HETEROSPECIFIC TRANSCRIPTION AS A CRITERION FOR THE

INVESTIGATION OF THE MOLECULAR EVOLUTION IN BACTERIA.

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TO MY PARENTS

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DECLARATION

I hereby declare that all the work in this thesis
(except where specifically stated) is my own.

Signed:

Date: 18- /12./1980.

ABSTRACT

In this thesis I present studies on the synthesis and
function of E.coli RNA polymerase subunits in different
gram-negative bacteria.   The primary purpose of this study
was to explore the extent to which RNA polymerase function
has been conserved in the course of evolution.   The plasmid
pZD100, constructed in vivo, provides a vehicle for rpoBC
transfer from E.coli to many bacteria.   This plasmid is
the product of the integration of λdrpoBC18, which carries
rpoBC genes, into the plasmid RP4 λatt.   The plasmids
pZD44 and pZD23 are derived from pZD100 and have deletion
removing some of λdrpoBC18 DNA.   In addition, pZD44 retains
and pZD23 lacks the determinant for ampicillin resistance.
The plasmid pZD23 is a better tool for my purpose as it is
not subjected to zygotic induction during transfer.   In vivo
as well as in vitro studies provided structural information
about these plasmids and RP4.   First, the orientation of
transfer of RP4 is in an anticlockwise direction;   in
otherwords, rpoBC plasmids transfer at O-rpoBC-Ap^r-Tc^r-Km^r..
Second, the repressor gene(s) for the transfer of RP4 is
located between the single EcoRI site and Ap^r gene.   Third,
the incompatibility, inc, gene(s) of RP4 is located between
Ap^r and Tc^r gene.   The Enterobacteriaceae bacteria chosen
for this study are: C.freundii, E.aerogenes, E.coli,
H.alvei møller, K.pneumoniae, P.morganii, P.vulgaris,
S.marcescens and S.typhimurium.   The non-Enterobacteriaceae
bacteria are: A.vinelandii, P.aeruginosa and R.leguminosarum.
Heterospecific gene expression can differentiate between

the genetically related species to <u>E.coli</u> and one could
conclude that it is possible to use this criterion for
the investigation of the genetic relatedness between
bacterial species.   In addition, promoters are highly
conserved parts of the DNA and thus the G + C content of
the DNA does not affect heterospecific gene expression.

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INTRODUCTION

Chapter 1 - Introduction

1. The Genetics of RNA Polymerase

A. Structural genes

E.coli RNA polymerase (nucleoside triphosphate: RNA nucleotidyl transferase EC 2.7.7.6) consists of at least four subunits α, β, β' and σ. The structural genes of these subunits have been discovered, mapped and some of them cloned into vectors in order to understand the nature of their role in RNA synthesis.

a. rpoBC

rpoB and rpoC are the structural genes for the $\beta\beta'$ subunits of E.coli RNA polymerase. They are located at 88.5 minutes on the chromosomal map of E.coli (Bachmann et al, 1976). They are contained in a single operon as they share a common promoter and they are cotranscribed in an rpoB → rpoC direction into a polycistronic mRNA (Matzura et al, 1971; Errington et al, 1974).

It was found that various antibiotics such as rifampicin, streptolydigin and streptovaricin inhibit RNA synthesis in E.coli and other bacteria by direct inter-action with RNA polymerase (Ezekiel and Hutchins, 1968; Mizuno et al, 1968; Tocchini Valentini et al, 1968; Schleif, 1969; Iwakura et al, 1973). The target of these antibiotics in E.coli was found to be the rpoB gene product (Schleif, 1969; Rabussay and Zillig, 1969; Heil and Zillig, 1970; Iwakura et al, 1973). This was shown to be true for the RNA polymerase of E.coli K12 (used in my work) as Boyd et al (1974) demonstrated that the

rifampicin resistant mutant of E.coli K12 has an altered

β subunit.   The understanding of the genetical structure

of the E.coli rpoBC genes was not possible till the

beginning of the 1970s, when the isolation methods for

mutations in these genes were available.   The rifampicin

resistance mutation was located near the argCBH operon

because of its cotransduction with the argH gene;   this

location was confirmed by Errington et al (1974), as they

have characterised a deletion fusing the argCBH operon to

the middle of the rpoB gene.

Some mutations in the structural genes for the enzyme

RNA polymerase will be lethal to the cell;   as the cell

will not be able to grow if the enzyme is unable to

function.   It is obvious that the presence of more than

one copy of the structural genes of RNA polymerase in a

cell will provide an opportunity for the isolation of

mutants and for the detailed investigation of these

mutations.   A merodiploid E.coli cell which has another

copy of rpoBC located on an extrachromosomal element,

will serve as an excellent source for the isolation of

mutants.

Amber mutations in the rpoBC operon have been isolated

(Austin et al, 1971);   they have been found to affect the

synthesis of the β and, in some cases, the β' subunits.

Some of the latter mutations were found to be polar in

rpoB and for this reason affect the synthesis of both

subunits;   amber mutations which are located in the

structural gene for the β subunit are recognised by their

failure to complement rpoB mutations (Hayward et al, 1974;

Miller et al, 1976; Oeschger, personal communication).

The exploitation of the merodiploid system in the isolation

of mutations was of great interest. In an E.coli cell

that is diploid for the rpoBC genes, if the chromosomal

gene is mutated so that it is unable to function under

certain conditions, then the product of the extrachromosomal

copy of the gene will permit growth of the cell. Austin

and Scaife (1970) have isolated a rifampicin resistant

merodiploid mutant strain in which the chromosomal rpoB

(rif-s) gene is inactivated in the presence of the

rifampicin, thus it allows the dominance of the extra-

chromosomal rif-r allele which is otherwise recessive.

Kirschbaum and Konrad (1973) isolated the rpoB3 or

rif^d mutation. They employed a partial diploid E.coli

cell which contained two copies of the rif^s allele; one

was chromosomal and the other was episomal. A rifampicin

resistant mutant was selected from this parent at a

frequency of 10^{-9}-10^{-10} and in which the rif^d allele was

dominant over the rif^s. This dominance is the distingu-

ishing character that separates it from the other

rifampicin resistant mutants. Rif^d is 52% cotransducible

with argH2 and 40% cotransducible with purD. These

frequencies are characteristic of rpoB mutations suggesting

that rif^d mutation is in or near the structural gene for

the β subunit. RNA polymerase when extracted from rif^d

mutant showed resistance to rifampicin (Kirschbaum, 1973).

The rif^d mutation has been of great value to my field

of interest as it simplifies the detection of the incorporation of E.coli rpoBC into a vector and the investigation of the expression of these genes in an alien cell.

The other class of mutations are the conditional lethal temperature sensitive mutations in the rpoB and the rpoC genes. They have been isolated in many laboratories (Panny et al, 1974; Oeschger and Berlyn, 1975; Claeys et al, 1976; Miller et al, 1976). They have been found to exhibit abnormal patterns of β and β' subunit synthesis and they influence the rate of subunit synthesis. Since they are the only type of mutations located in rpoC gene, they have contributed to the understanding of the role of β' subunit in RNA synthesis as well as the regulation of RNA polymerase synthesis in the cell (Gross et al, 1976; Sugiura et al, 1977; Kirschbaum, 1978).

The recent genetic studies have proved that the rpoBC genes are cotranscribed with the neighbouring ribosomal protein gene(s) (Linn and Scaife, 1978; Yamamoto and Nomura, 1978; Newman et al, 1979; Fiil et al, 1979). The newly developed in vitro genetical techniques permitted the cloning of the rpoBC genes into bacteriophage λ, the plasmid ColEl and its derivative pBR322 (Collins et al, 1976; Fiil et al, 1979). Furthermore, deletions, insertions and restriction analysis have confirmed that rplL is cotranscribed with rpoB and in addition the localisation of RNA polymerase binding site within the

rpoB gene whose function is not clear (Collins, 1979;
Taylor and Burgess, 1979).

I am interested in extending the genetic information
of E.coli RNA polymerase by introducing the structural
genes for the β and β' subunits into other bacterial
species.   It would be of great interest to investigate
the universality of E.coli RNA polymerase synthesis and
function in different bacteria.

b. rpoA

In addition to β and β' subunits there is a third
constituent of the core polymerase, α subunit.   It is
the product of the rpoA gene which is located at
72 minutes on the linkage map of E.coli (Jaskunas et al,
1975; Bachmann et al, 1976).   The rpoA gene is located
near a large group of ribosomal protein genes with which
it shares a common promoter (Jaskunas et al, 1976).

rpoA109 mutation in rpoA gene, has been isolated
(Sunshine and Sauer, 1975).   It produces an altered α
subunit in which histidine is substituted for a leucine
residue (Fujiki et al, 1976).

The biosynthesis of α subunits have been investigated
through the utilisation of rpoA mutants (Matzura and
Runzi, 1978).

There is no essential difference in the polypeptide
action of the free α subunit and the assembled one in the
intact RNA polymerase (Taketo et al, 1976a).   However, the
α subunit is modified in E.coli after T4 infection, and
this modification prevents the functioning of the enzyme

(Mailhammer et al, 1975). They concluded that α subunit is involved in the recognition of the promoter sequences.

c. rpoD

The α, β and β' subunits comprise the RNA polymerase core enzyme. Association of core with the sigma subunit generates holoenzyme. Recently, the structural gene for the sigma subunit, rpoD, has been mapped (Harris et al, 1977; Nakamura et al, 1977). The independent work of both laboratories has indicated that rpoD gene is mapped around 66 minutes on the current genetic map of E.coli (Bachmann et al, 1976). It is closer to the dnaG gene than to the tolC gene and is more than 90% cotransducible with it. Merodiploid E.coli cells for the dnaG region have higher levels of sigma subunits compared to the haploid. This rise in the rate of sigma subunit was due to gene dosage effects (Nakamura et al, 1977).

The other support for the localisation of the E.coli rpoD gene in these studies has come from the analysis of Salmonella typhimurium merodiploids for the same region of the E.coli chromosome. The sigma subunit of Salmonella typhimurium is distinguishable from E.coli in its mobility in sodium dodecyl sulphate polyacrylamide gel electrophoresis (Harris et al, 1977). The rpoD gene is more precisely mapped recently in the region between uxaAC and dnaG genes (Nakamura, 1978). By applying the localised mutagenesis technique Nakamura (1978) isolated mutation U303 in the rpoD gene; it produces a temperature sensitive RNA polymerase which is altered in optimal salt concentration.

In fact, all the isolated rpoD mutants are temperature

sensitive in vivo and/or in vitro; these are rpoD1,

U303 and alt-1 (rpoD2) mutations (Gross et al, 1978;

Nakamura, 1978; Travers et al, 1978). Burgess et al

(1979) have provided chemical evidence that the sigma

subunits of the rpoD1 and the rpoD2 are altered.   These

mutations have provided the first genetic evidence

emphasising the importance of sigma subunit in the process

of transcription.

What do we know about the role of the sigma subunit

in transcription?   Much biochemical evidence suggests

that sigma subunit of RNA polymerase plays a major role in

the initiation of transcription (Bautz et al, 1969;

Mueller, 1971; Hinkle and Chamberlin, 1972; Fukuda and

Ishihama, 1974; Chamberlin, 1976).   This is confirmed

by the genetical studies (Harris et al, 1977; Travers

et al, 1978).   The importance of sigma in promoter

selection is particularly clear from studies on a sigma

mutant which expresses catabolite sensitive operons in

the absence of cAMP-crp system (Travers et al, 1978).

B.   Specialised transducing phage λdrpoBC18

a.   Isolation

Specialised transducing bacteriophage for the

structural genes of E.coli RNA polymerase subunits have

been isolated (Kirschbaum and Konrad, 1973;   Konrad et al,

1973; Jaskunas et al, 1975; Bass et al, 1978).

One of these phages, namely λdrppBC18 (Kirschbaum

and Konrad, 1973) serves as a very widely used source for

the rpoBC genes and is of major importance for the purpose

of cloning of these specific genes into plasmids.   The

bacteriophage λdrpoBC18 is a rifampicin resistant

temperature inducible, defective phage that carries the

rpoBC genes.   It was isolated by the induction of a λ

lysogen in which λ had integrated near the rpoB3 (rif^d)

gene at the bfe site.   This insertion of λ was selected

in a bacterial mutant whose normal λ attachment site had

been deleted (Shimada et al, 1972).   The temperature

inducibility of λdrpoBC18 is conferred by the mutation

cI857 in the repressor gene (Sussman and Jacob, 1962).

The DNA of the λdrpoBC18 was isolated and its molecular

weight was first estimated by electron microscopy to be

33.5 x 10^6 daltons (Corrigenda of Kirschbaum et al

estimation, 1976; Meyers, R., 1979 personal communication).

Meyers (1979) has estimated a new size for the plasmid

pSC101 (unpublished results) which was used by Kirschbaum

et al (1976) as standard for the measurement of the size

of the λdrpoBC18 DNA.   A more recent estimate, taking

restriction analysis into account, is about 34 x 10^6 daltons

(Palmer et al, 1979; Tayler and Burgess, 1979).

Kirschbaum and Konrad (1973) presented no evidence concerning

the existence of the rpoC gene on the bacterial segment

that is carried by the phage.   This evidence, that

λdrpoBC18 carries the rpoC gene in addition to the rpoB

gene has come from the complementation test between this

phage and a polar amber mutation, rpoB38, in the E.coli

chromosome which affects the synthesis of β and β' subunits

(Kirschbaum and Scaife, 1974). An rpoB38 mutant cell
was able to grow in the absence of an amber suppressor
only when it was lysogenised with the bacteriophage
λdrpoBC18 or when another copy of the rpoBC genes is present.
When this mutant was made lysogenic for λdrpoBC18, the
resulting strain was viable without an amber suppressor,
showing that not only did the phage carry rpoB and rpoC, but
also the promoter (Kirschbaum and Scaife, 1974).

b. Restriction analysis and cloning

In addition to the genes for the specific subunits of
the enzyme RNA polymerase, it has been shown that the
λdrpoBC18 phage carries a ribosomal RNA gene (rrnB), a set
of genes for several tRNAs, an elongation factor EF-Tu
(tufB) and the following set of ribosomal protein genes
rplK (L11), rplA (L1), rplJ (L10) and rplL (L7/12)
(Lindahl et al, 1975; Lindahl et al, 1977). The order
of these genes on the bacterial segment of the λdrpoBC18
genome was concluded from the restriction analysis. The
physical map of the λdrpoBC18 genome was constructed by
digestion with restriction endonuclease enzymes EcoRI,
HindIII, HaeIII, and HpaII and by heteroduplex mapping
(Kirschbaum et al, 1976; Taylor and Burgess, 1979). This
map was extended by further analysis with the SmaI
(Lindahl et al, 1977), BamHI, SalI, HpaI (Boros and Sain,
1977) and KpnI, PstI, BglII (Fiil et al, 1979).

The construction of the physical map for the λdrpoBC18
genome was advantageous for many reasons:

First, it has allowed the in vitro cloning of the various

bacterial genes and promoters, that are present on the
phage genome, into various plasmids (Collins et al, 1976;
Glaser et al, 1977; Kiss et al, 1978). Second, it has
facilitated the localisation of the promoter for the
rpoBC genes and their cotranscription with the neigh-
bouring ribosomal protein genes (Linn and Scaife, 1978;
Yamamoto and Nomura, 1978; Fiil et al, 1979; Goldberg
et al, 1979; Newman et al, 1979; Taylor and Burgess,
1979). All these studies show that the rpoBC loci and
rplL gene are located in a single operon, cotranscribed
from a common promoter. Most probably the rplJ gene
is in the same operon (Linn and Scaife, 1978; Yamamoto
and Nomura, 1978; Taylor and Burgess, 1979). However,
the possibility that it is in a separate operon cannot yet
be excluded (Fiil et al, 1978). Newman et al (1979)
postulated the presence of a regulatory site, rpoU,
between the rpoB and the rplL genes.

Furthermore, the restriction of λdrpoBC18, DNA with
the restriction endonuclease enzymes such as HindIII,
has allowed the localisation of the bacterial genes
carried by the phage to specific restriction fragment.
Schweitzer and Matzura (1977) isolated the HindIII fragment
that contained the rpoB3 (rif^d) gene and used it for the
transformation of a rifampicin sensitive E.coli cell.
The construction of the specialised transducing phage
λdrpoBC18 has greatly enhanced the genetic analysis of
the transcriptional units that are carried on the bacterial
segment of the phage chromosome. Bass et al (1979) have

constructed in vitro the plasmid pOD162 which carries a
fragment of the rpoB gene and no other chromosomal DNA
regions.   It is a ColEl derivative plasmid which is
prepared from the DNA of the bacteriophage $\lambda drif^d 47$
(Mindlin et al, 1976).

2. The Subunits of the Enzyme RNA Polymerase

I would now like to consider the known physical
properties of the RNA polymerase subunits and their
contribution to enzyme function.

A. The β subunit

a. Properties

This subunit of the E.coli RNA polymerase is 145,000-
155,000 daltons polypeptide chain (Burgess, 1969b;   Zillig
et al, 1970a;   Berg et al, 1971).   It is one of the
largest polypeptides in the cell and this facilitates
its identification and its isolation.   The β subunit
contains about 1,400 amino acid residues (Iwakura et al,
1973).   It is the target for the antibiotics rifampicin,
streptolydigin and streptovaricin (Rabussay and Zillig,
1969; Heil and Zillig, 1970; Boyd et al, 1974).   The
β subunit contains the binding sites for these antibiotics
which are inhibitors for RNA synthesis;   the binding
sites for rifampicin and the streptolydigin on the
β subunit are close to each other and overlap (Iwakura
et al, 1973).

However, the evidence that the β subunit is the target
for these antibiotics was supported by the observation of

the alteration in the mobility of the β subunit from a rifampicin resistant mutant in comparison with the β subunit of the wild type E.coli cell (Heil and Zillig, 1970). This alteration is due to the exchange of the amino acid in the β polypeptide and not due to its size as seen from the comparison of the cyanogen bromide 2-dimensional electrophoresis (Boyd et al, 1974).

b. Assembly pathway

The β subunit is one part of the enzyme which unites with the other subunits (α and β') to form the core enzyme or the holoenzyme after the assembly of the σ subunit. It is present in equimolar amounts with the β' subunit in E.coli (Matzura et al, 1973). In vitro analysis of the course of assembly of the separated subunits of the enzyme RNA polymerase has revealed that the first step is the $\alpha_2\beta$ complex formation which is followed by the assembly of the β' subunit with the previous complex to form the core enzyme (Fukuda and Ishihama, 1974; Palm et al, 1975). Palm et al (1975) have shown that the first complex, $\alpha_2\beta$, formation is a rapid reaction that takes less than 2 minutes even a $0^{\circ}C$; in addition, this complex is unstable and degrades rapidly unlike the core enzyme which becomes stable after the addition of the β' subunit. This pathway of the subunit assembly in vitro proves to be identical to the order of the steps of their assembly in vivo (Saitoh and Ishihama, 1979; Ishihama et al, 1979).

c. Role

The β subunit as one of the constituents of the enzyme RNA polymerase has a vital role in the functioning of the enzyme. It is a multifunctional subunit of RNA polymerase and its role in the various steps of transcription is shown below.

1. DNA binding

Okada et al (1978) observed that the β subunit has a great affinity for binding to normal E.coli DNA and to DNA substituted with 5 bromodeoxyuridine and poly d(Br U-A) and it provides the contact between the enzyme RNA polymerase and the DNA. This observation has resulted from the close examination of the binding affinity of the separated subunits to the DNA which was tested by the cross linking of these subunits.

2. Selectivity of transcription

It has been concluded that the β subunit is involved in the recognition of the promoter or the terminator sites or both at least when σ is present as it is in vivo (Tessman and Peterson, 1976); the alterations in the gel patterns from phage S13 infected rifampicin resistant cell extracts reveals the specific role of the β subunit in the selectivity of transcription in vivo (Tessman and Peterson, 1976).

Lecocq and Dambly (1976) have provided another evidence for the role of β subunit in the selectivity of transcription. They observed an alteration in the lysogenisation of a rifampicin resistant mutant, rif501, with the bacteriophage λ.

3. Initiation

β subunit interacts with the antibiotic rifampicin suggesting that it is involved in the initiation step of the RNA synthesis which is inhibited by the specific antibiotic (Heil and Zillig, 1970).

4. Elongation

The β subunit directly interacts with the antibiotic streptolydigin which is an inhibitor for the elongation step of the RNA synthesis; this has led Iwakura et al (1973) to the conclusion that the β subunit is responsible for catalysing RNA chain elongation.

Besides its role in the process of transcription, the β subunit contains the binding site for the other subunits of the enzyme RNA polymerase such as α and σ subunits (Lill and Hartmann, 1975; Zillig et al, 1976; Okada et al, 1978).

Much of the data that I will present in the next section relates to the β subunit; it will show that the β subunit is an essential part of the enzyme RNA polymerase and the transcription of its structural gene of E.coli was investigated in different bacterial species.

B. The β' subunit

a. Properties

This subunit of the core enzyme and the holoenzyme RNA polymerase is a 150,000-165,000 molecular weight polypeptide (Burgess, 1969a; Zillig et al, 1970a; Berg et al, 1971). It is the largest subunit of the enzyme RNA polymerase. It is the most basic subunit and

this character has a direct effect on its function (Fujiki and Zurek, 1975; Zillig et al, 1976). Cross-linking studies have led to the conclusion that the β' subunit in conjunction with the β subunit, constitutes the backbone of the core enzyme and the holoenzyme (Hillel and Wu, 1977; Okada et al, 1978). The β' subunit contains 1.4 ± 0.5 g atoms/mol of tightly bound Zinc ion (Wu et al, 1977). The quantitative determination of the amino acid composition and the amino acid sequences of the N-terminal of the β' subunit as well as the other subunits were reported by Fujiki and Zurek (1975); they found that the β' subunit contains the highest molar percentage of lysine, histidine and arginine in comparison with the other subunits of the enzyme RNA polymerase. Although the β' subunit is similar in size to β it does not have the same primary sequence (Schachner and Zillig, 1971; Lowe and Malcolm, 1976).

Mutations in the rpoC gene, which produce altered subunits have been isolated in many laboratories (Ilyina et al, 1971; Panny et al, 1974; Dmitriev et al, 1976; Gross et al, 1976; Khesin et al, 1976; Bautz et al, 1977; Sugiura et al, 1977; Gragerov et al, 1978; Kirschbaum, 1978). Most of these mutants were temperature sensitive affecting the activity of the enzyme RNA polymerase at high temperatures such as 42°C retaining its activity at 30°C. One of these altered β' subunits which is the product of the rpoC92 mutation

(Sugiura et al, 1977) showed a reversible temperature
sensitivity which was not observed in the other cases;
it restored the activity of the enzyme when the
temperature is reshifted from $42^{\circ}C$ to $30^{\circ}C$.   The other
property of the altered temperature sensitive β' subunit
is that when it constitutes an enzyme it becomes salt
sensitive in vitro (Gross et al, 1976;   Khesin et al,
1976;   Bautz et al, 1977).

b.   Role

There are many studies in this field which reveal
that like β, the β' subunit is also multifunctional.
The majority of these studies is concerned with
altered β' subunit as described below.   The mutants
with an altered β' subunit have contributed to our
understanding of its function and particularly its role
in the process of transcription.

1.   DNA binding

As mentioned earlier (Fujiki and Zurek, 1975;   Zillig
et al, 1976) the β' subunit is the most basic subunit
of the enzyme RNA polymerase.   The basic proteins may
be expected to bind to polyanions like DNA (Zillig et al,
1976).   This explains the ability of the β' subunit to
bind to the polyanion heparin (Zillig et al, 1970b).
Moreover, Fukuda and Ishihama (1974) have exploited the
specific character of β' binding to polyanions, including
phosphocellulose to dissociate the β' subunit from the
premature core polymerase and release it into a polyanion
bound form.   They have also observed that the β' subunit

binds to the DNA and their quantitative analysis revealed the presence of more than one thousand β' binding sites on T7 DNA.

Moreover, the presence of the sigma subunit lowers the affinity of the β' subunit to DNA binding probably due to the competition between both, the sigma subunit and the DNA for binding to the β' subunit (Fukuda and Ishihama, 1974).

Finally, it has been shown that the mutation rpoC1 decreases the binding ability between the enzyme RNA polymerase and the DNA by lowering the affinity of the altered β' subunit for binding to DNA (Bass et al, 1978; Gragerov et al, 1978).

2. Selectivity of transcription

It is concluded from reconstitution studies with mutant XH56 or rpoC56 that the β' subunit plays an important role in the selection of ⌇ promoters (Gross et al, 1976). Sugiura and Yoshinaga (1978) have also come to a similar conclusion as that of Gross et al (1976) that the β' subunit plays an important role for the selective recognition of promoters. This conclusion had come from the comparison of the ratios of the enzymatic activity at $43^{O}C$ of the RNA polymerase from the mutant E.coli that has the rpoC92 mutation by using different DNA templates.

Recently, it was reported that an altered β' subunit was found to be defective in the termination of transcription at the λt_1 site in the absence of N protein,

suggesting that the β' subunit plays a role in the termination step of the process of transcription (Yura and Ishihama, 1979).

It has been concluded from the study of the T4 early transcription of the T4-infected rpoC1 E.coli mutant that the normal function of the β' subunit is required for T4 early transcription (Ito and Sekiguchi, 1978). They have observed the inhibition of the synthesis of various T4 early proteins in this mutant.

3. Elongation

Studies on the RNA polymerase in the mutant rpoC56 (Little and Dennis, 1979) have shown that the enzyme is defective in RNA chain elongation. Studies with another rpoC mutant, with the cold sensitive csl mutation, has led to the same conclusion (Gragerov et al, 1978).

4. Assembly pathway

Taketo and Ishihama (1976) have found that the two types of the temperature sensitive mutants with altered β' subunits have produced two kinds of assembly defective mutants; the first one, which carries the mutation rpoC4, accumulates the intermediate subassembly $\alpha_2\beta$ suggesting that the altered β' is defective in binding to the subassembly $\alpha_2\beta$. The second mutant, which carries the rpoC1 mutation accumulates the premature core, suggesting that it is defective in the activation of the premature core.

C. The α subunit

a. Properties

This constituent of the enzyme RNA polymerase is a

small polypeptide present in the ratio $\alpha:\beta:\beta' =$
2.4-6:1:1 (see Kawakami et al, 1979). It has a molecular
weight of 39,000-41,000 (Burgess, 1969b; Zillig et al,
1970a; Berg et al, 1971). There is only one type of
the α subunit in E.coli RNA polymerase (Fujiki et al,
1976; Taketo et al, 1976a; Ovchinnikov et al, 1977;
Yura and Ishihama, 1979) and the free α subunit is
identical to the assembled one in the RNA polymerase as
revealed by the analysis of the tryptic peptides from
the α subunit (Taketo et al, 1976a). The purified
subunit is present mostly in the dimeric state (Ito et al,
1975; Hillel and Wu, 1977).

The primary structure of the α polypeptide was
investigated by different methods and the amino acid
sequences was determined (Ovchinnikov et al, 1977;
Modyanov et al, 1978). It was found that the α subunit
consists of 329 amino acid residues (Ovchinnikov et al,
1977; Modyanov et al, 1978).

The isolated α subunit is very stable in vitro and
it is resistant to the attack of proteinases
(Palm et al, 1975; Lowe and Malcolm, 1976); it is
stable in vivo as well (Taketo et al, 1976a).

There is some evidence that the two α subunits are
close to each other in the core and in the holoenzyme RNA
polymerase. Firstly, oxidation of the core and the
holoenzyme at pH 9 causes linkage of the two polypeptides
(Zillig et al, 1976); secondly, treatment of the
holoenzyme and the core enzyme with bifunctional reagents

such as methyl 4-mercaptobutyrimidate (cleavable) and non-cleavable dimethyl suberimidate and N,N'-(1,4 phenylene) bismaleimide causes cross-linking of the isolated subunits (Hillel and Wu, 1977).

In vitro studies and in vivo analysis of the assembly pathway of the subunits of the enzyme RNA polymerase lead to the conclusion that $\alpha_2\beta$ complex is formed (Ito et al, 1975; Palm et al, 1975; Ishihama et al, 1976) which has a sedimentation coefficient of about 9S; it is rapidly formed and rapidly dissociated unless it is stabilised by the addition of the β' subunit (Palm et al, 1975). It has been found that the complex $\alpha_2\beta$ protects the β subunit from digestion by proteolytic enzymes in vitro (Lowe and Malcolm, 1976). The α subunit does not bind to DNA (Okada et al, 1978) but the $\alpha_2\beta$ complex can. There are 50-100 $\alpha_2\beta$ binding sites on T7 DNA (Fukuda and Ishihama, 1974). The $\alpha_2\beta$ complex binds to the antibiotic rifampicin (Zillig et al, 1976).

E.coli RNA polymerase is modified after T4 infection (Goff, 1975; Mailhammer et al, 1975; Zillig et al, 1975). Modification of the RNA polymerase is mainly due to the change in the α subunit which is achieved in two steps, "alteration" in which ADP is introduced into one of the two α subunits and "modification" which is the result of the ADP-ribosylation of the α subunit (Zillig et al, 1975). These changes result in the increase of the negative charge on the α polypeptide and thus alters its mobility on the SDS polyacrylamide gel (Goff, 1975). Ito and Sekiguchi

(1978) have reported recently that it is T4 alt or mod gene product which is responsible for the phosphorylation of the α subunit immediately after infection.

