promoters (de Boer et al., 1983), recA promoter (Shirakawa et al., 1984) and lpp/lac promoters (Masui et al., 1984).

The main disadvantage of using plasmids as cloning vehicles is the size limitation of inserted DNAs. The average insert size is usually about 5 kb and inserts of more than 20 kb are very unusual. This limits their usefulness for the construction of gene libraries, especially from an organism with a large genome. The construction of gene libraries involves the generation of a collection of cloned DNA fragments which comprise the entire genome of the organism. The number of clones required to contain all the genome depends on both the size of the genome and the average size of the DNA fragments cloned (Clark and Carbon, 1976). Cosmid vectors are more suitable for the construction of gene libraries because they can usually accommodate much larger DNA inserts than plasmids and therefore reduces the number of clones required to contain the genome.

Cosmids are vectors derived from plasmids containing the λ cohesive (cos) ends (Collins and Brüning, 1978; Collins and Hohn, 1979). Only a small region near the cos site is recognised by the λ packaging system, both in vitro and in vivo, thus for efficient packaging the cos sites should be 37-52 kb apart (75-105% of the size of λ DNA). This means that for a 5 kb cosmid, an insert of 32 to 47 kb is required for efficient packaging in vitro and can be transduced into E.coli. Once inside the bacterium, the cosmid circularises, but because it does not contain all the essential λ genes, it is unable to go through a lytic cycle and therefore

resembles a plasmid vector. Since cosmids need only to contain a replication origin, a selective marker and *cos* sites, they are usually small e.g. pJB8 (5.4 kb) (Ish-Horowicz and Burke, 1981) and c2XB (6.8 kb) (Bates and Swift, 1983).

3.3 Cutting and joining DNA molecules *in vitro*

Usually fragments that are to be cloned are generated by the cleavage of chromosomal DNA 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 its introduction into a host cell system. Prior to 1970, there was no method available for cleaving a duplex DNA molecule into discrete fragments. The breakthrough came in 1970 with the discovery in *Haemophilus influenzae* Rd of a restriction enzyme (Kelly and Smith, 1970; Smith and Wilcox, 1970) which became the prototype of a large number of restriction endonucleases now known as type II enzymes. Type II restriction endonucleases recognise a particular target sequence in the DNA molecule and break the polynucleotide chain within, or near to, that sequence to give rise to discrete DNA fragments. Many type II restriction endonucleases have been discovered and isolated from a wide variety of microorganisms and their number continues to grow as more bacterial genera are surveyed. Recently, a list of 475 enzymes was published (Roberts, 1984).

The vast majority of type II restriction endonucleases recognise and break DNA within a particular sequence of tetra, penta, hexa or hepta nucleotides which have an axis of rotational symmetry, generating 5'-cohesive ends, e.g. *Eco*RI (5'-G↓AATTC-3'),

3'- cohesive ends, e.g. PstI (5'-CTGCA↓G-3'), or blunt-ended fragments, e.g. NruI (5'-TCG↓CGA-3'). Enzymes from different sources which recognise the same sequence are called isoschizomers, although some of them e.g. SmaI and XmaI, cut their target in different places. Recently, a new kind of type II restriction enzyme e.g. HgaI has been identified which makes breaks in the two strands at a measured distance to one side of its asymmetric target sequence ($\begin{matrix}5'\text{-GACGCNNNNN}\downarrow & -3' \\ 3'\text{-CTGCGNNNNNNNNNN}\downarrow & -5'\end{matrix}$).

To create recombinant DNA molecules, the DNA fragments generated by restriction enzymes should be joined together. There are three methods available for joining DNA fragments in vitro. The first relies on the ability of DNA ligase from E.coli or phage T4 infected E.coli to join the annealed cohesive ends produced by certain enzymes, by the formation of phosphodiester linkage between adjacent nucleotides in a duplex DNA chain (Olivera et al., 1968), thus repairing the nicks on both strands to form an intact duplex. Joining DNA fragments with cohesive ends by DNA ligase is a relatively efficient process which has been used extensively to create artificial recombinants. The formation of recombinants can be improved by using the right vector-target ratio, a DNA concentration to minimize the circularisation of linear fragments and by minimizing the recircularisation of vector DNA by removing the 5'-phosphate from both ends of the linear vector DNA with bacterial alkaline phosphatase or calf intestinal phosphatase (Seeburg et al., 1977; Ullrich et al., 1977). In this case, circularisation of the vector can occur only by insertion of non-phosphate-treated foreign DNA which provides a 5'-terminal phosphate at each join. The optimum temperature for ligation of cohesive ends is a compromise

between the rate of enzyme action and association of the termini and usually 12.5° C is used (Rodriguez and Tait, 1983).

The second method for joining DNA fragments depends on the ability of T4 DNA ligase to join blunt-ended fragments by the formation of phosphodiester bonds between them (Sgaramella, 1972). The blunt-end ligation reaction is more complicated and significantly slower than cohesive-end ligation. It needs a higher DNA concentration to increase the number of juxtapositions and 10 to 30 times more T4 DNA ligase to achieve a ligation rate equivalent to that observed for cohesive-end ligation (Rodriguez and Tait, 1983). Since the thermal stability of annealed cohesive ends is not a consideration with blunt-end ligation, the reaction is less influenced by temperature and generally 23° C is used for blunt-end ligation. 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 third method of joining DNA fragments utilizes homopolymer tailing (Jackson et al., 1972). This involves adding a sufficiently long homopolymer extension to the 3' ends of the vector DNA and a complementary extension to the 3' ends of the passenger DNA such that they are able to form a stable hydrogen-bonded structure. For more information see Deng and Wu (1981) and Michelson and Orkin (1982).

3.4 Transformation of E.coli

Early attempts to transform E.coli with chromosomal DNA were unsuccessful suggesting that this bacterium does not possess a natural mechanism for transformation. However, Mandel and Higa

(1970) found that treatment with $CaCl_2$ allowed E.coli to take up bacteriophage λ DNA while Cohen et al. (1972) showed that $CaCl_2$-treated E.coli cells were effective recipients for plasmid DNA. Since restriction enzymes in the host cell degrade foreign DNA, the presence of restriction enzyme systems can influence the efficiency of transformation. For this reason it is usual to use a restrictionless E.coli mutant as a host for plasmid transformation. Since transformation of E.coli is an essential step in many cloning experiments, several groups of workers have examined the factors affecting the efficiency of transformation. However, Hanahan (1983) has devised a set of conditions applicable to most E.coli K-12 strains which results in efficiences of 10^7 to 10^8 transformants per μg DNA.

3.5 Selection of recombinants

Selection of a desired recombinant from a population of bacteria can be done by the selection of vectors carrying fragments that give a selectable phenotype to the host, i.e. complementation of a mutation. The requirements for this are that the cloned gene can be transcribed and expressed in the host, e.g. Nagahari et al. (1980). If the inserted gene sequence is expressed in the host but without conferring a selectable property, then immunochemical methods (e.g. Broome and Gilbert, 1978) can be very useful for selecting such recombinants. Other techniques e.g. nucleic acid hybridisation, hybrid-released and hybrid-arrested translation, have been developed to detect recombinants of interest. These approaches do not require that a gene be expressed, only that the particular DNA sequence of interest is present (for a review see

Dahl et al., 1981).

3.6 Detecting expression of cloned genes

When it is known that a cloned gene codes for a particular protein, then suitable tests can be devised to determine if that gene is expressed in its new host. However, with genes of unknown function, the only way to detect their expression is to look for synthesis of novel proteins in cells carrying the recombinant molecules. Three systems are commonly used to observe the protein products encoded by recombinant plasmids: mini-cells (Meagher et al., 1977), maxi-cells (Sancar et al., 1979) and the in vitro transcription-translation system (Yang and Zubay, 1978). Only the maxi-cell system was used in this study and it will be considered in some detail.

3.6.1 Maxi-cells technique

Maxi-cells are plasmid-containing E.coli cells whose chromosomes have been extensively degraded (Sancar et al., 1979). They are used for specifically labelling plasmid-encoded proteins. Maxi-cells are produced by taking advantage of the difference in size and copy number of the plasmid compared with the chromosome, to selectively degrade the chromosome but not the plasmid by UV-irradiation. Thus when recA , uvr cells are UV-irradiated, about 80% of their DNA becomes acid-soluble within 3h because photoproducts in the chromosome act as substrates for exonucleases (Howard-Flanders, 1968). However, if these cells contain a small multicopy plasmid, some of the plasmid molecules will not receive a lethal UV hit because of their smaller size and will continue to replicate. When E.coli

CSR603 (recA1, uvrA6, phr-1) harbouring pBR322 is UV irradiated with a dose of 30 JM^{-2} and incubated at 37° C for 6 h, over 80% of the chromosomal DNA is degraded while the plasmid DNA increases about 10-fold (Sancar et al., 1979). Addition of protein label to the medium after the completion of chromosome degradation results in the selective labelling of the plasmid-encoded proteins.

E.coli CSR603 is usually used for making maxi-cells because it is possible to handle the culture under ordinary light after UV irradiation. However, any recA or recA , uvr strain can be used provided the cells are protected from photoreactivating light after irradiation.

CHAPTER 2

MATERIALS AND METHODS

BACTERIAL STRAINS AND PLASMIDS

The strains of *Deinococcus* spp. used are listed in Table 2.1, *Escherichia coli* strains in Table 2.2, Plasmids and their hosts are listed in Table 2.3.

GROWTH AND MAINTENANCE OF CULTURES

TGY medium (Anderson *et al.*, 1956) was used to grow strains of *Deinococcus* spp. other than *D.radiophilus* which was grown in Nutrient broth No. 2 (NB2) (Oxoid). L-broth was used to grow *Bacillus subtilis* and *E.coli* strains. Liquid cultures were grown with shaking. *Deinococcus* strains were incubated at 30°C, *B.subtilis* and *E.coli* strains at 37°C. Media were solidified by adding 12g agar (Oxoid No. 1) l^{-1}. Genetically-marked strains and those carrying plasmids were routinely subcultured onto supplemented media for characterisation of their phenotypes.

MEDIA

1. TGY medium

	gl^{-1}
Tryptone (Difco)	5
D-glucose	1
Yeast extract (Difco)	3

2. Nutrient broth for the growth of *D.radiophilus*

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

Table 2.1

Strains of *Deinococcus* used

Strain	Relevant genotype	Reference
D.radiodurans 302	*mtcA*	Moseley and Copland, 1978
" krase*	*kan*, *rif*, *acr*, *str*, *ery*	Tirgari and Moseley, 1980
" Sark	Wild type	Murray and Robinow, 1958
" UVS9	*uvsC*, *mtcA*	Moseley and Evans, 1983
" UVS25	*uvsD*, *mtcA*	"
" UVS78	*uvsE*, *mtcA*	"
" 262	*mtcB*	Moseley and Copland, 1978
" 2-1	*asp*-1	This study
" 5-1	*pdx*-1	"
" 18-1	*trp*-1	"
" 22-1	*trp*-2	"
" 27-1	*trp*-3, *rif*	"
" 30-1	*tyr*, *rif*	"
" 34-1	*arg*, *rif*	"
" 40-1	*ura*-1, *acr*	"
" 41-1	*trp*-4, *acr*	"
" 44-1	*trp*-5	"
" 62-1	*ura*-2, *acr*	"
" 66-1	*trp*-6	"
" 67-1	*trp*-7, *acr*	"
D.radiophilus	Wild type	Lewis, 1971
" Rif^R	*rif*	This study
D.radiopugnans	Wild type	Davis *et al.*, 1963
D.proteolyticus	Wild type	Kobatake *et al.*, 1973

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

Table 2.2

Strains of *E.coli* used

Strain of *E.coli*	Relevant genotype	Reference or source
HB101	F^-, *pro*, *leu*, *thi*, r^-_k, m^-_k, *recA*	Boyer and Roulland-Dussoix (1969)
CSR603	F^-, *thr*, *leu*, *pro*, *phr*-1, *recA*, *arg*, *thi*, *uvrA*	Sancar and Rupert (1978)
JA221	*trpE*, *leuB*, *hsdR*(r^-m^+)	Dr David B.Yelton*
W3110trpA33	*trpA*	"
W3110trpB9579	*trpB*	"
W3110trpC670	*trpC*	"
W3110trpD562	*trpD*	"

* Department of Microbiology, West Virginia University Medical Centre, Morgantown, USA

Table 2.3

Plasmids used

Plasmid	Host	Selectable marker[a]	Source[b]
pAT153	E.coli HB101	Tc, Ap	Dr M Mackay
pHV33	E.coli HVC181	Tc, Ap, Cm	"
pC194	B.subtilis HVS62	Cm	"
pUB110	B.subtilis HVS89	Kn	"

[a] Abbreviations and selection concentrations:

Ap - Ampicillin, $20\mu g ml^{-1}$

Cm - Chloramphenicol, $10\mu g ml^{-1}$

Kn - Kanamycin, $20\mu g ml^{-1}$

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

[b] Department of Molecular Biology, University of Edinburgh.

3. Luria broth (L-broth) (Lennox, 1955)

	gl^{-1}
Tryptone	10
Yeast extract	5
NaCl	5
D-glucose	1
2ml of 1N NaOH	

4. Minimal medium for D.radiodurans

This medium was developed by Diana Sweet (cited in Tirgari, 1977).

Basal medium

	gl^{-1}
D-glucose	2
Monosodium glutamate	2
Tris-base	2
Ammonium acetate	1

To this medium 1ml each of solutions A, B and C, 0.1ml of solution D and 2ml of solution E were added, and the pH adjusted to 7.8 using acetic acid. These solutions were made up as follows and stored at 4°C until required.

Solution A

Potassium acetate	20.0g
Magnesium acetate $.4H_2O$	10.0g
Sodium acetate	0.5g
Calcium chloride $.2H_2O$	0.5g
Distilled water	to 100ml

Solution B

Ammonium phosphate	8.7g
Ammonium sulphate	2.0g
Distilled water	to 100ml

Solution C

Nicotinic acid	50mg
Vitamin B1 (aneurin HCl)	50mg
Biotin	1mg
Vitamin B12 (cyanocobalamine)	0.1mg
Distilled water	to 100ml

Solution D

Boric acid	50mg
Manganese acetate $.4H_2O$	50mg
Zinc acetate $.2H_2O$	40mg
Ferric chloride	20mg
Ammonium molybdate	10mg
Potassium iodide	10mg
Cupric acetate $.H_2O$	4mg
Citric acid	100mg
Distilled water	to 100ml

Solution E

L-Cysteine	1200mg
L-Methionine	800mg
L-Lysine monohydrochloride	915mg
L-Histidine	780mg
Distilled water	to 100ml

5. M9 salts (x10) for E.coli minimal medium

	gl^{-1}
$NaHPO_4.H_2O$	60
KH_2PO_4	30
NaCl	5
NH_4Cl	10

pH was adjusted to 7.4 and sterilized by autoclaving.

6. M9 minimal medium for E.coli

M9 salts (x10)	100ml
20% Glucose	20ml
0.1M $MgSo_4$	10ml
0.01M $CaCl_2$	10ml
Distilled water	to 1000ml

Each solution was sterilized separately and components mixed aseptically before use.

7. Regeneration medium (RM)

TGY broth in the case of D.radiodurans and NB2 broth for D.radiophilus containing the following

	gl^{-1}
Casamino acids	5.0
$MgCl_2.6H_2O$	5.1
$CaCl_2.2H_2O$	3.7
Agar	20.0

8. K Medium

M9 minimal medium supplemented with 1% (w/v) casamino acids and 0.1μg ml^{-1} thiamine hydrochloride.

9. Hershey salts

	gl^{-1}
NaCl	5.4
KCl	3.0
NH_4Cl	1.1
$CaCl_2.2H_2O$	0.015
$MgCl_2.6H_2O$	0.2
$FeCl_3.6H_2O$	0.0002
KH_2 PO4	0.087
Tris-base	12.1
Distilled water	to 1000ml

pH was adjusted to 7.4 and sterilized by autoclaving.

10. Hershey medium

Hershey salts	100ml
20% (w/v) Glucose	2ml
2% (w/v) Threonine	0.5ml
1% (w/v) Leucine	1ml
2% (w/v) Proline	1ml
2% (w/v) Arginine	1ml
0.1% (w/v) Thiamine hydrochloride	0.1ml

Each solution was sterilized separately and components mixed aseptically before use.

BUFFERS AND SOLUTIONS

1. 0.1M Phosphate buffer, pH5.7

	gl^{-1}
$NaH_2PO4.2H_2O$	14.35
Na_2HPO_4	1.14

2. 0.067M phosphate buffer, pH7.0

	gl^{-1}
Na_2HPO4	4.73
KH_2PO4	4.56

3. Phosphate-EDTA (PE) buffer

	gl^{-1}
Na_2HPO4	4.73
KH_2PO4	4.56
EDTA-disodium salt	0.34

4. Butanol-saturated phosphate-EDTA buffer

Phosphate-EDTA buffer contained 6% (v/v) n-butanol.

5. PS buffer

0.067M phosphate buffer pH7.0 containing $171gl^{-1}$ (0.5M) sucrose.

6. SMM buffer, pH 6.5 (Wyrick and Rogers, 1973)

	gl^{-1}
Sucrose	171.0
Sodium maleate	3.56
Magnesium chloride $6H_2O$	4.1

7. TS buffer

0.01M Tris HCl pH 8.0 containing $171gl^{-1}$ (0.5M) sucrose.

8. TGYS

TGY broth containing 171gl^{-1} (0.5M) sucrose.

9. TST1

A mixture of equal volumes of 2x TS and 2x TGYS.

10. TST2

A mixture of equal volumes of 2x TS and 4x TGYS.

11. TE buffer, pH7.4

	gl^{-1}
Tris-base	1.2
EDTA-disodium salt	0.4

12. Loading buffer for agarose gels

Sodium dodecyl sulphate (SDS)	0.09g
EDTA-disodium salt	0.04g
Bromophenol blue	0.005g
Ficoll	2.0g
20mM Tris-HCl, pH8.0	10ml

13. Sample buffer for Maxi-cells

SDS	0.23g
2-Mercaptoethanol	0.5ml
Glycerol	1.0ml
Bromophenol blue	0.005g
1M Tris-HCl, pH6.8	0.625ml
Distilled water	to 10ml

14. SSC buffer (x 20)

	gl^{-1}
NaCl	175.3
Sodium citrate	88.2

pH was adjusted to 7.0 with NaOH

15. SET buffer (x 40)

	gl^{-1}
NaCl	350.64
EDTA-disodium salt	14.9
0.08M Tris-HCl, pH7.8	to 1000ml

16. Denhardt's solution (x 40)

	gl^{-1}
Ficoll	8.0
Polyvinylpyrrolidone	8.0
Bovine serum albumin	8.0

The solution was sterilised by filtration.

17. Prehybridisation solution

SET buffer (x 40)	1.25ml
Denhardt's solution (x 40)	1.25ml
10% (w/v) SDS	0.1ml
10% (w/v) NaPPi (Sodium pyrophosphate)	0.1ml
Denatured sonicated salmon sperm DNA	1mg
Distilled water	to 10ml

18. Hybridisation solution

SET buffer (x 40)	1.25ml
Denhardt's solution (x 40)	1.25ml
10% (w/v) SDS	0.1ml
10% (w/v) NaPPi	0.1ml
^{32}P-Labelled denatured DNA	≈0.7ml
20% Dextran sulphate	5.0ml
Distilled water	to 10ml

19. Nick-translation buffer (x 10)

	gl^{-1}
$MgSO4.7H_2O$	24.65
Dithiothreitol	1.54
Bovine serum albumin	0.5
0.5M Tris-HCl pH7.2	to 1000ml

The stock buffer was divided into small aliquots and stored at -20°C.

CHEMICALS

Sodium dodecyl sulphate (SDS), ethylenediamine tetra-acetic acid, disodium salt (EDTA), hydroxymethyl methylamine (Tris base), Tris-hydrochloride, caesium chloride, polyethylene glycol (PEG) 6000, bromophenol blue, ethidium bromide, 3-(N-Morpholino) propanesulphonic acid (MOPS), acrylamide and bisacrylamide (electrophoretic grade) were obtained from BDH Chemicals Ltd., England; ficoll, agarose type 1 (low EEO), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), deoxyadenosine 5-triphosphate (dATP) and bovine serum-albumin (BSA) from Sigma Chemical Co. Ltd., London; dithiothreitol (DTT), tetramethylethylenediamine (Temed) (high purity) and ultra-pure phenol from BRL Bethesda Research Laboratories (UK) Ltd., Cambridge; sephadex G-50 was obtained from Pharmacia (GB) Ltd , Middlesex.

ANTIBIOTICS AND NUTRITIONAL SUPPLEMENTS

The antibiotic D-cycloserine was purchased from Glaxo. All other antibiotics and nutritional supplements were obtained from Sigma.

RADIOACTIVELY-LABELLED COMPOUNDS

[α-^{32}P] labelled deoxycytosine 5'-triphosphate, deoxythymidine 5'-triphosphate and [^{35}S] L-methionine (Amersham International p.l.c., England) were obtained as gifts from Dr Edwin Southern, MRC Mammalain Genome Unit, Edinburgh and Dr Martin Mackay, Universtiy of Edinburgh.

ENZYMES

T4 DNA ligase, calf intestinal alkaline phosphatase and S1 nuclease were purchased from Boehringer Mannheim, Lewes, England; pancreatic RNase, pancreatic DNase and lysozyme from Sigma; E.coli DNA polymeraseI from Cambridge Biotechnology, England; restriction endonucleases AccI, BamHI, ClaI, HindIII, KpnI, StuI and XbaI from Boehringer Mannheim; PstI, SalI and SstII from BRL; BalI, MluI, NruI and XmaI from New England Biolabs, Bishops Stortford, England; EcoRI from NBL Enzymes Ltd., Cramlington, Northumberland and DraI was a gift from I.J.Purvis, University of Edinburgh. Enzymes were used in accordance with the manufacturer's instructions.

MEASUREMENT OF BACTERIAL TURBIDITY

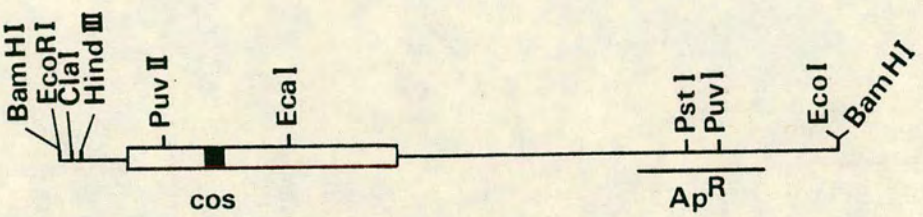
Bacterial turbidity was measured in a nephlometer (Evans Electroselenium Ltd., Halstead) with orange filter.

GENE LIBRARY

A gene library from D.radiodurans krase constructed in the cosmid vector pJBFH (3.7kb) (Fig.2.1), a derivative of pJB8 (Ish-Horowicz and Burke, 1981), was obtained as an in vitro packaged phage mix from Dr Martin Mackay, University of Edinburgh. The average size of inserts in the cosmid vector was calculated to be 35kb (Mackay, 1983).

Fig.2.1

Cosmid pJBFH (3.7kb) a derivative of pJB8 (Ish-Horowicz and Burke, 1981), made by deletion of the HpaI-AvaI fragment (F.G.Grosveld, personal communication).



MUTAGENESIS OF DEINOCOCCUS RADIODURANS

Cells of D.radiodurans 302 were harvested from 10ml of an exponential phase culture with a turbidity reading of 30 (~1.0×10^8 viable units ml^{-1}), washed once and resuspended in 10ml phosphate buffer, pH5.7. MNNG was added to a final concentration of 20$\mu g ml^{-1}$, and the bacterial suspension incubated at 30°C with shaking for 45 min. 0.2ml of the treated culture was added to 20ml fresh TGY broth and incubated overnight with shaking at 30°C.

To isolate auxotrophs, the culture was diluted appropriately, 0.1ml samples spread on TGY plates and the plates incubated at 30°C for three days. Colonies growing on TGY plates were replicated onto minimal medium and TGY plates. The master plates were kept at 4°C and replica plates incubated for 4 days at 30°C. All putative auxotrophs were picked from the master plates and tested for their growth requirements by plating each one on a series of twelve supplemented minimal plates using the method of Holliday (1956). Media were supplemented with amino acids at 20$\mu g ml^{-1}$, nucleotides at 10$\mu g ml^{-1}$ and vitamins at 1$\mu g ml^{-1}$.

For selecting antibiotic-resistant mutants, 0.1ml samples of appropriate dilutions of the mutagenised culture were spread on TGY plates containing appropriate antibiotics. Colonies which grew on antibiotic plates were streaked on TGY agar containing increasing concentrations of the antibiotic to ascertain their level of resistance.

For selecting double mutants, putative auxotrophs were replicated onto TGY plates containing appropriate antibiotics, while the antibiotic-resistant mutants were replicated onto minimal medium and TGY plates containing other kinds of antibiotics. Single and

double mutants were selected and kept on TGY plates.

SPHEROPLAST FORMATION AND REGENERATION

Different kinds of spheroplasting buffers, regeneration media and several procedures were used in an attempt to find a suitable protocol for both the formation of spheroplasts from D.radiodurans and the regeneration of their cell walls. The protocol described below was then adopted since it gave the best results.

0.1ml of an 18h culture of D.radiodurans 302 in TGY broth was inoculated into 50ml of fresh TGY broth and incubated with shaking at 30°C until the culture reached late exponential phase (turbidity reading = 80-90). Cells from 30ml were harvested by centrifugation at room temperature (10,000g for 5 min), washed once with an equal volume of PS buffer and resuspended in 10ml of the same buffer. A sample from the suspension was diluted appropriately in TGYS and spread onto TGY agar plates for a viable count. Fresh lysozyme (in PS buffer) was added to the suspension to a final concentration of $2mgml^{-1}$, EDTA added to a final concentration of 0.002M and the mixture incubated at 37°C with gentle shaking. Samples were removed at intervals and tested for spheroplast formation by microscopic examination and osmotic shock. Unfixed cell suspensions were examined to determine the conversion rate to spheroplasts and ghost cell formation after the lysozyme-EDTA treatment and photographed in a Leitz Orthoplan phase microscope with Orthomat camera. Osmotic sensitivity was determined by observing the decrease in turbidity of samples from the cell suspension after the addition of NaOH to a final concentration of 0.1M. After 5 to 6 h of lysozyme-EDTA treatment, more than 99% of the cells were converted into sphero-

plasts (according to microscopic examination and osmotic shock experiments). These were collected from 6ml by centrifugation at room temperature (10,000g for 10 min), washed once with an equal volume of TGYS and resuspended in 2 ml TGYS. Samples were diluted appropriately in TGYS and distilled water and samples from both dilutions spread onto the regeneration medium (RM). Plates were incubated at 37°C for 6 days.

The number of osmotic-resistant (OR) cells remaining in the spheroplasting buffer after the completion of treatment was considered to be the same as the number of colonies appearing on RM plates inoculated with the spheroplast suspension diluted in distilled water. The number of spheroplasts in the spheroplasting buffer was calculated by subtracting the number of OR cells from the total viable count before the treatment with lysozyme-EDTA. The percentage of spheroplast formation was the number of spheroplast times 100 divided by the total viable count. The number of regenerants (colonies developed from regenerated spheroplasts) was the number of colonies appearing on RM plates inoculated with the spheroplast suspension diluted in TGYS (colonies developed from both spheroplasts and OR cells) minus the number of colonies developed from OR cells. The regeneration frequency was the ratio of regenerants to spheroplasts. The percentage of regenerants was the number of regenerants times 100 divided by the number of colonies appearing on RM plates inoculated with the spheroplast suspension diluted in TGYS.

