Studies on the Genetics of *Micrococcus radiodurans*

Simin Tirgari

A thesis presented for the degree of Doctor of Philosophy

University of Edinburgh

1977

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

CONTENTS

ACKNOWLEDGEMENTS

I would like to thank Dr. B. E. B. Moseley, my supervisor, for all his help and encouragement throughout this project. I am also grateful for the help of the following:-

Mr. Philip R. Tempest and Miss Deirdre A. Cunningham for their valuable reading and criticism of this manuscript; Dr. I. W. Dawes for his useful suggestions; Miss Lindsay Riddick for typing this thesis; Mr. G. Finnie for preparing the figures and photographs; and Professor J. F. Wilkinson for permitting this work to be done in his department.

SUMMARY

An attempt has been made to develop the genetics of Micrococcus radiodurans by analysing transformation and conjugation processes. The nature of the transformation process in M. radiodurans was studied by investigating the effect of various factors on the efficiency of transformation. M. radiodurans can be transformed by exogenous DNA at a frequency of 10^{-6} without special treatment. The presence of glucose or amino acids during growth or transformation had no significant effect on the frequency. Treatment of recipient cells with calcium chloride at the optimum concentration of 30 mM increased the frequency of transformation up to 10,000-fold for some markers, e.g. rifampicin^{r} resistance. Magnesium and strontium ions could not replace calcium while zinc ions completely inhibited transformation. The efficiency of transformation was also affected by the pH, pH 7.0 being the most effective with $CaCl_2$-treated cells. The minimum time required for phenotypic expression varied with different markers, e.g. for acriflavin^{r} resistance and streptomycin^{r} resistance it was 35 and 170 minutes respectively. To obtain the maximum number of transformants further incubation was necessary before the addition of antibiotics. This time also varied for different markers.

The high frequency of transformation obtained using the new protocol enabled studies on genetic linkage to be started. Four linked pairs of markers were identified, kanI-glyA, kan2-ts4, tyrA-rnaA and trpB-stsI.

In order to study conjugation in M. radiodurans, crosses were performed in an attempt to transfer plasmids with known sex factor activity such as RP4 and pJH2 from Escherichia coli and Streptococcus faecalis respectively to M. radiodurans. Although

these R factors transfer between other gram-negative and gram-positive bacteria respectively, crosses between *E. coli* or *S. faecalis* and *M. radiodurans* were unsuccessful in demonstrating plasmid transfer. Because *M. radiodurans* is able to undergo transformation at a high frequency, attempts were made to transfer pure plasmid DNA of RP4 and pJH4 into *M. radiodurans*. No transformants were obtained which had the phenotype conferred in their normal host by either plasmid DNA when transformed to *M. radiodurans* although some evidence was obtained that RP4 DNA entered *M. radiodurans*.

INTRODUCTION

PART I

Development of Competence

Micrococcus radiodurans is a red-pigmented bacterium which has a high level of resistance to ultraviolet (U.V.) and ionizing radiations (Anderson et al., 1956; Duggan et al., 1959). Prior to 1956, vegetative bacteria were thought to be relatively sensitive to radiation and spores to be relatively resistant. In 1956, during studies on the effect of gamma (γ) radiation on the sterilization of meat, spoilage occurred in irradiated meat and M. radiodurans was isolated in pure culture (Anderson et al., 1956). M. radiodurans was subsequently shown to be approximately 0.05 to 0.1% of the total microbial population of the unirradiated meat. A culture of this non-spore forming micrococcus was shown to survive a γ-ray dose of 4×10^6 roentgen equivalent physical (rep) in ground meat and tryptone, glucose, yeast extract (TGY) broth and up to 6×10^6 rep on TGY agar. M. radiodurans represents a single species closely related to Micrococcus roseus ATCC 19172 (Davis et al., 1963), Micrococcus rubens tetragenus ATCC 186, Micrococcus radiophilus (Lewis, 1971) and Micrococcus radioproteolyticus (Kobatake et al., 1973) having similar morphology, cell wall structure and radio resistance.

M. radiodurans is relatively large with a spherical shape of diameter 1.5 - 3 μm. It occurs in broth as tetrads, pairs or occasionally as single cells. Besides its unusual resistance to radiation, it has an unusual cell wall structure. The wall contains relatively large amounts of lipid and lipoprotein similar to that found in gram-negative bacteria. The cell wall mucopeptide contains ornithine, lysine, meso-diaminopimelic acid and an unidentified amino acid (Work, 1964). This cell wall composition causes variable gram stains in the bacteria.

In the early stages of growth, it stains gram-positive and in other stages is gram-variable.

M. radiodurans grows well in TGY broth and on agar at 30°C colonies visible to the naked eye appear after 48 hours incubation. At 37°C in TGY broth, the bacteria aggregate to form large clumps and cannot be used for quantitative studies. No growth occurs at 45°C. Vigorous aeration on a rotary shaker accelerates the growth of the bacteria. Sodium chloride at a final concentration of 2% inhibits the growth of cultures in TGY broth (Anderson et al., 1956).

The extremely high radiation resistance of M. radiodurans to the lethal action of ionizing and ultraviolet radiation (Anderson et al., 1956; Duggan et al., 1959) has been attributed to pigmentation (Kilburn et al., 1958), to the presence of mercapto alkylamine (Anderson et al., 1956) and to multicellularity (Stapleton, 1960). However, because of its high level of resistance to the lethal action of ionizing and ultraviolet radiation and also to chemical agents which damage DNA, such as N-methyl-N'-nitro-N-nitrosoguanidine (Moseley, 1967), nitrous acid and hydroxylamine (Sweet and Moseley, 1976), it is believed that this micro-organism has very efficient excision repair mechanisms (Boling and Setlow, 1966) and a post-replication recombination repair mechanism (Moseley and Mattingley, 1971).

The discovery of genetic transformation in M. radiodurans (Moseley and Setlow, 1968) was the beginning of an attempt to develop an experimental genetic system. Although the frequency of transformation was not as high as in other transformation systems, e.g. Diplococcus pneumoniae, the process of transformation provided a useful tool for studying repair

mechanisms. However, transformation is not the best method for genetic mapping since only small regions of the chromosome are transferred and to develop a genetic map, there is a need to transfer larger portions of the genome to allow mapping over larger areas. The most suitable method for transferring large parts of the genome is conjugation.

Transformation

The pathogenic effects of capsulate Diplococcus pneumoniae on mice led Griffith to the discovery of transformation and to the idea of a transforming principle which he thought might be a protein (Griffith, 1928). Avery, Macleod and MacCarty (1944) identified the chemical nature of transforming principle and concluded that DNA is a carrier of hereditary information.

In 1957, Hotchkiss showed that DNA extracted from a penicillin-resistant strain of D. pneumoniae could be transformed into the penicillin-sensitive strain of this bacterium. The discovery that drug-sensitive bacteria could be transformed to drug-resistance by exogenous DNA meant that the efficiency of transformation could be measured.

Most information on transformation has come from studies on the transformation of antibiotic-sensitive bacteria to antibiotic-resistance in bacteria such as Diplococcus pneumoniae, Haemophilus influenzae and Bacillus subtilis (Alexander and Leidy, 1951; Spizizen, 1958). Since Escherichia coli is the most thoroughly studied micro-organism, the development of a transformation system in this bacterium is of great interest (Cosloy and Oishi, 1973).

A transformation system is useful not only as an aid to genetic analysis in bacteria, but also as a test for

characterizing the mechanism of recombination and integration (Notani and Setlow, 1974). Transformation is also useful for investigating the action of UV light and other DNA damaging agents so that the development of a transformation system in M. radiodurans by Moseley and Setlow (1968) was useful in the study of extreme resistance to radiation damage.

The Development of Competence

Before a bacterial cell is able to take up transforming DNA and incorporate it into its genome, the cell must be in a certain physiological state known as competence. Competence has been defined as the capacity of the cell to undergo transformation (Tomasz, 1966).

The occurrence of this physiological state in some species depends on the presence of a competence factor (CF) which enables non-competent bacteria to take up DNA and become transformed (Tomasz, 1966). This factor loses activity on treatment with proteases. In D. pneumoniae, the CF has a molecular weight of about 10,000 (Tomasz and Mosser, 1966) and in a group H strain of Streptococcus the molecular weight is about 5,000 (Leonard and Cole, 1972; Ranhand et al., 1971). The CF may be free in the medium or bound to the cell. It is possible to prepare CF from D. pneumoniae or S. sanguis (Kohoutová et al., 1968; Ranhand et al., 1971). These authors showed that highly purified preparation of the CF contains a high proportion of basic amino acids and could be obtained from culture supernatant fluids. The addition of competence factor to related species resulted in increased frequencies of transformation. A mutant of D. pneumoniae has been isolated which does not require CF for competence since it has an ability to become competent in the

presence of trypsin (Lacks and Greenberg, 1973).

Competence can be induced in different ways for different bacteria. For instance, in H. influenzae and B. subtilis, competence involves subjecting cultures to conditions that might be expected to cause drastic changes in cell physiology, such as near-stationary growth, deprivation of oxygen or a change from a rich to a minimal medium (Goodgal and Herriot, 1961; Spencer and Herriot, 1965; Anagnostopoulos and Spizizen, 1961). However, these situations do not induce competence in all bacteria. In some, such as M. radiodurans and Neisseria species, competence is not associated with any particular stage of growth (Moseley and Setlow, 1968; Catlin, 1960). However, with most transformable bacteria, the duration of competence is limited to a small part of the growth cycle, e.g. B. subtilis becomes competent at an early stage during spore formation (Young and Spizizen, 1961) and H. influenzae at the end of exponential phase growth (Alexander and Leidy, 1953). In H. influenzae, however, when the competent culture was centrifuged, washed and resuspended in saline, the bacteria lost 80 to 90% of their capacity to undergo transformation (Goodgal and Herriot, 1961). This observation shows that there might be a factor in, or on the cell which was important for transformation and which is removed by washing. Goodgal and Herriot (1961) also concluded that transformation is not restricted to a genetically distinct portion of the transforming population and that competence is not due to genetic factors. This does not mean that competence is not genetically controlled, but places a restriction on the probable frequency of cells with different potential competence in a given culture.

Environmental Factors

Competence is also affected by factors other than competence factors and the growth stage, e.g. pH, media, salt concentrations and temperature. Some compounds which are capable of stimulating competence in some bacteria are inhibitors in others. For instance, valine is an inhibitor of transformation in H. influenzae, while acting as a stimulator in B. subtilis and D. pneumoniae (Spencer and Herriot, 1965; Wilson and Bott, 1968; Tomasz and Mosser, 1966). Thus the development of a competent stage is different from system to system.

Role of the Cell Wall in the Development of Competence

In a competent cell DNA penetrates through the cell wall to bind to the membrane, both reactions requiring an energy source (Strauss, 1970). However, removal of the cell wall by the action of lysozyme after exposure to radioactive DNA indicated that each DNA molecule binds to the cell surface (Miller et al., 1967 reported in Notani and Setlow, 1974). In competent B. subtilis, cells which were subjected to complete removal of the cell wall before exposure to transforming DNA, gave no transformants, whilst part removal of the wall prior to exposure to DNA stimulated the uptake of transforming DNA (Wilson and Bott, 1970).

Also an important factor in the development of competence is the composition of the cell wall. For example, in B. subtilis competence has also been correlated with an increased level of galactosamine in the cell wall, while poorly transformable strains of this bacterium have an excess amount of glucose in the cell wall (Young, 1965).

Effect of Glucose

An effect of glucose has been observed in other

transformation systems. In Azotobacter vinelandii, although glucose shift-down was not effective in inducing competence, shifting glucose-grown wild type cells to a medium containing 0.2% β-hydroxybutyrate induced competence (Page and Sadoff, 1976). These authors also observed that the addition of 0.1 mM cyclic adenosine 3',5'-monophosphate (c-AMP) to a non-competent culture in early exponential phase growth resulted in a slower growth rate and the immediate induction of competence with a transformation frequency at least 1,000-fold greater than before the addition of c-AMP.

Effect of Divalent Cations

The requirement for divalent cations for the uptake of DNA in several transformation and transfection systems in bacteria such as D. pneumoniae and E. coli have been investigated (Fox and Hotchkiss, 1957; Oishi and Cosloy, 1972; Cosloy and Oishi, 1973; Cohen et al., 1972). Although the presence of divalent cations, such as Mg^{2+} and Ca^{2+} increase the irreversible uptake of DNA, genetic transformation can also be inhibited by such cations. In competent D. pneumoniae conversion of the cell-associated DNA to a nuclease-resistant form requires calcium ions (Seto and Tomasz, 1976). Transformation of competent D. pneumoniae is inhibited by a divalent cation chelating agent such as ethylenediaminetetra acetate (EDTA), this inhibition being overcome by the addition of calcium, but not magnesium ions (Seto and Tomasz, 1974; 1976). Although normal culture growth does not seem to require Ca^{2+}, the presence of Ca^{2+} is necessary for transformation and transfection in many bacteria. The concentration of Ca^{2+} is important. The optimal concentration of Ca^{2+} required for transformation and transfection of E. coli

K12 is 30 mM (Oishi and Cosloy, 1972; Cosloy and Oishi, 1973), while in D. pneumoniae and A. vinelandii maximum concentrations of 5 mM and 0.29 mM respectively are required (Seto and Tomasz, 1976; Page and Sadoff, 1976). However, in all these systems when the concentration of Ca^{2+} ions was increased to a higher level, some inhibition was observed. Increasing the concentration of Ca^{2+} to 1 M in E. coli, and in D. pneumoniae and A. vinelandii increasing the concentrations of Ca^{2+} to 10 and 2.32 mM respectively, reduced the transformation frequency. Although the presence of Mg^{2+} ions is required for growth and for activation to competence of D. pneumoniae and Ca^{2+} ions cannot replace this requirement, Mg^{2+} ions have no effect in stimulating transformation in this bacterium (Seto and Tomasz, 1976). On the other hand, A. vinelandii requires Mg^{2+} ions, both for growth and for stimulating competence (Page and Sadoff, 1976).

Effect of pH on Competence

The effect of pH on the development of competence has been investigated in many bacteria, e.g. B. subtilis, H. influenzae, S. sanguis and Neisseria gonorrhoeae (Young and Spizizen, 1963; Barnhart and Herriot, 1963; Ranhand, 1976; Biswas et al., 1977). The pH optima for transformation and for DNA binding varies for different bacteria. In D. pneumoniae cells do not develop competence below pH 7.0, the competence factor not being synthesised at pH 6.8 (Tomasz, 1966). S. sanguis can develop competence at pH 7.0, but not at pH 6.5, although once competence has been achieved maximum transformation occurs at pH 6.5 (Ranhand, 1976). In S. sanguis two pH optima are observed for transformation which are dependent on the buffer. In phosphate the optimum is pH 7.5, whereas in Tris the pH optimum is 7.0

(Schlissel and Sword, 1966; Ranhand, 1976). In N. gonorrhoeae in Tris hydrochloride, the pH optimum for transformation is 7.5. Transformation frequency falls sharply at pH values below 7.0 and above 8.0 (Biswas et al., 1977).

Mechanism of Nucleic Acid Uptake by Recipient Cells

The efficiency of uptake of transforming DNA in a population of bacteria depends on the size of the DNA as well as the number of competent bacteria. Non-competent cells compete with competent cells in binding transforming DNA to the extent that this can cause some double-strand breaks to occur in the DNA, as observed in B. subtilis (Cahn and Fox, 1968; Haseltine and Fox, 1971). It seems likely that DNA suffers double-strand nucleolytic cleavage and loses biological activity. When competent bacteria are exposed to DNA, the latter is sensitive to the action of DNase. Transforming DNA can be taken up by the recipient bacteria and become resistant to DNase (Gabor and Hotchkiss, 1966).

Although in D. pneumoniae competent bacteria can take up foreign DNA such as calf thymus DNA as well as homologous DNA (Hotchkiss, 1954), other bacteria such as B. subtilis take up E. coli DNA with less efficiency than B. subtilis DNA (Bodmer and Ganesan, 1964). This observation indicates that there may be discrimination against foreign DNA at the cell surface of some bacterial species.

The size of the transforming fragments is predetermined by the degree to which the chromosomal DNA is broken down during extraction from the donor bacteria and subsequent purification. There is a lower limit to the size of double stranded homologous DNA required for transformation (Cato and Guild, 1968). In

D. pneumoniae transformation is a linear function of intracellular donor DNA length, extrapolating to zero at about 7.7×10^4 daltons (Morrison and Guild, 1972). Single-stranded DNA has an efficiency of less than 1% of native DNA in transforming D. pneumoniae (Miao and Guild, 1970).

Shortly after uptake the double-stranded donor DNA is denatured or degraded to a single-stranded form which in D. pneumoniae is believed to be an intermediate (Lacks, 1962; Fox and Allen, 1964). In H. influenzae, a single-stranded donor DNA intermediate has not been detected (Notani and Goodgal, 1966), although after uptake of transforming DNA, 10 to 15% of the donor DNA is sensitive to S1 endonuclease, which degrades only single-stranded DNA (Leclerc and Setlow, 1974). In B. subtilis, a four-stranded DNA is postulated to be an intermediate (Kohnlien and Hutchison, 1976). The incorporation of transforming DNA into the recipient chromosome requires a recombination event so that genetic transformation offers certain advantages over other methods of genetic exchange in that donor DNA can be re-extracted from recipients and examined both with respect to its physico-chemical aspects and its biological activity (Fox and Hotchkiss, 1960; Venema et al., 1965).

Transformation as a Function of DNA Concentration Saturating Level

There is a linear relationship between the number of bacteria transformed for a single marker and the amount of DNA irreversibly bound by the cell, indicating that a single DNA molecule can produce a transformed cell (Lerman and Tolmach, 1957). Populations of recipient D. pneumoniae and H. influenzae were exposed to different concentrations of DNA between 1 and

1,000 μg/ml. For concentrations up to 100 μg/ml the number of transformants were proportional to the DNA concentration. At a concentration of 100 μg/ml, it was estimated that there were about 10 molecules of DNA per bacterium. Above this concentration, the number of transformants was constant and the system was said to be saturated (Goodgal and Herriot, 1957; Lerman and Tomach, 1957; Schaeffer, 1958).

Phenotypic Expression

Phenotypic expression of most transformational events is due to resultant changes in enzyme activities. In D. pneumoniae, the time of expression of the gene responsible for the production of amylomaltose is the same time as that required for incorporation of the gene (Lacks and Hotchkiss, 1960). The time for antibiotic resistance markers to be expressed is longer, e.g. 90 minutes for erythromycin resistance and 120 minutes for streptomycin resistance (Butler and Smiley, 1970). One reason for the difference is the dominant and recessive nature of the genes involved. Thus genes conferring prototrophy to an auxotrophic recipient are dominant (Lacks and Hotchkiss, 1960) and resistance to antibiotics is generally recessive to sensitivity (Lederberg, 1949; Hayes, 1970; Austin et al., 1971).

Aim of Project: To Develop Competence

The aim of this study was to analyse the transformation process in M. radiodurans. Specifically, attempts were made to increase the frequency of transformation. Many mutants have been isolated in M. radiodurans with defective DNA repair (Moseley, 1967; Moseley and Copland, 1975), but lack of genetic analysis has restricted a further understanding of them. A

high frequency of transformation would enable further studies to be made of this problem in future. Since transformation is a product of information transfer of donor DNA through complicated cellular processes which start at the uptake of DNA and end at the completion of recombination, it was reasonable to investigate factors which could affect any of these processes and hence affect the efficiency of transformation.

PART II

The Establishment of Linkage by Transformation

Discovery of Linkage Groups

Morgan's investigation led him to the physical basis of the chromosome as a linkage group (from Strickberger, 1972). He found that the number of linkage groups in Drosophila corresponded to the number of chromosomes in the somatic cells of this organism.

Although in the first half of the 20th century, genetic studies were mainly concerned with higher organisms, such as Drosophila, and Zea mays, genetic linkage by transformation started with the study of Hotchkiss and Marmur (1954) in bacteria. They investigated by transformation a number of mutant characters of D. pneumoniae and discovered that genes responsible for streptomycin resistance and mannitol fermentation were strongly linked. This was shown by extracting DNA from a streptomycin-resistant, mannitol-fermenting donor ($\underline{str}^r$ $\underline{mtl}^+$) D. pneumoniae and transforming a strain which was streptomycin-sensitive and unable to ferment mannitol ($\underline{str}^s$ and $\underline{mtl}^-$). Most transformants received both donor characters, i.e. str^r and mtl^+, indicating that the genes were usually on the same molecule of DNA.

When they transformed the same recipient with DNA carrying the two markers ($\underline{str}^s$ and $\underline{mtl}^+$), but extracted from two donors each carrying only one of the genes, so that each marker had to be on separate DNA molecules, there was no evidence of linkage. Thus the transfer of genetic markers located on a molecule of DNA provides a mechanism by which closely linked genetic loci may be ordered in a segmental map. Consequently, when a pair of genes shows an appreciable degree of joint transformation, it points directly to their genetic contiguity.

Use of Transformation in Constructing a Chromosome Map

In D. pneumoniae following the first reports of linkage (Hotchkiss and Marmur, 1954; Ravin, 1966, reported in Butler and Nicholas, 1973), the construction of a chromosome map by searching for linkage groups involving antibiotic markers has been carried out (Butler and Nicholas, 1973; Butler and Smiley, 1973). Thus the genes responsible for erythromycin and tetracycline resistance are co-transformable and the gene responsible for streptomycin resistance is situated between the two. Since streptomycin resistance is known to be closely linked to mannitol fermentation, the four markers are in a linkage group. In this way, a map can be built up. Such procedures have established linkage in many bacterial species, e.g. Bacillus subtilis and Staphylococcus aureus (Anagnostopoulos and Crawford, 1961; Pattee, 1975; 1976).

Assessment of Linkage

Three criteria can be used for linkage:-

1. The Appearance of the DNA Dilution Curve

There is a linear relationship between the number of bacteria transformed for a single marker and the amount of DNA irreversibly bound by the cell (Lerman and Tolmach, 1957; Goodgal and Herriot, 1957). Thus each transformant is the result of an interaction between a single molecule of DNA with a recipient bacterium (Hotchkiss, 1957 reported in Hayes, 1970; Goodgal and Herriot, 1957). Therefore, if the frequency of single transformants is 1% for each marker, then both markers, if unlinked, would be expected to be transformed into the same recipient bacterium at a frequency of 0.01% (1% x 1%) assuming all the population to be capable of taking up DNA. When the DNA concentration is reduced, the frequency of double transformants for unlinked markers falls

more rapidly than those due to linked markers and the shapes of the dilution curves are quite different for the two situations. In the case of very closely linked markers, the frequency of both single and double transformants is in direct proportion to the reduction in the DNA concentration. When there is linkage between genes in bacterial transformation, it means that the two genes concerned are frequently situated on the same piece of transforming DNA. Conversely, unlinked genes are those which occur only on different molecules of DNA and double transformants obtained from such DNA preparations must be derived by the integration of two separate molecules of DNA resulting from independent events. The avoidance of shearing of the DNA in its preparation is important for the investigation of linkage (Kelly and Prichard, 1965), since if care is taken to prevent excessive shearing, markers which appear unlinked in normal preparation can be shown to be linked.

2. Frequency of Double Transformants Among Clones Selected for Only One of the Markers (Clonal Analysis)

A useful assessment for the degree of linkage is the number of clones (colonies) selected for only one of the markers which also carry the second, unselected marker. The assessment of linkage is independent of the number of competent bacteria. It has been used in both cultures of D. pneumoniae which were highly competent and B. subtilis which were not (Fox and Hotchkiss, 1957; Nester and Stocker, 1963; Butler and Nicholas, 1973; Cahn and Fox, 1968).

3. The Values of Co-Transfer and Linkage Indices

The linkage relationship can be expressed numerically as a co-transfer index (Nester and Lederberg, 1961) or as a linkage

index (Butler and Nicholas, 1973). The co-transfer index compares the frequency of double transformants with the total number of recombinant genotypes measured by the transformation studies. It is defined as $\frac{AB}{Ab + aB + AB}$ (Formula 1). The experimental value obtained for genotype A consists of both single (Ab) and double (AB) transformants.

Thus, $A = Ab + AB$ - Equation 1.

Similarly, $B = aB + AB$ - Equation 2.

Substituting Equations 1 and 2 into Formula 1 gives:-

Co-transfer index = $\frac{AB}{A + B - AB}$.

In this method, very close linkage gives an index almost equal to 1 whereas unlinked markers give values approaching zero. Thus, as the numerical value decreases, so does the closeness of the markers being considered.

The linkage index is defined as $\frac{A \times B}{AB}$. Using this method, values approaching zero are indicative of very close linkage and a value of 1 indicates no linkage. Although this formula is used in studies on D. pneumoniae where the fraction of the bacteria which are competent is taken into account (Butler and Nicholas, 1973), it can also be used for other bacteria, e.g. M. radiodurans, where the percentage of competence is not known.

N-methyl-N'-nitro-N-nitrosoguanidine (NTG) as a Mutagenic Agent for the Investigation of Linkage

NTG is a powerful mutagen under conditions of high survival in E. coli (Mandel and Greenberg, 1960; Cerdá-Olmedo and Hanawalt, 1968) and in other biological systems. Treatment with NTG provides an approach to the study of the chromosome map and to the pattern of replication (Cerdá-Olmedo et al., 1968). Comparison of mutagenesis in exponentially growing cells and in cells with

completed rounds of DNA synthesis (resting phase) gave a five-fold difference in the numbers of mutants obtained. This suggested that 80% of the mutants are induced in the vicinity of the replication fork (Cerdá-Olmedo et al., 1968) and that many of such mutations are linked (Guerola and Ingraham, 1971; Metzer and Schurman, 1974).

M. radiodurans, which is generally very resistant to mutation by other mutagens can be mutated by NTG (Sweet and Moseley, 1976). NTG would seem useful, therefore, for causing multiple mutations at the replication fork so that any double mutations isolated would have a high probability of being mutant in closely linked genes.

Aim of Project: To Determine Linkage

The reason for carrying out this study was to isolate double mutants induced by the action of NTG and to determine, by transformation, whether the mutations were linked or not. The identification of such linked genes would be the beginning of a long study in which linkage groups would be identified and finally a map of the chromosome established.

PART III

Transfer of Plasmids

Plasmids

A plasmid is defined as a covalently-closed circular element of DNA which is physically separated from the chromosome of the cell and is able to be perpetuated stably in this condition (Silver and Ozeki, 1962; Watanabe, 1963; Clowes, 1963; 1972). This definition therefore includes all plasmid DNA such as the sex factor F, R factors, Col factors and P1 prophage but would exclude lambda prophage or the DNA of abortive transductants (Burton and Sinsheimer, 1965; Fiers and Sinsheimer, 1962; Clowes, 1972).

During the past few years, investigation of an increasing number of bacterial properties suggested that they were controlled by plasmids and not by the chromosome. An important trait determined by certain bacterial plasmids, e.g. the F factor, some Col factors and some resistance factors, is the ability to promote gene transfer. The genetic material transferred can include, as well as the plasmid, other plasmid or chromosomal DNA (Clowes, 1972).

Resistance Transfer Factors (RTFs)

Resistance transfer factors are defined as a group of plasmids that carry genetic information for resistance to antibiotics and/or other antibacterial drugs. They were first isolated in a large number of strains of Shigella in Japan which showed multiple resistance to streptomycin, chloramphenicol, tetracycline and sulphonamide (Watanabe, 1963; 1964). The multiple resistance was transferable to sensitive bacteria, not only among Shigella strains, but also among Enterobacteriaceae as a whole and also to species such as Vibrio cholerae and Pseudomonas, by cell to cell contact (Watanabe, 1963; Meynell,

Meynell and Datta, 1968; Cohen and Miller, 1970; Haapala and Falkow, 1971). The transferable plasmids are divided into two general groups, the F, or sex factor-like plasmids and the I or ColI bp9 type. The drug resistance factors R1 to R6 and some Col factors such as ColV and ColI k94 are all F-type plasmids. They can produce on the surface of their hosts pili that agglutinate with F pilus-specific antiserum and they adsorb F-specific phage (Meynell et al., 1968; Novick, 1969; Watanabe, 1971; Brinton, 1971; Curtiss, 1969).

Class P R-Factors

Among a group of R factors studied in E. coli, a minority of the fi^- plasmids, including RP4, do not appear to produce either F or I pili (Lawn et al., 1967). This group of R factors derived from Pseudomonas aeruginosa S8, which is highly resistant to carbenicillin, has been studied in E. coli (Lowbury et al., 1969; Black and Girdwood, 1969; Holloway and Richmond, 1973; Grinsted et al., 1972). RP4 and RP1, which are thought to be identical, can be transferred from P. aeruginosa to other gram-negative bacteria such as E. coli, Salmonella typhimurium, Klebsiella aerogenes, Rhizobium leguminosarum and Agrobacterium tumefaciens (Olsen and Gonzaler, 1974; Hedges et al., 1975; Olsen and Wright, 1976; Chakrabarty, 1976). RP4 is also transmissible by conjugation from E. coli to Chromobacterium violaceum D254, Rhizobium trifolii 24G and Rhizobium meliloti at an efficiency of 10^{-6} and to P. aeruginosa PAO2 with a frequency of 2.3×10^{-3} (Datta et al., 1971). Although it can be transferred with 100% efficiency among E. coli K12 strains, transfer of RP4 to Agrobacterium tumefaciens B6 occurred with very low efficiency (10^{-8} to 10^{-9}), while no other R-factors could be

introduced into this organism (Datta et al., 1971).

Existence of Plasmids in Gram-Positive Bacteria

The presence of plasmids has also been studied in gram-positive bacteria, such as Staphylococcus spp., Streptococcus spp., Bacillus megaterium and Micrococcus luteus (Novick, 1969; Ehrlich, 1977; Lee and Davidson, 1970). Although the presence of R factors among Streptococcus strains has been demonstrated (Toala et al., 1969; Dunney et al., 1973; Clewell et al., 1974; Cords et al., 1974), recent investigations of S. faecalis have shown the presence of R factors capable of promoting their own transfer (Jacob and Hobbs, 1974; Dunney and Clewell, 1975). For example, in S. faecalis var. zymogenes (strain JH1), the transferable plasmid of molecular weight 50×10^6 determining high levels of resistance to kanamycin, neomycin, erythromycin and tetracycline, can be transferred to other strains of S. faecalis (Jacob and Hobbs, 1974).