Another altered α subunit is the product of the rpoA109 mutation and it is found to be defective in supporting the growth of the bacteriophage P2 (Sunshine and Sauer, 1975) suggesting that α is perhaps important in the selectivity of transcription.

b. Role

The clear function of the α subunit has not yet been understood. It could play an indirect role in the catalysis of RNA synthesis such as keeping the linkage between the β and the β' subunits (Yura and Ishihama, 1979). I have reviewed, in the previous section, evidence showing that α subunit has a binding site for another α subunit ($2\alpha \rightarrow \alpha_2$) and another binding site for the β subunit ($\alpha_2 + \beta \rightarrow \alpha_2\beta$) (Ito et al, 1975; Zillig et al, 1975). On the other hand evidence does exist suggesting that the α subunit plays a role in the interaction of the enzyme RNA polymerase with promoters (Mailhammer et al, 1975; Zillig et al, 1976; Yura and Ishihama, 1979). Mailhammer et al (1975) observed that changes in the α subunit clearly result in changes in the ability of the enzyme RNA polymerase to recognise the different classes of promoters as shown by the comparison between the normal RNA polymerase and the T4-modified one for their ability to express different genes such as the lac operon, the trp operon, and the tRNA genes.

D. The σ subunit

a. Properties

The σ subunit binds to core enzyme ($\alpha_2 \beta\beta'$) to form the holoenzyme ($\alpha_2 \beta\beta'\sigma$) (Burgess et al, 1969; Chamberlin, 1974). It has a molecular weight of 82,000-95,000 (Burgess, 1969b; Zillig et al, 1970a; Berg et al, 1971; Lowe et al, 1979). This subunit is the most acidic one among the other other subunits of the enzyme RNA polymerase (Fujiki and Zurek, 1975) and because of its acidity it is proved to be more sensitive to the chymotrypsin than to the trypsin (Lill and Hartmann, 1975). The amino acid composition and the N-terminal sequences were determined (Fujiki and Zurek, 1975; Lowe et al, 1979). Its amino acid contents is characterised by the high level of GLX and ASX and by low level of cysteine/cystine residues (Low et al, 1979).

The sigma subunit is released from the holoenzyme after the initiation of the RNA chain (Travers and Burgess, 1969; Ruet et al, 1970; Hinkle and Chamberlin, 1972; Campbell and Lowe, 1977).

Altered sigma subunits were observed in vitro and in vivo (Gross et al, 1978; Harris et al, 1978; Travers et al, 1978). Most studies lead to the conclusion that the σ subunit has no DNA binding ability (Fukuda and Ishihama, 1974; Yura and Ishihama, 1979), although a low affinity has been reported by Okada et al (1978).

b. Role

It has been shown that the σ subunit plays an important

role in the promoter selection of the holoenzyme RNA polymerase (Bautz et al, 1972; Chamberlin, 1976; Harris et al, 1977; Travers et al, 1978; Yura and Ishihama, 1979). The σ subunit interacts with $\alpha_2 \beta$, β, and β' components of the enzyme and lowers the β' affinity for binding to DNA (Fukuda and Ishihama, 1974; Okada et al, 1978). The σ factor is close to β and β' polypeptides in holoenzyme since it protects them against tryptic cleavage (Lill and Hartmann, 1975).

The σ subunit enhances the reconstitution of the holoenzyme in vitro (Zillig et al, 1976; Fukuda and Ishihama, 1974). The binding of the σ subunit to the core enzyme was assumed to cause conformational changes in the enzyme which alters its binding affinity to the DNA nucleotide sequences (Mueller, 1971; Yura and Ishihama, 1979). In his study, Mueller (1971) has determined the amounts of RNA polymerase which is attached to T4 DNA in the presence or absence of sigma factor and found that the presence of σ restricts the attachment of the enzyme to only a small number of specific template sites.

Finally, Fukuda et al (1974) have observed a polypeptide of 56,000 molecular weight, which has a similar function to the σ subunit and it was designated as σ' subunit.

3.A. Biosynthesis of RNA polymerase

So far, we have had an idea about the structural

properties and function of each subunit of the enzyme RNA polymerase. In this part of the text, I will deal with the synthesis of the enzyme RNA polymerase through the description of the synthesis of its individual subunits. Later, the description of the regulation of RNA polymerase synthesis will follow.

a. Synthesis of β subunit

It is proved that the rate of synthesis of the β subunit is proportional to the number of the rpoB genes present in the cell (Hayward et al, 1974). They have observed a 40% increase in the rate of synthesis of the β and the β' subunits compared to the haploid parent. Bass et al (1978) observed a doubling in the rate of synthesis of the β subunit in merodiploid cells for the rpoB gene; however, no gene dosage effect was observed when rpoC1 was doubled in the cell (Bass et al, 1977) even though the rpoC1 mutant overproduces the β and the β' subunits at low and high temperatures of $30^{o}C$ and $42^{o}C$ (Taketo et al, 1978). The extra subunits produced in the meroliploids degrade rapidly due to the attack of the proteolytic enzymes (Hayward et al, 1974). Recently, Fukuda et al (1978) have demonstrated that the in vitro synthesis of the β subunit is repressed by at least two oligomers, the holoenzyme and the $\alpha_2\beta$ complex. The synthesis of the β subunit is coordinated with the synthesis of the β' subunit (Matzura et al, 1971; Errington et al, 1974) as well as the α subunit (Matzura, 1979), though it is not coordinated with the synthesis of

the α subunit (Kawakami et al, 1979) under certain

circumstances.

b. Synthesis of β' subunit

Ishihama et al (1976) observed an increase in the

rate of synthesis of the α, β and the β' subunits upon

temperature shift up to 42°C in the mutant E.coli cells

which had the rpoC4 mutation. However, the increase in

the rate of synthesis of the α subunit stopped at a time

where the increase of the rate of the β and the β'

subunits was continuing; this overproduction of the

β and the β' subunits was due to an increased rate of

specific mRNA synthesis as most of the mRNA produced in

this mutant corresponds to the ββ' mRNA (Miller et al,

1976). This is similar to the 5-6 fold increase in the

rate of β and β' subunits which was observed in the rpoC56

temperature sensitive mutant at 39°C (Dennis, 1977).

These results are consistent with the observation of

Taketo et al (1976b) who noticed a five fold increase in

the relative rate of the β and the β' subunit synthesis

at 42°C compared to the wild type; a three fold increase

in the rate of β and β' synthesis was observed even at

low temperature of 30°C. Moreover, this effect of

mutation on the β and the β' subunit synthesis is recessive

to the wild type allele because it has been found that in

a rec⁻ derivative of this mutant (rpoC4) which contains

the episome KLF10, the synthesis of the β and the β'

subunits was at the wild type rate both at 30°C and at

42°C (Taketo et al, 1976b).

It has been observed that the missense mutation rpoC1 also accelerates the synthesis of the β and the β' subunits (Khesin et al, 1976; Dmitriev et al, 1976) both at the non-permissive temperatre ($42^{\circ}C$) as well as at the permissive temperature ($30^{\circ}C$). Another temperature sensitive mutation called rpoC110 was described by Kirschbaum (1978); it stimulates the rates of the β and the β' subunit synthesis at $30^{\circ}C$ where the rate of their synthesis is about three times higher than the wild type; and it accelerates the rate of these subunit synthesis at $42^{\circ}C$ as it has been found that the rate of the β and the β' subunit synthesis was 5-10 fold higher than the wild type. Recently, Gragerov et al (1978) have produced cold sensitive mutants which were found to have altered β' subunit; these mutants do have a great influence on the rate of synthesis of the core constituents including the β' subunit.

In all cases so far described, the extra amounts of the β and the β' subunits degraded rapidly (Hayward et al, 1974; Dmitriev et al, 1976; Ishihama et al, 1976; Taketo et al, 1976b); Kirschbaum, 1978).

Mutations in the neighbouring gene rpoB may affect the rate of the synthesis of the β' polypeptide as described in the following case: in a mutant cell with the rpoB22 temperature sensitive amber mutation, the rate of the β' polypeptide synthesis was stimulated in the presence of the Su2 suppressor; whereas in the presence of more efficient suppressor, namely Su1, the β' subunit

synthesis is decreased and the β subunit increased
(Dmitriev et al, 1976; Khesin et al, 1976). However,
the increase in the β' subunit synthesis in the first
case was due to the depression of the formation of the
β subunit.

c. Synthesis of α subunit

As mentioned earlier, the rpoA gene, the structural
gene for the α subunit, is located at 72 minutes on the
chromosomal map of E.coli (Bachmann et al, 1976) near
a cluster of ribosomal genes (Jaskunas et al, 1975).
It is not located in the same operon as the β and β'
subunits and there is extensive evidence that the
regulation of the α subunit synthesis is independent of
the ββ' synthesis. First, the molar ratio of the
α:β:β' is 2.4-6:1:1 (see Kawakami et al, 1979) which
indicates that there is an excess of the α subunit
compared to the amount of the β and β'. Second, the
amount of α synthesised in the exponentially growing
phase is more than ββ' and in the approach to the
stationary phase the differential rate of synthesis
decreases first for α and the ribosomal proteins
(Kawakami et al, 1979). Third, the rate of synthesis
of α subunit in the RNA polymerase temperature sensitive
mutants such as rpoC1, rpoC4 and rpoB2.rpoB7 (Taketo
et al, 1976b) is not affected. Thus, in the β' mutants
which have the rpoC1 and rpoC4 mutations, the rate of
synthesis of both subunits β and β' is increased several
fold at 42°C compared to the wild type; in the β mutant

with the rpoB2·rpoB7 the rate of synthesis of both

subunits β and β' is slightly reduced; however, in all

the above cases of rpoC1, rpoC4 and rpoB2·rpoB7 the

rate of synthesis of the α subunit is not significantly

altered and it is similar to the wild type (Taketo et al,

1976b).

In contrast to these mutants it was concluded that

the synthesis of the α subunit in the wild type is

coordinated with the synthesis of the β and β' subunits

(Iwakura et al, 1974; Taketo et al, 1976b). Guanosine

tetraphosphate, ppGpp, inhibits the synthesis of the

α subunit, rRNA, many ribosomal proteins and elongation

factors EFG and EFTu but it does not inhibit the

synthesis of the β and β' subunits (Maher and Dennis, 1977).

d. Synthesis of σ subunit

The rate of σ subunit synthesis is increased under

various conditions; it is increased in the presence of

the rifampicin (Hayward and Fyfe, 1978) and in the E.coli

mutant with the ts125 mutation (sig-1) (Nakamura and

Yura, 1975). In addition, it has been found that the

alteration of the transcription termination factor ρ

induces the σ subunit synthesis and the N gene product

of the bacteriophage λ interacts with the host RNA

polymerase and results in the induction of σ (Nakamura

and Yura, 1976). An increase in σ synthesis due to

gene dosage effect was observed in E.coli merodiploids

for the rpoD region (Nakamura et al, 1977). It is very

interesting to observe an increase in the rate of the σ

subunit synthesis in merodiploids for the rpoBC region which is distant from the rpoD region (Nakamura et al, 1977; Hayward and Fyfe, 1978) and in both cases there was a suggestion of the existence of a regulatory mechanism in that part of the E.coli genome which affects the synthesis of the σ subunit; it is noteworthy that the temperature sensitive (sig-1) mutation is located in that area as well (Nakamura and Yura, 1975). The synthesis of σ subunit is accelerated during the induction of the wild type λ lysogen with mitomycin C and when E.coli cells are infected by λvir phage (see Matzura, 1979).

B. Autogenous regulation of the enzyme RNA polymerase

According to the definition of Goldberger (1974) the autogenous regulation is the mechanism in which the product of a structural gene regulates the expression of the operon in which that structural gene resides. The mechanism of regulation has been recently proposed for the regulation of the synthesis of the enzyme RNA polymerase (Scaife, 1976; Fukuda et al, 1978; Kirschbaum, 1978; Taketo et al, 1978; Yura and Ishihama, 1979).

It has been shown that the synthesis of the enzyme RNA polymerase is stimulated under various conditions such as the presence of low effective concentrations of rifampicin (Hayward et al, 1973; Hayward and Fyfe, 1978; Fukuda et al, 1978; Bass et al, 1979). In the presence of the antibiotic rifampicin, an increase in the rate of

synthesis of the β and the β' subunits was observed both in vivo and in vitro (Fukuda et al, 1978; Bass et al, 1979) in the haploid as well as in the heterodiploid E.coli cells (Hayward et al, 1973; Nakamura and Yura, 1976). The synthesis of α and σ subunits is also induced by the rifampicin (Hayward and Fyfe, 1978; Matzura, 1979).

Most interesting is the observation of the stimulation of RNA polymerase synthesis in strains harbouring mutations in the structural genes of the enzyme (Dmitriev et al, 1976; Taketo and Ishihama, 1976; Kirschbaum, 1978). An increase in the rate of the ββ' subunit synthesis was observed in the temperature sensitive mutant with the rpoC110 mutation at the permissive temperature (30°C) as well as the non-permissive temperature (42°C) (Kirschbaum, 1978). The mutation rpoC1 is another example for the E.coli mutant cell which stimulates the synthesis of the β and the β' subunits (Bass et al, 1977; Dmitriev et al, 1976). However, the temperature sensitive mutation rpoC4 is found to increase the rate of the synthesis of the α, β and the β' subunits for the first hour after the shift to the non-permissive temperature which is then followed by a continuation of the rise in only the rate of the ββ' subunit synthesis whereas the rate of the α subunit synthesis remains constant (Taketo and Ishihama, 1976).

The β' subunit synthesis is stimulated in the case of the significant suppression of the rpoB22 amber

mutation (Dmitriev et al, 1976). In this case, the depression in the synthesis of the β subunit have resulted in the activation of the β' subunit synthesis (Dmitriev et al, 1976; Bass et al, 1977). Taketo and Ishihama (1977) have reported recently the depression of the synthesis of the β and the β' subunits in the mutant E.coli cell which has the rpoB2·rpoB7 mutation.

The most widely accepted mechanism for the control of synthesis of the enzyme RNA polymerase is the autogenous regulation where the RNA polymerase itself is the repressor of its own structural or regulator gene (see Scaife, 1976). The mutations described above might cause a transient change in the concentration of the holoenzyme or its subassembly complexes which result in the repression or the derepression of the synthesis of the RNA polymerase subunits (Fukuda et al, 1978). Recent in vitro studies of the effect of the RNA polymerase and its subassemblies on the synthesis of the β subunit whose structural gene is carried by the bacteriophage λdrif$^+$-6 have provided a clear evidence that the two oligomers, the holoenzyme and the $\alpha_2\beta$ have repressed the synthesis of the β subunit (Fukuda et al, 1978).

Some of the mutations described above are proved to be defective in the assembly pathway of the enzyme (Taketo and Ishihama, 1976; Taketo and Ishihama, 1977). E.coli cells which have the rpoC4 mutation are defective in the assembly of the $\alpha_2\beta$ complex with the altered β' subunit resulting in the accumulation of the $\alpha_2\beta$ complex (Taketo

and Ishihama, 1976). In addition, another rpoC mutation namely, rpoC1, was found to be defective in the last step of the core formation and resulting in the accumulation of the premature core enzyme (Taketo and Ishihama, 1976). The analysis of the rpoB2.rpoB7 has revealed the accumulation of the free $\alpha, \alpha_2\beta$ and premature core at $30^\circ C$ (Taketo and Ishihama, 1977; Taketo et al, 1978). It is more likely that the depression in the rate of the β and the β' subunit synthesis following the shift of the temperature from $30^\circ C$ to $42^\circ C$ was caused by the accumulation of some of these assembly intermediates which act as a repressor for the expression of the rpoBC operon.

Taketo et al (1978) have investigated the effect of the assembly defective mutations on the expression of the rpoBC operon which was introduced to these mutants by the transducing phage λdrif$^+$-6 after the UV irradiation of the mutant cells.

The inhibition of the β subunit synthesis was observed in the rpoB2.rpoB7 mutant and the stimulation of its synthesis was reported in the rpoC1 as well as in the rpoC4 assembly defective mutations (Taketo et al, 1978). It is noteworthy that the expression of the rpoBC neighbouring genes which are carried by the phage such as EF-Tu, L1, L7/L12 was not affected by these mutations (Taketo et al, 1978).

The autogenous regulation mechanism has been strongly suggested for at least the rpoBC operon by the RNA polymerase subunits or by its subassembly complexes (Taketo et al, 1978);

they have suggested that the β and the α subunits act as part of the regulatory protein. The experiments described by Errington et al (1974) have shown that the β' subunit does not take part in the repression of the expression of the rpoBC operon because the excess of the β' subunit that is synthesised in the cell under certain conditions had no effect on the inhibition of the synthesis of the β subunit; this effect of the β' subunit was investigated in an argR⁻/KLF10Δ18 heterodiploid E.coli cell in which the β' subunit synthesis is under the control of arginine and which is deficient in the formation of the repressor for arginine synthesis.

There are other described examples of systems that are autogenously regulated such as the bacteriophage T4 gene 32 (Krisch and Houwe, 1976), the araC system in E.coli (Lee, 1978), histidine utilisation system, hut, in Salmonella typhimurium (Goldberger, 1974; Magasanik, 1978) and the λcI repressor synthesis (Ptashne, 1978).

In my study of heterospecific gene expression, the rpoBC operon of E.coli was transferred into an alien cell and this will open a new opportunity for the investigation of the regulation of the synthesis of the E.coli RNA polymerase in these cells.

4. The Process of Transcription

It is convenient to divide the transcription process into different steps, namely, RNA polymerase template binding, RNA chain initiation, RNA chain elongation, and

RNA chain termination.   I will treat each one separately in different sections as follows.

a.   RNA polymerase template binding

It is the first step of the transcriptional cycle in which the enzyme RNA polymerase interacts with the DNA template and forms a binary complex locating a specific site of the genome called the promoter (see Scaife, 1973; Travers, 1974).   At this site the initiation of RNA synthesis can occur and its position is just before the genes to be transcribed.   This step is also called the promoter site selection and activation (see Chamberlin, 1976).

It has been found that about 40-45 bases of the DNA template are protected by the enzyme RNA polymerase binding (Krakow et al, 1976; Lathe, 1978) against DNAase digestion.   This complex is called the "closed complex" and it is followed by the formation of the "open complex" which is characterised by the melting of the double helix; this allows the direct interaction of the enzyme with the DNA template which is followed by the initiation process of RNA synthesis (Chamberlin et al, 1976; Lathe, 1978). More details of the template binding of the enzyme are discussed by Krakow et al (1976).

b.   RNA chain initiation

This step is characterised by the binding of the ATP or the GTP to the enzyme RNA polymerase and the binding of another nucleoside triphosphate (purine or pyrimidine) to a second site on the enzyme; as a result an inter-nucleotide 3'5' phosphodiester bond is synthesised and a

dinucleoside tetraphosphate of the structure pppPupX is generated by the elimination of the inorganic pyrophosphate (Chamberlin, 1974). The rate of the initiation of the RNA synthesis is very rapid compared to the rate of the promoter selection and it is affected by the concentration of the ATP, GTP, salt and by the temperature (Chamberlin et al, 1976; Krakow et al, 1976). Following this stage the σ subunit is released either due to conformational changes in the enzyme which reduces its affinity or due to the displacement by the nascent RNA chain (Ruet et al, 1970; Krakow et al, 1976).

c. RNA chain elongation

For the elongation of the nascent RNA chain, the nucleotide monophosphate residues are added to the 3'OH terminus of the dinucleoside tetraphosphate (Chamberlin, 1976; Chamberlin et al, 1976). At this stage the enzyme forms a ternary complex with the DNA template and the nascent RNA chain (Krakow et al, 1976).

d. RNA chain termination

This is another step of the transcription which involves the specific interaction of the RNA polymerase with the DNA template (Zillig et al, 1976). There are at least two mechanisms for the termination of RNA synthesis (see Lathe, 1978). The first one involves the recognition of the DNA signals (terminator) by the enzyme only and in the absence of any additional factor; this mechanism is called direct termination. The second mechanism involves the presence of a termination factor

called ρ which mediates the termination at additional
sites on the DNA template.   Mutants of ρ have been
isolated which proved the interaction of the RNA poly-
merase with ρ during the transcription termination
(Das et al, 1978).

At this stage of transcription, the synthesised RNA
chain and the enzyme RNA polymerase are released from the
template (Adhya and Gottesman, 1978; Lathe, 1978).   The
termination of the transcription process is extensively
discussed by Roberts (1976) and by Adhya and Gottesman
(1978).

5.   Rifampicin: The Inhibitor of the RNA Polymerase

a.   Definition

Rifampicin is a member of the group of the anti-
biotic rifamycins produced by Streptomyces mediterranei
which is found to affect the prokaryotic and the eukaryotic
RNA polymerases (see Riva and Silvestri, 1972; Franklin
and Snow, 1975; Scaife, 1976; Wehrli, 1977b; Matzura,
1979).

b.   Subunit binding

Rifampicin binds to the enzyme RNA polymerase tightly
and the rifampicin:enzyme ratio is variously estimated
between 1:1-2:1 (Fietta and Silvestri, 1975; Wehrli, 1977a).
The target has been shown to be the β subunit (Heil and
Zillig, 1970; Rabussay and Zillig, 1970).   More recently,
Lowe and Malcolm (1976) have tested the ability of the RNA
polymerase and its subunit mixtures to bind rifampicin and

they found that among α, β, β', $\alpha_2\beta$, $\beta\beta'$, $\alpha_2\beta'$ and $\alpha_2\beta\beta'$ only $\alpha_2\beta$ and $\alpha_2\beta\beta'$ bound rifampicin; it is suggested that the ability of $\alpha_2\beta$ to bind rifampicin is brought by a conformational change in the β subunit induced by α (Lowe and Malcolm, 1976).

c. Chemical structure

Riva and Silvestri (1972) have described the various chemical structures of the rifamycins. Below is the chemical structure of rifampicin as appeared in Riva and Silvestri (1972), in Franklin and Snow (1975), and Wehrli (1977b).
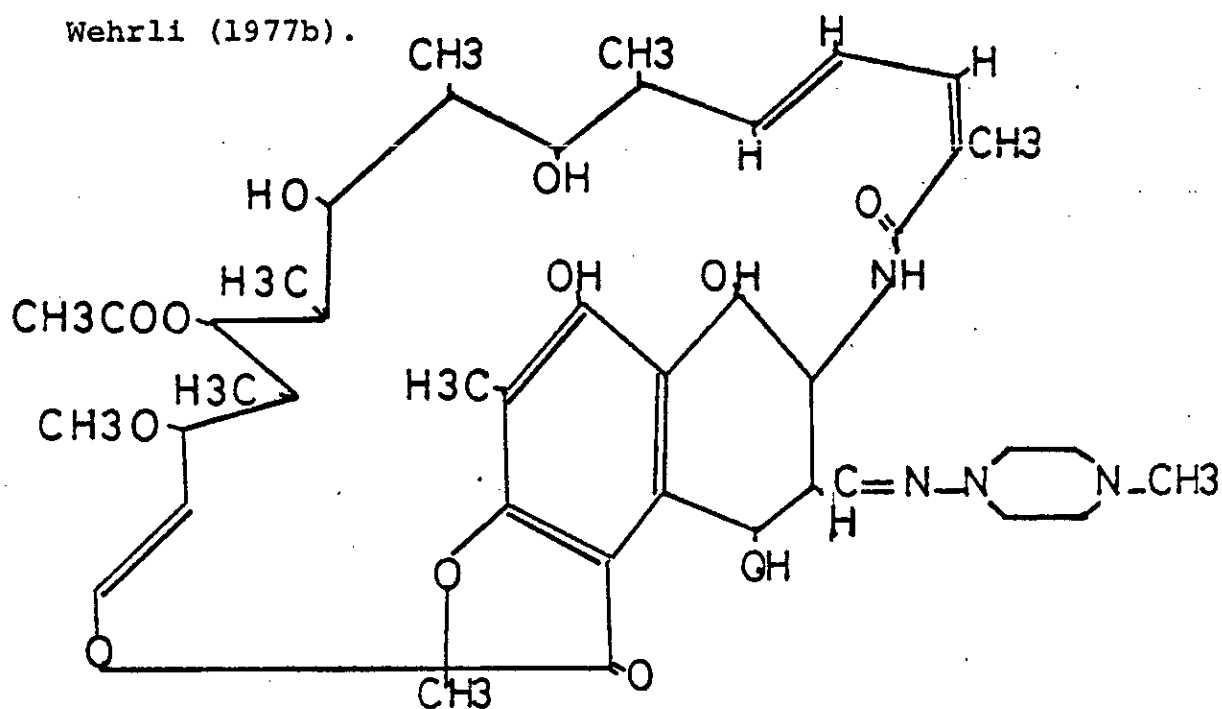
d. Sensitivity of different bacteria

Rifampicin is an antituberculosis drug (Tanaka et al, 1978; Matzura, 1979); it has a wide range of antibacterial activity against gram negative and gram positive bacteria (Bals and Filipesco, 1969; Reinlein et al, 1973; Shorin and Shapolova, 1974). As the various E.coli strains differ in their permeability for the antibiotic rifampciin

(Matzura, 1979) one would expect that the different

bacterial species do vary in their permeability to

rifampicin.   Page and Sadoff (1976) have used 10 µg/ml

of rifampicin for the isolation of the rifampicin resistant

mutant of Azotobacter vinelandii, whereas only 4 µg/ml

final concentration of rifampicin was used for the

selection of rifampicin resistant Serratia marcescens

(Parish, 1975) and 50 µg/ml was used for the selection

of Achromobacter rifampicin resistant mutant (Robb

et al, 1977).   Arioli et al (1977) have used 100 µg/ml of

rifampicin for the isolation of the Salmonella typhimurium

rifampicin resistant mutant.

In E.coli, spontaneous rifampicin resistant mutants

occur at a frequency of 10^{-7}-10^{-8} (Ezekiel and Hutchins,

1968; Matzura, 1979).   Interestingly, Tanaka et al (1978)

have demonstrated that almost all E.coli strains isolated

from tuberculosis patients treated with rifampicin

antibiotic for a month were resistant to the antibiotic

and were highly resistant to the rifampicin after 2-6 months

treatment.

e.   Action

The antibiotic rifampicin inhibits RNA synthesis

(Hartmann et al, 1967; Scaife, 1976) and the initiation of

the transcription is resumed upon the release of this kind

of inhibition (Engbaek et al, 1976).   The inhibition of

RNA synthesis by rifampicin is achieved by blocking the

formation of the preinitiation complex (Sippel and

Hartmann, 1968).

In addition to the RNA synthesis inhibition, rifampicin has other actions: first, the inhibition of the replication of a number of DNA viruses in mammalian cells (Franklin and Snow, 1975); second, the elimination of F factor from the rifampicin sensitive E.coli cells but not from the rifampicin resistant one which may be attributed to the requirement of certain RNA species for F plasmid replication (Falkow, 1975); third, it alters the dependence of the ØX174 replicative form synthesis on the dnaC protein activity as in E.coli cells the dnaC protein is not required for the bacteriophage ØX174 parental replicative form synthesis but only when rifampicin inhibits the E.coli DNA dependent RNA polymerase (Dumas et al, 1975); fourth, rifampicin is immunosuppressive both in animals and in man (Bass et al, 1978) and finally, rifampicin and arginine induce the synthesis of acety-lornithine δ-transaminase in an arginine inducible E.coli W rifampicin resistant mutant (Wozny et al, 1975).

The interaction between the enzyme RNA polymerase and the rifampicin has been investigated thoroughly (Stender and Scheit, 1976; Wehrli et al, 1976; Wehrli, 1977a). The mode of action of the rifampicin antibiotic has been reviewed (Wehrli and Staehelin, 1971; Riva and Silvestri, 1972; Wehrli, 1977b) and recently investigated (Kessler and Hartmann, 1977; McClure and Cech, 1978; Kassavetis et al, 1979). It has recently been discovered that the RNA polymerase-rifampicin complex retains its ability to recognise the promoters on the T7 DNA though it is unable

to initiate RNA chains; the drug-enzyme complex also blocks transcription by drug-free molecules which have initiated RNA chains upstream (Kassavetis et al, 1979).

It has been observed that the affinity of the rifampicin for binding the enzyme is reduced in the presence of the DNA and that the action of the rifampicin at low concentration is very slow on RNA polymerase engaged in catalytic activity compared to its high activity at higher concentrations at which the rifampicin overcomes the protecting effect of the DNA (Kessler and Hartmann, 1977). The study of McClure and Cech (1978) have shown that there is no effect of the antibiotic rifampicin on the selectivity of the RNA polymerase for binding to the promoters and that rifampicin blocks RNA chain elongation.

f.   Other inhibitors of RNA synthesis

The antibiotics, streptolydigin and streptovaricin (Mizuno et al, 1968; Schleif, 1969; Iwakura et al, 1973) and many other inhibitors of RNA synthesis have been reported (Wehrli et al, 1968; Chou et al, 1978; De Lorbe et al, 1979; Schleif, 1969) such as actinomycin, olivamycin, cinerubin, polyriboinosinic acid (polyI), serminal plasmin ... etc. These inhibitors share this character with rifampicin but some of them are different from the rifampicin antibiotic in respect to their mode of action, for example, actinomycin interacts with the DNA and not with the RNA polymerase (Reich and Golgberg, 1964; Wehrli et al, 1968); polyriboinosinic

acid (polyI) competes with the DNA for the polynucleoside binding sites on RNA polymerase (Hirschbein et al, 1967; De Lorbe et al, 1979).