The same procedure was used to produce spheroplasts from D.radiophilus. The final concentration of lysozyme and EDTA were $1 mg ml^{-1}$ and 0.001M respectively, and the treatment was stopped after 1 h. TGY was replaced by NB2 in growth medium, dilutents and

regeneration medium.

SPHEROPLAST FUSION

The protocol used for spheroplast fusion was similar to that used for protoplast fusion of members of the genera Bacillus (Fodor and Alföldi, 1976, 1979; Gabor and Hotchkiss, 1979) and Streptomyces (Baltz and Matsushima, 1981). Spheroplasts were prepared separately from two mutants, D.radiodurans 30-1 (Rif^R, Tyr^-) and D.radiodurans 40-1 (Acr^R, Ura^-), as described. Equal volumes (0.5ml) of the two spheroplast suspensions in PS buffer were mixed and centrifuged for 45 s at room temperature in a microcentrifuge (Hettich Mikroliter). The pellet was resuspended in 0.1ml PS buffer and mixed gently with 0.9ml of 40% (w/v in PS buffer) PEG6000 for 1-2 min. Samples were diluted appropriately in TGYS and distilled water and 0.1ml amounts from both dilution series were spread onto RM plates. Mixtures of the two spheroplast suspensions without centrifugation or addition of PEG as well as spheroplasts of each mutant separately were diluted and spread onto to RM plates in the same way, to determine the frequency of spontaneous mutation. DNase to a final concentration of $10\mu g ml^{-1}$ was present in all solutions and RM to inhibit transformation. In other experiments, $CaCl_2$ to a final concentration of 25mM was added to the fusion mixture. Plates were incubated at 37°C for 10-12 h to allow cell wall regeneration, 8ml of soft RM (0.4% agar) containing $10\mu g ml^{-1}$ of both acriflavine and rifampicin were overlaid on the top of the inoculated plates which were then incubated at 37°C for 8 days. Alternatively, colonies were allowed to grow fully on RM, then replicated onto selective media and incubated at 37°C for 6 days.

In other experiments, spheroplast suspensions were mixed together (1ml each), centrifuged and the pellet resuspended in 1ml PS buffer. 3ml of 40% (w/v) PEG6000 and $CaCl_2$ to a final concentration of 25mM were added simultaneously. After 2 min of mixing, 6ml of PS buffer were added to stop the action of PEG. Samples from appropriate dilutions were spread onto RM plates and selection for fusants made as before.

ISOLATION OF CHROMOSOMAL DNA

A modification of Marmur's (1961) method was used for the isolation of chromosomal DNA from members of the genus Deinococcus. Cells in stationary phase were harvested from 2l cultures by centrifugation at 10,000g for 5 min, resuspended in 50ml butanol-saturated phosphate-EDTA buffer and left for 45 min at room temperature. This treatment renders the cell wall sensitive to lysozyme degradation (Driedger and Grayston, 1970). Cells were collected by centrifugation, washed once and resuspended in 50ml of phosphate-EDTA (PE) buffer. Lysozyme was added to a final concentration of $2mgml^{-1}$ and the mixture was incubated at 37°C with shaking until a 1ml sample lysed on the addition of 0.1ml of 11% SDS in PE buffer. This was usually about 1 h. The bacteria were lysed by adding 5ml of 11% SDS solution. After swirling the flask to ensure complete lysis, 13ml of sodium perchlorate (70.25g $NaClO_4.H_2O$ and 4.4g NaCl in 1l distilled water) and 73ml of chloroform: isoamyl alcohol (24:1 v/v) were added and the mixture shaken gently by hand for 30 min before centrifugation at 30,000g for 20 min at 4°C. The mixture separated into three layers. The aqueous top layer that contained the nucleic acids was removed carefully using a 10ml

pipette and added slowly to two volumes of absolute ethanol and the precipitated DNA wound onto clean glass rods and air dried. The DNA was redissolved in 10ml TE buffer and treated with RNase (final concentration of 200$\mu g ml^{-1}$) for 1 h at 37°C. An equal volume of chloroform: isoamyl alcohol was added and the mixture shaken for 10 min. The phases were separated by centrifugation at 12,000g for 30 min at 4°C and the upper aqueous phase collected. The DNA was precipitated as before, wound out, dried and dissolved in TE buffer. It was stored at 4°C for use.

Chromosomal DNA from D.radiodurans krase strain used for DNA-DNA hybridisation was further purified by the dye-buoyant density gradient centrifugation technique which will be described later.

ISOLATION OF PLASMID DNA

1. From members of the genus Deinococcus

A modification of the Birnboim and Doly (1979) procedure was used for the rapid screening of members of the genus Deinococcus for the presence of plasmid DNA.

Cells were harvested from 1.5ml cultures in stationary phase by centrifugation at room temperature in a microcentrifuge for 45 s and resuspended in 0.5ml butanol-saturated phosphate-EDTA buffer and left at room temperature for 30 min. Cells were collected by centrifugation, washed once with 0.5ml lysis buffer and the pellet resuspended in 100µl of lysis buffer (25mM Tris-HCl, pH8.0, 10mM EDTA, 50mM glucose) containing lysozyme to a final concentration of $2 mg ml^{-1}$. The contents of the tube were mixed by inversion and held on ice for 30 min. 200µl of 0.2N NaOH containing 1% SDS solution was added, mixed by inversion and placed on ice. After 5 min the

suspension became almost clear and viscous. 150µl of 3M sodium acetate (pH4.8) was added and the contents of the tube gently mixed by repeated inversion for about 1 min. The tube was kept on ice for 1 h which allowed most of the protein, high molecular weight RNA and chromosomal DNA to precipitate. Centrifugation in a microcentrifuge for 5 min at 4°C yielded a cloudy supernatant which was removed, using a pipette, and added to 1ml cold absolute ethanol and held at -20°C for 30 min. The DNA was collected by centrifugation in a microcentrifuge for 5 min at 4°C and resuspended in 100µl of 0.1M sodium acetate (pH6.0). The DNA was ethanol-precipitated and collected by centrifugation as before, dried in a vacuum and resuspended in 40µl distilled water before applying to an agarose gel.

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

Cells were collected from 1l stationary-phase cultures by centrifugation at 12,000g for 10 min and resuspended in 30ml of butanol-saturated phosphate-EDTA buffer. The suspension was left at room temperature for 30 min. The cells were collected as before, washed once and resuspended in 30ml lysis buffer. 10ml of fresh lysozyme solution ($8mgml^{-1}$ in lysis buffer) were added, mixed gently and placed on ice for 30 min. 80ml of 0.2N NaOH containing 1% SDS solution were added, mixed by inversion and held on ice for 5 min. 60ml of 3M sodium acetate (pH4.8) were added to the mixture, mixed gently but thoroughly and placed on ice for 1 h. The mixture was centrifuged at 16,000g for 10 min at 15°C and the supernatant passed through a plastic tea-strainer to remove any clots. DNA was precipitated in two volumes of absolute ethanol, collected by centrifugation at 12,000g for 10 min at 4°C and resuspended in 25ml of

0.1M sodium acetate pH6.0. The DNA was once again ethanol-precipitated and collected as before. The pellet was dried under vacuum before being dissolved in 5ml TE buffer. Plasmid DNA was purified by the dye-buoyant density gradient centrifugation technique (Radloff et al., 1967).

2. From E.coli

The rapid boiling method (Holmes and Quigley, 1981) was used to screen small culture volumes for the presence of plasmids or recombinant DNA molecules in E.coli.

Cells were harvested from 1.5ml of an 18 h culture in L-broth, containing the selective antibiotic, by centrifugation for 45 s in a microcentrifuge and resuspended in 350µl of STET buffer (10mM Tris-HCl, pH8.0, 50mM EDTA, 8% (w/v) sucrose, 0.5% (v/v) Triton X 100). 25µl of fresh lysozyme solution ($10 mg ml^{-1}$ in STET buffer) was added, mixed by inversion and placed immediately in a boiling water bath for 40 s, followed by centrifugation in a microcentrifuge at 4°C for 5 min. The gelatinous pellet was removed from the microcentrifuge tube with a toothpick and 40µl of 4M sodium acetate added to the supernatant. 410µl of cold propan-2-ol was added and the DNA collected by centrifugation in a microcentrifuge at 4°C for 5 min. The pellet was rinsed with 100µl of 70% ethanol, dried in a vacuum and resuspended in 40µl distilled water. A sample of this preparation was used directly for electrophoresis, restriction analysis or transformation.

For a large scale preparation of plasmid DNA from E.coli, cells were harvested from a 1l 18 h culture in L-broth, containing a selective antibiotic, and plasmid DNA prepared using the same

procedure used for members of the genus Deinococcus (a scale-up modification of Birnboim and Doly (1979) technique) but without treating the cells with butanol-saturated phosphate-EDTA buffer.

3. From B.subtilis

Plasmids pC194 (Ehrlich, 1977) and pUB110 (Gryczan et al., 1978) were isolated from B.subtilis using the method of Gryczan et al. (1978).

Cells were harvested from 250ml of an 18 h culture in L-broth containing an appropriate antibiotic, washed with 100ml of a buffer containing 0.1M NaCl, 0.05M Tris, pH7.4 and 0.001M EDTA and resuspended in 10ml of 25% (w/v) sucrose, 0.1M NaCl and 0.05M Tris pH7.4. Lysozyme to a final concentration of $0.5mgml^{-1}$ 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 pH8.5 and 12.5ml of freshly-prepared 0.7M NaCl containing 2% SDS. The suspension was gently, but thoroughly, mixed and left overnight at 4°C. The lysate was centrifuged for 45 min at 15,000g at 10°C. The supernatant was collected and adjusted to 0.3M by adding 4M sodium acetate. DNA was precipitated in absolute ethanol and collected by centrifugation at 10,000g for 10 min, dried and dissolved in 5ml TES buffer (30mM Tris pH7.5, 50mM NaCl, 5mM EDTA). Pancreatic RNase to a final concentration of $50\mu gml^{-1}$ was added and incubated for 30 min, followed by another 30 min incubation with predigested pronase at a concentration of $500\mu gml^{-1}$. Plasmid DNA was purified by the dye-buoyant density gradient centrifugation technique.

DYE-BUOYANT DENSITY GRADIENT CENTRIFUGATION

DNA prepared from different bacterial strains was purified using

dye-buoyant density centrifugation in caesium chloride gradients (Radloff et al., 1967). Gradients were made by adding 1.1g of caesium chloride and 0.1ml of ethidium bromide solution ($10 mg ml^{-1}$ in TE buffer) to each ml of the DNA preparation. The refractive index of the gradients was adjusted to 9.4 measured in an Abbe 60 Refractometer (Bellingham and Stanley Ltd., England) by adding further caesium chloride, if necessary. The mixture was transferred to a 10ml polypropylene tube (MSE), overlaid with liquid paraffin to prevent evaporation and centrifuged at 45,000 rpm for 60 h at 18°C in a Prespin 65 MSE ultracentrifuge. When both chromosomal and plasmid DNA were present, two bands formed in the gradients. The lower was the covalently-closed circular (ccc) plasmid DNA, while the upper band was the chromosomal and nicked plasmid DNA. The desirable bands were removed carefully by puncturing the side of the tube with a 19 gauge needle attached to a 1ml syringe. Ethidium bromide was removed from the DNA by several extractions with two volumes of propan-2-ol which was saturated with NaCl-saturated distilled water. The preparation was then dialysed against three changes of 1l cold TE buffer and the DNA precipitated, dried in a vacuum and resuspended in TE buffer.

TRANSFORMATION

1. Transformation of members of genus Deinococcus with chromosomal and plasmid DNA

The procedure used was that developed by Tirgari and Moseley (1980). 1ml of an 18 h culture was diluted into 20ml of fresh TGY or NB2 in the case of D.radiophilus and incubated at 30°C with shaking. 10ml of the exponential culture, with a turbidity reading

of 30 (~1.0×10^8 viable units ml^{-1}), was centrifuged at 5,000g for 5 min at room temperature and cells resuspended in 5ml TGY (or NB2 in the case of D.radiophilus). 2ml of 0.1M CaCl2 solution was added and the culture placed on ice for 10 min. 0.3ml of the chilled culture was added to 50µl of TE buffer containing 5µg of chromosomal DNA or 1-5µg of plasmid DNA. The mixture was incubated with gentle shaking at 30°C for 90 min, then diluted tenfold with the same culture medium and allowed to grow for 5 h for phenotypic expression. Appropriate dilutions were plated onto TGY or NB2 agar and selective media. For the selection of UV-resistant (UV^R) transformants of D.radiodurans UVS78, cells were spread on TGY plates and irradiated with a dose of $190 Jm^{-2}$, 254nm UV light, from a Hanovia germicidal lamp (Hanovia Lamps Ltd., Slough, Bucks) at a dose rate of $1.05 Jm^{-2}S^{-1}$. UV^R transformants of both D.radiodurans UVS9 and UVS25 were selected by irradiating at a dose of $440 Jm^{-2}$.

2. Transformation of D.radiodurans and D.radiophilus spheroplasts with chromosomal DNA

Spheroplasts were prepared from D.radiodurans 302 cells in their late exponential phase as described. The spheroplast suspension was centrifuged, washed once with 5ml TGYS, resuspended in 2.5ml TGYS and 0.1ml of 1M $CaCl_2$ and the mixture held on ice for 10 min. 5µg of chromosomal DNA from D.radiodurans 62-1 (Acr^R, Ura^-) in 100µl TE buffer was added to 0.6ml of the spheroplast suspension and incubated with gentle shaking at 30°C for 90 min. Samples were diluted in both TGYS and distilled water, plated onto RM plates which were incubated at 30°C for 6 days for a viable count and to determine the percentage of regenerated colonies. Also, samples

from the TGYS dilution were spread onto RM plates and incubated at 30°C for 10-12 h to allow the regeneration of cell walls. The plates were overlaid with 8ml amounts of soft RM containing acriflavin ($10\mu g ml^{-1}$) and further incubated at 30°C for 8 days. As a control, 0.6ml of the spheroplast suspension was treated in the same way but without adding DNA, to determine the level of spontaneous mutation.

In other experiments, 5μg of chromosomal DNA in 100μl TE buffer was mixed with 0.6ml of spheroplast suspension. 2ml of 40% (w/v) PEG6000 in PS buffer was added and mixed gently for 2 min. The mixture was diluted with 7.3ml of TGYS to stop the action of PEG. Spheroplasts were collected by centrifugation, resuspended in 0.7ml TGYS and incubated at 30°C with gentle shaking for 90 min, before proceeding as described above.

Spheroplasts of D.radiophilus were transformed with chromosomal DNA from a rifampicin-resistant (Rif^R) strain of D.radiophilus, in the presence and absence of PEG, as described for D.radiodurans. TGYS was replaced by NB2S which also was present in RM instead of TGY.

3. Transformation of D.radiodurans and D.radiophilus spheroplasts with plasmid DNA

A modification of Chang and Cohens' (1979) procedure was used to transform the spheroplasts of D.radiodurans 302 and D.radiophilus with plasmid DNA.

Spheroplasts from both species were prepared as described and suspended in 2ml PS buffer. $CaCl_2$, to a final concentration of 30mM, was added and the suspension held on ice for 10 min. 1-5μg plasmid DNA in 50μl TE buffer was mixed with an equal volume of 2xPS buffer

in a sterile 20ml bottle and added to 0.5ml of the spheroplast suspension. 1.5ml of 40% PEG6000 solution in PS buffer was added immediately and the contents gently mixed. After 2 min treatment with PEG, 5ml of TGYS (NB2S in the case of D.radiophilus) was added to the mixture to dilute the PEG and spheroplasts were recovered by centrifugation. The treated spheroplasts were resuspended in 1ml of TGYS or NB2S and incubated for 90 min at 30°C with shaking. Samples were diluted in distilled water and TGYS or NB2S and spread onto RM plates which were incubated at 30°C for 8 days to determine the percentage of regenerated colonies. Also samples from dilutions in TGYS or NB2S were spread onto RM plates, incubated at 30°C for 12 h to allow regeneration of the cell wall, overlaid with 8ml amounts of soft RM containing the appropriate antibiotic and further incubated at 30°C for 8 days.

Alternatively, samples of treated spheroplasts were spread onto RM plates, incubated at 30°C and the resulting colonies replicated onto selective media to score transformants.

4. Transformation of E.coli with plasmid DNA

Strains of E.coli were transformed with plasmid or recombinant DNA according to the procedure of Humphreys et al. (1979).

An overnight culture was diluted 1:30 with fresh pre-warmed L-broth and incubated at 37°C with shaking. 10ml of the exponential culture (turbidity reading = 30-40) were centrifuged in a micro-centrifuge for 45 s at room temperature. Cells were washed once with 5ml of 10mM $CaCl_2$ at 0°C and resuspended in 0.5ml 75mM $CaCl_2$, 10mM MOPS, 0.5% (w/v) glucose, pH6.5. Plasmid DNA in 0.1ml of 0.1M Tris-HCl pH7.2 was added to 0.2ml of the recipient cells, then 0.2ml of

fresh 75mM $CaCl_2$, 10mM MOPS, 0.5% (w/v) glucose, pH6.5 was added and the mixture incubated at 0°C for 45 min. The transformation mixture was transferred to 42°C for 10 min and 1ml of fresh L-broth added before incubating at 37°C for 2 h for phenotypic expression. Samples were diluted appropriately and spread onto selective media. Plates were incubated at 37°C for 1-3 days.

GEL ELECTROPHORESIS

1. Agarose gel electrophoresis

Agarose gels (0.8-1.0%) were run either horizontally or vertically using home-made, perspex gel tanks. Gels were run in TAE (0.04M Tris-acetate, 0.001M EDTA, pH8.0), TBE (0.089M Tris-borate, 0.002M EDTA, pH8.1) or TPE (0.08M Tris-phosphate, 0.008M EDTA, pH8.0) buffers. Samples of DNA were mixed with 1/10 volume of the loading buffer and added to the wells in the gel. Running conditions depended on the nature of the material to be electrophoresed. In general, gels were run either for 3-4 h at $5vcm^{-1}$ and the gel buffer added up to the level of the horizontal gel surface or for 15-18 h at $1-2vcm^{-1}$ by submerging the gel in buffer.

2. Polyacrylamide gel electrophoresis

Polyacrylamide gels of dimensions 110x150x1mm were cast in cassettes made from 4mm glass and perspex spacers and sealed with paraffin wax. The 7-15% gradient was produced by the controlled mixing of the solutions shown in Table 2.4. The mixture was pumped into the cassette, overlaid with n-butyl alcohol to prevent the gel drying out and left overnight at room temperature. Shortly before use, the n-butyl alcohol was removed and the resolving gel overlaid

Table 2.4

Relative composition of polyacrylamide mixing solutions

Solution	Solution A(15%)	Solution B(7%)	Stacking gel
Tris 1.5M, pH8.8	3.25ml	3.25ml	-
Tris 0.5M, pH6.8	-		2.5ml
Acrylamide solution (29.2% acrylamide, 0.8% bisacrylamide)	6.5ml	3.0ml	1.5ml
1% SDS	1.3ml	1.3ml	1.0ml
H_2O	0.5ml	5.4ml	5.0ml
Glycerol	1.3ml	-	-
Temed	15µl	15µl	25µl
10% Ammonium persulphate	20µl	20µl	25µl

with 5ml of stacking gel, into which a comb was inserted, and left for 30 min to set. Slots were loaded with 0.5-1.0μg of DNA or 5-10μg of protein which were mixed with 1/10 volume of the loading buffer (0.125M Tris-HCl pH6.8, 2% (w/v) SDS, 10% (w/v) glycine, 0.05% (w/v) bromophenol blue, pH8.0) and the gel run at a constant current of 20mA for 5-6 h in a vertical tank. The running buffer used consisted of 0.2M glycine, 0.01% (w/v) SDS and 0.05M Tris base, pH8.4.

STAINING AND VISUALIZATION OF DNA IN GELS

Agarose gels were stained with ethidium bromide either by incorporation of the dye to a final concentration of $0.5\mu g ml^{-1}$ into the gel and running buffer or by immersing them in distilled water containing the dye in the same concentration for 45 min. Polyacrylamide gels were stained by immersing them in distilled water containing ethidium bromide at $0.5\mu g ml^{-1}$ for 10-15min.

DNA bands were visualized by UV illumination at 302nm on an UV transilluminator (U.V. Products Inc., Cambridge). Agarose gels were destained in distilled water for 1 h, and polyacrylamide gels for up to 10 h to get rid of the background before photographs were taken using a Polaroid MP-4 camera with Polaroid type 55 film and a Kodak 22A Wrattan filter.

SIZING OF DNA FRAGMENTS FROM GEL ELECTROPHORESIS

Sizes of DNA fragments were measured according to their mobilities on gels compared with standards (Southern, 1979). The relationship between the molecular weight of a DNA fragment and its mobility was represented by simple equations (Fig.2.2.). DNA fragments were run on a gel in the presence of a standard sizing

Fig.2.2. Calculation of size of DNA molecules and fragments from their mobilities in gels.

$$(a)\quad M_0 = \frac{M_3 - M_1[(L_1-L_2)/(L_2-L_3)\times(M_3-M_2)/(M_2-M_1)]}{1 - [(L_1-L_2)/(L_2-L_3)\times(M_3-M_2)/(M_2-M_1)]}$$

$$(b)\quad K_1 = \frac{L_1-L_2}{1/(M_1-M_0)-1/(M_2-M_0)}$$

$$(c)\quad K_2 = L_1-K_1/M_1-M_0$$

$$(d)\quad L = K_1/(M-M_0) + K_2$$

marker (usually λ DNA digested with HindIII). The log mobility of the different λ DNA fragments was plotted against its log molecular weight. Three points connected by a straight line were chosen corresponding to sizes L_1, L_2 and L_3 with mobilities M_1, M_2 and M_3 respectively. These values were used in equations a, b and c; then equation d was used to calculate the size of a fragment L with mobility M. This method was more accurate than the graphical method.

ELUTION OF DNA FROM AGAROSE GELS

The smallest plasmid of D.radiophilus viz. pUE1 and particular restriction fragments were eluted from agarose gels according to the method of McDonnell et al. (1977).

Intact plasmids or restricted DNA were applied to a 0.8% agarose gel. The gel was run horizontally in TBE buffer at $2.0 vcm^{-1}$ for 15-18 h. Ethidium bromide was present in the gel and the running buffer to a final concentration of $0.5 \mu g ml^{-1}$. DNA bands were visualised by UV light and the slice of the gel containing the band of interest was cut out using a sharp scalpel. The gel slice was slipped into a dialysis bag filled with 0.5xTBE buffer and allowed to sink to the bottom of the bag. Most of the buffer was removed from the bag leaving just enough fluid to keep the gel slice in constant contact with the electrophoresis buffer. The bag was tied just above the gel slice without trapping air bubbles, and immersed in a shallow layer of 0.5xTBE buffer in an electrophoresis tank. An electric current of 100v was passed through the bag until the DNA was electroeluted out of the gel and onto the inner wall of the dialysis bag. This was determined by illuminating the gel slice inside the dialysis bag with UV light. The polarity of the current was reversed

for 1-2 min to release the DNA from the wall of the dialysis bag. The bag was opened at one side and the gel slice removed carefully using sterile forceps. The buffer containing the eluted DNA was removed from the dialysis bag and passed through a column of sterilised, siliconised glass-wool packed in a 1ml disposable pipette tip, to remove any gel particles. The eluate was extracted twice with phenol, once with phenol-chloroform (1:1 v/v) and once with chloroform-isoamyl alcohol (24:1 v/v). The DNA was precipitated by adding 1/10 volume of 4M sodium acetate and two volumes of absolute ethanol, collected by centrifugation in a microcentrifuge for 5 min at 4°C and dried in a vacuum. The pellet was dissolved in an appropriate volume of TE buffer or distilled water and stored at 4°C. DNA eluted by this method was suitable for restriction analysis and cloning.

CURING D.RADIOPHILUS OF ITS PLASMIDS BY SPHEROPLAST FORMATION AND REGENERATION

Spheroplasts of D.radiophilus were prepared as described and plated on RM. One hundred regenerated colonies were selected and screened for missing plasmids. Plasmid DNA was prepared from a 1.5ml culture of each colony using a modification of the Birnboim and Doly (1979) method. Lysates were run on 0.8% agarose gels. Gels were stained with ethidium bromide and DNA visualised under UV light looking for missing plasmid DNA bands.

SINGLE AND DOUBLE DIGESTION OF DNA WITH RESTRICTION ENDONUCLEASES

Chromosomal and plasmid DNA were digested with various restriction endonucleases in 20 or 40µl of reaction mixture and incubated

for 2-4 h for complete digestion. Enzymes were used in accordance with the manufacturers' instructions. When the two enzymes required different buffers, double digestions were performed in two stages with an ethanol precipitation step in between.

CLONING IN PLASMID DNA

1. Cloning by sticky-end ligation

Vector (pAT153) and target DNA were digested separately with a suitable restriction endonuclease. The linearised pAT153 was then ethanol-precipitated and resuspended in 40µl of alkaline phosphatase buffer (50mM Tris-HCl, 0.1mM EDTA, pH8.0). The vector had the 5' terminal phosphate groups removed by the addition of 1U of calf intestinal alkaline phosphatase and incubation for 30 min at 37°C. This treatment prevented the recircularisation of the vector unless target DNA was inserted. Vector and target DNA were deproteinised by two phenol and two phenol-chloroform extractions followed by washing with distilled water-saturated ether and ethanol-precipitated at -20°C. Vector and target DNA were resuspended in the ratio of 3:1 and to a final concentration of $50\mu g ml^{-1}$ in 40µl ligase buffer (20mM Tris-HCl, pH7.6, 10mM MgCl2, 10mM DTT, 0.6mM ATP). Ligation was carried out by adding 0.2U of T4 DNA ligase and incubating the mixture at 12°C for 16 h. The ligated DNA was ethanol-precipitated, resuspended in 100µl Tris-HCl pH7.2 and used to transform E.coli HB101.

2. Cloning by blunt-end ligation

Ligation of DNA fragments with non-compatible ends was performed by the treatment of both the vector and target DNA with 50-100U of

nuclease S1 for 30 min at 37°C. This treatment removed the single-strand extensions producing blunt-end fragments. After ethanol-precipitation, blund-end vector and target DNA were resuspended and mixed together in 20ul of ligase buffer at a concentration of >200μgml^{-1}. 2U of T4 DNA ligase was added and the mixture incubated at 23°C for 15 h.

TRANSDUCTION OF E.COLI HB101 WITH IN VITRO PACKAGED λ PHAGE PARTICLES

The in vitro packaged λ phage particles, obtained from Martin Mackay, were used to transduce E.coli HB101 in order to select the colonies to represent the gene library.

50μl of the packaging mix was added to 50μl of E.coli HB101 culture growing in L-broth containing 0.4% (w/v) maltose. The phage particles were allowed to adsorb by incubating the culture at 37°C. After 20 min, 1ml L-broth was added and the incubation continued for a further 45 min for expression. The culture was then spread onto L-plates containing 40μgml^{-1} ampicillin (Ap) and incubated overnight at 37°C. 320Ap^{R} colonies were selected to represent the gene library. Since the average size of the inserts in the cosmid vector was 35kb (Mackay, 1983) and the size of D.radiodurans genome is 3,000kb, screening of these 320 colonies would give a 97.7% probability of finding a unique D.radiodurans sequence according to the formula of Clarke and Carbon (1976).