The molecular weight of the S. faecalis chromosome is estimated to be 1.47×10^9 (Back et al., 1970) and the molecular weight of the plasmids pJH1 and pJH4 to be 50×10^6 and 38×10^6 respectively (Jacob and Hobbs, 1974). Assuming that the specific radioactivity of closed circular and chromosomal DNA is the same, the amount of plasmid DNA isolated expressed as a percentage of isolated chromosome, gave values ranging between 2.6 and 6.6% corresponding to between 0.43 and 1.1 copies per chromosome (Jacob and Hobbs, 1974). This value is approximately the same as for RP4 (Holloway and Richmond, 1973; Grinsted et al., 1972; Saunders and Grinsted, 1972).

R-Factor-Mediated Resistance to UV

Plasmids such as some Col factors, several enteric R-

factors and also the sex factor F may confer increased resistance to the lethal effects of UV irradiation on bacteria such as E. coli, Salmonella typhimurium and Pseudomonas aeruginosa (Howarth, 1965; 1966; Drabble and Stocker, 1968; Marsh and Smith, 1969; Krishnapillai, 1975; Macphee, 1973). Some R-factors such as pPL1 and pMG2 in P. aeruginosa confer protection against the lethal effects of UV- and γ-irradiation, methyl methanesulphonate and N-methyl-N'-nitro-N-nitrosoguanidine treatment while increasing the susceptibility of the host to the mutagenic effects of these agents (Lehrbach et al., 1977).

Incompatibility of Plasmids

Incompatibility involving the sex factor F was first reported by Scaife and Gross (1962), who introduced F'-lac into an Hfr strain of E. coli K12 and observed that F'-lac was unstable and could not establish itself in the progeny and therefore was lost. R plasmids belonging to the same incompatibility group, designated group H in E. coli K12, displace the F factor from E. coli K12 F^+ (Smith et al., 1973). Incompatibility is explained as a property exhibited by isogenic plasmids for those functions, such as the maintenance genes which are essential for autonomy (Jacob et al., 1963; Novick, 1969; Datta and Hedges, 1971). In this case, incompatibility among isogenic plasmids can be due to a limitation of a single plasmid membrane site leading to competition for this site, so that only one of two isogenic plasmids can attach and be stably inherited. For example, in S. faecalis, plasmid PJH1 cannot stably exist in the same cell as the plasmid of 6.1×10^6 molecular weight (Jacob and Hobbs, 1974). Thus these two plasmids belong to the same incompatibility group.

Incompatibility is also observed among plasmids which are not isogenic such as ColV2, K94 and F in E. coli K12 (Macfarren and Clowes, 1967). So the loss of plasmid DNA when transferred to another strain can be the result of the presence of incompatible plasmids within the same host.

Transforming Plasmid DNA

The availability of a system for the genetic transformation of host bacteria by purified R-factor DNA enables a study to be made of important aspects of R-factor biology. The transformation of plasmid DNA is observed in both gram-negative and gram-positive bacteria (Cohen et al., 1972; LeBlanc and Hassell, 1976; Ehrlich, 1977). It does not require the integration of the transforming DNA into the chromosome since the plasmid DNA replicates autonomously.

In E. coli K12, although attempts to transform the cell with chromosomal DNA were unsuccessful, cells were capable of taking up phage and plasmid DNA (Mandel and Higa, 1970; Cohen et al., 1972). The capability of E. coli cells to take up phage or plasmid DNA is due to the covalent circularity of these DNAs which is required for its biological activity in the host. Closed circular and open circular forms of plasmid DNA have equal transforming ability, but sonication of the R-factor DNA to smaller fragments or denaturation of the DNA destroys its ability to transform E. coli (Cohen et al., 1972). Although most studies have been restricted to those plasmids that express easily-selected or identifiable properties, such as antibiotic resistance, colicin immunity or conjugal fertility, transformation using plasmid DNA is necessary for analysing non-self-transmissible plasmids. For example, the R-factor 219, a non-

transmissible plasmid carrying tetracycline (Tc) resistance in E. coli (Embden and Cohen, 1973), was transformed into E. coli minicells, which enables a study to be made of the mechanism of plasmid-borne tetracycline resistance (Embden and Cohen, 1973). Also, the non-transmissible tetracycline and chloramphenicol resistance plasmids pT127 and pC194 from S. aureus can be transformed into B. subtilis (Ehrlich, 1977). In contrast, when a plasmid from S. faecalis was transformed into S. sanguis, it was observed that some of the plasmid was rapidly converted to a linear form in the new host cell (LeBlanc and Hassell, 1976). Although the reason for the instability of some of the circular DNA during transformation is not known, it seems possible that this is due to specific nuclease activity. However, transformation is a simple procedure that permits selection of any plasmid replicon capable of being transformed into relevant bacteria.

Restriction and Modification Mechanism

Identification of the enzymes involved in the restriction and modification mechanism came from the experimental work of Arber and Dussoix (1962). A general concept is that two enzymes, an endonuclease and a DNA methylase, are responsible for the restriction and modification of DNA (Fujimoto et al., 1965; Gold and Hurwitz, 1963; Boyer, 1971) and that the unique specificity of a restriction and modification mechanism is generally controlled either by chromosomal alleles or occasionally by plasmids. The investigation of restriction and modification enzymes has been mainly in gram-negatives, e.g. E. coli strains which have at least 6 and possibly 7 different host specificities (Arber and Morse, 1965; Bannister, 1970; Lark and Arber, 1970). Similar mechanisms of restriction and modification have been

reported in gram-positive bacteria, e.g. Staphylococcus sp. and Streptococcus cremoris (Rowntree, 1956; Collins, 1956). It is important to be aware that restriction mechanisms may well operate against an introduced plasmid.

Isolation of Plasmid DNA

The physical analysis of plasmid structure has been carried out using two different techniques. The first includes various forms of ultracentrifugal analysis, notably isopycnic density gradient centrifugation in CsCl, by which macromolecules are separated according to their density, and zone sedimentation in preformed gradients, usually of sucrose, where the macromolecules are separated according to their shape and molecular weight. The second technique has been electron microscopy, which has enabled estimations of the size and configuration of plasmid DNA molecules from centrifugation experiments to be verified by di-direct observation.

For genetic and physical analysis of plasmid DNA, pure homogenous plasmid DNA is required.

A method of plasmid DNA extraction used by Helinski et al. (1971) and Bazaral and Helinski (1968) consists first of the formation of sphaeroplasts by the addition to the bacteria of a mixture of lysozyme, ribonuclease and EDTA in sucrose followed by lysis with an ionic detergent such as sodium lauryl sarcosinate. Protein can be removed by extraction with buffered phenol and the phenol removed by dialysis. In an improved method, gentler lysis was accomplished with a non-ionic detergent, i.e. Brij 58, in the presence of sodium deoxycholate so that chromosomal DNA remained attached to a cellular component during low speed centrifugation and most of the plasmid DNA remained

in the supernatant fluid (Godson and Sinsheimer, 1967; Clewell and Helinski, 1969). The supernate from such lysed preparations is termed cleared lysate and has been extensively used for separation of plasmid DNA.

Guerry et al. (1973) used a method for preparing cleared lysates in which high molecular weight chromosomal DNA is selectively precipitated from crude lysates by the presence of sodium dodecyl sulphate and a high concentration of sodium chloride. Another method for the separation of plasmid DNA from the bulk of chromosomal DNA, is to subject the DNA to shearing forces, so that the chromosomal DNA is fragmented and the covalent closed circular (ccc) DNA which has smaller size is left intact (Fiers and Sinsheimer, 1962). Shearing is also possible by drawing the DNA through a fine-needle syringe (Bazaral and Helinski, 1968) after exposing the DNA to a high temperature or high pH. Under these conditions, the hydrogen bonds between the two strands of the DNA duplex are broken and the strands of the linear (chromosomal) DNA are separated by denaturation, while the ccc (plasmid) DNA is left interlocked.

These techniques for purification of plasmid DNA depend on the covalently closed circular nature of plasmid DNA and its consequent resistance to denaturation (Helinski and Clewell, 1971).

Centrifugation of lysates of plasmid-bearing strains on alkaline sucrose gradient has also been used for purifying plasmid DNA (Freifelder, 1968). Under these conditions, ccc DNA sediments faster than denatured DNA.

Early experiments separating plasmid DNA by virtue of its difference in base ratio from host DNA (Cohen and Miller, 1969; Nisioka et al., 1969) consistently indicated molecules with

super-coiled ccc structure. The super-coiling of closed circular DNA enabled it to be separated from nicked duplex or from linear duplex DNA (e.g. chromosomal fragments) by the technique of dye-buoyant density centrifugation. This depends on isopycnic density gradient centrifugation in CsCl in the presence of an acridine dye (Bauer and Vinograd, 1968; Strim et al., 1967). Acridine derivatives, in particular ethidium bromide, are believed to intercalate into polynucleotide chains, becoming inserted between adjacent bases (Waring, 1968; Bauer and Vinograd, 1968). The effect is first to diminish the super-coiling of closed circular DNA (ccc), so that as the amount of dye per molecule is increased, the super-coiling decreases and a relaxed circle is formed. When the amount of dye is further increased, the relaxed form becomes super-coiled in the opposite direction (Radloff et al., 1967). However, intercalation of ethidium bromide into ccc DNA is limited because of the restriction of the rotation of the two strands about each other, due to the absence of a free end of rotation. Thus, in the presence of ethidium bromide, the extension of ccc DNA is less than that of the linear DNA forms, and its density is thus lowered to a lesser extent. Hence, in ethidium bromide, the ccc form is denser and can be separated from the other linear forms even in the absence of any differences in base ratio (Radloff et al., 1967). However, these methods can be used only with the small volumes of bacterial cultures.

A method has recently been developed by Humphreys et al. (1975) for the isolation of large quantities of plasmid DNA which is independent of the molecular weight of the plasmids. A low concentration of polyethylene glycol 6000 (PEG) (10% w/v)

is used to precipitate the DNA from the cleared lysate. The PEG is removed by the addition of a high concentration of CsCl. However, up to 50% of the plasmid DNA can be lost at this stage. The remaining DNA can be subjected to dye buoyant density gradient centrifugation.

Aim of Project: To Transfer a Plasmid

The aim was to transfer a well-characterized plasmid DNA such as RP4 or pJH1 into M. radiodurans either by conjugation or transformation. There have been no reports of plasmid transfer from Enterobacteriaceae to any gram-positive bacteria since these studies were started. RP4 is not only a plasmid with three readily selectable antibiotic resistance markers, but can be transferred from E. coli to Rhizobium spp. and Agrobacterium (Datta et al., 1971). Additionally, members of the same compatibility group as RP4 promote chromosomal transfer in P. aeruginosa, R. leguminosarum and E. coli (Stanisich and Holloway, 1971; Beringer and Hopwood, 1976; Jacob et al., 1977).

Plasmids pJH1 and pJH4 are also capable of bringing about conjugation between strains of streptococci. These plasmids were used in order to optimize the chance of getting M. radiodurans strain containing a plasmid species, because of closer morphological relationship between the two strains.

MATERIALS AND METHODS

Bacterial Strains

The bacterial strains used, their relevant phenotypes and sources are listed in Tables 1, 2 and 3.

Inhibitory Concentration of Antibiotics

Sensitive and resistant strains of Micrococcus radiodurans, Escherichia coli and Streptococcus faecalis have different levels of sensitivity and resistance to antibiotics and other growth inhibitors. The minimum inhibitory concentrations (MIC) are also shown in Tables 1, 2 and 3.

Maintenance of Cultures

Strains of M. radiodurans were maintained on TGY agar plates or stabs. Strains of E. coli and S. faecalis were maintained on nutrient agar (NA) plates or stabs. All strains were kept at 4°C.

Complex Media

TGY medium was used for most experiments with M. radiodurans and contained: Bactotryptone (Difco), 5 g; Glucose, 1 g; Yeast extract (Difco), 3 g; Distilled water to 1 l. TGY agar was made by solidifying this medium with 15 g Bacto agar per l.

TY medium contained Yeast extract (Difco), 3 g; Bactotryptone (Difco), 5 g; Distilled water to 1 l.

Nutrient broth (NB), used as a rich medium for E. coli and S. faecalis contained Oxoid nutrient broth No. 2, 25 g; Distilled water to 1 l. Nutrient agar (NA) was made by solidifying this medium with 15 g Bactoagar (Difco) per l.

L-broth (LB) used during the transformation procedure for E. coli contained Bactotryptone (Difco), 10 g; Yeast extract (Difco), 5 g; NaCl, 10 g; Distilled water to 1 l.

Minimal Media

Table 1: Strains of Micrococcus radiodurans

Minimum inhibitory concentrations (μg/ml) of antibiotics

Strain	Relevant Phenotype	Kan	Rif	Acr	Str	Tet	Ery	Pen	MTC	Source
Wild type	Prototrophic, sensitive to antibiotics	<5	<2	<1	<1	<0.5	<1	<1	<0.5	B.E.B. Moseley
302	Prototrophic, highly mutable by alkylating agents	<5	<2	<1	<1	<0.5	<1	<1	<0.02	B.E.B. Moseley
KRASE	Prototrophic, resistant to some antibiotics	30	>100	5	200	25	25	<1	<0.5	B.E.B. Moseley
Rif^r	Prototrophic, resistant to rifampicin	<5	>100	<1	<1	<0.5	<1	<1	<0.5	By this thesis

The minimum inhibitory concentration (MIC) of antibiotics for M. radiodurans were obtained on TGY plates containing varying concentrations of antibiotics.

Abbreviations: Kan = kanamycin; Rif = rifampicin; Acr = acriflavin; Str = streptomycin; Tet = tetracycline; Ery = erythromycin; Pen = penicillin; MTC = mitomycin C.

Table 2: Strains of *Escherichia coli*

Minimum inhibitory concentrations (μg/ml) of antibiotics

Strain	Relevant Phenotype	Chromosomal Resistance		Plasmid Borne Resistance			Plasmid	Molecular Weight	Parental Strain	Source
		Nal	Str	Pen	Kan	Tet				
J5-3	*pro-met*	50	-	>1000	>25	>20	RP4	$40_6 \times 10$ *	K12	B.E.B. Moseley
W1103	*thy-trp*	-	-	>1000	>25	>20	RP4	$40_6 \times 10$	K12	B.E.B. Moseley
W1106	*arg*	-	50	<5	<5	<2	-	-	K12	R.W. North
"655"	*met*	-	-	<5	<5	<2	-	-	K12	W.J. Brammar

Minimum inhibitory concentration of antibiotics shown by bacteria growing on complete medium (*E. coli* K12 data from Beringer (1973)).

Abbreviations: Nal = naladixic acid; Str = streptomycin; Pen = penicillin; Kan = kanamycin; Tet = tetracycline.

* = Datta *et al*. (1971).

Growth requirements: pro = Proline; met = Methionine; thy = Thymine; trp = Tryptophan; arg = arginine.

Table 3: Strains of Streptococcus faecalis

Minimum inhibitory concentrations (μg/ml) of antibiotics

Strain	Relevant Phenotype	Chromosomal Resistance			Plasmid Borne Resistance					Plasmid	Molecular Weight	Reference	Source
		Fus	Rif	Gen	Kan	Str	Nm	Tet	Ery				
JH1-2	thy	4	1.25	80	40000	10000	>40000	320	5000	pJH1	$50_6 \times 10$	Jacob & Hobbs, 1974	A.E. Jacob
JH2-14	thy	-	1280	80	>65536	>65536	>65536	None	4096	pJH4	$25_6 \times 10$	Jacob & Hobbs, 1974	A.E. Jacob
JH2-2	thy	256	1280	20	40	40	80	1.25	0.125	-	-		A.E. Jacob

Minimum inhibitory concentration of antibiotics shown by bacteria growing on lysed blood plates using Oxoid urine multodisks.

(Data from Jacob and Hobbs, 1974).

Abbreviations: Fus = fusidic acid; Rif = rifampicin; Gen = gentamycin; Kan = kanamycin; Str = streptomycin; Nm = neomycin; Tet = tetracyclin; Ery = erythromycin.

Growth requirements: thy = thymine.

Minimal Medium for M. radiodurans

This medium was developed for M. radiodurans by Miss Diana Sweet (personal communication). The basal medium contained: Glucose, 2 g; Monosodium glutamate, 2 g; Tris(hydroxymethyl)-aminomethane, 2 g; Ammonium acetate, 1 g; Distilled water to 1 l. To this 1 ml of each solution A, B, C and D and 2 ml of solution E were added. These solutions were made up as follows and could be stored at 4°C until required.

A:	Potassium acetate	20 g
	Magnesium acetate, 4 H_2O	20 g
	Sodium acetate	0.5 g
	Calcium chloride, H_2O	0.5 g
	H_2O (distilled)	100 ml
B:	Ammonium phosphate	8.7 g
	Ammonium sulphate	2.0 g
	H_2O (distilled)	100 ml
C, Vitamins:		
	Nicotinic acid	50 mg
	Vitamin B1 (aneurine HCl)	50 mg
	Biotin	1 mg
	Vitamin B12 (cyanocobalamin)	0.1 mg
	H_2O (distilled)	100 ml
D, Trace Elements:		
	Boric acid	50 mg
	Manganese acetate, 4 H_2O	50 mg

Zinc acetate, 2 H_2O	40 mg
Ferric chloride, 6 H_2O	20 mg
Ammonium molybdate VI	15 mg
Potassium iodide	10 mg
Cupric acetate H_2O	10 mg
Citric acid H_2O	100 mg
H_2O (distilled)	100 ml

E, Amino Acids:

L-cysteine	1200 mg
L-methionine	800 mg
L-lysine monohydrochloride	915 mg
L-histidine	955 mg
H_2O (distilled)	100 ml

The pH of the medium was adjusted to 7.8 by adding glacial acetic acid. The medium was then autoclaved. Minimal agar was made by adding 15 g Bactoagar per 1.

M. radiodurans minimal medium was supplemented when necessary as follows:-

Individual amino acids as required	100 µg/ml
Pyridoxin	100 µg/ml
Adenine and uracil	50 µg/ml

Minimal Medium for E. coli

Medium M9 was used and contained the following:-

$Na_2HPO_4 \cdot 2\ H_2O$	7 g
KH_2PO_4	3 g
NaCl	0.5 g
NH_4Cl	1 g
$CaCl_2$	0.011 g

$MgSO_4 \cdot 7\ H_2O$	0.25 g
Glucose	2 g
H_2O (distilled)	1 l

pH was adjusted to 7.0.

Glucose was made as a 20% solution and autoclaved separately.

E. coli M9 medium was supplemented as follows:-

Proline	100 µg/ml
Methionine	100 µg/ml
Thymine	2 or 0.5 µg/ml

Agar media were distributed as 100 or 200 ml amounts in flasks and autoclaved (see below). Before use, the agar was melted in either an autoclave for 10 minutes or in a boiling water bath, cooled to 47°C and supplemented as needed before pouring plates.

Sterilization

Sterilization of media was by autoclaving at 15 lb for 15 minutes. Tryptophan, tyrosine, methionine, pyridoxine and vitamins and antibiotics were sterilized by filtration. Other amino acids and growth factors were autoclaved. Antibiotics were made up as fresh solutions. Most antibiotics and antimicrobial compounds were soluble in water. However, rifampicin was dissolved in dimethyl sulphoxide at a concentration of 50 mg/ml and erythromycin was suspended in water and a few drops of HCl added until it dissolved.

Buffers

Standard Saline-Citrate (SSC) contained:-

NaCl	9 g (0.15 M)
Na_3 citrate	4 g (0.015 M)

H_2O (distilled)	1 l

pH adjusted to 7.0.

Phosphate buffer contained:

Na_2HPO_4	4.73 g
KH_2PO_4	4.5 g
H_2O (distilled)	1 l

pH = 7.0.

Sodium phosphate buffer contained:

Na_2HPO_4	17.7 g (0.1 M)
NaH_2PO_4	15.6 g (0.1 M)
H_2O (distilled)	1 l

pH = 7.0.

Ethylenediaminetetra-acetic acid (EDTA) was used as 93.06 g in 1 l distilled water (0.25 M); pH adjusted to 8.0.

TES buffer contained:

Tris	6.05 g (0.05 M)
EDTA	1.86 g (0.005 M)
NaCl	2.92 g (0.05 M)
H_2O (distilled)	1 l

pH adjusted to 8.0.

Measurement of Bacterial Growth

Liquid cultures of bacteria were grown in 50 ml amounts of medium in 250 ml Erlenmeyer flasks and their turbidity measured in a nephelometer (Evans Electroselenium Ltd., Halstead) with an orange filter. A plot of turbidity against time allowed the growth rate to be measured. A culture having a turbidity of 0.30 was equal to 1.0×10^8 viable units M. radiodurans/ml, 5.0×10^8 viable units E. coli/ml or 1.0×10^9 viable units S. faecalis/ml.

Preparation of Transforming DNA from M. radiodurans

A fully grown culture of a strain of M. radiodurans was centrifuged in an MSE Mistral 6L at 3000 r.p.m. for 30 minutes. The bacteria were washed in 50 ml SSC, resuspended in 40 ml butanol-saturated phosphate buffer and left at room temperature for one hour. This treatment removes lipoprotein from the cell wall which protects the mucopolysaccharide from the action of lysozyme (Driedger and Grayston, 1970). The bacteria were centrifuged in an MSE high speed 18 for 10 minutes at 10,000 r.p.m., washed and resuspended in 40 ml SSC. Lysozyme at 1 mg/ml in SSC buffer was prepared just before adding to the mixture to a final concentration of 20 mg/ml. The mixture was incubated at 37°C for 15 to 30 minutes or until a small sample (0.5 ml) lysed on addition of 0.05 ml of sodium dodecyl sulphate (SDS). The bacteria were lysed by the addition of 20% SDS in water to a final concentration of 2%. The flask was swirled until the mixture was completely lysed. DNA was then purified by the method of Marmur (1961). Five ml sodium perchlorate (70.25 g $NaClO_4.H_2O$ and 4.4 g NaCl in 100 ml distilled water) for each 20 ml of lysate was added and an equal volume of chloroform : isoamyl alcohol mixture (24 : 1) was added for deproteinization. The mixture was shaken by hand vigorously for 30 minutes before being centrifuged at 18,000 r.p.m. in an MSE high speed 18 for 35 minutes. The mixture separated into two layers with an interface protein layer. The aqueous top layer contained DNA and RNA. The top layer was removed carefully to avoid shearing the DNA. The removal of the aqueous layer was not allowed to disturb the bottom chloroform layer. The nucleic acid was precipitated by pouring the aqueous component carefully into 1.7 times its volume of absolute alcohol. The nucleic acids were wound onto clean glass rods,

left to dry at room temperature (when the nucleic acids change from a white to a clear appearance), resuspended in a minimum volume (2 to 3 ml) of SSC and dissolved by shaking gently at 37° or by being left overnight at 4°C.

For further purification, RNase (10 mg/ml) was added to a final concentration of 1 mg/ml and the preparation left shaking at 37°C for 1 hour. An equal volume of chloroform : isoamyl alcohol was added and the mixture shaken for 10 minutes. It was centrifuged at 18,000 r.p.m. for 30 minutes, the aqueous layer removed and the DNA precipitated as before. Finally, the DNA was dissolved in 2 to 3 ml of SSC and kept at 4°C for use.

Procedure for Transformation

Development of a New Method for Transformation

The transformation procedure used initially was that of Moseley and Mattingly (1971), but a new procedure was devised during the course of this work, which will be described in more detail in the Results section.

For all the transformation studies, an overnight culture of M. radiodurans in TGY medium was diluted into 20 ml of fresh TGY in a 250 ml conical flask and incubated with aeration at 30°C to give an exponential culture with approximately 3×10^8 viable units/ml. Ten ml of the culture was centrifuged at 3,000 r.p.m. for 5 minutes, resuspended in 5 ml of fresh pre-warmed TGY (held at 30°C) and 1.8 ml of 0.1 M $CaCl_2$ solution. 0.30 ml amounts of this culture were then added to 0.05 ml amounts of transforming DNA in sterile test tubes. The mixtures were shaken gently in a water bath at 30°C for 2 hours and appropriately diluted with TGY, TY or minimal medium. One ml of the sample was plated into 10 ml agar, which was melted and cooled in the water

bath to about 46°C before mixing with the bacteria. The plates were then incubated at 30°C for expression of the transformed marker and overlaid with 10 ml of the same melted TGY agar containing the antibiotics or antibacterial compounds. The plates were incubated at 30°C for between 2 to 3 days or until transformant colonies were countable.

Divalent Cation

All divalent cations apart from Ca^{2+} were used at 0.1 M in transformation studies in M. radiodurans. These were $MgCl_2$, $SrCl_2$ and $ZnCl_2$. The final concentration in assay tubes was 0.035 M.

Dilution Curve

A standard DNA preparation was diluted with SSC to 3×10^{-1}, 10^{-1}, 3×10^{-2}, 10^{-2} and 3×10^{-3}. 0.3 ml of a competent culture prepared with 0.1 M $CaCl_2$ (0.035 M in assay tube) was added to 0.05 ml of each dilution of DNA. After incubation for 2 hours, the bacteria were plated in triplicate in TGY agar and left for expression time. When the double mutant was used, full expression was allowed for the gene having the longest expression time. The top layer containing a single or two antibiotics for selecting single or double transformants was added and the plates were incubated for 4 days before colonies were counted.

Clonal Analysis

When it was not possible to select double transformants, i.e. temperature-sensitive and auxotrophic markers, clones of single transformants were tested for the presence of a second marker. A large number of each transformant was analysed for the transfer of the second marker and the frequencies of each transformant class were calculated.

The frequency of all transformant types at the varying concentrations of DNA were compared using the linkage index (Butler and Nicholas, 1973) and co-transfer index (Nester and Lederberg, 1961) methods.

Minimum Time for Uptake of DNA in M. radiodurans

Cultures of M. radiodurans (0.3 ml) were exposed to 0.05 ml amounts of transforming DNA for increasing periods of time (0 - 90 minutes) before 0.05 ml DNase (2.5 mg/ml) was added to inactivate the DNA. Samples were appropriately diluted and plated. Colonies were counted after 3 days.

Expression Times

Competent bacteria were exposed to transforming DNA for 90 minutes and diluted appropriately. A 1 ml sample was mixed with TGY agar in several plates and the plates incubated from 0 to 8 hours. Every hour, the plates were overlaid with TGY agar containing the appropriate antibiotic or antibacterial compound. All the plates were incubated for 3 days before transformant colonies were counted. For the expression of prototrophy, transformants were allowed to express in TGY medium. After 90 minutes incubation of the bacteria and transforming DNA, the culture was diluted tenfold with TGY and incubated with shaking at 30°C. After 0 to 5 hours, the culture was suitably diluted and 0.1 ml of the culture plated on the surface of the minimal medium. For the double mutants, plates were incubated at 39°C for temperature sensitivity, and for the auxotrophs plates were incubated at 30°C. After 4 days, the number of single and double transformants was counted, and transformation frequency for each class calculated.

Mutagenesis of Bacteria

The procedure used was based on mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine (NTG). To enhance the mutation frequency, an exponential culture of turbidity 0.30, was centrifuged and the bacteria washed and resuspended in phosphate buffer at pH = 5.7. NTG was added to a final concentration of 100 µg/ml and the bacterial suspension incubated for 30 minutes at 30°C. The bacteria from 1 ml of culture were washed twice, resuspended in 20 ml fresh TGY and incubated at 30°C until growth reached a turbidity of 0.50. To isolate auxotrophs, the culture was diluted appropriately and 0.1 ml samples spread on TGY plates to give about 80 to 100 colonies per plate. Plates were incubated at 30°C for 2 days. To identify the auxotrophic mutants the colonies were replicated onto minimal and TGY agars. The master plates were kept at 4°C and replicates incubated for 3 days at 30°C. All possible auxotrophs were picked from the master plates.

The putative auxotrophs were tested for growth requirements by plating each on a series of 12 supplemented minimal plates, using the method of Holliday (1956).

For selecting antibiotic resistant mutants, 0.1 ml amounts of the culture were spread on TGY agar plates containing appropriate antibiotics. Colonies which grew on antibiotic plates were isolated and plated on TGY containing varying amounts of antibiotic to ascertain their levels of resistance.

Test for Temperature-Sensitivity

Auxotrophic and antibiotic-resistant mutants were grown on TGY to 10^8 viable units/ml, diluted to give 10^3 viable units/ml, plated, incubated to give colonies, and the colonies replicated onto 2 TGY plates. One plate was incubated at 30°C and the other at 39°C (permissive and restrictive temperatures

respectively) for 2 days. Colonies which grew at 30°C, but not at 39°C were selected as double mutants.

Incorporation of Radioactively-Labelled Compounds into Bacteria

DNA, RNA and protein biosynthesis were studied by following the incorporation of [6-^{3}H] thymidine (^{3}HTdR) (specific activity 25 Ci/mmol), [5,6-^{3}H] uridine (^{3}HU) (specific activity 46 Ci/mmol) and L-[4,5-^{3}H] leucine (specific activity 57 Ci/mmol) respectively into trichloroacetic acid (TCA) insoluble material.

The temperature-sensitive mutants were grown in TGY to a turbidity of 0.10 and an appropriate radioactive compound (10 μCi) added to 0.2 ml of the culture.