A more powerful, dimeric rifampicin called rifamazine has been reported to be active against the RNA polymerases extracted from the rifampicin resistant mutants of E.coli (Fietta and Silvestri, 1975).   It is well known that the rifampicin resistant mutations in the E.coli map at 88.5 minutes in the rpoB gene (Bachmann et al, 1976; Matzura, 1979).

I found that the antibiotic rifampicin is a useful tool for the detection of the presence of the E.coli rpoB3 gene product in the bacterial cell.

6.   Comparative Studies between RNA Polymerases of E.coli and other Gram Negative Bacteria

It is interesting to compare the E.coli RNA polymerase with the RNA polymerase of other gram negative bacteria because all of the bacteria chosen for this work are of the gram negative group.   It has been found that the RNA polymerases of these bacteria also consist of the subunits α,β,β' and σ and some of them have similar molecular weights as E.coli and the others are slightly variated (Burgess, 1976):

Bacterial species	β'	β	σ	α_2
E.coli	165	155	95	39
	150	145	86	41
	160	155	85	40
Azotobacter vinelandii	165	155	95	39
Pseudomonas putida	165	155	98	44
Pseudomonas BAL-31	165	155	89	38

The above table extracted from Burgess (1976) shows no
difference in molecular weights of the RNA polymerase
subunits between E.coli and Azotobacter vinelandii whereas
Pseudomonas putida RNA polymerase is characterised by
higher molecular weights of α and σ subunits.

Serratia marcescens subunits of RNA polymerase have
the same migration as E.coli subunits on the same gel
(Konze-Thomas and Rüger, 1976).   In their study,
Konze-Thomas and Rüger (1976) have allowed the
reconstitution of the intergeneric hybrid RNA polymerases
from the interaction of the subunits of E.coli and
S.marcescens RNA polymerases;   these enzymes are able to
recognise the promoters and the terminators of the DNA
template;   it is likely that the β subunit structure of
Serratia marcescens is very similar to E.coli because the
β subunit of the RNA polymerase enzyme from both bacteria
can fully substitute each other and the hybrid enzymes
yielded 92% and 96% of the activity of the homologous
reconstituates whereas the replacement of the $\beta'_{E.coli}$
by the $\beta'_{S.marcescens}$ have resulted in 50% loss of the
activity.   On the other hand the reciprocal replacement
of $\beta'_{S.marcescens}$ by $\beta'_{E.coli}$ has yielded an active hybrid
enzyme with 85% of the activity of the homologous
reconstituate;   this led to the conclusion that no decisive
difference is detected, in vitro, between the RNA polymerase
of E.coli and S.marcescens (Konze-Thomas and Rüger, 1976).

The sigma subunits of Salmonella anatum and Proteus
mirabilis are larger than the sigma subunit of E.coli

whereas the α subunit of <u>Proteus mirabilis</u> is smaller than the α subunit of <u>E.coli</u> (Fukuda <u>et al</u>, 1977). A comparative analysis between the RNA polymerases of <u>Salmonella typhimurium</u>, <u>Salmonella anatum</u>, <u>Serratia marcescens</u>, <u>Aerobacter aerogenes</u>, <u>Proteus mirabilis</u> and <u>Bacillus subtilis</u> had led to the conclusion that all the RNA polymerases from these bacteria cross-react with anti-<u>E.coli</u> holoenzyme and were recovered in antibody precipitates (Fukuda <u>et al</u>, 1977). In addition, the enzyme activity of all the examined bacteria was inhibited by anti-<u>E.coli</u> β antiserum which suggests that the structure of the β subunit is conserved in these bacteria (Fukuda <u>et al</u>, 1977).

The RNA polymerase of <u>Azotobacter vinelandii</u> is able to recognise the T7 DNA promoters and terminators that are recognised by the <u>E.coli</u> RNA polymerase (Wiggs <u>et al</u>, 1979) and this suggests that there is a conservation of the promoter and the terminator recognition function among the two bacteria (Wiggs <u>et al</u>, 1979).

Although the studies with the RNA polymerases of the gram negative bacteria, other than <u>E.coli</u>, are not much developed, one can conclude from the above studies that in spite of the divergence of the genomes of these bacteria and <u>E.coli</u> during evolution, their RNA polymerases seem to have some properties that are common with <u>E.coli</u> and that are not changed during evolution.

In my thesis, I have carried this further by the <u>in vivo</u> investigation of the <u>E.coli</u> <u>rpoB</u>3 gene expression in these bacteria.

7.   The Heterospecific Gene Expression in Prokaryotes

The heterospecific gene expression phenomenon is applied to the introduction of the genes into an alien cytoplasm which belongs to a phylogenetically distant species followed by their functioning in the alien host (Ehrlich and Sgaramella, 1978).   It has been well established and developed during the last few years as a result of the discovery of the wide host range plasmids and the progress of the DNA cloning techniques.

In 1974, Chang and Cohen demonstrated that a gene from Staphylococcus aureus can be expressed in E.coli. The expression of the E.coli histidine genes in Pseudomonas aeruginosa and Salmonella typhimurium was detected after the transfer of the plasmid RP1I-42 from E.coli into the histidine auxotrophic bacterial species (Olsen and Gonzalez, 1974).   They have observed a lower transfer of the histidine genes compared to the drug resistant markers carried by the plasmid and they have attributed this phenomenon to the restriction of the E.coli histidine genes in the recipient alien cell. Dunican and Tierney (1974) have demonstrated the transfer of the nitrogen fixing ability from Rhizobium trifolii to Klebsiella aerogenes by plasmid R1-19 drd;   neither E.coli nor Pseudomonas aeruginosa were able to reduce acetylene when tested for nitrogen fixing ability in the same way as Klebsiella aerogenes.   The genetic complement-ation of the nitrogen fixation mutants (nif⁻) of Azotobacter vinelandii by the Klebsiella nif genes carried

by the plasmid RP41 was demonstrated (Cannon and Postgate, 1976). _Azotobacter vinelandii_, in contrast to _Agrobacterium_, was able to transcribe and translate the _Klebsiella_ _nif_ genes (Cannon and Postgate, 1976). In addition, the _Klebsiella_ _nif_ genes are fully expressed in _S.typhimurium_ (Postgate and Krishnapillai, 1977).

RP41 is derived from the P-plasmid RP4 and it harbours _nif-his_ and RP4 drug resistant determinants (Dixon _et al_, 1976). The cotransfer of the _Rhizobium_ _trifolii_ _nif_ genes with the P-plasmid RP1 was observed (Stanley and Dunican, 1979); in their study of the heterospecific gene expression they have detected the expression of the _Rhizobium_ _trifolii_ _nif_ genes in _Agrobacterium_ _tumefaciens_ as well as in the _Klebsiella_ _pneumoniae_ _nif_ mutants. A ribosomal DNA fragment of _E.coli_ was introduced into _Proteus_ _mirabilis_ by the F-prime F14 (Morgan and Kaplan, 1976), transcribed, and the products were packaged into ribosomes which exhibit normal functional activities in the hybrid _Proteus_ _mirabilis_ strain (Morgan and Kaplan, 1976). The FN68 plasmid of the F-compatibility group which carries the _his_ and the _nif_ genes of _Klebsiella_ _pneumoniae_ was used for intergeneric matings (Cannon _et al_, 1976). The FN68 plasmid was transferable to _Klebsiella_ _aerogenes_, _E.coli_ and _Salmonella_ _typhimurium_ but not to _Proteus_ _mirabilis_ and both the _his_ and the _nif_ genes were expressed in these bacterial species (Cannon _et al_, 1976; Kennedy and Postgate, 1977).

Furthermore, Boucher _et al_ (1977) have introduced

the bacteriophage Mu into the taxonomically distant

bacteria Pseudomonas solanacearum and Rhizobium meliloti

by means of the plasmid RP4::Mu cts.   In the two alien

hosts, the Mu genome is expressed and the plaque forming

Mu phages are produced (Boucher et al, 1977; Dénarié

et al, 1977).   By contrast the transferability of the

RP4::Mu plasmid and its derivative RP4::Mu cts62 r23

(pGM17) from E.coli to Agrobacterium tumefaciens was

proved to be very low and the development of this phage

Mu cts62 r23 in this host was very poor compared to E.coli

(Van Vliet et al, 1978).

The plasmid RP4-trp which carries the complete

tryptophan operon of E.coli was constructed and was

transferred from E.coli into Pseudomonas aeruginosa

(Nagahari et al, 1977).   The expression of the E.coli

tryptophan operon was constitutive in Pseudomonas

aeruginosa implying the inability of the Pseudomonas trp

repressor. to bind to E.coli trp operator (Nagahari

et al, 1977).   Recently, the same plasmid RP4-trp was

transferred from E.coli into four Rhizobium leguminosarum

trp mutant recipients (Nagahari et al, 1979) which were

converted into the trp$^+$ phenotype due to the expression of

the E.coli trp genes and they were able to transfer back

the plasmid into E.coli trp and Pseudomonas putida trpB

recipients (Nagahari et al, 1979).   As in the Pseudomonas

aeruginosa case, the expression of the E.coli trp genes in

Rhizobium leguminosarum as well as in Pseudomonas putida

was constitutive and the enzymatic activity of the trp genes

were not repressed by exogenous tryptophan (Nagahari et al, 1979). In contrast, low gene expression of the Pseudomonas trpAB genes, carried by a derivative of the plasmid R68.44, was observed in E.coli K12 trp mutants (Hedges et al, 1977).

Johnston et al (1978) have constructed derivatives of the plasmid R68.45 which carry the different trp genes of Rhizobium meliloti and they were able to suppress the different trp mutants of R.leguminosarum and P.aeruginosa but none of the E.coli trp mutants. Many strains of Azotobacter vinelandii that are unable to fix nitrogen (nif⁻) were transformed into nif⁺ with the DNA of Rhizobium trifolii (Bishop et al, 1977). Tryptophan auxotrophy of an A.tumefaciens mutant was suppressed by RP4-Ø80trp plasmid on which Ø80trp transducing phage had been transposed (Mergeay and Gerits, 1978). Various mutations in Pseudomonas fluorescens can be complemented by the E.coli chromosomal genes carried by the F-prime plasmids, for example, arginine, isoleucine, valine, histidine, leucine and methionine (Mergeay and Gerits, 1978); in addition they have described the dominance of the rpoB allele carried by KLF10 over the rif^r allele of the P.fluorescens thus converting the rifampicin resistant P.fluorescens into the rifampicin sensitive phenotype.

It has been reported that the B.subtilis leu genes which are cloned on the plasmid RSF2124-B.leu can be expressed in E.coli cells whereas the E.coli leu genes were not expressed in B.subtilis cells when transferred

(Nagahari and Sakaguchi, 1978); this is not the only example of the inability of the E.coli genes to express in B.subtilis because there are several other E.coli genes such as trpE and the genes coding for the resistances to ampicillin, kanamycin, chloramphenicol and tetracycline were tested for their ability to function in B.subtilis but none of them did (Ehrlich and Sgaramella, 1978).

It is evident that not every gene can be expressed in any alien cell because of the existence of the evolutionary barriers that can prevent the operation of the heterospecific gene expression system. These barriers could be nuclease barriers, replication unit barriers, transcription barriers and translation barriers (Ehrlich and Sgaramella, 1978; Sakaguchi and Nagahari, 1978).

In my study of the heterospecific gene expression the structural genes for the E.coli RNA polymerase were introduced by plasmids into many bacterial species which are phylogenetically distant or close to E.coli and their functioning in these cells was investigated.

8.A   The Plasmid RP4

a.   Isolation

RP4 is a plasmid that is originally derived from a strain of P.aeruginosa which was highly resistant to the antibiotic carbenicillin (Lowbury et al, 1969); at different times and in different laboratories it has been designated at RP1, R1822, R18 and RK2 (Holloway and Richmond, 1973; Grinsted et al, 1977; Meyer et al, 1977;

Pühler et al, 1978; Burkardt et al, 1979; Thomas et al, 1979). The plasmid RP4 confers resistance to the ampicillin/carbenicillin, kanamycin/neomycin and the tetracycline antibiotics (Datta et al, 1971). RP4 has a buoyant density of 1.719 g/cm^3 which is equivalent to about 60% Guanine + Cytosine ratio (Grinsted et al, 1972; Holloway, 1975).

b. Compatibility

RP4 belongs to the P-compatibility group of plasmids and it can coexist with the F-like or the I-like plasmids (Datta et al, 1971).

c. Sensitivity to bacteriophages

RP4 specifies the sensitivity to many bacteriophages such as PRR1, PRD1, PR3 and Pf3 (Olsen and Shipley, 1973; Stanisich, 1974; Olsen et al, 1977).

d. Size

The molecular weight of RP4 is estimated between 36-40 x 10^6 daltons (Grinsted et al, 1972; Bennett and Richmond, 1976; Holloway, 1975; Barth and Grinter, 1977; De Picker et al, 1977; Burkardt et al, 1978; Sakanyan et al, 1978; Broda, 1979). Recently it has been found that the plasmid RP4 is about 19 µm long (Sakanyan et al, 1978; Burkardt et al, 1979).

e. Physical map

The physical map of the RP4 plasmid, which is shown below, was the product of the restriction analysis, deletion mutants, and insertion of transposon C (Barth and Grinter, 1977; De Picker et al, 1977; Grinsted et al, 1977; Sakanyan et al, 1978; Thomas et al, 1979).
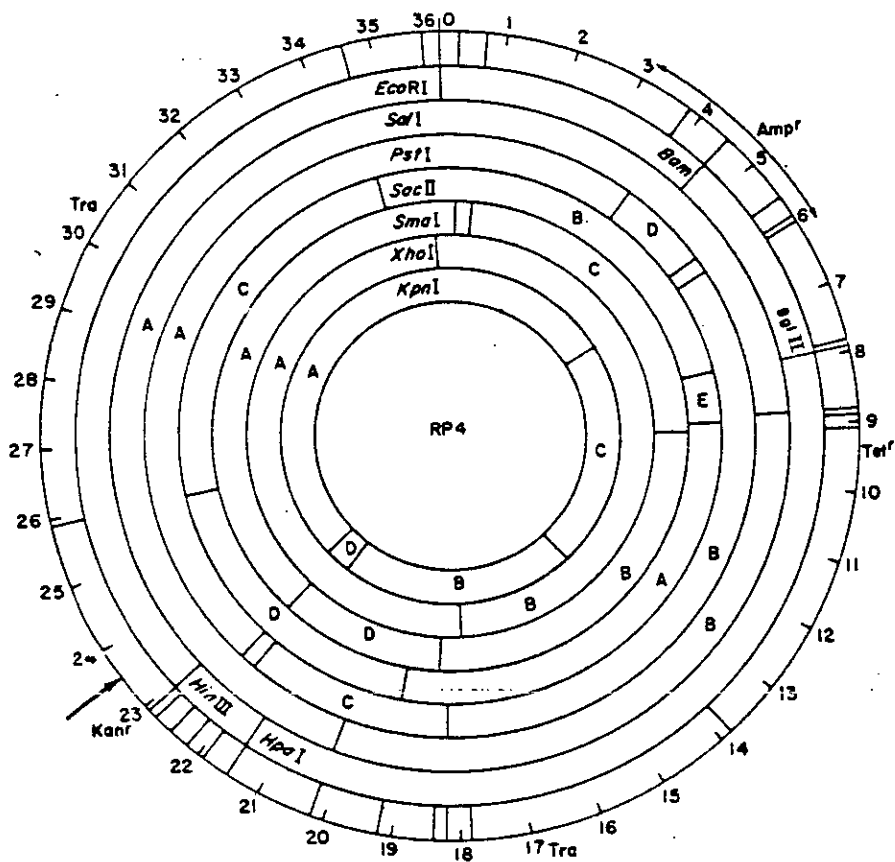
Figure 1. The physical map of RP4, extracted from Bukhari et al (1977).

This map (Fig. 1) shows the various restriction enzymes that can cleave RP4 DNA and it is worth noting that the XbaI enzyme does not cleave RP4 (De Picker et al, 1977).

Sakanyan et al (1978) have mapped the replication gene(s), rep, in the region between amp and tet or after the tet gene and the incompatibility gene(s), inc, between amp and the EcoRI site or between amp and tet genes; these results support the previous mapping of Barth and Grinter (1977) of the rep gene(s). Deletions

of the plasmid RP4 were produced _in_ _vivo_ and _in_ _vitro_
and were designated RP4-δl (Hedges _et_ _al_, 1976) and
mini RP4 (Haque, 1978; Haque, 1979; Pühler _et_ _al_,
1978) to serve as cloning vehicles.

f.   Host range

Most interesting is the wide host range of the
plasmid RP4 as it had been transfered by the various
mating methods as shown in Table 1.

The broad host range of RP4 makes it useful for the
transfer of genes between bacterial species
for the investigation of heterospecific gene
expression.

An RP4 derivative has been constructed which carries
the _his_ operon of _E.coli_ and has been designated as
RP1I-42 (Olsen and Gonzalez, 1974).   Three derivatives
of RP4 designated as RP1-PM1,2 and 3 have incorporated
fragments of the _P.mirabilis_ chromosomal DNA (Jacob _et_ _al_,
1976) and another two RP4 variants have incoporated
R.leguminosarum chromosomal DNA and were designated
as RP4-R1 and RP4-R2 respectively (Jacob _et_ _al_, 1976).

Dixon _et_ _al_ (1976) have constructed the plasmid RP41
which harbours the _nif_ _his_ region of the _K.pneumoniae_
chromosome.   An RP4-colE1 hybrid plasmid was obtained
_in_ _vitro_ (Stepanov _et_ _al_, 1976).   The genomes of the

A.calcoaceticus	A.calcoaceticus	1.5×10^{-1}	Olsen & Shipley (1973)
	P.aeruginosa	1.2×10^{-4}	
	P.fluorescens	1.8×10^{-2}	
	E.coli	2.1×10^{-4}	
	A.vinelandii	7.5×10^{-6}	
A.vinelandii	A.calcoaceticus	O	
	P.aeruginosa	2.8×10^{-6}	
	P.fluorescens	3.6×10^{-5}	
	E.coli	2.0×10^{-4}	
E.coli	P.aeruginosa	4.9×10^{-4}	
	P.fluorescens	3.0×10^{-4}	
	E.coli	2.4×10^{-3}	
	A.calcoaceticus	1.0×10^{-4}	
	A.vinelandii	3.8×10^{-4}	
	E.coli	3.6×10^{-4}	
E.coli	R.leguminosarum	6.2×10^{-5}	Beringer (1974)[*]
	E.coli	4.9×10^{-3}	
R.leguminosarum	R.leguminosarum	2.2×10^{-3}	
	E.coli	2.8×10^{-4}	
E.coli	P.solanacearum	10^{-2}	Message et al (1975)[*]
E.coli	A.calcoaceticus	$1 \times 10^{-5} - 1.3 \times 10^{-5}$	Towner and Vivian (1976)[*]
A.calcoaceticus	A.calcoaceticus	$1.1 \times 10^{-4} - 2.8 \times 10^{-4}$	
A.tumefaciens	A.tumefaciens	$1.3 \times 10^{-3} - 1.6 \times 10^{-1}$	Van Larebeke et al (1977)
	A.radiobacter	$1.7 \times 10^{-5} - 5 \times 10^{-3}$	
P.aeruginosa	E.coli	$3.3 \times 10^{-5} - 1.6 \times 10^{-4}$	Alexander & Jollick (1977)
	P.aeruginosa	$1 \times 10^{-5} - 8.9 \times 10^{-4}$	
	Caulobacter vibrioides	$5.3 \times 10^{-4} - 1.1 \times 10^{-3}$	
	Caulobacter crescentus	$4.4 \times 10^{-4} - 6.9 \times 10^{-4}$	
R.meliloti	R.meliloti	10^{-4} **	Meade & Signer (1977)
	R.meliloti	10^{-1} ***	

Table 1.

Donor	Recipient	Frequency	Reference
E.coli J5-3	E.coli J6-2	8.6×10^{-4}	Datta et al (1971)
	P.aeruginosa PAO2	2.3×10^{-3}	
	C.violaceum D254		
	R.trifolii 24G	10^{-6} or greater	
	R.meliloti 1.5		
	A.tumefaciens B6	$10^{-8} - 10^{-9}$	
P.aeruginosa	P.aeruginosa	5×10^{-4}	Grinsted et al (1972)
	E.coli	7×10^{-4}	
	P.mirabilis	1×10^{-5}	
E.coli	E.coli	5×10^{-4}	
	P.aeruginosa	1×10^{-5}	
	P.mirabilis	4×10^{-4}	
P.mirabilis	E.coli	5×10^{-4}	
	P.aeruginosa	2×10^{-5}	
R.lupini	R.lupini	not mentioned	Pühler et al (1972)
P.aeruginosa PAL	P.aeruginosa PAT	5.6×10^{-3}	Olsen & Shipley (1973)
	A.calcoaceticus	8.0×10^{-7}	
	R.rubrum	3.0×10^{-5}	
	R.spheroides	1×10^{-4}	
	E.coli	3.3×10^{-3}	
	V.cholera	2.6×10^{-4}	
P.aeruginosa PAT P.fluorescens	P.fluorescens	1.2×10^{-3}	
	P.putida	1.4×10^{-6}	
	A.vinelandii	7×10^{-6}	
	S.typhimurium	1.7×10^{-4}	
	S.boydii	4.1×10^{-6}	
	N.perflava	4.2×10^{-4}	

Legend to Table 1

This Table illustrates the transfer of RP4 from various donors. All the frequencies of transfer are estimated per donor except where * is present. Liquid culture matings and plate matings are represented by ** and *** respectively.

bacteriophage Mu or its mutants Mu cts62 and Mu cts64 r23 have been inserted into the plasmid RP4 (Dénarié et al, 1977; Boucher et al, 1977; Van Vliet et al, 1978). The complete trp operon of E.coli has been incorporated into RP4 forming the plasmid RP4-trp (Nagahari et al, 1977). The degradative plasmid Tol can integrate into the RP4 plasmid forming the complex RP4-Tol (Jacoby et al, 1978; Nakasawa et al, 1978). Holsters et al (1978) have reported the cointegration of the A.tumefaciens tumour inducing plasmids, Ti, with the plasmid RP4. Recently, Barth (1979) has prepared various RP4-primes carrying different segments of the E.coli genome.

g.   Transposon insertion

Transposons which are the DNA segments which can insert into several sites in a genome (Campbell et al, 1977) have been reported to insert into RP4   RP4 has thirty six insertion sites for Tn7 (Barth et al, 1978), twenty three insertion sites for Tn501 (Stanisich et al, 1977; Grinsted et al, 1978) and eight sites for the insertion of Tn951 (Cornelis et al, 1979).

h.   Chromosome mobilisation

The ability of transferring chromosomal genes is possessed by various plasmids (Holloway, 1979). The plasmid RP4 is found to promote chromosomal mobilisation at the low frequency of 10^{-7}-10^{-8} in rec$^+$ or recA E.coli donor strains (Dénarié et al, 1977; Barth, 1979). RP4 chromosomal mobilisation ability, though poor, was reported in A.calcoaceticus (Towner and Vivian, 1976), in

P.glycinea (Lacy and Leary, 1976), in R.meliloti (Meade and Signer, 1977) and in R.lupini (Pühler and Burkardt, 1978). However, Hedges and Jacob (1977) have reported their consistent failure to mobilise ⟨⟩ chromosomal genes using RP4 (unpublished results). Danilevich et al (1978a, 1978b) have found that the efficiency of ⟨⟩ integration of RP4 into the E.coli chromosome is increased in the presence of a homologous region such as Tn1 in a rec$^+$ cell.

It has been found recently that the frequency of transfer of the chromosomal genes by the plasmid RP4 has become higher under various conditions such as the presence of the normal attachment site of the bacteriophage λ on RP4 λatt (Watson and Scaife, 1978) and when RP4 carries a homologous piece of DNA that is present on the chromosome; this was demonstrated when the genome of the bacteriophage Mu was inserted into RP4 and the chromosome both in E.coli and K.pneumoniae (Dénarié et al, 1977) and when the various segments of the E.coli chromosome were inserted into RP4 (Barth, 1978; Barth, 1979; Casse et al, 1978). Yet, Jacob et al (1976) were unable to see this increase in chromosome mobilising ability in Proteus mirabilis or R.leguminosarum.

B. RP4 λatt

The plasmid RP4 λatt is interesting to me for two main reasons: first, it serves as an excellent source for the wide host range plasmid RP4 and second, it allows the lysogenisation of the λb2-like bacteriophage λdrpoBC18 (Kirschbaum and Konrad, 1973).

a.   Construction

The presence of only one EcoRI site (Jacob and Grinter,
1975) in the plasmid RP4 has allowed the insertion of the
EcoRI generated fragment of the bacteriophage λ DNA which
resulted in the formation of the recombinant plasmid
RP4 λatt (Pastrana, 1976).   RP4 λatt has incorporated the
EcoRI fragment (srIλ2-3) which harbours the genes
att-int-xis and contains one target for the HindIII
restriction endonuclease (Pastrana, 1976).   Furthermore,
the int gene carries the amber mutation int-29 and further
studies of the expression of the λ genes carried by the
RP4 λatt revealed that the promoter for xis gene is not
present (Watson and Scaife, 1978);   they have also
provided evidence that the RP4 λatt does promote chromo-
somal mobilisation by int-promoted integration into the
chromosomal λ attachment site.   RP4 λatt serves as a
vehicle for the transfer of the λ sequence P.P' whose
presence was confirmed by testing its ability to
lysogenise λb2 (Zichichi and Kellenberger, 1963;
Pastrana, 1976).

9.   Thesis Plan

The subsequent part of the thesis is the experimental
investigation Chapter which consists of basically three
parts.   In the first part, I will describe the construction
of the RP4 derivative, pZD100, from the lysogenisation
process of RP4 λatt with λdrpoBC18 which carries the
structural genes for the ββ' subunits of RNA polymerase.

I will show that on transfer into a λ non-lysogen recipient, this plasmid is subjected to zygotic induction. To overcome zygotic induction , which results in the apparent reduction of the efficiency of transfer of the plasmid into a λ sensitive recipient, deletant mutants which consist mostly of RP4 and carry the rpoB3 gene were derived. One of these mutant plasmids pZD23 serves as a better tool for my purpose of the investigation of heterospecific gene expression as it will be shown that this plasmid transfers into the λ non-lysogen recipient without the subjection to zygotic induction.. The original plasmid, pZD100 and the derivatives were characterised in vivo and in vitro; the detailed information about their genetical and physical structure are shown in part II.

The construction of the original plasmid and its derivatives was carried out in E.coli but their utilisation for the heterospecific gene expression was carried out in various bacterial species as will be shown in part III. In part III, I shall discuss the utilisation of these plasmids as vectors for E.coli rpoB3 gene and how the various bacterial species which belong to many families responded to the presence of the foreign E.coli gene in their cells. The gene codes for the dominance of the rifampicin resistance converting the rifampicin sensitive E.coli cell into rifampicin resistant. This is the criterion upon which the detection of heterospecific gene expression in an alien cell was based.

Heterospecific gene expression results in the production of $\beta\beta'$ subunits of E.coli RNA polymerase in the alien cell allowing them to interact with the native subunits of the enzyme converting the cell into the rifampicin resistant phenotype. It will be shown, in this part, that the heterospecific gene expression operates successfully in the species that are closely related to E.coli.

EXPERIMENTAL   INVESTIGATIONS

Chapter 2 - Experimental Investigations

Part I The Construction of the Plasmids carrying the
E.coli rpoB gene

My aim here is to construct a plasmid for the E.coli structural genes of the ββ' subunits of the enzyme RNA polymerase. It will serve as a vector for the rpoBC genes that can be transferred into a broad range of bacterial species which belong to taxonomically different families. The majority of the chosen bacterial species are members of the Enterobacteriaceae; others belong to the Azotobacteriaceae, Pseudomonadaceae and Rhizobiaceae.

There are specialised transducing phages, such as λdrpoBC18, which may be utilised as vectors for that region of the bacterial chromosome (rpoBC) which contains the structural genes for the ββ' subunits of RNA polymerase. However, the phages are not suitable for heterospecific gene expression studies since they have a limited host range.

Recently, broad host range plasmids have been used as a tool for genetic engineering. The P-group plasmid RP4 has been extensively used in construction of derivative plasmids with DNA pieces of phage or bacterial origin (Dixon et al, 1976; Dénarié et al, 1977; Nagahari et al, 1977; Van Vliet et al, 1978).

The incorporation of the E.coli rpoBC genes into RP4 will be discussed in two sections. Firstly, I will present the evidence for the construction of the plasmid pZD100 by the lysogenisation of the RP4 derivative, RP4 λatt,

with λd<u>rpoBC</u>18.   Later I will discuss the derivation of

several <u>rpoBC</u> plasmids from the original plasmid pZD100.