SELECTION OF HYBRID COSMIDS CARRYING GENES FROM D.RADIODURANS CAPABLE OF FUNCTIONAL EXPRESSION IN E.COLI

E.coli HB101 was transduced with packaging mix as described. After 45 min of incubation for expression, 10ml of L-broth containing Ap (40μgml^{-1}) was added to the transduced population and incubation continued overnight at 37°C. Samples were diluted appropriately and

spread on M9 supplemented agar lacking either leucine or proline and on L-plates containing erythromycin ($10\mu gml^{-1}$). Samples were also spread on L-plates and irradiated with UV light ($31.5Jm^{-2}$). Plates were incubated at 37°C for 3 days.

SELECTION OF HYBRID COSMIDS CARRYING GENES COMPLEMENTING D.RADIODURANS MUTATIONS

The 320 ampicillin-resistant (Ap^R) E.coli HB101 clones containing the D.radiodurans gene library were divided into 8 groups of 40 colonies. Each group of colonies growing on one L-plate containing Ap was susupended in 3ml L-broth. These suspensions were used to inoculate 1l amounts of L-broth containing Ap which were incubated at 37°C with shaking for 18 h and used for DNA preparation. Hybrid cosmid DNA from each group was used in the ccc form or after linearisation with BamHI to transform the UV-sensitive (UV^S), mytomycin c-senstive (Mtc^S) mutant D.radiodurans UVS78 to antibiotic, UV and/or Mtc resistance, and several auxotrophic mutants of D.radiodurans to prototrophy. Samples from transformed D.radiodurans UVS78 were diluted and spread onto TGY plates containing one of the following: erythromycin ($15\mu gml^{-1}$), mitomycin c ($0.04\mu gml^{-1}$), rifampicin ($30\mu gml^{-1}$) and streptomycin ($5\mu gml^{-1}$). Samples were also spread on TGY plates and irradiated with UV light at a dose of $190Jm^{-2}$ for the selection of clones transformed to UV resistance by the acquisition of either the mtcA or uvsE genes. Samples from the transformed auxotrophs were diluted and spread on mininmal medium. Plates were incubated at 30°C for 4-5 days.

MAXI-CELL PREPARATION AND LABELLING OF PROTEINS

The procedure described by Sancar and Rupp (1983) was used to detect the expression of D.radiodurans genes in E.coli using the maxi-cell technique.

E.coli CSR603 was transformed with the hybrid plasmid to be analysed and checked for the presence of the plasmid by the rapid boiling method. 0.3ml of an 18 h culture of E.coli CSR603 carrying the plasmid of interest in K medium was inoculated into 10ml of fresh K medium and incubated with shaking at 37°C. 2.5 ml of the exponential culture (~1.0×10^8 cells ml^{-1}) was transferred into a sterile glass petri dish and irradiated with UV light at a dose of $6Jm^{-2}$, while stirring the culture magnetically. 2ml of the irradiated culture was transferred to a 20ml bottle and incubated at 37°C for 1 h to allow the cell wall and septum synthesis to be completed. 15µl of freshly-prepared D-cycloserine solution ($15mgml^{-1}$) was added and the culture incubated at 37°C for 10-16 h. Cycloserine (like penicillin) acts on the growing cell wall and division septum and will therefore kill dividing and growing cells, thus eliminating any surviviors left in the culture. Cells were collected from 1.5ml of culture by centrifugation in a microcentrifuge for 45 s at room temperature, washed twice with 1ml of Hershey salts and resuspended in 0.8ml of Hershey medium. After incubation at 37°C for 1 h, 0.2ml of Hershey medium containing 6µCi [^{35}S]-methionine was added, mixed gently and incubated for a further 2 h at 37°C. Labelled cells were harvested by centrifugation in a microcentrifuge for 45 s and washed twice with 1ml of 100mM NaCl. The pellet was resuspended in 50µl of sample buffer by vortexing and placed in a boiling water bath for 3-5 min to lyse cells and denature proteins. 35µl samples were loaded on 7-15% SDS-polyacrylamide gels and run as described. E.coli CSR603 containing no plasmid and E.coli CSR603 harbouring the plasmid pAT153 were treated in the same way and acted as controls. The gels were also loaded with 5-10µg of the

following non-labelled proteins: thyroglobulin (MW=330000), bovine serum albumin (MW=68000), ovalbumin (MW=43000) and trypsin inhibitor (MW=20000) as standard molecular weight markers.

After the completion of electrophoresis, proteins were fixed by soaking the gel with 400ml of 40% methanol, 10% acetic acid solution for 30 min, followed by soaking for another 30 min with 400ml of 10% ethanol, 5% acetic acid solution. Lanes which contained the standard molecular weight markers cut out by a sharp scalpel and stained overnight with kanacid blue stain (0.25% (w/v) kanacid brilliant blue, 45% (v/v) methanol, 8% (v/v) acetic acid in distilled water). The stain was discarded and the gel destained overnight with destaining solution (5% methanol, 7.5% acetic acid). The part of the gel containing the labelled protein was soaked overnight with 30% methanol, 3% glycerol solution to prevent the gel cracking during the subsequent vacuum drying at 80°C. The dried gel was wrapped in Saran wrap (Dow Chemical Company, Michigan) and applied to X-ray film (Fuji Rx X-ray film) for 48 h at -80°C to obtain an autoradiographic image.

NICK-TRANSLATION

DNA of plasmids pAT153 and pUE441 were nick-translated using a scaled down version of the procedure described by Maniatis *et al.* (1982). The nick-translation mixture (20µl) was set up as follows.

10 x nick-translation buffer	2µl
DNA	1µl (100ng)
[α-^{32}P]dCTP	1µl (10µCi)
[α-^{32}P]dTTP	1µl (10µCi)
Unlabelled dATP	1µl (0.4nmol)
Unlabelled dGTP	1µl (0.4nmol)

DNase I	1µl (0.1ng)
E.coli DNA polymerase 1 .	0.3µl (2U)
Distilled water	to 20µl

The mixture was incubated at 16°C for 1 h. The proportion of [α-^{32}P]dNTPs incorporated into the DNA was determined by spotting 0.3µl of the mixture onto the centre of a Whatman CF/C glass microfiber disc (2.1cm) and assaying the radioactivity using a hand-held monitor. The distance between the monitor and the disc was adjusted to give a reading of 100. The disc was then washed four times with 10% trichloroacetic-acid (TCA) solution to remove the unincorporated dNTPs and the radioactivity measured again with the same monitor. The difference in reading of the monitor before and after washing indicated the proportion of [α-^{32}P]dNTPs incorporated into DNA. The nick-translated DNA was separated from unincorporated dNTPs by passing the mixture through a column of Sephadex G50 fine. The Sephadex G50 was prepared in TE buffer and packed in a disposable 5ml pipette plugged with a piece of sterile, siliconised glass wool. The column was washed several times with TE buffer and the DNA sample, in a volume of 100µl (20µl nick-translation mixture + 80µl TE buffer), was applied to the column. The progress of the incorporated and unincorporated [α-^{32}P]dNTPs was followed down the column using a hand-held monitor. The leading peak which consisted of the nucleotides incorporated into DNA was collected into sterile Eppendorf tubes and stored at -20°C. Before adding to the hybridisation solution, the nick-translated DNA was denatured by placing it in boiling water for 10 min and then transferring it to 0°C.

SOUTHERN TRANSFER

Chromosomal DNA (2.5µg) of D.radiodurans krase was digested in

20µl reaction mixture with EcoRI or SmaI and the digests run on a vertical 0.8% agarose gel at 1.5vcm^{-1} for 15 h. DNA was stained with ethidium bromide after the completion of electrophoresis and the gel photographed. Unused areas of the gel were trimmed away with a sharp scalpel and the DNA denatured by soaking the gel in two volumes of 1.5MNaCl, 0.5MNaOH solution for 1 h at room temperature with constant shaking. The gel was washed with distilled water and neutralized by soaking it in two volumes of 1M Tris-HCl pH8.0, 1.5M NaCl solution at room temperature for 30-60 min. A stack of glass plates was covered with a piece of Whatman 3MM paper (wick) and placed inside a large dish. 20 x SSC buffer was poured into the dish almost to the top of the glass stack and the air bubbles trapped between the 3MM paper and the stack removed with a glass rod. The gel was placed on the damp 3MM paper without trapping air bubbles and covered with a piece of wet (soaked in 2 x SSC for 2 min) nitrocellulose filter (Schleicher & Schuell BA85), so that one edge extended just over the line of slots at the top of the gel. All air bubbles under the nitrocellulose filter were removed and two wet pieces (in 2 x SSC) of Whatman 3MM (cut to the same size as the gel) were placed on the top of the nitrocellulose filter without trapping air bubbles. A stack of paper towels (about 10cm high) was placed on the 3MM paper and weighed down with a 500g weight. To prevent short circuiting of fluid between the paper towels and the 3MM paper under the gel, the gel was surrounded with Nesco film (Nippon Shoji Kaisha Ltd., Japan). DNA transfer was allowed to proceed for 15 h when the paper towels and the 3MM papers above the nitrocellulose filter were removed and the position of the gel slots marked on the filter with a soft pencil. The nitrocellulose filter was removed

from the top of the gel and washed with 2 x SSC for 5 min at room temperature. Excess fluid was allowed to drain from the filter which was set to dry on a sheet of 3MM paper. The dry filter was baked for 2 h at 80°C in a vacuum.

DNA-DNA HYBRIDISATION

The baked filter (to which the DNA was transferred) was floated on the surface of 6 x SSC to wet it from beneath and then immersed in the buffer for 2 min. The wet filter was slipped into a roll tube and stuck to the inner surface of the tube without trapping air bubbles. 10ml of prehybridisation solution was added to the bottom of the tube which was closed tightly and incubated while rolling slowly at 68°C. After 7 h the prehybridisation solution was removed and 10ml of the hybridisation solution was added to the bottom of the tube and incubated as before for 15 h. The filter was removed from the roll tube and washed at 55°C as follows, 1 h in 4 x SSC then 30 min each in 3 x, 1 x and 0.1 x SSC. All solutions contained 0.1% (w/v) SDS and 0.1% (w/v) NaPPi. The filter was dried between 2 3MM papers at room temperature, wrapped in Saran wrap and applied to X-ray film for 3 h at -80°C to obtain an autoradiographic image.

CHAPTER 3

RESULTS

1. MUTAGENESIS

Auxotrophic and antibiotic-resistant mutants were isolated from D.radiodurans 302 after treatment with MNNG. Some of them appeared to be double mutants i.e. auxotrophic and antibiotic-resistant or resistant to two antibiotics. The characterised mutants are listed in Table 3.1. The nutritional requirements for some putative auxotrophs could not be identified. Of course it was not possible to select for auxotrophs which needed nutritional requirements already incorporated in the minimal medium for the growth of the wild type. The characterised mutants were stable upon repeated subculturing and some of them were used in a later part of this study.

2. SPHEROPLAST GENETICS

2.1 Spheroplast formation

2.1.1 Spheroplast formation from D.radiodurans

Treatment of D.radiodurans 302 with lysozyme-EDTA in PS buffer for 5-6 h converted more than 99% of the cells to an osmotic-sensitive form (spheroplasts). Spheroplast formation was determined by microscopic examination and osmotic shock. Cells were osmotically sensitive when single cells or groups of cells appeared to be surrounded by a membrane-like structure (Fig.3.1-b). Addition of NaOH at such a stage turns the spheroplast suspension into a clear one. Samples examined under the microscope after osmotic shock comprised of mainly ghost cells (Fig.3.1-c). Only cells that retained the morphology of normal cells (Fig.3.1-a) remained intact after osmotic shock. Cells were harvested from spheroplasting buffer when more

Table 3.1

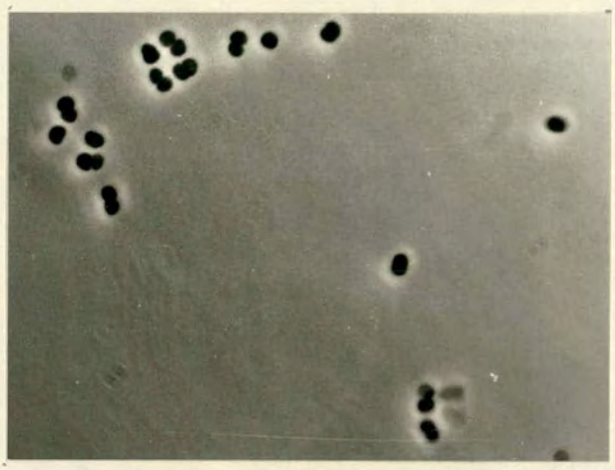
Characterised mutants isolated from *D.radiodurans* 302

Strain of *D.radiodurans* 302	Relevant genotype	Phenotype
2-1	*asp*-1	Required asparagine
3-1	*asp*-2	" "
5-1	*pdx*-1	Required pyridoxine
15-1	*asp*-3	Required asparagine
18-1	*trp*-1	Required tryptophan
21-1	*pdx*-2	Required pyridoxine
22-1	*trp*-2	Required tryptophan
27-1	*trp*-3, *rif*	" " , Rif^R
28-1	*rif*	Rif^R
30-1	*tyr*, *rif*	Required tyrosine, Rif^R
34-1	*arg*, *rif*	Required arginine, Rif^R
40-1	*ura*-1, *acr*	Required uracil, Acr^R
41-1	*trp*-4, *acr*	Required tryptophan, Acr^R
42-1	*acr*	Acr^R
44-1	*trp*-5	Required tryptophan
62-1	*ura*-2, *acr*	Required uracil, Acr^R
66-1	*trp*-6	Required tryptophan
67-1	*trp*-7, *acr*	" " , Acr^R
73-1	*kan*, *acr*	Kan^R, Acr^R
84-1	*kan*, *rif*	Kan^R, Rif^R
114-2	*ser*	Required serine

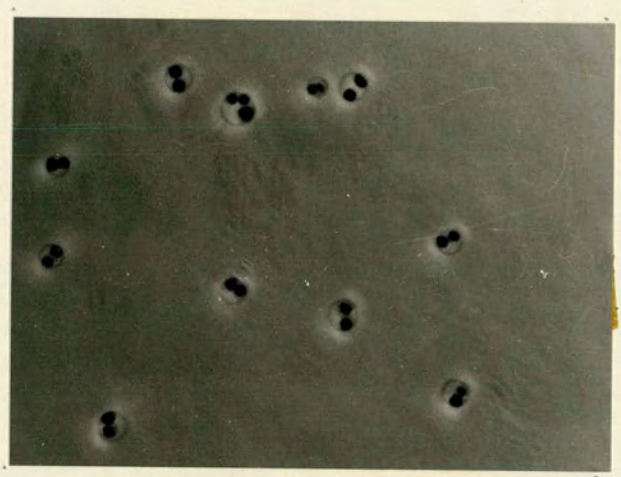
Fig.3.1

Spheroplast formation from D.radiodurans 302
(a) Normal cells growing in TGY broth
(b) Spheroplasts produced after 5 h treatment with lysozyme-EDTA in PS buffer
(c) Ghost cells (lysed spheroplasts) formed by osmotic shock

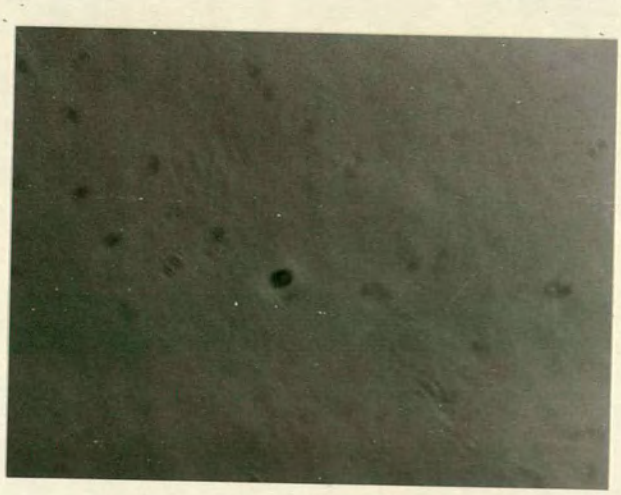
a



b



c



than 99% of them had been converted into spheroplasts. The time required to achieve this percentage varied from one experiment to another. In general 5-6 h of treatment was needed. Further incubation with lysozyme-EDTA after spheroplast formation affected their viability and resulted in poor regeneration frequencies. Treatment of cells with lysozyme-EDTA for 15 h did not remove the membrane-like structure and resulted in complete loss of viability.

In addition to PS, other buffers were used for spheroplast formation from D.radiodurans. Using TS buffer, more than 99.9% of the cells were converted into spheroplasts within a very short time (15 min), even with lower concentrations of lysozyme and EDTA, but this treatment badly affected cell viability and resulted in a poor regeneration frequency (Table 3.2). A longer time (2 h) was needed to convert more than 99% of the cells into spheroplasts using TST1, but gave a much better regeneration frequency compared with that obtained using TS buffer. TST2 and SMM were not suitable buffers for spheroplast formation; after 7 h of treatment, only 66% and 57% of the cells respectively were converted to spheroplasts. As a result only a small percentage of the colonies appearing on RM plates were developed from spheroplasts. TGYS was also a very poor medium for spheroplast formation. Thus PS buffer, compared with other buffers, gave much better results and so was used as the spheroplasting buffer for D.radiodurans. The RM used for the regeneration of spheroplasts was the one described in Materials and Methods.

2.1.2 Effect of growth phase on spheroplast formation

Cells of D.radiodurans 302 from different growth phases were harvested and used for spheroplast formation, to determine which

Table 3.2

Spheroplast formation from D.radiodurans 302 using different spheroplasting media

Spheroplasting medium	Time of treatment (h)	Lysozyme concentration ($mgml^{-1}$)	EDTA concentration (M)	Spheroplast percentage	Regeneration frequency	Regenerant percentage
TS	0.25	1	0.001	>99.9	$4.8x10^{-4}$	99.7
TST1	2	2	0.002	99.8	$1.8x10^{-2}$	98
TST2	7	2	0.002	66	$1.3x10^{-1}$	26
SMM	7	2	0.002	57	$2.3x10^{-1}$	23
TGYS	7	2	0.002	40	$2.2x10^{-1}$	13
PS	6	2	0.002	99.2	$1.2x10^{-1}$	90

growth phase is more suitable for spheroplast formation and regeneration. As shown in Table 3.3, cells from the early exponential phase of growth (turbidity = 30) were less susceptible to the lysozyme-EDTA treatment. After 7 h, 91% of the cells were converted to spheroplasts and subsequently only 74% of the colonies developed on RM plates were actually from spheroplasts. For the same time of treatment, cells from the mid-exponential phase of growth (turbidity = 60) showed a greater susceptibility to lysozyme-EDTA treatment and 96% being converted to spheroplasts. Cultures from the late exponential phase of growth (turbidity = 90) needed only 5 h for the conversion of more than 99% of the cells to spheroplasts with 96% of the colonies developing on RM plates being from spheroplasts.

2.1.3 Spheroplast formation from D.radiophilus

Cells of D.radiophilus in the late exponential phase of growth were more susceptible to the lysozyme-EDTA treatment in PS buffer than those of D.radiodurans. Spheroplast formation was completed within 40-60 min using half the concentration of lysozyme and EDTA used for the spheroplasting of D.radiodurans. In the early stages of the treatment, cells became more round and surrounded by the membrane-like structure (Fig.3.2-a-arrow). Later, membranes were broken down leaving round cells (Fig.3.2-b) which were osmotic-sensitive, and these were converted into ghost cells after osmotic shock. As shown in Table 3.4, spheroplast formation in SMM buffer was poor and after 6 h of treatment, only 67% of the cells were converted to spheroplasts. On RM plates the percentage of colonies developed from spheroplasts was very low.

Table 3.3

Effect of growth phase on spheroplast formation in D.radiodurans

Turbidity	Time of treatment (h)	Spheroplast percentage	Regeneration frequency	Regenerant percentage
30	7	91	2.7×10^{-1}	74
60	7	96	2.0×10^{-1}	85
90	5	>99	1.6×10^{-1}	96

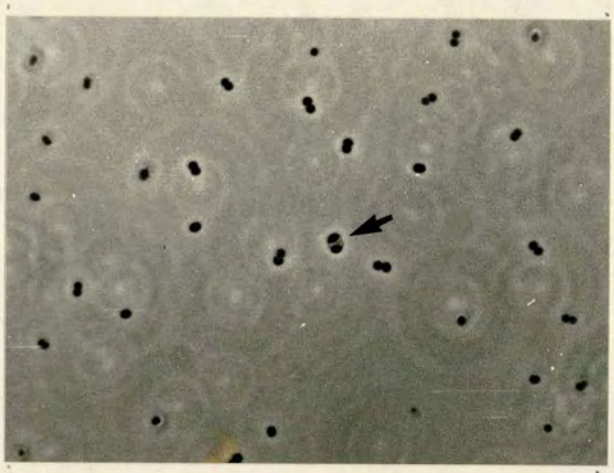
Fig.3.2

Spheroplast formation from D.radiophilus

(a) Cells in the early stages of treatment with lysozyme-EDTA in PS buffer. Most of the cells are similar in morphology to normal cells, while others have started to change and have become surrounded by a membrane-like structure (arrow)

(b) Spheroplasts produced after 1 h of treatment with lysozyme-EDTA in PS buffer. The membrane-like structure which surrounded the cells in the early stages of treatment has broken down leaving single cells.

a



b

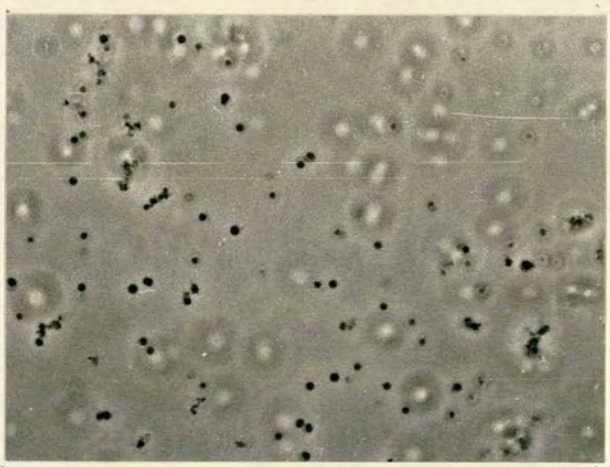


Table 3.4

Spheroplast formation from D.radiophilus using PS and SMM as spheroplasting media

Spheroplasting medium	Time of treatment (h)	Lysozyme concentration ($mgml^{-1}$)	EDTA concentration (M)	Spheroplast percentage	Regeneration frequency	Regenerant percentage
PS	1	1	0.001	>99	$3.6x10^{-2}$	88
SMM	6	2	0.002	67	$1.1x10^{-2}$	27

2.2 Regeneration of spheroplasts

Several recipies for regeneration media were used for the regeneration of D.radiodurans spheroplasts. The regeneration medium (RM) described in Materials and Methods was the most suitable. The regeneration frequencies on this medium ranged between 10 and 20%. Colonies appeared on RM plates at different times. Some colonies appeared after two days, while others appeared after three or four days of incubation. There was no significant difference in the regeneration frequency when plates were incubated at 30°C or 37°C, but colonies grew faster at 37°C. Spheroplasts were plated onto RM plates in two different ways. Samples were either spread onto RM plates or added to 4ml of warm (45°C) soft RM (0.4% agar) and overlaid on the solid RM. Use of the overlaying method did not improve the regeneration frequency and so the spreading method was adopted as routine. The addition of 0.5% (w/v) of casamino acids (Difco) to RM doubled the number of regenerants. Increasing the amount of casamino acids to 1.0% (w/v) did not increase the number of regenerants, so 0.5% was used in the RM. Supplementation of RM with different concentrations (0.01%, 0.05% and 0.1% w/v) of Bovine Serum Albumin (BSA) did not enhance regeneration. Spheroplasts of D.radiodurans were plated on TGY, RM and RM supplemented with materials which are known to improve the regeneration frequency in other microorganisms (Table 3.5). The regeneration frequency and the regenerants percentage on TGY agar were much lower than those on RM which indicated that spheroplasts were not stable on TGY. The addition of BSA, gelatin or BSA and gelatin to RM did not alter the regeneration frequency significantly, but regenerants grew faster and bigger in the presence of gelatin. The presence of osmotic

Table 3.5

Regeneration of D.radiodurans spheroplasts on different regeneration media

Regeneration medium	Regeneration frequency	Regenerant percentage
TGY	6.0×10^{-3}	56
RM[1]	1.4×10^{-1}	94
RMB[2]	1.3×10^{-1}	94
RMG[3]	1.2×10^{-1}	94
RMBG[4]	1.5×10^{-1}	93
RMS1[5]	6.0×10^{-2}	96
RMS2[6]	0	0
RMS3[7]	0	0

[1]RM described in materials and methods
[2]RM containing 0.08% BSA
[3]RM containing 5% gelatine (Oxoid)
[4]RM containing BSA and gelatine
[5]RM containing 0.25M sucrose
[6]RM containing 0.5M sucrose
[7]RM containing 0.5M sodium succinate

stabilisers which are normally used in regeneration media for other microorganisms affected the regeneration frequency. The presence of 0.5M sucrose or 0.5M sodium succinate caused complete inhibition of growth of both spheroplasts and normal cells of D.radiodurans. 0.25M sucrose also affected cell growth and reduced the regeneration frequency. However, the presence of 0.5M sucrose as an osmotic stabiliser in spheroplasting medium and all diluents was very important for the stabilisation of spheroplasts before plating them onto the solid RM.

Regeneration frequencies of D.radiophilus spheroplasts prepared in PS buffer and regenerated on RM containing NB2 instead of TGY, were lower than those of D.radiodurans and ranged from 2-3%.

2.3 Spheroplast fusion

Spheroplasts of two different D.radiodurans mutants were mixed together in an attempt to fuse them in the presence of PEG6000 as described in Materials and Methods. Upon addition of PEG, aggregation of cells was observed microscopically. Two fusion experiments were run. In one of them, DNase was present in the spheroplasting buffer, diluents and RM at a concentration of $10\mu g ml^{-1}$ to prevent transformation, while no DNase was present at any stage of the other experiment. Results obtained from spreading samples from the different treatments onto RM plates which were then overlaid with soft RM containing rifampicin (Rif) and acriflavine (Acr) are shown in Table 3.6. Presence of PEG affected the viability of spheroplasts and resulted in reducing the regeneration frequencies in both experiments. In the absence of DNase, Rif^R, Acr^R colonies were obtained in similar numbers from both the treated (with PEG) and

Table 3.6

Regeneration frequencies and number of $Rif^R Acr^R$ colonies after attempted fusion of D.radiodurans mutants 30-1 (Tyr^-, Rif^R) and 40-1 (Ura^-, Acr^R) in the presence and absence of DNase

Presence of DNase	Regeneration frequency of the fused mixture	Regeneration frequency of the control mixture	Number of Rif^R, Acr^R colonies from the fused mixture ml^{-1}	Number of Rif^R, Acr^R colonies from the control mixture ml^{-1}	Number of Rif^R, Acr^R colonies from spheroplasts of D.radiodurans 30-1 ml^{-1}	Number of Rif^R, Acr^R colonies from spheroplasts of D.radiodurans 40-1 ml^{-1}
+	2.1×10^{-2}	7.1×10^{-2}	0	0	0	0
-	2.3×10^{-2}	9.0×10^{-2}	2.3×10^{2}	1.9×10^{2}	0	0

untreated spheroplast mixtures. In both experiments, no Rif^R, Acr^R colonies developed on plates inoculated with spheroplasts of either D.radiodurans 30-1 or D.radiodurans 40-1. The Rif^R, Acr^R colonies from the treated and untreated spheroplast mixtures were replicated onto minimal media, and none of them was prototrophic. The results indicate that the Rif^R, Acr^R colonies developed as a result of transformation, rather than fusion or spontaneous mutation. Replication of fully grown colonies on RM plates from different treatments onto TGY containing rifampicin and acriflavine gave results similar to those shown in Table 3.6.