To measure synthesis, two cultures were incubated at 30°C for 30 minutes before 1 tube was removed and incubated at the restrictive temperature. The amount of radioactivity in the TCA-insoluble fraction of the bacteria was measured as a function of time by the method of Bollum (1959). Ten μl of culture was placed onto a Whatman No. 1 filter disc, diameter 1.8 cm. When the liquid was absorbed by the paper, the disc was immersed in cold 5% TCA. After all the samples had been taken, the discs were left in 5% TCA on ice for 10 minutes and washed twice with fresh cold TCA. All the procedures were carried out in ice. The discs were then washed twice in absolute alcohol to remove the TCA and spread on polyethylene backed absorbent paper to dry at room temperature. Each disc was placed in a scintillation vial and 5 ml of toluene-based liquid scintillant NE 233 (Nuclear Enterprises, Edinburgh, Scotland) added to each vial. The amount of radioactivity on each disc was measured in a Packard Tricarb Liquid Scintillation Spectrometer.

U.V. Irradiation

All the strains were U.V.-irradiated to test their level of sensitivity or resistance compared with the wild type. Mutants were streaked on TGY plates and irradiated with a Hanovia germicidal lamp (model 12) for varying times from 2 to 15 minutes.

Crosses of M. radiodurans with E. coli and S. faecalis

Several R-factors were received in bacterial strains and were used as donors in crosses with M. radiodurans. These R-factors, their host strain and the relevant phenotype conferred on the host are summarized in Tables 2 and 3.

Techniques of Conjugation

Crosses between strains were carried out using two different methods.

1. In Liquid Media

Cultures of the donor and recipient were prepared by static overnight incubation at 37°C in nutrient broth for E. coli or S. faecalis and at 30°C in TGY for M. radiodurans, followed by a tenfold dilution into fresh broth 4 to 6 hours before crossing. 0.5 ml of donor was mixed with 4.5 ml of recipient and incubated for 90 minutes at 30°C or 37°C. The mixture was suitably diluted and plated on selective media.

For crosses between M. radiodurans and E. coli carrying RP4, transconjugants were selected on 100 µg/ml rifampicin and 25 µg/ml kanamycin or 100 µg/ml rifampicin and 5 µg/ml tetracycline or 100 µg/ml rifampicin and 10 µg/ml penicillin. Cultures were also suitably diluted and plated on TGY agar for the viable counts of the donor and recipient.

Crosses between M. radiodurans and S. faecalis strain JH1-2 carrying pJH1 were performed in liquid media as above. To select transconjugants, TGY agar plates were used containing 100

μg/ml rifampicin and 25 μg/ml kanamycin or 100 μg/ml rifampicin and 20 μg/ml erythromycin and/or 100 μg/ml rifampicin and 20 μg/ml streptomycin or 5 μg/ml tetracycline.

Crosses between E. coli J5-3 and W1106 and S. faecalis JH1-2 and JH2-2 were also performed to test the frequency of transconjugants obtained.

To select the transconjugants of E. coli, a culture was plated on 25 μg/ml kanamycin and 50 μg/ml nalidixic acid. To show that colonies which were growing on kanamycin were not W1103 which had mutated to kanamycin-resistance, colonies were tested for the acquisition of the other antibiotic resistances present on the plasmid.

To select the transconjugants of S. faecalis, a culture was plated on 200 μg/ml tetracycline together with 200 μg/ml kanamycin. Colonies were then tested for the acquisition of resistance to the other two antibiotics.

2. On Membrane Filters

Cultures of the donor and recipient were prepared as described. 0.5 ml of donor and 4.5 ml of recipient were mixed and 2 ml taken up into a sterile polypropylene syringe. The mixture was passed through a sterile Millipore-Swinnex-25 filter. The filter membrane to which the bacteria were attached was placed on an NA plate and incubated for 24 hours at 37°C. The filter membranes were then removed and washed in 5 ml broth. The suspension was suitably diluted and plated on selective media.

Transfer of Resistance Factor by Transformation

Plasmid DNA extracted from E. coli J5-3, W1103 and S. faecalis JH1-2 and JH2-14 was used to transform

M. radiodurans and E. coli "655".

Isolation and Purification of RP4 DNA from E. coli

Preparation of the Cleared Lysate

E. coli J5-3 containing the resistance plasmid RP4 was grown at 30°C to 10^9 cells/ml in 1 litre of M9 medium, supplemented with 100 µg/ml of proline and methionine, in an orbital incubator. The bacteria were then quickly chilled and harvested by centrifugation at 4°C at 2,500 r.p.m. for 1 hour in an MSE Mistral 6L or 10,000 r.p.m. for 15 minutes in an MSE High Speed 18. The pellet was kept in ice and washed in 500 ml of sodium phosphate buffer pH 7.0. The suspension was centrifuged at 10,000 r.p.m. for 10 minutes and the pellet resuspended in 33 ml ice cold sucrose solution (25% sucrose in 0.05 M Tris pH 8.0). Fifteen ml freshly prepared lysozyme (5 mg/ml in 0.25 M Tris pH 8.0) was added and the solution shaken gently to mix the contents. After 5 minutes at ice temperature 13.5 ml of cold EDTA (0.25 M pH 8.0) was added and the mixture left in ice for another 10 minutes with occasional swirling. Fifty-four ml of lysis mixture (1% Brij 58, 0.4% sodium deoxycholate, 0.0625 M EDTA in 0.05 M Tris, pH 8.0) was added and immediately mixed gently. The mixture was left for 10 minutes or until it cleared.

The lysate was transferred into 50 ml polypropylene MSE tubes, centrifuged at 18,000 r.p.m. for 35 minutes at 4°C and the supernatant carefully poured off into a measuring cylinder. The resulting cleared lysate was kept frozen until used.

Concentrating the DNA

To concentrate the DNA present in the cleared lysate, the method of Humphrey et al. (1975) was used. To the cleared

lysate (usually about 110 ml at this stage), 3.2 g sodium chloride was added (molarity of 0.5) and after the sodium chloride was dissolved, 11 g polyethylene glycol 6000 (PEG) was added. The mixture was then left to allow the PEG to dissolve (the PEG comes in very large flakes and was ground with a mortar and pestle before adding to the cleared lysate).

The mixture was left overnight at 4°C to precipitate the DNA. The precipitate was centrifuged at 3,000 r.p.m. for 5 minutes, the supernatant poured off and the flocculent precipitate dissolved in 2 ml of TES buffer. This was made up to 3.8 g by adding distilled H_2O.

Dye-Buoyant Density Centrifugation

Caesium chloride (5.2 g) was added to give a molarity of 7.7. This molarity of CsCl removed the PEG, which appeared as a layer on the top of the liquid, after centrifugation at 2,000 r.p.m. for 2 minutes. Ethidium bromide (0.3 ml of 10 mg/ml in TES buffer) was added to a final concentration of 500 µg/ml. The refractive index was adjusted using a refractometer (Bellingham and Stanley) to 1.3925 (density 1.625 g/cm^3) by adding distilled H_2O. The mixture was then transferred into two 5.5 polycarbonate tubes (MSE). The tubes were centrifuged at 33,000 r.p.m. (average 95,000 g) for 60 hours at 18°C in the Superspeed 65,000 MSE centrifuge.

Two bands were formed in the middle of the tubes, separated by a distance of approximately 5 mm. The lower band was covalently closed circular plasmid DNA and the upper band was linear plasmid and chromosomal DNA.

The lower band was removed carefully using a 2 ml disposable syringe with a 19 gauge needle inserted through the side of

the tube and dialysed against 10 ml Dowex 50 W-X in 50 ml TES buffer for 24 hours. The resin of the Dowex was pre-washed with 2 M HCl followed by 1 M NaOH to convert to the Na^+ form.

The DNA was finally dialysed against SSC buffer and transferred into small vials and kept at 4°C to be used.

Isolation of Plasmid DNA from S. faecalis

S. faecalis was grown in 1 litre of TGY at 30°C with aeration until the stationary phase was reached (about 10^9 bacteria/ml). The bacteria were harvested, washed twice in TES buffer pH 8.0 at 4°C. The pellet was resuspended in 30 ml of TES buffer containing 100 mg of sucrose/ml and the bacteria lysed using the method of Basaral and Helinski (1968) as modified by Jacob and Hobbs (1974) for use with S. faecalis. The mixture was left in ice for 5 minutes and 30 ml of TES buffer containing 100 mg of sucrose/ml and 2 mg of lysozyme/ml was added. The mixture was incubated for 30 minutes at 37°C and cooled for 5 minutes in ice. The bacteria were lysed by the addition of 30 ml sodium lauryl sarcosinate (SLS, 2.4% in water) and the mixture gently and rapidly mixed until it cleared. The lysate was passed through an 18-gauge needle 6 to 7 times to shear the chromosomal DNA while stirring slowly on a magnetic stirrer. Sodium hydroxide (1N) was added until the pH of the lysate was raised to between 12.1 and 12.4 (LeBlanc and Hassell, 1976). Stirring was continued for 10 minutes and the pH quickly lowered to 8.5 by the addition of 2 M Tris-hydrochloride pH 7.0 (Currier and Nester, 1976). NaCl and PEG were added as before, the precipitate dissolved in TES buffer and made up to 9 ml.

Dye-Buoyant Density Centrifugation

The gradient was prepared by adding 8.9 g CsCl to 9 ml of

the sample in TES buffer. Ethidium bromide was added to a final concentration of 300 μg/ml and the refractive index adjusted to 1.3867 using the refractometer. The mixture was transferred to a 10 ml polycarbonate MSE tube and centrifuged at 35,000 r.p.m. (average 90,000 g) in an MSE 10 x 10 ml fixed angle rotor for 60 hours.

The plæmid band was collected and dialysed.

Procedure for Isolation of Possible Plasmid DNA from M. radiodurans

Micrococcus radiodurans was grown in 1 litre of TGY and lysed as previously described. The lysate was passed through an 18-gauge needle 10 to 12 times to shear the chromosomal DNA. Because it was difficult to prepare a cleared lysate by centrifugation, PEG precipitation of the normal lysate was carried out as described before and caesium chloride and ethidium bromide added as for S. faecalis. The refractive index was adjusted to 1.3867 and centrifugation carried out at 33,000 r.p.m. in 5.5 ml polycarbonate tubes (MSE) for 60 hours.

Preparation of Radioactively-Labelled Plasmid DNA from E. coli for Transformation

For radioactive labelling of the plasmid DNA, E. coli W1103 was used. An overnight culture of W1103 in NB was inoculated into 500 ml M9 medium containing 1 mCi of [6-^{3}H] thymidine and supplemented with 100 and 0.5 μg/ml of tryptophan and thymine respectively. The culture was incubated overnight at 37^{o}C and centrifuged at 10,000 r.p.m. for 10 minutes at 4^{o}C.

The pellet was washed in cold sodium phosphate buffer and resuspended into 100 ml of cold sucrose solution.

The lysis of the bacteria was exactly as described for

E. coli J5-3, as was the preparation of the plasmid DNA.

Small samples (100 or 10 µl) were removed at each stage of the preparation and assayed for radioactive counts in the TCA-insoluble material.

Preparation of Radioactively-Labelled Plasmid DNA of M. radiodurans

For the incorporation of [6-^{3}H] thymidine into putative plasmid DNA, an overnight culture of M. radiodurans was inoculated into 50 ml of TGY containing 100 µCi of [6-^{3}H] thymidine. The culture was incubated at 30°C for 18 hours. Lysis of the bacteria has been described previously. To shear the chromosomal DNA, the lysate was passed through an 18-gauge needle 6 or 7 times while being stirred slowly on a magnetic stirrer. The procedure for banding plasmid DNA was as described.

After centrifugation, the tube was pierced and 10-drop fractions collected on a filter paper strip. The paper was dried, washed in ice-cold 5% TCA and alcohol and the activity of each fraction measured in a Packard Tricarb Liquid Scintillation Spectrometer.

Transformation of M. radiodurans by Plasmid DNA

Purified plasmid DNA from E. coli and S. faecalis was incubated with M. radiodurans using the new method developed for transformation as described.

For selecting possible transformants of M. radiodurans containing RP4 DNA, 25, 25 and 10 µg/ml of tetracycline, kanamycin, and penicillin were used respectively. The antibiotics were always used singly and not in combination.

For selecting possible transformants containing S. faecalis plasmid DNA, 25, 25, 10 and 100 µg/ml of kanamycin, erythromycin,

tetracycline and streptomycin were used respectively.

Assay for the Biological Activity of RP4 DNA

Plasmid DNA extracted from E. coli J5-3 and W1103 was transformed into E. coli strain "655" using the method of Cosloy and Oishi (1973).

Bacteria were inoculated into 20 ml of L-broth and incubated in a water bath at 37°C with aeration. The culture was then diluted 20 to 50 times or to a turbidity of 0.04 and incubated at 37°C in a conical flask until it reached 10^8 cells/ml. The culture was immediately chilled in ice and 10 ml of the culture centrifuged at 10,000 r.p.m. for 10 minutes at 4°C. The pellet was resuspended in 10 ml cold 0.1 M $MgCl_2$, washed and resuspended in 5 ml cold 0.1 M $CaCl_2$ and kept in ice for 20 minutes. The bacteria were then centrifuged, resuspended in 0.25 ml cold 0.1 M $CaCl_2$ and 0.1 ml SSC buffer. To this, 10 µl of the plasmid DNA preparation was added. The mixture was then incubated at 42°C for 2 minutes and placed in ice for 20 minutes, diluted with 2.7 ml of L-broth (10 times dilution) and incubated for 3 hours to allow expression time. 0.1 ml samples were plated on selective media each containing 25, 25, 100 µg/ml of tetracycline, kanamycin and penicillin respectively in TGY agar one at a time. Plates were incubated for 48 hours at 37°C and transformant colonies counted.

RESULTS

PART I

Transformation Studies in M. radiodurans

Effect of Nutritional and Physiological Factors on the Frequency of Transformation of Micrococcus radiodurans

Glucose:

M. radiodurans was grown in TY medium with glucose at 0, 0.1, 1 and 5 mg/ml. The bacteria were centrifuged and resuspended in 5 ml of media containing the same concentration of glucose. To 0.3 ml samples of these cultures, 0.05 ml DNA was added.

The presence or absence of glucose in the growth medium had no significant effect on either growth or transformation. Doubling time of the cultures was 80 minutes and the frequencies of transformation in the presence of Ca^{2+} were between 1.7×10^{-3} and 2.5×10^{-3}.

Amino Acids:

M. radiodurans needs four amino acids, L-cysteine, L-methionine, L-lysine and L-histidine for growth in minimal medium (Diana Sweet, personal communication). These and other amino acids, including tryptophan, arginine, alanine, tyrosine and glutamic acid were added individually to minimal or TGY medium to test their effect on transformation. They had no effect in increasing the transformation frequency, although arginine reduced it by between 1.5 and 2-fold.

Divalent Cations:

The influence of the salts of 4 divalent cations, $MgCl_2$, $CaCl_2$, $SrCl_2$ and $ZnCl_2$ during transformation of M. radiodurans was investigated.

Exponential cultures of M. radiodurans grown in TGY or TY medium were centrifuged and resuspended in half the volume of the same medium plus 0.03 M cation. Small volumes (0.3 ml) of

each were exposed to 0.05 ml of transforming DNA. The frequency of transformation was compared with that obtained in the absence of any factors.

The frequency of transformation was increased several hundred to several thousand-fold, depending on the marker used, by the presence of $CaCl_2$. The highest frequency obtained was with the rifampicin marker in which 2 to 3% of the bacteria were transformed. The lowest frequency of transformants (0.01%) was obtained using kanamycin resistance as the marker. Comparing the number of transformants with and without treatment with $CaCl_2$ showed a 10,000-fold increase in rifampicin resistance and 1,000-fold in kanamycin resistance. An increase was observed with all markers tested (Table 4).

To determine the concentration of $CaCl_2$ which gives the maximum transformation frequency, cells of M. radiodurans were treated with concentrations of $CaCl_2$ ranging between 0 and 0.3 M during exposure to transforming DNA. The maximum frequency of transformation was obtained when cells were exposed to 0.03 M $CaCl_2$ during transformation (Table 5).

Other Divalent Cations:

The divalent cations Mg^{2+} and Sr^{2+} did not increase the frequency of transformation. In fact, Zn^{2+} completely inhibited transformation.

pH:

Bacteria were grown in TGY medium, centrifuged and resuspended in TGY at pH 5.7, 7.0 or 8.0. $CaCl_2$ was added to a concentration of 0.03 M and the bacteria exposed to transforming DNA.

The markers used were acriflavin and rifampicin resistance.

Table 4: Effect of 0.03 M $CaCl_2$ in TGY and TY Medium on the Frequency of Transformation of M. radiodurans

Growth Medium	$CaCl_2$ Addition to Assay Tube	Frequency of Transformation					
		Kan	Rif	Acr	Str	Ery	Aux
TGY	-	1.5×10^{-7}	3.0×10^{-6}	5.0×10^{-7}	2.0×10^{-7}	1.0×10^{-6}	2.0×10^{-6}
TGY	+	1.0×10^{-4}	2.5×10^{-2}	1.5×10^{-3}	1.5×10^{-4}	3.0×10^{-4}	2.0×10^{-3}
TY	-	1.5×10^{-7}	1.5×10^{-6}	8.0×10^{-7}	2.1×10^{-7}	1.0×10^{-6}	2.5×10^{-6}
TY	+	1.5×10^{-4}	3.0×10^{-2}	1.5×10^{-3}	2.0×10^{-4}	3.0×10^{-4}	2.5×10^{-3}

Markers used in this assay to select the transformants were: kanamycin (Kan); rifampicin (Rif); acriflavin (Acr); streptomycin (Str); erythromycin (Ery); and adenine-requiring auxotroph (Aux).

Table 5: Effect of the Concentration of $CaCl_2$ on the Frequency of Transformation in M. radiodurans

Final Concentration of Added $CaCl_2$ (M)	Frequency of Transformation $\times 10^6$
0.3	8
0.25	9
0.15	75
0.075	75
0.03	190
0.025	90
0.015	20
0.003	10
0	0.1

2.5 µg/ml acriflavin was used to select the transformants.

The frequencies of transformation for the acriflavin marker were 1.5×10^{-4} at pH 5.7, 3.5×10^{-3} at pH 7.0 and 2×10^{-3} at pH 8.0. For the rifampicin marker the frequencies of transformation were 2×10^{-4} at pH 5.7, 4.5×10^{-3} at pH 7.0 and 3.8×10^{-3} at pH 8.0. The results indicated that at pH 7.0 more transformants were obtained than at the other two pH values.

Time Required for the Uptake of Transforming DNA

As a control for this experiment, 0.03 ml of 3 mg DNase/ml was added to 0.3 ml of the bacterial culture and 0.05 ml DNA added to this mixture.

To several other tubes each containing 0.3 ml of the culture, DNA was added and then DNase added from 2 seconds to 90 minutes later to inactivate the transforming DNA. All the tubes were then incubated to 90 minutes at $30^{o}C$. One ml of each sample was mixed with 10 ml TGY agar and incubated for expression of the markers used.

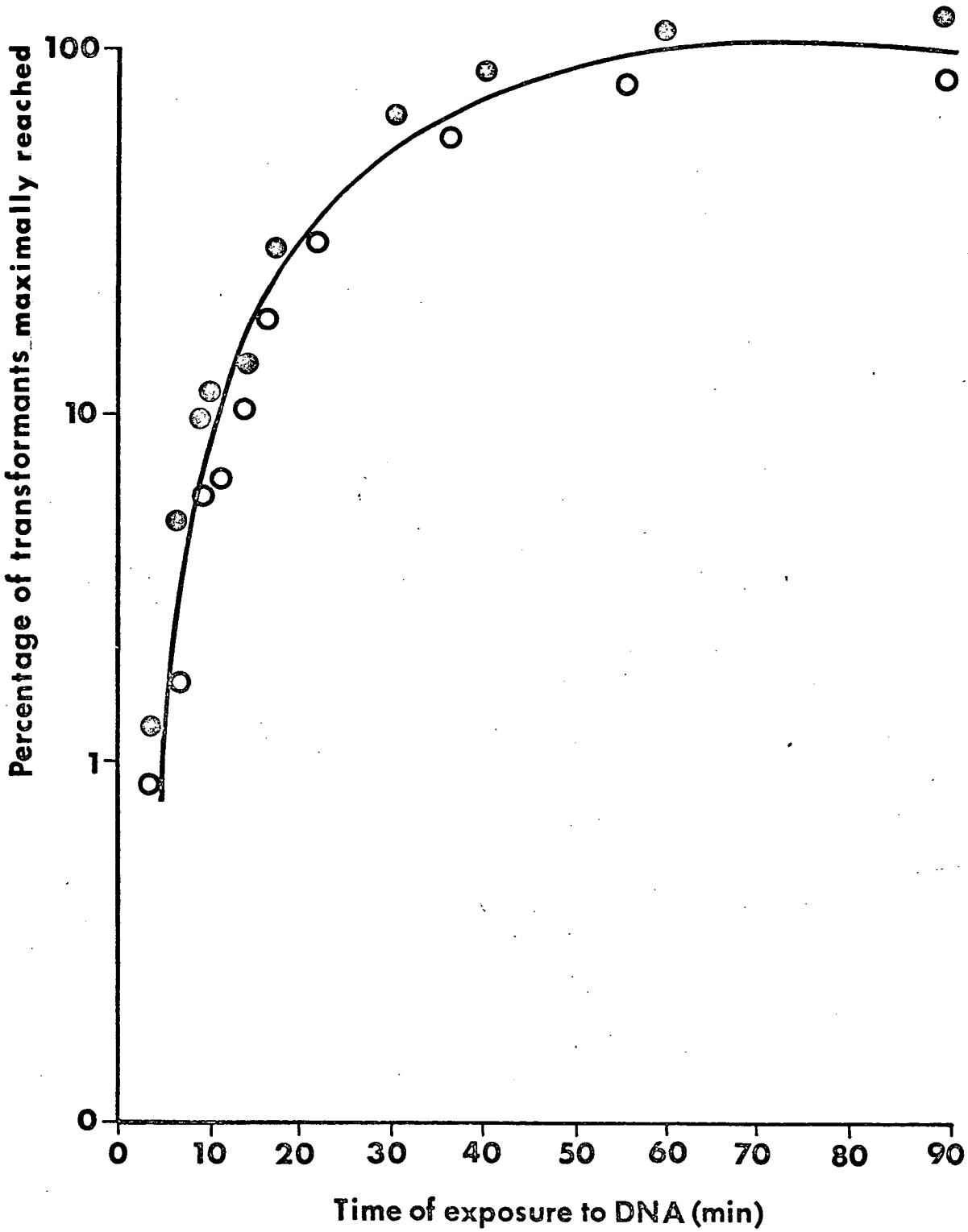
The minimum time for the uptake of transforming DNA was 2.5 minutes for both acriflavin and rifampicin resistance markers. The frequencies of transformation at 2.5 minutes for acriflavin and rifampicin resistance markers were 1×10^{-7} and 1.2×10^{-7} respectively. The number of transformants then increased up to 90 minutes, in 20 minutes 30% of the final number of transformants being obtained (Fig. 1).

Time Required for Phenotypic Expression

The acriflavin and streptomycin markers were used for measuring the time required for these markers to be phenotypically expressed.

M. radiodurans was grown in TGY medium. 0.05 ml DNA was added to several tubes containing 0.3 ml of culture treated with

Fig. 1: Frequency of transformation as a function of the time of incubation of recipient bacteria with DNA. Cells were incubated with DNA from 2 to 90 minutes, inactivated with DNase and after this time 3 and 4 hours incubation allowed for expression of acriflavin and rifampicin markers respectively. The frequencies of transformation obtained after 90 minutes were 8.2×10^{-4} for acriflavin (○) and 4.1×10^{-3} for rifampicin (◌).


Percentage of transformants maximally reached
100
10
1
0
0
10
20
30
40
50
60
70
80
90
Time of exposure to DNA (min)

$CaCl_2$. DNase was added after 20 minutes and the culture incubated for a further 10 minutes. A large number of samples were each mixed with 10 ml TGY agar in a plate and incubated at 30°C to allow for expression for 5 minutes to 3 hours before a top layer of acriflavin agar was added or from 30 minutes to 6 hours before streptomycin agar was added. Colonies were counted after incubation for 4 days and the number of transformants as a function of the expression time allowed plotted (Fig. 2).

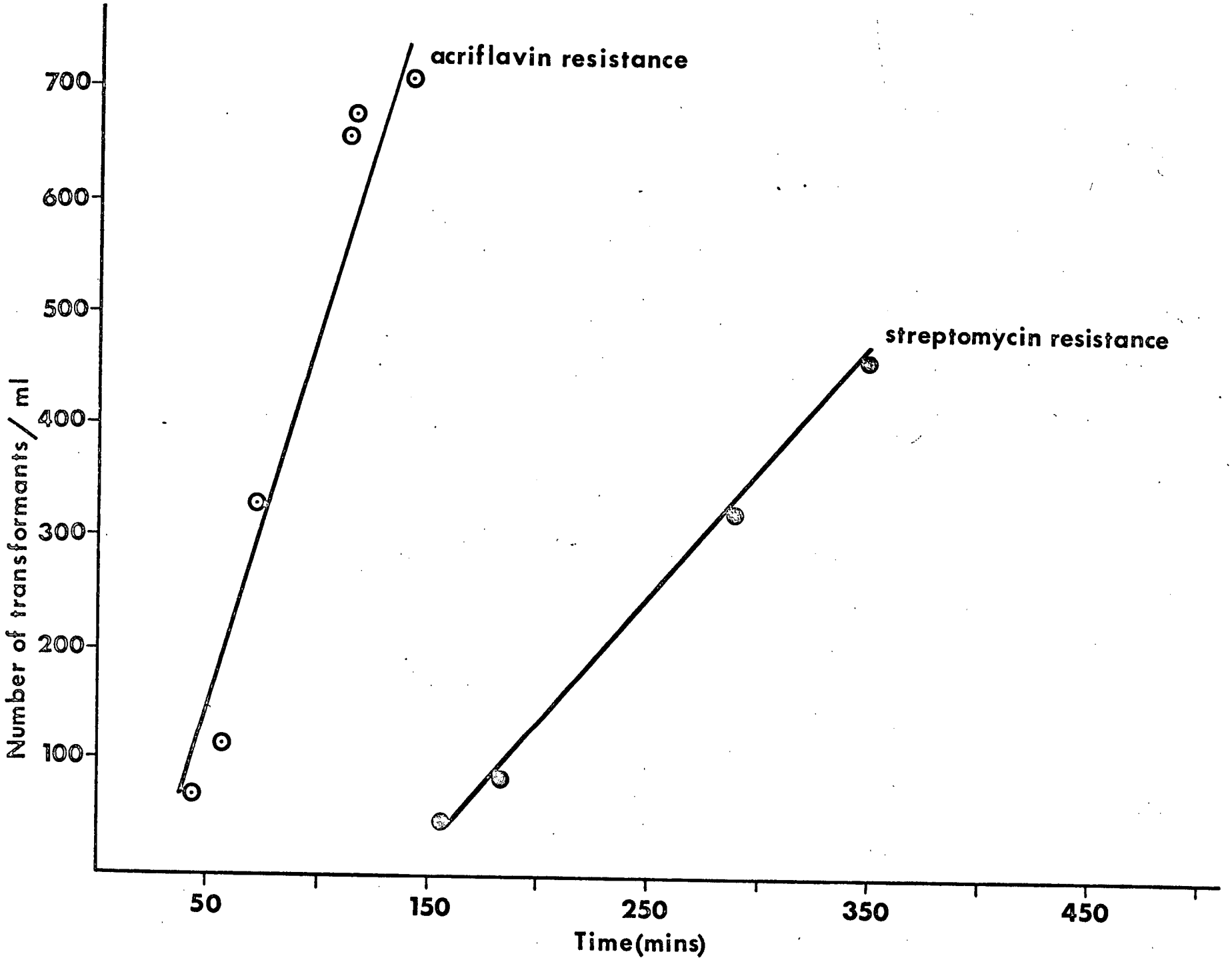
For acriflavin resistance, the minimum time required for phenotypic expression was 15 minutes after exposure to DNA for 20 minutes, i.e. 35 minutes. The frequency of transformation at this time was 1.6×10^{-7} and after a full expression time of 200 minutes, i.e. 180 minutes incubation after 20 minutes exposure to DNA, the frequency was 2.2×10^{-5}. Comparing these two frequencies indicates that 0.7% of the final number of transformants were obtained in 35 minutes.

The minimum time required for phenotypic expression of streptomycin resistance was 170 minutes (20 + 150). The frequency of transformation at this time was 8.0×10^{-7}. After an expression time of 7 hours, the frequency of transformation was 2.5×10^{-5}, which indicates that 3% of the final number of transformants were expressed in 170 minutes (Fig. 2).

Expression Time Required for Maximum Transformation Frequencies

An exponentially growing culture of M. radiodurans was centrifuged, resuspended in TGY containing 0.03 M $CaCl_2$ and exposed to KRASE DNA. After 2 hours incubation at 30°C to allow maximum uptake of DNA by the cell, 1 ml amounts of the transformed culture were mixed with 10 ml amounts of agar in several plates and the plates incubated from 0 to 8 hours. Every hour, a top layer

Fig. 2: λ_{minimum} Time required for phenotypic expression of acriflavin (⊙) and streptomycin (⊛) resistance. DNA was added to cells for 20 minutes, DNase was added for 10 minutes, samples diluted 10^{-1} and/or 10^{-2}. Maximum number of transformants obtained for acriflavin (200 minutes) was 1,080, and for streptomycin (440 minutes) was 1,250.


acriflavin resistance
streptomycin resistance
Number of transformants / ml
700
600
500
400
300
200
100
50
150
250
350
450
Time(mins)

containing an appropriate concentration of antibiotic was added and further incubation was carried out at 30°C for between 3 to 4 days for the formation of colonies. In the case of auxotrophic markers, selection of the prototrophs in TGY was not possible, so the transformed culture was diluted 10 times in TGY and each sample was washed in minimal medium and plated on minimal agar to score the prototrophs. The results (Fig. 3) showed that the number of transformants increased with the time at which selection was applied until a maximum level was reached. Table 6 shows the time for the maximum expression of each marker. Nutritional markers gave maximum values rapidly while streptomycin resistance needed an expression time of 7 to 8 hours to reach the maximum value.