A.   <u>The Construction of the Plasmid pZD100</u>

a.   <u>Introduction</u>

The construction of a vector for the <u>E.coli</u> <u>rpoBC</u>

genes to provide a means for their transfer from <u>E.coli</u>

into an alien cytoplasm where their expression can be

investigated.   I have used λd<u>rpoBC</u>18 as a source for the

<u>rpoBC</u> genes.   It is a defective specialised transducing

phage which confers two properties on <u>E.coli</u>, resistance

to the antibiotic rifampicin and temperature sensitivity

which is a result of the <u>c</u>I857 mutation in the repressor gene of

the phage (see Introduction).   The plasmid RP4 λ<u>att</u> is a

derivative of the wide host range plasmid RP4 and it is

suitable for <u>in</u> <u>vivo</u> cloning as it provides the

attachment region of the bacteriophage λ.

The essential step for the construction of the plasmid

pZD100 is the lysogenisation of RP4 λ<u>att</u> with λd<u>rpoBC</u>18

which results in the formation of a plasmid molecule

comprising RP4 and λd<u>rpoBC</u>18.

There are three possibilities for the integration of

the λd<u>rpoBC</u>18 in an <u>E.coli</u> cell harbouring the plasmid

RP4 λ<u>att</u>; it may integrate into the chromosome through the

λ integration site or through recombination between the

homologous regions on the phage and the chromosome;   the

third possibility is that it may integrate into the plasmid

RP4 λ<u>att</u> through P.P' sequence of the <u>att</u>λ.   To exclude

the possibility of the integration of the phage into the
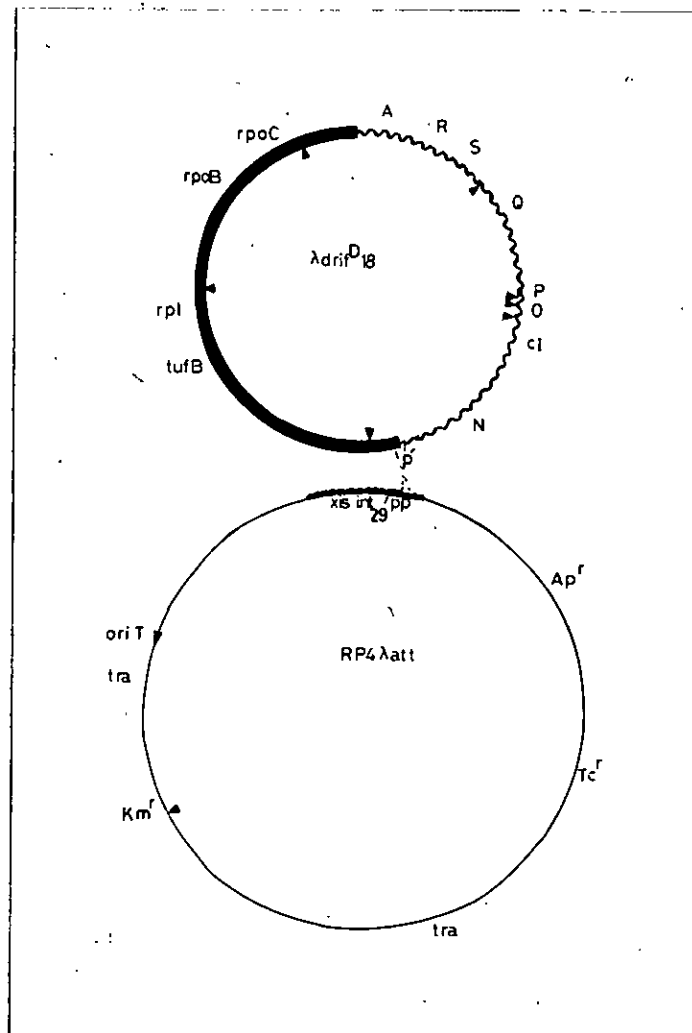
Fig. 2. The lysogenisation of RP4 λatt with λdrpoBC18.
The serrated part of λdrpoBC18 represents the λ genome and
the solid part represents the bacterial chromosome
segment which contains the rpoB3 and the rpoC genes. The
serrated portion of RP4 λatt represents attλ and the solid
part represents the RP4 molecule. This figure illustrates
the orientation of the λatt fragment with respect to RP4
genome which will be discussed in Part II.

chromosome through the recombination of the homologous regions, the construction process was carried out in a rec⁻ cell.

b. Results

The transduction of ZD7 (metB B_1^- recA56 str-s λ^S/ RP4 λatt) with λdrpoBC18 is shown in Table 2. The constructed plasmid pZD100 is the result of the lysogen- isation of RP4 λatt with λdrpoBC18 (Fig. 2). The resistance of ZD7 transductants to rifampicin cannot be attributed to the presence of the constructed plasmid (Fig. 3) unless it is proved that it is associated with plasmid transfer, i.e. the transfer of the resistance to ampicillin, kanamycin and tetracycline antibiotics. I will provide evidence, later, that the plasmid pZD100 is temperature sensitive.

Transfer of the plasmid pZD100 was shown by mating E.coli ZD100 (metB B_1^- recA56 λcI857S7/pZD100) with J62 (Pro trp his λ^+ str-r) as shown in Table 3. A parallel mating experiment to transfer RP4 λatt from ZD7 (metB B_1^- recA56 str-s λ^S/RP4 λatt) into the same recipient was run at the same time as a control. The transconjugants were selected on glucose minimal media supplemented with the required amino acids and the antibiotics ampicillin, kanamycin, tetracycline and streptomycin. A random selection of 100 transconjugants of the mating involving the transfer of the plasmid pZD100 were tested for their resistance to rifampicin by patching them on two selective plates, one of them containing rifampicin.

Table 2   The construction of the plasmid pZD100

Recipient	Transduction to rifampicin resistant with λdrpoBC18	Location of λdrpoBC18
ZD6 (RP4)	+	Chromosomal
ZD7 (RP4 λatt)	+	Plasmid and chromosomal

ZD7 (metB B_1^- recA56 str-s λ^S/RP4 λatt) was transduced with λdrpoBC18 (10^{10}/ml p.f.u. and 4×10^5 transductants/ml). The transduction was carried out as described by Kirschbaum (1973). 0.2 ml of fresh late log phase ZD7 in 0.01 M $MgSO_4$ was mixed with 0.01 ml of phage lysate and the mixture was incubated at $30^{\circ}C$ for 15 mins. 0.1 ml of the mixture was whirlimixed with 2 mls BBL top agar and was plated over fresh BBL plate (see Media). After six hours incubation at $30^{\circ}C$, the rifampicin was layered in top agar at 100 µg/ml. Transduction of ZD6 (metB B_1^- recA56 str-s λ^S/RP4) was carried out in the same way. 0.1 ml of ZD7, ZD6 and λdrpoBC18 [0.2 ml of 0.01 M $MgSO_4$ + 0.01 ml phage lysate] were plated separately as controls too.

The yield of the transductants (above) was rather high (+). The untransduced rifampicin resistant colonies of ZD6 and ZD7 appeared at a frequency of 10^{-7}-4×10^{-7}.

The transductants and the untransduced colonies of ZD6 and ZD7 on the rifampicin plate were tested for their temperature sensitivity as well as their resistance to rifampicin. The untransduced colonies of ZD6 and ZD7 were resistant to rifampicin at $42^{\circ}C$. The temperature sensitive rifampicin resistant transductants of ZD6 and ZD7 comprised 56% and 100% respectively.

The temperature sensitive rifampicin resistant transductants (e.g. ZD100) were tested for the location of λdrpoBC18 (whether plasmid or chromosomal) by spot mating nine of them with the following recipients: TGL1 (metB his argH recA str-r) and TGL2 (metB his argH recA str-r λcI857). Recombinants were selected in the presence of streptomycin and the ampicillin. ZD6 transductants were spot mated in the same way as a control. The transfer of ampicillin resistance was reduced in only three of the ZD7 matings into the TGL1 recipient. Therefore only 33% of the ZD7 transductants represent cells in which λdrpoBC18 is on the plasmid.

Table 3. The transfer of pZD100 and its zygotic induction.

Donor	Recipient	Frequency of transfer per donor	ratio	Transfer into lysogen / Transfer into non-lysogen
ZD7 ($\underline{metB}$ B$_1^-$ $\underline{recA}$56 $\underline{str}^S$ λ^S/RP4 $\lambda\underline{att}$)	J62 ($\underline{pro}$ $\underline{trp}$ $\underline{his}$ λ^+ $\underline{str-r}$)	7×10^{-3}		2.7
ZD7 ($\underline{metB}$ B$_1^-$ $\underline{recA}$56 $\underline{str}^S$ λ^S/RP4 $\lambda\underline{att}$)	X240 ($\underline{metB}$ $\underline{his}$ B$_1^-$ $\underline{strA}$ $\underline{lac}$)	2.6×10^{-3}		
ZD100 ($\underline{metB}$ B$_1^-$ $\underline{recA}$56 $\lambda\underline{c}$I857S7/pZD100)	J62 ($\underline{pro}$ $\underline{trp}$ $\underline{his}$ λ^+ $\underline{str-r}$)	3.5×10^{-3}		120.7
ZD100 ($\underline{metB}$ B$_1^-$ $\underline{recA}$56 $\lambda\underline{c}$I857S7/pZD100)	X240 ($\underline{metB}$ $\underline{his}$ B$_1^-$ $\underline{strA}$ $\underline{lac}$)	2.9×10^{-5}		

The transfer of RP4 derivatives pZD100 and RP4 $\lambda\underline{att}$ was carried out by liquid culture mating for two hours at 30°C (see Methods). The frequency of transfer per donor is the number of the transconjugants divided by the number of the donor cells/ml at the beginning of the matings. This table illustrates that pZD100 is transmissible to a λ-lysogen recipient. However, its zygotic induction on transfer into a λ non-lysogen recipient is shown by the significant reduction in its frequency of transfer

Legend to Fig. 3

This is a model for the genetical structure of the plasmid pZD100.   It confers the

resistance to ampicillin, tetracycline, kanamycin and rifampicin.   This map is

deduced *from* the physical studies of the plasmid which will be discussed in Part II.

POc857 N

Ap^r

Q

oriV
Tc^r

R

tra3

A

rpoC

pZD100

123.9 Kb

tra2

rpoB

L
J
rpl { A
K

tufB

rrnB

Km^r

tra1

oriT
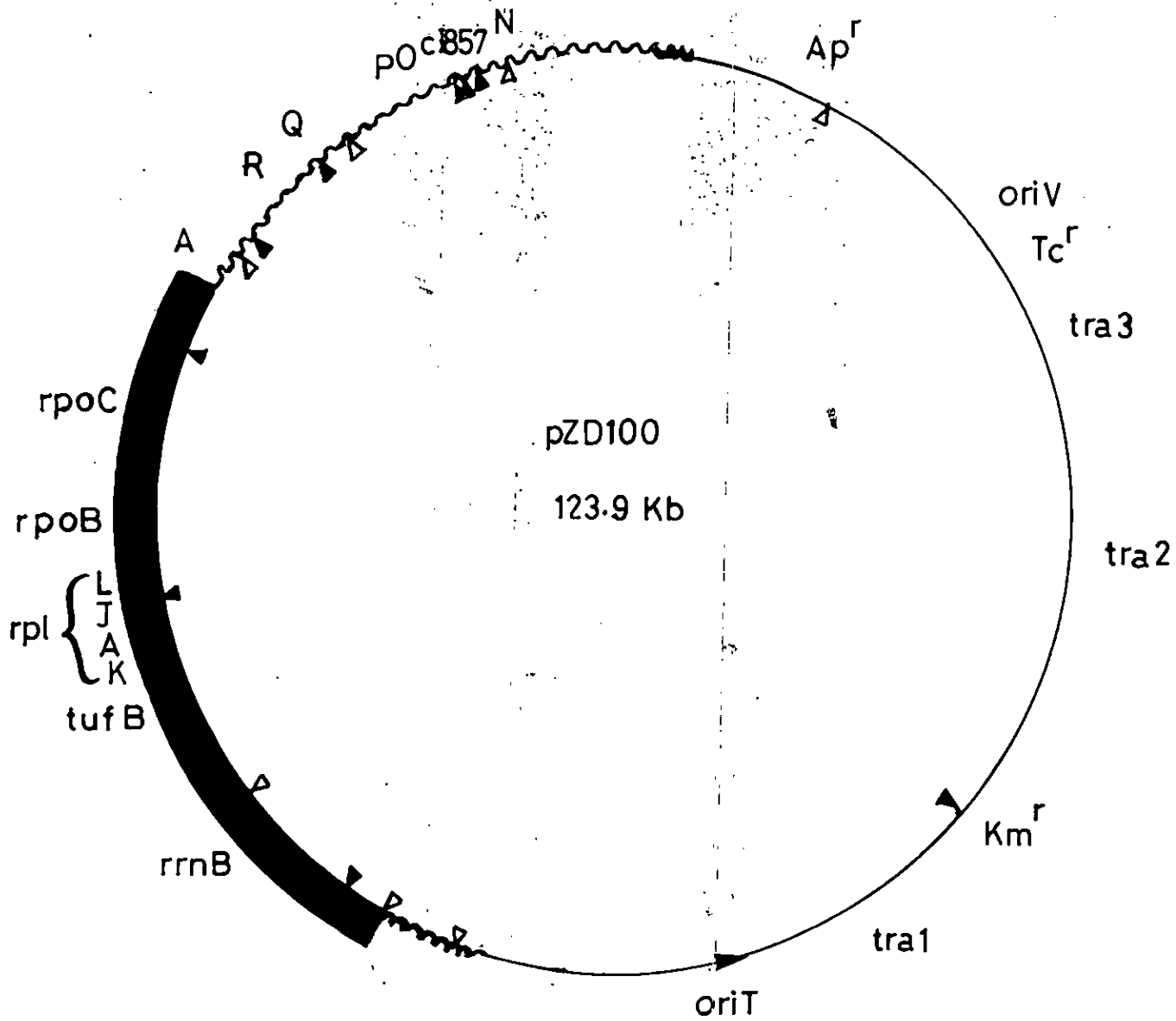
The transfer of the plasmid pZD100 is affected by the presence or the absence of λ in the recipient. This was shown by mating the same donors (see above) with a λ non-lysogen recipient, X240 (metB his BI⁻ strA lac) (see Table 3). The transconjugants of the two matings were selected as described above. A hundred colonies of pZD100 transconjugants were also tested for rifampicin resistance in the same way.

I found that on transfer of pZD100 into the λ lysogen recipient, J62, 100% of the transconjugants were resistant to rifampicin whereas in the case where pZD100 was transferred into the λ non-lysogen recipient, X240, the rifampicin resistant transconjugants comprises only 17%. The frequencies of transfer of pZD100 and RP4 λatt into both recipients are shown in Table 3. There is about 120 fold difference in the frequency of transfer of pZD100 into a λ non-lysogen recipient.

The reduction in the transfer of the rifampicin resistance to the λ non-lysogen recipient, X240, provides evidence that λdrpoBC18 is carried by the plasmid pZD100.

c. Discussion

The phage λb2 partially deleted for the attachment site (Δ.P') shows an int-directed recombination with an intact phage attachment site (P.P'), such as is carried by RP4 λatt (Gottesman and Weisberg, 1971; Pastrana, 1976; Pastrana and Brammar, 1979). This was originally used by Pastrana (1976) to reveal the presence of the att region of λ fragment (srI λ2-3) in the recombinant plasmid RP4 λatt.

It has been shown that the frequency of recombination between the Δ.P' and the P.P' is higher than that of the recombination between Δ.P' and the bacterial B.B' sequences of the normal integration site of the bacteriophage λ (Zichichi and Kellenberger, 1963; Signer et al, 1969).

λdrpoBC18 is likely to be similar to λb2 in that it is deleted in the P sequence of the att region due to the replacement by the bacterial chromosome segment (see Introduction).

The (srI λ2-3) λatt fragment contains int29 amber mutations so that the integration of the plasmid RP4 λatt into the chromosome through the recombination between P.P' sequence of the plasmid and B.B' on the chromosome is prevented in the absence of the suppressor. For this reason, the construction of the plasmid pZD100 was carried out under a suppressor-free cellular environment. The prophage λdrpoBC18 has its own int gene.

In addition, there exist other requirements which are essential for the in vivo construction of the plasmid pZD100. First, the defect in the recombination system of the cell or the rec⁻ condition is required to prevent recombination between the homologous regions of the bacteriophage λdrpoBC18 and the chromosome. Second, the sensitivity of the cell to the bacteriophage λ is required for the introduction of λdrpoBC18 into it. Third, the absence of any extrachromosomal element, but RP4 λatt, to avoid any complication which may interfere with the construction of the plasmid pZD100.

The transduction of ZD7 ($\underline{metB}$ B_1^- $\underline{recA}56$ $\underline{str\text{-}s}$ λ^s/ RP4 $\lambda\underline{att}$) with $\lambda\underline{drpoBC}18$ was carried out at 30°C because of the presence of the $\underline{c}I857$ temperature sensitive mutation in the repressor genes of both $\lambda\underline{drpoBC}18$ and its helper phage $\lambda\underline{c}I857$ (Sussman and Jacob, 1962). The transductants were selected for their resistance to the rifampicin antibiotic. However, not all of the rifampicin resistant transductants need to be cells in which $\lambda\underline{drpoBC}18$ has associated with RP4 $\lambda\underline{att}$; some of them may have had $\lambda\underline{drpoBC}18$ integrated into the chromosome. However, the temperature resistant colonies of ZD6 ($\underline{metB}$ B_1^- $\underline{recA}56$ $\underline{str\text{-}s}$ λ^s/RP4) that appeared among the transductants (see legend to Table 2) are spontaneous rifampicin resistant mutants. ZD6 transductants that were temperature sensitive and rifampicin resistant comprised only 56% and represent the cells in which $\lambda\underline{drpoBC}18$ has integrated into the chromosome. All the tested ZD7 ($\underline{metB}$ B_1^- $\underline{recA}56$ $\underline{str\text{-}s}$ λ^s/RP4 $\lambda\underline{att}$) transductants were temperature sensitive and rifampicin resistant but they need not be similar; some of them may have the constructed plasmid, pZD100, while the others may have $\lambda\underline{drpoBC}18$ integrated into the chromosome.

The plasmid pZD100 has been transferred from ZD100 ($\underline{metB}$ B_1^- $\underline{recA}56$ $\lambda\underline{c}I857S7$/pZD100) into two recipients, one is a λ lysogen and the other is a λ non-lysogen. It is demonstrated (Table 3) that the frequency of transfer of pZD100 into the λ lysogen, J62, is significantly higher

than its transfer into the λ non-lysogen, X240. It would

have been ~~better~~ to use ~~two~~ recipients ~~[illegible]~~

~~of~~ the same strain ~~differing~~ only ~~in~~ [illegible]

the presence of λ in one of them. The comparison of the

transfer of the plasmid RP4 λatt into both recipients

serves as a control; there is only $\stackrel{a}{\wedge}$ 2.7 fold difference

in the efficiency of transfer of RP4 λatt into J62 and

X240 and this difference reflects the variation in the

mating ability of ZD7 (metB B$_1^-$ recA56 str-s λ^S/RP4 λatt)

with both recipients (Table 3). The reduction in the

frequency of transfer of pZD100 into the λ non-lysogen

recipient $\stackrel{can}{[illegible]}$ be attributed to the zygotic induction

phenomenon. The bacteriophage λ which is present in $\stackrel{the}{\wedge}$ J62

strain in the lysogenic phase codes for the repressor

protein which in turn stabilises the λdrpoBC18 on pZD100

and for this reason higher number of transconjugants were

recovered which were resistant to ampicillin, kanamycin,

tetracycline and rifampicin. The low $\stackrel{number}{[illegible]}$ of the

rifampicin resistant transconjugants of X240 is evidence

for the presence of λdrpoBC18 on this plasmid; this

reduction is attributed to the $\stackrel{induction}{[illegible]}$ of λdrpoBC18

in the absence of the repressor. However, zygotic induction

of $\stackrel{an}{\wedge}$ RP4 λatt derivative carrying λb2 has been recently

demonstrated (Pastrana and Brammar, 1979). ZD100 (Table 3)

was able to be a donor for ampicillin, kanamycin,

tetracycline and rifampicin antibiotic resistant determinants.

Thus the evidence suggests that a transmissible plasmid

has been formed, composed of RP4 drug resistant determinants

and which harbours at least the rpoB3 (rif^D) and the cI857 genes of λdrpoBC18. However, the possibility of the presence of the helper phage λcI857S7 in tandem cannot be excluded on this evidence alone (see below).

B.   The Derivation of the Temperature Resistant Plasmids

a.   Introduction

It has been shown above that the plasmid pZD100, constructed in E.coli, is temperature sensitive and confers resistances to ampicillin, kanamycin, tetracycline and rifampicin.   It transfers at a low frequency of 2.9×10^{-5} per donor into a λ non-lysogen E.coli recipient.   However, the aim is to introduce pZD100 into λ non-lysogenic bacterial species and because of the zygotic induction barrier, the experiments described in this section were carried out.   The bacterial species are non-lysogens because they are not the normal hosts for the bacteriophage λ (except E.coli).   I wanted deletion mutants of pZD100 to overcome zygotic induction.

The selection of the temperature resistant derivatives of pZD100 was not carried out while residing in ZD100 (metB B$_1^-$ recA56 str-s λcI857/pZD100) because I found that in such a strain the majority of the mutants are revertants which contain a λ repressor which has regained its heat stability.   To avoid the complication that may be caused by the presence of two copies of cI857, one on the chromosome and the other on the plasmid pZD100, I con- structed a single-lysogen cell that contains only pZD100. However, the derivativation of the temperature resistant

Table 4. The construction of the single-lysogen parent as a first step towards the isolation of the temperature resistant derivatives.

Plate no.	No. of transconjugants (ZD27) at various dilutions			
	0	10^{-1}	10^{-2}	10^{-3}
1	+	496	80	10
2	+	450	91	12
3	+	543	67	13
Average	+	496	79	12
Frequency of transfer per donor		2.5×10^{-4}	4×10^{-4}	6×10^{-4}

This table illustrates the transferability of the plasmid pZD100 from ZD100 (metB B_1^- recA56 λcI857S7/pZD100) into TGL10 (F^- his$^-$ trpA9761 argE171 recA56). The mating mixture was incubated for six hours in a shaking water bath at $30^{\circ}C$. The transconjugants were selected on glucose minimal medium with arginine, tryptophan and histidine (at 20 µg/ml) and the antibiotics which select for RP4, i.e. ampicillin, kanamycin and tetracycline. ZD100 and TGL10 were streaked onto the transconjugants selective plates as a control for the absence of their growth; in addition, they were tested for their resistances to ampicillin, kanamycin, tetracycline and rifampicin by using the Oxoid Multodisk (see Methods).

The frequency of transfer of pZD100 into the λ non-lysogen recipient, TGL10, is 4.2×10^{-4} per donor.

plasmids was achieved by the selection of ⟨λ⟩ temperature resistant mutants from the single-lysogen temperature sensitive parents harbouring pZD100; this selection will probably lead to deletion in the original plasmid pZD100.

Here, I will first describe the construction of the single lysogen cell that harbours the temperature sensitive plasmid and second the isolation of the temperature resistant mutants.

b. Results

1. The construction of the single-lysogen parents

The plasmid pZD100 was transferred from ZD100 (metB B$_1^-$ recA56 λcI857S7/pZD100) into a non-lysogenic recipient TGL10 (F$^-$ his$^-$ trpA9761 argE171 recA56) by liquid culture mating (see Methods) for six hours at 30°C (see legend to Table 4). It is shown (Table 4) that pZD100 transfers into the λ non-lysogen recipient, TGL10, at a frequency of 4.2 x 10^{-4} per donor. The selected transconjugants were tested for their resistance to rifampicin to estimate the survival of the phage λdrpoBC18 in the transfer; for this test a sample of fifty trans-conjugants of the above mating experiment were tested by streaking them onto the original selective medium and a similar plate supplemented with rifampicin (100 µg/ml). Forty eight out of the tested fifty were rifampicin resistant, i.e. 96% of the transconjugants.

2. The characterisation of the single-lysogen parents

The rifampicin resistant transconjugants of the above mating were characterised prior to their utilisation for

the isolation of the temperature resistant mutants.
Fifteen L-broth cultures were prepared by inoculating them
from the selective plates and they were used for the
following tests:

(i) λ immunity test

It was carried out as described in the methods.
ZD100 (metB B_1^- recA56 str-s λcI857S7/pZD100) and
TGL10 (F^- his$^-$ trpA9761 argE171 recA56) were used
as controls for the λ lysogen and the λ non-lysogen
cells respectively.   A sterility control was done
by spotting λv and λcl at many dilutions on BBL
plate with no bacterial lawn.   All the fifteen
tested cultures were λ immune plating λv but not
λcl (see Table 5).

(ii)   The stability of the antibiotic resistances

The fifteen cultures were checked for their
resistance to ampicillin, kanamycin, tetracycline
and rifampicin antibiotics as described in the
methods.   ZD100 (metB B_1^- recA56 str-s λcI857S7/
pZD100) and TGL10 (F^- his$^-$ trpA9761 argE171 recA56)
were used as controls for the antibiotic resistant
and the antibiotic sensitive bacteria respectively.
All the tested cultures were resistant to ampicillin,
kanamycin, tetracycline and rifampicin to which
ZD100 was resistant and TGL10 was sensitive (Table 5).

(iii)   Temperature sensitivity

This test was done by streaking the bacterial culture
onto two similar selective plates;   one is incubated

Table 5. Properties of the temperature sensitive parents.

Parent strain	λ immunity	Stability of antibiotic resistance	Temperature sensitivity 42°C	30°C	Single lysogeny
ZD27-1	+	+	−	+	+
ZD27-2	+	+	−	+	+
ZD27-3	+	+	−	+	+
ZD27-4	+	+	−	+	+
ZD27-5	+	+	−	+	+
ZD27-6	+	+	−	+	+
ZD27-7	+	+	−	+	+
ZD27-8	+	+	−	+	+
ZD27-9	+	+	−	+	+
ZD27-10	+	+	−	+	+
ZD27-11	+	+	−	+	+
ZD27-12	+	+	−	+	+
ZD27-13	+	+	−	+	+
ZD27-14	+	+	−	+	+
ZD27-15	+	+	−	+	+
ZD100	+	+	−	+	−
TGL10	−	−	N.D.	N.D.	N.D.

These are the four employed tests for the temperature sensitive parents which will be used for the selection the temperature resistant mutants.

+ is λ immune, resistant to all the antibiotics of the Multodisk, temperature sensitive and a single lysogen.

− is λ sensitive, sensitive to all the antibiotics of the Multodisk, temperature sensitive and a double lysogen.

N.D. Not done.

at 30°C and the other at 42°C. All the tested

cultures were temperature sensitive as they were

unable to grow at 42°C but only at 30°C (see Table 5).

(iv) Single lysogeny for λdrpoBC18

This test was employed to check that the above

cultures were single lysogens for the λdrpoBC18 present

on the plasmid. One or more of them could have

picked up free helper from the conjugation mixture.

Lysates were prepared from the fifteen cultures by

heat induction (see Methods). A Lysate from ZD100

(metB B$_1^-$ recA56 str-s λcI857S7/pZD100) culture was

prepared as a double lysogen control. Double

lysogens should yield viable plaque forming phage.

Heat-induced lysates of each culture were plated on

an appropriate indicator (Ymel). In contrast to a

lysate of ZD100, none of these gave plaques, showing

that the strains only contained the defective

transducing phage (see Table 5).

3. The selection of the temperature resistant mutants

To isolate the temperature resistant mutants, a sample

of 0.2 ml of midlog phase of each of the above fifteen

L-broth cultures was plated onto a selective plate and was

incubated at 42°C; another 0.2 ml sample of the same culture

was plated on LB plate (see Media) and was incubated at the

same temperature. The number of temperature resistant

mutants that have arisen on the selective plate and on the

non-selective LB plate are shown in Table 6. It is

evident that the number of the temperature resistant colonies

Table 6. The selection for the temperature resistant mutants.

Parental strains	Frequency of the temperature resistant colonies on the selective minimal plate	Frequency of the temperature resistant colonies on the LB plate
ZD27-1	6.6×10^{-5}	+
ZD27-2	6.3×10^{-5}	+
ZD27-3	1.3×10^{-4}	+
ZD27-4	2×10^{-4}	+
ZD27-5	-	2.8×10^{-4}
ZD27-6	-	2.4×10^{-4}
ZD27-7	-	4.3×10^{-4}
ZD27-8	1.3×10^{-6}	-
ZD27-9	5×10^{-6}	+
ZD27-10	9.6×10^{-5}	+
ZD27-11	1.3×10^{-6}	1.7×10^{-4}
ZD27-12	9.6×10^{-5}	+
ZD27-13	6.6×10^{-5}	+
ZD27-14	1.3×10^{-6}	2.6×10^{-4}
ZD27-15	9.8×10^{-5}	+

The temperature resistant mutants were selected by incubating the selective plates and LB plates at 42°C. It is illustrated that the temperature sensitive parental strains which were used for the selection of the temperature resistant mutants fall into two groups; one group produces the temperature resistant mutants at higher frequency when compared with the second group.

- where no temperature resistant mutants appeared
+ where the frequency of the temperature resistant mutant is very high, between $1.3 \times 10^{-3} - 1.3 \times 10^{-2}$.