The addition of $CaCl_2$ to the fusion mixture, or the use of different proportions of PEG and spheroplast mixture (see Materials and Methods) did not enhance the fusion of the two mutant spheroplasts.

2.4 Transformation of spheroplasts with chromosomal DNA

Spheroplast suspensions of D.radiodurans 302 were transformed with chromosomal DNA carrying the Acr^R marker in the presence or absence of PEG6000 as described in Materials and Methods. Normal cells of D.radiodurans 302 were also transformed with chromosomal DNA in the same way, as a control. After incubation for 8 days, the Acr^R colonies from the different treatments were counted and the transformation frequencies and regenerant percentages calculated (Table 3.7). Transformation frequencies obtained from spheroplast suspensions were the ratio of the Acr^R colonies to the total number of colonies that appeared on RM inoculated from spheroplast suspension diluted in TGYS (colonies developed from both spheroplasts and OR cells), and were very low compared with those obtained from normal

Table 3.7

Transformation of spheroplast suspension and normal cells of *D.radiodurans* 302 with 5μg of chromosomal DNA carrying an Acr^R marker in the presence and absence of PEG6000

Cells	Presence of PEG6000	Transformation frequency	Control (no DNA)	Regenerant percentage
Spheroplasts	-	1.0×10^{-6}	$<1.7 \times 10^{-8}$	97
Spheroplasts	+	6.0×10^{-7}	$<9.0 \times 10^{-7}$	93
Normal	-	1.6×10^{-3}	$<3.0 \times 10^{-8}$	-
Normal	+	2.9×10^{-4}	$<1.5 \times 10^{-8}$	-

cells. The presence of PEG reduced the transformation frequency of both the spheroplast suspensions and normal cells. No Acr^R colonies were obtained from controls (spheroplast suspensions or normal cells treated in the same way but without the addition of DNA), which indicated that the Acr^R colonies obtained from the spheroplast transforming mixtures were transformants and not spontaneous mutants. Cell suspensions with lower spheroplast percentages (and therefore with higher OR percentages) transformed to Acr^R with higher frequencies (data not shown) which indicated that the Acr^R colonies obtained from the spheroplast suspensions arose as the result of transformation of normal cells in the suspension rather than of spheroplasts. Increasing the DNA concentrations in the presence of PEG, did not increase the spheroplast transformation frequency. On the contrary, the transformation frequencies obtained using higher DNA concentrations were lower than those resulting from the use of lower DNA concentrations (Table 3.8).

D.radiophilus spheroplasts were transformed with different amounts of chromosomal DNA carrying a Rif^R marker (0.5μg, 1.0μg, 2.0μg, 5.0μg) in the presence or absence of PEG. No Rif^R colonies were obtained from any of the treatments (transformation frequency $<1.0 \times 10^7$). This result indicated that D.radiophilus spheroplasts, like normal vegetative cells, are not transformable (using the described procedure) with homologous DNA, even in the presence of PEG.

2.5 Transformation of spheroplasts with plasmid DNA

Results of transformation of D.radiodurans spheroplasts with different plasmids are shown in Table 3.9. All plasmids tested

Table 3.8

Transformation of a D.radiodurans 302 spheroplast suspension with different amounts of chromosomal DNA, carrying Acr^R marker, in the presence of PEG6000

Amount of DNA (μg)	No. of Acr^R transformants (ml^{-1})	Control (no DNA)
0	0	0
1	$6.0x10^1$	0
5	$2.0x10^1$	0
10	$2.0x10^1$	0
15	$1.0x10^1$	0
20	$1.0x10^1$	0

Table 3.9

Transformation of a D.radiodurans 302 spheroplast suspension with plasmid DNA

Plasmid DNA (2μg)	Antibiotic resistance selected for and concentration used ($\mu g ml^{-1}$)	Transformation frequency
pHV33	Ap 1	C*
	Tc 1	C
	Cm 5	C
pAT153	Ap 1	C
	Tc 1	C
pC194	Cm 5	C
pUB110	Kn 10	C

* C = control level = $< 6.0 \times 10^{-7}$

failed to yield any transformants, and unlike transformation with chromosomal DNA, normal cells in the spheroplast suspension did not transform with any plasmid DNA used. Varying the amount of plasmid DNA used for transformation to 1μg or 5μg did not have any effect on transformation. Also, when treated spheroplasts were allowed to grow fully on RM and were then replicated onto TGY agar containing the appropriate antibiotic, the results were negative.

Plasmid DNA used to transform D.radiophilus spheroplasts also failed to yield any transformants (Table 3.10).

2.6 Curing of plasmids by spheroplast formation and regeneration

D.radiophilus cells were spheroplasted and allowed to regenerate their cell walls as described. Since plasmids of D.radiophilus and those of other Deinococcus strains are cryptic (Mackay et al., 1985) the only way to detect cured strains was to prepare plasmid DNA from the regenerants and to analyse it on agarose gels. One hundred regenerants were screened for missing plasmid bands, but they all showed plasmid DNA band patterns similar to that of the wild type (Fig.3.3). This indicated that spheroplast formation and regeneration is not an effective method of curing D.radiophilus of its plasmids.

Table 3.10

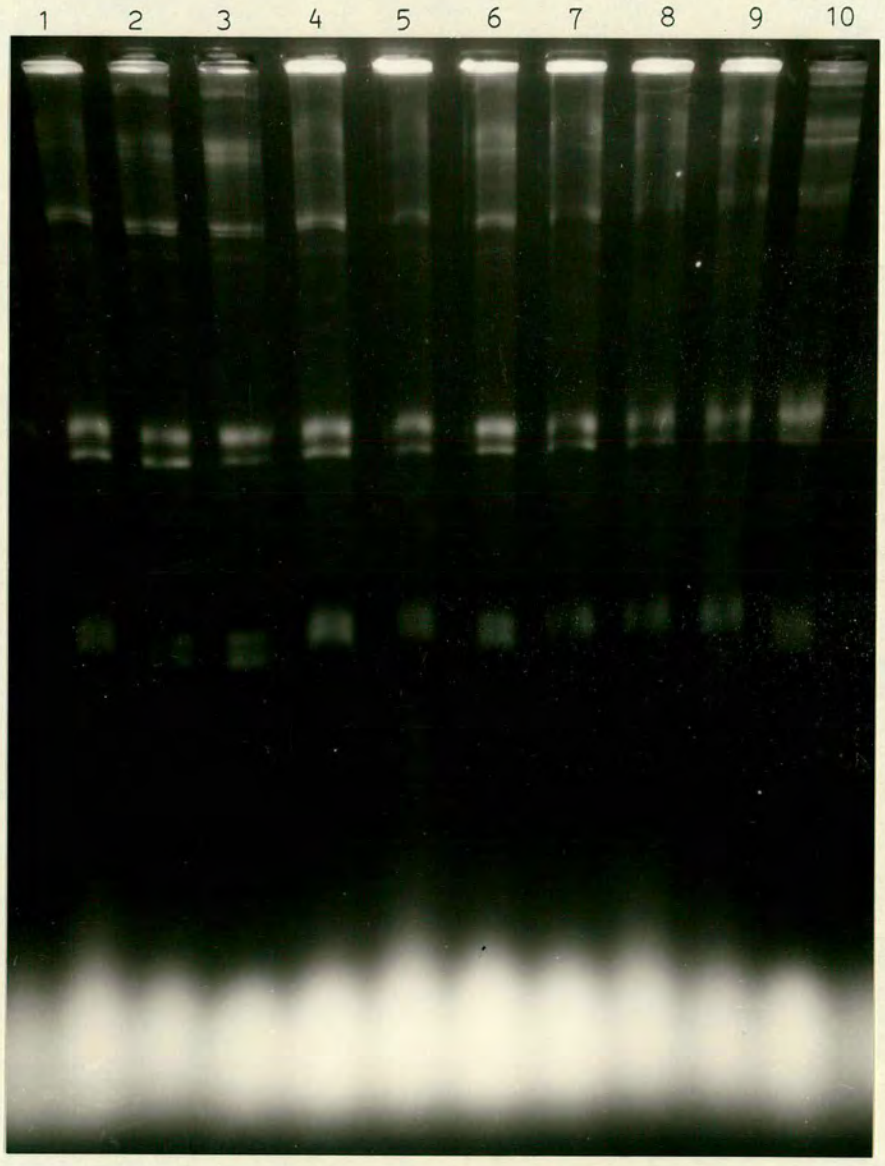
Transformation of a D.radiophilus spheroplast suspension with plasmid DNA

Plasmid DNA (2μg)	Antibiotic resistance selected for and cocncentration ($\mu g ml^{-1}$)	Transformation frequency
pAT153	Ap 3	C*
	Tc 3	C
pC194	Cm 5	C
pUB110	Kn 5	C

* C = control level = $< 1.5 \times 10^{-7}$

Fig.3.3

Agarose gel electrophoresis of plasmid DNA prepared from D.radiophilus by the rapid method of Birnboim and Doly (1979). Lane 1, plasmid DNA prpepared from normal cells. Lanes 2-10, plasmid DNA prepared from regenerated spheroplasts.



3. CONSTRUCTION OF THE HYBRID PLASMID pUE109

3.1 Elution, cloning and restriction analysis of pUE1

From the range of plasmids detected in the four species of the genus Deinococcus (Mackay et al., 1985), the smallest plasmid of D.radiophilus, pUE1, was chosen in an attempt to develop a cloning vector for the genus. Its advantages were its relatively small size (10.8kb) and the fact that it gave reasonably good yields of plasmid DNA for elution from gels and for restriction studies. The smallest plasmid in the genus viz. pUE30 (2.5kb) of D.radiopugnans gave very poor yields of plasmid DNA, while the smallest plasmid of the transformable strain D.radiodurans Sark viz. pUE10 (37.0kb) was not chosen because it was too large to be suitable as a cloning vector or to be eluted efficiently from gels or to be cloned into a plasmid vector and it gave a very poor yield of plasmid DNA.

Using the procedure described in Materials and Methods, about 50-60% of pUE1 was recovered after elution from agarose gels. The eluted pUE1 was free from contamination by other plasmid DNAs present in D.radiophilus (Fig.3.4) and was suitable for restriction analysis.

The eluted pUE1 was completely digested with several restriction endonucleases and the digests run on agarose or polyacrylamide gels, to measure the sizes of the different fragments generated. The size of pUE1 after linearisation with PstI, in comparison with HindIII-digested λ DNA was calculated to be 10.72kb. The numbers of cleavage sites and the sizes of fragments produced after digestion with various restriction enzymes are shown in Table 3.11.

pUE1 was ligated into the PstI site of the vector pAT153 and the mixture used to transform E.coli HB101 to tetracycline resistance.

Fig.3.4

Agarose gel electrophoresis of plasmid pUE1 after its elution from an agarose gel (lane 1) and the plasmids prepared from D.radiophilus (lanes 2 and 3).

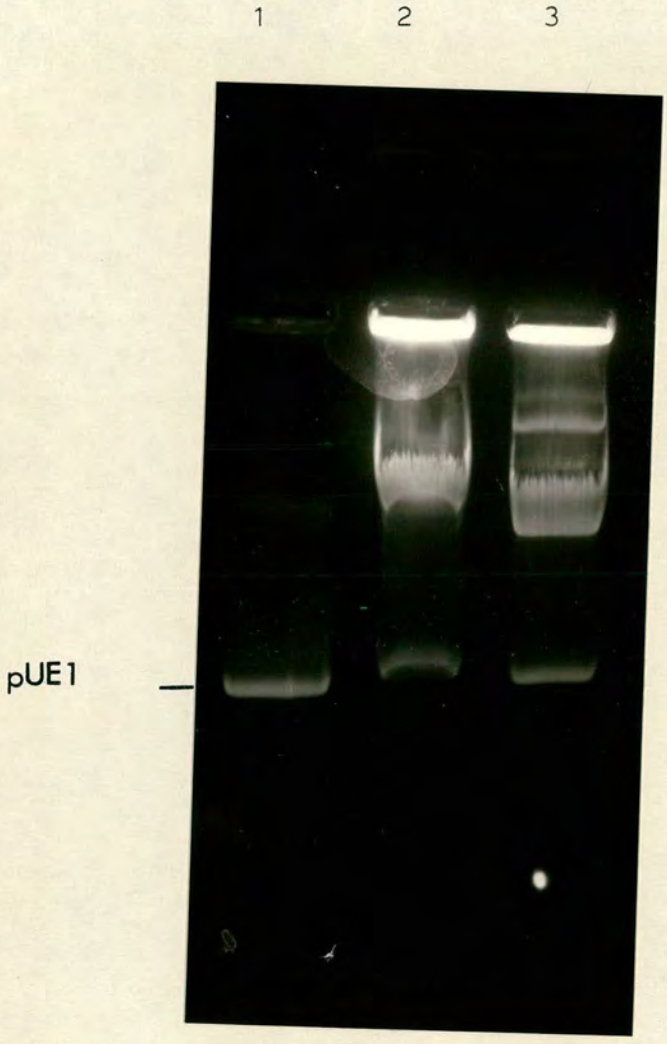


Table 3.11

Fragments of pUE1 produced by cleavage with various restriction endonucleases

Restriction endonuclease	No. of cleavage sites	Sizes of fragments (kb)
SalI	0	
DraI	0	
PstI	1	10.72
SstII	1	10.72
BamHI	2	9.16, 1.60
EcoRI	4	6.56, 2.76, 0.95, 0.37
HindIII	4	6.40, 2.15, 1.45, 0.6

The transformation frequency was $4.1 \times 10^{3} \mu g^{-1}$ vector DNA. Two out of ten tetracycline-resistant (Tc^{R}), ampicillin-sensitive (Ap^{S}) colonies screened contained a recombinant plasmid with the predicted size of pUE1 plus pAT153. The two hybrid plasmids had identical restriction patterns and so only one of them, pUE109, was used for further study. The amplification of pUE1 within pAT153 in E.coli HB101 made the task of constructing a restriction map of pUE1 much easier, since its elution from gels was time-consuming and yielded only small amounts of plasmid DNA. The hybrid plasmid pUE109 was subjected to single and double digestions with various restriction endonucleases. Digestion with PstI produced two fragments, one the size of pAT153, the other the size of plasmid pUE1. The numbers of cleavage sites and the sizes of fragments generated from pUE109 by the various restriction endonucleases are shown in Table 3.12. DraI has three cleavage sites in pAT153 (Purvis and Moseley, 1983) and one in pUE1 but it did not cleave pUE1 after elution from gels (Table 3.11) because DraI is a restriction endonuclease from the same strain as pUE1, viz. D.radiophilus (Purvis and Moseley, 1983). Thus the sensitive site for this enzyme would be modified in D.radiophilus but not in E.coli HB101. The restriction analysis of pUE109 indicated that no rearrangement of pUE1 had occurred during the construction of pUE109. After single and double digestions of pUE109, the cleavage sites were mapped and are shown in Fig.3.5.

Five colonies of E.coli HB101 into which pUE109 had been introduced were tested for increased resistance to UV radiation by streaking each of them and the original strain on agar plates and applying increasing doses of UV radiation along the streak. There was no increase in resistance of the plasmid-carrying strains.

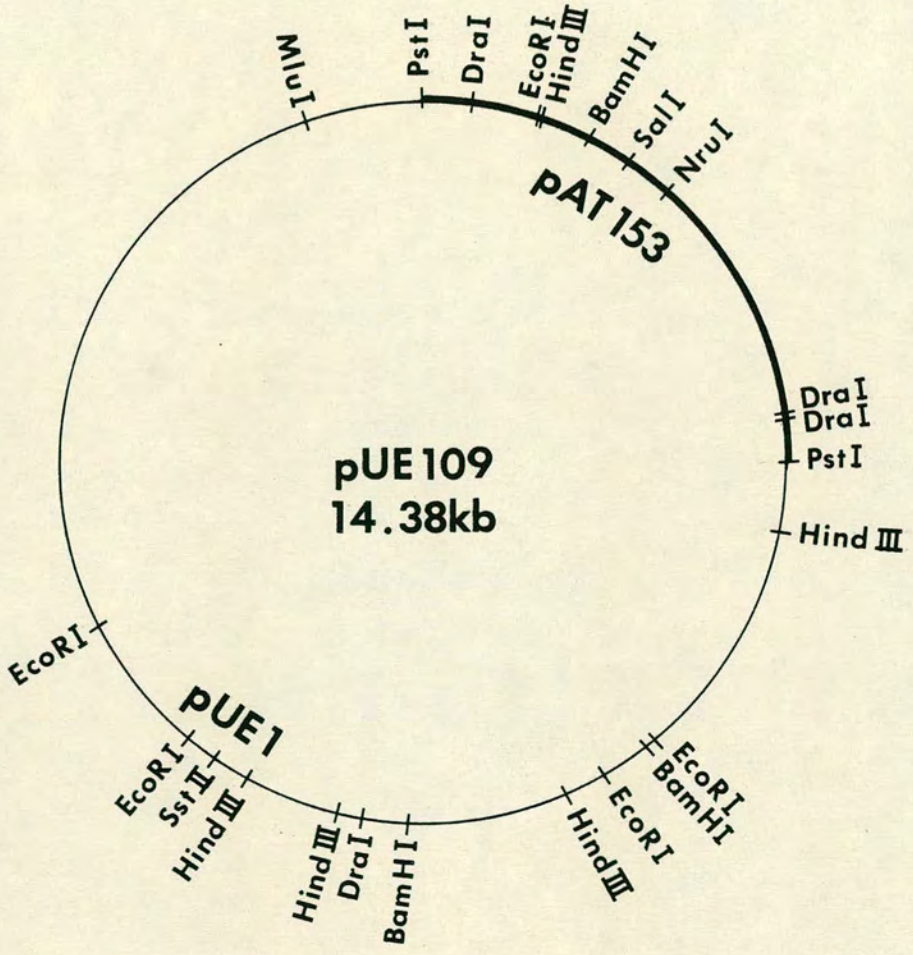
Table 3.12

Fragments of pUE109 produced by cleavage with various restriction endonucleases

Restriction endonucleases	No. of cleavage sites	Sizes of fragments (kb)
MluI	1	14.38
NruI	1	14.38
SalI	1	14.38
SstII	1	14.38
PstI	2	10.72, 3.66
BamHI	3	8.25, 4.51, 1.60
DraI	4	7.14, 4.26, 2.97, 0.02
EcoRI	5	5.41, 4.81, 2.76, 0.95, 0.37
HindIII	5	6.83, 3.23, 2.15, 1.45, 0.60

Fig.3.5

Restriction map of the hybrid plasmid pUE109.



3.2 Transformation of *Deinococcus* strains with pUE109 DNA

Covalently-closed circular DNA of pUE109 was used in an attempt to transform all strains of the genus *Deinococus* to Tc^R using the method described in Materials and Methods. pUE109 failed to transform any of the strains to Tc^R as shown in Table 3.13.

Table 3.13

Attempted transformation of Deinococcus strains with pUE109 selecting for tetracycline resistance

Strain	Concentration of Tc used for selection ($\mu g ml^{-}$)	Transformation frequency
D.radiodurans 302	1.0	$< 1.9 \times 10^{-8}$
D.radiodurans Sark	1.0	$< 1.3 \times 10^{-8}$
D.proteolyticus	1.0	$< 4.8 \times 10^{-8}$
D.radiopugnans	2.0	$< 1.6 \times 10^{-8}$
D.radiophilus	2.0	$< 3.2 \times 10^{-8}$

4. GENE CLONING

4.1 Selection of a hybrid cosmid carrying the leuB gene of D.radiodurans

When E.coli HB101 was transduced with the packaging mix (cosmid bank), and selection made for Pro^+, Leu^+, erythromycin-resistant (Ery^R) and UV^R transductants as described in chapter 2, no colonies developed on supplemented M9 agar lacking proline, on TGY agar supplemented with erythromycin or on TGY plates irradiated with UV. Colonies were detected only on supplemented M9 agar lacking leucine. Fifty of these Leu^+ colonies were tested for ampicillin resistance and all were resistant. The hybrid cosmids prepared from 10 Leu^+Ap^R colonies had the same size (51.5kb). Hybrid cosmid (pUE40) DNA prepared from one of these colonies transformed E.coli HB101 to Leu^+ at a frequency of $2.2x10^3 \mu g^{-1}$ DNA. One hundred Leu^+ transformants were streaked on L-plates containing Ap and all were Ap^R. This indicated that the leucine independence was the result of the presence of the leuB gene of D.radiodurans (so called because it is an analogue to the leuB gene of E.coli) in the insert of the hybrid cosmid and not a reversion phenomenon in the E.coli gene.

DNA of pUE40 was digested with several restriction endonucleases. The number of cleavage sites and the sizes of fragments produced by each of the enzymes are shown in Table 3.14. Although the cosmid pJBFH has one site for BamHI, this enzyme did not cut the hybrid cosmid pUE40 because the BamHI site had been lost as a result of ligating an MboI fragment of D.radiodurans DNA to the BamHI site of the cosmid during the construction of the gene library. One of the seven fragments (3.6kb) generated by EcoRI represented almost the

Table 3.14

Fragments of pUE40 produced by cleavage with various restriction endonucleases

Restriction endonuclease	No. of cleavage sites	Sizes of fragments (kb)
BamHI	0	
SstII	0	
DraI	1	51.5
PstI	1	51.5
HindIII	3	24.0, 14.8, 12.3
SalI	3	34.0, 13.1, 2.8
MluI	5	24.2, 9.5, 7.9, 4.7, 2.8
EcoRI	7	14.9, 9.37, 8.1, 7.36, 6.8, 3.6, 1.5

whole cosmid pJBFH since it has two EcoRI sites flanking the insertion site, BamHI (see Fig.2.1).

4.2 Subcloning of the D.radiodurans leuB gene in pAT153

The hybrid cosmid pUE40 was digested with EcoRI and the fragments generated ligated to pAT153. E.coli HB101 was transformed to leucine independence with the ligated mixture at a frequency of $3.7 \times 10^{2} \mu g^{-1}$ vector DNA. One hundred Leu^{+} colonies tested were all Ap^{R}, Tc^{R}. Two different sizes of insert were obtained following EcoRI digestion of hybrid plasmids prepared from several of these colonies viz. 6.9kb (pUE44) and 8.4kb (pUE47). The latter resulted from the ligation of two EcoRI-generated fragments (6.9 and 1.5kb) of pUE40 into pAT153 (Fig.3.6). There were no cleavage sites in the 6.9kb insert of pUE44 for BclI, DraI, HindIII, PstI or SalI. MluI had one site that generated fragments of 0.6 and 6.3kb, while SmaI had two sites that generated fragments of 1.5, 1.9 and 3.5kb. Cleavage sites for MluI and SmaI were mapped and are shown in Fig.3.7-a.

For further reduction in the size of the leuB insert, pUE44 DNA was doubly digested with EcoRI and SmaI, and the sticky ends generated by EcoRI removed by treatment with nuclease S1. Blunt-ended fragments were ligated to linearised pAT153 in which the EcoRI-generated sticky ends were removed by nuclease S1. E.coli HB101 was transformed to Ap^{R} with the ligated mixture at a frequency of $2.5 \times 10^{2} \mu g^{-1}$ DNA. 20% of the Ap^{R} colonies were Leu^{+}. Three out of ten $Ap^{R}Leu^{+}$ colonies screened harboured a hybrid plasmid of 7.16kb, while the others harboured hybrid plasmids of an identical size to that of pUE44. Restriction analysis of one of the 7.16kb hybrid plasmids (pUE441) (Fig.3.7-b) showed that it had no EcoRI, SmaI or MluI sites and one cleavage site for each of SalI, HindIII and BamHI. This indicated

Fig.3.6

Agarose gel electrophoresis of fragmented hybrid plasmids pUE44 and pUE47, carrying the leuB gene of D.radiodurans. Lane 1, λ DNA digested with HindIII, Lanes: 2, pUE40; 3, pUE47; 4, pUE44 and 5, pAT153 all digested with EcoRI.

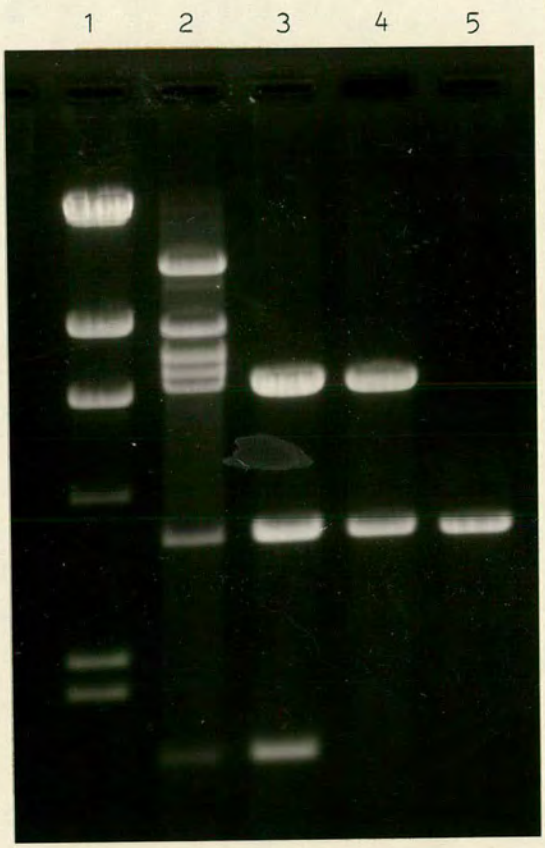
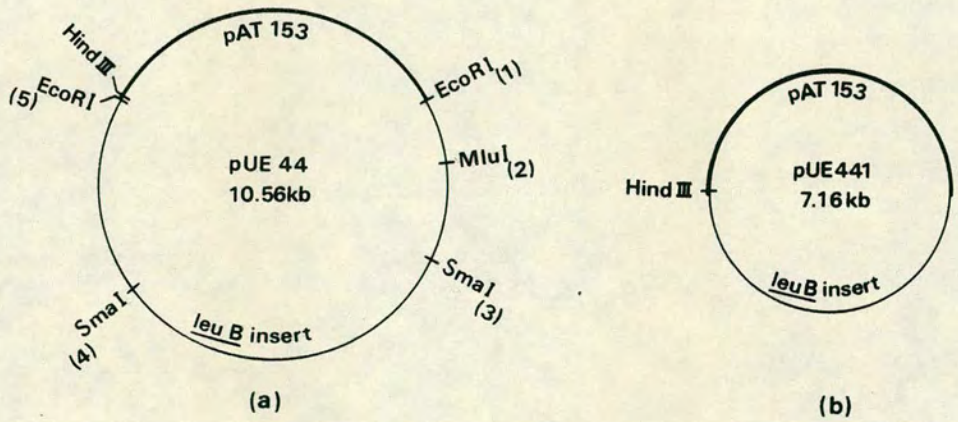


Fig.3.7

(a) Hybrid plasmid pUE44 consisting of a 6.9kb D.radiodurans DNA segment (thin line) carrying the leuB gene inserted into the EcoRI site of pAT153 (heavy line). (b) Hybrid plasmid pUE441. Carrying the leuB gene constructed by ligating the 3.5kb SmaI fragment generated from pUE44 into the EcoRI site of pAT153, after removal of the sticky ends by the treatment with nuclease S1. Size of fragments (kb); 1 to 2, 0.6; 2 to 3, 0.9; 3 to 4, 3.5; 4 to 5, 1.9.



that the 3.5kb fragment generated by SmaI from pUE44 was cloned into the EcoRI site of pAT153 after removal of the sticky ends. pUE441 DNA transformed E.coli HB101 to $Ap^{R}Leu^{+}$ at a frequency of $2.1x10^{4}\mu g^{-1}$ DNA which indicated that the 3.5kb insert carried the leuB gene of D.radiodurans.