Fig 3: Time required for maximum phenotypic expression of markers.

Markers: kanamycin ▣ , rifampicin □ , acriflavin △ , streptomycin ⊘ and erythromycin ○ resistance and prototrophy trp$^+$ ▲ .

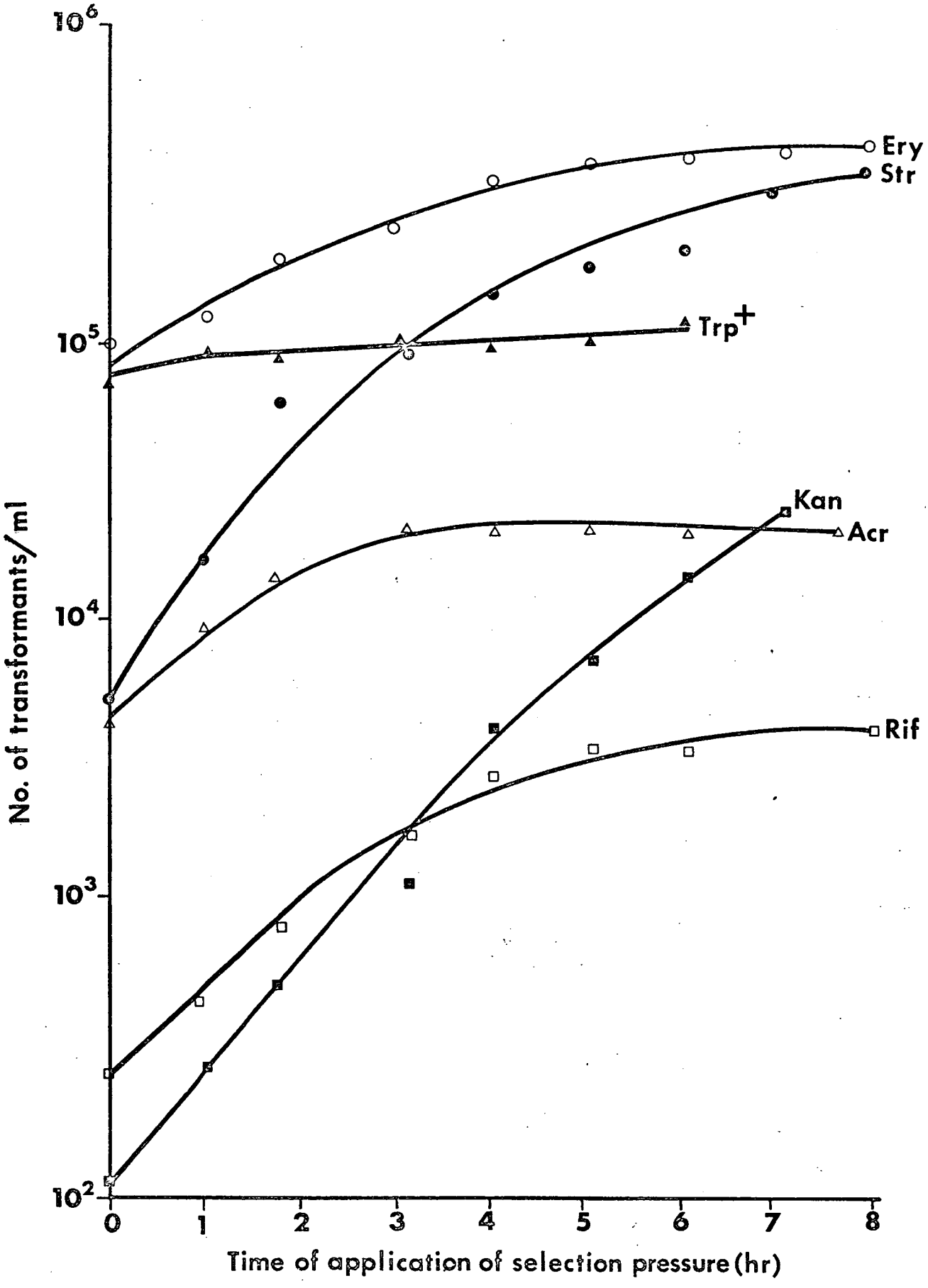

10^6
10^5
10^4
10^3
10^2
No. of transformants/ml
Ery
Str
Trp+
Kan
Acr
Rif
0
1
2
3
4
5
6
7
8
Time of application of selection pressure (hr)

Table 6: Time Required Before Applying Selection for Maximum Phenotypic Expression of Markers

Selected Markers	Time to Reach Maximum Expression (hr)
Adenine$^+$	1 - 2
Tryptophan$^+$	1 - 2
Acriflavin resistance	2 - 3
Rifampicin "	4 - 5
Erythromycin "	4 - 5
Kanamycin "	>7
Streptomycin "	7 - 8

PART II

Linkage Studies

Linkage Studies

The high transformation frequencies for individual markers resulting from treatment of M. radiodurans with $CaCl_2$ made it possible to attempt to find evidence of genetic linkage as the first step in constructing a map.

In transformation studies evidence for linkage is obtained by showing that molecules of transforming DNA which are quite small compared with the whole chromosome carry two or more individual genes on the same molecule. Since NTG causes multiple mutations near the replicating fork (Cerdá-Olmedo et al., 1968), it is reasonable to suppose that a high proportion of double mutants, obtained following NTG mutagenesis, should be linked. The criteria used for linkage were based on:-

a) The appearance of the dilution curve in which the numbers of single and double transformants are plotted as a function of the transforming DNA concentration. Double events, requiring two individual DNA molecules to give rise to double transformants of unlinked genes, should become very rare as the DNA concentration is reduced. Hence the number of such transformants will fall off more steeply than those due to linked genes which require only a single DNA molecule carrying both genes (Butler and Nicholas, 1973).

b) Calculation of the co-transfer index of Nester and Lederberg (1961) and the linkage index of Butler and Nicholas (1973) from the results.

c) The clonal analysis of double transformants.

Isolation of Double Mutants

All auxotrophs isolated were tested on minimal medium supplemented with different growth factors to detect those mutants

which had a double auxotrophic requirement. Antibiotic-resistant mutants were tested for auxotrophy by streaking the colonies on minimal medium. The number of auxotrophs detected after mutagenesis of the wild type with NTG was 3% of the surviving population. Mutants resistant to kanamycin, rifampicin, streptomycin, erythromycin and spectinomycin comprised 0.5% of the population, the majority being spectinomycin-resistant.

Auxotrophs and antibiotic-resistant mutants were plated at permissive (30°C) and restrictive (39°C) temperatures. Nineteen out of 500 auxotrophic or antibiotic-resistant mutants (c. 4%) did not grow at 39°C.

All double mutants were tested for possible linkage between markers. Evidence was obtained for linkage of two genes in each of 4 such mutants and is shown below, together with a description of each strain.

1. Mutant ST9, which is Temperature-Sensitive and Requires Tyrosine for Growth

This mutant required 100 µg/ml of tyrosine for growth and was temperature sensitive, being unable to grow at the restrictive temperature (39°C) on TGY agar or minimal medium supplemented with tyrosine.

It was transformed with DNA extracted from the wild type and transformants selected for either prototrophy or temperature-resistance or both.

Evidence from the dilution curves, co-transfer and linkage indices and clonal analysis showed that about 2.6% of the DNA molecules which carry the gene involved in tyrosine synthesis also carry the gene for temperature resistance.

Dilution Curves

The standard DNA concentration was 500 μg/ml and dilutions were made to 150, 50, 15, 5 and 1.5 μg/ml. Small volumes (0.3 ml) of competent cultures of ST9 were transformed with constant volumes of DNA (0.05 ml) and transformants selected. With standard DNA the frequency of single transformants to prototrophy and temperature-resistance were 1.2×10^{-3} and 1.3×10^{-3} respectively and the frequency of double transformants 1.4×10^{-4}. The spontaneous frequency of temperature-resistant mutants was 2×10^{-8}. When cells were plated on unsupplemented minimal medium, no spontaneous mutation was observed. The dilution curve and linkage relationships are shown in Fig. 4 and Table 7.

Eight hundred and fifty colonies of *tyr*$^+$ transformants, obtained from transformation of ST9 with DNA from the wild type, were tested at the restrictive temperature (39^{o}C) and 22 of them grew giving a value of 2.6% linkage between the genes.

Calculation of Linkage Indices

The degree of linkage of two genes (*tyr* and ts) can be expressed as a co-transfer index (Nester and Lederberg, 1961) or as a linkage index (Butler and Nicholas, 1973). The values of linkage and co-transfer indices for standard DNA are 0.011 and 0.059 respectively. The values for lower concentrations of DNA are shown in Table 7. The linkage relationship of two markers (*tyr* and ts), their frequency and the percentage of linkage at varying concentrations of DNA and by clonal analysis for pairs of markers are summarized in Tables 7 and 12.

Measurement of DNA, RNA and Protein Synthesis in Mutant ST9

Cultures of the temperature sensitive mutant ST9 and the wild type were grown in TGY. The uptake of [^{3}H] thymidine, [^{3}H] uridine and [^{3}H] leucine into TCA-insoluble material as an

Fig. 4: Dilution curves resulting from transforming mutant ST9 (tyr and ts) with DNA from the wild type. Selected transformants: tyr$^+$ (◐), ts$^+$ (O) and tyr and ts$^+$ (◘).

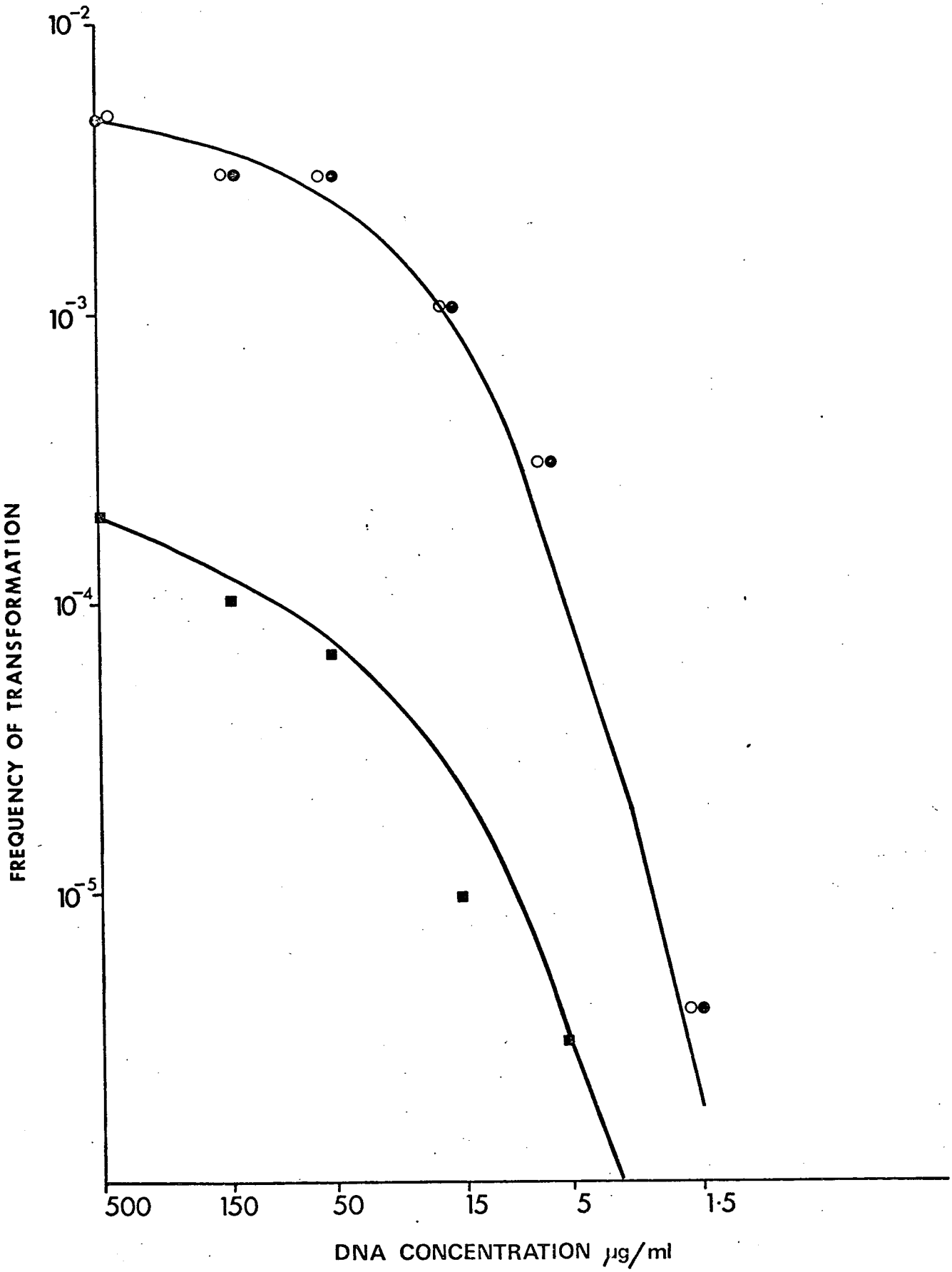

10^{-2}
10^{-3}
10^{-4}
10^{-5}
FREQUENCY OF TRANSFORMATION
500
150
50
15
5
1·5
DNA CONCENTRATION µg/ml

Table 7: The Frequencies of Transformation for Single and Double Transformants at Varying Concentrations of DNA

Wt DNA Concentration µg/ml	Frequency of $\underline{tyr}^+$ Transformants	Frequency of ts^+ Transformants	Frequencies of $\underline{tyr}^+ ts^+$ Transformants	Linkage Index	Co-Transfer Index	Clonal Analysis Co-transformant / No. Tested
500	1.2×10^{-3}	1.3×10^{-3}	1.4×10^{-4}	0.011	0.05	$\frac{5}{150}$
150	9×10^{-4}	8.5×10^{-4}	7.5×10^{-5}	0.010	0.04	$\frac{4}{150}$
50	8.5×10^{-4}	7.8×10^{-4}	1.3×10^{-5}	0.051	0.008	$\frac{5}{150}$
15	1.3×10^{-4}	1.1×10^{-4}	9.5×10^{-6}	0.0015	0.04	$\frac{4}{150}$
5	8.1×10^{-5}	7.8×10^{-5}	1.5×10^{-6}	0.004	0.01	$\frac{2}{150}$
1.5	2.9×10^{-6}	1.8×10^{-6}	5×10^{-8}	0.0001	0.01	$\frac{2}{100}$

The values for the linkage and co-transfer indices were calculated from the data.

indication of DNA, RNA and protein synthesis respectively was measured. All the cultures were incubated at 30°C for 30 minutes then shifted to 39°C and the uptake of the radioactive compounds followed. When the temperature was raised to 39°C, the synthesis of RNA ceased after a short period of time (60 minutes), but synthesis of DNA and protein continued (Fig. 5 and 6).

2. Mutant ST12, which Requires Tryptophan and is Temperature-Sensitive

This mutant required 100 µg/ml tryptophan. Pyridoxin could substitute for tryptophan at 30°C, but not at 39°C. Clonal analysis of 800 colonies of the mutant by streaking them onto plates containing anthranilic acid, indole, tryptophan or pyridoxin showed good growth on tryptophan or pyridoxin, but no growth on anthranilic acid or indole. It is probable that the biochemical block has occurred in the tryptophan pathway between indole and tryptophan where pyridoxal phosphate is required as a cofactor. Mutant ST12 grew well in TGY or minimal medium supplemented with tryptophan or pyridoxin at 30°C.

The mutant was also temperature sensitive for some unknown reaction in which folic acid satisfies a requirement. Thus the presence of folic acid enabled the strain to grow at the restrictive temperature (39°C). The spontaneous mutation frequency to temperature resistance was 2.5×10^{-7}. The dilution curve, co-transfer and linkage indices and clonal analysis of transformants indicated a very close linkage of about 84% between the genes required for tryptophan synthesis and temperature resistance.

Dilution Curve

The mutant was transformed with varying concentrations of DNA from the wild type and single and double transformants

Fig. 5: Uptake of [^{3}H] thymidine at 30°C (▲) and 39°C (△), [^{3}H] uridine at 30°C (●) and 39°C (○) and [^{3}H] leucine at 30°C (■) and 39°C (□) into the TCA-insoluble fraction of mutant ST9 as a function of time. The temperature was raised to 39°C after 30 minutes at 30°C and is indicated by the arrow.

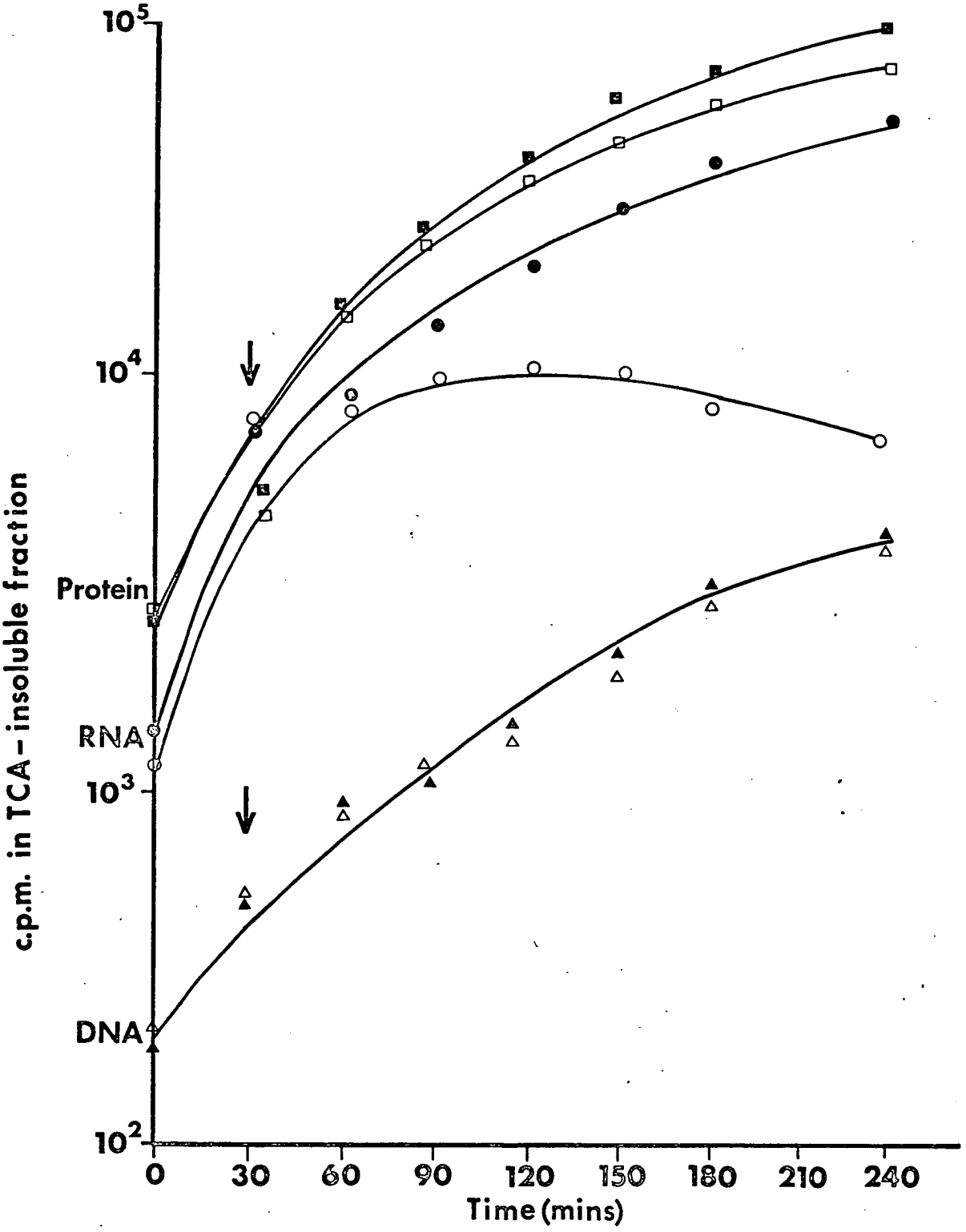
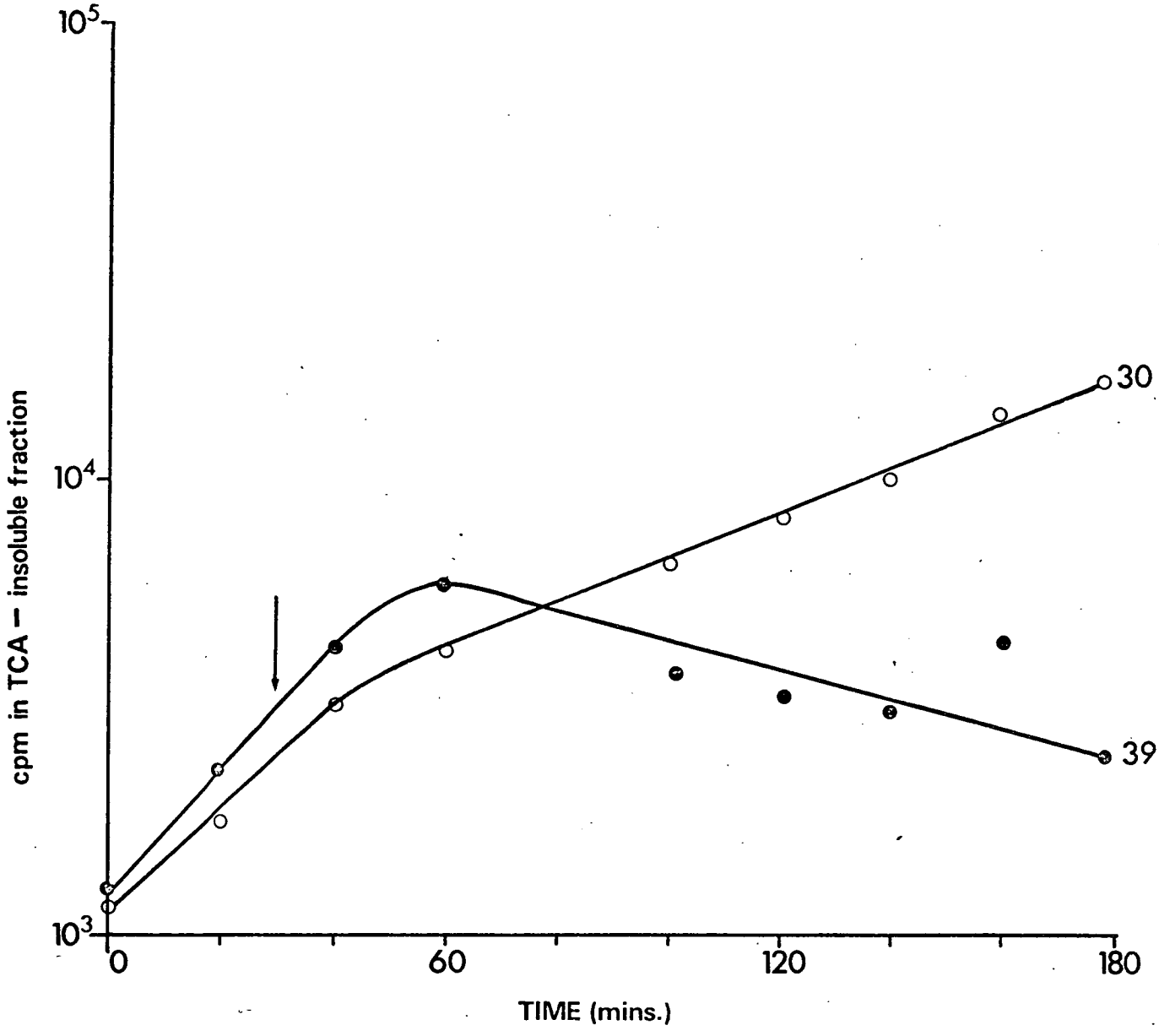

10^5
10^4
10^3
10^2
Protein
RNA
DNA
c.p.m. in TCA – insoluble fraction
0
30
60
90
120
150
180
210
240
Time (mins)

Fig. 6: Uptake of [^{3}H] uridine at 30°C (O) and 39°C (●) into the TCA-insoluble fraction of mutant ST9 as a measure of RNA synthesis. The temperature was raised to 39°C after 30 minutes at 30°C as indicated by the arrow.


10^5
10^4
10^3
cpm in TCA – insoluble fraction
0
60
120
180
TIME (mins.)
30
39

selected in the same manner as described for mutant ST9.

With standard DNA (500 µg/ml), the transformation frequencies obtained for the two single markers were 1.5×10^{-3} and 1.8×10^{-3}, whilst the frequency of double transformants was 1.2×10^{-3}. These frequencies and the curves obtained with varying concentrations of DNA indicates very close linkage between two markers (Fig. 7, Table 8).

The values for the co-transfer and linkage indices were 0.6 and 0.0022 for standard DNA respectively.

Clonal analysis of 800 colonies of transformants selected for prototrophy showed that 672 (84%) were also carrying the temperature-resistance gene.

All the results for mutant ST12 are summarized in Table 8 and 12.

3. Mutant ST1, which is Kanamycin Resistant and Requires Glycine for Growth

This strain was isolated as a mutant resistant to 30 µg/ml kanamycin. It was also an auxotroph requiring 100 µg/ml of glycine for growth. After transformation of ST1 with DNA extracted from the wild type, transformants were selected for prototrophy and tested for their sensitivity to kanamycin. The wild type was also transformed with DNA from ST1, kanamycin-resistant transformants selected and these tested for auxotrophy.

Dilution Curves

Mutant ST1 was transformed with various concentrations of wild type DNA as described previously. Selection, of course, could only be made for prototrophic transformants. Such transformants occurred at a frequency of 3×10^{-4} for the standard DNA and 6×10^{-7} for the lowest concentration (1.5 µg/ml) (Fig.

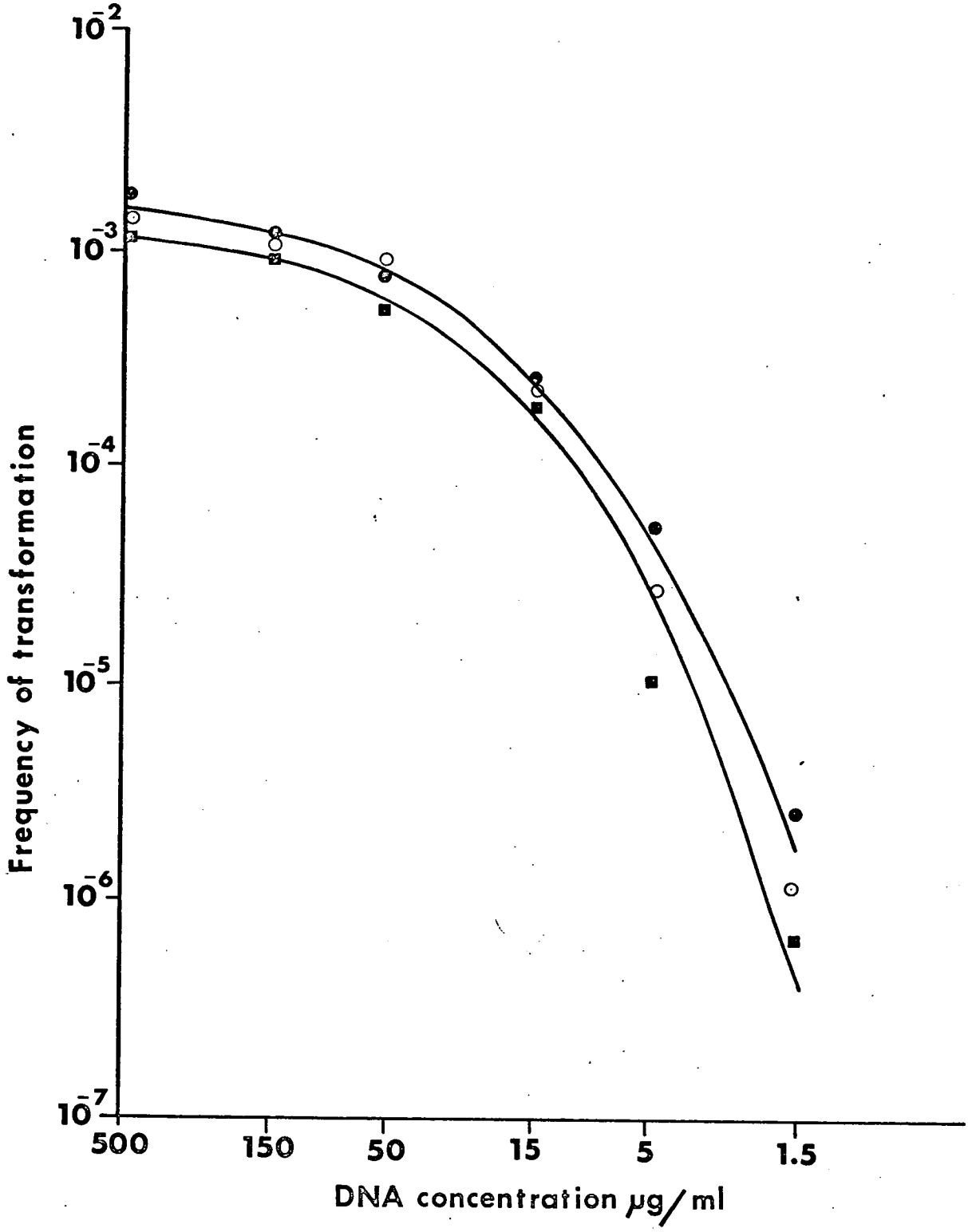

10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
10^{-7}
Frequency of transformation
500
150
50
15
5
1.5
DNA concentration µg/ml

Table 8: The Frequencies of Transformation for Single and Double Transformants at Various Concentrations of Transforming DNA

Wt DNA Concentration µg/ml	Frequency of trp^+ Transformants	Frequency of ts^+ Transformants	Frequency of trp^+ ts^+ Transformants	Linkage Index	Co-Transfer Index	Clonal Analysis Co-Transformants / No. Tested
500	1.8×10^{-3}	1.5×10^{-3}	1.2×10^{-3}	0.0022	0.6	171/200
150	1.1×10^{-3}	1.1×10^{-3}	9×10^{-4}	0.0013	0.7	159/200
50	8×10^{-4}	8.2×10^{-4}	6.2×10^{-4}	0.0010	0.6	180/200
15	2.5×10^{-4}	2.1×10^{-4}	1.8×10^{-4}	0.0002	0.6	80/100
5	5×10^{-5}	2.6×10^{-5}	1×10^{-5}	0.0001	0.1	82/100
1.5	2.5×10^{-6}	1.2×10^{-6}	6.5×10^{-7}	0.000005	0.2	-

The values for linkage and co-transfer indices were calculated from the data.