The average number of the plated cells was 8×10^{-7} and the frequencies were estimated by dividing the number of the temperature resistant colonies by 8×10^{-7} and then multiplying by a hundred.

on the non-selective LB-plates is higher than the number on the antibiotic selective plates. On the basis of the comparison of the numbers of temperature resistant colonies, (Table 6) it is apparent that the temperature sensitive parental cells are divided into two groups: one of them tends to produce temperature resistant colonies at higher frequencies on the antibiotic selective plate which ranges between $6.3 \times 10^{-5} - 2 \times 10^{-4}$, and the other group has a very low tendency to produce the temperature resistant mutants as their frequencies range between $0-5 \times 10^{-6}$ (see Table 6). There seems to be differences between the temperature sensitive parental strains and there are several explanations which I will discuss later.

4. Properties of the temperature resistant mutants

The isolation of the temperature resistant mutants was followed by testing their sensitivity to the bacteriophage λ and by testing the stability of their resistances to ampicillin, kanamycin, tetracycline and rifampicin antibiotics (see Methods). For these tests, a maximum of ten temperature resistant colonies of each line of the isolates were inoculated from the selective plate into L-broth and incubated at 42°C. It was found that the temperature resistant cultures are divided into five groups as far as the resistance to the antibiotics is concerned. The majority of them (43%) have stably maintained the resistances to the ampicillin, kanamycin, tetracycline and rifampicin

antibiotics. The second group (36%) includes the cells which are resistant to ampicillin only. The third group (12.8%) represent the cells which have lost the resistance to rifampicin but maintained RP4 drug resistant determinants. A small group (3.5%) includes the cells which have lost the ampicillin resistance but are resistant to kanamycin, tetracycline and rifampicin. However, there are some cells (4.7%) which are sensitive to all the tested antibiotics.

On the other hand, the λ sensitivity test has revealed that 90.8% of the temperature resistant mutants were λ sensitive and the rest fall into three classes; one class comprises 7% of the temperature resistant mutants and represents the cells which remained λ immune. Partially resistant or totally resistant to λ are the second and the third classes comprising 1.1% each.

As far as the λ sensitivity of the 43% group, which is described above is concerned, I have found that its individual cultures fall into four groups. The majority, 74.2% are λ sensitive; the second group is λ immune and it comprises 19.4%; the third and fourth group, each comprising 3.2% are partially resistant and totally resistant to λ.

However, the 3.5% group of the temperature resistant mutants which are sensitive to ampicillin but resistant to kanamycin, tetracycline and rifampicin are found to be sensitive to the bacteriophage λ. Since many temperature resistant mutants which are sensitive appeared

to the bacteriophage λ, therefore the occurrance of a deletion event in λdrpoBC18 part of pZD100 can be suggested.

c. Discussion

The main interest in the isolation of the temperature resistant derivatives of the plasmid pZD100 is to remove the major part of the λ genome and to produce a derivative which consists largely of the RP4 genome and that part of λdrpoBC18 which contains at least rpoB3.

Two stages have been involved for the derivation of the temperature resistant plasmids: the first one is the transfer of the plasmid pZD100 into a λ non-lysogen recipient; for the plasmid pZD100 to escape zygotic induction there must be either a mutation or a deletion that would maintain the plasmid in the cell; there could be rare cases where the prophage either does not come out (or replicate) at all, or does but returns and gets repressed.

However, the single lysogen parental cells (F⁻ his⁻ trpA9761 argE171 recA56/pZD100) were λ immune, temperature sensitive and they were resistant to ampicillin, kanamycin, tetracycline and rifampicin. This would suggest the possibility of the presence of the whole pZD100 in these cells (see Fig. 3). The resistance to the ampicillin, kanamycin and tetracycline indicates the presence of the whole RP4 part of pZD100 whereas the immunity to λ and the resistance to the rifampicin suggests the presence of λdrpoBC18 part of the plasmid.

It is unlikely that the protection against
zygotic induction of pZD100 is caused by a mutation on
the plasmid or by a deletion in the λ part of the plasmid;
in this case this structural change of the pZD100 plasmid
must be permanent and therefore if the plasmid is transfered
again into a λ non-lysogen recipient it should not be
subjected to zygotic induction similar to its transfer
into a λ lysogen recipient; in other words, the protection
from the zygotic induction should be permanent; however,
this is not the case as will be illustrated in Part III.

It may be considered that the maintenance of the
plasmid in the λ non-lysogen recipient is accomplished by a
repressor building up mechanism which stabilises λdrpoBC18
on pZD100. It has been shown that 96% of the trans-
conjugants (F⁻ his⁻ trpA9761 argE171 recA56/pZD100) of
the mating between ZD100 (metB B₁⁻ recA56 str-s λcI857S7/
pZD100) and TGL10 (F⁻ his⁻ trpA9761 argE171 recA56) were
resistant to the four antibiotics but only 4% were
rifampicin sensitive and maintained the resistances to
ampicillin, kanamycin and tetracycline. This suggests
that they represent the cells in which RP4 λatt plasmid
has segregated from the plasmid pZD100 or the cells in
which spontaneous excision of λdrifD18 has occurred; the
loss of the phage could happen spontaneously before the
mating or during the mating.

A total of fifteen of the above transconjugants were
chosen for the isolation of the temperature resistant
mutants as it was not clear at this point that all the

temperature sensitive parents harbour the same plasmid whether it is the original pZD100 plasmid or a mutated derivative.

The second stage which is the selection of the temperature resistant mutants from the chosen fifteen L-broth cultures has proved that they harbour different plasmids because they fell mainly into two groups, one produces the temperature resistant mutants at significantly higher frequency than the other, which is more capable of killing the cell at the non-permissive temperature.

Certainly this difference cannot be due to the cI gene of λ, because the plasmids of the two groups are temperature sensitive due to the cI857 mutation. However, structural differences between the temperature sensitive parent plasmids could be detected by hybridisation with λdrpoBC18 and with RP4 λatt. On the other hand, it is possible to conduct mating experiments with two recipients, one a λ-lysogen and the other a λ sensitive, and compare the efficiency of zygotic induction in these matings involving both types of temperature sensitive plasmids.

Also, the difference between the two types of temperature sensitive parents could be chromosomal; in this case, curing one of them of the plasmid and transferring the other temperature sensitive plasmid into the cured cells followed by the selection of temperature resistant mutants will answer the question of whether the difference is due to the plasmid or not.

Part II   The Characterisation of the Plasmid pZD100 and

its Derivatives

a.   Introduction

Full structural and genetic characterisation of the

plasmids is important because the aim is to utilise them

to transfer the rpoB gene of E.coli into different species.

It is thus important to be able to detect any kind of

change in the properties of the plasmid after its

introduction into an alien cell.

The temperature resistant λ sensitive mutants that

are of importance to my studies are the rifampicin

resistant ones.   For this reason, two groups of these

mutants have been chosen for this study;   one includes

those which are resistant to ampicillin, kanamycin,

tetracycline and rifampicin, and the other represents the

mutants that are sensitive to ampicillin but resistant

to kanamycin, tetracycline and rifampicin.

Here, I present the structural and genetic properties

of the plasmids from the temperature resistant mutants as

compared with the original plasmid pZD100.   The comparative

studies will include the in vitro characterisation of the

plasmids such as the determination of their molecular

weights and their physical structure by restriction mapping.

But first I will present an account of their genetic

markers.

b.   Results

1.   In vivo characterisation of pZD100 and its derivatives

Several tests were employed for the chosen temperature

Table 7.   The in vivo characterisation of the temperature resistant plasmids

E.coli temperature resistant mutants	RD 100 µg/ml	The Oxoid Antibiotic Multodisk			K 25 µg/ml	PRR1 sensitivity	λ immunity
		PN 50 µg/ml	TE 10 µg/ml	PY 100 µg/ml			
ZD2714	+	+	+	+	+	+	−
ZD2742	+	+	+	+	+	+	−
ZD2743	+	+	+	+	+	+	−
ZD2744	+	+	+	+	+	+	−
ZD2745	+	+	+	+	+	+	−
ZD2746	+	+	+	+	+	+	−
ZD2723	+	−	+	−	+	+	−
ZD27154	+	−	+	−	+	+	−
ZD27135	+	−	+	−	+	+	−
ZD100	+	+	+	+	+	+	+
ZD7	−	+	+	+	+	+	N.D.
TGL10	−	−	−	−	−	−	−

The temperature resistant mutants were inoculated from the antibiotics selective plates into three mls of sterile L-broth and were incubated at 37°C;   the mid log phase cultures have been used for the tests of this table.

+/−   is resistant/senstive to the antibiotic;   sensitive/resistant to the RP4-specific phage PRR1;   immune/sensitive to the bacteriophage λ.

RD   is rifampicin, PN is ampicillin, TE is tetracycline, PY is carbenicillin, and K is kanamycin.

N.D.   not done.

resistant mutants to characterise the plasmids that they harbour and to compare their genetical structures with that of pZD100.

(i) Checking the antibiotic resistances

The employed method is as described in the legend to Table 7 and in Methods.   It is shown (Table 7) that the chosen temperature resistant mutants are in two groups: one is resistant to ampicillin/carbenicillin, kanamycin, tetracycline and rifampicin like the parent pZD100 (metB B_1^- recA56 str-s λcI857S7/pZD100);   the second group is sensitive,to ampicillin/carbenicillin but resistant to the other antibiotics.

(ii) Sensitivity to RP4-specific phage PRR1

This test was carried out to detect the production of RP4 coded pili which are specific for the bacterio-phage PRR1.   The lysate of PRR1 was plated at various dilutions (see Methods) on lawns of the temperature resistant mutants.   Like the control strains which harbour pZD100 and RP4 λatt plasmids (see Table 7), the temperature resistant mutants were sensitive to the bacteriophage PRR1, whereas TGL10 was resistant to the tested phage.

(iii) λ immunity test

This test was employed for the same cultures of the above tests as described in the Methods.   It is demonstrated (Table 7) that like the λ non-lysogen TGL10 control, all the tested temperature resistant mutants were sensitive to the bacteriophage   plating both phages λv and λcI;   the control strain ZD100 (metB B_1^- recA56 str-s λcI857S7/pZD100) was sensitive to λv but immune to λcI.

ZD100 (<u>metB</u> B$_1^-$ <u>recA</u>56 λ<u>c</u>I857S7/pZD100)

Temperature sensitive
parents

Temperature resistant
mutants

ZD27-1

ZD27-2 —————————————— ZD2723

ZD27-3

ZD27-4

ZD2741
ZD2742
ZD2743
ZD2744
ZD2745
ZD2746

ZD27-5 - ZD27-15

TGL10 (<u>his</u>$^-$ <u>trpA</u>9761 <u>argE</u>171 <u>recA</u>56)
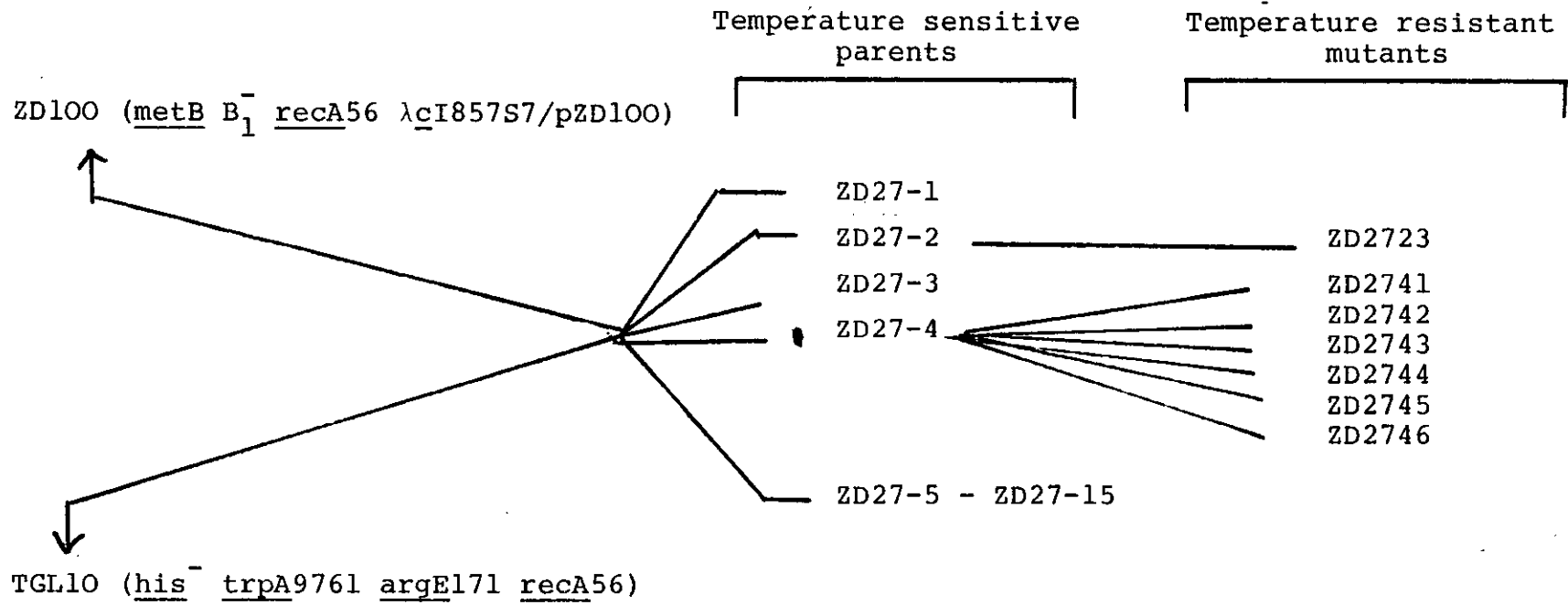
<u>Fig</u>. 4.   This scheme illustrates the derivation of the temperature resistant mutants

from the λ non-lysogen/pZD100 temperature sensitive parents.

In summary, it is concluded from Table 7, that in
addition to the resistances to the antibiotics, the
temperature resistant mutants are sensitive to the
bacteriophage PRR1 which means that RP4 has survived.
The sensitivity·of these mutants to λ indicated that some
of the bacteriophage genes have been removed.

(iv)   Zygotic induction

It has been shown earlier (Table 3) that the original
plasmid pZD100 transfers at a lower frequency into a λ
non-lysogen recipient than ⌐‾‾‾‾‾⌐ into a λ lysogenic
one.   It is a matter of interest to investigate
primarily the zygotic induction of these temperature
resistant plasmids by comparing their transfer into E.coli
λ lysogen^{ic} and λ non-lysogen^{ic} recipients.   The transfer
ability of the temperature resistant mutants was compared
individually with that of their temperature sensitive parent
(Fig. 4; Table 8).

The temperature resistant and the temperature sensitive
plasmids were transferred from their donors (Table 8)
into the λ lysogen TGL6 (metB B$_1^-$ recA56 str-s λ^+ λ^R) and
λ non-lysogen AW1 (metB B$_1^-$ recA56 str-s λ^S) recipients by
liquid culture matings for two hours at the appropriate
temperatures i.e. at 37°C for the matings of the temperature
resistant mutants and at 30°C for the matings of the
temperature sensitive parent.   RP4 λatt was transferred
from ZD28 (F$^-$ his$^-$ trpA9761 argE171 recA56/RP4 λatt) into
the same recipients as a control for the absence of zygotic
induction.   Neither the donors nor the recipients grew

Table 8.   The zygotic induction test of the temperature
            resistant plasmid and its temperature
            sensitive parent.

Donor	Recipient	Frequency of transfer per donor	Zygotic induction ratio	% rifampicin resistant transconjugants
ZD27-2 (ts$^-$)	AW1 (λ^-)	6.3×10^{-4}	3.8	87
ZD27-2	TGL6 (λ^+)	2.4×10^{-3}		100
ZD2723 (ts$^+$)	AW1 (λ^-)	3.5×10^{-2}	0.1	100
ZD2723	TGL6 (λ^+)	5.3×10^{-3}		100
ZD28	AW1 (λ^-)	2.2×10^{-3}	1.5	N.D.
ZD28	TGL6 (λ^+)	3.4×10^{-3}		N.D.

AW1 (metB B$_1^-$ recA56 str-s λ^s) is the λ non-lysogen recipient;
TGL6 (metB B$_1^-$ recA56 str-s λ^+ λ^R) is the λ lysogen recipient.
See Fig. 4 for details of the donors.   ZD28 (F$^-$ his$^-$
trpA9761 argE171 recA56/RP4 λatt) serves as a control for
the transfer of RP4 λatt into AW1 and TGL6.

0.1 ml of -0, 10^{-1}, 10^{-2} and 10^{-3} dilutions of the mating
mixture were plated with top agar over the selective plates;
each plate contains methionine, B1 and either kanamycin for
the ZD2723 matings or ampicillin and kanamycin for the rest
of the matings.

The donors and the recipients were streaked on the selective
plates as a control and they were tested for their
resistances to the antibiotics by the Multodisk.

Zygotic induction ratio, is the ratio between the frequency
of transfer of the plasmid to the λ lysogen recipient to that
of its transfer to the λ non-lysogen recipient. However,
there is 55.6-fold difference between the frequencies of
transfer of the temperature sensitive plasmid from ZD27-2 and
its temperature resistant derivative from ZD2723 into the λ
non-lysogen recipient; whereas only 2.2-fold difference
between their transfer into the λ lysogen one.
The transconjugants were examined for their resistance to
rifampicin by patching a maximum of fifty transconjugants
onto two selective plates, with and without rifampicin.

N.D. Not done.

on the selective plates; both recipients, AW1 ($\underline{metB}$ B_1^-
$\underline{recA}56$ $\underline{str-s}$ λ^S) and TGL6 ($\underline{metB}$ B_1^- $\underline{recA}56$ $\underline{str-s}$ λ^+ λ^R)
were sensitive to ampicillin, kanamycin, tetracycline and
rifampicin to which all the temperature resistant donors
(Table 8) except ZD2723 were resistant.   ZD28 (F⁻ $\underline{his}^-$
$\underline{trpA}9761$ $\underline{argE}171$ $\underline{recA}56$/RP4 $\lambda\underline{att}$) was sensitive to the
rifampicin but resistant to all the antibiotics which
resistances are conferred by RP4.

Like the transfer of RP4 $\lambda\underline{att}$ into the λ lysogen
and the λ non-lysogen recipients, there is no
zygotic induction of the temperature resistant plasmid
(Table 8).   However, there is a significant reduction
in the transfer of the parental temperature sensitive
plasmid when transferred into the λ non-lysogen recipient
AW1 (Table 8), which I ascribe to its continued
susceptibility to zygotic induction.   The reduction in
the transferability of the plasmids is indicated by the
zygotic induction ratio (see Table 8).

All the transconjugants which have the temperature
resistant plasmid are resistant to rifampicin (Table 8)
whereas not all the transconjugants which have the
plasmids from the temperature sensitive donors were
rifampicin resistant.

(v)   Transferability

The temperature resistant plasmids were transferred
into $\underline{E.coli}$, TGL6 ($\underline{metB}$ B_1^- $\underline{recA}56$ $\underline{str-s}$ λ^+ λ^R) by liquid
culture mating for two hours at 37°C.   The transferability
of the temperature sensitive parent plasmids was tested

Table 9   The transferability test of the temperature
           resistant plasmids and their temperature
           sensitive parent plasmids.

Donor	Frequency of transfer per donor	% rifampicin resistant transconjugants
ZD27-2 (ts$^-$)	2.4 x 10^{-3}	100
ZD2723 (ts$^+$)	5.3 x 10^{-3}	100
ZD27-4 (ts$^-$)	3.6 x 10^{-4}	100
ZD2742 (ts$^+$)	4 x 10^{-8}	N.D.
ZD2743 (ts$^+$)	2.4 x 10^{-7}	100
ZD2744 (ts$^+$)	0	N.D.
ZD2745 (ts$^+$)	1.6 x 10^{-6}	100
ZD2746 (ts$^+$)	7.4 x 10^{-8}	N.D.

The temperature sensitive donors (F$^-$ his$^-$ trpA9761 argE171
recA56/pZD100?) are phenotypically similar.   The
temperature resistant donors fall into two groups which
are derived from two lines.   ZD2723 (F$^-$ his$^-$ trpA9761
argE171 recA56/pZD23) is ampicillin sensitive but
kanamycin, tetracycline and rifampicin resistant; it has
been derived from ZD27-2.   The second group of the
temperature resistant plasmids was derived from ZD27-4
and includes the mutants that are resistant to ampicillin,
kanamycin, tetracycline and rifampicin.   All the above
donors were mated with TGL6 (metB B$_1^-$ recA56 str-s λ$^+$ λ^R)
by liquid culture mating (see Methods).   The trans-
conjugants were selected in the presence of methionine,
thiamine, ampicillin and kanamycin (except the pZD23 mating).
The transconjugants which have pZD23 were selected in the
presence of kanamycin only in addition to methionine and
thiamine.   All transconjugants were tested for their
rifampicin resistance by patching them on two selective
plates, with and without rifampicin.

N.D.   Not done.

Some of the above data, concerning pZD23 and its temperature
sensitive parent plasmid, are extracted from Table 8.

at 30°C by the same method. It is shown (Table 9) that the temperature resistant plasmids are also divided into two groups as far as their transferability is concerned. One group, such as pZD23 (of ZD2723) is tra⁺ while the second group transfers at a very low frequency (tra⁻).

So far, it is concluded that the temperature resistant plasmid pZD23 contains that part of the RP4 genome which confers the resistance to kanamycin and tetracycline and which contains the tra genes which are responsible for the transfer of the plasmid. In addition, the plasmid pZD23 has that part of λdrpoBC18 which contains the rpoB3 (rif^D) conferring the resistance to the rifampicin.

In addition I infer that the deletion in the temperature resistant plasmid, pZD23, ends either within or beyond Ap^r gene (see Fig. 3); the other end of the deletion can be defined by testing for the presence of the rpoC gene.

(vi) Does pZD23 harbour rpoC?

The following complementation test was employed in order to fix the other end of the λ deletion of pZD100 (Fig. 3). The plasmid pZD23 was transferred from ZD2723 (F⁻ his⁻ trpA9761 argE171 recA56/pZD23) into two rpoC temperature sensitive mutants. One of them, X242 (metB B$_1$⁻ his⁻recA56 strA lac rpoC4) is rec⁻ whilst the other, 397C (argG B$_1$⁻ rpoCts) is rec⁺. Crosses were done in liquid culture (see Methods) for three hours at 30°C. The donor, ZD2723 (F⁻ his⁻ trpA9761 argE171 recA56/pZD23) and the recipient X242 (metB B$_1$⁻ his⁻ recA56 strA lac rpoC4)

Table 10. Does the plasmid pZD23 harbour the rpoC gene?

Donor	Recipient	Frequency of transfer per donor	% rifampicin resistant transconjugants (30°C)	% ts$^+$ rifampicin resistant transconjugants
ZD2723	397C (RecA$^+$)	7.1×10^{-3}	100	100
ZD2723	X242 (RecA$^-$)	6.2×10^{-5}	100	20
ZD7	397C	8.6×10^{-2}		
ZD28	X242	8.6×10^{-5}		

The genotypes of the donors are: ZD2723 (F$^-$ his$^-$ trpA9761 argE171 recA56/pZD23)

ZD7 (metB B$_1^-$ recA56 str^S λ^S/RP4 λatt)

ZD28 (F$^-$ his$^-$ trpA9761 argE171 recA56/RP4 λatt)

The recipients are rec$^+$ and rec$^-$ temperature sensitive mutants for the rpoC gene of E.coli; their genotypes are: 397C (argG B$_1^-$ rpoCts)

X242 (metB B$_1^-$ his$^-$ recA56 strA lac rpoC4)

The transconjugants of the X242 mating were selected in the presence of the methionine, thiamine, histidine and kanamycin. The transconjugants of the second mating were selected in the presence of arginine, thiamine and kanamycin.

Forty purified transconjugants which have pZD23 were patched onto two similar selective plates with the exception that one of them contains rifampicin; the donor ZD2723 and the recipients were streaked onto the same plates as controls for the absence of their growth. X242 has a high tendency for reversion as it should be purified many times before starting the experiment.

and 397C ($\underline{argG}$ B$_1^-$ $\underline{rpoC}$ts) were streaked onto the selective

plates as controls for the absence of their growth; the

second control was to test their antibiotic resistances

by the Multodisk (see Methods); thirdly, control crosses

were made in which the plasmid RP4 $\lambda\underline{att}$ was transferred

to both recipients; the donor strain used for the transfer

of RP4 $\lambda\underline{att}$ into 397C was ZD7 ($\underline{metB}$ B$_1^-$ $\underline{recA}$56 $\underline{str-s}$ λ^S/

RP4 $\lambda\underline{att}$) and for its transfer into X242 was ZD28

(F$^-$ $\underline{his}^-$ $\underline{trpA}$9761 $\underline{argE}$171 $\underline{recA}$56/RP4 $\lambda\underline{att}$).

The transconjugants from the pZD23 crosses (Table 10)

were tested for their rifampicin resistance (legend -

Table 10). Of the tested transconjugants from both

matings all are rifampicin resistant at 30°C.

Ten rifampicin resistant $\underline{rec}^+$ transconjugants (397C)

were tested for their temperature sensitivity in the

presence and absence of rifampicin. Table 10 shows that

100% of these $\underline{rec}^+$ transconjugants ($\underline{argG}$ B$_1^-$ $\underline{rpoC}$ts/pZD23)

were temperature resistant in the presence and the

absence of the rifampicin at 42°C and at 30°C.

A Similar test for the temperature sensitivity of the

X242 rifampicin resistant transconjugants ($\underline{metB}$ B$_1^-$ $\underline{his}^-$

$\underline{recA}$56 $\underline{strA}$ $\underline{lac}$ $\underline{rpoC}$4/pZD23) was carried out in the same

way on supplemented glucose minimal media. It is

demonstrated (Table 10) that only two out of the ten

tested X242 ($\underline{rec}^-$) transconjugants, i.e. 20%, were

temperature resistant.

It can be possibly concluded that there is a deletion

in the $\underline{rpoC}$ gene of the plasmid, pZD23, such that in a

rec$^+$ background only, it can recombine with the temperature
sensitive rpoC mutation.   It is possible to find the
explanation of these results from in vitro analysis.
The conclusions from these tests are likely to be
informative about the structure of the original plasmid
pZD100 (see Fig. 3) and about how the RP4 λatt genome is
oriented with respect to the λdrpoBC18 genome on the
plasmid pZD100.

2.   In vitro characterisation of pZD100 and its
derivatives

The physical analysis of the temperature resistant
plasmids and the original plasmid pZD100 will contribute
to our understanding of the structure of these plasmids.
The physical analysis includes two tests which will be
described below.

(i)   Determination of the molecular weights of the
plasmids

The comparison of the molecular sizes of these plasmids
demonstrates the existence of the deletions in these
plasmids and allows me to determine the size of these
deletions.   I chose three temperature resistant plasmids
for this study.   Two of them, pZD23 and pZD135, confer
resistance to kanamycin, tetracycline and rifampicin but
not ampicillin.   The other plasmid, pZD44, confers the
resistance to ampicillin in addition to the above three
antibiotics and was isolated in the same way in the
presence of ampicillin, kanamycin, tetracycline and
rifampicin.   The plasmid pZD135 was isolated mainly for

the comparison with the pZD23 plasmid. The original
plasmid pZD100 (Fig. 3), the plasmids RP4 and RP4 λatt
as well as the temperature resistant plasmids, mentioned
above, were isolated in the presence of the antibiotics
to which they confer resistance (see Methods).

The molecular weight of each plasmid was estimated
by measuring the contour length of the plasmid DNA
molecule relative to the nearest standard plasmid molecule
under the electron microscope. The plasmids pBR313
and pSC101 were separately used as standards. The
molecular weights of the tested plasmids are shown in
Table 11. It is shown that the molecular weights of
the plasmids pZD23 and pZD135 are exactly the same,
whilst pZD44 is 3.7 megadaltons bigger.

In all the plasmid DNA preparations of Table 11,
except pZD100, there were only molecules of a single
size. However, in pZD100 preparation, there were
different classes of molecules, one of them corresponds
to the predicted molecular weight of the plasmid pZD100
(81.8 megadaltons). The predicted size of pZD100 should
equal to the total molecular weights of RP4 λatt
(46.04 Md) and λdrpoBC18 (about 35 Md).

(ii) Restriction analysis of pZD44 and pZD23

This analysis was carried out to establish the
physical maps of both plasmids which will allow me to
conclude the genetical structure of the temperature
sensitive plasmid pZD100 from which they were derived.
The restriction analysis will determine the size and

Table 11   The electron microscopic determination of the
molecular weights of the plasmids.

Plasmid	No. of molecules measured	Standard used	Molecular Weight (Megadaltons)
RP4	12	pSC101	42.5 ± 0.6
RP4 λatt	10	pSC101	46.04 ± 0.6
pZD23	8	pBR313	54.8 ± 0.4
pZD135	11	pBR313	54.8 ± 0.6
pZD44	11	pBR313	58.5 ± 0.7
pZD100	* 6 * 5	pSC101	81.8 ± 0.8 60.1 ± 1.2

*   In addition to these there was one molecule of each of

the following: 45.5 Md, 69.2 Md, 102.5 Md, 134.2 Md, 141.7 Md.