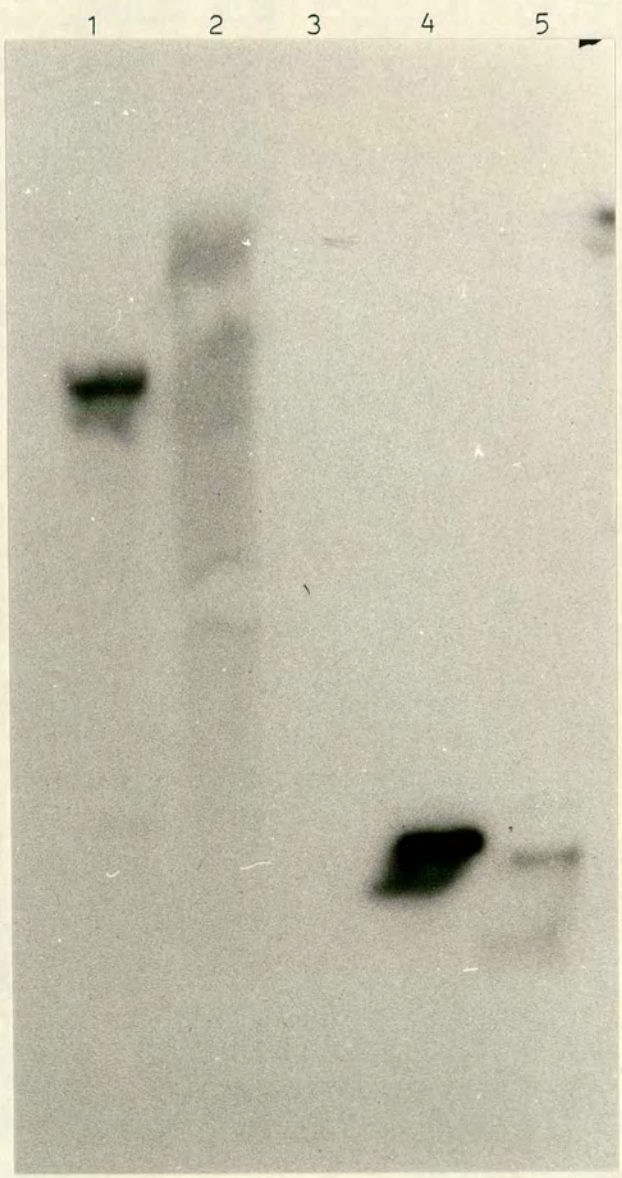
4.3 Homology between the hybrid plasmid pUE441 and chromosomal DNA of D.radiodurans krase

In spite of several attempts, a Leu^{-} mutant could not be obtained from D.radiodurans and it was not possible therefore to use the cloned leuB gene to complement such a mutation as proof that this gene was derived from D.radiodurans. As an alternative DNA-DNA hybridisation was used.

Hybrid plasmid pUE441 DNA, containing the 3.5kb SmaI fragment carrying the leuB gene was labelled with [α-^{32}P]dNTPs by nick-translation and hybridised to chromosomal DNA from D.radiodurans, strain krase, digested with either EcoRI or SmaI, chromosomal DNA from E.coli digested with HindIII (as a control) and linearised pAT153 (as a size marker) as described in Materials and Methods. The autoradiographic image (Fig.3.8) showed that the labelled pUE441 hybridised to 3.5 and 14.5kb fragments generated from D.radiodurans chromosomal DNA by SmaI and EcoRI respectively, and also to pAT153 since the pUE441 contains this vector. There was no hybridisation between the labelled pUE441 and E.coli DNA. When pAT153 was labelled and hybridised to chromosomal DNA from D.radiodurans and E.coli in same way, no hybridisation was detected (data not shown). Thus the 3.5kb insert of pUE441 which carries the leuB gene was derived from D.radiodurans.

Fig.3.8

Homology between the hybrid plasmid pUE441 and chromosomal DNA. Labelled pUE441 hybridised with: Lane 1, D.radiodurans chromosomal DNA digested with EcoRI; lane 2, E.coli chromosomal DNA digested with HindIII; lane 3, λ DNA digested with HindIII (bands of this size marker needed a longer time of exposure to appear); lane 4, plasmid pAT153 digested with EcoRI; lane 5, D.radiodurans chromosomal DNA digested with SmaI.



It was expected that labelled pUE441 would hybridise to a 6.9kb fragment of the EcoRI digest of D.radiodurans DNA rather than to a 14.5kb fragment, since the hybrid plasmid pUE44 (Fig.3.7-a) contained a 6.9kb EcoRI fragment carrying the leuB gene. This was to be expected if the insert in pUE44 was a continuous EcoRI fragment in its native form. Hybridisation to 14.5kb EcoRI fragment was probably because the 6.9kb insert of pUE44 was a junction fragment of the insert DNA in the hybrid cosmid pUE40, i.e. it was generated as a result of EcoRI cutting in the insert of pUE40 on one side and in the cosmid pJBFH (which has two EcoRI sites flanking the cloning site, see Fig.2.1) on the other side.

4.4 Selection of hybrid cosmids carrying mtcA, uvsE, trp or asp genes

The results of transforming the Mtc^S, UV^S strain D.radiodurans UVS78 (mtcA, uvsE) with linearised hybrid cosmid DNAs from each of the eight groups of 40 colonies are shown in Table 3.15. Similar transformation frequencies were obtained for UV resistance when the ccc form of DNA was used (data not shown). UV^R transformant colonies were obtained using DNA from each of the groups, while Mtc^R colonies were obtained from four groups. UV^R transformants can be obtained from D.radiodurans UVS78 either because of its acquiring mtcA or uvsE, since the repair of pyrimidine dimers formed by UV irradiation in this microorganism can be initiated by either UV endonuclease α (which requires functional mtcA and mtcB genes and also repairs the damage caused by mitomycin c in DNA) or by UV endonuclease β (which requires functional uvsC, uvsD and uvsE genes and repair only UV induced pyrimidine dimers) (Evans and Moseley, 1983; Moseley and Evans, 1983).

Table 3.15

Transformation of Deinococcus radiodurans UVS78 (mtcA, uvsE) with DNA prepared from the different groups of the gene library

Group of 40 colonies from which DNA was prepared	Transformation frequency for resistance to					Gene(s) present in the inserts
	UV	Mtc	Str	Ery	Rif	
1	2.8×10^{-3}	2.1×10^{-5}	C[a]	C	C	uvsE, mtcA
2	2.6×10^{-3}	C	C	C	C	uvsE
3	2.0×10^{-3}	C	C	C	C	uvsE
4	2.0×10^{-3}	1.9×10^{-5}	3.2×10^{-8}	C	C	uvsE, mtcA, str
5	1.5×10^{-4}	C	C	C	C	uvsE
6	1.8×10^{-5}	C	C	C	C	uvsE
7	1.3×10^{-4}	1.7×10^{-6}	C	C	C	uvsE, mtcA
8	1.2×10^{-3}	2.1×10^{-5}	C	C	C	uvsE, mtcA
Control (no DNA)	2.9×10^{-7}[b]	$< 1 \times 10^{-9}$	$< 1 \times 10^{-9}$	$< 1 \times 10^{-9}$	$< 1 \times 10^{-9}$	

[a] C = Control level

[b] Represents spontaneous reversions and cells that did not receive an adequate UV exposure

If the UV^R transformants are all Mtc^S, this means that they have acquired only the uvsE gene. If all are Mtc^R, this means that they have acquired only the mtcA gene. When some UV^R clones are Mtc^R it indicates that the UV^R transformants arose as a result of acquiring both mtcA and uvsE genes. The transformation frequencies for UV resistance in groups 1, 4, 7 and 8 were much higher than those for Mtc resistance and it was concluded that some cells received the uvsE gene while others received the mtcA gene. The str gene was present in group 4 DNA and the low transformation frequency observed was expected, since this gene is expressed at a much lower frequency per gene copy than, for example, rifampicin or erythromycin resistance (Tirgari and Moseley, 1980). However, DNA from all eight groups failed to transform D.radiodurans UVS78 to rifampicin or erythromycin resistance.

Hybrid cosmid DNA was prepared from each of twelve colonies from groups 1 and 4 by the rapid boiling method and used to transform D.radiodurans UVS78 to UV and Mtc resistance. One colony (1-1) from group 1 was found to carry the mtcA gene, and four colonies, one from group 1 (1-10) and three from group 4 (4-4, 4-5 and 4-11) carried the uvsE gene. Results of transformation of D.radiodurans UVS78 with DNA from 1-1, 4-4 and 4-11, purified from caesium chloride gradients are shown in Table 3.16. pUE50 DNA transformed D.radiodurans UVS78 to both UV and Mtc resistance at approximately the same frequency. All 100 colonies selected for UV resistance were also resistant to Mtc, i.e. they all contained the MtcA gene. pUE60, which transformed D.radiodurans UVS78 to UV resistance did not transform it to Mtc resistance. Thus pUE60 carried only the uvsE gene. pUE70 DNA transformed D.radiodurans UVS78 to both UV and Mtc resistance, and it

Table 3.16

Transformation of Deinococcus radiodurans UVS78 (mtcA, uvsE) with DNA derived from colonies harbouring different hybrid cosmids

Colony	Hybrid cosmid designation	Size of hybrid cosmid (kb)	Transformation frequency for		Gene(s) present in the insert
			UV resistance	Mtc resistance	
1-1	pUE50	37.9	7.6×10^{-3}	8.0×10^{-3}	mtcA
4-4	pUE60	46.0	8.8×10^{-3}	C[a]	uvsE
4-11	pUE70	52.5	5.7×10^{-3}	3.4×10^{-6}	uvsE, mtcA
Control (No DNA)	-	-	2.0×10^{-6}[b]	$< 1 \times 10^{-8}$	-

[a] C = Control level

[b] Represents spontaneous reversions and cell that did not receive an adequate UV exposure

was concluded that pUE70 carried both the uvsE and MtcA genes. The very low transformation frequency to Mtc^R was probably because the mtcA gene is located at the end of the insert and this resulted in inefficient recombination. Restriction analysis of pUE50 and pUE70 with EcoRI supported this conclusion since there was no pUE70 fragment corresponding to the 5.6kb EcoRI fragment of pUE50 shown to carry the mtcA gene (data not shown). Restriction analysis of pUE60 and pUE70 with several restriction enzymes (Table 3.17) showed that they share a common fragment in their inserts.

The results of transforming the different auxotrophic mutants of D.radiodurans with hybrid cosmid DNAs prepared from each of the eight groups of the gene library are shown in Table 3.18. D.radiodurans 2-1 (asp-1) was transformed to Asp^+ using DNA from groups 1 and 8, while D.radiodurans 18-1 (trp-1) was transformed to Trp^+ using DNA from groups 3, 4, 6 and 7. DNA from all eight groups failed to transform the other D.radiodurans auxotrophs to prototrophy.

Hybrid cosmid DNA prepared from each of the 40 colonies of group 8 by the rapid boiling method was used to transform D.radiodurans 2-1 (asp-1) to prototrophy. Hybrid cosmid pUE90 from colony 8-17 transformed it at a frequency of 1.3×10^{-4}. The size of pUE90 was calculated to be 13.0kb and its restriction with EcoRI produced two fragments, one of 3.7kb which represented the cosmid pJBFH and one of 9.3kb which represented the insert. HindIII had two sites in the hybrid cosmid producing fragments of 6.4 and 6.6kb in size. Two (4-9 and 4-28) out of the 40 colonies of group 4 screened harboured hybrid cosmids which transformed D.radiodurans 18-1 to Trp^+ at frequencies of 4.4×10^{-4} and 7.2×10^{-4} respectively. Hybrid cosmids pUE80 and pUE100 prepared from colonies 4-28 and 4-9 respectively,

Table 3.17

Fragments of pUE60 and pUE70 produced by cleavage with various restriction endonucleases

Restriction endonuclease	Hybrid cosmid	No. of cleavage sites	Sizes of fragments (kb)
BamHI	pUE60	0	
	pUE70	1	52.5
BclI	pUE60	0	
	pUE70	0	
SstII	pUE60	1	46.0
	pUE70	1	52.5
PstI	pUE60	1	46.0
	pUE70	1	52.5
MluI	pUE60	3	23.8, 17.9, 2.9
	pUE70	3	20.45, 17.9, 14.18
HindIII	pUE60	4	24.5, 12.2, 5.6, 3.7
	pUE70	4	24.5, 12.2, 10.2, 5.6
EcoRI	pUE60	7	16.36, 13.40, 8.38, 3.70, 1.65, 1.40, 1.10
	pUE70	7	17.50, 15.18, 8.38, 5.03, 3.70, 1.65, 1.10

Table 3.18

Transformation of D.radiodurans auxotrophs with DNA prepared from the different groups of the gene library

Strain of D.radiodurans	Mutant gene	Transformation frequency to prototrophy using DNA from group								Control (no DNA)
		1	2	3	4	5	6	7	8	
2-1	asp-1	8.6×10^{-5}	C*	C	C	C	C	C	9.1×10^{-5}	$< 7.5 \times 10^{-7}$
5-1	pdx-1	C	C	C	C	C	C	C	C	$< 1.2 \times 10^{-8}$
18-1	trp-1	C	C	3.4×10^{-4}	5.1×10^{-4}	C	4.0×10^{-4}	2.3×10^{-4}	C	$< 1.0 \times 10^{-8}$
30-1	tyr	C	C	C	C	C	C	C	C	$< 1.3 \times 10^{-8}$
34-1	arg	C	C	C	C	C	C	C	C	$< 2.3 \times 10^{-8}$

*C = Control level

had the same size (52.1kb) and their restriction patterns with EcoRI were identical (Fig.3.9). Hybrid cosmid pUE80 was prepared from colony 4-28 and used for further studies.

4.5 Selection of mtcB, uvsC and uvsD genes

The presence of uvsE and mtcA genes in the insert of the hybrid cosmid pUE70 indicated the possibility that the DNA repair genes mtcA, mtcB, uvsC, uvsD and uvsE are located close to each other on the chromosome of D.radiodurans. pUE50 DNA was used to transform D.radiodurans 262 (mtcB) to Mtc resistance and pUE60 and pUE70 to transform D.radiodurans strains UVS9 (uvsC) and UVS25 (uvsD) to UV resistance and D.radiodurans 262 (mtcB) to Mtc resistance. The results in Table 3.19 show that the mtcB gene was present in the insert of pUE50 in addition to the mtcA gene; that the insert of pUE60 carried the uvsC and uvsD genes as well as the uvsE gene and that the insert of pUE70 carried the uvsC and uvsD genes in addition to uvsE and mtcA. The low transformation frequencies were caused by the poor transformability of the recipient strains compared with D.radiodurans UVS78.

4.6 Subcloning of mtcA and mtcB into pAT153

pUE50 DNA carrying both mtcA and mtcB genes was restricted with EcoRI. Part of the digest was used to transform D.radiodurans UVS78 and D.radiodurans 262 to Mtc resistance, while the other part was run on an agarose gel to determine the restriction pattern. The digested DNA transformed D.radiodurans UVS78 to Mtc resistance at a frequency of 1.2×10^{-4} compared with 6.8×10^{-3} for the same concentration of unrestricted DNA, and transformed D.radiodurans 262 at a

Fig.3.9

Agarose gel electrophoresis of hybrid cosmids pUE80 and pUE100 which carry a *trp* gene of *D.radiodurans*. Lane 1, λ DNA restricted with *HindIII*; lane 2, pUE80 restricted with *EcoRI*; lane 3, unrestricted pUE80; lane 4, unrestricted pUE100; lane 5, pUE100 restricted with *EcoRI*.

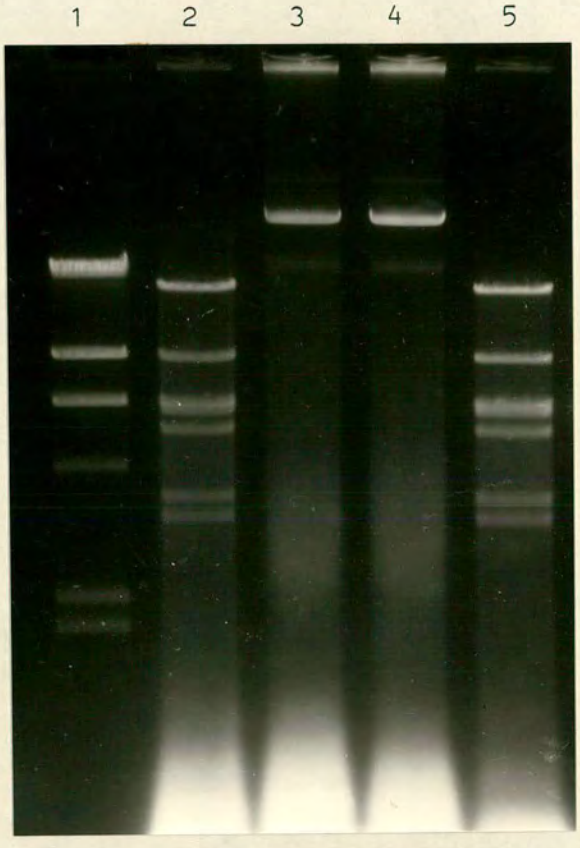


Table 3.19

Transformation of different strains of Deinococcus radiodurans to Mtc or UV resistance with hybrid cosmid DNAs, selecting for mtcB, uvsC or uvsD genes

DNA	Strain of D.radiodurans	Mutation in	Transformation frequency	Control (No DNA)
pUE50	262	mtcB	4.5×10^{-5}	$< 2 \times 10^{-9}$
pUE60	UVS9	uvsC	3.8×10^{-4}	3.9×10^{-5}[a]
pUE60	UVS25	uvsD	1.4×10^{-3}	2.1×10^{-5}[a]
pUE60	262	mtcB	C[b]	$< 2 \times 10^{-9}$
pUE70	UVS9	uvsC	4.7×10^{-4}	3.9×10^{-5}[a]
pUE70	UVS25	uvsD	1.0×10^{-3}	2.1×10^{-5}[a]
pUE70	262	mtcB	C	$< 2 \times 10^{-9}$

[a] Represents spontaneous reversions and cells that did not receive an adequate UV exposure

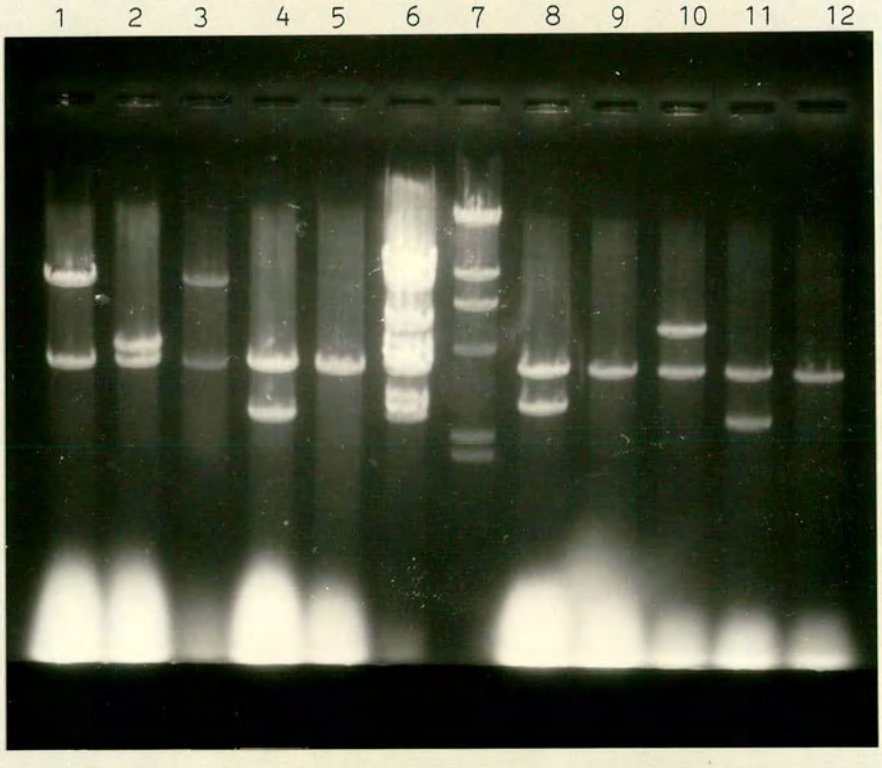
[b] C = Control level

frequency of 2.0×10^{-7} compared with 1.7×10^{-4} for unrestricted DNA. Seven fragments ranging in size from 2.7 to 10.1kb were generated from pUE50 by EcoRI and were ligated to pAT153 in order to subclone the mtcA and mtcB genes. E.coli HB101 was transformed with the ligated DNA and screened for $Ap^R Tc^R$ transformants. Plasmid DNA from twenty of these transformants was prepared by the boiling method, restricted with EcoRI and the digests run on agarose gels to measure the sizes of the inserts. Six different sizes viz. 2.7kb (pUE59), 2.9kb (pUE56), 3.7kb (pUE55), 4.4kb (pUE52), 5.6kb (pUE58) and 8.5kb (pUE51), representing six of the seven EcoRI-generated fragments from pUE50 were identified (Fig.3.10). Hybrid plasmids from colonies harbouring different sizes of insert were used to transform D.radiodurans UVS78 and D.radiodurans 262 to Mtc resistance. DNA of pUE58 transformed the former at a frequency of 9.5×10^{-4} while DNA of pUE59 transformed D.radiodurans 262 at a frequency of 2.5×10^{-5}. Thus, the mtcA gene was present in the 5.6kb insert of pUE58 and mtcB in the 2.7kb insert of pUE59.

When pUE50 DNA was restricted with EcoRI and used to transform D.radiodurans 262 to Mtc resistance, the transformation frequency was very low compared with that of unrestricted DNA. It was thought that perhaps EcoRI cut inside the mtcB gene, but when the EcoRI-generated 2.7kb fragment from pUE50 was ligated to pAT153 (pUE59) and used to transform D.radiodurans 262 the transformation frequency increased 100-fold and this appears to rule out the possiblity of an EcoRI site in mtcB. The possibility of plasmid maintenance in strain 262 was ruled out for three reasons. Firstly, the transformation frequencies would be expected to be inversely related to plasmid size (for a similar DNA concentration). However, the transformation frequency to

Fig.3.10

Agarose gel electrophoresis of fragmented hybrid plasmids resulting from digestion and cloning of pUE50 DNA into the EcoRI site of pAT153. All hybrid plasmids, hybrid cosmid pUE50 and pAT153 were digested with EcoRI to determine the size of the inserts, using λ DNA digested with HindIII as a standard. Lanes: 1, pUE51; 2, pUE52; 3, pUE53; 4, pUE54; 5, pUE55; 6, pUE50; 7, λ DNA; 8, pUE56; 9, pUE57; 10, pUE58; 11, pUE59; 12, pAT153.



Mtc resistance of D.radiodurans 262 using pUE50 DNA (37.9kb) was about 10 times greater than that with pUE59 (6.4kb). Secondly, twenty Mtc^R transformants of D.radiodurans 262 were screened for the presence of the hybrid plasmid pUE59. Half of the prepared cleared lysate was used to transform E.coli HB101 to Tc^R and the other half run on agarose gels. E.coli HB101 was not transformed to Tc^R, and no band corresponding to the plasmid was detected on the gel. Finally, when pUE59 DNA was linearised with SalI and used to transform D.radiodurans 262, the transformation frequency obtained, 2.2×10^{-5}, was similar to that obtained using the ccc form of pUE59. The most likely explanation is that the 2.7kb fragment, following its uptake into the cell, is susceptible to the action of exonuclease so that the mtcB gene is inactivated by digestion, while its insertion into pAT153 gives it protection from exonucleases prior to successful chromosomal integration, and this indicated that the recombination process in D.radiodurans is effective even over a short length of homologus DNA.

In an attempt to clone mtcA and mtcB genes on one fragment to see how close they are on the chromosome, pUE50 DNA was restricted with HindIII. The three generated fragments (3.7, 8.1 and 26.0kb) were eluted from the agarose gel and each used to transform strains UVS78 and 262 of D.radiodurans to Mtc resistance. The largest fragment (26.0kb) transformed both strains at frequencies of 2.4×10^{-3} and 1.2×10^{-5} respectively. The 26.0kb HindIII fragment was ligated to pAT153 after elution from an agarose gel and the ligated mixture used to transform E.coli HB101 to $Ap^R Tc^S$. Two out of seven $Ap^R Tc^S$ colonies screened carried a hybrid molecule capable of transforming both D.radiodurans strains to Mtc resistance. The two hybrid molecules

were found to have different sizes; 26.0kb (pUE502) and 29.9kb (pUE505).

The restriction analysis of pUE502 and pUE505 with some restriction enzymes is shown in Fig.3.11. Digestion of pUE505 with HindIII produced two fragments, one of 26.0kb representing the insert and one of 3.66kb representing the vector pAT153. pUE505 has one site for each of ClaI and SalI which are situated in the pAT153 portion. Digestion of pUE502 with HindIII produced only one linear fragment of 26.0kb which has no ClaI or PstI sites. Digestion with EcoRI showed that pUE502 and pUE505 have exactly the same restriction pattern, apart from a 3.7kb fragment missing from pUE502. This indicated that pUE502 resulted from the recircularisation of the 26.0kb-HindIII fragment of pUE50 which appears to carry the cosmid pJBFH minus the small HindIII-BamHI fragment which contains the ClaI site (see Fig. 2.1).

The number of cleavage sites and the size of fragments generated from pUE505 by various restriction enzymes are shown in Table 3.20. When the four fragments generated by KpnI were eluted from an agarose gel, in the form of pure 11.2kb and pure 8.3kb fragments and a mixture of the 5.3 and 5.1kb fragments, and used to transform strains UVS78 and 262 of D.radiodurans to Mtc resistance, only the mixture of 5.3 and 5.1kb fragments transformed UVS78 at a frequency of 2.3×10^{-4}. This indicated that one of these two fragments carries the mtcA gene, while the mtcB gene was inactivated probably as a result of cutting with KpnI.