8, Table 9 and 12.

The frequency of double transformants was determined by clonal analysis of the single transformants. Clonal analysis was made of 1,000 colonies of single transformants. The transformants were obtained both from transforming ST1 with wild type DNA and selecting for prototrophy and transforming wild type with ST1 DNA and selecting for kanamycin resistance. Forty of the 1,000 colonies possessed both characters, indicating that 4% of the DNA molecules which carry the gene for kanamycin sensitivity also carry a gene involved in glycine synthesis. The results are shown in Table 9 and 12. Because it was not possible to select double transformants, values for the co-transfer and linkage indices could not be calculated.

4. Mutant ST5, which is Kanamycin Resistant and Temperature Sensitive for Growth

This mutant was resistant to kanamycin at concentrations up to 50 μg/ml. Transformation of ST5 with varying concentrations of wild type DNA and clonal analysis of temperature-resistant transformants, and transformation of the wild type with varying concentrations of ST1 DNA and clonal analysis of transformants showed 10% linkage between genes for kanamycin sensitivity and temperature resistance.

Dilution Curves

Transformants selected for temperature resistance occurred at a frequency of 6.5×10^{-4} for standard DNA and 2.5×10^{-6} for the lowest concentration of DNA (1.5 μg/ml) (Fig. 9 and Table 10).

Transformants selected for kanamycin resistance occurred at a frequency of 3×10^{-5} for standard DNA and 1×10^{-7} for the dilution of 5 μg/ml.

Fig. 8: Dilution curves, resulting from transforming mutant ST1 (*kan*^{r}, *gly*) with DNA from the wild type. Selected transformants are: *gly*$^+$ (⊛).

The number of double transformants was calculated from clonal analysis of the prototrophic transformants for kanamycin sensitivity (O).

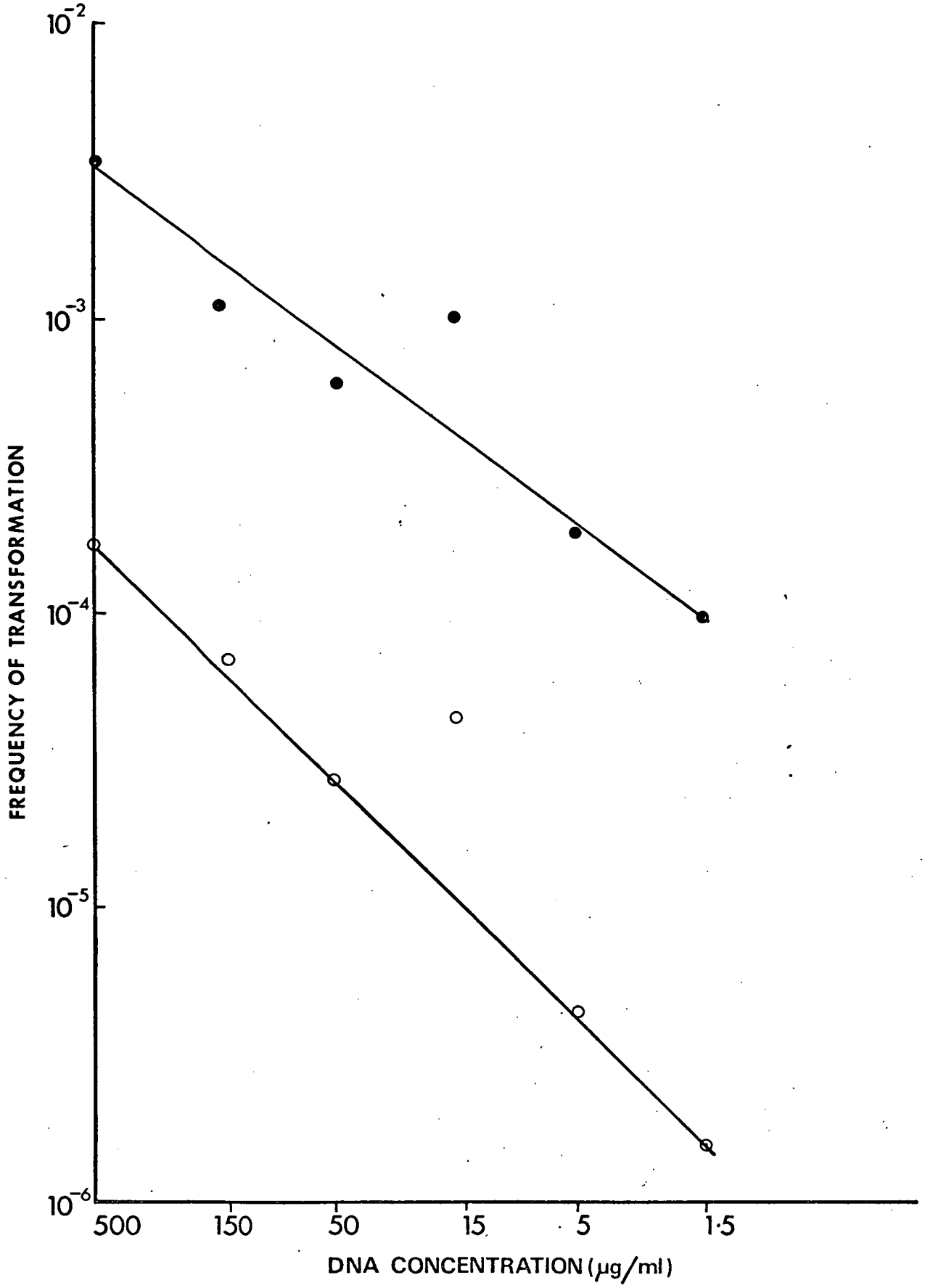

10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
FREQUENCY OF TRANSFORMATION
500
150
50
15
5
1·5
DNA CONCENTRATION (μg/ml)

Table 9: Mutant ST1. Frequencies of Single Transformants Selected for Kanamycin Resistance or Prototrophy in Appropriate Experiments, at Various Concentrations of DNA

ST1 DNA Concentration µg/ml	Frequency of kan^r Transformants	Clonal Analysis Co-Transformants No. Tested	Wt DNA Concentration µg/ml	Frequency of gly^+ Transformants	Clonal Analysis Co-Transformants No. Tested
500	3×10^{-4}	4/110	500	9.4×10^{-4}	6/120
150	1.5×10^{-5}	5/110	150	2.1×10^{-4}	4/110
50	5×10^{-6}	5/110	50	8.2×10^{-5}	4/110
15	2.1×10^{-6}	4/110	15	2.1×10^{-5}	4/110
5	1×10^{-6}		5	5×10^{-6}	4/110
1.5	6×10^{-7}		1.5	1.5×10^{-6}	

The number of double transformants determined by clonal analysis is shown.

Of 500 single transformants to temperature resistance, 55 were also sensitive to kanamycin and of 500 single transformants to kanamycin resistance, 45 were also temperature sensitive. The average value obtained from clonal analysis indicated 10% linkage between the two genes (Table 10 and Table 12).

Values of the co-transfer and linkage indices could not be calculated.

Measurement of DNA, RNA and Protein Synthesis in ST5

The mutant was incubated at 30°C for 30 minutes in the presence of [^{3}H] thymidine, [^{3}H] uridine or [^{3}H] leucine and then shifted to 39°C and the uptake of radioactivity into TCA-insoluble material was followed as before. Although synthesis of DNA, RNA and protein did not stop immediately after raising the cultures to the restrictive temperature, all ceased after varying times (Fig. 10). It is not clear what the primary cause of the defect was which caused the mutant to be temperature sensitive (Fig. 10).

Ilv Mutants

Among auxotrophs which were tested for double growth requirements, 5 out of 100 required both isoleucine and valine. These mutants were grown in TGY broth, diluted and plated on minimal medium supplemented with either isoleucine or valine. Three hundred colonies of each were tested for the second requirement. All the colonies required both isoleucine and valine.

Unlinked Markers

Varying dilutions of DNA extracted from the KRASE strain of M. radiodurans, having two unlinked markers (resistance to 50 and 5 µg/ml of rifampicin and acriflavin respectively) were used

Fig. 9: Dilution curves, resulting from transforming ST5 mutant (*kan*^{r}, ts) with wild type DNA. Transformants are: ts$^+$ (●) and ts$^+$ and *kan*^{s} (O).

The number of double transformants was calculated from clonal analysis of the temperature resistance transformants for kanamycin sensitivity (O).

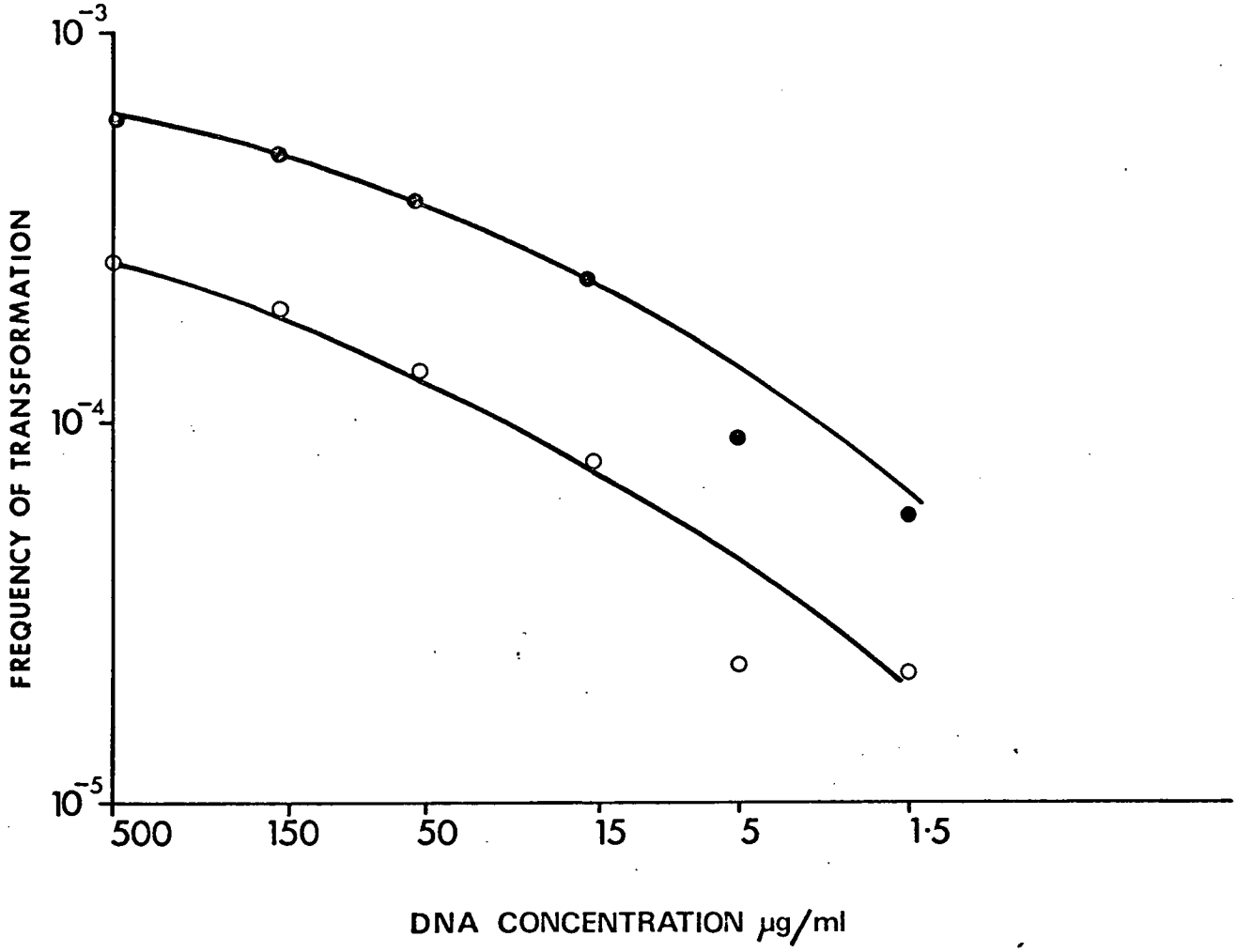
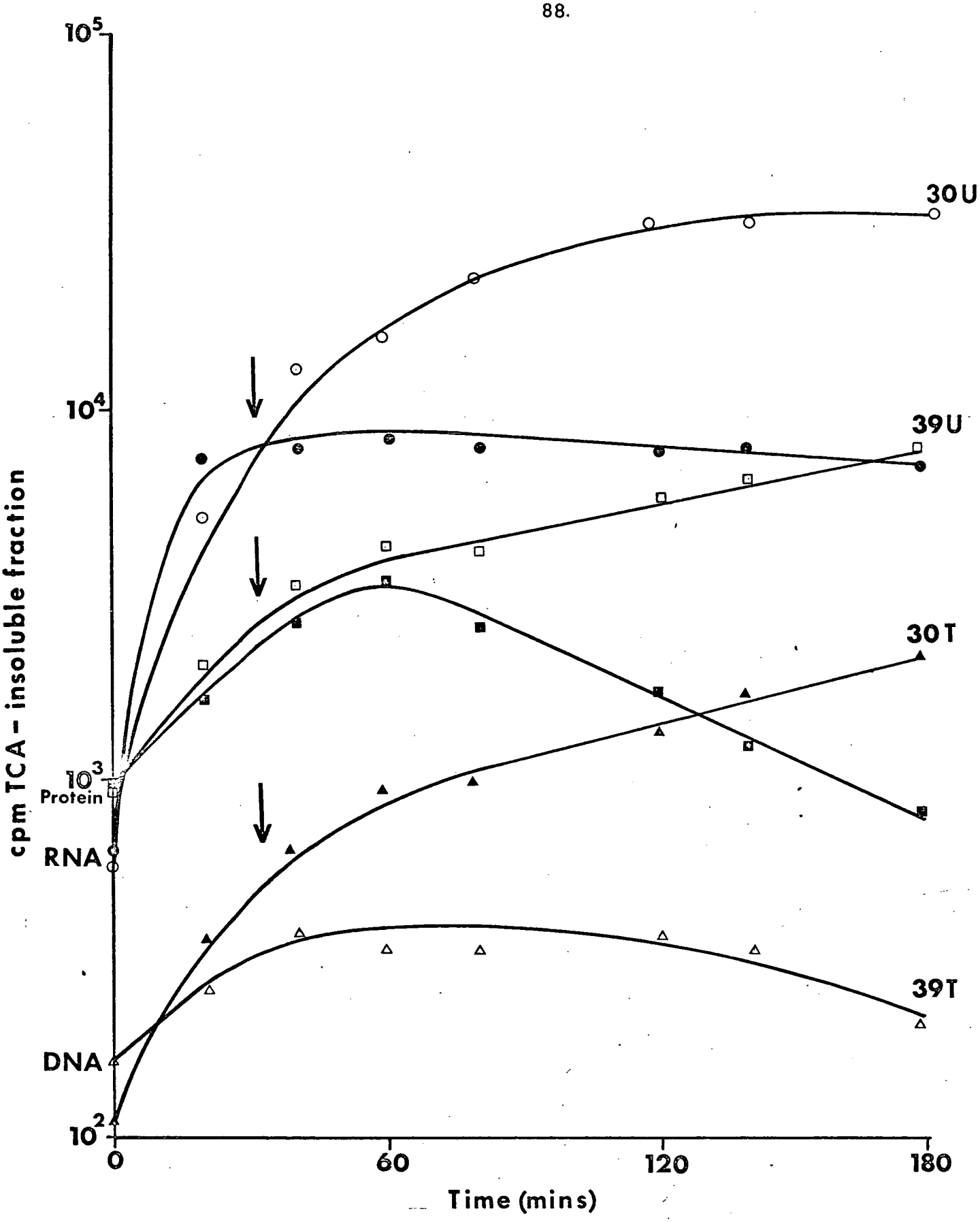

10^{-3}
10^{-4}
10^{-5}
FREQUENCY OF TRANSFORMATION
500
150
50
15
5
1·5
DNA CONCENTRATION μg/ml

Table 10: Mutant ST5. Frequencies of Single Transformants Selected for Kanamycin Resistance or Temperature Resistance in Appropriate Experiments, at Various Concentrations of DNA

ST5 DNA Concentration µg/ml	Frequency of kan^r Transformants	Clonal Analysis Co-Transformants No. Tested	Wt DNA Concentrations µg/ml	Frequency of ts^+ Transformants	Clonal Analysis Co-Transformants No. Tested
500	1.5×10^{-4}	20/200	500	6.5×10^{-4}	16/110
150	8×10^{-5}	15/200	150	2.1×10^{-4}	13/110
50	3×10^{-5}	10/100	50	8×10^{-5}	15/110
15	7.5×10^{-6}	-	15	2×10^{-5}	8/110
5	1.5×10^{-6}	-	5	5.5×10^{-6}	3/60
1.5	4.5×10^{-7}	-	1.5	2.5×10^{-6}	

The number of double transformants determined by clonal analysis is shown.

Fig. 10: Uptake of [^{3}H] thymidine at 30^o (▲) and 39^o (Δ), [^{3}H] uridine at 30^o (O) and 39^o (●) and [^{3}H] leucine at 30^o (□) and 39^o (■) into TCA-insoluble fraction of ST5 mutant as a measure of DNA, RNA and protein synthesis. The temperature was raised to 39^{o}C after 30 minutes at 30^{o}C as indicated by the arrow.


10^5
10^4
10^3
10^2
Protein
RNA
DNA
30U
39U
30T
39T
0
60
120
180
Time (mins)
cpm TCA – insoluble fraction

to transform a wild type recipient. Transformants were selected which were resistant to either or both antibiotics.

The frequency of rifampicin-resistant transformants was 3 x 10^{-3} and for acriflavin resistance was 1.3 x 10^{-3} with concentrations of 500 µg DNA/ml, whilst for double transformants the frequency obtained was 4 x 10^{-6}. The number of double transformants fell off rapidly as the DNA concentration decreased (Fig. 11), until at the lowest concentrations (1.5 and 5 µg/ml) no double transformants were obtained.

Clonal analysis was carried out on 200 colonies of each single transformant to detect if they had been co-transformed with the other marker. No double transformants were detected.

The values of co-transfer and linkage indices of these two unlinked genes for standard DNA are 0.9 and 0.0009 respectively (Table 11). These values are compared with linked markers in Table 12.

Relationship of Two Kanamycin Resistance Mutants

The two mutants ST1 and ST5 were both selected as kanamycin resistant and shown to be linked to an auxotroph and temperature sensitive gene respectively. Since these two mutants had different levels of resistance, it was necessary to determine whether these two mutants were alleles of the same gene. If they were, then the gene involved in glycine synthesis and the gene producing a temperature sensitive product would also be linked.

DNA extracted from ST5 was used to transform mutant ST1 and transformants selected for prototrophy.

Five hundred of these were streaked onto TGY plates and incubated at the restrictive temperature to detect any temperature

Fig. 11: Dilution curves, resulting from transforming the wild type strain with DNA from the KRASE strain. Selected transformants: <u>rif</u>^{r} (●), <u>acr</u>^{r} (O) and <u>rif</u>^{r} and <u>acr</u>^{r} (■).

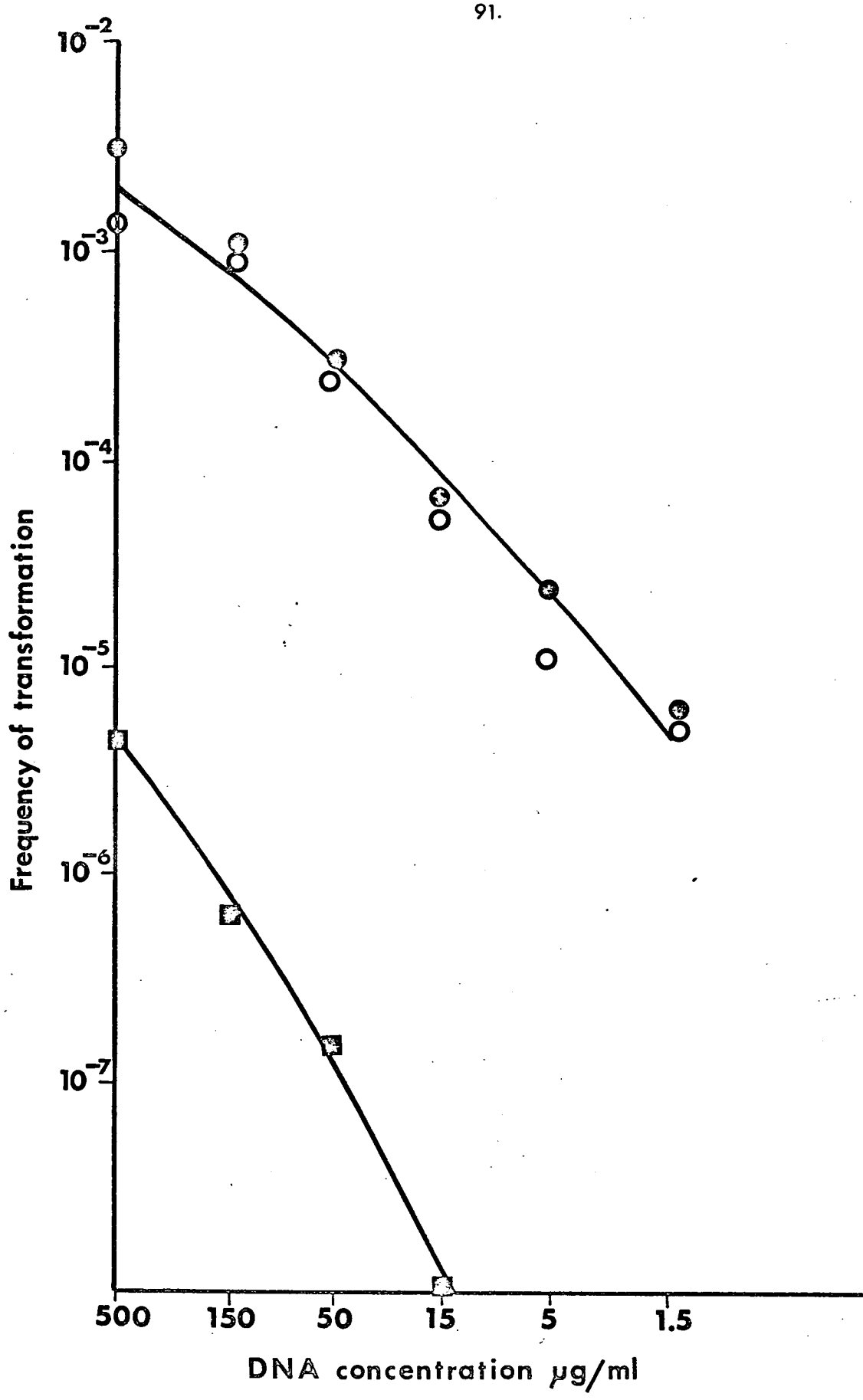

Frequency of transformation
10⁻²
10⁻³
10⁻⁴
10⁻⁵
10⁻⁶
10⁻⁷
500
150
50
15
5
1.5
DNA concentration µg/ml

Table 11: The Frequencies of Transformation for Single and Double Transformants as a Function of the Concentration of Transforming DNA

KRASE DNA Concentration µg/ml	Frequency to $\underline{Acr}^r$	Frequency to $\underline{Rif}^r$	Frequencies to $\underline{Rif}^r$ and $\underline{Acr}^r$	Linkage Index	Co-Transfer Index	Clonal Analysis Co-Transformants / No. Tested
500	1.3×10^{-3}	3×10^{-3}	4×10^{-6}	0.9	0.0009	$\frac{0}{100}$
150	8.5×10^{-4}	9.2×10^{-4}	7.2×10^{-7}	1.0	0.0004	$\frac{0}{100}$
50	2.5×10^{-4}	3.1×10^{-4}	1.5×10^{-7}	0.5	0.0002	$\frac{0}{50}$
15	5.8×10^{-5}	6.9×10^{-5}	1×10^{-8}	0.4	0.00005	$\frac{0}{50}$
5	1.1×10^{-5}	2.3×10^{-5}	-	-	-	-
1.5	5×10^{-6}	6.1×10^{-6}	-	-	-	-

The values for linkage and co-transfer indices were calculated from the data.

Table 12: Percentage Linkage of Markers as Determined by Clonal Analysis and the Values of Linkage and Co-Transfer Indices where Appropriate

Recipient	DNA Standard	% of Linkage by Clonal Analysis	Linkage Index	Co-Transfer Index
ST9 (*tyr*$^-$ *ts*)	Wt (*tyr*$^+$ *ts*$^+$)	2.5	0.01	0.05
ST12 (*trp*$^-$ *ts*)	Wt (*trp*$^+$ *ts*$^+$)	85	0.0022	0.5
ST1 (*kan*^{r} *gly*$^-$)	Wt (*kan*^{s} *gly*$^+$)	4.0	-	-
ST5 (*kan*^{r} *ts*)	Wt (*kan*^{s} *ts*$^+$)	10	-	-
Wt (*rif*^{s} *acr*^{s})	KRASE (*rif*^{r} *acr*^{r})	0	0.9	0.0001

sensitive clones. Among 500 colonies, none were temperature sensitive.

U.V. Sensitivity of the Mutants

Mutants were streaked on TGY plates and irradiated with doses of 675, 1350 and 2250 J/m^2. All mutants had the same level of resistance as the wild type.

PART III

Transfer of Plasmids

Plasmid

Although there is no report of plasmid transfer from members of the Enterobacteriaceae to any gram-positive bacterium, or Micrococcus radiodurans, a group of plasmids with known resistance factor activity was tested in crosses with M. radiodurans.

The first plasmid used was RP4, which has both a wide host range and carries three selectable antibiotic resistance markers.

As a control, crosses were first carried out between strains of E. coli in order to measure the frequency of transfer of the plasmid under conditions where it is known to transfer. Crosses were made between the donor J5-3 Nal^r, carrying RP4 which confers resistance to kanamycin, tetracycline and penicillin and the recipient W1106 Str^r, which carries a chromosomal gene responsible for streptomycin resistance. Crosses were performed both in liquid and on solid media.

Crosses Between Strains of Escherichia coli in Liquid Medium

The first crosses were carried out in NB or TGY as described in the Materials and Methods section. After each cross, dilutions of donor, recipient and donor/recipient mixture were plated on antibiotic-free medium and on selective medium. After 24 hours of incubation viable counts of donors, recipients and R^+ recipients were determined. The crosses carried out in NB or TGY had a frequency of transfer varying from 10^{-4} to 10^{-3} per recipient plated (Table 13). Transfer was observed in all donor/recipient conjugation experiments.

All putative R^+ recipients (W1106) selected for kanamycin were plated on NA or TGY agar containing 100 µg/ml penicillin or 25 µg/ml tetracycline to test the clones selected on one antibiotic for resistance to the other antibiotics carried by the

plasmid. Since transfer occurred in liquid medium, it seemed that this was a useful mating procedure to use.

Crosses Between Strains of E. coli on Plates

To improve the frequency of transfer, crosses were performed between J5-3 Nal^r and W1106 Str^r on a filter disc on an agar plate. Donor and recipient were mixed and passed through a filter as described in Materials and Methods. The transfer of RP4 was then followed. Fifty per cent of the recipients received RP4 and became R^+. The frequencies of transfer resulting from two different crosses are summarized in Table 13.

No differences in frequencies were observed when crosses were made in TGY or NB.

Crosses Between Strains of Streptococcus faecalis

Crosses were performed between JH1-2, carrying plasmid pJH1, as donor and JH2-2 as recipient on a filter disc on a NA plate. 4×10^9 recipients were mixed with 10^9 donors and transconjugants were selected on a plate containing kanamycin and rifampicin. 0.05% of the recipients received the plasmid. The frequency was lower than that observed in crosses between strains of E. coli. All transconjugants obtained on plates containing kanamycin were tested for resistance to other antibiotics (streptomycin, tetracycline and penicillin) carried by the plasmid.

Crosses Between M. radiodurans and E. coli or S. faecalis

E. coli J5-3 Nal^r carrying RP4 was crossed with one strain of M. radiodurans resistant to rifampicin (Rif^r) on a filter disc on TGY agar plates. In five crosses performed, the number of recipient cells in the mixture was between 9 to 12×10^8/ml and the number of donor cells about 10^9/ml.

Table 13: Transfer of the R-Factor RP4 in Strains of E. coli

Strains of E. coli	Kan (25) µg/ml	Str (50) µg/ml	Kan (25) + Str (50) µg/ml	Frequency of Trans-conjugants per Recipient
	Number of Cells/ml	Number of Cells/ ml	Number of Transconju-gants/ml	
J5-3 Nal^r donor	5×10^8	0	-	
W1103 Str^r recipient	0	3.5×10^8	-	
Cross be-tween J5-3 and W1106 in broth	-	1.3×10^8	2×10^5	1.5×10^{-3}
Cross be-tween J5-3 and W1106 on plates	-	1.3×10^8	7×10^7	5.4×10^{-1}

After incubation, the mixture was diluted and 10^9 recipients plated on plates containing individual antibiotics to which RP4 confers resistance. In all five experiments, no colonies were detected on any of the antibiotic plates. The data indicate that the frequency of transconjugants was less than 1×10^{-9} in each cross.