The plasmid DNA preparation is described in the Methods. The
conditions that were used for the spreading of the plasmids
are the same as Davis et al, 1971; three copper grids
(small circular copper pieces of copper mesh) were prepared
for each plasmid. They were covered with a film of plasmid
DNA + standard/cytochrome C, washed in uranyl acetate stain
(0.05 M uranyl acetate, 0.05 M HCl) for 30 seconds and finally
rinsed in 90% ethanol for 10 seconds then dried with filter
paper. The copper grids were then shadowed with platinum and
then with carbon and finally washed with absolute ethanol for
1 min before viewing under the electron microscope (Siemens 101).
Photographs were taken at 20,000x magnification and they were
magnified 5x when they were put in De Vere 54 varicon projector
for tracing. The tracings of the contour length were
measured with a map measure. By measuring the standard pSC101
and pBR313 it was possible to estimate the molecular weight
of each plasmid. pSC101 is 6.16 megadaltons and pBR313 is
5.8 megadaltons.

Legend to Fig. 5

The lysogenisation of RP4 λatt with λdrpoBC18 results in the construction of the plasmid pZD100. The plasmid pZD100 consists of the genomes of RP4 λatt and λdrpoBC18; Δ44 and Δ23 represent the two deletions which result in the derivation of the temperature resistant plasmids pZD44 and pZD23 from pZD100.

Black and white arrows (triangles) are HindIII and BamHl sites respectively.
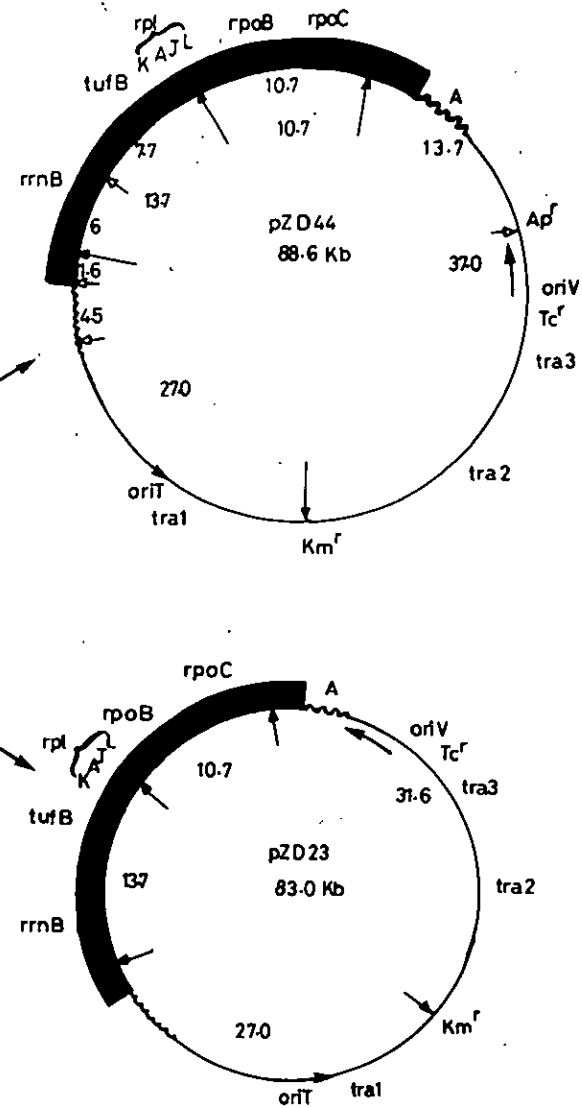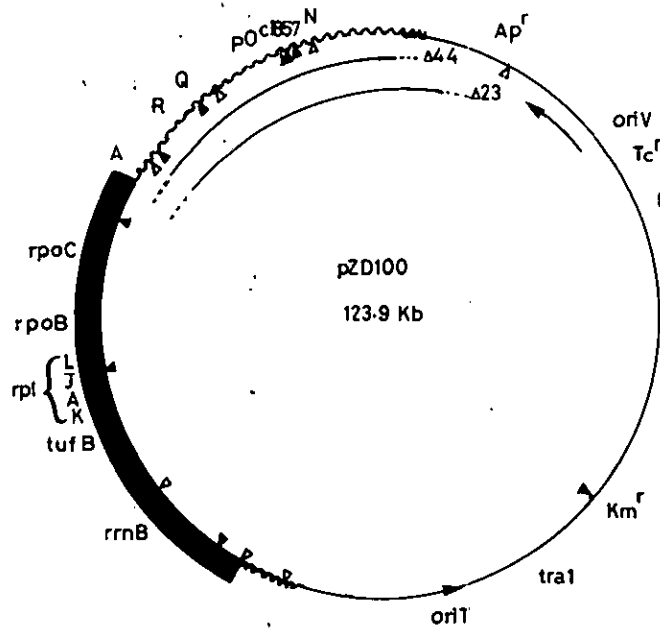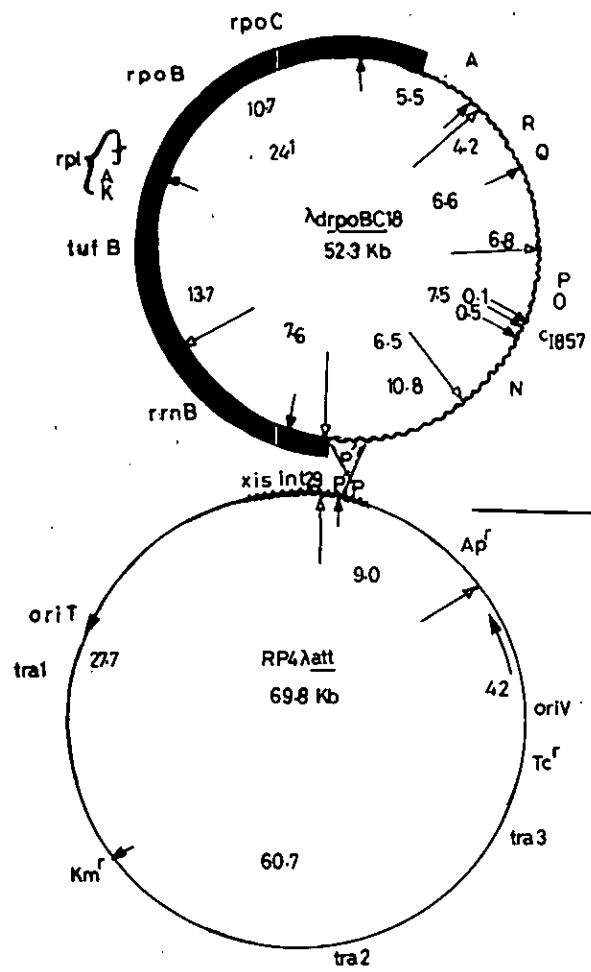
the location of the deletions in the temperature

resistant plasmids pZD23 and pZD44.

The physical maps of the plasmids (see Fig. 5) were

deduced from the digestion of both plasmids with

endoR.HindIII enzyme and from further analysis of pZD44

with endoR.BamHl which will be described later.

A.   HindIII restriction of pZD23

The DNA of the plasmid pZD23 was digested with the

restriction endonuclease enzyme HindIII and the fragments

were electrophoretically fractionated through agarose

gel (see Methods; Legend to Fig. 6a).   HindIII fragments

of RP4 λatt and λdrpoBC18 were fractionated on the same

gel (Fig. 6a).   As expected, RP4 λatt produced two DNA

bands and λdrpoBC18 produced five bands.   In addition,

extra band appeared in the λdrpoBC18 track which comes from

the contaminating helper DNA.   The molecular weights

of the HindIII fragments of λdrpoBC18 were extracted from

Taylor and Burgess (1979).   It appears that there are

three HindIII fragments of pZD23 but actually the first

band is a doublet as revealed by the densitometer (Fig. 7).

The migration of the fragments is shown in Fig. 8 and

their sizes are demonstrated in Table 12.

If we take the molecular weight of the plasmid pZD23

to be 83 kb then the molecular weight of the doublet will

be 58.6 kb.   It is now possible to conclude the sizes

of the HindIII fragments of RP4 λatt (Fig. 8; Table 12)

whose total molecular weights is 69.7 kb;   this is

Kb

13·7
10·8
10·7
6·8
5·5
4·2
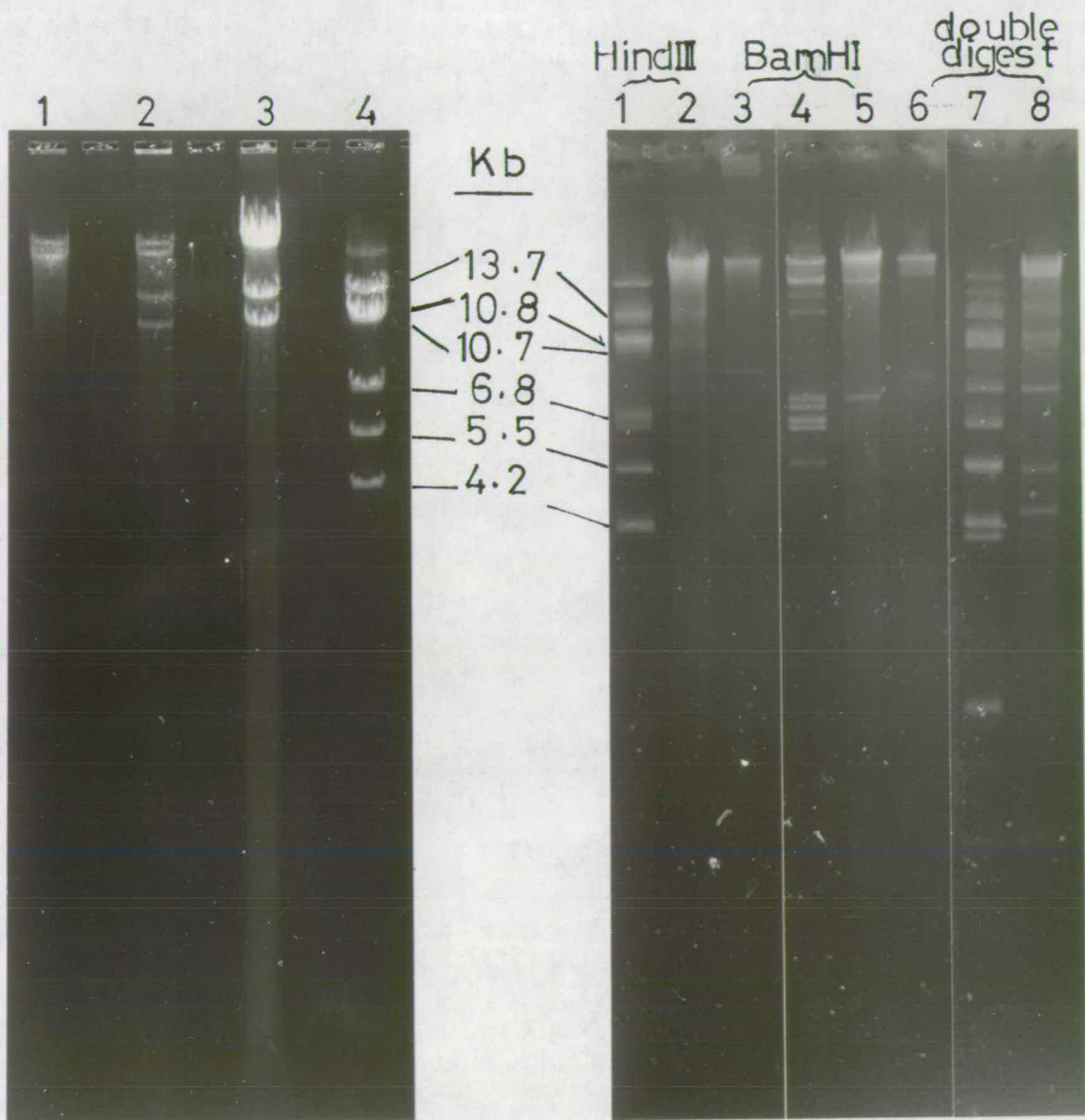
a) The digestion of the plasmids pZD44 and pZD23 DNA with the endoR.HindIII using RP4 λatt DNA and λdrpoBC18 DNA as controls. 1) RP4 λatt, 2) pZD44, 3) pZD23 4) λdrpoBC18.

b) pZD44 DNA restriction with endoR.HindIII, BamHl, and the double digestion with both enzymes using RP4 λatt DNA and λdrif^{D}18 DNA as controls. 3,6 (RP4 λatt), 1,4,7 (λdrif^{D}18), 2,5,8 (pZD44).

Fig. 6. 0.7% agarose gel containing ethidium bromide (3 μg/ml); the buffer is tris-acetate. The gel system is horizontal as described by McDonell et al (1977) and is photographed under long UV exposure for 45 seconds.
Unlabelled fragments of λdrif^{D}18 are from the contaminating λ helper DNA.

Legend to Fig. 7

The endoR.HindIII restriction analysis of the plasmid pZD23
as shown by the densitometer and has resulted in the
production of four fragments; the two largest fragments
have appeared as a doublet (Fig. 6a) on the 0.7% agarose
horizontal gel.

Density
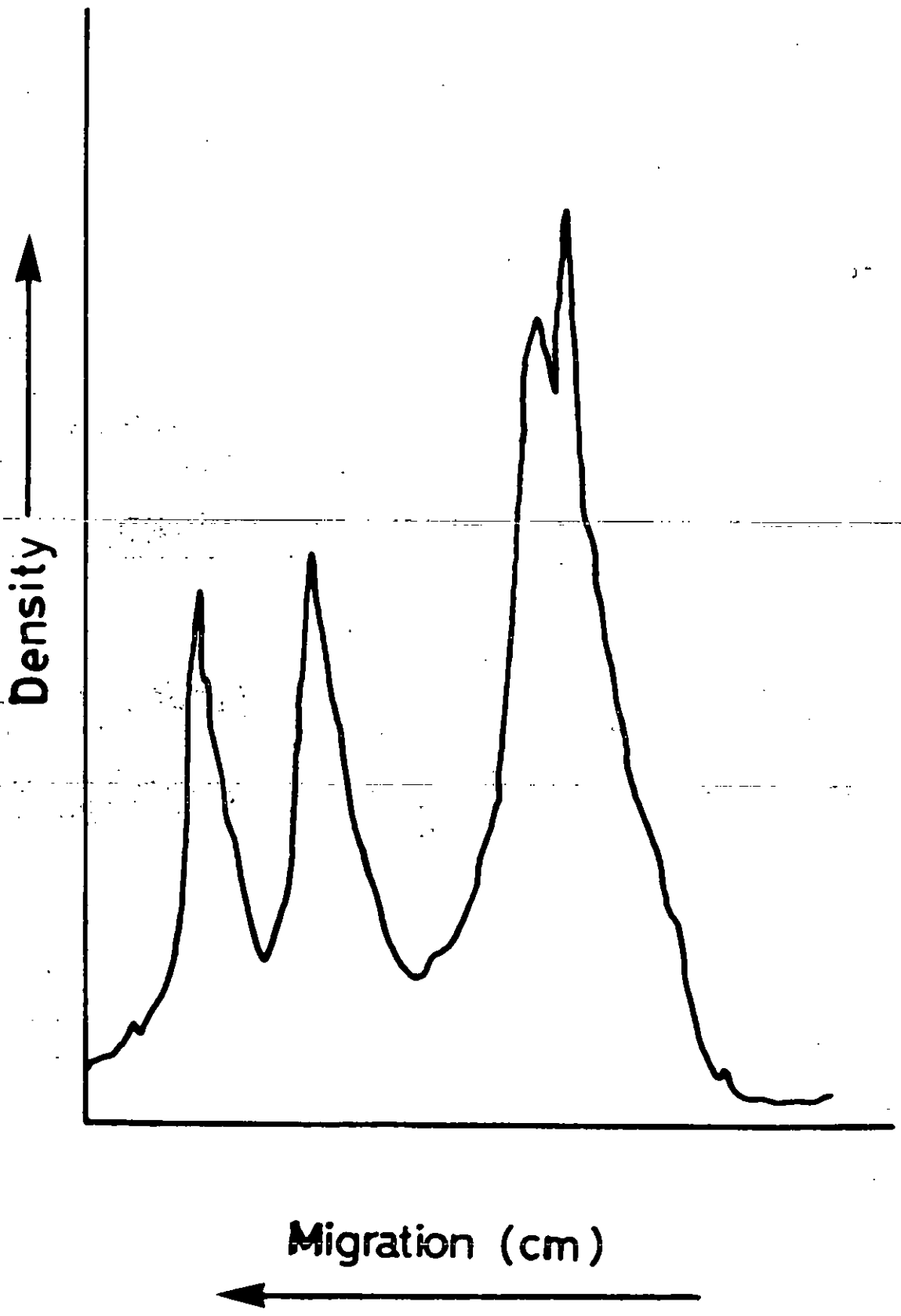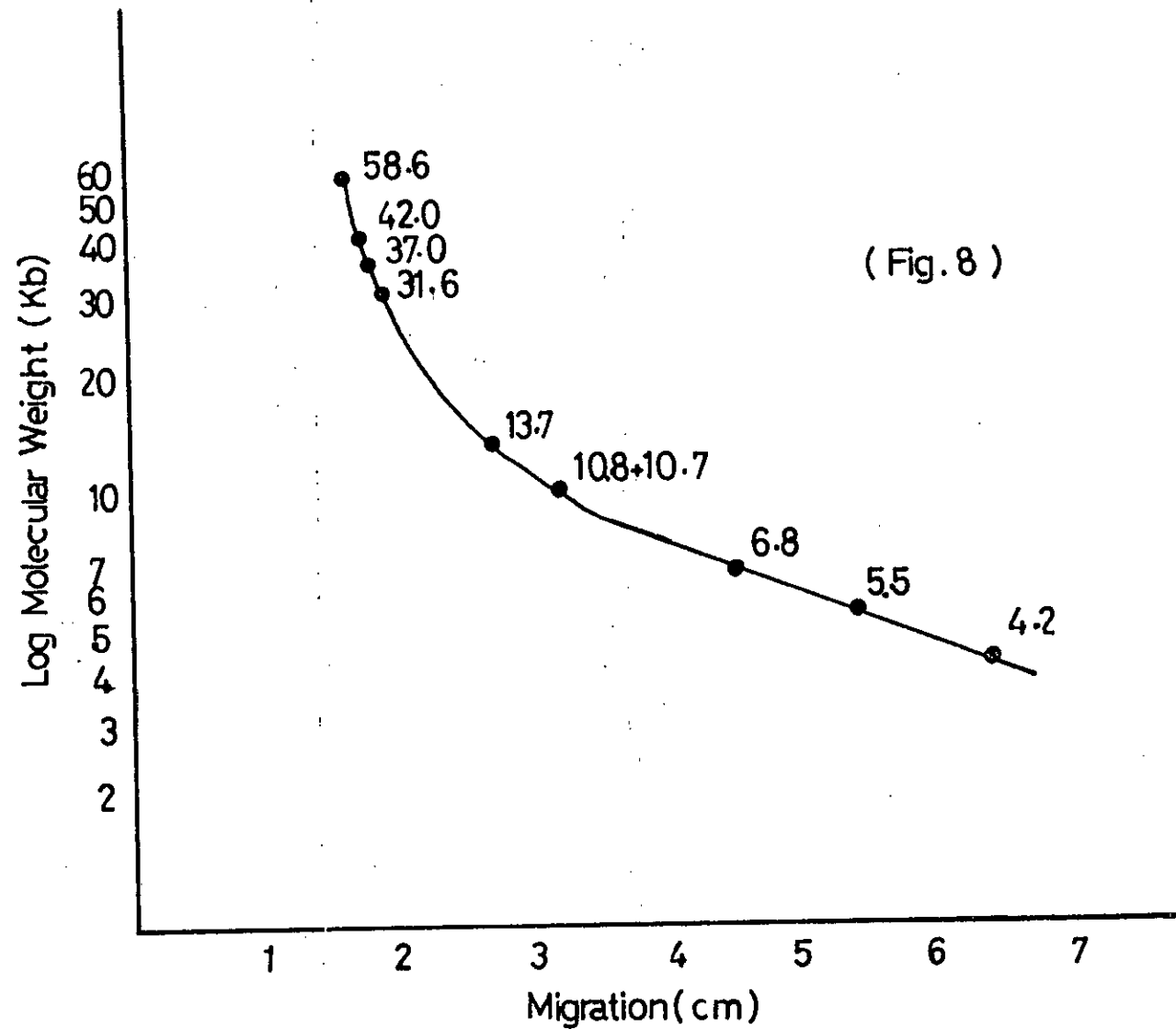
Migration (cm)

Fig. 8. The relation between the size of the restricted DNA fragment and its migration through the 0.7% agarose gel; it represents the endoR.HindIII fragments of pZD44, pZD23, λdrpoBC18 and RP4 λatt.

Table 12   The HindIII analysis of pZD44 and pZD23.

Fragments	λdrpoBC18	pZD44	pZD23	RP4 λatt
A	13.7	37.0	31.6	42
			58.6	
B	10.8 + 10.7	27.0	27.0	27.7
C	6.8	13.7	13.7	
D	5.5	10.7	10.7	
E	4.2			
*F	0.5			
*G	0.1			

The molecular weights (in kb) of the HindIII fragments of
pZD44 and pZD23 (Fig. 6a) are obtained by comparison with
the sized fragments of λdrpoBC18; the sizes of λdrpoBC18
HindIII fragments are based on the data of Taylor and
Burgess (1979) which are consistent with the size of λ
(Szybalski and Szybalski, 1979; Daniels et al, 1980).
These fragments (*) have not appeared on the gel (Fig. 6a).

similar to the estimation by the electron microscopy

which is 69.76 kb or 46.04 Md (see Table 11).

The conclusion about the size of each band in a doublet

will come after the analysis of the HindIII restriction

of pZD44.

B.   Restriction analysis of pZD44

1.   HindIII restriction

It is expected (Fig. 5) that pZD44 produced four

HindIII fragments.   It is shown (Fig. 6) that there are

four HindIII fragments of pZD44.   The sizes of the

fragments are concluded from Fig. 8 and are listed in

Table 12.   Two small fragments (13.7 kb and 10.7 kb)

are present in λdrpoBC18.   They represent the bacterial

chromosome part of the phage.   The two large HindIII

fragments are of RP4 λatt origin.   As shown in Fig. 5

there are three common HindIII fragments in pZD23 and

pZD44;   the gel patterns of Fig. 6 have revealed two of

them, so the third fragment is the 27 kb one.   It is

concluded that this is part of the doublet of pZD23.

Therefore the fourth fragment should have a molecular

weight of 31.6 kb;   the size of this fragment has been

concluded by subtracting the total of the three fragments

from the molecular weight of pZD23 which is 83 kb or

54.8 Md (see Table 11).

2.   BamHl restriction

Further analysis of pZD44 was carried out with the

restriction enzyme BamHl.   The restriction procedure
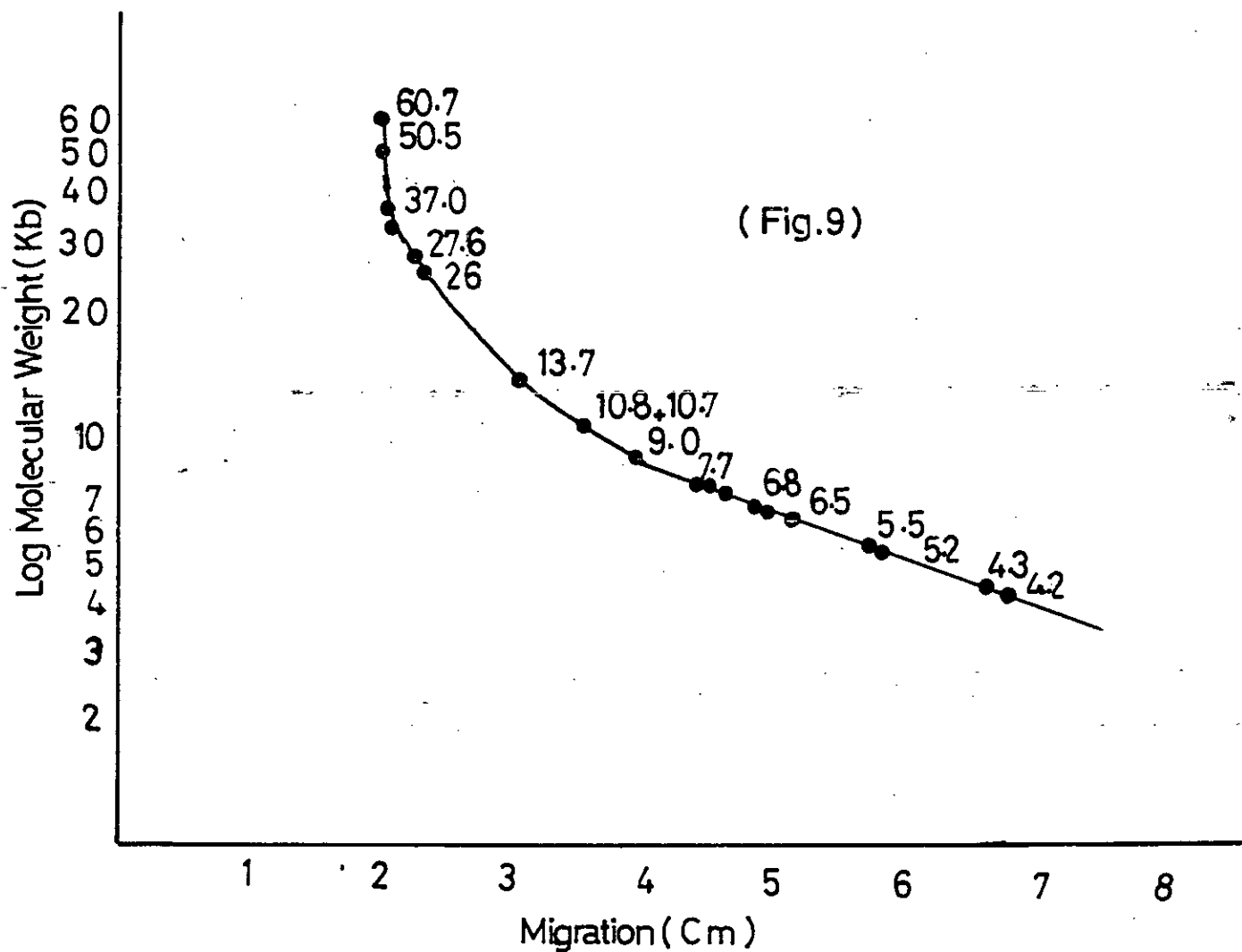
Fig. 9. The relation between the electrophoretically separated fragments (Fig. 6b) and their molecular weights. They represent the endoR.HindIII, the BamHl and the double digests of pZD44, λdrpoBC18 and RP4 λatt.

Table 13.   The restriction analysis of the plasmid pZD44 using the DNA of λdrpoBC18 and RP4 λatt as controls.

Fragments	HindIII			BamH1 + HindIII		BamH1		
	λdrpoBC18	RP4 λatt	pZD44	pZD44	RP4 λatt	λdrpoBC18	pZD44	RP4 λatt
A	13.7	42.0	37.0	Doublet	33.1	24.1	50.5	60.7
B	10.8+ 10.7	27.7	27.0	13.7	27.6	7.6	26.0	9.0
C	6.8		13.7	10.7	8.9	7.5	7.6	
D	5.5		10.7	7.7	0.1*	6.6	4.3*	
E	4.2			5.2		6.5		
F	0.5*			4.3				
G	0.1*			1.6				

The electrophoretic properties of the digested pZD44 with endoR.HindIII, BamH1 or both HindIII and BamH1 (see Fig. 6b).   The sizes of the fragments are in kb.   The HindIII data of pZD44 and RP4 λatt are extracted from Table 12.   BamH1 fragments of λdrpoBC18 are extracted from Boros and Sain (1977) and corrected according to the recent estimation of the size of λ (Szybalski and Szybalski, 1979;   Daniels et al, 1980).

*   These fragments have not appeared on the gel.

is described in detail (see Methods). The BamHl fragments

of pZD44, λdrpoBC18 and RP4 λatt were fractionated through

the same gel where their HindIII fragments were separated

(see Fig. 6b). The controls, RP4 λatt and λdrpoBC18

produced two and five BamHl fragments respectively. Extra

BamHl fragments of λdrpoBC18 are from the contaminating λ

helper DNA. The sizes of the fragments of λdrpoBC18 are

extracted from Boros and Sain (1977) and are calibrated to

the recently estimated size of λ (Szybalski and Szybalski,

1979; Daniels et al, 1980). All fragments are plotted

(see Fig. 9) in order to conclude the unknown sizes and

they are listed in Table 13. The restriction of pZD44

with BamHl yields four fragments (Fig. 5); three of

them can be seen on the gel (Fig. 6). There is only one

fragment (7.6 kb) in common with λdrpoBC18.

3. The double digestion of pZD44 with HindIII and BamHl

From the previous results of the single digestion of

pZD44 with BamHl or HindIII, it is possible to expect

the production of eight fragments from the double digestion

of pZD44 with both enzymes (see Fig. 5). The double

restriction of RP4 λatt (see Methods; Fig. 6b) produced

three fragments whose sizes are concluded from Fig. 9.

However, the size of the largest fragment was also

concluded from knowing the size of RP4 λatt by electron

microscopy (see Tables 11 and 12). The double restriction

of pZD44 with HindIII and BamHl has resulted in its

fragmentation into eight pieces as shown in Fig. 6b.