4.7 Subcloning of the uvsE into pAT153

HindIII-generated fragments of pUE60 (hybrid cosmid carries the

Fig.3.11

Agarose gel electrophoresis of pUE505 and pUE502. Lanes: 1, unrestricted pUE505; 2, unrestricted pUE502; 3 and 4, pUE505 and pUE502 respectively, restricted with SalI; 5 and 6, pUE505 and pUE502 respectively restricted with ClaI; 7, λ DNA restricted with HindIII; 8 and 9, pUE505 and pUE502 respectively, restricted with HindIII; 10 and 11, pUE505 and pUE502 respectively, restricted with EcoRI.

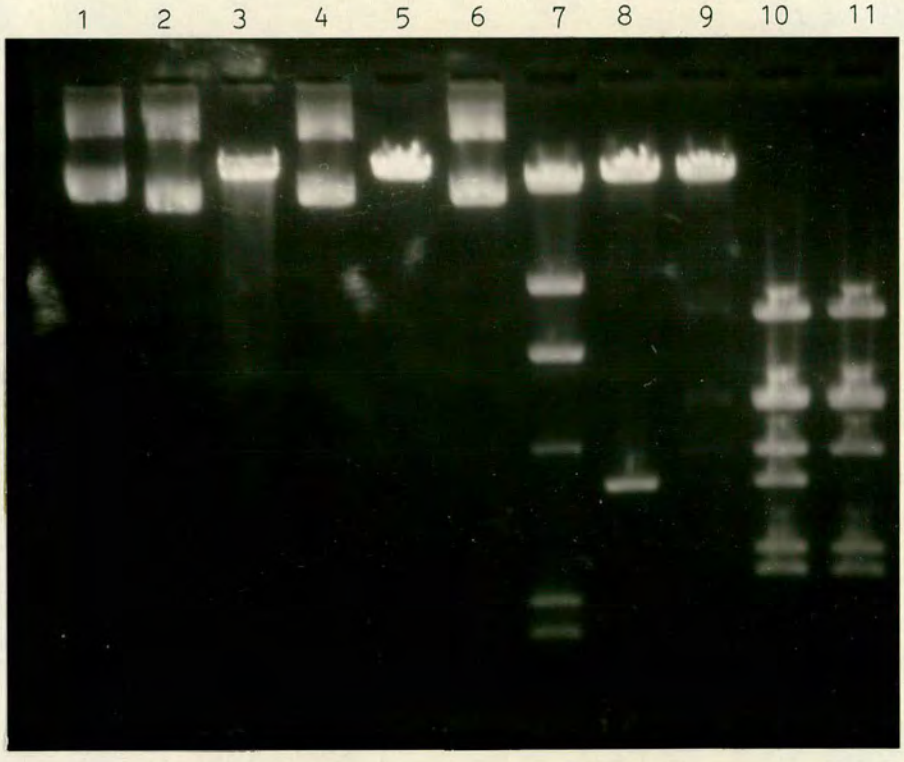


Table 3.20

Fragments of pUE505 produced by cleavage with various restriction endonucleases

Restriction endonuclease	No. of cleavage sites	Sizes of fragments (kb)
BalI	0	
XbaI	0	
AatI	2	25.73, 3.6
MluI	2	25.98, 3.77
PstI	2	25.35, 3.67
StuI	2	22.4, 7.3
KpnI	4	11.2, 8.3, 5.3, 5.1
AccI	6	13.9, 5.6, 3.2, 2.6, 2.5, 1.5
SmaI	6	10.1, 6.4, 5.2, 3.8, 2.7, 1.8

uvsC, uvsD and uvsE genes) were ligated into pAT153 and used to transform E.coli HB101. Hybrid plasmid DNA from several $Ap^R Tc^S$ colonies were restricted with HindIII and run on agarose gels. As shown in Fig.3.12, three of the four possible sizes of insert were identified viz. 3.7kb (pUE61), 5.6kb (pUE63) and 12.2kb (pUE62). pUE61, pUE62 and pUE63 DNAs were used in an attempt to transform D.radiodurans strains UVS78 (uvsE), UVS25 (uvsD) and UVS9 (uvsC) to UV resistance. pUE62 DNA transformed D.radiodurans UVS78 at a frequency of 8.3×10^{-3}. All three DNAs failed to transform D.radiodurans UVS9 and UVS25 to UV resistance which indicated that the pUE62 carried the uvsE gene only, while uvsC and uvsD were probably present in the fourth HindIII fragment (24.5kb) which could not be cloned. There was no cleavage site in the insert of pUE62 for EcoRI, MluI, PstI or SstII, while SalI had only one cleavage site, and this was shown by double digestion with HindIII and SalI, to be in the middle of the insert. pUE62 (Fig.3.13-a) was restricted with SalI and religated with T4 DNA ligase. The religated DNA was used to transform E.coli HB101 to Ap^R. Hybrid plasmid DNAs from eleven Ap^R colonies were restricted with SalI and run on an agarose gel in a search for those which had only one sensitive site to SalI, producing one fragment of about 9.0kb. Five out of the eleven had such a segment. These hybrid plasmids e.g. pUE621 (Fig.3.13-b) consisted of a 6.1kb fragment from the insert of pUE62 ligated into pAT153 minus 0.62kb (the fragment between the SalI and HindIII sites).

pUE621 DNA transformed D.radiodurans UVS78 to UV resistance at a frequency of 7.1×10^{-3} which showed that it carried the uvsE gene.

The genes uvsC, uvsD and uvsE in the insert of pUE60, and uvsE alone in the insert of pUE621 were introduced into E.coli CSR603

Fig.3.12

Agarose gel electrophoresis of hybrid plasmids resulting from digestion and cloning of pUE60 DNA into the HindIII site of pAT153. All hybrid plasmids, hybrid cosmid pUE60, pAT153 and λ DNA were digested with HindIII. Lanes: 1, pUE61; 2, pUE62; 3, pUE63; 4, pUE64; 5, pUE65; 6, pUE66; 7, pUE60; 8, λ DNA; 9, pUE67; 10, pUE68; 11, pUE69; 12, pAT153.

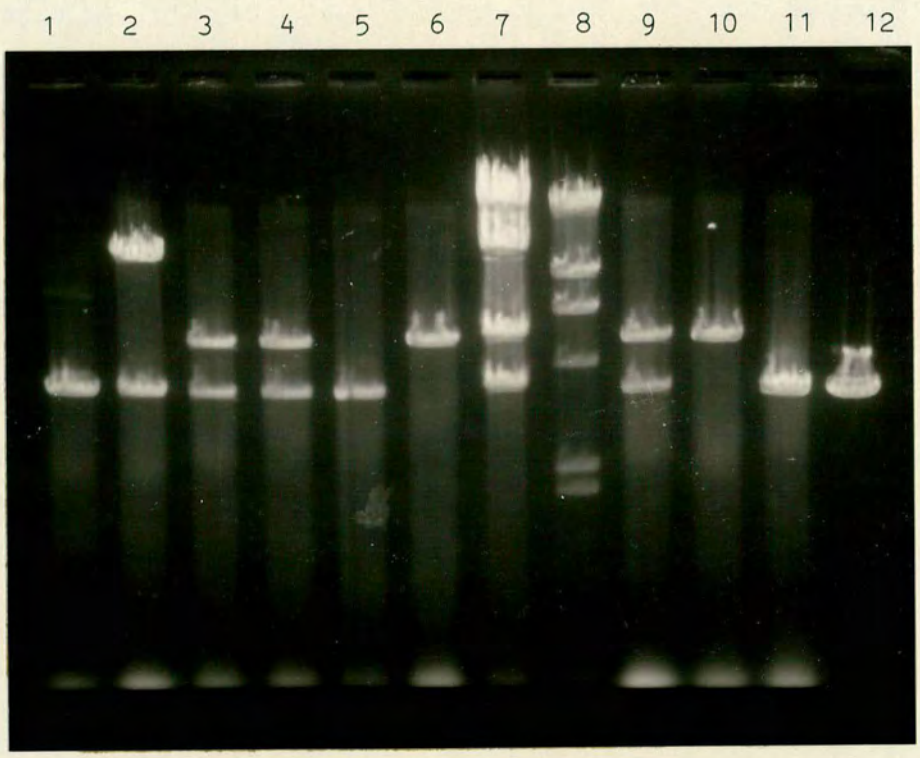
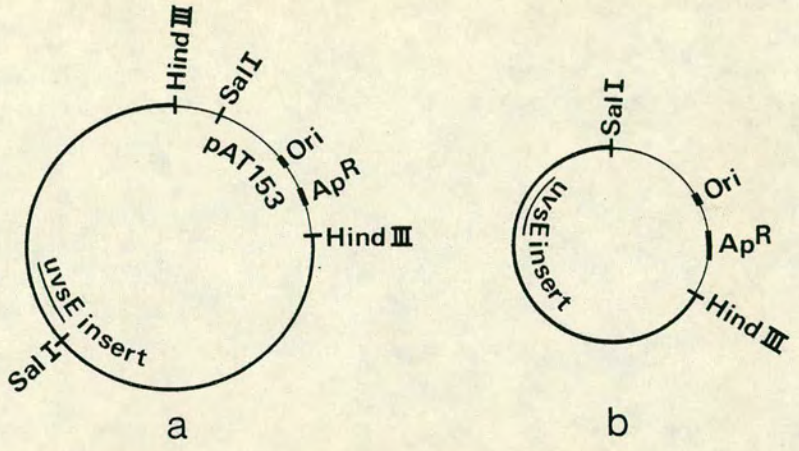


Fig.3.13

(a) Hybrid plasmid pUE62 consisting of a 12.2kb D.radiodurans DNA segment (heavy line) carrying the uvsE gene inserted into the HindIII site of pAT153 (thin line). (b) Hybrid plasmid pUE621 carrying the uvsE gene constructed by SalI digestion of pUE62 which allowed in vitro deletion of 6.1kb of the insert along with the 625 bp HindIII-SalI segment of the vector.



(uvrA) by transformation. None of them was able to complement the repair defect in this strain.

4.8 Subcloning of a trp gene into pAT153

Seven fragments ranging in size from 3.5 to 17.62kb were generated by EcoRI digestion of pUE80 DNA (Fig.3.9) which carried the trp gene of D.radiodurans. These fragments were ligated into pAT153 and the ligated mixture transformed E.coli HB101 to $Ap^R Tc^R$ at a frequency of $1.8 \times 10^3 \mu g^{-1}$ vector DNA. Plasmid DNA from twenty transformants was prepared using the boiling method, restricted with EcoRI, and the digests run on agarose gels to measure the sizes of the inserts. All seven different fragments viz. 3.5kb (pUE84), 3.7kb (pUE83), 5.5kb (pUE81), 6.33kb (pUE82), 6.43kb (pUE88), 8.96kb (pUE89) and 17.62kb (pUE87) generated by EcoRI from pUE80 were identified. Hybrid plasmid DNA from colonies harbouring different sizes of insert was used to transform D.radiodurans 18-1 (trp-1) to Trp^+. Only DNA of pUE81 transformed it, at a frequency of 6.2×10^{-4}. There were no cleavage sites in the insert of pUE81 for HindIII, BamHI, SalI or MluI. SstII had one site producing fragments of 3.7 and 1.8kb. Further reduction in the insert size was not made.

4.9 Transformation of D.radiodurans Trp^- mutants with pUE80 and pUE81

Seven Trp^- mutants of D.radiodurans were transformed with pUE80 and pUE81 to determine whether these strains are mutated in the same trp gene or not, and to see if pUE80 carries more than one trp gene of D.radiodurans. Results of the transformation experiment are shown in Table 3.21. The transformation frequencies obtained using pUE80

Table 3.21

Transformation of different Trp^- mutants of D.radiodurans with pUE80 and pUE81

Strain of D.radiodurans	Transformation frequency to Trp^+ using		Control (no DNA)
	pUE80	pUE81	
18-1	2.4×10^{-4}	5.3×10^{-4}	$< 1.0 \times 10^{-8}$
22-1	C*	C	$< 1.3 \times 10^{-8}$
23-1	C	C	$< 7.8 \times 10^{-7}$
27-1	8.0×10^{-7}	5.5×10^{-7}	$< 1.0 \times 10^{-8}$
41-1	1.8×10^{-4}	6.0×10^{-4}	2.1×10^{-8}
66-1	C	C	1.5×10^{-8}
67-1	1.7×10^{-4}	4.2×10^{-4}	1.3×10^{-8}

* C = Control level

were similar to those resulting from the use of pUE81. The strains of D.radiodurans 22-1, 23-1 and 66-1 were not transformed to Trp^+ using either pUE80 or pUE81 indicating that they were mutated in trp gene(s) other than that of D.radiodurans 18-1, 27-1, 41-1 and 67-1.

4.10 Transformation of different E.coli trp mutants with pUE80 and pUE81

The five different trp E.coli mutants listed in Materials and Methods were transformed to Ap resistance with pUE80 and pUE81 DNAs to determine if the cloned trp gene of D.radiodurans is capable of complementing any one of them. Results of transformation are shown in Table 3.22. E.coli JA221 (trpE) was transformed to Ap resistance at 40 to 50-fold higher frequencies than the other strains probably because it is a restrictionless strain and the others are not. Also the transformation frequencies to Ap resistance using pUE81 were higher than those resulting from the use of pUE80 because pUE81 (11.16kb) is smaller than pUE80 (52.1kb). Ten Ap^R transformants from each mutant were streaked on supplemented M9 agar lacking tryptophan. None of the transformants were able to grow in the absence of tryptophan which indicated that the cloned trp gene of D.radiodurans present in the inserts of pUE80 and pUE81 was not capable of functional expression in E.coli.

Table 3.22

Transformation of different E.coli trp mutants to Ap resistance using pUE80 and pUE81

Strain	Transformation frequency μg^{-1} DNA using	
	pUE80	pUE81
E.coli trpA	6.0×10^{1}	7.0×10^{2}
E.coli trpB	9.1×10^{1}	9.0×10^{2}
E.coli trpC	6.0×10^{1}	9.5×10^{2}
E.coli trpD	8.0×10^{1}	9.3×10^{2}
E.coli JA221 trpE	3.2×10^{3}	1.0×10^{5}

5. DETECTION OF PROTEIN PRODUCTION USING THE MAXI-CELL TECHNIQUE

To try to identify the product of the cloned leuB gene of D.radiodurans and to detect whether there are any translational products of the uvsE, mtcA and mtcB genes in an E.coli host, the maxi-cell system of Sancar et al. (1979) was employed using E.coli CSR603 as described in Materials and Methods.

As shown in Fig.3.14, maxi-cells containing pUE44 (the hybrid plasmid carrying leuB in a 6.9kb insert) produced two proteins of approximate molecular weight 73000 and 45500 (lane 2) while cells containing pUE621 (the hybrid plasmid carrying uvsE in a 6.1kb insert) produced proteins with approximate molecular weights of 47600 and 45000 (lane 3). None of these proteins were coded for by either the vector pAT153 (lane 1) or the host strain E.coli CSR603 (lane 6). However maxi-cells carrying pUE44 produced a further two proteins which were coded for by pAT153, i.e. the ampicillin and tetracycline-resistance gene products, while cells carrying pUE621 produced only the ampicillin-resistance gene product, since the tetracycline-resitance gene was inactivated by ligation of the D.radiodurans DNA into the HindIII site of pAT153. Maxi-cells containing pUE58 (the hybrid plasmid carrying mtcA in a 5.6kb insert) or pUE59 (the hybrid plasmid carrying mtcB in a 2.7kb insert) coded only for the ampicillin and tetracycline-resistance gene products (lanes 4 and 5 respectively).

In the case of maxi-cells containing pUE441 (the hybrid plasmid carrying the leuB gene in a 3.5kb insert), the 73000 MW protein was not present, leaving only the 45500 MW protein band (Fig.3.15) which represented the product of the leuB gene of D.radiodurans.

Fig.3.14

Maxi-cell analysis of the translational product of hybrid plasmids with genes of D.radiodurans present. E.coli CSR603 carried the following plasmids: lane 1, pAT153 (Ap^RTc^R); lane 2, pUE44 (Ap^RTc^R leuB); lane 3, pUE621 (Ap^RuvsE); lane 4, pUE58 (Ap^RTc^R mtcA); lane 5, pUE59 (Ap^RTc^R mtcB); lane 6, E.coli CSR603 alone.

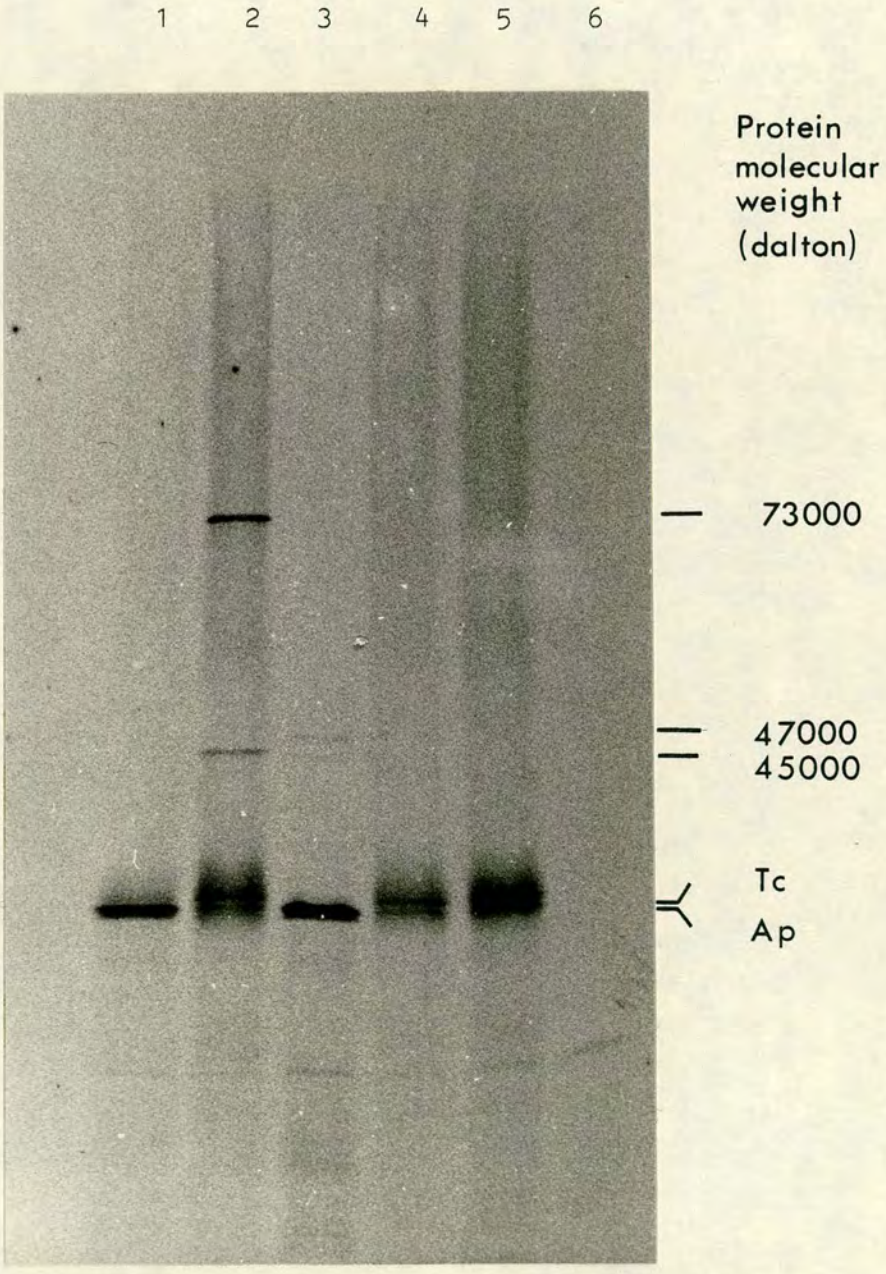
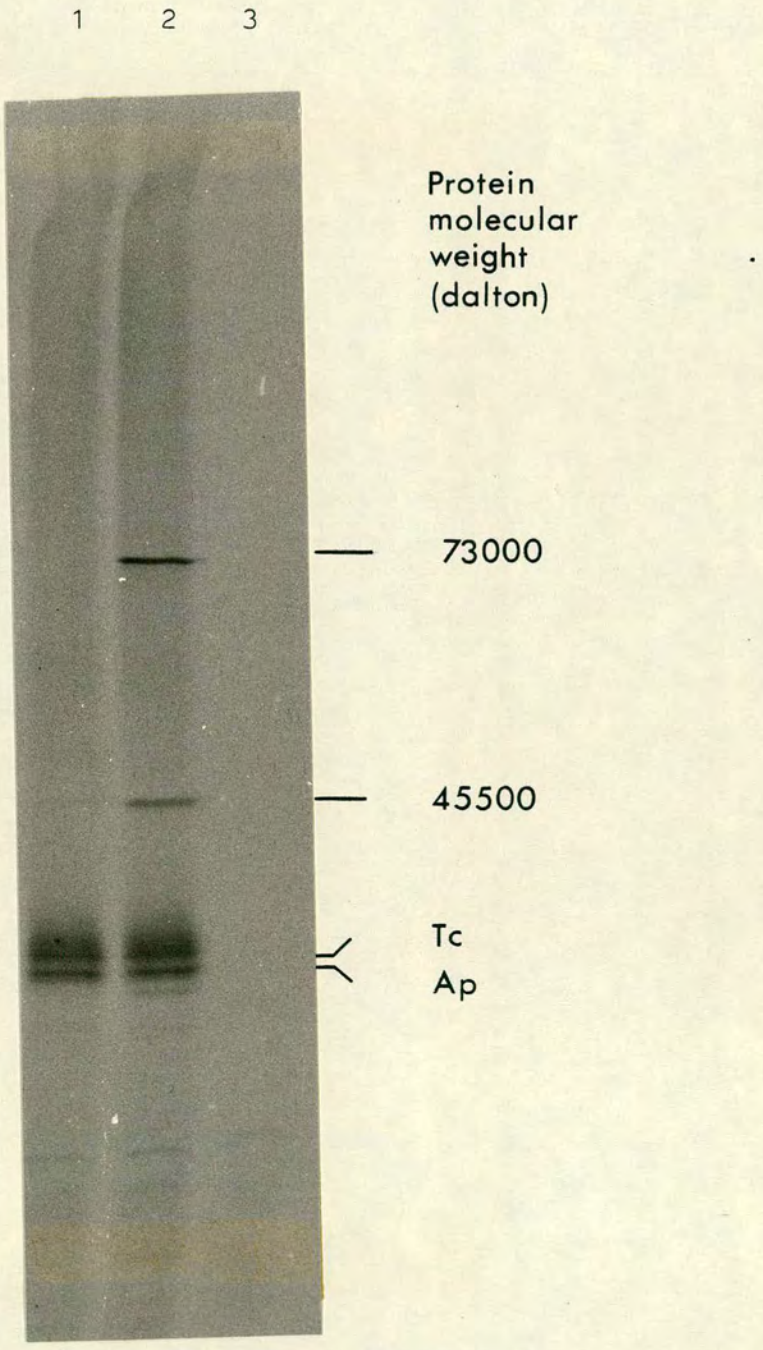


Fig.3.15

Maxi-cell analysis of the translational products of two hybrid plasmids carrying the leuB gene of D.radiodurans. E.coli CSR603 carried the following plasmids: lane 1, pUE441 (Ap^RTc^R leuB); lane 2, pUE44 (Ap^RTc^R leuB); lane 3, E.coli CSR603 alone.



CHAPTER 4

DISCUSSION

1. SPHEROPLAST GENETICS

1.1 Spheroplast formation and regeneration

The fact that Gram-positive bacteria can be converted, relatively easily, to true protoplasts by using hydrolytic enzymes (e.g. Fodor and Alföldi, 1976; Novick et al., 1980) did not apply to D.radiodurans. Although D.radiodurans is considered to be a Gram-positive bacterium (Brooks et al., 1980) its cell envelope is completely different from that of a typical Gram-positive one. It has a very complicated multilayered structure (see Fig.1.1) and a very unusual chemical composition showing some features corresponding to those of Gram-negative bacteria (Thornley et al., 1965; Work and Griffiths, 1968). Treatment with lysozyme, of D.radiodurans cells, disrupted by shaking with glass beads or by ultrasonic vibrations, resulted in their losing rigidity as a result of solubilisation of the mucopeptide layer (the holey layer) (Work, 1964; Thornley et al., 1965). However, intact cells showed more resistance to lysozyme action indicating that some compounds in the cell envelope prevented the enzyme reaching its target. In order to get a rapid and gentle lysis of cells, needed for DNA preparation, Driedger and Grayston (1970) treated D.radiodurans with n-butanol prior to lysozyme treatment. They suggested that extraction of certain compounds e.g. lipoproteins, from the cell envelope by n-butanol allowed the lysozyme to reach the mucopeptide layer as previously reported for Gram-negative bacteria (Noller and Hartsell, 1961). In Gram-negative bacteria, the outer membrane prevents lysozyme from reaching its substrate (the mucopeptide layer) and this makes them resistant to lysozyme (Weidel et al., 1960; Herzberg and Green, 1964). The role

of EDTA in mediating lysozyme lysis of Gram-negative bacteria lies in its ability as a chelating agent to uncover the mucopeptide layer by destabilising the cell envelope. Once this has been accomplished, lysozyme can reach its substrate and lyse the cell wall (Weidel et al., 1960; Voss, 1964). Work with several species has shown that EDTA causes loss of lipoprotein and lipopolysaccharide from the cell wall (Noller and Hartsell, 1961; Gray and Wilkinson, 1965; Leive, 1965). The presence of lipids and lipopolysaccharides in the cell envelope of D.radiodurans (Work and Griffiths, 1968; Brooks et al., 1980), may thus prevent lysozyme from reaching its target (the holey layer). For this reason, EDTA was used in this study in combination with lysozyme in an attempt to produce protoplasts from D.radiodurans and D.radiophilus.

Compared with other treatments, cells of D.radiodurans treated with lysozyme-EDTA in TS buffer were converted to spheroplasts in a very short time but the resulting regeneration frequency was poor (Table 3.2). The reason for this rapid conversion is that Tris buffer itself acts as a destabilising agent by degrading the compartmentalised layer in the cell envelope of D.radiodurans (Lancy and Murray, 1978), thus exposing the holey layer to lysozyme. It seems that the combination of Tris and EDTA had more effect on the destabilisation of the cell envelope of D.radiodurans than EDTA alone. Goldschmidt and Wyss (1967) found that the Tris -EDTA combination is a more powerful chelating agent than EDTA alone. In fact, a combination of Tris and EDTA is usually used to render Gram-negative bacteria sensitive to lysozyme (Repask, 1958; McQuillen, 1960; Herzberg and Green, 1964; Voss, 1964). The low regeneration frequency was probably due to the toxicity of Tris-EDTA combination as has been found with

some other bacteria (Goldschmidt and Wyss, 1967).

The SMM buffer of Wyrick and Rogers (1973) which is very widely used as a protoplasting buffer for Gram-positive bacteria (e.g. Schaeffer et al., 1976; Novick et al., 1980) was not suitable for spheroplast production from D.radiodurans (Table 3.2) and D.radiophilus (Table 3.4). The reason for this could be the presence of Mg^{++} in the buffer which might reverse the effect of EDTA in the same way that it reverses the effect of Tris (Hollinger and Wardlaw, 1971). EDTA destabilises the cell envelopes of Gram-negative bacteria by binding to, and removing, the divalent cations, especially Mg^{++} and Ca^{++} which are required for the integrity of the lipoproteins and lipopolysaccharides. Removing these cations results in breaking the bridges which hold these macromolecules together (Brown, 1964; Leive, 1965; Kushner and Onishi, 1966; Hollinger and Wardlaw, 1971). It might be expected therefore that Mg^{++} in SMM would bind to the EDTA and prevent it destabilizing the cell envelopes of D.radiodurans and D.radiophilus, so protecting the holey layer from lysozyme.