Crosses between M. radiodurans (Rif^r) and S. faecalis (JH1-2) were carried out with the same method described above. The number of recipients plated on selected media containing individual antibiotics was 10^9 cells/plate. In three crosses performed, no transconjugants were obtained, which indicates that the frequency of conjugation is again less than 10^{-9}.

Transformation of M. radiodurans with Plasmid DNA

The usefulness of transforming bacteria with purified plasmid DNA has been demonstrated among transformable strains of E. coli and S. faecalis (Cohen et al., 1972; LeBlanc and Hassell, 1976). It seemed reasonable, therefore, to attempt to transform M. radiodurans using purified plasmid DNA.

Purification of Plasmid DNA

Purified RP4 DNA was prepared from E. coli J5-3 using the methods of both Cohen and Miller (1969, 1970) and Humphreys et al. (1975).

The method of Humphreys and his co-workers using polyethylene glycol 6000 (PEG) for the preparation of large quantities of plasmid DNA was successful (Plate 1). One litre of culture containing 10^9 cells/ml was used for plasmid DNA preparation. If one copy of RP4 with a molecular weight of 4×10^7 (Datta et al., 1971; Barth and Grinter, 1974) is present in each cell of E. coli, the amount of RP4 DNA present in one litre of culture is

approximately 67 μg.

In the method of Humphreys et al. (1975), assuming that all the plasmid DNA was precipitated with PEG, 67 μg of plasmid DNA would be present in the centrifuge tube. In fact, from one litre of culture 44 μg plasmid DNA was recovered and the plasmid band was clearly visible (Plate 1B).

In the method of Cohen, PEG precipitation was not used and the amount of DNA in the gradient was much less (3.5 μg DNA/ tube), so the plasmid band was not visible (Plate 1A).

Isolation of Radioactively-Labelled Plasmid DNA

Although radioactive labelling of DNA was unnecessary in large scale preparation since the plasmid band was clearly visible in the gradient (Plate 1B), for further studies on transformation and estimation of the loss of DNA during preparation, plasmid DNA was labelled.

For the incorporation of [^{3}H] thymidine for labelling E. coli, strain W1103 (R^-) was used, which had a requirement for 5 μg/ml of thymine and 100 μg/ml of tryptophan for growth. For transferring the plasmid from strain J5-3 containing RP4 into strain W1103, crosses were performed using a filter method between donor and recipient. Clones of transconjugants were $RP4^+$, thy^- and trp^- which were then used for labelling purposes.

Strain W1103 ($RP4^+$, thy^-, trp^-) was tested for growth and uptake of labelled thymine in M9 minimal medium supplemented with 100 μg/ml of tryptophan and varying concentrations of thymine. This experiment was used to determine the lowest concentration of thymine which allows the maximum incorporation of [^{3}H] thymidine in this strain. Several tubes, each containing 1 ml of M9 medium supplemented with 5 μCi of [6-^{3}H] thymidine, 100 μg/ml of

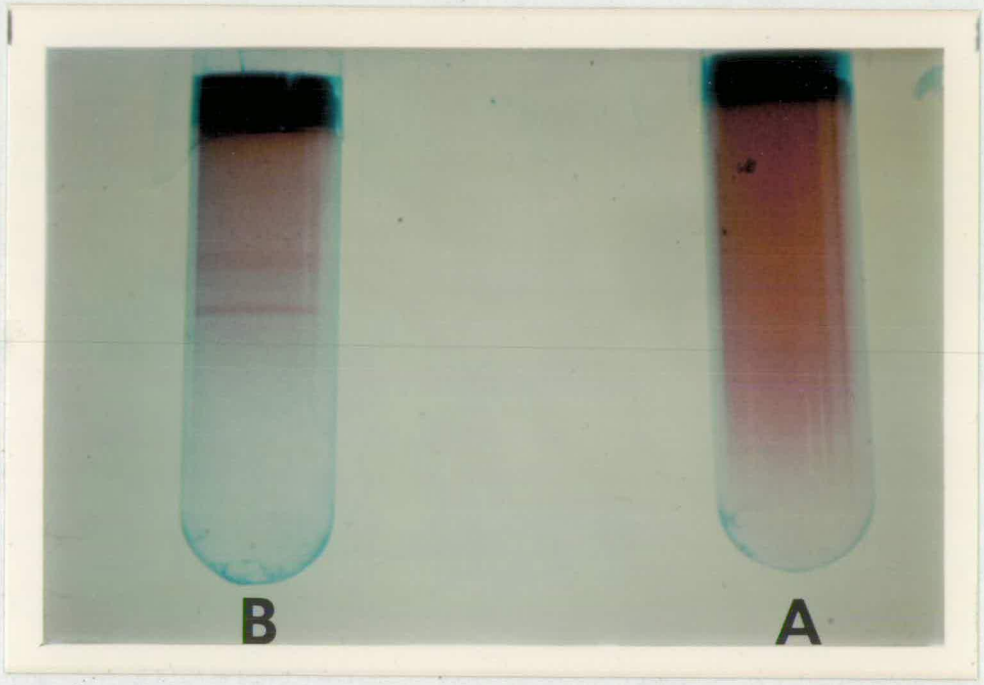


Plate 1: Tubes A and B containing plasmid and chromosomal DNA.

Tube A: Prepared without using PEG precipitation. No bands visible.

Tube B: Prepared by PEG precipitation, showing two visible bands. The upper band contains chromosomal DNA and the lower band plasmid DNA.

tryptophan and increasing concentrations of thymine were incubated with an overnight culture of strain W1103. (Before incubation, 10 ml of the culture was washed with an unsupplemented M9 medium to remove all the residual thymine.)

The cultures were grown by shaking them in a water bath at 37°C for 18 hours. The turbidity of the cultures was measured by nephelometry and indicated the minimum concentration of thymine required for growth. 0.5 µg/ml of thymine was shown to be adequate for the growth of the culture (Table 14). A sample (100 µl) of each culture was removed to a paper disc and the amount of radioactivity on each disc was measured after washing in 5% ice-cold TCA. Table 14 shows the amount of incorporation of [^{3}H] thymidine with varying concentrations of thymine.

Amount of Labelled Plasmid DNA Obtained

To measure the amount of DNA present at each stage of preparation, 500 ml of medium containing 1 mCi [^{3}H] thymidine, 0.5 µg/ml thymine and 100 µg/ml tryptophan was inoculated with *E. coli* W1103 ($RP4^+$, thy^-, trp^-) and incubated for 18 hours. The culture was grown to 10^9 cells/ml, the bacteria harvested and lysed as described. The DNA was precipitated with PEG and subjected to CsCl density gradient centrifugation (Plate 2). The radioactivity in the TCA-soluble and -insoluble fractions of samples taken during the preparation was measured. The percentage loss of TCA-insoluble counts was then calculated as a fraction of the starting material (Table 15).

The specific activity of the plasmid DNA in this preparation was 2.8×10^5 cpm/µg.

It seemed that extracting plasmid DNA using isopropanol for removing the ethidium bromide, reduced the biological activity

Table 14: Growth, and Incorporation of [^{3}H] Thymidine into E. coli W1103, Grown in Supplemented Medium Containing Different Concentrations of Thymine

Concentration of Thymine µg/ml	Turbidity After 18 Hr of Growth	TCA-Insoluble Counts/100 min/µl
0	0	2463
0.2	0	8203
0.5	120	67680
1.0	110	49567
2.0	80	19263

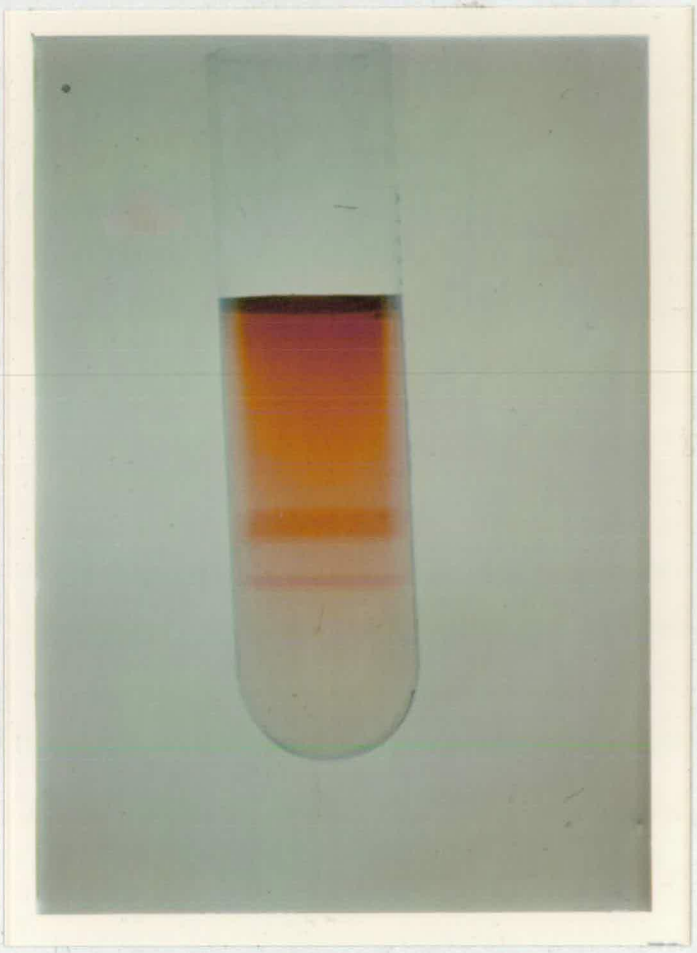


Plate 2: The radioactively-labelled preparation of RP4 using PEG precipitation. The upper band is chromosomal DNA and the lower band plasmid DNA.

Table 15: Amount of Labelled DNA Obtained at Each Stage During Preparation

Sample	Counts per Minute per Volume (µl)	Total Volume (ml)	Total Count	Amount of Incorporation
Total culture after bacterial growth. TCA-soluble material	60174/100	500	3.01×10^8	74%
Supernate after centrifuging out the bacteria	15624/100	500	7.81×10^7	
Total culture. TCA-insoluble counts (chromosomal and plasmid DNA)	44550/100	500	2.23×10^8	100%
Cleared lysate (plasmid DNA and some fraction of chromosomal DNA)	24723/100	110	2.72×10^7	12%
Polyethylene glycol precipitated DNA	112014/10	1.5	1.68×10^7	8.0%
The amount of DNA obtained in gradient after removal of PEG	43523/10	4	1.74×10^7	8.0%
Plasmid DNA (covalent closed circular)	30508/1	0.4	1.22×10^7	5.5%

of the DNA. So ethidium bromide was removed by dialysing the DNA against Dowex and buffer.

Isolation of Plasmid DNA from S. faecalis

To isolate plasmid DNA from S. faecalis, strain JH2-14, containing the plasmid pJH4 was lysed by a method of Currier and Nester (1976). The lysate was passed through an 18-gauge needle, in order to shear the chromosomal DNA. The pH was raised to 12.4 and immediately reduced to 8.0, as described before. The DNA was then concentrated by PEG precipitation and subjected to CsCl density gradient centrifugation.

The method for obtaining a cleared lysate was not very satisfactory so PEG precipitation was performed on both lysate and cleared lysate in two different experiments.

In the process of isolating the pJH4 plasmid DNA, the plasmid molecules were at a position very close to the chromosomal DNA. Although two bands were observed, it was difficult to remove the plasmid band without it being contaminated with chromosomal DNA.

Plasmid Analysis in M. radiodurans

Preliminary experiments were carried out to determine if wild type M. radiodurans already harboured extrachromosomal DNA.

Cultures were incubated for 18 hours in the presence of [^{3}H] thymidine. The use of a thymine requiring strain was deemed unnecessary since the incorporation of [^{3}H] thymidine occurs to the same extent in both wild type and thy$^-$ strains (Moseley, personal communication). Cell lysis was carried out as described in Materials and Methods. The lysate was passed through an 18-gauge needle in order to shear chromosomal DNA (Currier and Nester, 1976). The method for obtaining a cleared lysate by high

speed centrifugation was unsuccessful, so PEG was added to the lysate to precipitate the DNA. To separate possible plasmid DNA from the bulk of chromosomal DNA, the precipitate was subjected to CsCl density gradient centrifugation in the presence of ethidium bromide. Only a single wide band was observed (Plate 3).

Also, to detect the presence of any plasmid DNA in M. radiodurans, a lysate of bacteria prelabelled with [^{3}H] thymidine was subjected to CsCl density gradient centrifugation without prior PEG precipitation. The DNA was centrifuged and fractions collected and counted for radioactivity. In one experiment, one major peak (A) and one minor peak (B) were observed (Fig. 12). Peak (B) was not detected in another experiment. This observation suggested that the minor peak was an artifact and it seemed unnecessary to investigate it further.

These results suggest that M. radiodurans contained no native plasmids or, if it did, they did not behave in a manner typical of plasmids in E. coli and S. faecalis.

The advantage of using PEG precipitation was to concentrate DNA from a large volume of culture, which proved successful for the E. coli strain. This method was unsuitable for isolating plasmid DNA from S. faecalis or M. radiodurans.

Transformation of Plasmid DNA into Bacteria

In the study of the transformation of R-factors into M. radiodurans, it was necessary to test the biological activity of the R-factors.

Transformation of E. coli "655" with RP4 DNA

Experiments were performed in which purified plasmid DNA from E. coli J5-3 or W1103 (RP4$^+$) extract was added to competent E. coli "655" as described.

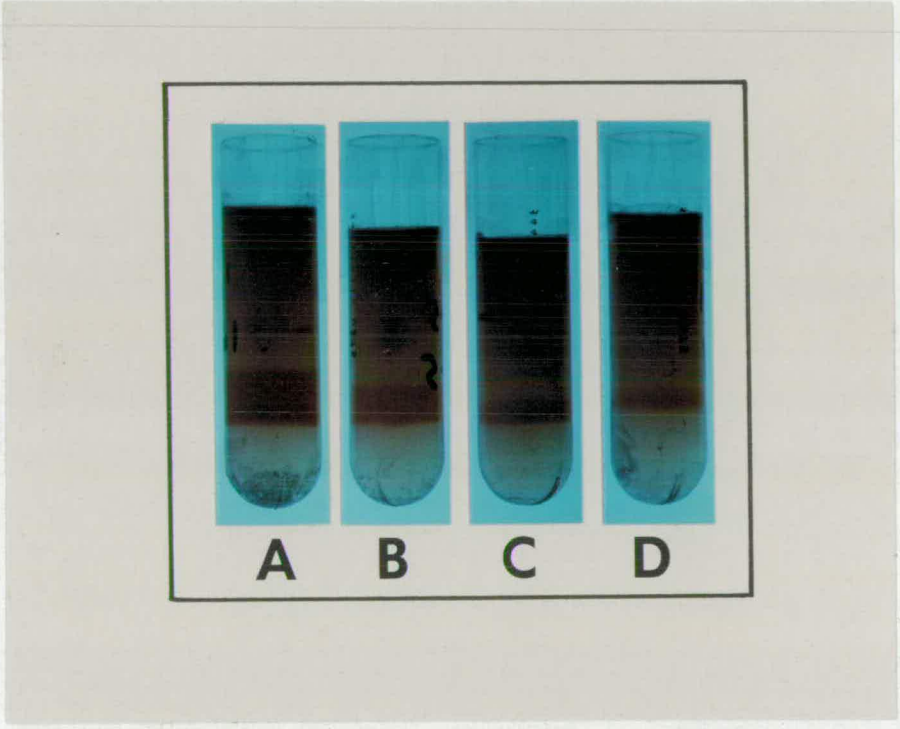
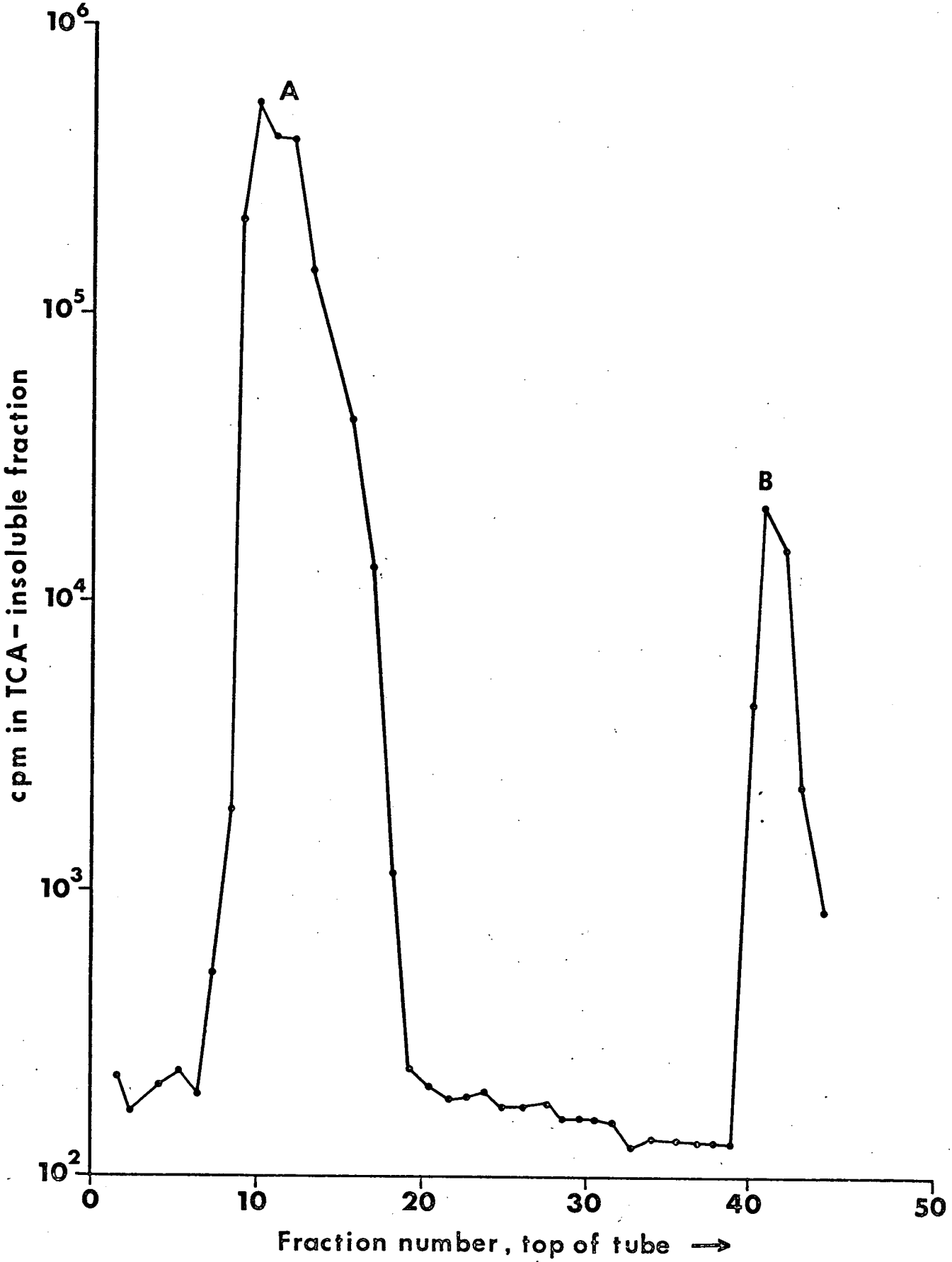


Plate 3: Tube A, B, C and D containing 250, 167, 50 and 25 µg DNA from M. radiodurans respectively.


10^6
10^5
10^4
10^3
10^2
A
B
cpm in TCA - insoluble fraction
0
10
20
30
40
50
Fraction number, top of tube ⟶

In all experiments a constant volume of 5 µl of transforming DNA with concentrations of 110 or 140 µg DNA/ml was added to 0.3 ml of bacterial culture and transformants selected on agar plates containing tetracycline or kanamycin. The frequency of transformation was between 10^{-5} and 10^{-6} (Table 16). All transformants were resistant to penicillin.

Attempted Transformation of M. radiodurans with RP4 DNA

A culture of wild type M. radiodurans treated with $CaCl_2$ as described before was exposed to RP4 DNA. The transformation procedure was exactly the same as for chromosomal DNA except a longer expression time was employed in two experiments. It was thought that expression of the plasmid DNA in transformed bacteria might require more extensive incubation in antibiotic-free medium than was used in E. coli. The concentration of DNA used varied between 110 and 140 µg/ml and the biological activity of the DNAs was tested previously in E. coli. After each transformation, the culture was diluted ten times in TGY and the cells were allowed a long expression time. Agar plates containing varying concentrations of each individual antibiotic, i.e. kanamycin, tetracycline and penicillin were used to select for transformants. 0.1 ml amounts of the transformed culture (about 10^9 cells/ml) were plated on ten TGY plates containing individual antibiotics but no antibiotic resistant transformants were obtained. Under these conditions, the frequency of transformation was less than 1×10^{-9}.

Since M. radiodurans lacks natural biomethylated bases (Schein et al., 1972), it might be that the wild type can recognise and excise methylated bases. The RP4 DNA was prepared from E. coli K12 which possesses a restriction and modification system

Table 16: Transformation of E. coli "655" with RP4 Plasmid DNA

Source and Concentration of plasmid DNA	No of Transformants per 10^{-1} Dilution per Plate		Frequency of Transformation	Resistance to 100 µg/ml Penicillin
	tet 25 µg/ml	kan 25 µg/ml		
DNA from J5-3 (RP4) (110 µg/ml)	52	47	5×10^{-6}	+
Labelled DNA from W1103 (RP4) (110 µg/ml)	61	51	1.5×10^{-5}	+
DNA from J5-3 (RP4) (from Dr. W. Brammar) (140 µg/ml)	60	59	1.1×10^{-5}	+

(Arber and Dussoix, 1962). Thus the plasmids are likely to have methylated bases and the failure to observe transformants of wild type M. radiodurans might be due to degradation of the DNA caused by the presence of methylated bases.

Strain 302, a mutant of M. radiodurans which is probably deficient in excision of some alkylated bases (Tempest and Moseley, 1977) was used as a recipient on the assumption that it would not excise the methylated bases and the plasmids would remain intact.

Transformation of 302 with RP4 DNA with the same concentration of DNA and the same number of cells gave no transformants and thus the number of transformants must be less than 1 in 10^9 cells.

Measurement of Radioactivity of Transforming DNA

Since there was no biological evidence for the transformation of M. radiodurans with RP4, wild type and strain 302 were exposed to [^{3}H] labelled RP4.

Thirty µl of [^{3}H] RP4 with a specific activity of 2.8×10^5 cpm/µg DNA was mixed with 0.3 ml of culture. For the control, 2 ml of a culture of M. radiodurans was heated at 70°C for 10 minutes to kill the bacteria. 0.2 ml of heated bacterial culture was mixed with 20 µl of plasmid DNA. Cultures were incubated for 2 hours. Three mg/ml of DNase was added to inactivate the DNA not taken up. Cultures were washed 3 times with SSC buffer. Several samples of each culture were placed on discs and the amount of radioactivity in TCA-insoluble material was measured.

The amount of the uptake of labelled RP4 after transformation of M. radiodurans and E. coli was compared. The bacteria were exposed to labelled RP4 DNA and the radioactivity followed.

The amount of radioactivity incorporated for each treatment is shown in Table 17. The data show that both strains of M. radiodurans take up plasmid DNA into DNase resistant state as well as E. coli.

Attempted Transformation of M. radiodurans with S. faecalis Plasmid DNA

Experiments were performed in which partially purified plasmid DNA from S. faecalis strain JH2-14 (pJH4) was added to M. radiodurans as described before. After a 2 hour incubation period, a long expression time (8 hours) in the antibiotic-free medium was performed to allow putative transformants to express.

0.1 ml of culture (10^9 cells/ml) was plated on a total of 20 agar plates containing the individual antibiotics kanamycin, streptomycin, erythromycin and tetracycline. The plates were incubated for 6 days.

Since no colonies appeared on any antibiotic plates, the frequency of transformation must be less than 1×10^{-8} per plate. This plasmid was not tested in S. faecalis for biological activity.

Table 17: Uptake of Labelled RP4 into TCA-Soluble and Insoluble Fraction of Wild Type and Strain 302 of *M. radiodurans* and *E. coli* ("655")

Samples (100 µl)	Total Counts per Minute (TCA-Insoluble + Soluble)	TCA Insoluble Counts per Minute
E. coli "655" treated with DNase	410	403
M. radiodurans (Wt) treated with DNase	773	641
302 treated with DNase	705	690
M. radiodurans heated cells treated with DNase	39	33
Blank	26	26

DISCUSSION

PART I

Transformation

Transformation is the simplest known form of sexuality and is an important tool for investigating the genetics of M. radiodurans. Transformation experiments following the procedure reported by Moseley and Setlow (1968), gave relatively low frequencies of transformants compared with transformation systems in other micro-organisms. Transformable bacteria acquire the ability to take up DNA in response to specific environmental alterations. Some changes in the environment of cells of M. radiodurans exposed to DNA were successful in increasing the frequency of transformation, while others made no difference or were inhibitory. The experiments presented in this study have revealed several unique aspects of the genetic transformation system in M. radiodurans.

The environmental changes studied were based on published transformation experiments in other micro-organisms. For example, elimination or increasing the amount of glucose from TGY medium did not have any effect either on frequency of transformation or the growth rate of M. radiodurans. Although the result is similar to that obtained with A. vinelandii when grown in complete medium, it is in contrast with those obtained with B. subtilis, where competence has been correlated with a decreased level of glucose in the cell wall resulting from growth in a glucose-free medium (Page and Sadoff, 1976; Young, 1965).

It was thought that some amino acids might stimulate competence in M. radiodurans, as they do in other systems. Thus competence in B. subtilis is stimulated by the presence of histidine, arginine or tryptophan (Bott and Wilson, 1967). However, none of the amino acids used stimulated competence in M. radiodurans. In fact, the presence of 50 or 100 µg/ml arginine in the assay tubes

reduced the number of transformants by a factor of two. The inhibitory effect of 100 μg/ml of arginine in transformation has been reported in B. subtilis and H. influenzae (Espinosa et al., 1976; Spencer and Herriot, 1965). The dramatic inhibition of competence (300-fold) by arginine in B. subtilis in a chemostat is due to interference with an early stage of transformation, since arginine-grown bacteria only bind about 15% of the amount of DNA that is bound by comparable bacteria grown without arginine (Espinosa et al., 1976). Although the inhibitory effect of arginine on transformation of M. radiodurans is not known, it is possible that arginine blocks the production of a competence-stimulating activity in M. radiodurans by a similar mechanism to B. subtilis.

Although the uptake of DNA into a DNase-sensitive state is the first step in the transformation process (Tomasz, 1969) it is quickly followed by a DNase-resistant state which in several transformation systems requires Ca^{2+}, Mg^{2+} or other divalent cations (Tomasz, 1969; Young and Spizizen, 1963). Thus the effect of these and other cations on transformation in M. radiodurans was studied. Experiments using recipient M. radiodurans cells which had been treated with various concentrations of $CaCl_2$ showed the optimum concentration to be approximately 0.03 M. When $CaCl_2$ was added to a final concentration of 0.03 M, the frequency of transformation increased up to 10,000 times for some markers, e.g. rifampicin resistance, and up to 1,000 times for other markers, e.g. kanamycin resistance. In the case of the rifampicin resistance marker, the frequency of transformants obtained was as high as 3%. Calcium ions have a similar effect on other bacteria such as E. coli, D. pneumoniae

and S. aureus (Mandel and Higa, 1970; Fox and Hotchkiss, 1957; Thompson and Pattee, 1977).

Although it is not known how Ca^{2+} ions increase the frequency of transformation, several lines of evidence suggest that they affect the permeability of the cell wall to DNA and facilitate the transport of DNA. Although a precise description of how changes in the cell wall affect the development of competence requires further genetical and biochemical evidence, there are some indications. For instance, phage DNA or bacterial DNA can be taken up by E. coli or S. aureus in the presence of a compatible helper phage (Mandel, 1967; Kaiser and Hogness, 1960). Although the helper phage alters the cell wall permeability, it appears to play several other roles such as protecting the transforming DNA from intracellular DNA-inactivation process and providing cohesive ends for the transfecting DNA in E. coli (Thompson and Patee, 1977; Kaiser and Inman, 1965; Epstein and Mahler, 1968). However, E. coli and S. aureus can be transfected or transformed in the absence of helper phage, but, in these cases, the presence of Ca^{2+} ions is necessary. Thus it is possible that Ca^{2+} ions affect the frequency of transformation of bacteria in the same way as helper phage does.

Although the biochemical nature of the Ca^{2+} stimulated step in M. radiodurans is not known, it may be effective in the conversion of cell-bound DNA to a form resistant to added nuclease and this process is probably due to the transport of DNA through the cell membrane. But it would not appear to involve inactivation of any exo- and/or endonuclease responsible for degrading incoming DNA, since Ca^{2+} ions have no effect on crude or dialysed pneumococcal exo- or endonuclease (Seto and Tomasz,

1976). It is also possible that the presence of 0.03 M $CaCl_2$ may neutralize the negative charge of the cell surface or may partially dissociate the membrane structure in the cell. Although in M. radiodurans 0.03 M $CaCl_2$ is necessary to increase the number of transformants, the latter is markedly decreased by lower or higher concentrations of $CaCl_2$. This effect is similar to that in E. coli K12, where the optimal concentration of $CaCl_2$ is also 0.03 M (Cosloy and Oishi, 1973). Other salts of divalent cations such as $MgCl_2$ and $SrCl_2$ did not stimulate an increase in the transformation frequency. This is similar to the situation in D. pneumoniae, where magnesium ions cannot replace calcium in stimulating the uptake of DNA into a nuclease-resistant form (Seto and Tomasz, 1976). In fact, in D. pneumoniae, Mg^{2+} ions cause the loss of cell-bound DNA into the medium where it is degraded. Both of these processes are catalyzed by a surface-located nuclease which requires magnesium ions (Lacks et al., 1974).