The first band is a doublet which corresponds to the

RP4 λatt part of pZD44; the next two smaller fragments
have the same size as the HindIII fragments of pZD44
(13.7 kb and 10.7 kb), but there is one BamHl site in
the 13.7 kb HindIII fragment (see Fig. 5), therefore, it
is concluded that the 13.7 kb fragment that has been
produced from the double restriction of pZD44 is the
located fragment between rpoC and Ap^r genes.

It seems from the results of the restriction analysis
that the models (Fig. 5) for the temperature resistant
plasmids pZD23 and pZD44 are most likely the accepted one
for the genetical and physical maps of these plasmids.
It has been described earlier (Part I.B) that these
plasmids are derivatives of the original plasmid pZD100,
therefore it is possible to conclude that the genetical
structure of the plasmid pZD100 is as shown in Figs. 3 and 5
and that the orientation of λatt fragment with respect
to the RP4 genome in the plasmid RP4 λatt is as shown in
Fig. 2.

C. Discussion

There are two reasons to suppose that the temperature
resistant mutants harbour RP4 derivatives.   Firstly they
show resistance to antibiotics whose determinants are
carried by RP4 (Table 7).   In fact, these mutants are
divided into two groups, some of them are resistant to
ampicillin, kanamycin, tetracycline and rifampicin while
others are ampicillin sensitive but resistant to kanamycin,
tetracycline and rifampicin.   Secondly, they are sensitive
to the bacteriophage PRR1 which is RP4 specific (Olsen

and Shipley, 1973; Olsen and Thomas, 1973). Although
all of them are PRR1 sensitive, they still fall into two
groups as far as their transferability is concerned which
will be discussed later.

In addition, the sensitivity of these mutants to the
bacteriophage λ indicates that the plasmids they harbour
are deleted in that part of λ genome which is responsible
for the immunity.

It is apparent, from the in vivo comparative studies showing
that the temperature resistant plasmids share the
properties with pZD100, that they consist of an RP4 genome
and that they confer resistance to rifampicin.

As far as the transferability of the temperature
resistant plasmids is concerned, it is shown in Tables 8
and 9 that the mutant ZD2723 (F⁻ his⁻ trpA9761 argE171
recA56/pZD23) which is ampicillin sensitive but resistant
to kanamycin, tetracycline and rifampicin, transfers its
plasmid pZD23 at higher frequency than the mutants which
are resistant to the four antibiotics. The frequency
of pZD23 transfer is in fact similar to that of RP4 λatt;
its transfer is not subjected to zygotic induction when
compared with its temperature sensitive parent plasmid.
This feature is due to the deletion of the λ genome in pZD23.

The second group of temperature resistant plasmids
is characterised by its very low frequency of transfer to
both λ lysogen and λ non-lysogen recipients. When
compared with their temperature sensitive parent plasmid
which resides in ZD27-4 (Fig. 4; Table 9) they have been

found to transfer at a significantly lower rate; this
alteration in transferability of the plasmids must be
due to the structural differences between the two types
of ⟨⟩temperature resistant plasmids (e.g. pZD23 and
pZD44). The question arises *as to* whether a deletion in the
tra operon of pZD44 ⟨⟩ exist**S**⟨⟩⟨⟩. There are three
tra operons in RP4 that ⟨⟩ flank*the* Km^r gene (Barth
and Grinter, 1977; Grinsted et al, 1977; Thomas et al,
1979). ⟨A⟩ deletion in tra cannot be in the region
between Km^r and Tc^r because the plasmid pZD44 confers
the resistance to both antibiotics kanamycin and
tetracycline. If there is a deletion in the tra operon
it should be in the large tra operon which is located
between *the* EcoRI site of RP4 and *the* Km^r gene (Barth and Grinter,
1977; De Picker et al, 1977; Thomas et al, 1979). The
deletion in pZD44 should not include the gene(s) that
code for the production of the specific pili to which PRR1
bacteriophage adsorbs (Olsen and Shipley, 1973;

Olsen and Thomas, 1973) because it
remains sensitive to the RP4 specific phage PRR1 (Table 7).

In addition, the restriction analysis of pZD44 indicates
that there is no⟨⟩ deletion in the tra region between *the* EcoRI
site of RP4 and *the* Km^r gene (Fig. 5; Table 13). This
inference is drawn from the following observations. It
has been shown from the electron microscopy that the
difference in molecular weight between the temperature
resistant plasmids pZD44 and pZD23 is 3.7 Md (Table 11)
which corresponds to the size of the fragment between

The EcoRI site of RP4 and the Ap^r gene (De Picker et al, 1977;
Thomas et al, 1979).   It is apparent that the difference
between pZD23 and pZD44 is that the latter contains the
region between EcoRI site of RP4 and its Ap^r gene. It is Possible that
part of the plasmid genome may contain a repressor gene(s)
for the tra operon which is under a different control in
pZD44 than in the RP4 plasmid and which is deleted in
pZD23.   The presence of a tra regulator gene(s) in
pZD44 Could explain its low transferability.

Apart from the region between the EcoRI site of RP4
and its Ap^r gene, the plasmids pZD23 and pZD44 are similar
to each other and the in vitro restriction analysis
(Fig. 6) has revealed that both of them harbour the rpoC
gene in addition to the rpoB3 gene.   However, it has been
demonstrated that on transfer of pZD23 into a rec$^+$ and a
rec$^-$ rpoCts mutant, all the transconjugants of the rec$^+$
transconjugants were temperature resistant whereas none
of the rec$^-$ genotype were temperature resistant
(Table 10).   The temperature resistance of the 397C
(argG B$_1^-$ rpoCts) rec$^+$ transconjugants are explained on the
basis of the dominance of the rpoC allele of pZD23 over
rpoCts of 397C (argG B$_1^-$ rpoCts);   the temperature
resistance of these transconjugants may also be due to
homogenotisation, where the rpoC allele is transferred onto
the chromosome which in turn is segregated with an rpoC
plasmid.   The dominance of the wild type rpoC allele
over an rpoC mutation is possible as it has been reported
that rpoC is dominant over the rpoC1 allele in merodiploids
(Bass et al, 1977).

However, the majority of the X242 (metB B$_1^-$ his$^-$
recA56 strA lac rpoC4) rec$^-$ transconjugants were temper-
ature sensitive and only 20% were temperature resistant;
this mutant has a different temperature sensitive
mutation than the 397C (argG B$_1^-$ rpoCts) mutant and it is
possible that it is dominant over the wild type allele
rpoC which is carried by the plasmid.   However, 20% of
the transconjugants were temperature resistant because
they are revertants of the rpoC4 mutation;   I found that
this strain had a high tendency to reversion as it should
be purified many times before starting the experiment.

The temperature resistant plasmid pZD23 is a suitable
vector because it harbours rpoB3, two of the RP4 drug
resistant determinants, it transfers at a similar frequency
to RP4 plasmid (Olsen and Shipley, 1973) irrespective of
the presence or absence of the bacteriophage λ in the
recipient and it is stable as there are molecules of only
one size in its DNA preparation (Table 11).

The molecular weights of the plasmid pZD100 and its
derivatives pZD23, pZD135 and pZD44 were estimated by
the electron microscopical methods (Legend, Table 11).
The molecular weights of the plasmids RP4 and RP4 λatt
were estimated by the same method.   The size of RP4
(see Table 11) is slightly higher than the published size
(Grinsted et al, 1972;   Meyer et al, 1977;   Burkardt
et al, 1978;   Thomas et al, 1979).   This could be
attributed to the fact that I am using a different
technique than the sucrose gradient centrifugation used by

Grinsted et al (1972) or the electron microscopic

procedure used by Burkardt et al (1978). In fact, the

higher value of the molecular weight of RP4 is due to

the use of the most recent estimation of the molecular

weight of the standard, pSC101, which is 6.16 Md or

9.34 Kilobases (R. Meyers, 1979, personal communication).

However, if we use the previously estimated size of

pSC101 which is 5.5 Md or 9.09 kb (Brevet et al, 1977 in Bukhari et al)

then we obtain the molecular weight of RP4 as 38 Md which

is the published size (Broda, 1979). The difference in

molecular weight between RP4 λatt and RP4 is 3.5 Md

(5.2 kb) which corresponds to the size of the λatt fragment

(Watson and Scaife, 1978; Szybalski and Szybalski, 1979;

Daniels et al, 1980).

It is very likely that the plasmid pZD135 which

confers resistance to kanamycin, tetracycline and

rifampicin is the same plasmid as pZD23, because they

confer the same phenotype and they have exactly the same

molecular weights (Table 11). However, on the basis of

the applied techniques, the possibility that they are

different cannot be ruled out. However, the difference

in molecular weight between these plasmids and pZD44 is

about 4 Md which corresponds to that part of RP4 genome

which extends from EcoRI site to the Ap^r gene (De Picker

et al, 1977; Meyer et al, 1977).

All the plasmid DNA preparations (Table 11) except

pZD100 contained Single sized molecules. The plasmid pZD100

preparation had various sizes of molecules; some of them

correspond to the expected size of the plasmid pZD100

(81.8 Md) which consists of the whole RP4 λatt genome

(46.04 Md) and λdrpoBC18 (about 35 Md) [see Introduction

for estimation of the size of λdrpoBC18]. Smaller

molecules of about 60 Md in the pZD100 preparation may

represent the derivatives of the pZD100 which have lost

the bacterial DNA segment which is about 20 Md

(Kirschbaum et al, 1976); it may have been lost due to

the expression of the xis gene carried by the phage

λdrpoBC18 or by the RP4 λatt.   The λatt fragment

(srI λ2-3) lacks the normal promoter for the xis gene

(Watson and Scaife, 1978); in this case the expression

of xis may be the result of a read-through mechanism from

an upstream promoter.   The preparation of the pZD100

DNA was performed in the presence of all the four

antibiotics whose resistance is conferred by pZD100.

If there is any molecule which represents a deletion in

the plasmid pZD100 such as the molecule with 45.5 Md

size (Table 11), it should not exist on its own in the

cell but with another copy of the plasmid which confers

the resistance to all the antibiotics.   This implies that

the plasmid pZD100 is less stable than its temperature

resistant derivatives.

The physical maps of the temperature resistant plamids

pZD23 and pZD44 are deduced from the restriction analysis

of both plasmids with endoR.HindIII and pZD44 restriction

with endoR.BamH1 and its double restriction with HindIII

and BamH1.   From this analysis it appeared that the λatt

fragment is oriented in RP4 λatt as shown in Figs. 2 and 5
and the genetical structure of the temperature sensitive
plasmid pZD100 is as shown in Figs. 3 and 5.

The endoR.HindIII restriction analysis (Fig. 5;
Table 12; Fig. 6a) has revealed the presence of three
common HindIII fragments in pZD23 and pZD44 plasmids;
two of them are present in the HindIII digest of λdrpoBC18
and they represent the bacterial chromosomal segment
which is carried by the phage and the plasmids. The
third common fragment is 27 kb (Fig. 6; Table 12). This
size can be accepted if the plasmids are as shown in
Fig. 5, because the size of the fragment between Km^r gene
and EcoRI site of RP4 (clockwise direction) is 21.4 kb
and the part of the plasmids between the remnant of the
attλ region and the nearest HindIII site is 6.1 kb (Fig. 5).
It is demonstrated that apart from the remnant of the
λatt fragment, the bacterial segment is located within
the BamHl fragment (7.6 kb) of λdrpoBC18 (Fig. 5).

Models of the plasmids which are contrasting to those
of Fig. 5 cannot be accepted for the following reasons:
first, the 27 kb fragment should be located between rpoC
and Km^r and this is not possible because as mentioned
earlier the size of the fragment between Km^r and EcoRI
site of RP4 is 21.4 kb and if there is an extra 6.1 kb
towards rpoC gene then it should contain another HindIII
site which produces a 5.5 kb fragment (Fig. 5). Second,
the size of the largest HindIII fragment (Table 12)
should be bigger than 37 kb and third, if the Ap^R gene is

present then the same fragment should appear in pZD23 HindIII digest.

The digestion of the plasmid pZD44 with endoR.BamHl has resulted in the production of four fragments (Figs. 5 and 6) whose molecular weights are shown in Table 13. This has provided another support for the deduced maps of Fig. 5 because if the orientation of RP4 part is reversed so that Ap^r gene is near the remnant of λatt then the large fragment should be much larger in size than 50.5 kb, the 26 kb fragment should disappear and a new fragment of about six kilobases should appear which represent the region from Ap^r gene to the remnant of the attλ region where another site of endoR.BamHl is located (Davies et al, 1978; Szybalski and Szybalski, 1979; Daniels et al, 1980).

The double restriction of pZD44 has provided additional support for the models of Fig. 5 as it has resulted in the production of eight fragments (Fig. 6b) whose molecular weights are listed in Table 13. Although reversing of the orientation (shown in Fig. 5) will also result in the production of eight fragments when double restriction occurs, the sizes of these fragments do not correspond to the sizes shown in Table 13 such that the fragment which has 13.7 kb molecular weight will disappear and another fragment of about six kilobases appear as described earlier. The restriction analysis of the temperature resistant plasmids has contributed to our understanding of the genetical structure

of the original plasmid pZD100 (Figs. 3 and 5), the
temperature resistant plasmid (Fig. 5) as well as the
plasmid RP4 λatt (Fig. 2).

The plasmid RP4 λatt can integrate into the normal
λ prophage integration site on the chromosome to form
stable Hfr strains and can promote chromosomal transfer
in the order: O-lac-leu-thr-trp (Watson and Scaife, 1978).

Combining this result with the restriction analysis will lead us to
conclude RP4 λatt orientation in the Hfr.

The origin of transfer, oriT, has been mapped at
48 kb (31.7 Md) on the map of the plasmid RK2 (Thomas
et al, 1979). It is concluded that pZD100, the
derivative of RP4 λatt, transfers its markers in the
order: O-rpoB-Ap^r-Tc^r-Km^r and that the tra genes are the
last to enter the recipient cell similar to the tra gene
transfer characteristic of the F plasmid (Guyer and Clark, 1977).

Part III   The Utilisation of the pZD23 and the pZD100
            Plasmids as Vectors for the E.coli rpoB3 Gene

a.   Introduction

This part of the thesis will describe the utilisation
of one of the temperature resistant plasmids, pZD23, as
vector for the structural gene for the β subunit of the
enzyme RNA polymerase of E.coli.   A comparison was
carried out between this plasmid and the original
temperature sensitive pZD100 plasmid

The majority of the bacterial species to which the plasmids are transferred are members of the Enterobacteriaceae; some of them are known to receive the plasmid RP4 such as E.coli and Salmonella typhimurium while others such as Citrobacter freundii and Hafnia alvei møller were tested for the first time in this respect. In addition, I tested species which belong to families other than the Enterobacteriaceae such as Rhizobium leguminosarum, Pseudomonas aeruginosa and Azotobacter vinelandii.

The data will be presented as follows. Firstly, the bacterial species will be characterised by the standard microbiological tests. Secondly, they will be used as recipients for pZD23 and pZD100. Finally, the plasmid-bearing derivatives will be analysed for heterospecific expression. The main index of the latter is the conversion of the rifampicin sensitive phenotype of the plasmid-bearing species into a rifampicin resistant one, indicating expression of the E.coli-rpoB3 gene in their cells; their failure to acquire the rifampicin resistant phenotype has many implications which will be discussed.

Part of the results were presented at the XIV International Congress of Genetics, 1978.

b. Results

Prior to the construction of the hybrids which harbour the plasmid, the bacterial species were tested for their natural resistance to the antibiotics against which

Table 14. The classification of the various bacterial genera that have been chosen for the studies of the heterospecific gene expression.

Family	Enterobacteriaceae	Azotobacteriaceae	Pseudomonadaceae	Rhizobiaceae
Species	E.aerogenes	A.vinelandii	P.aeruginosa	R.leguminosarum
	* E.coli			
	C.freundii			
	H.alvei møller			
	K.pneumoniae			
	P.morganii			
	P.vulgaris			
	S.marcescens			
	* S.typhimurium			
Source	J. Fleming	R. Olsen	P. Clarke	J. Beringer
	* J. Scaife			

The twelve bacterial species that have been chosen for the heterospecific gene expression studies are classified here, according to the system of Buchanan et al, 1974. The table also contains the sources from which these bacteria were obtained. All the bacterial species that came from J. Fleming were received as lyophils which were prepared in 1960 except Hafnia which was prepared in 1952 and Serratia which was prepared in 1971.
Other strains were received as fresh nutrient slants.

<u>Legend to Table 15.</u>   This table illustrates the natural resistance of the bacterial species

of Table 12 to the following concentrations of the antibiotics: 100 μg/ml rifampicin (RD);

50 μg/ml ampicillin (PN);   10 μg/ml tetracycline (TE);   100 μg/ml carbenicillin (PY);

25 μg/ml kanamycin (K).

They have been tested by the use of the Oxoid Antibiotic Multodisk as described in the

Methods.

+/- is resistant/sensitive to the specific concentration of the tested antibiotic.

The non-Enterobacteriaceae species were tested for their sensitivity to a series of

concentrations of rifampicin.

It has been found that <u>A.vinelandii</u>, <u>P.aeruginosa</u> and <u>R.leguminosarum</u> are sensitive to

10, 25, and 4 μg/ml rifampicin respectively.

Table 15   The natural resistance of the gram-negative bacteria to the antibiotics.

Bacterial species	RD (100 µg/ml)	PN (50 µg/ml)	TE (10 µg/ml)	PY (100 µg/ml)	K (25 µg/ml)
E.aerogenes	-	+	-	-	-
E.coli	-	-	-	-	-
C.freundii	-	+	-	-	-
H.alvei møller	-	-	-	-	-
K.pneumoniae	-	-	-	-	-
P.morganii	-	-	-	-	-
P.vulgaris	-	-	-	-	-
S.marcescens	-	+	+	-	-
S.typhimurium	-	-	-	-	-
R.leguminosarum	-	-	-	-	-
P.aeruginosa	-	+	-	-	-
A.vinelandii	-	+	-	-	-

Table 16. The differentiation between the various members of the Enterobacteriaceae

Bacterial species	Citrate	Methyl red	Voges Proskauer	H_2S	Gelatin hydrolysis	Sorbitol	% G + C
E.aerogenes	+	-	+	-	-	+	56.1
E.coli	-	+	-	-	-	+	
C.freundii	+	+	-	+	-	+	
H.alvei møller	-	-	-	-	-	-	49.0
K.pneumoniae	+	-	-	-	-	+	
S.marcescens	+	-	+	-	+	N.D.	60.0

This table summarises the properties of the various members of the Enterobacteriaceae as revealed by the employed microbiolgical tests. Citrate media is described earlier (see Media). Methyl red, Voges Proskauer, H_2S production, Gelatin hydrolysis and Sorbitol utilisation tests are described in the Methods.

+ indicates positive results such as the utilisation of citrate as a sole carbon source or the production of acid and gas from the Sorbitol fermentation.

- indicates negative results such as the inability of the bacterial species to hydrolyse gelatin or to produce H_2S.

N.D., not done.

The G + C contents of the DNAs of three bacterial species were estimated as follows: the chromosomal DNA was prepared as described in the methods. It was mixed with the standard DNA. Caesium chloride was added to a final concentration of 1.7 g/cm^3. The samples were ultra-centrifuged in a Beckman Analytical Ultracentrifuge (model E) at 40 Krpm for 20 hrs at 25°C. Results of ultracentrifugation and calculation of the G + C content are described in detail (Fig. 10; Fig. 11).

of the production of H$_2$S and S.mercescens can hydrolyse
gelatin.   In addition, some of these bacteria have
distinctive colonial morphology.   S.marcescens, for
example, is red in colour due to the production of the
prodigiosin red pigment and the intensity of the red
pigmentation varies depending on the type of the growth
medium and age of the colony.   The colour of S.marcescens
colonies on Simmon's citrate medium is dark iridescent
red.

Klebsiella pneumoniae is characterised by its dry
mucoid colonies on all utilised growth media.   Proteus
morganii and Proteus vulgaris have a strong putrifactive
odour.   P.vulgaris is characterised by its swarming on
nutrient agar unlike P.morganii which is unable to swarm
on nutrient agar and· which does not have the tendency to
swarm spontaneously.   However, both species have the
ability to. deaminate phenylalanine into phenyl pyruvic
acid which will result in the production of the green
colour due to the reaction between phenyl puruvic acid
and the ferric salt (see Methods).   None of the tested
species of the Enterobacteriaceae have this property.
The strain of Salmonella typhimurium which I used is arg$^-$;
I used this feature to identify it.

The bacterial species which are outside the
Enterobacteriaceae such as Rhizobium leguminosarum,
Pseudomonas aeruginosa and Azotobacter vinelandii are
easily recognised by their gross features.
R.leguminosarum colonies are mucoid on nutrient and minimal

media but their mucoid appearance differs from that of
K.pneumoniae in that the latter is more dry.
R.leguminosarum grows at a much lower rate than the other
bacterial species and its generation time is about twelve
hours; its growth temperature is 28°C. Pseudomonas
aeruginosa is characterised by the production of the
pyocyanin pigment which is fluorescent under ^{an} ultra-
violet source.

Azotobacter vinelandii produces water soluble
fluorescent pigment which diffuses into the medium and
appears green in ultraviolet light (wavelength 280 nm).
Under the light microscope the cells appear in pairs and
cysts are formed.

Some of the representatives of the Enterobacteriaceae
were characterised for the percent G + C content of their
DNA. These were E.aerogenes, H.alvei møller and
S.marcescens. The method is described in legends to
Table 16 and Fig. 11. The separation of the DNA band of
the tested species from that of the known standard species
after CsCl centrifugation (Fig. 10) and the estimation of
the percent G + C content of the DNA is described in Fig. 11.

3. Construction of hybrid strains

Various types of mating experiments were carried out
in which the plasmids pZD23 and pZD100 were transferred
from E.coli into the different bacterial species after
their characterisation. For all the matings, the plasmid
pZD23 was transferred from ZD2723 (F⁻ his⁻ trpA9761 argE171
recA56/pZD23) and pZD100 was transferred from ZD100

Legend to Fig. 10

The chromosomal DNA bands of the tested bacteria and the standard DNA as they appear under the ultraviolet source. The DNAs of Enterobacter aerogenes (a - right) and Hafnia alvei møller (a - left) are separated from the standard DNA of Micrococcus lysodikteus (lower bands). The chromosome DNA band of Serratia marcescens (b - right) is separated from the E.coli standard DNA band whereas Hafnia DNA band is not separated from E.coli.

(a)   (b)

Legend to Fig. 11

The densitographs reveal the distances of the separated DNA bands.   The separation between

the E.aerogenes DNA and M.lysodikteus DNA is 34 mm (Fig. 11a);   the separation between

H.alvei møller DNA and M.lysodikteus DNA is 49 mm (Fig. 11b).   Fig. 11c illustrates the

separation of the Serratia DNA from E.coli DNA which is 19 mm.   It is known that 87 mm =

0.040 ρ (Purdom, personal communication).   Therefore the G + C content of:

1.   E.aerogenes: $\frac{34 \times 0.04}{87} = 0.016$

$$\rho = 1.731 - 0.016$$
$$= 1.715 \text{ g/cm}^3 \text{ buoyant density}$$
$$\rho = 1.660 + 0.098 \text{ (GC) (Adams et al, 1976)}$$
$$GC\% = \frac{1.1715 - 1.660}{0.098} = 56.1\%$$

2.   H.alvei møller: $\frac{49 \times 0.040}{87} = 0.023$

$$\rho = 1.731 - 0.023 = 1.708 \text{ g/cm}^3 \text{ buoyant density}$$
$$GC\% = \frac{1.708 - 1.660}{0.098} \text{ (see above)}$$
$$GC\% = 49\%$$

3.   S.marcescens: $\frac{19 \times 0.040}{87} = 0.009$

$$E.coli \ \rho = 1.710 \text{ g/cm}^3$$
$$S.marcescens \ \rho = 1.710 + 0.009 = 1.719 \text{ g/cm}^3$$
$$GC\% = \frac{1.719 - 1.660}{0.098} = 60\%$$

(a)

(b)

(c)
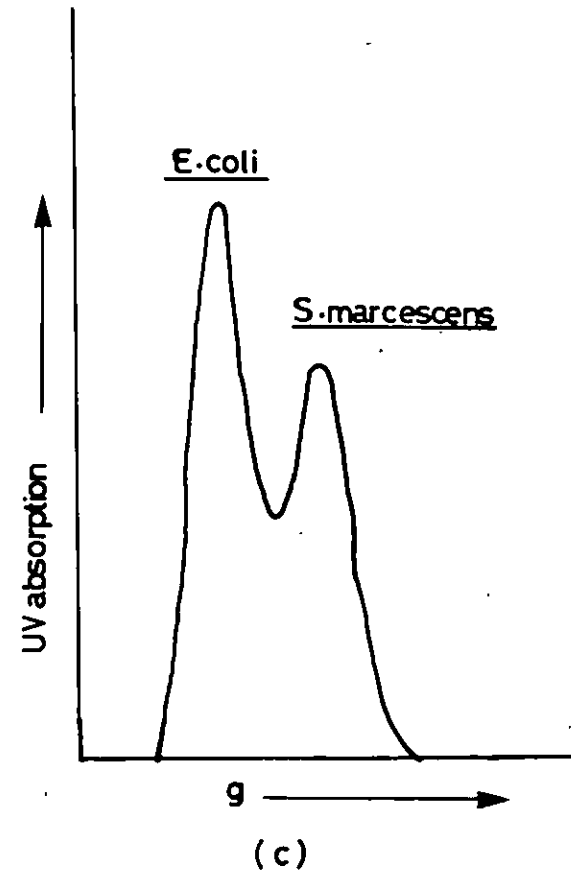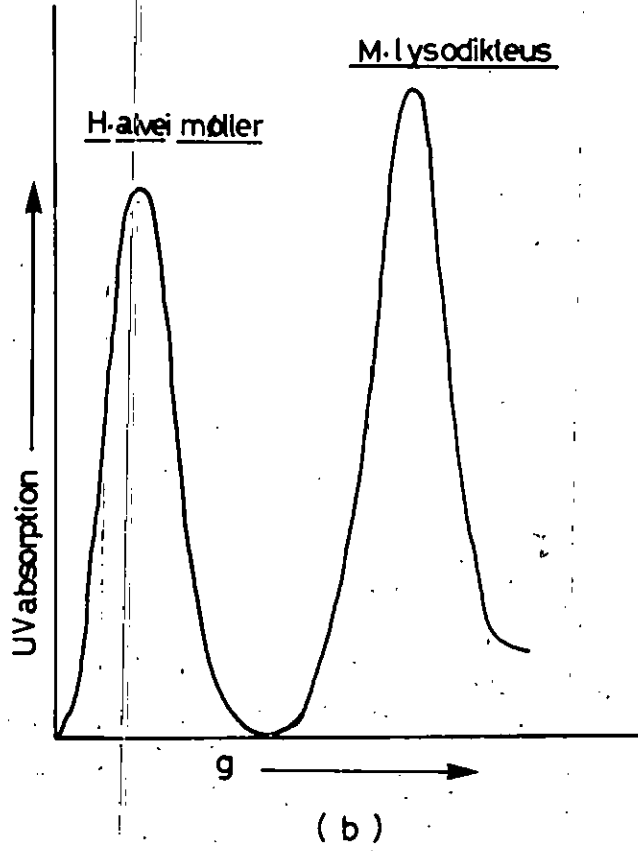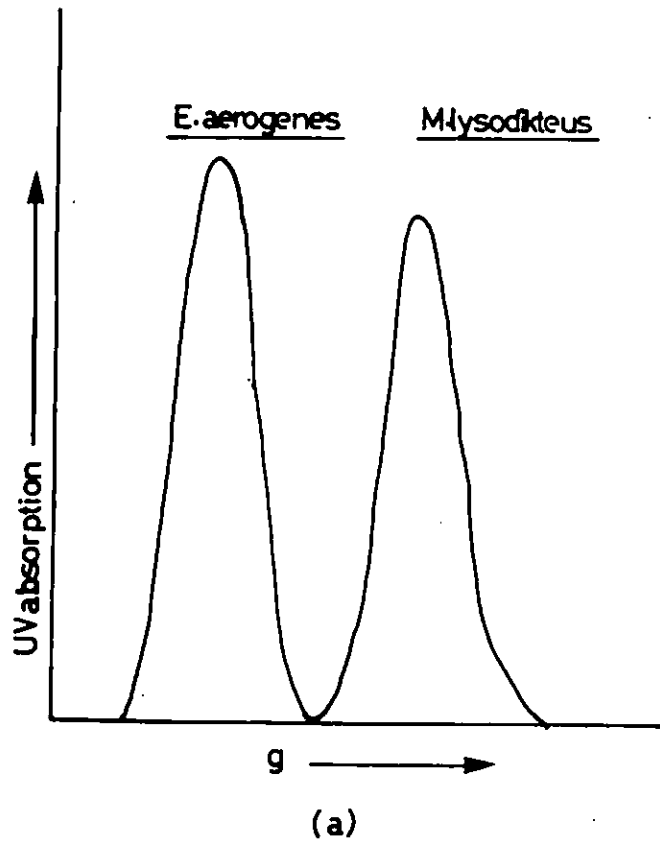
(metB B$_1^-$ recA56 λcI857S7/pZD100).   The construction of
the hybrids was firstly based on the selection of transfer
of RP4 markers.   The hybrids were tested later for the
presence of E.coli rpoB3 gene by testing their resistance
to rifampicin and by testing their plasmid donor ability.

a. Construction of hybrids of the Enterobacteriaceae

The representatives of the Enterobacteriaceae that
are included in this study are listed in Table 14.   Liquid
culture matings for five hours (see Methods) were carried
out for the transfer of pZD23 and pZD100 into E.aerogenes,
C.freundii, H.alvei møller, K.pneumoniae and S.marcescens.
In E.coli, two hours of mating time were enough for the
transfer of pZD23 from ZD2723 (F$^-$ his$^-$ trpA9761 argE171
recA56/pZD23) into AW1 (metB B$_1^-$ recA56 str-s λ^S) whereas
six hours were required for the transfer of pZD100 from
ZD100 (metB B$_1^-$ recA56 λcI857S7/pZD100) into TGL10
(F$^-$ his$^-$ trpA9761 argE171 recA56).   The transfer of both
plasmids into S.typhimurium was carried out by Millipore
filter matings (see Methods);   the same techique was
employed for the transfer of the plasmid pZD23 into both
species of Proteus.