Cells of D.radiodurans were converted to an osmotically-sensitive form after treatment with lysozyme-EDTA as a result of losing their mucopeptide and compartmentalised layers. The membrane-like structure which remained around the osmotically-sensitive cells (Fig.3.1-b) was most probably the hexagonal subunit layer, attached to the outer membrane of the cell envelope (R.G.E.Murray, personal communication). Further treatment of cells with lysozyme-EDTA did not solubilize this membrane-like structure. It is known that the hexagonal subunit layer is very strongly attached to the outer membrane and is very resistant to chemical disruption (Lancy and Murray, 1978; Thompson et al., 1982). Since the complete removal of

the cell envelope of D.radiodurans was not achieved, the term "spheroplast" has been used in this study instead of "protoplast". In the case of D.radiophilus the membrane-like structure appeared only in the early stages of treatment with lysozyme-EDTA (Fig.3.2-a) and was dissolved by further incubation, leaving round, osmotically-sensitive cells (Fig.3.2-b). It is possible that true protoplasts were produced from D.radiophilus cells but no electron microscopic studies were done to prove this. It is difficult to explain the difference in the sensitivity of cell envelopes of D.radiodurans and D.radiophilus to the lysozyme-EDTA treatment because of lack of information about the cell envelope of the latter. However, it is clear that there is a difference in the structure, chemical composition or both of the two cell envelopes.

The difference in the regeneration frequencies of D.radiodurans spheroplasts obtained from RM and TGY plates indicates that $CaCl_2$ and $MgCl_2$ present in the RM are important for regeneration, most probably because they stabilise the spheroplasts as they do for other bacteria, e.g. E.coli (Tabor, 1962) and Clostridium acetobutylicum (Allcock et al., 1982).

The RM used for the regeneration of D.radiophilus spheroplasts was that used for D.radiodurans but with replacement of TGY by NB2. Since different species and even closely-related strains of bacteria require different nutritional materials and conditions for maximum regeneration, it might have been possible to increase the regeneration frequency of D.radiophilus spheroplasts by trying different nutritional materials in the RM. However, the regeneration frequency obtained (2-3%) was suitable for the purpose of the experiment (curing plasmids by regeneration of spheroplasts) and an attempt to improve the

regeneration frequency was not made.

1.2 Spheroplast fusion

The failure to remove completely the cell envelope of D.radiodurans, i.e., to dissolve the membrane-like structure surrounding osmotically-sensitive cells, was probably the reason for the absence of complementation after the fusion experiments. The presence of this membrane-like structure prevented direct contact of the cytoplasmic membranes of the two different mutants which is crucial for successful cell fusion in the presence of PEG. Unless this membrane-like structure can be removed without affecting cell viability, the development of a cell fusion protocol for this micro-organism is not possible.

The development of such a system could possibly be very useful as a tool for chromosomal mapping. It has been successfully used for genetic mapping in Streptomyces (Baltz, 1978; Hopwood and Wright, 1978) and in Staphylococcus (Stahl and Pattee, 1983). However, protoplast fusion has not been useful for chromosomal mapping in some other microorganisms, e.g. B.megaterium (Fleisher and Vary, 1985). This technique could also be used as a means for inter- and intra-species transfer of plasmids (Dancer, 1980; Fleisher and Vary, 1985).

The development of a spheroplast-fusion system for D.radiophilus seems more promising, since it was relatively easy to remove the membrane-like structure with lysozyme-EDTA treatment. However, fusion experiments were not made because of the lack of suitable mutants in this species. Attempts to isolate a variety of antibiotic-resistant mutants either by spontaneous mutations or using MNNG were not successful. Only Rif^R mutants were obtained, and the development

of suitable mutagenic procedures would be necessary for the isolation of others before fusion experiments could be done with this species.

1.3 Transformation of spheroplasts with plasmid DNA

One of the reasons suggested for the failure to transform D.radiodurans with foreign plasmid vector is the possiblity that foreign DNA may not be taken up by the cells (Mackay, 1983; Purvis, 1984). Since the protoplast of some bacteria, e.g. B.subtilis (Chang and Cohen, 1979) and Streptomyces coelicolor (Bibb et al., 1978) show a greater ability to be transformed with plasmid DNA, in the presence of PEG, than the normal cells, and in others, e.g. B.megaterium (Brown and Carlton, 1980) it is possible to transform protoplasts but not normal cells with plasmid DNA, it was thought that spheroplasts of D.radiodurans and D.radiophilus could be transformed with plasmid DNA more successfully than their normal cells. However, spheroplasts of both species were not transformed with any of the plamsid vectors used. It is possible that the plasmids were taken up by the spheroplasts, with the aid of PEG, and the failure to obtain transformants due to the degradation of the plasmid DNA on entry to the cell by the host restriction endonucleases, or to the failure of the plasmids to replicate or to express their genetic marker(s) in the new host. Incompatibility with the host plasmids could be another reason.

1.4 Curing of plasmids by the regeneration of spheroplasts

As in the case of using different chemical and physical agents (Mackay, 1983), the process of spheroplast formation and regeneration was not effective in eliminating any of the plasmids in D.radiophilus.

According to the hypothesis of Novick *et al*. (1980), plasmids that are not cured by protoplast formation and regeneration depend for their partition on an association with the chromosome or some structural cell component that is obligatorily partitioned with the chromosome, and thus the intact cell envelope is not required for their partition. It is possible, of course, that plasmids of *D.radiophilus* carry genes for essential functions and their loss results in cell death.

2. DEVELOPMENT OF A CLONING VECTOR FOR THE GENUS DEINOCOCCUS

All previous attempts to transform D.radiodurans with a wide variety of foreign plasmid DNAs were unsuccessful (Mackay, 1983; Purvis, 1984), as were attempts to transform spheroplasts prepared from either D.radiodurans or D.radiophilus (this study). From these results it is obvious that conventional vectors derived from both Gram-positive and Gram-negative bacteria are ineffective as cloning vectors for this genus, and thus it was decided to develop a novel cloning vector using the native plasmid pUE1 of D.radiophilus.

The cloning and amplification of pUE1 within pAT153 (to give pUE109) in E.coli HB101 and the construction of its restriction map was the first step in developing such a cloning vector and showed that pUE1 has the advantage of having four unique restriction sites which will be useful for cloning purposes.

The failure to transform D.radiopugnans, D.radiophilus and D.proteolyticus to Tc^R with pUE109 was not unexpected, because none of them has been successfully transformed using homologous chromosomal DNA and may, therefore, be unable to take up any form of DNA. However, D.radiodurans can take up both linear (Moseley and Setlow, 1968) and covalently-closed-circular DNA (this study) and the failure, therefore, to obtain Tc^R transformants from either of the two strains of D.radiodurans, i.e., R1 and Sark, could be due to

i) Failure of expression of the Tc^R marker in D.radiodurans.

ii) Restriction of pUE109 on entry to the cells by the host restriction endonucleases, especially by the SstII isoschizomer, MraI present in D.radiodurans R1 (Wani et al., 1982).

iii) Failure of the replication origin of pUE1 to function in D.radiodurans, or the presence of the PstI site in the pUE1 replica-

tion origin.

Until these reasons are investigated, it is not possible to decide whether pUE1 is a potential cloning vector for D.radiodurans or not. Attempts to introduce a selectable genetic marker from D.radiodurans into the SstII or MluI sites of pUE109 using shotgun experiments were unsuccessful (data not shown) and because of lack of cloned genetic markers from D.radiodurans at that stage of the study, no more work was done in this direction. Several genetic markers were cloned from D.radiodurans in the later stages of this study and are now available for introduction into pUE1. Also the SstII site of pUE1 can be removed by treatment with, for example, nuclease S1. Such improvements could result in getting a suitable cloning vector for D.radiodurans, and the hybrid plasmid pUE109 may be useful also as a shuttle vector between E.coli and D.radiodurans.

The isolation of a recombination-deficient and restrictionless mutant from D.radiodurans would certainly improve chances of developing an efficient cloning system.

3. GENE CLONING AND EXPRESSION IN E.COLI

The construction of a gene library from D.radiodurans in a cosmid vector and the availability of different mutants of this species (some of them isolated during this study) made the cloning of several genes from this interesting microorganism possible. Using the cosmid gene bank as a phage mix, it was possible to select directly for hybrid cosmids carrying a gene from D.radiodurans capable of functional expression in E.coli, e.g. the leuB gene. The large inserts in the cosmid vectors also made it possible to identify other genes by screening the relatively small number of clones representing the genome by "complementation" of mutant genes in D.radiodurans, and, in some cases, to identify more than one gene of interest in the same insert. This will help in mapping these genes on the chromosome and in studying their structure, function and regulation.

Eight genes were cloned from D.radiodurans and only one of them (the leuB gene) was shown to be capable or functional expression in E.coli. The expression of the leuB gene of D.radiophilus in E.coli HB101 when introduced as a hybrid plamsid with pAT153 has also been demonstrated (Purvis, 1984).

The biosynthesis of leucine in E.coli is controlled by a cluster of four structural genes, leuA, B, C and D. The gene leuA codes for α-isopropylmalate (α-IPM) synthetase, leuB for β-IPM dehydrogenase and leuC and leuD for α-IPM isomerase. Leucine genes that complement E.coli leuB have been cloned from several other bacteria, e.g. Thermus thermophilus (Nagahari et al., 1980), Azotobacter vinelandii (Medhora et al., 1983) and alkalophilic Bacillus No.221 (Honda et al., 1984). Of the three leucine genes (leuA, B and C) of B.subtilis, only the leuC (which

corresponds to leuB in E.coli) expresses in E.coli, while none of the E.coli leucine genes express in B.subtilis (Nagahari and Sakaguchi, 1978).

Considering the large size of the insert (about 48kb) in pUE40, it is possible that leucine biosynthesis genes other than leuB from D.radiodurans would be present. However their presence and functional expression in E.coli could not be investigated because neither leuA, leuC or leuD mutants of E.coli were available (a set of such mutants has been reported by Honda et al. (1984) and a request made for them to be sent but they have not arrived), nor have Leu$^-$ mutants of D.radiodurans been isolated.

In addition to the leuB gene, two other auxotrophic markers were cloned from D.radiodurans; an asp gene and a trp gene. The size of the insert in the hybrid cosmid pUE90 which carried the asp gene of D.radiodurans was very small (9.3kb) compared with the average size of inserts in other hybrid cosmids of the gene library. Such a size was not expected, and theoretically it is not possible to package such a small hybrid cosmid into a λ phage particle, since the λ cos sites should be at least 37kb (75% of the size of the λ DNA) apart for efficient packaging. The most likely explanation is that a part of the insert was deleted during the successive sub-culturing of the E.coli strain which harboured pUE90, especially since the isolation of pUE90 was done in later stages of this study. The functional expression of the asp gene of D.radiodurans in E.coli was not investigated because no suitable E.coli mutant was available.

The results of transforming the seven Trp$^-$ mutants of D.radiodurans with the hybrid cosmid pUE80 and the hybrid plasmid pUE81 which carried a trp gene from D.radiodurans (Table 3.21)

indicated that there are at least two different trp mutants of D.radiodurans. The similar transformation results obtained with pUE80 (with an insert of 48.0kb) and pUE81 (with an insert of 5.5kb) could have alternative explanations

i) The insert of the hybrid cosmid pUE80 contained only one trp gene from D.radiodurans which was separated from the rest of trp genes by digestion with MboI during the preparation of the gene library, and so it is situated at one end of the insert and that the four Trp^- mutants which were transformed to prototrophy are mutated in the same gene.

ii) The insert of pUE80 contained more than one trp gene from D.radiodurans and these genes are clustered on a chromosomal region of not more than 5.5kb. This means that the four Trp^- mutants which transformed to prototrophy are possibly mutated in different trp genes. The very low transformation frequency of D.radiodurans 27-1 to prototrophy can be explained according to this assumption. The gene which "complements" the mutation in this strain is located at the end of the insert of pUE80 or pUE81 and this resulted in a very low transformation frequency. It is possible of course that this strain has the same trp mutation as the others, but for some reason it is a poorly transformable strain. It is possible to find out whether these four trp mutants are mutated in the same gene or in different ones by using DNA from each one to transform the others to see if any two mutations can complement each other or not and this would indicate whether the inserts of pUE80 and pUE81 contain one trp gene or more.

The failure to complement any of the five different trp mutants of E.coli using either pUE80 or pUE81 could have been because the

trp gene of D.radiodurans was transcribed and translated but its product was not functional in E.coli, or because the promoter of D.radiodurans is not recognised by E.coli DNA polymerase, or because the inserts of pUE80 and pUE81 contained only the structural gene without the promoter.

The maxi-cell technique was not used in this case to indicate whether there was a translational product for the cloned trp gene in E.coli or not.

The DNA repair genes mtcA, mtcB, uvsC, uvsD and uvsE were all cloned. Transformation data published previously indicated that neither mtcA and mtcB (Moseley and Copland, 1978) nor uvsC, uvsD and uvsE (Moseley and Evans, 1983) are closely linked. However, these data did not clarify the distribution of these genes on the chromosome. Results of this study, i.e., presence of mtcA and mtcB in the insert (22.0kb) of pUE502; uvsC, uvsD and uvsE in the insert (34.0kb) of pUE60; and uvsC, uvsD, uvsE and mtcA in the insert (49.0kb) of pUE70, indicated that these genes are located on a fragment of chromosome of not more than 71.0kb. The close location of some of the DNA repair genes have since been demonstrated by the size reduction of fragments carrying them. The size of the fragment carrying the mtcA and mtcB genes has now been reduced to 18.0kb (I.Masters, personal communication), while the size of the DNA fragment carrying uvsC, uvsD and uvsE has been reduced to 8.0kb (D.Evans and I.Masters, personal communication). It should soon be possible to map the five DNA repair genes relative to each other on the chromosome since the link between the mtc and the uvs genes is available in the insert of the hybrid cosmid pUE70.

Using the maxi-cell technique, no translational products for

the cloned mtcA or mtcB were detected, while the hybrid plasmid pUE621 which carries the uvsE gene (in a 6.1kb insert) coded for two proteins of approximate molecular weight 45000 and 47000 in addition to the product of the ampicillin gene of pAT153. It is not possible to decide, at this stage, whether either of these proteins is the product of the uvsE gene or not, since both molecular weights are higher than the 36000 calculated for UV endonuclease β from gel filtration studies (Evans and Moseley, 1985). Considering the difference in the methods used to measure the molecular weights of the proteins in the two studies, there is a possibility that one of the two proteins is the product of the uvsE gene. It should be possible to determine if either of the two proteins is the product of the uvsE gene by reducing the size of the 6.1kb insert to the smallest possible fragment carrying the uvsE gene and to see whether it codes for either of the two proteins or not.

The successful mapping of the five genes relative to each other, and their transcription and translation in another host, e.g. E.coli should help to illuminate the nature of excision repair of UV-induced damage in D.radiodurans.

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Cloning of the DNA repair genes *mtcA, mtcB, uvsC, uvsD, uvsE* and the *leuB* gene from *Deinococcus radiodurans*

(Recombinant DNA; gene library in cosmid vector; hybrid plasmids; expression of foreign gene in *Escherichia coli*)

Ghalib H. Al-Bakri[a], Martin W. Mackay[a]*, Paul A. Whittaker[b] and B.E.B. Moseley[a]**

[a]Department of Microbiology, University of Edinburgh, School of Agriculture, Edinburgh EH9 3JG, Tel. (031) 667 1041, ext. 266, and [b]M.R.C. Mammalian Genome Unit, Department of Zoology, University of Edinburgh, Edinburgh EH9 3JT (U.K.) Tel. (031) 667 1081, ext. 3462

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SUMMARY

A gene library from *Deinococcus radiodurans* has been constructed in the cosmid pJBFH. A 51.5-kb hybrid cosmid, pUE40, that transduced *Escherichia coli* HB101 from leucine dependence to independence was selected, and a 6.9-kb fragment which carried the *leuB* gene from *D. radiodurans* was subcloned into the *Eco*RI site of pAT153. The DNA repair genes *mtcA, mtcB, uvsC, uvsD* and *uvsE*, which code for two *D. radiodurans* UV endonucleases were identified by transforming appropriate repair-deficient mutants of *D. radiodurans* to repair proficiency with DNA derived from the gene library. Hybrid cosmid pUE50 (37.9 kb) containing an insert carrying both the *mtcA* and *mtcB* genes was selected and 5.6- and 2.7-kb DNA fragments carrying *mtcA* and *mtcB*, respectively, i.e., the genes that code for UV endonuclease α, were subcloned into the *Eco*RI site of pAT153. The three genes *uvsC, uvsD* and *uvsE*, that code for UV endonuclease β, were all present in the 46.0-kb hybrid cosmid pUE60. The *uvsE* gene in a 12.2-kb fragment was subcloned into the *Hin*dIII site of pAT153 and the size of the insert reduced to 6.1 kb by deletion of a 6.7-kb fragment from the hybrid plasmid pUE62. None of the *uvs* genes introduced into the UV-sensitive *E. coli* CSR603 (*uvrA*$^-$) was able to complement its repair defect. The *mtcA, uvsC, uvsD* and *uvsE* genes were found in the 52.5-kb hybrid cosmid pUE70. It is concluded that the DNA repair genes *mtcA, mtcB, uvsC, uvsD* and *uvsE* are located within an 83.0-kb fragment of the *D. radiodurans* genome.

*Present address: Department of Molecular Biology, University of Edinburgh, Edinburgh EH9 3JR (U.K.) Tel. (031) 667 1081 ext. 2719.

**To whom all correspondence and reprint requests should be addressed.

Abbreviations: Ap, ampicillin; ccc, covalently closed circular; Em, erythromycin; kb, kilobase pairs; Mtc, mitomycin c; ^{R}(superscript), resistance; ^{S}(superscript), sensitivity; Tc, tetracycline; TGY, see MATERIALS AND METHODS, section **a**; u, unit(s); UV, ultraviolet.

INTRODUCTION

D. radiodurans (formerly *Micrococcus radiodurans*: Brooks and Murray, 1981) is the type species of a small number of exceptionally radiation-resistant, red-pigmented cocci considered to comprise one of the eight major groups of the Eubacteria (Fox et al., 1980). Although *D. radiodurans* is extremely resistant to both the lethal and mutagenic effects of a variety

of DNA-damaging agents (Sweet and Moseley, 1976), it has been shown to possess only two mechanisms for the repair of UV-irradiated DNA: excision repair (Boling and Setlow, 1966) and recombination repair (Moseley and Copland, 1975; Moseley et al., 1972). Excision repair of pyrimidine dimers in *D. radiodurans* can proceed through two distinct, equally efficient pathways (Moseley and Evans, 1983). One pathway requires functional *mtcA* and *mtcB* genes, while the other requires functional *uvsC, uvsD* and *uvsE* genes. Only when both pathways are blocked by mutations, are the bacteria incapable of excising pyrimidine dimers, although strains blocked only in *mtcA* or *mtcB* are Mtc^S (Moseley and Copland, 1978). The genetics of DNA repair processes in *D. radiodurans* have been analysed by transformation since, to date, this is the only way of effecting gene transfer in this species. However, transformation has extreme limitations when used as a means of mapping chromosomal genes and all attempts to develop transduction, conjugation or a protoplast fusion technique have been unsuccessful (Moseley, 1983; unpublished results).

As an alternative approach we have chosen to construct a gene library from *D. radiodurans* in a cosmid vector in order to isolate various genes, especially those involved in DNA repair as large DNA fragments.

MATERIALS AND METHODS

(a) Bacterial strains and growth media

The bacterial strains used are listed in Table I. TGY medium for the growth of *D. radiodurans* was as described by Sweet and Moseley (1976). L-broth for the growth of *E. coli*, contained 10 g tryptone (Difco), 5 g yeast extract (Difco), 5 g NaCl, and 1 g glucose in 1 litre distilled water. For in vitro packaging of cosmid DNA and transduction, *E. coli* was grown in L-broth containing 0.4% maltose. M9 medium was used as a minimal medium for *E. coli*, supplemented as required with L-leucine (50 μg/ml), L-proline (50 μg/ml) and thiamine · HCl (5 μg/ml). Media were solidified by adding 12 g agar (Oxoid No. 1)/l.

(b) Enzymes

The restriction enzymes *Hin*dIII, *Bam*HI and *Mbo*I were purchased from Boehringer Mannheim, Lewes, Sussex; *Pst*I, *Sal*I and *Sst*II were from Bethesda Research Laboratories (U.K.) Ltd.; *Mlu*I was from New England Biolabs, Bishops Stortford; *Eco*RI was from NBL Enzymes Ltd., Cramlington, Northumberland; *Dra*I was a gift from I.J. Purvis. T4 DNA ligase and calf intestine alkaline phosphatase were purchased from Boehringer Mannheim. Enzymes were used in accordance with the manufacturers' instructions.

TABLE I

Strains of bacteria used in this study

Strain	Relevant genotype	Reference
D. radiodurans Krase	*kan, rif, acr, str, ery*	Tirgari and Moseley (1980)
D. radiodurans UVS9	*uvsC, mtcA*	Moseley and Evans (1983)
D. radiodurans UVS25	*uvsD, mtcA*	Moseley and Evans (1983)
D. radiodurans UVS78	*uvsE, mtcA*	Moseley and Evans (1983)
D. radiodurans 262	*mtcB*	Moseley and Copland (1978)
E. coli HB101	F^-, *pro*$^-$, *leu*$^-$, *thi*$^-$, *str*^{r}, r_K^-, m_K^-, *recA*$^-$	Boyer and Roulland-Dussoix (1969)
E. coli BHB2688	N205 *recA*$^-$ (λ *Eamb2red*$^-$*imm*434*c*Its*S*am) λ^R	Hohn and Murray (1977)
E. coli BHB2690	N205 *recA*$^-$ (λ *Damb2red*$^-$*imm*434*c*Its*S*am) λ^R	Hohn and Murray (1977)
E. coli CSR603	F^-, *thr*$^-$, *leu*$^-$, *pro*$^-$, *phr*$^-$, *recA*$^-$, *arg*$^-$, *thi*$^-$, *uvrA*$^-$	Sancar and Rupert (1978)

(c) DNA preparation

Chromosomal DNA from *D. radiodurans* for construction of the library was prepared by a modification of Marmur (1961) method. This involved suspending cells in butanol-saturated phosphate, EDTA buffer ($Na_2HPO_4 \cdot 2H_2O$, 4.7 g; KH_2PO_4, 4.6 g; EDTA, 3.7 g; *n*-butanol, 60 ml; distilled water to 1 l). Butanol pretreatment rendered the cells sensitive to lysozyme degradation (Driedger and Grayston, 1970). The rapid boiling method (Holmes and Quigley, 1981) was used to screen for hybrid DNA molecules in *E. coli*. Screening for hybrid molecules in *D. radiodurans* was performed by a modification of the Birnboim and Doly (1979) method. This involved pretreatment of cells with butanol as described above. The large-scale preparation of plasmids was performed using a 1-litre scale-up of the Birnboim and Doly (1979) technique. Ethanol-precipitated lysates were purified for ccc plasmid DNA by the dye buoyant-density-gradient centrifugation technique (Radloff et al., 1967).

(d) Gel electrophoresis

Agarose gels (0.5–0.8%) were run in Tris · acetate buffer (0.04 M Tris · acetate, 0.001 M EDTA, pH 8.0) and stained with ethidium bromide. DNA bands were visualised by UV illumination at 302 nm, and photographed using a Polaroid MP-4 camera with Polaroid type 55 film and Kodak 22A Wratten filter. Sizes of DNA fragments were measured according to their mobility on gels compared to standards (Southern, 1979).

(e) Restriction and ligation

Target DNA from the antibiotic-resistant strain *D. radiodurans* Krase (10 μg) was partially digested by incubating it at 37°C with 0.04 u *Mbo*I for 1 h. The average size of DNA at this stage, as determined by agarose gel electrophoresis (0.5% agarose) was 40 kb. Digestion was halted by heating to 65°C for 10 min, and the DNA was incubated at 37°C with alkaline phosphatase. 10 μg of cosmid pJBFH (3.75 kb), a derivative of pJB8 (Ish-Horowicz and Burke, 1981) made by deletion of the *Hpa*I-*Ava*I fragment (F.G. Grosveld, personal communication), was linearised by incubating it at 37°C with 50 u of *Bam*HI for 1 h. After phenol and chloroform extraction vector and target DNAs were mixed and ligated with T4 DNA ligase at 14°C for 16 h. DNA was then packaged as described in Maniatis et al. (1982), and 50 μl of the packaging mix added to 50 μl *E. coli* HB101 growing in the presence of 0.4% maltose. The phage particles were allowed to adsorb by incubating the culture at 37°C. After 20 min, 1 ml L-broth was added and the incubation continued for a further 45 min for expression. The culture was then spread onto L-plates containing 100 μg Ap/ml and incubated overnight at 37°C.

(f) Transformation

Transformation of *D. radiodurans* was carried out according to the method of Tirgari and Moseley (1980). No DNase was added and the expression time allowed in TGY broth was 4 h. The protocol of Humphreys et al. (1979) was used to transform *E. coli* with hybrid molecules.

(g) Selection of hybrid cosmids carrying genes from *D. radiodurans* capable of functional expression in *E. coli*

E. coli HB101 was transduced with the packaging mix as described. After 45 min for expression, 10 ml of L-broth containing 40 μg Ap/ml was added to the transduced population, and incubation continued for 18 h at 37°C. Samples were diluted appropriately and spread on M9-supplemented agar lacking either leucine or proline and on L-plates containing 10 μg Em/ml. Samples were also spread on L-plates and irradiated with UV light (31.5 J/m^2). Plates were incubated at 37°C for 4 days.

(h) Selection of hybrid cosmids carrying *mtcA* or *uvsE* genes

Transduced Ap^R *E. coli* HB101 colonies were divided into eight groups of 40 colonies. Each group of colonies growing on one L-plate containing Ap was suspended in 3 ml L-broth. These suspensions were used to inoculate 1-litre amounts of L-broth containing Ap, which were incubated at 37°C with shaking for 18 h and used for DNA preparation. Hybrid cosmid DNA from each group was used after linearisation with *Bam*HI to transform the mutant

D. radiodurans UVS78. Samples were diluted appropriately and spread on TGY plates containing 0.04 μg Mtc/ml, for the selection of clones transformed to Mtc^R by the acquisition of the *mtcA* gene. Samples were also spread on TGY plates and irradiated with UV light at a dose of 190 J/m^2 for the selection of clones transformed to UV^R by the acquisition of either the *mtcA* or *uvsE* genes. Plates were incubated at 30°C for 3 days.

(i) Cloning in pAT153

Two μg of pAT153 (Twigg and Sherratt, 1980) and 1 μg of the target DNA were digested separately with a suitable restriction endonuclease for 2 h at 37°C, and the digested pAT153 treated with alkaline phosphatase to prevent recircularisation. Vector and target DNA were mixed at a final concentration of 50 μg/ml and ligated for 18 h with T4 DNA ligase at 12°C. *E. coli* HB101 was transformed to Ap^R, or Ap^R and Tc^R depending on the site of cloning in pAT153.