$ZnCl_2$ at the non-lethal concentration of 0.03 M, completely inhibited transformation, but it is not known whether it affects the uptake of DNA or has an inhibitory effect on growth. However, the presence of 10^{-3} M Zn^{2+} ions during mating blocks the formation of mating pairs of E. coli by acting on the F pili of the male (Caro and Schnos, 1966; Ou and Anderson, 1972), whilst it produces more recombinants if the F^- E. coli is treated before mating. It seems that Zn^{2+} treatment affects the ability of females to attach to males by increasing the area of lipopolysaccharide on the cell surface which is believed to contain the receptor site of the female for mating (Lancaster et al., 1965; Ou, 1973).

Effect of pH

In the transformation of M. radiodurans without using $CaCl_2$, some increase in transformation frequency was observed when the pH was lowered in the assay tubes from 7.0 to 5.7 (Moseley, personal communication). When the effect of pH on $CaCl_2$ treated cells was investigated in this study, the maximum frequency obtained was at pH 7.0.

In S. sanguis, although cells bind CF at pH values 5.5 and 6.0, DNA was optimally bound to cells in a DNase-resistant form at pH values between 7.0 and 8.5 (Ranhand, 1976). Also, in D. pneumoniae, CF is synthesised at pH 7.0, but not 6.8 (Tomasz and Mosser, 1966). However, the effect of pH on the transformation of M. radiodurans may be the same as in these micro-organisms and possibly is due to the nature of DNA binding protein and/or the negative charge on the DNA molecules.

Uptake and Expression of Transforming DNA

Following various periods of incubation of M. radiodurans with transforming DNA with subsequent DNase treatment of the mixture, the uptake of DNA did not extrapolate linearly to zero at zero time, but exhibited a lag of 2.5 minutes. This lag period is observed to be only a few seconds in H. influenzae and one minute in B. subtilis (Stuy and Stern, 1964; Levine and Strauss, 1965). The reason for the different times of uptake of DNA in M. radiodurans and other micro-organisms is not known.

Kinetic measurements of the interaction between cells and radioactively labelled DNA to produce transformants has given estimates for the average number of uptake sites per cell in some bacteria, e.g. 30 to 80 in D. pneumoniae (Fox and Hotchkiss, 1957), 2 to 8 in H. influenzae (Stuy and Stern, 1964) and 20 to

50 in B. subtilis (Singh, 1972). Thus, a high number of binding sites does not necessarily lead to a faster uptake of DNA. These observations indicate that the relatively slow uptake of DNA by M. radiodurans need not reflect a low number of DNA binding sites.

Expression

The time of expression for each marker varied in M. radiodurans. The minimum time required for expression was measured only for acriflavin and streptomycin resistance markers. In these experiments, the bacteria were allowed 20 minutes of incubation with DNA and the minimum phenotypic expression time then was measured. The first acriflavin resistant transformants were detected when acriflavin was added at 35 minutes and the first streptomycin resistant transformants when streptomycin was added at 170 minutes. The time of maximum expression was measured for all the markers. When wild type DNA was used to transform alanine and tyrosine requiring auxotrophs to prototrophy, a period of only one hour was required for phenotypic expression of these auxotrophic markers at a maximum frequency. This is expected, since the wild type gene product will be effective in the presence of the mutant alleles, as in D. pneumoniae (Lacks and Hotchkiss, 1960). In contrast, most antibiotic resistant markers especially resistance to erythromycin, kanamycin and streptomycin are the result of alteration to the ribosomal subunit and are recessive to sensitivity because for a mutant cell to be detected, most, if not all, of the ribosomes must be of the mutant type, (Nomura, 1970; Funatsu and Wittman, 1972; Apirion and Schlessinger, 1969; Tanaka et al., 1968; Hanson and Corcoran, 1969). Thus in M. radiodurans, the time required

for the maximum frequency of phenotypic expression for these three markers was 5, 6 and 8 hours respectively. The recessive/dominant relationships with regard to rifampicin and acriflavin resistance are less clear in M. radiodurans. In E. coli, all known rifampicin resistance mutations are due to alterations in the subunit structure of DNA-dependent RNA polymerase (Ezekiel and Hutchin, 1968) and mutations are recessive (Austin et al., 1971). Although the nature of the biochemical defect in the rifampicin resistant mutant of M. radiodurans is not known, the mutant lesion may not reside in an RNA polymerase subunit, since the maximum phenotypic expression for this marker occurs between 3 to 4 hours and 50% of the transformants are obtained after one hour (Moseley, personal communication).

PART II

Linkage Studies

The high frequency of transformation obtained by treating M. radiodurans cells with $CaCl_2$ enabled a study of linkage to be made.

To analyse linkage in any organism necessitates the isolation and at least partial identification of mutants. NTG is the only mutagen known to be capable of mutating wild type M. radiodurans significantly (Sweet and Moseley, 1974; 1976). To begin the construction of a chromosomal map, a search for linked markers was made.

Four pairs of linked genes trpB1-sts, kanI-ts4, kan2-glyA and tyrA-rnaA were identified. However, the map position of one pair with respect to any of the other pairs could not be determined. The frequency of transformation obtained in this study is high enough to give co-transformation of two unlinked markers (rif^r and acr^r).

Characterization and Designation of Mutations

Temperature Sensitive Mutants

The three temperature sensitive mutants isolated in this study were unable to grow at 39°C and could be distinguished from each other by the nature of their defects. The defects in these strains were probably single mutations since they could be transformed to temperature resistance with wild type DNA at frequencies associated with the transformation of a single marker.

The mutation leading to temperature sensitivity (ts4) of mutant ST5 was not clear since DNA, RNA and protein synthesis all became defective when the temperature was increased. The nature of the defect is unknown, but it could be, for example, in the cell membrane which is associated with the synthesis of

DNA, RNA and protein.

Strain ST9 has a mutation in rnaA causing RNA synthesis to be temperature sensitive. RNA synthesis is inhibited at 39°C, but cessation is not immediate and synthesis continues for up to 30 minutes after the increase in temperature. It is possible that either promoter binding or the assembly of the polymerase is affected by the high temperature.

The gene responsible for the temperature sensitivity of mutant ST12 is a starvation temperature sensitive gene (sts). Although folic acid provides conditions for growth of this mutant at 39°C, the nature of the defect is not clear.

Auxotrophic Mutants

Three auxotrophic markers involved in three of the linked pairs of genes (trpB1-sts, tyrA-rnaA, kan1-glyA) were isolated.

The tryptophan requiring mutant could grow well on either tryptophan or pyridoxin. Pyridoxal phosphate is a co-enzyme in a variety of reactions involved in the synthesis of many amino acids. In tryptophan synthesis pyridoxal phosphate is involved in the convertion of indole glycerol phosphate to tryptophan, a reaction catalyzed by tryptophan synthetase. Since this mutant does not grow on anthranilic acid or indole glycerol phosphate, it seems that the lesion resides in tryptophan synthetase.

The biosynthesis of tryptophan via anthranilic acid is shown in Fig. 13. The step affected by that mutation is indicated. Following the nomenclature used for E. coli, this gene is designated trpB1.

Tyrosine arises from prephenic acid (Pittard and Wallace, 1966; Taylor and Thoman, 1964) which is a branch point for the synthesis of phenylalanine and tyrosine by separate pathways.

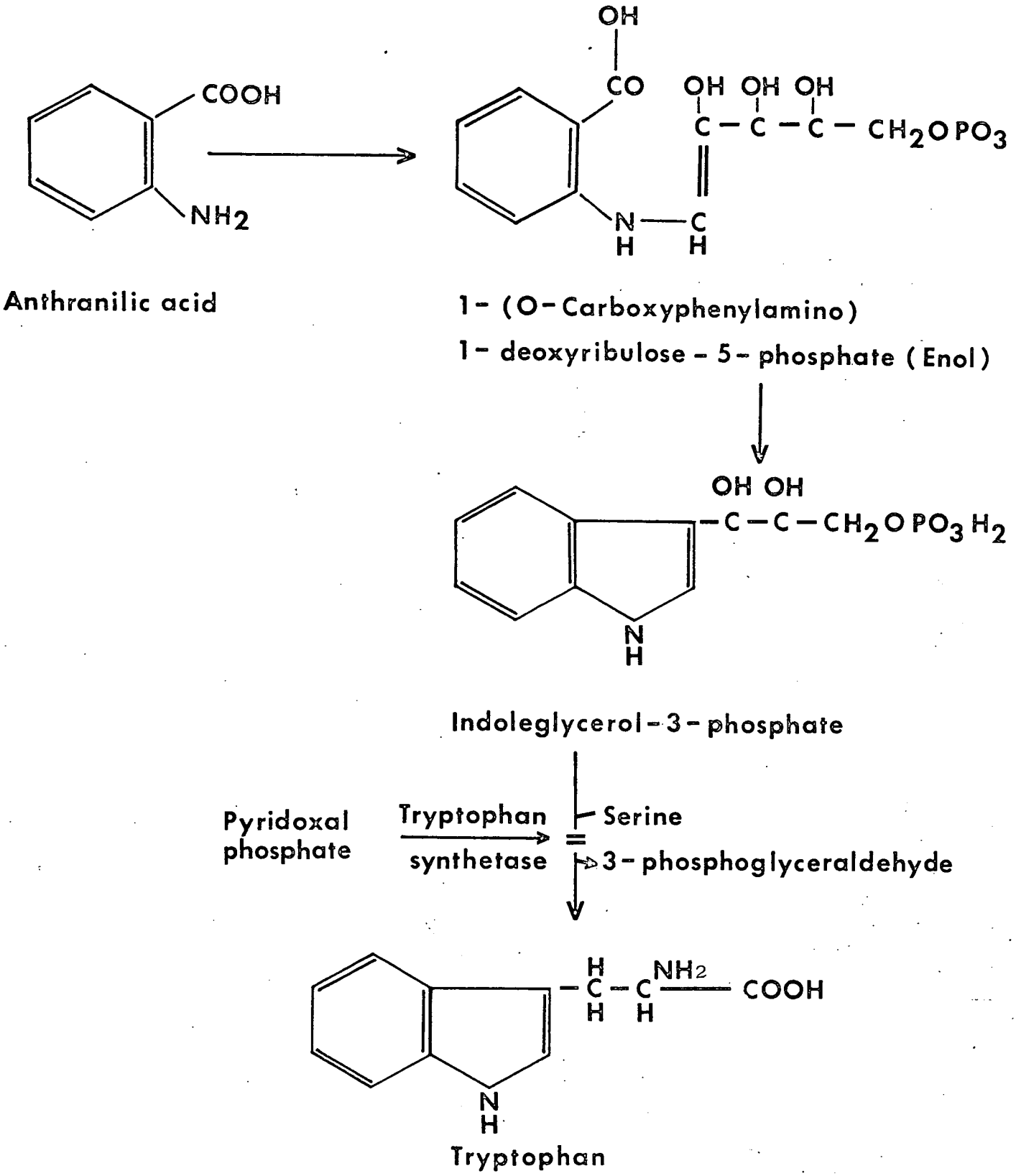


Fig. 13. (after Yanofsky and Rachmeler (1958))

Since mutant ST9 requires tyrosine, but not phenyalanine, the mutation is probably in the pathway between prephenic acid and tyrosine where prephenate dehydrogenase acts in the terminal branched pathway. This effect is similar to tyrA of E. coli (Pittard and Wallace, 1966; Taylor and Trotter, 1967).

Similarly, the gene which is mutated in ST1 and causing a glycine requirement is designated glyA. Because serine is the precursor of glycine and ST1 does not grow on serine, it is probable that the lesion resides in serine hydoxymethyl transferase which catalyses the reaction converting serine to glycine (Pittard and Wallace, 1966).

The enzymes of the common isoleucine-valine biosynthetic pathway are coded by four closely-linked genes in the Enterobacteriaceae (Pittard et al., 1963; Ramakrishnam and Adelberg, 1965). In M. radiodurans, the routes for synthesis of valine, leucine and isoleucine are also closely related and all arise through a common pathway. If any one of these amino acids is not present in the medium, the enzymes specified by these genes are derepressed. Linkage between genes responsible for isoleucine and valine biosynthesis and the four linked groups was not determined.

Kanamycin Resistant Mutants

Strain ST1 and ST5 were isolated as kanamycin resistant mutants. Since these two mutations are involved in two linkage groups (kan1-glyA, kan2-ts4), the question arose of whether the two mutations are in the same gene. Evidence suggests that this was not the case. When DNA extracted from mutant ST5 (kan2-ts4) was used to transform mutant ST1 (kan1-glyA) none out of 500 prototrophic transformants carried the gene responsible for

temperature sensitivity. If the mutations were in the same gene and glyA and ts4 mutations were on either side of the kanamycin resistance gene then 0.4% of prototroph transformants would carry the gene responsible for temperature sensitivity. Since statistically insignificant numbers of transformants were tested, the data cannot rule out the possibility that the two mutations are in the same gene. But since the two mutants have different levels of resistance to kanamycin, this may reflect their different locations. Kanamycin resistant mutants of E. coli have an altered ribosome subunit (Apirion and Schlessinger, 1969; Gale et al., 1972). No reconstitution experiments have been reported to see if mutations causing kanamycin resistance affect the 30S or 50S subunit. Since kanamycin causes miscoding in extracts of sensitive but not resistant E. coli (Tanaka et al., 1967) and the ability of drugs such as kanamycin to cause miscoding varies markedly with the drug/ribosome ratio, it may be that this compound interacts at multiple sites on the ribosome. This would explain the difficulty of obtaining single-step ribosomal mutants of high resistance to this drug.

While the orientation of four pairs of linked genes relative to the replication origin has not been determined and the entire question of the overall organisation of the M. radiodurans genome remains unclear, the linkage reported here is presumed to represent part of the chromosomal organisation rather than extrachromosomal. Since of the genes studied six were necessary for the survival of the cell it is unlikely that these would be located on extrachromosomal molecules. The two kanamycin resistance mutations are linked to two essential genes and thus they must also be chromosomal.

PART III

Transfer of Plasmids to M. radiodurans

The reason for this study was to extend the methodology for the genetic analysis of M. radiodurans. The two strains used in crosses with M. radiodurans were E. coli J5-3 containing RP4 and S. faecalis JH1-2 containing plasmid pJH1, gram negative and gram positive bacteria respectively. The choice of plasmid and mating conditions used was a personal one, there being no precedent. The criterion for transfer of the plasmid had to be the expression of its genes, which allowed the selection of recipients. Therefore, for the experiments to be considered successful, the plasmid had not only to be transferred, but also to be expressed in the new host.

Although crosses between strains of E. coli in liquid media were successful and plasmid transfer occurred at a frequency of 10^{-3}, crosses performed on plates were even more successful, yielding up to 50% transconjugants carrying RP4. Thus crosses between M. radiodurans and E. coli were made on plates to give maximum cell to cell contact. However, such crosses gave no indication of RP4 having been transferred from E. coli to M. radiodurans. Recipient cells have several specific roles in gene transmission during conjugation. These functional roles are postulated to be (1) specific-union formation which is mediated by donor pili; (2) effective-union formation; (3) DNA transfer; (4) stable inheritance of an extrachromosomal plasmid (establishment and maintenance) (Brinton, 1971; Curtiss, 1969). The failure to observe transfer of RP4 may be due to failure at any one of the above steps. It is possible that the special wall structure of M. radiodurans which is neither gram-negative nor gram-positive (Work, 1964) might be the reason for the failure to observe any conjugational transfer. Thus although it is the

unique structure of the donor pilus which forms a specific union (Ou, 1973), it follows that M. radiodurans must have a specific receptor for the pilus tip if transfer of the plasmid is to occur.

Whenever RP4 is transferred between bacteria of different species, the frequency of transfer is very much lower than when transfer is between bacteria of the same species, e.g. RP4 can be transferred between strains of P. aeruginosa at a frequency of 10^{-5}, but when transferred from P. aeruginosa S8 to a strain of E. coli or Proteus mirabilis, the frequency is 10^{-8} and 10^{-7} respectively. Having been transferred, the host, M. radiodurans, might have difficulty in maintaining it. Thus Saunders and Grinsted (1972) observed that RP4 is not maintained in E. coli J5-3 on transfer from P. aeruginosa S8 unless appropriate drug selection is applied immediately, but is maintained without such treatment in other strains of E. coli. However, the reason for the differences is not known. The failure to observe conjugational transfer to M. radiodurans may thus be due to either an extremely low frequency of transfer (less than 10^{-9} for instance) or to an inability to maintain the plasmid.

Although strain JH1-2 of S. faecalis was used as a gram-positive bacterium with a closer morphological relationship to cross with M. radiodurans, there were no transconjugants. The reasons for failure to observe transconjugants could be the same as with the cross between E. coli and M. radiodurans.

Transforming Plasmid DNA in M. radiodurans

Since M. radiodurans is capable of undergoing transformation at a high frequency, an attempt to transform a plasmid into M. radiodurans was made using purified plasmid DNA. The choice

of plasmid DNAs used in developing a plasmid transformation system in M. radiodurans was based on three criteria: (1) the plasmid could be readily purified and transformed into other species; (2) its physical properties in the native host were well characterized; (3) it carried markers which could easily be selected. These criteria were satisfied by the plasmids RP4 and pJH2.

Although Cohen et al. (1972) obtained a maximum transformation efficiency of 10^{-6} with purified R-factor among strains of E. coli, partly purified RP4 can be transformed into Rhizobium trifolii with a much higher frequency of about 1.3×10^{-4} (O'Gara and Dunican, 1973). Apart from RP4, plasmid pJH4 from S. faecalis can also be transformed into S. sanguis at a frequency of 1.8×10^{-5} (LeBlanc and Hassell, 1976).

Purification of Plasmid DNA

Although the preparation of plasmid DNA in this study is based on the techniques devised in recent years for purification of plasmid DNA, partly purified plasmid DNA can also be used for transformation. For example, partly purified RP4 DNA can be transformed into Rhizobium trifolii with the same efficiency as purified DNA (O'Gara and Dunican, 1973) and S. aureus can be transformed with DNA preparation from phenol extracts of crude lysates from S. aureus 8325 (Lindberg et al., 1972). The reason for using purified plasmid DNA in this study was to maximize the chance of getting a transformant with plasmid DNA by eliminating competition with chromosomal DNA. Although the amount of R-factor DNA in E. coli is 1.3% of the total DNA present (Barth and Grinter, 1974), the preparation of putative RP4 plasmid DNA in this study comprised 5% of total DNA. This amount is higher

than expected and is almost certainly due to contamination of the plasmid form by chromosomal DNA. The method of preparing cleared lysates in which high molecular weight chromosomal DNA is selectively centrifuged from crude lysates of plasmid-bearing strains is applicable to many species of bacteria (Guerry et al., 1973). Cleared lysates ordinarily contain less than 1% of the chromosomal DNA and 60 to 90% of the plasmid DNA originally present in the cell.

Attempted Transformation of Plasmid DNA into M. radiodurans

An attempt was made to transform both RP4 and pJH2 plasmid DNAs into M. radiodurans. Although RP4 can be expressed immediately after transformation (Cohen et al., 1972) and pJH2 expresses itself within 2 hours (LeBlanc and Hassell, 1976), a long expression time was allowed before selection for the plasmid markers was applied. Using 4 different preparations of RP4 DNA of known biological activity and similarly 2 pJH2 DNA preparations of unknown biological activity, no transformants were obtained in M. radiodurans. It was possible that this result could have been due to degradation of the plasmid DNA by restriction enzymes which identify and degrade foreign DNA. Although the reaction of wild type M. radiodurans towards foreign DNA is not known, it seemed reasonable to attempt to transform plasmid DNAs into strain 302 of M. radiodurans which is defective in either N-glycosidase or endonuclease activity towards alkylated DNA (Tempest and Moseley, 1977). Since M. radiodurans has no natural biomethylated bases (Schein et al., 1972), it might be that the bacteria can excise any methylated base which has been chemically or naturally produced. Plasmid RP4 which has come from an E. coli strain that has naturally methylated bases as a

part of a modification and restriction system (Arber and Morse, 1965; Bannister, 1970) would contain such bases. M. radiodurans may attempt to repair the methylated bases in RP4 and degrade the DNA. However, strain 302 did not yield any transformants containing either plasmids (RP4 or pJH4).

Since neither the wild type nor strain 302 of M. radiodurans gave any transformants, it was possible that plasmid DNA was not taken up by the strains. However, when radioactively labelled and biologically active RP4 was transformed into E. coli and incubated with strains of M. radiodurans, 0.04% of the label appeared in E. coli and 0.07% in M. radiodurans. The DNA did not appear to be degraded to TCA-soluble material. However, no transformants were obtained. The reason that RP4 DNA does not yield any transformants in M. radiodurans could be one of the following.

(1) There is not a specific attachment site for RP4. Since the presence of labelled RP4 was followed only for up to 2 hours in M. radiodurans, it might be that the DNA is degraded or excluded from the cell after this time.

(2) M. radiodurans may harbour a plasmid which is incompatible with RP4 so that RP4 is excluded.

(3) Expression of the plasmid DNA is repressed because either the transcriptional machinery of M. radiodurans is unable to transcribe RP4 DNA or the gene products are unable to produce their phenotypic effect.

(4) M. radiodurans with RP4 present might produce plasmid-borne gene products able to produce their phenotype, but unable to support replication of the plasmid. This would lead to unilinear inheritance of the plasmid DNA. Such a situation has been

described by LeBlanc and Hassell (1976) where, after transforming a plasmid DNA extracted from S. faecalis into S. sanguis, tetracycline resistant transformants could not be selected. This is analogous to the fate of DNA in abortive transduction (Stocker, 1956; Lederberg, 1956). However, microscopical observations indicated no small slow growing colonies of M. radiodurans.

Analysis of Putative Plasmid DNA in M. radiodurans

It is possible that M. radiodurans could harbour a plasmid(s) with unknown phenotype similar to that described in Rhodopseudomonas spheroides (Saunders et al., 1976). Apart from its unusually high resistance to U.V. radiation, there was no particular reason to relate any known phenotype of M. radiodurans to the presence of a plasmid.

To isolate putative cryptic plasmid, a cleared lysate was made, but it contained no DNA. This is also observed in Rhizobium trifolii which contains a large plasmid of 130 to 400 x 10^6 MW (Dunican and Tierny, 1974; Tshitenge et al., 1975; Nuti et al., 1977). Failure to detect the large plasmid seemed to involve the stable attachment of this plasmid to membrane. PEG precipitation of whole lysates also gave no indication of any plasmid DNA.

The presence of a cryptic plasmid cannot be excluded. If it had a high molecular weight, it might act like chromosomal DNA and remain attached to the membrane during the low speed centrifugation. Alternatively, if the plasmid was much less than 1% of the size of the chromosome, it would be difficult either to visualise it in an ethidium bromide CsCl gradient or to detect it as a peak of radioactivity in an excess of chromosomal DNA.

REFERENCES

Alexander, H. E. and G. Leidy (1951). Determination of inherited traits of Hemophilus influenzae by desoxyribonucleic acid fractions isolated from type-specific cells. Journal of Experimental Medicine 93: 345-359.

Alexander, H. E. and G. Leidy (1953). Induction of streptomycin resistance in sensitive Hemophilus influenzae by extracts containing desoxyribonucleic acid from resistant Hemophilus influenzae. Journal of Experimental Medicine 97: 17-31.

Anagnostopoulos, C. and I. P. Crawford (1961). Transformation studies on the linkage of markers in the tryptophan pathway in Bacillus subtilis. Proceedings of National Academy of Sciences of the United States of America 47: 378-390.

Anagnostopoulos, C. and J. Spizizen (1961). Requirements for transformation in Bacillus subtilis. Journal of Bacteriology 81: 741-746.

Anderson, A. W., H. C. Norden, R. F. Cain, G. Parrish and D. Duggan (1956). Studies on a radio-resistant micrococcus. I. Isolation, morphology, cultural characteristics and resistance to gamma radiation. Food Technology 10: 575-578.

Apirion, D. and D. Schlessinger (1969). Functional interdependence of ribosomal components of Escherichia coli. Proceedings of the National Academy of Sciences of the United States of America 63: 794-799.

Arber, W. and D. Dussoix (1962). Host specificity of DNA produced by Escherichia coli. I. Host controlled modification of bacteriophage . Journal of Molecular Biology 5: 18-36.

Arber, W. and M. L. Morse (1965). Host specificity of DNA produced by Escherichia coli. VI. Effects on bacterial conjugation. Genetics 51: 137-148.

Austin, S. J., I. P. B. Tittawella, R. S. Hayward and J. G. Scaife (1971). Amber mutations of Escherichia coli RNA polymerase. Nature New Biology 232: 133-136.

Avery, O. T., C. M. Macleod and M. McCarty (1944). Studies on the chemical nature of the substance inducing transformation of pneumococcal types. Induction of transformation by a desoxyribonucleic acid fraction isolated from Pneumococcus type III. Journal of Experimental Medicine 79: 137-157.

Back, A. L., C. Christiansen and A. Stenderup (1970). Bacterial genome sizes determined by DNA renaturation studies. Journal of General Microbiology 64: 377-380.

Bannister, D. (1970). Explanation of the apparent association of host specificity determinants with fi$^+$ R factors. Journal of General Microbiology 61: 283-287.

Barnhart, B. J. and R. M. Herriot (1963). Penetration of deoxyribonucleic acid into Hemophilus influenzae. Biochimica et Biophysica Acta 76: 25-39.

Barth, P. J. and N. J. Grinter (1974). Comparison of the deoxyribonucleic acid molecular weights and homologies of plasmids conferring linked resistance to streptomycin and sulfonamides. Journal of Bacteriology 120: 618-630.

Bauer, W. and J. Vinograd (1968). The interaction of closed circular DNA with intercalative dyes. I. The super-helix density of SV40 DNA in the presence and absence of dye. Journal of Molecular Biology 33: 141-171.

Bazaral, M. and D. R. Helinski (1968). Circular DNA forms of colicinogenic factor E1, E2 and E3 from Escherichia coli. Journal of Molecular Biology 36: 185-194.

Beringer, J. E. (1973). Ph.D. Thesis, University of East Anglia.

Beringer, J. E. and D. A. Hopwood (1976). Chromosomal recombination and mapping in Rhizobium leguminosarum. Nature 264: 291-293.

Biswas, G. O., T. Sox, E. Blackman and P. F. Sparling (1977). Factors affecting genetic transformation of Neisseria gonorrhoeae. Journal of Bacteriology 129: 983-992.

Black, W. A. and R. W. A. Girdwood (1969). Carbenicillin resistance in Pseudomonas aeruginosa. British Medical Journal ii: 234.

Bodmer, W. F. and A. T. Ganesan (1964). Biochemical and genetic studies of integration and recombination in Bacillus subtilis transformation. Genetics 50: 717-738.

Boling, M. E. and J. K. Setlow (1966). The resistance of Micrococcus radiodurans to ultraviolet radiation. III. A repair mechanism. Biochimica et Biophysica Acta 123: 26-33.

Bollum, F. J. (1959). Thermal conversion of non-pairing deoxyribonucleic acid to primer. Journal of Biological Chemistry 234: 2733-2744.

Bott, K. F. and G. A. Wilson (1967). Development of competence in the Bacillus subtilis transformation system. Journal of Bacteriology 94: 562-570.

Boyer, H. W. (1971). DNA restriction and modification mechanisms in bacteria. Annual Review of Microbiology 25: 153-176.

Brinton, C. C., Jr. (1971). The properties of sex pili, the viral nature of "conjugal" genetic transfer systems and some possible approaches to the control of bacterial drug resistance. CRC Critical Reviews in Microbiology 1: 105-160.

Burton, A. and R. L. Sinsheimer (1965). The process of infection with bacteriophage ØX 174. VII. Ultracentrifugal analysis of the replicative form. Journal of Molecular Biology 14: 327-347.

Butler, L. O. and G. Nicholas (1973). Mapping of the Pneumococcus chromosome. Linkage between the genes conferring resistances to erythromycin and tetracycline and its implication to the replication of the chromosome. Journal of General Microbiology 79: 31-44.

Butler, L. O. and M. B. Smiley (1970). Characterization by transformation of an ampicillin-resistant mutant of Pneumococcus. Journal of General Microbiology 61: 189-195.

Butler, L. O. and M. B. Smiley (1973). Mapping of the Pneumococcus chromosome: application of the density-shift method. Journal of General Microbiology 76: 101-113.

Cahn, F. H. and M. S. Fox (1968). Fractionation of transformable bacteria from competent cultures of Bacillus subtilis on renograffin gradients. Journal of Bacteriology 95: 867-875.

Caro, L. G. and M. Schnos (1966). The attachment of the male-specific bacteriophage F1 to sensitive strains of Escherichia coli. Proceedings of the National Academy of Sciences of the United States of America 56: 126-132.

Catlin, B. W. (1960). Transformation of Neisseria meningitidis by deoxyribonucleates from cells and from culture slime. Journal of Bacteriology 79: 579-590.

Cato, A. and W. R. Guild (1968). Transformation and DNA size. I. Activity of fragments of defined size and a fit to a random double cross-over model. Journal of Molecular Biology 37: 157-180.

Cerdá-Olmedo, E., P. C. Hanawalt and N. Guerola (1968). Mutagenesis of the replication point by nitrosoguanidine: map and pattern of replication of the Escherichia coli chromosome. Journal of Molecular Biology 33: 705-719.

Chakrabarty, A. M. (1976). Plasmids in pseudomonas. Annual Review of Genetics 10: 7-51.

Clewell, D. B. and D. R. Helinski (1969). Supercoiled circular DNA-protein complex in Escherichia coli: purification and induced conversion to an open circular DNA form. Proceedings of the National Academy of Sciences of the United States of America 62: 1159-1166.

Clewell, D. B., Y. Tagi, G. M. Dunny and S. K. Schultz (1974). Characterization of three plasmid deoxyribonucleic acid molecules in a strain of Streptococcus faecalis. Identification of a plasmid determining erythromycin resistance. Journal of Bacteriology 117: 283-289.

Clowes, R. C. (1963). Colicin factors and episomes. Genetical Research 4: 162-165.

Clowes, R. C. (1972). Molecular structure of bacterial plasmids. Bacteriological Reviews 36: 361-405.