RESULTS AND DISCUSSION

(a) Construction of the gene library

The library was constructed as described. The number of Ap^R transductants was $8.6 \times 10^4/\mu g$ of pJBFH. Hybrid cosmids were isolated from 24 colonies using the boiling method, and *Eco*RI restriction analysis was performed on small samples derived from these. The insert sizes ranged from 30–46 kb with an average of 35 kb. Screening of 320 clones would therefore give a 97.7% probability of finding a unique *D. radiodurans* sequence (Clarke and Carbon, 1976).

(b) Selection of a hybrid cosmid carrying the *leuB* gene of *D. radiodurans*

When *E. coli* HB101 was transduced with the packaging mix, and selection made for various markers as described, colonies were detected only on M9 agar lacking leucine. The hybrid cosmids prepared from ten Leu^+ Ap^R colonies had the same size (51.1 kb). Hybrid cosmid (pUE40) DNA prepared from one of these colonies transformed *E. coli* HB101 to Leu^+ at a frequency of $2.2 \times 10^3/\mu g$ of DNA. 100 Leu^+ transformants streaked on L-plates containing Ap were all Ap^R. This indicated that the leucine independence was the result of the presence of the *leuB* gene of *D. radiodurans* (so called because it is an analogue of the *leuB* gene of *E. coli*) in the insert of the hybrid cosmid and not a reversion phenomenon in the *E. coli* gene. This is the first gene from *D. radiodurans* shown to express in *E. coli* and suggests that the expression of other genes will be possible. Functional genetic expression of the analogous gene from *Azotobacter vinelandii* (Medhora et al., 1983), *Bacillus subtilis* (Nagahari and Sakaguchi, 1978) and *Thermus thermophilus* (Nagahari et al., 1980) in *E. coli leuB*$^-$ has been reported. The expression of the *leuB* gene from *Deinococcus radiophilus* in *E. coli* HB101 when introduced as a hybrid plasmid with pAT153 has also been demonstrated in this laboratory (I.J. Purvis, personal communication). Subcloning of the *D. radiodurans leuB* gene from pUE40 into pAT153 localized the gene to a 6.9-kb *Eco*RI fragment.

(c) Selection of hybrid cosmids carrying *mtcA* or *uvsE* genes

DNA from four groups (2, 3, 5 and 6) of 40 colonies transformed the Mtc^S, UV^S *D. radiodurans* UVS78 (*mtcA, uvsE*) to UV^R at frequencies ranging from 1.8×10^{-5} to 2.6×10^{-3}, but not to Mtc^R. This indicated that the transformants received the *uvsE* gene and that the *mtcA* gene was absent from these DNA pools. When DNA from the other four groups (1, 4, 7 and 8) was used to transform *D. radiodurans* UVS78, both UV^R transformants (frequencies from 1.3×10^{-4} to 2.8×10^{-3}) and Mtc^R transformants (frequencies from 1.7×10^{-6} to 2.1×10^{-5}) were isolated. The majority of the colonies selected for UV^R were Mtc^S, indicating that these had acquired the *uvsE* gene. All colonies selected for Mtc^R were, of course, also UV^R as a result of acquiring the *mtcA* gene. Thus both *uvsE* and *mtcA* were present in each of the four pools: 1, 4, 7 and 8.

Hybrid cosmid DNA from twelve individual colonies from two of these groups (1 and 4) was prepared by the rapid boiling method and used to transform *D. radiodurans* UVS78 to UV^R or Mtc^R. One colony (1-1) from group 1 was found to carry the *mtcA* gene,

and four colonies, one from group 1 (1-10) and three from group 4 (4-4, 4-5 and 4-11) carried the *uvsE* gene. pUE50 DNA (from colony 1-1) transformed *D. radiodurans* UVS78 to both UV^R and Mtc^R at approximately the same frequency (about 8×10^{-3}). All of 100 colonies selected for UV^R were also Mtc^R, i.e., they all contained the *mtcA* gene. pUE60 (from colony 4-4), which transformed *D. radiodurans* UVS78 to UV^R at a frequency of 8.8×10^{-3} did not transform it to Mtc^R. Thus pUE60 carried only the *uvsE* gene. In the case of pUE70 DNA (from colony 4-11), the transformation frequency to Mtc^R (3.4×10^{-6}) was much lower than that to UV^R (5.7×10^{-3}). It was concluded that pUE70 DNA carried both the *uvsE* and *mtcA* genes, but that the latter was probably located at the end of the insert and this resulted in inefficient recombination and a low transformation frequency. Restriction analysis of pUE50 and pUE70 supported this conclusion (not shown). These results also indicated that the *mtcA* and *uvsE* genes lie within a chromosomal region of about 49 kb.

(d) Selection of *mtcB*, *uvsC* and *uvsD* genes

Transformation data published previously indicated that neither *mtcA* and *mtcB* (Moseley and Copland, 1978) nor *uvsC*, *uvsD* and *uvsE* (Moseley and Evans, 1983) are closely linked. However, these data did not clarify the distribution of these genes on the chromosome. pUE50 DNA was used to transform *D. radiodurans* 262 (*mtcB*) to Mtc^R and pUE60 and pUE70 DNAs to transform *D. radiodurans* strains UVS9 (*uvsC*) and UVS25 (*uvsD*) to UV^R. The results indicated that the *mtcB* gene was present in the insert of pUE50 in addition to the *mtcA* gene, that the insert of pUE60 carried the *uvsC* and *uvsD* genes as well as the *uvsE* gene, and that the insert of pUE70 carried the *uvsC* and *uvsD* genes in addition to *uvsE* and *mtcA*. The relatively low transformation frequencies in these experiments, from 4.5×10^{-5} to 1.4×10^{-3}, were caused by the poor transformability of the recipient strains compared with *D. radiodurans* UVS78. It appears from these results that the *mtcA* and *mtcB* genes are located on a fragment of chromosomal DNA of about 34 kb, while *uvsC*, *uvsD*, *uvsE* and *mtcA* are located on a fragment of about 49 kb.

(e) Subcloning of *mtcA* and *mtcB* into pAT153

Six of the seven *Eco*RI-generated fragments from pUE50 were subcloned into pAT153. Hybrid plasmid DNA from colonies harbouring different sizes of insert was used to transform *D. radiodurans* UVS78 (*mtcA*) and *D. radiodurans* 262 (*mtcB*) to Mtc^R. pUE58, containing a 5.6-kb insert, transformed the former at a frequency of 9.5×10^{-4}, while DNA of pUE59, containing a 2.7-kb insert, transformed *D. radiodurans* 262 at a frequency of 2.5×10^{-5}.

When pUE50 DNA was restricted with *Eco*RI and used to transform *D. radiodurans* 262 to Mtc^R, the transformation frequency was 2.0×10^{-7} compared with 1.7×10^{-4} for unrestricted DNA. It was thought that perhaps *Eco*RI cut inside the *mtcB* gene, but when an *Eco*RI-generated 2.7-kb fragment from pUE50 was ligated to pAT153 (pUE59) and used to transform *D. radiodurans* 262, the transformation frequency increased 100-fold, and this appears to rule out the possibility of an *Eco*RI site in *mtcB*. No evidence could be obtained for plasmid maintenance in strain 262 (not shown), and the most likely explanation is that the 2.7-kb fragment, following its uptake into the cell, is susceptible to the action of exonucleases so that the *mtcB* gene is inactivated by digestion, while its insertion into pAT153 gives it protection from exonucleases prior to successful chromosomal integration.

(f) Subcloning of the *uvsE* gene into pAT153

*Hin*dIII fragments of pUE60, which carried the *uvsC*, *uvsD* and *uvsE* genes, were inserted into pAT153, and three of the four possible sizes of inserts were identified, viz. 3.7 kb (pUE61), 5.6 kb (pUE63) and 12.2 kb (pUE62). pUE61, pUE62 and pUE63 DNAs were used in an attempt to transform *D. radiodurans* UVS78 (*uvsE*), UVS25 (*uvsD*) and UVS9 (*uvsC*) to UV^R. pUE62 DNA transformed *D. radiodurans* UVS78, at a frequency of 8.3×10^{-3}. All three DNAs failed to transform *D. radiodurans* UVS9 and UVS25 to UV^R, which indicated that pUE62 carried the *uvsE* gene only, while *uvsC* and *uvsD* were probably either present in the fourth *Hin*dIII fragment (24.5 kb) which could not be cloned or contained internal *Hin*dIII sites. pUE621, derived from pUE62 (Fig. 1), transformed *D. radiodurans* UVS78 to UV^R at a frequency of 7.1×10^{-3}, which showed that it carried the *uvsE* gene.

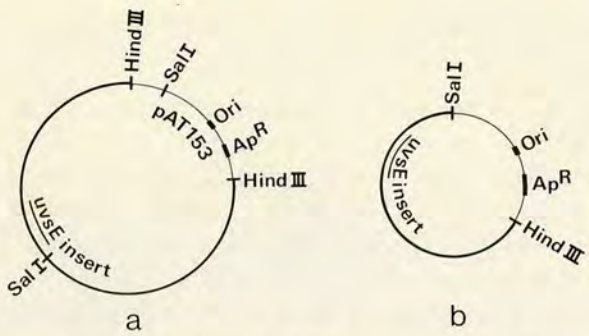

Fig. 1. (a) Hybrid plasmid pUE62 consisting of a 12.2-kb *D. radiodurans* DNA segment (heavy line) carrying the *uvsE* gene inserted into the *Hin*dIII site of pAT153 (thin line). (b) Hybrid plasmid pUE621 carrying the *uvsE* gene constructed by *Sal*I digestion of pUE62 which allowed in vitro deletion of 6.1 kb of the insert along with the 625-bp *Hin*dIII-*Sal*I segment of the vector.

The genes *uvsC, uvsD* and *uvsE* in the insert of pUE60, and *uvsE* alone in the insert of pUE621, were introduced into *E. coli* CSR603 (*uvrA*$^-$) by transformation. None of them was able to complement the repair defect in this strain.

The relatively large inserts in the constructed gene library have enabled us to locate the *D. radiodurans* repair genes, *mtcA* and *mtcB*, coding for UV endonuclease α, and *uvsC, uvsD* and *uvsE* coding for UV endonuclease β (Evans and Moseley, 1983). The presence of *mtcA* and *mtcB* on the insert of pUE50 (37.9 kb) and *uvsC, uvsD, uvsE* and *mtcA* on the insert of pUE70 (52.5 kb) indicated that these genes are located on a fragment of chromosome of not more than 83.0 kb. The successful mapping of these genes relative to each other, and their transcription and translation in *E. coli* should help to illuminate the nature of excision repair of UV-induced damage in *D. radiodurans*.

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The plasmids of *Deinococcus* spp. and the cloning and restriction mapping of the *D. radiophilus* plasmid pUE1

Martin W. Mackay*, Ghalib H. Al-Bakri, and B. E. B. Moseley

Department of Microbiology, University of Edinburgh, School of Agriculture, Edinburgh, EH9 3JG, UK

Abstract. Plasmids were found in strains representing all four species of the genus *Deinococcus* viz. *D. radiodurans, D. radiopugnans, D. radiophilus* and *D. proteolyticus* but were not found in the most intensively-investigated strain of the genus, *D. radiodurans* R1. Their sizes were calculated from electron micrographs. *D. radiophilus* yielded three size classes of plasmid while *D. radiodurans* Sark, *D. proteolyticus* and *D. radiopugnans* each yielded two. Attempts to cure *D. radiophilus* and *D. radiodurans* Sark of any of their plasmids, using a variety of methods, were unsuccessful. A 10.8 kbase pair (kb) plasmid from *D. radiophilus*, pUE1, was cloned into the PstI site of pAT153 and propagated in *Escherichia coli* HB101. The recombinant plasmid, pUE109 was subjected to single and double digestion with various restriction endonucleases and its restriction map constructed. The resistance of *E. coli* HB101 to ultraviolet radiation was not increased when pUE109 was introduced into it. Attempts to transform *D. radiodurans* with pUE109 failed to detect tetracycline-resistant transformants.

Key words: Cloning – *Deinococcus radiophilus* – *Deinococcus* spp. – Plasmids – Restriction mapping

The genus *Deinococcus* contains four species that were formerly considered to be part of the genus *Micrococcus* (Brooks and Murray 1981), namely *D. radiodurans, D. radiopugnans, D. radiophilus* and *D. proteolyticus*. The four species are characterised by their morphology, red pigmentation and extreme resistance to both the lethal (Anderson et al. 1956; Davis et al. 1963; Lewis 1971; Kobatake et al. 1973) and mutagenic (Sweet and Moseley 1974, 1976; Tempest and Moseley 1982) effects of ionizing and ultraviolet (UV) radiation.

A thorough genetic analysis of radiation resistance in these bacteria is limited by the fact that transformation using homologous chromosomal DNA is, so far, the only method available for genetic exchange in the two strains of *D. radiodurans* (Moseley and Setlow 1968; Tirgari and Moseley 1980) while none is available for the other three species. Although transformation has been useful for investigating the mechanisms that confer resistance to lethal and mutagenic damage in *D. radiodurans*, e.g. Moseley and Copland (1978), it has extreme limitations when used as a means of mapping chromosomal genes. In spite of a long search, no phages have been isolated that plaque on *Deinococcus* spp., and hence no transduction system is available while all attempts to develop a conjugation system or a protoplast fusion technique have likewise been unsuccessful (Moseley 1983; Al-Bakri, unpublished results). Attempts to mobilise the chromosome by introducing plasmids from other species of bacteria have been unsuccessful (Tirgari 1977; I. J. Purvis, personal communication; unpublished results). To overcome this impasse, a search has been made for native plasmids that might provide the basis for constructing a chromosome-mobilising element and which might also be useful as a cloning vehicle in this group of microorganisms.

Materials and methods

Bacteria. Those used were *Deinococcus radiodurans* R1 (Anderson et al. 1956), *D. radiodurans* Sark (Murray and Robinow 1958), *D. radiopugnans* (Davis et al. 1963), *D. radiophilus* (Lewis 1971), *D. proteolyticus* (Kobatake et al. 1973) and *Escherichia coli* HB101 (Boyer and Roulland-Dussoix 1969).

Media and growth conditions. TGY medium (Anderson et al. 1956) was used for the growth of *D. radiodurans* R1, *D. radiodurans* Sark, *D. radiopugnans* and *D. proteolyticus* and nutrient broth (Oxoid no. 2) for the growth of *D. radiophilus*. L-Broth (Lennox 1955) was used for the growth of *E. coli* HB101. Liquid cultures were incubated with shaking at 30° C for *Deinococcus* strains and 37° C for *E. coli* HB101.

Enzymes. The restriction enzymes *Pst*I and *Sst*II were purchased from BRL Bethesda Research Laboratories (UK) Limited, Cambridge; *Mlu*I and *Nru*I from New England Biolabs, Bishops Stortford; *Eco*RI from NBL Enzymes Limited, Cramlington, Northumberland; *Hin*dIII and *Bam*HI from Boehringer-Mannheim, Lewes, Sussex; *Dra*I was a gift from I. J. Purvis. The enzymes T4 DNA ligase (EC 6.5.1.1) and calf intestine alkaline phosphatase (EC 3.1.3.1) were purchased from Boehringer-Mannheim. Enzymes were used in accordance with the manufacturers' instructions.

Isolation of plasmids. Rapid screening of members of the genus *Deinococcus* for the presence of plasmids was performed using a modification of the Birnboim and Doly

* *Present address:* Department of Molecular Biology, University of Edinburgh, Mayfield Road, Edinburgh, EH9 3JR, UK
Offprint requests to: B. E. B. Moseley

(1979) technique. This involved harvesting bacteria from 1.5 ml cultures and resuspending them in butanol-saturated phosphate, ethylene diamine tetra-acetic acid-disodium salt (EDTA) buffer (Na_2HPO_4, 4.7 g; KH_2PO_4, 4.6 g; EDTA, 3.7 g; n-butanol, 60 ml; distilled water to 1 l). Butanol pretreatment rendered the cells sensitive to lysozyme degradation (Driedger and Grayston 1970). Large-scale preparations of plasmids were performed using a 2 l scale-up of the previous procedure. The Ish-Horowicz and Burke (1981) procedure was employed for the isolation of pAT153 (Twigg and Sherratt 1980). Recombinant plasmids were screened using the rapid boiling method (Holmes and Quigley 1981). Ethanol or propan-2-ol precipitated lysates from *Deinococcus* spp. and *E. coli* were purified for covalently-closed circular plasmid DNA by the dye-buoyant, density gradient centrifugation technique (Radloff et al. 1967).

Agarose gel electrophoresis and elution of pUE1 from gels. Plasmids and restriction endonuclease-cleaved fragments were analysed by agarose gel electrophoresis (Meyers et al. 1976). Sizes of fragments were measured according to their mobility on gels compared with a *Hin*dIII digest of λ DNA as a standard (Southern 1979). The *D. radiophilus* plasmid pUE1 was eluted from agarose gels, according to the method of McDonnell et al. (1977).

Electron microscopy. Plasmids were examined in an electron microscope (Siemens Elmiskop 101) using the protein film technique (Kleinschmidt 1968) as modified by Davis et al. (1971). The contour lengths of the molecules were measured from photographic enlargements with a Ferranti digitiser and Olivetti P6040 minicomputer. Plasmid pAT153, 3.66 kb, was used as an internal standard.

Curing strains of plasmids. Cultures from flasks containing ethidium bromide (1 $\mu g\ ml^{-1}$), sodium dodecyl sulphate (SDS) (32 $\mu g\ ml^{-1}$), rifampicin (0.2 $\mu g\ ml^{-1}$) or novobiocin (0.2 $\mu g\ ml^{-1}$), the highest concentrations of these agents that did not prevent growth of the test organisms, were diluted and spread on agar. In addition, cultures of the test organisms were grown at 38.5° C, the temperature just below the minimum that inhibited growth, for 24 h before spreading on agar. Cultures were also UV irradiated at a dose of 1.1×10^3 J m^{-2} before growing up and plating on agar. In the case of *D. radiophilus*, cells were protoplasted and allowed to regenerate their walls on nutrient broth (Oxoid no. 2) medium containing per litre: casamino acids, 5 g; $MgCl_2 \cdot 6\ H_2O$, 5.1 g; $CaCl_2 \cdot 2\ H_2O$, 3.7 g; agar, 20 g. At least 100 single colonies from each treatment were subcultured into fresh broth, grown for 18 h and screened for plasmids using the rapid screening technique.

Construction of the recombinant plasmid pUE109. pUE1 and pAT153 were digested separately with *Pst*I. The linearised pAT153 was then ethanol precipitated, resuspended in 40 µl of alkaline phosphatase buffer (1 M Tris, pH 9.5; 20 mM EDTA; 1 mM spermidine) and 1 u of calf intestine alkaline phosphatase (BCL) added. The mixture was incubated for 30 min at 37° C and then for 30 min at 56° C. After phenol extraction and ethanol precipitation, vector and target DNA were resuspended, in the ratio of 3:1 and to a final concentration of 50 $\mu g\ ml^{-1}$, in 40 µl ligase buffer (10 mM Tris HCl, pH 7.8; 10 mM Mg Cl_2, 20 mM DTT, 0.6 mM ATP). T4 DNA ligase (2.5 u ml^{-1}) was added and the mixture incubated at 12° C for 16 h. *E. coli* HB101 was transformed to tetracycline resistance with the ligated mixture, according to the method of Humphreys et al. (1979) and tetracycline-resistant, ampicillin-sensitive colonies screened for the presence of a recombinant plasmid having the predicted size of pUE1 plus pAT153.

Single and double digestion of plasmids with restriction endonucleases. Plasmid DNA was digested with various restriction endonucleases at 37° C for 2 h. When the two enzymes required different buffers, double digestions were performed in two stages with an ethanol precipitation step in between.

Results and discussion

Plasmids in members of the genus Deinococcus. Initially small-volume cultures were used for the rapid screening of *Deinococcus* spp. for the presence of plasmids. Cleared lysates, produced by a modification of the Birnboim and Doly (1979) technique, were precipitated with ethanol and examined on agarose gels. Plasmid bands were detected in tracks loaded with DNA from *D. radiodurans* Sark and *D. radiophilus* but not from *D. radiodurans* R1, *D. radiopugnans* or *D. proteolyticus*. However, when cleared lysates produced from 2 l cultures were precipitated with ethanol 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.

The purified plasmids were examined by electron microscopy and their measured sizes are shown in Table 1. *D. radiophilus* harboured three plasmid class-sizes while *D. radiodurans* Sark, *D. radiopugnans* and *D. proteolyticus* each contained two. A third plasmid class size of 74.0 kb was isolated from *D. radiodurans* Sark. However, restriction analysis of this molecule and pUE10 showed it to be a dimer of pUE10 (data not shown). There was a wide diversity of plasmid size within the group, varying from the 2.5 kb molecule of *D. radiopugnans* to the 138.8 kb molecule of *D. proteolyticus*. Only pUE2 and pUE31 from *D. radiophilus* and *D. radiopugnans* respectively shared a similar size, although it is not known whether their restriction digest patterns are the same or whether they share any other characteristics.

Table 1. Sizes of plasmids in species of *Deinococcus* as determined by electron microscopy

Bacterial species and strain	Plasmid designation	Plasmid size ± SD (kb)	No. 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
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

No plasmids were isolated from *D. radiodurans* R1 in spite of an intensive search. It is difficult to argue from a negative result such as this because it may reflect inadequate technique rather than a real absence of plasmid, but a preliminary conclusion would be that the native plasmids do not appear to play a significant role in radiation resistance of *Deinococcus* spp.

The chemical agents ethidium bromide, SDS, rifampicin and novobiocin and physical techniques of growth at elevated temperatures, UV irradiation and protoplast formation and regeneration, all failed to cure *D. radiodurans* Sark or *D. radiophilus* of any of their plasmids. It is possible, of course, that these plasmids carry genes for essential functions, the loss of which results in cell death.

Cloning and restriction mapping of pUE1. From the range of plasmids detected in the four species the smallest plasmid of the non-transformable species *D. radiophilus*, pUE1, was chosen in an attempt to develop a cloning vector for the genus. Its advantages were its relatively small size (10.8 kb) and the fact that it gave reasonably good yields of plasmid DNA which were required for DNA elution from gels and for the restriction studies. The smallest plasmid viz. pUE30 (2.5 kb), from *D. radiopugnans* gave very poor yields of plasmid DNA while the smallest plasmid of a transformable species viz. pUE10 (37 kb), from *D. radiodurans* Sark was considered to be too large to be suitable as a cloning vector.

Plasmid pUE1 was eluted from a gel and digested with several restriction endonucleases. Its size, after linearisation with *Pst*I was calculated to be 10.72 kb. It contained no cleavage sites for *Sal*I or *Dra*I, single sites for *Pst*I and *Sst*II, two sites for *Bam*HI (yielding fragments of 9.10 and 1.60 kb) and four sites for each of *Eco*RI (6.56, 2.76, 0.95 and 0.37 kb) and *Hin*dIII (6.40, 2.15, 1.45 and 0.60 kb).

pUE1 was ligated into the *Pst*I site of pAT153 and the mixture used to transform *E. coli* HB101 to tetracycline resistance. The transformation frequency was 4.1×10^3 μg^{-1} vector DNA. Two out of ten tetracycline-resistant, ampicillin-sensitive colonies screened contained a recombinant plasmid with the predicted size of pUE1 plus pAT153. The two recombinant plasmids had identical restriction patterns and so only one of them, pUE109, was used for further study. The amplification of pUE1 within pAT153 in *E. coli* HB101 made the task of constructing a restriction map of pUE1 much easier since its elution from gels was time-consuming and yielded only small amounts of plasmid DNA. The recombinant plasmid pUE109 was subjected to single and double digestion with various restriction endonucleases. Digestion with *Pst*I produced two fragments, one the size of pAT153, the other the size of plasmid pUE1. There were single cleavage sites for *Mlu*I, *Nru*I, *Sal*I and *Sst*II, three sites for *Bam*HI (yielding fragments of 8.25, 4.51 and 1.60 kb), four for *Dra*I (7.14, 4.26, 2.97 and 0.02 kb) and five for each of *Eco*RI (5.41, 4.81, 2.76, 0.95 and 0.37 kb) and *Hin*dIII (6.83, 3.23, 2.15, 1.45 and 0.60 kb). *Dra*I has three cleavage sites in pAT153 and one in pUE1 but did not cleave pUE1 after elution from gels because *Dra*I is a restriction endonuclease from the same strain as pUE1, viz. *D. radiophilus* (Purvis and Moseley 1983). Thus the sensitive site for this enzyme would be modified in *D. radiophilus* but not in *E. coli* HB101. The restriction analysis of pUE109 indicated that no rearrangement of pUE1 had occurred during its construction. After single and double digestions, the cleavage sites were mapped and are shown in Fig. 1.

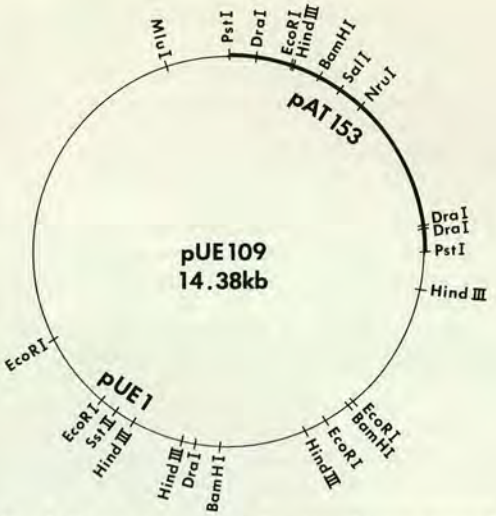

Fig. 1. Restriction map of the recombinant plasmid pUE109

Five clones of *E. coli* HB101 into which pUE109 had been introduced were tested for increased resistance to UV radiation by streaking each of them and the original strain on agar plates and applying increasing doses of UV radiation along the streaks. There was no increase in resistance of the plasmid-carrying strains. Attempts to transform *Deinococcus* strains to tetracycline resistance with pUE109 were unsuccessful. This was not unexpected for *D. radiopugnans*, *D. radiophilus* or *D. proteolyticus* because none of these strains has been successfully transformed using homologous chromosomal DNA and they may, therefore, be unable to take up any form of DNA. However, *D. radiodurans* can take up both linear (Moseley and Setlow 1968) and covalently-closed circular DNA (unpublished results). We assume therefore that the PUE109 plasmid, grown in *E. coli* HB101 is restricted on entry to *D. radiodurans* by the *Sst*II isoschizomer *Mra*I (Wani et al. 1982), although it is also possible that there is a failure of transcription or that the *Pst*I site of pUE1 is in its replication origin.

The amplification of plasmid pUE1 in *E. coli* and the construction of its restriction map is the first step in developing a cloning vehicle for *Deinococcus* spp. The main advantage of using this plasmid as a cloning vehicle is the presence of four unique restriction sites viz. *Pst*I, *Mlu*I, *Sst*II and *Dra*I. This will enable a selectable genetic marker to be introduced. The main disadvantage is that it is larger than most cloning vectors but there is no reason why it should not be reduced in size. The recombinant plasmid pUE109 may be useful as a shuttle vector between *E. coli* and *Deinococcus* spp. particularly when a genetic marker from *Deinococcus* spp. is introduced. In addition a potential chromosome-mobilising element might be developed from pUE109.

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