Cohen, S. N., A. C. Y. Chang and L. Hsu (1972). Nonchromosomal antibiotic resistance in bacteria: genetic transformation of Escherichia coli by R-factor DNA. Proceedings of the National Academy of Sciences of the United States of America 69: 2110-2114.

Cohen, S. N. and C. A. Miller (1969). Multiple molecular species of circular R-factor DNA isolated from Escherichia coli. Nature 224: 1273-1277.

Cohen, S. N. and C. A. Miller (1970). Nonchromosomal antibiotic resistance in bacteria. II. Molecular nature of the R-factors isolated from Proteus mirabilis and Escherichia coli. Journal of Molecular Biology 50: 671-687.

Collins, E. B. (1956). Host-controlled variations in bacteriophages active against lactic streptococci. Virology 2: 261-271.

Cords, B. R., L. L. McKay and P. Guerry (1974). Extrachromosomal elements in group N streptococci. Journal of Bacteriology 117: 1149-1152.

Cosloy, S. D. and M. Oishi (1973). Genetic transformation in Escherichia coli K12. Proceedings of the National Academy of Sciences of the United States of America 70: 84-87.

Currier, T. and E. W. Nester (1976). Evidence for diverse types of large plasmids in tumor-inducing strains of Agrobacterium. Journal of Bacteriology 126: 157-165.

Curtiss, R. (1969). Bacterial conjugation. Annual Review of Microbiology 23: 69-136.

Datta, N. and R. W. Hedges (1971). Compatibility groups among f1 R factors. Nature 234: 222-223.

Datta, N., R. W. Hedges, E. J. Shaw, R. B. Sykes and M. H. Richmond (1971). Properties of an R factor from Pseudomonas aeruginosa. Journal of Bacteriology 108: 1244-1246.

Davis, N. S., G. J. Silverman and E. B. Masurovsky (1963). Radiation-resistant, pigmented coccus isolated from Haddock tissue. Journal of Bacteriology 86: 294-298.

Drabble, W. T. and B. A. D. Stocker (1968). R (transmissible drug resistance) factors in Salmonella typhimurium: pattern of transduction by phage P22 and ultraviolet-protection effect. Journal of General Microbiology 53: 109-123.

Driedger, A. A. and M. J. Grayston (1970). Rapid lysis of cell walls of Micrococcus radiodurans with lysozyme: effect of butanol treatment on DNA. Canadian Journal of Microbiology 16: 889-893.

Duggan, D. E., A. W. Anderson, P. R. Elliker and R. F. Cain (1959). Ultraviolet exposure studies on a gamma radiation-resistant micrococcus isolated from food. Food Research 24: 376-382.

Dunican, L. K. and A. B. Tierney (1974). Genetic transfer of nitrogen fixation from R. trifolii to K. aerogenes. Biochemical and Biophysical Research Communications 57: 62-72.

Dunney, G. M., N. Birch, G. Hascall and D. B. Clewell (1973). Isolation and characterization of plasmid deoxyribonucleic acid from Streptococcus mutants. Journal of Bacteriology 114: 1362-1364.

Dunney, G. M. and D. B. Clewell (1975). Transmissible toxin (hemolysin) plasmid in Streptococcus faecalis and its mobilization of a noninfectious drug resistance plasmid. Journal of Bacteriology 124: 784-790.

Easterling, S. B., E. M. Johnson, J. A. Wohlhieter and L. S. Baron (1969). Nature of lactose-fermenting Salmonella strains obtained from clinical sources. Journal of Bacteriology 100: 35-41.

Ehrlich, S. D. (1977). Replication and expression of plasmids from Staphylococcus aureus in Bacillus subtilis. Proceedings of the National Academy of Sciences of the United States of America 74: 1680-1688.

Embden, J. V. and S. N. Cohen (1973). Molecular and genetic studies of an R factor system consisting of independent transfer and drug resistance plasmids. Journal of Bacteriology 116: 699-709.

Epstein, H. T. and I. Mahler (1968). Mechanisms of enhancement of SP82 transfection. Journal of Virology 2: 710-715.

Espinosa, M., R. Lopez, M. T. Pérez-Ureña, E. Garcia and A. Portolès (1976). Competence in continuous culture of Bacillus subtilis: inhibition by arginine and reversal of the inhibition by Mn^{2+}. Journal of General Microbiology 97: 297-301.

Ezekiel, D. H. and J. E. Hutchins (1968). Mutations affecting RNA polymerase associated with rifampicin resistance in Escherichia coli. Nature 220: 276-277.

Fiers, W. and R. L. Sinsheimer (1962). The structure of DNA of bacteriophage ØX 174. III. Ultracentrifugal evidence for a ring structure. Journal of Molecular Biology 5: 424-434.

Fox, M. S. and M. K. Allen (1964). On the mechanism of deoxyribonucleate integration in pneumococcal transformation. Proceedings of the National Academy of Sciences of the United States of America 52: 412-419.

Fox, M. S. and R. D. Hotchkiss (1957). Initiation of bacterial transformation. Nature 179: 1322-1325.

Fox, M. S. and R. D. Hotchkiss (1960). Fate of transforming deoxyribonucleate following fixation by transformable bacteria. Nature 187: 1002-1006.

Freifelder, D. (1968). Studies on Escherichia coli sex factors. III. Covalently closed F'lac DNA molecules. Journal of Molecular Biology 34: 31-38.

Fujimoto, D., P. R. Srinivasan and E. Borek (1965). On the nature of the deoxyribonucleic acid methylases. Biological evidence for the multiple nature of the enzymes. Biochemistry 4: 2849-2855.

Funatsu, G. and H. G. Wittman (1972). Ribosomal proteins. XXXIII. Location of amino acid replacements in protein S12 isolated from Escherichia coli mutants resistant to streptomycin. Journal of Molecular Biology 68: 547-550.

Gabor, M. and R. D. Hotchkiss (1966). Manifestation of linear organization in molecules of pneumococcal transforming DNA. Proceedings of the National Academy of Sciences of the United States of America 56: 1441-1448.

Gale, E. F., E. Cundliffe, P. E. Reynolds, M. H. Richmond and M. J. Waring (1971). The molecular basis of antibiotic action, London, John Wiley & Sons.

Godson, G. N. and R. L. Sinsheimer (1967). Lysis of Escherichia coli with a neutral detergent. Biochimica et Biophysica Acta 149: 476-488.

Gold, M. and J. Hurwitz (1963). The enzymatic methylation of the nucleic acids. Cold Spring Harbor Symposia of Quantitative Biology 28: 149-156.

Goodgal, S. H. and R. M. Herriot (1957). A study of linked transformations in Haemophilus influenzae. Genetics 42: 372-373.

Goodgal, S. H. and R. M. Herriot (1961). Studies on transformation of Haemophilus influenzae. I. Competence. Journal of General Physiology 44: 1201-1227.

Griffith, F. (1928). The significance of pneumococcal types. Journal of Hygiene 27: 113-159.

Grinsted, J., J. R. Saunders, L. C. Ingram, R. B. Sykes and M. H. Richmond (1972). Properties of an R factor which originated in Pseudomonas aeruginosa 1822. Journal of Bacteriology 110: 529-537.

Guerola, N., J. L. Ingraham and E. Cerdá-Olmedo (1971). Induction of closely linked multiple mutations by nitrosoguanidine. Nature New Biology 230: 122-125.

Guerry, P., D. J. LeBlanc and S. Falkow (1973). General method for the isolation of plasmid DNA. Journal of Bacteriology 116: 1064-1066.

Haapala, D. K. and S. Falkow (1971). Physical studies of the drug-resistance transfer factor in Proteus. Journal of Bacteriology 106: 294-295.

Hanson, A. and J. W. Corcoran (1969). Alteration in a ribosomal protein of Bacillus subtilis associated with sensitivity to erythromycin. Federation Proceedings 28: 725.

Haseltine, F. P. and M. S. Fox (1971). Bacterial inactivation of transforming deoxyribonucleate. Journal of Bacteriology 107: 889-899.

Hayes, W. (1970). The genetics of bacteria and their viruses, Second Edition, Oxford, Blackwell Scientific Publications.

Hedges, R. W., A. E. Jacob, N. Datta and J. N. Coetze (1975). Properties of plasmids produced by recombination between R factors of groups J and FII. Molecular and General Genetics 140: 289-302.

Helinski, D. R. and D. B. Clewell (1971). Circular DNA. Annual Review of Biochemistry 40: 899-942.

Holliday, R. (1956). A new method for the identification of biochemical mutants of micro-organisms. Nature 178: 987.

Holloway, B. W. and M. H. Richmond (1973). R-Factors used for genetic studies in strains of Pseudomonas aeruginosa and their origin. Genetical Research 21: 103-105.

Hotchkiss, R. D. and J. Marmur (1954). Double marker transformants as evidence of linked factor in desoxyribonucleate transforming agents. Proceedings of the National Academy of Sciences of the United States of America 40: 55-60.

Howarth, S. (1965). Resistance to the bactericidal effect of ultraviolet radiation conferred on enterobacteria by the colicin factor ColI. Journal of General Microbiology 40: 43-55.

Howarth, S. (1966). Increase in frequency of ultraviolet induced mutation brought about by the colicin factor ColI in Salmonella typhimurium. Mutation Research 3: 129-134.

Humphreys, G. O., A. Willshaw and E. S. Anderson (1975). A simple method for the preparation of large quantities of pure plasmid DNA. Biochimica et Biophysica Acta 383: 457-463.

Jacob, A. E., J. M. Cresswell and R. W. Hedges (1977). Molecular characterization of the P group plasmid R68 and variants with enhanced chromosome mobilizing ability. Federation of European Microbiological Societies Letters 1: 71-74.

Jacob, A. E. and S. J. Hobbs (1974). Conjugal transfer of plasmid-borne multiple antibiotic resistance in Streptococcus faecalis var. zymogenes. Journal of Bacteriology 117: 360-372.

Jacob, F., S. Brenner and F. Cuzin (1963). On the regulation of DNA replication in bacteria. Cold Spring Harbor Symposium of Quantitative Biology 28: 329-348.

Kaiser, A. D. and D. S. Hogness (1960). The transformation of Escherichia coli with deoxyribonucleic acid isolated from bacteriophage λ dg. Journal of Molecular Biology 2: 392-415.

Kaiser, A. D. and R. B. Inman (1965). Cohesion and the biological activity of bacteriophage lambda DNA. Journal of Molecular Biology 13: 78-91.

Kelly, M. S. and R. H. Pritchard (1965). Unstable linkage between genetic markers in transformation. Journal of Bacteriology 89: 1314-1321.

Kilburn, R. E., W. D. Bellamy and S. A. Terni (1958). Studies on a radiation-resistant pigmented Sarcina spp. Radiation Research 9: 207-215.

Kobatake, M., S. Tanabe and S. Haseyawa (1973). Nouveau Micrococcus radiorésistant à pigment rouge, isolé de fèces de Lama glama, et son utilisation comme indicateur microbiologique de la radiostérilisation. Comptes Rendu de la Société de Biologie (Paris) 167: 1506-1510.

Kohnlein, W. and F. Hutchinson (1976). A four stranded DNA from Bacillus subtilis which may be an intermediate in genetic recombination. Molecular and General Genetics 144: 323-331.

Kohoutová, M., H. Braná and I. Holubová (1968). Isolation and purification of a substance inducing competence and inactivating transforming DNA in pneumococcus. Biochemical and Biophysical Research Communications 30: 124-129.

Krishnapillai, V. (1975). Resistance to ultraviolet light and enhanced mutagenesis conferred by Pseudomonas aeruginosa plasmids. Mutation Research 29: 363-372.

Lacks, S. (1962). Molecular fate of DNA in genetic transformation of Pneumococcus. Journal of Molecular Biology 5: 119-131.

Lacks, S. and B. Greenberg (1973). Competence for deoxyribonucleic acid uptake and deoxyribonuclease action external to cells in the genetic transformation of Diplococcus pneumoniae. Journal of Bacteriology 114: 152-163.

Lacks, S., B. Greenberg and M. Neuberger (1974). Role of a deoxyribonuclease in the genetic transformation of Diplococcus pneumoniae. Proceedings of the National Academy of Sciences of the United States of America 71: 2305-2309.

Lacks, S. and R. D. Hotchkiss (1960). Formation of amylomaltose after genetic transformation of Pneumococcus. Biochimica et Biophysica Acta 45: 155-162.

Lancaster, J. H., E. P. Goldschmidt and O. Wyss (1965). Characterization of conjugation factors in Escherichia coli cell walls. I. Inhibition of recombination by cell walls and cell extracts. Journal of Bacteriology 89: 1478-1480.

Lark, C. and W. Arber (1970). Host specificity of DNA produced by Escherichia coli. XIII. Breakdown of cellular DNA upon growth in ethionine of strains with r^+_{15}, r^+_{p1} or r^+_{n3} restriction phenotypes. Journal of Molecular Biology 52: 337-348.

Lawn, A. M., E. Meynell, G. G. Meynell and N. Datta (1967). Sex pili and the classification of sex factors in the Enterobacteriaceae. Nature 216: 343-346.

LeBlanc, D. J. and F. P. Hassell (1976). Transformation of Streptococcus sanguis challis by plasmid deoxyribonucleic acid from Streptococcus faecalis. Journal of Bacteriology 128: 347-355.

LeClerc, J. E. and J. K. Setlow (1974). Transformation in Haemophilus influenzae. In R. F. Grell (ed). Mechanisms in Recombination, pp 187-207. New York, Plenum Press.

Lederberg, J. (1949). Aberrant heterozygotes in Escherichia coli. Proceedings of the National Academy of Sciences of the United States of America 35: 178-184.

Lederberg, J. (1956). Linear inheritance in transductional clones. Genetics 41: 845-871.

Lee, C. S. and N. Davidson (1970). Physico-chemical studies on the minicircular DNA in Escherichia coli 15. Biochimica et Biophysica Acta 204: 285-295.

Lehrbach, P., A. H. C. Kung, B. T. O. Lee and G. A. Jacoby (1977). Plasmid modification of radiation and chemical-mutagen sensitivity in Pseudomonas aeruginosa. Journal of General Microbiology 98: 167-176.

Leonard, C. C. and R. M. Cole (1972). Purification and properties of streptococcal competence factor isolated from chemically defined medium. Journal of Bacteriology 110: 273-280.

Lerman, L. S. and L. J. Tolmach (1957). Genetic transformation. I. Cellular incorporation of DNA accompaying transformation in Pneumococcus. Biochimica et Biophysica Acta 26: 68-82.

Levin, J. S. and N. Strauss (1965). Lag period characterizing the entry of transforming deoxyribonucleic acid into Bacillus subtilis. Journal of Bacteriology 89: 281-287.

Lewis, N. F. (1971). Studies on a radio-resistant coccus isolated from Bombay duck (Harpodon nehereus). Journal of General Microbiology 66: 29-35.

Lindberg, M., J. E. Sjöström and T. Johanson (1972). Transformation of chromosomal and plasmid characters in Staphylococcus aureus. Journal of Bacteriology 109: 844-847.

Lowbury, E. J. L., A. Kidson, H. A. Lilly, G. A. J. Ayliffe and R. J. Jones (1969). Sensitivity of Pseudomonas aeruginosa to antibiotics: emergence of strains highly resistant to carbinicillin. Lancet II: 448-452.

Macfarren, A. C. and R. C. Clowes (1967). A comparative study of two F-like colicin factors, ColV2 and ColV3, in Escherichia coli K12. Journal of Bacteriology 94: 365-377.

MacPhee, D. G. (1973). Effect of rec mutations on the ultraviolet protecting and mutation enhancing properties of the plasmid R-Utrecht in Salmonella typhimurium. Mutation Research 19: 357-359.

Mandel, M. (1967). Infectivity of phage P2 DNA in presence of helper-phage. Molecular and General Genetics 99: 88-96.

Mandel, M. and A. Higa (1970). Calcium-dependent bacteriophage DNA-infection. Journal of Molecular Biology 53: 159-162.

Mandell, J. and J. Greenberg (1960). A new chemical mutagen for bacteria, 1-methyl-3 nitro-1-nitrosoguanidine. Biochemical and Biophysical Research Communications 3: 575-577.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from micro-organisms. Journal of Molecular Biology 3: 208-218.

Marsh, E. B. and D. H. Smith (1969). R Factors improving survival of Escherichia coli K-12 after ultraviolet irradiation. Journal of Bacteriology 100: 128-139.

Metzer, E., V. Shurman and A. W. Ravin (1974). A nitrosoguanidine-induced cluster of linked mutations near the streptomycin locus of Streptococcus. Journal of General Microbiology 82: 153-162.

Meynell, E., G. G. Meynell and N. Datta (1968). Phylogenetic relationships of drug-resistance factors and other transmissible bacterial plasmids. Bacteriological Reviews 32: 55-83.

Miao, R. M. and W. R. Guild (1970). Competent Diplococcus pneumoniae accept both single- and double-stranded deoxyribonucleic acid. Journal of Bacteriology 101: 361-364.

Morrison, D. A. and W. R. Guild (1972). Transformation and deoxyribonucleic acid size: extent of degradation on entry varies with size of donor. Journal of Bacteriology 112: 1157 - 1168.

Moseley, B. E. B. (1967). The isolation and some properties of radiation sensitive mutants of Micrococcus radiodurans. Journal of General Microbiology 49: 293-300.

Moseley, B. E. B. and H. J. R. Copland (1975). Isolation and properties of a recombination-deficient mutant of Micrococcus radiodurans. Journal of Bacteriology 121: 422-428.

Moseley, B. E. B. and A. Mattingly (1971). Repair of irradiated transforming deoxyribonucleic acid in wild type and a radiation -sensitive mutant of Micrococcus radiodurans. Journal of Bacteriology 105: 976-983.

Moseley, B. E. B. and J. K. Setlow (1968). Transformation in Micrococcus radiodurans and the ultraviolet sensitivity of its transforming DNA. Proceedings of the National Academy of the United States of America 61: 176-183.

Nester, E. W. and J. Lederberg (1961). Linkage of genetic units of Bacillus subtilis in DNA transformation. Proceedings of the National Academy of Sciences of the United States of America 47: 52-55.

Nester, E. W. and B. A. D. Stocker (1963). Biosynthetic latency in early stages of deoxyribonucleic acid transformation in Bacillus subtilis. Journal of Bacteriology 86: 785 -796.

Nisioka, T., M. Mitani and R. C. Clowes (1969). Composite circular forms of R-factor deoxyribonucleic acid molecules. Journal of Bacteriology 97: 376-385.

Nomura, M. (1970). Bacterial ribosome. Bacteriological Reviews 34: 228-277.

Notani, N. K. and S. H. Goodgal (1966). On the nature of recombinants formed during transformation of Haemophilus influenzae. Journal of General Physiology 49: 197-209.

Notani, N. K. and J. K. Setlow (1974). Mechanism of bacterial transformation and transfection. Progess in Nucleic Acid Research and Molecular Biology 14: 39-100.

Novick, R. P. (1969). Extrachromosomal inheritance in bacteria. Bacteriological Reviews 33: 210-263.

Nuti, M. P., A. M. Ledeboer, A. A. Lepidi and R. A. Schilperoot (1977). Large plasmids in different Rhizobium species. Journal of General Microbiology 100: 241-248.

O'Gara, F. and L. K. Dunican (1973). Transformation and physical properties of R-factor RP4 transferred from Escherichia coli to Rhizobium trifolii. Journal of Bacteriology 116: 1177-1180.

Oishi, M. and S. D. Cosloy (1972). The genetic and biochemical basis of the transformability of Escherichia coli K12. Biochemical and Biophysical Research Communications 49: 1568-1572.

Olsen, R. H. and C. Gonzaler (1974). Escherichia coli gene transfer to unrelated bacteria by a histidine operon-RP1 drug resistance plasmid complex. Biochemical and Biophysical Research Communications 59: 377-385.

Olsen, R. H. and C. D. Wright (1976). Interaction of Pseudomonas and Enterobacteriaceae plasmids in Aeromonas salmonicida. Journal of Bacteriology 128: 228-234.

Ou, J. T. (1973). Effect of Zn^{2+} on bacterial conjugation: increase in ability of F^- cells to form mating pairs. Journal of Bacteriology 115: 648-654.

Ou, J. T. and T. F. Anderson (1972). Effect of Zn^{2+} on the adsorption of male-specific filamentous deoxyribonucleic acid and isometric ribonucleic acid bacteriophages. Journal of Virology 10: 869-871.

Page, W. J. and H. L. Sadoff (1976). Physical factors affecting transformation of Azotobacter vinelandii. Journal of Bacteriology 125: 1080-1087.

Pattee, P. A. (1976). Genetic linkage of chromosomal tetracycline resistance and pigmentation to a purine auxotrophic marker and the isoleucine-valine-leucine structural genes in Staphylococcus aureus. Journal of Bacteriology 127: 1167-1172.

Pattee, P. A. and D. S. Nevelu (1975). Transformation analysis of three linkage groups in Staphylococcus aureus. Journal of Bacteriology 124: 201-211.

Pittard, J., J. S. Loutit and E. A. Adelberg (1963). Gene transfer by F' strains of Escherichia coli. I. Delay in initiation of chromosome transfer. Journal of Bacteriology 85: 1394-1401.

Pittard, J. and B. J. Wallace (1966). Distribution and function of genes concerned with aromatic biosynthesis in Escherichia coli. Journal of Bacteriology 91: 1494-1508.

Radloff, R., W. Bauer, and J. Vinograd (1967). A dye-buoyant-density method for the detection and isolation of closed circular duplex DNA: the closed circular DNA in Hela cells. Proceedings of the National Academy of Sciences of the United States of America 57: 1514-1520.

Ramakrishnan, T. and E. A. Adelberg (1965). Regulatory mechanisms in the biosynthesis of isoleucine and valine. II. Identification of two operator genes. Journal of Bacteriology 89: 654-660.

Ranhand, J. M. (1976). Effect of pH on competence development and deoxyribonucleic acid uptake in Streptococcus sanguis (Wicky). Journal of Bacteriology 126: 205-212.

Ranhand, J. M., C. G. Leonard and R. M. Cole (1971). Autolytic activity associated with competent group H streptococci. Journal of Bacteriology 106: 257-268.

Rountree, P. M. (1956). Variations in a related series of staphylococcal bacteriophages. Journal of General Microbiology 15: 266-279.

Saunders, J. R. and J. Grinsted (1972). Properties of RP4, an R factor which originated in Pseudomonas aeruginosa S8. Journal of Bacteriology 112: 690-696.

Saunders, V. A., J. R. Saunders and P. M. Bennett (1976). Extrachromosomal deoxyribonucleic acid in wild-type and photosynthetically incompetent strains of Rhodopseudomonas spheroides. Journal of Bacteriology 125: 1180-1187.

Scaife, J. and J. D. Gross (1962). Inhibition of multiplication of an F-lac factor in Hfr cells of Escherichia coli K12. Biochemical and Biophysical Research Communications 7: 403-407.

Schaeffer, P. (1958). Interspecific reactions in bacterial transformation. Symposia of the Society for Experimental Biology 12: 60-74.

Schein, A., B. J. Berdahl, M. Low and E. Borek (1972). Deficiency of the DNA of Micrococcus radiodurans in methyladenine and methylcytosine. Biochimica et Biophysica Acta 272: 481-485.

Schlissel, H. J. and C. P. Sword (1966). Conditions affecting transformation of a group H Streptococcus. Journal of Bacteriology 92: 1357-1363.

Seto, H. and A. Tomasz (1974). Early stages in DNA binding and uptake during genetic transformation of pneumococci. Proceedings of the National Academy of Sciences of the United States of America 71: 1493-1499.

Seto, H. and A. Tomasz (1976). Calcium-requiring step in the uptake of deoxyribonucleic acid molecules through the surface of competent pneumococci. Journal of Bacteriology 126: 1113-1118.

Silver, S. and H. Ozeki (1962). Transfer of deoxyribonucleic acid accompanying the transmission of colicinogenic properties by cell mating. Nature 195: 873-874.

Singh, R. N. (1972). Number of deoxyribonucleic acid uptake sites in competent cells of Bacillus subtilis. Journal of Bacteriology 110: 266-272.

Smith, H. R., N. D. F. Grindley, G. O. Humphreys and E. S. Anderson (1973). Interactions of group H resistance factors with the F factor. Journal of Bacteriology 115: 623-628.

Spencer, H. T. and R. M. Herriot (1965). Development of competence of Haemophilus influenzae. Journal of Bacteriology 90: 911-920.

Spizizen, J. (1958). Transformation of biochemically deficient strains of Bacillus subtilis by deoxyribonucleic acid. Proceedings of the National Academy of Sciences of the United States of America 44: 1072-1078.

Stanisich, V. A. and B. W. Holloway (1971). Chromosome transfer in Pseudomonas aeruginosa mediated by R Factors. Genetical Research 17: 169-172.

Stapleton, G. E. (1960). Protection and recovery in bacteria and fungi. In A. Hollaender (ed), Radiation Protection and Recovery, p.89, New York, Pergamon Press.

Stocker, B. A. D. (1956). Abortive transduction of motility in Salmonella, a non-replicating gene transmitted through many generations to a single descendant. Journal of General Microbiology 15: 575-598.

Strauss, N. (1970). Transformation of Bacillus subtilis using hybrid DNA molecules constructed by annealing resolved complementary strands. Genetics 66: 283-293.

Strickberger, M. W. (1972). Genetics, p.305, New York, The Macmillan Company.

Strim, S., F. Ørskov, I. Ørskov and A. Brich-Anderson (1967). Episome-carried surface antigen K88 of Escherichi coli. III. Morphology. Journal of Bacteriology 93: 740-748.

Stuy, J. H. (1976). Restriction enzymes do not play a significant role in Haemophilus homospecific or heterospecific transformation. Journal of Bacteriology 128: 212-220.

Stuy, J. H. and D. Stern (1964). The kinetics of DNA uptake by Haemophilus influenzae. Journal of General Microbiology 35: 391-400.

Sweet, D. M. and B. E. B. Moseley (1974). Accurate repair of ultraviolet-induced damage in Micrococcus radiodurans. Mutation Research 23: 311-318.

Sweet, D. M. and B. E. B. Moseley (1976). The resistance of Micrococcus radiodurans to killing and mutation by agents which damage DNA. Mutation Research 34: 175-186.

Tanaka, N., T. Kinoshita and H. Masukawa (1968). Mechanism of protein synthesis inhibition by fusidic acid and related antibiotics. Biochemical and Biophysical Research Communications 30: 278-283.

Tanaka, N., K. Sashikata and H. Umezawa (1967). Antibiotic-sensitivity of ribosomes from kanamycin-resistant E. coli. Journal of Antibiotics 20: 115-119.

Taylor, A. L. and M. S. Thoman (1964). The genetic map of Escherichia coli K12. Genetics 50: 659-677.

Taylor, A. L. and C. D. Trotter (1967). Revised linkage map of Escherichia coli. Bacteriological Reviews 31: 332-353.

Tempest, P. R. and B. E. B. Moseley (1977). A mutant of Micrococcus radiodurans which is very sensitive to mutagenesis by some monofunctional alkylating agents. Proceedings of the Society for General Microbiology 4: 137-138.

Thompson, N. E. and P. A. Pattee (1977). Transformation in Staphylococcus aureus: role of bacteriophage and incidence of competence among strains. Journal of Bacteriology 129: 778-788.

Toala, P., A. McDonald, C. Wilcox and M. Finland (1969). Susceptibility of group D Streptococcus (enterococcus) to 21 antibiotics in vitro with special reference to species difference. Journal of Medical Science 258: 416-430.

Tomasz, A. (1966). Model for the mechanism controlling the expression of competent state in Pneumococcus cultures. Journal of Bacteriology 91: 1050-1061.

Tomasz, A. (1969). Some aspects of the competent state in genetic transformation. Annual Review of Genetics 3: 217-232.

Tomasz, A. and J. L. Mosser (1966). On the nature of the pneumococcal activator substance. Proceedings of the National Academy of Sciences of the United States of America 55: 58-62.

Tshitenge, C., N. Luyindula, P. F. Lurquin and L. Ledoux (1975). Plasmid DNA in R. vigna and R. trifolii. Biochimica et Biophysica Acta 414: 357-361.

Venema, G., R. H. Pritchard and T. Venema-Schroder (1965). Properties of newly introduced transforming Deoxyribonucleic acid in Bacillus subtilis. Journal of Bacteriology 90: 343-346.

Waring, M. J. (1968). Drugs which affect the structure and function of DNA. Nature 219: 1320-1325.

Watanabe, T. (1963). Infective heredity of multiple drug resistance in bacteria. Bacteriological Reviews 27: 87-115.

Watanabe, T. (1971). The origin of R factors. Annals of the New York Academy of Sciences 182: 126-140.

Watanabe, T., C. Ogata and S. Sato (1964). Episome-mediated transfer of drug resistance in Enterobacteriaceae. VIII. Six drug-resistance R factors. Journal of Bacteriology 88: 922-928.

Wilson, G. A. and K. F. Bott (1968). Nutritional factors influencing the development of competence in the Bacillus subtilis transformation system. Journal of Bacteriology 95: 1439-1449.

Wilson, G. A. and K. F. Bott (1970). Effect of lysozyme on competence for Bacillus subtilis transfection. Biochimica et Biophysica Acta 199: 464-475.

Work, E. (1964). Amino acids of walls of Micrococcus radiodurans. Nature 201: 1107-1109.

Yanofsky, C. and M. Rachmeler (1958). The exclusion of free indole as an intermediate in the biosynthesis of tryptophan in Neurospora crassa. Biochimica et Biophysica Acta 28: 640-641.

Young, F. E. (1965). Variation in the chemical composition of the cell walls of Bacillus subtilis during growth in different media. Nature 207: 104-105.

Young, F. E. and J. Spizizen (1961). Physiological and genetic factors affecting transformation of Bacillus subtilis. Journal of Bacteriology 81: 823-829.

Young, F. E. and J. Spizizen (1963). Incorporation of deoxyribonucleic acid in the Bacillus subtilis transforming system. Journal of Bacteriology 86: 392-400.