The selective plates for the transconjugants varied
with respect to the recipient of each mating.   Glucose
minimal media (see Media) supplemented with kanamycin were
used for the selection of pZD23 hybrids in all matings of
the members of the Enterobacteriaceae family except
S.typhimurium, P.morganii and P.vulgaris.   Glucose minimal
media used for the selection of the Salmonella transconjugants

Legend to Table 17.   This table illustrates the frequencies of transfer (per donor) of the plasmids RP4 λatt, pZD23 and pZD100.   It differentiates between the bacterial genera which have recognised E.coli rpoB3 gene and have become resistant to the rifampicin (100 µg/ml) and the bacterial genera in which no expression of the E.coli rpoB3 gene is detected.

*   The rifampicin resistant transconjugants (rif-r) are small in size when compared with the normal colony size indicating that they show poor expression of the E.coli rpoB3.

**   The mating technique which was employed for these matings is the millipore filter mating which is described in the Methods.

Table 17. Heterospecific expression of E.coli rpoB3 gene in some gram-negative bacteria.

Bacterial strains	RP4 λatt	pZD100	% rifampicin resistant hybrids	pZD23	% rifampicin resistant hybrids
Escherichia coli	2.6×10^{-3}	4.2×10^{-4}	58-88	5.2×10^{-2}	100
Enterobacter aerogenes	4.5×10^{-4}	1×10^{-5}	98	4.5×10^{-4}	100
Citrobacter freundii	1.4×10^{-4}	7.5×10^{-5}	98	1.1×10^{-2}	100
Hafnia alvei møller	4.7×10^{-6}	8.7×10^{-6}	77	1.1×10^{-5}	100
Klebsiella pneumoniae	6.3×10^{-6}	6×10^{-7}	58	1.1×10^{-4}	100
Proteus morganii		Not done		3.5×10^{-2}**	26 70 }*
Proteus vulgaris				2.3×10^{-2}**	18 80 }*
Serratia marcescens	2.9×10^{-6}	2.8×10^{-5}	0	2.6×10^{-5}	0
Salmonella typhimurium	5.3×10^{-7}	1.8×10^{-4}**	0	7.6×10^{-3}**	100
Rhizobium leguminosarum	1×10^{-6}	3×10^{-4}**	0	1.4×10^{-5}**	0
Pseudomonas aeruginosa	1.6×10^{-4}	6.2×10^{-4}	0	N.D.	
Azotobacter vinelandii	2.8×10^{-5}	1.5×10^{-4}	0	3.6×10^{-5}	0

were supplemented with arginine in addition to kanamycin.

The same type of the selective media has been used, with the addition of carbenicillin, for the selection of pZD100 hybrids.

Grabow and Smit minimal medium (see Media) supplemented with kanamycin and tetracycline was used for the selection of P.morganii and P.vulgaris transconjugants.

The incubation temperature for the matings which involve the transfer of pZD23 and pZD100 was 37°C and 30°C respectively except Proteus. The transfer of pZD23 into both species of Proteus was carried out at 30°C.

The frequencies of transfer of both plasmids, per donor, into the tested species are illustrated in Table 17. The parental plasmid RP4 λatt was transferred from ZD7 (metB B_1^- recA56 str-s λ^S/RP4 λatt) into these species as a control for their ability to receive the derivatives pZD23 and pZD100. The frequencies of transfer of RP4 λatt, per donor, are shown in Table 17.

b. Construction of hybrids of the non-Enterobacteriaceae

Three bacterial species (see Table 14) were chosen for this investigation. They were representatives of the Azotobacteriaceae, Pseudomonadaceae and Rhizobiaceae.

Liquid culture matings for eight hours (see Methods) were set for the transfer of pZD23 from ZD2723 (F⁻ his trpA9761 argE171 recA56/pZD23) and pZD100 from ZD100 (metB B_1^- recA56 str-s λcI857S7/pZD100) into Azotobacter vinelandii at 37°C and 30°C respectively.

However, for transfer of pZD100 into Pseudomonas

<u>aeruginosa</u>, a spot mating technique (see Methods) was carried out at 30°C for 24 hours.

Millipore matings (see Methods) were carried out for transfer of both plasmids into <u>Rhizobium leguminosarum</u> at 28°C.

As described in section (a), the first selection of the transconjugants was for the transfer of RP4 drug resistant determinants which are carried by pZD23 and pZD100.

Sherwood minimal medium (see Media) supplemented with phenylalanine, tryptophan, carbenicillin, kanamycin and streptomycin (500 µg/ml) was used for the selection of pZD100 hybrids of <u>R.leguminosarum</u>; the same medium, with the elimination of carbenicillin, was employed for the selection of pZD23 transconjugants. It is demonstrated in Table 17 that the non-Enterobacteriaceae species have received both plasmids as well as RP4 λ<u>att</u>.

4. Is <u>rpoB3</u> present in the hybrid?

Testing the rifampicin resistance of the hybrids is the answer for two questions; the first is whether <u>rpoB3</u> of <u>E.coli</u> is present in the alien cell or not, and the second is whether it can function effectively or not. About fifty purified transconjugants were tested on selective plates supplemented with rifampicin and on similar plates with no rifampicin as a control.

a. <u>The rifampicin resistance of the Enterobacteriaceae hybrids</u>

i. <u>General view</u>

All the Enterobacteriaceae hybrids were tested for

their resistance to 100 µg/ml rifampicin.   They were

all resistant to rifampicin except Serratia and

pZD100 hybrids of Salmonella.

Table 17 demonstrates that the percentage of the

rifampicin resistant hybrids is always higher when

the plasmid pZD23 is transferred into the cell, in

comparison with the percentage of the rifampicin

resistant transconjugants which have received the

plasmid pZD100.

As far as the resistance to rifampicin is concerned,

members of the Enterobacteriaceae are similar to

each other apart from three features which arose in

the following representatives of the family.

ii.   Serratia marcescens

The transconjugants of S.marcescens which have

received pZD23 were resistant to kanamycin but they

were sensitive to rifampicin.   The same result applies

to the pZD100 hybrids of Serratia as they were

resistant to carbenicillin and kanamycin but sensitive

to rifampicin.   Serratia hybrids were also tested

for their resistance to 30 µg/ml and 40 µg/ml of

rifampicin.   The results with these hybrids were

similar to that with Serratia itself as both of them

were sensitive to the 40 µg/ml final concentration

of rifampicin and both of them were resistant to

30 µg/ml of the same antibiotic.   In other words, as

far as the resistance to rifampicin is concerned there

is no difference between Serratia which have the

plasmids and those which lack them.

iii. __Hafnia alvei møller__

pZD23 hybrid colonies of this bacterium have very
small sizes at 37°C on all media tested.   They can
be observed with the naked eye.   However, they grow
into normal size when the incubation temperature
is 30°C.

iv. __Proteus spp.__

The following feature of the __Proteus__ hybrids applies
to both species, __P.morganii__ and __P.vulgaris__.   On
transfer of pZD23, two types of __Proteus__ hybrids which
are resistant to kanamycin and tetracycline were
obtained.   One type comprises 70-80%, is characterised
by its small colonial size on the rifampicin plate
indicating the poor expression of the __E.coli__ __rpoB3__
gene.   The second type shows the normal colony
growth size in the presence of rifampicin.

b.   __The rifampicin resistance of the non-__

__Enterobacteriaceae hybrids__

__Pseudomonas aeruginosa__ and __Rhizobium leguminosarum__
hybrids were tested on a series of decreasing rifampicin
concentrations ranging from 100 μg/ml, 75, 50 and 25 μg/ml.
In addition, the hybrids of __R.leguminosarum__ were tested
at even lower concentrations of rifampicin of 15, 10
and 4 μg/ml.

__Azotobacter vinelandii__ hybrids were tested for the
resistance to 100 μg/ml and 10 μg/ml rifampicin.

None of the hybrids of the bacterial species in
different taxonomic families from __E.coli__ became rifampicin

resistant on receiving E.coli rpoB3 plasmids. They remained sensitive to even very low concentrations of the drug.

c. Further investigation on the effect of rifampicin on growing cultures of P.aeruginosa and R.leguminosarum.

This experiment was carried out to see the effect of the rifampicin on the growing cells of the non-Enterobacteriaceae pZD100 hybrid. The tested species were hybrids of P.aeruginosa and R.leguminosarum. The optical density of the growing cultures was measured continuously at a fixed time interval. The cultures were later divided into two aliquots and the rifampicin antibiotic was added to one of them. Immediate reading of the optical density was taken and the procedure was repeated several times for both types of cultures.

The same procedure was repeated with RP4 λatt hybrids of both species and with spontaneous rifampicin resistant mutants of the same RP4 λatt hybrids as controls for the rifampicin sensitive and the rifampicin resistant cell. The effect of rifampicin on the growing cultures is as described below:

i. Pseudomonas aeruginosa

The growth of Raf3 (Pseudomonas/pZD100) and Raf4 (Pseudomonas/RP4 λatt) is inhibited immediately after the addition of rifampicin to the L-broth cultures (Fig. 12). A rifampicin resistant mutant of P.aeruginosa, Raf8 (Pseudomonas rif^r/RP4 λatt),

<u>Legend to Fig. 12</u>

This demonstrates the effect of the rifampicin on the growing cultures of <u>Pseudomonas</u>

<u>aeruginosa</u>.

L-broth cultures of Raf3 (<u>Pseudomonas</u>/pZD100), Raf4 (<u>Pseudomonas</u>/RP4 λ<u>att</u>) and Raf8

(<u>Pseudomonas</u> <u>rif</u>^{r}/RP4 λ<u>att</u>) were prepared by the inoculation of 30 mls of L-broth, in a

side-armed flask, with <u>Pseudomonas</u> strain from glucose minimal media containing carbenicillin

(100 µg/ml) and kanamycin (25 µg/ml) for Raf3 and Raf4 and in addition rifampicin (100 µg/ml)

for Raf8.

The log-phase cultures were counted for the number of bacterial cells/ml at 20 minute

intervals by the Klett spectrophotometer. Rifampicin (100 µg/ml) was added after 40 mins

and immediate spectrophotometer reading was taken followed by the subsequent reading at

20 minute intervals.
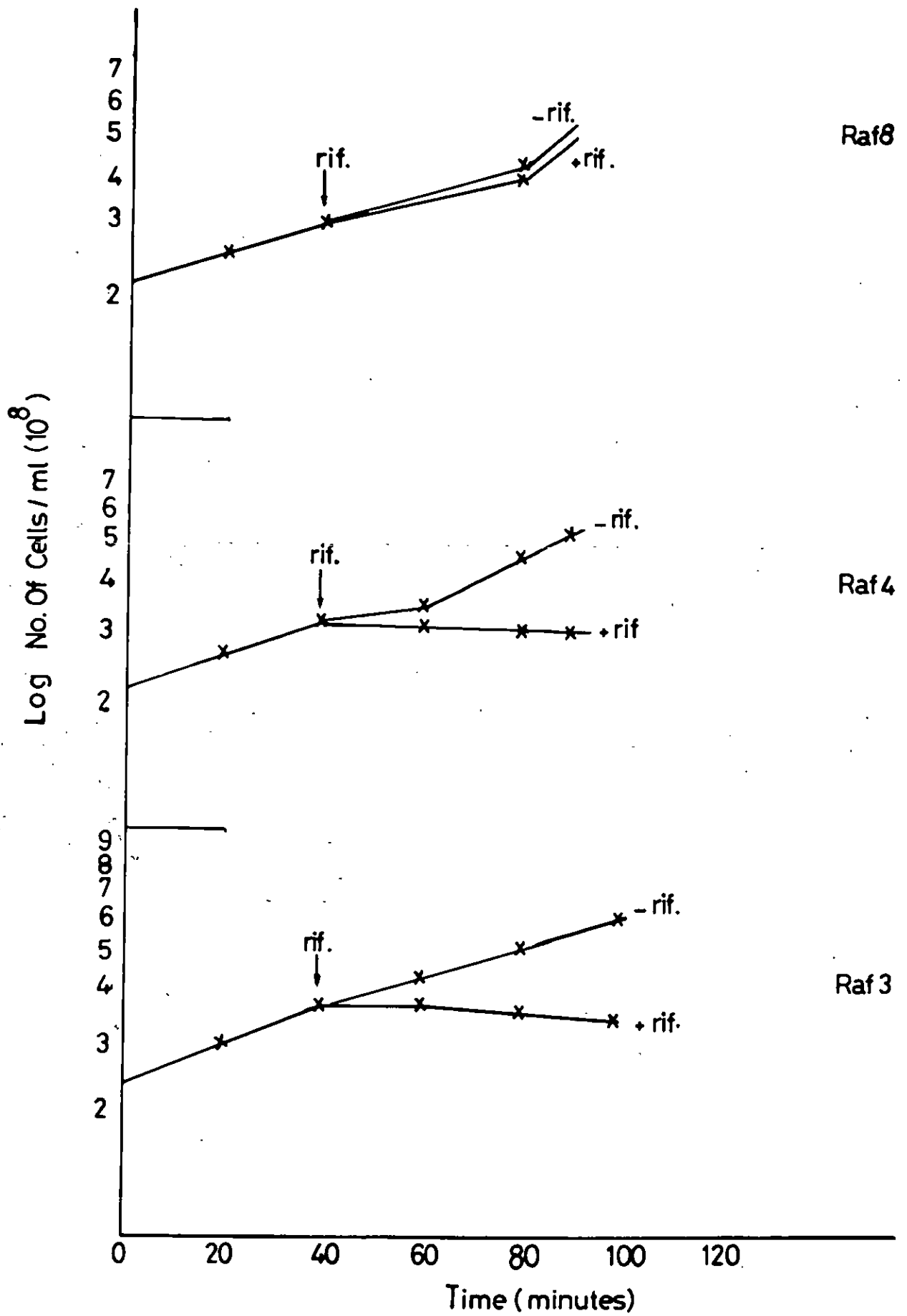
serves as a control and its growth is not inhibited by the rifampicin.

ii. Rhizobium leguminosarum

Like Pseudomonas, this organism shows no difference between hybrids with (ZDR19 (Rhizobium/pZD100)) and without (ZDR17 (Rhizobium/RP4 λatt)) the rpoB3 gene. They are both inhibited by the rifampicin (25 μg/ml) as shown in Fig. 13. It is worth noting that in contrast to Pseudomonas, rifampicin only affects growth of Rhizobium after a lag period of about four hours (Fig. 13). A rifampicin resistant mutant (Fig. 13) ZDR18 (Rhizobium rif^r/RP4 λatt) is not affected by rifampicin.

It is concluded that pZD100 hybrids of Pseudomonas and Rhizobium behave in the same way as the RP4 λatt hosts which do not carry the E.coli rpoB3 gene.

5. Transfer of pZD23 and pZD100 from the g(-ve) hybrids into E.coli

This experiment was conducted mainly to understand the rifampicin sensitive phenotype of pZD23 and pZD100 hybrids of the g(-ve) bacteria. Are they rifampicin sensitive due to the loss of the E.coli rpoB3 gene or due to the inability of this gene to express or to interact in their cells. In addition, it allows the detection of the modification of these plasmids in the hybrid cell. For this, the plasmids pZD23 and pZD100 were transferred from the hybrids into E.coli; the resistance of E.coli transconjugants to rifampicin is evidence for the maintenance of the rpoB3 gene on the plasmid.

Legend to Fig. 13

This illustrates the effect of the rifampicin on the growing cultures of Rhizobia.

The method is as described in the legend to Fig. 12 except that TY broth cultures were used, the spectrophotometer reading was taken at an interval of one hour, and rifampicin (25 μg/ml) was added after four hours.

(*) means that the spectrophotometer reading is the same for the two cultures, with or without the rifampicin.

From this figure it appears that the generation time for Rhizobium is 12.5 hrs. This was calculated by the application of the following equation:

No. of cells at time t = No. of cells at time t_o x $2^{(t/\zeta)}$

where ζ is the generation time.
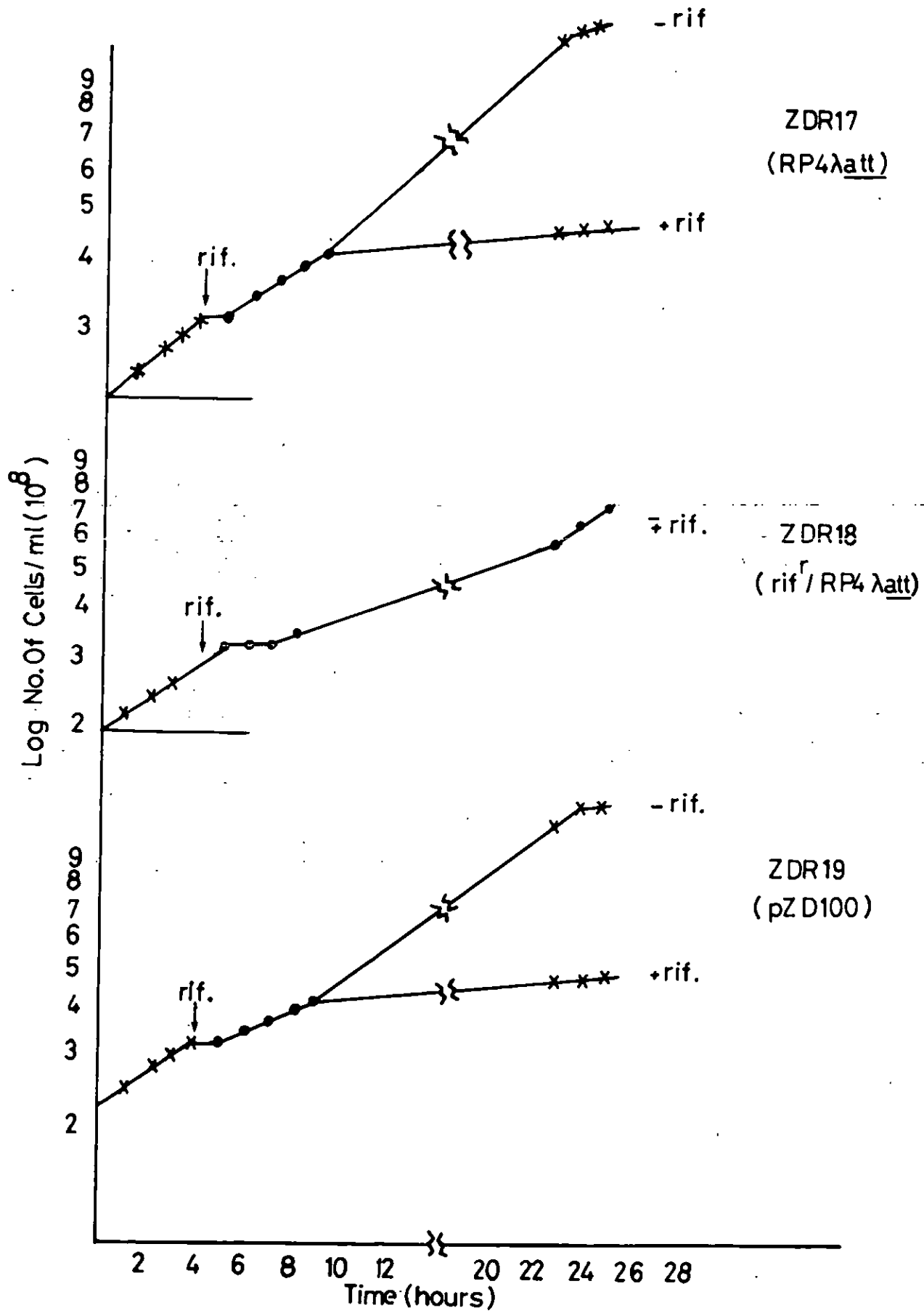
a. Transfer of the plasmids from the Enterobacteriaceae
     hybrids into E.coli

All the Enterobacteriaceae matings (Table 18) were
carried out using the E.coli λ-lysogen recipient ZD162
(metB B_1^- recA56 str-r λ^+). Various types of mating
techniques were employed which will be described later
(see Methods; legend to Table 18). The first selection
was for the transfer of the drug resistant determinants
of the plasmid RP4 which are carried by pZD23 and pZD100.
E.coli transconjugants were tested, then, for their
resistance to rifampicin.

For the transfer of pZD23 from the various hybrids
into E.coli, the Millipore filters (loaded with the mating
mixture) were incubated for two days except for
C.freundii and S.typhimurium matings there the incubation
time was 24 hours. In addition, spot mating technique
was applied for pZD23 hybrids of Proteus. However, for
pZD100 transfer, the Millipore filters were incubated for
three days except C.freundii and S.typhimurium matings
where the incubation time was one day.

All the Enterobacteriaceae hybrids were able to
transfer   pZD23 and pZD44 (Table 18) back to E.coli
except S.marcescens. In other words, neither pZD23 nor
pZD100 have transferred back from S.marcescens into E.coli
despite changes of the incubation time of the mating
mixture for one, two or three days (Millipore mating) or
even shorter incubation time of the mating mixture for
five hours only (liquid culture mating); this will be
discussed later.

Legend to Table 18. The attempted transfer of the plasmids pZD23 and pZD100 from the gram-negative bacteria back into an E.coli recipient. The first selection was for the transfer of RP4 markers of these plasmids then the transconjugants were checked for their resistance to the rifampicin as a test for the maintenance of rpoB3 (rif^D) gene on the plasmids.

RP4 markers have transferred back (+) from all the bacterial species except from Serratia (-).

rpoB3 (rif^D) has not transferred back from either Serratia or the Rhizobium species.

Various mating techniques were employed which were described in the methods:

  * Spot mating.

 ** Both spot mating and millipore filter mating.

*** Liquid culture mating.

The selective plates for transconjugants were glucose minimal media supplemented with methionine, thiamine, streptomycin (200 μg/ml) and either kanamycin (for pZD23 transfer) or both of ampicillin and kanamycin. Selective plates for the transconjugants of the transfer back of pZD100 from Raf3 (P.aeruginosa/pZD100) into J62 (Pro$^-$ trp$^-$ his$^-$ str-r λ$^+$) contained proline, tryptophan and histidine as well as the antibiotic streptomycin (200 μg/ml), ampicillin and kanamycin.

The incubation temperature for all the matings was 30°C except for R.lequminosarum matings where the incubation temperature was 28°C.

Table 18. Transfer of pZD23 and pZD100 from the g(-ve) hybrids into E.coli.

Bacterial donors of pZD100 or pZD23	Transfer of RP4 markers of pZD23 or pZD100	% rifampicin resistant transconjugants	
		pZD100	pZD23
Escherichia coli (λ^+)	+	100***	100***
Enterobacter aerogenes	+		100
Citrobacter freundii	+	90	100
Hafnia alvei møller	+	14	100
Klebsiella pneumoniae	+	10	100
Proteus morganii	+		100*
Proteus vulgaris	+		100*
Serratia marcescens	-	0	0
Salmonella typhimurium	+	55**	100
Rhizobium leguminosarum	+	0	0*
Pseudomonas aeruginosa	+	100*	
Azotobacter vinelandii	+	100**	100*

It is noteworthy that pZD23 has transferred into E.coli ZD162 (metB B_1^- recA56 str-r λ^+) from the rifampicin sensitive P.morganii and P.vulgaris hybrids converting E.coli into the rifampicin resistant phenotype.

b. Transfer of the plasmids from the non-Enterobacteriaceae hybrids into E.coli

E.coli ZD162 (metB B_1^- recA56 str-r λ^+) was the recipient in all the matings of the non-Enterobacteriaceae hybrids except P.aeruginosa. Raf2 (P.aeruginosa/pZD100) was spot-mated with J62 (Pro⁻ trp⁻ his⁻ str-r λ^+) recipient. The mating technique used for each of the non-Enterobacteriaceae matings is indicated in Table 18. The selection for the transconjugants was exactly as described in section (a). All Millipore filters were incubated for two days. In addition to Millipore matings, spot-matings were carried out (see Methods) when many donors for a particular mating were employed. In the case of A.vinelandii, ten donors of the rifampicin sensitive ZAV23 (A.vinelandii/pZD100) and another ten donors of the rifampicin resistant ZAV21 (A.vinelandii rif^r/ pZD100) were spot-mated with ZD162 (metB B_1^- recA56 str-r λ^+) as described in the Methods. However, only five Raf3 (P.aeruginosa/pZD100) were spot-mated with the J62 (Pro⁻ trp⁻ his⁻ str-r λ^+) recipient for the transfer of pZD100 back into E.coli.

In addition, spot-mating was applied for the transfer back of pZD23 from Rhizobium leguminosarum. Hybrids of R.leguminosarum such as ZDR19 (phe1/trp12 str37/pZD100)

and ZD R21 (<u>phe</u>1 <u>trp</u>12 <u>str</u>37 /pZD23) have transferred back
only the RP4 drug resistant determinants but not the
<u>rif</u>^{D} phenotype.

Another attempt for the transfer back of <u>rpo</u>B3
(<u>rif</u>^{D}) to <u>E.coli</u> was from the few rifampicin resistant
<u>R.leguminosarum</u> harbouring the plasmid pZD23 and the few
rifampicin resistant <u>A.vinelandii</u> harbouring either pZD100
or pZD23. As mentioned earlier, a spot-mating technique
was carried out. Ten L-broth cultures of each of the
following donors were prepared from the rifampicin
resistant <u>A.vinelandii</u>/pZD100 or ZAV21, the rifampicin
sensitive <u>A.vinelandii</u>/pZD100 or ZAV23 and the rifampicin
sensitive <u>A.vinelandii</u>/pZD23 or ZAV24. TY broth cultures
(see Media) were prepared from the rifampicin sensitive
and the rifampicin resistant <u>R.leguminosarum</u>/pZD23 donors.
After 24 hours incubation of the mating mixture at 30°C
and 28°C for the <u>Azotobacter</u> and the <u>Rhizobium</u> matings
respectively, <u>E.coli</u> transconjugants were streaked out of
each spot onto two fresly prepared selective plates; one
of them containing rifampicin.

None of the <u>E.coli</u> transconjugants of ZDR21 (<u>phe</u>1
<u>trp</u>12 <u>str</u>37/pZD23) were rifampicin resistant; all <u>E.coli</u>
transconjugants of ZAV21 (<u>A.vinelandii</u> <u>rif</u>^{r}/pZD100),
ZAV23 (<u>A.vinelandii</u>/pZD100) and ZAV24 (<u>A.vinelandii</u>/pZD23)
were resistant to rifampicin.

This had led to the conclusion of the existence of a
third possibility for the expression of <u>E.coli</u> <u>rpo</u>B3 (<u>rif</u>^{D})
in the alien cell (see Fig, 14) for different ways of
explaining these results).

Legend to Fig. 14

There are four different possibilities for the explanation of the phenotype of the transconjugants which have received one of the plasmids which harbours $\underline{E.coli}$ $\underline{rpoB}3$ ($\underline{rif}^D$).

I.   The presence of the plasmid has not altered the rifampicin sensitive phenotype into the rifampicin resistant; this indicates any of the following:

     1.   The inability of the host to express $\underline{E.coli}$ $\underline{rif}^D$.

     2.   The loss of the $\underline{E.coli}$ $\underline{rpoB}3$ ($\underline{rif}^D$) gene.

     3.   The cessation of the dominance of the rifampicin resistant allele over the rifampicin sensitive chromosomal allele.

II.   The possession of the $\underline{rpoB}3$ ($\underline{rif}^D$) plasmid has converted the recipient into the rifampicin resistant phenotype.

III & The rifampicin resistant phenotype of the transconjugants may be due to the presence
IV   of a spontaneous chromosomal rifampicin resistant mutation only (III) or due to both, the chromosomal $\underline{rif}^r$ mutation and the expression of the $\underline{E.coli}$ $\underline{rpoB}3$ ($\underline{rif}^D$) present on the plasmid (IV).

Phenotype

I.

chromosome — plasmid ×rif^d — rifampicin sensitive

rif^s×

II.

chromosome — plasmid ×rif^d — rifampicin resistant

rif^s×

III & IV.

chromosome — plasmid ×rif^d — rifampicin resistant

rif^r×

chromosomal   chromosomal
                     and
                     plasmid
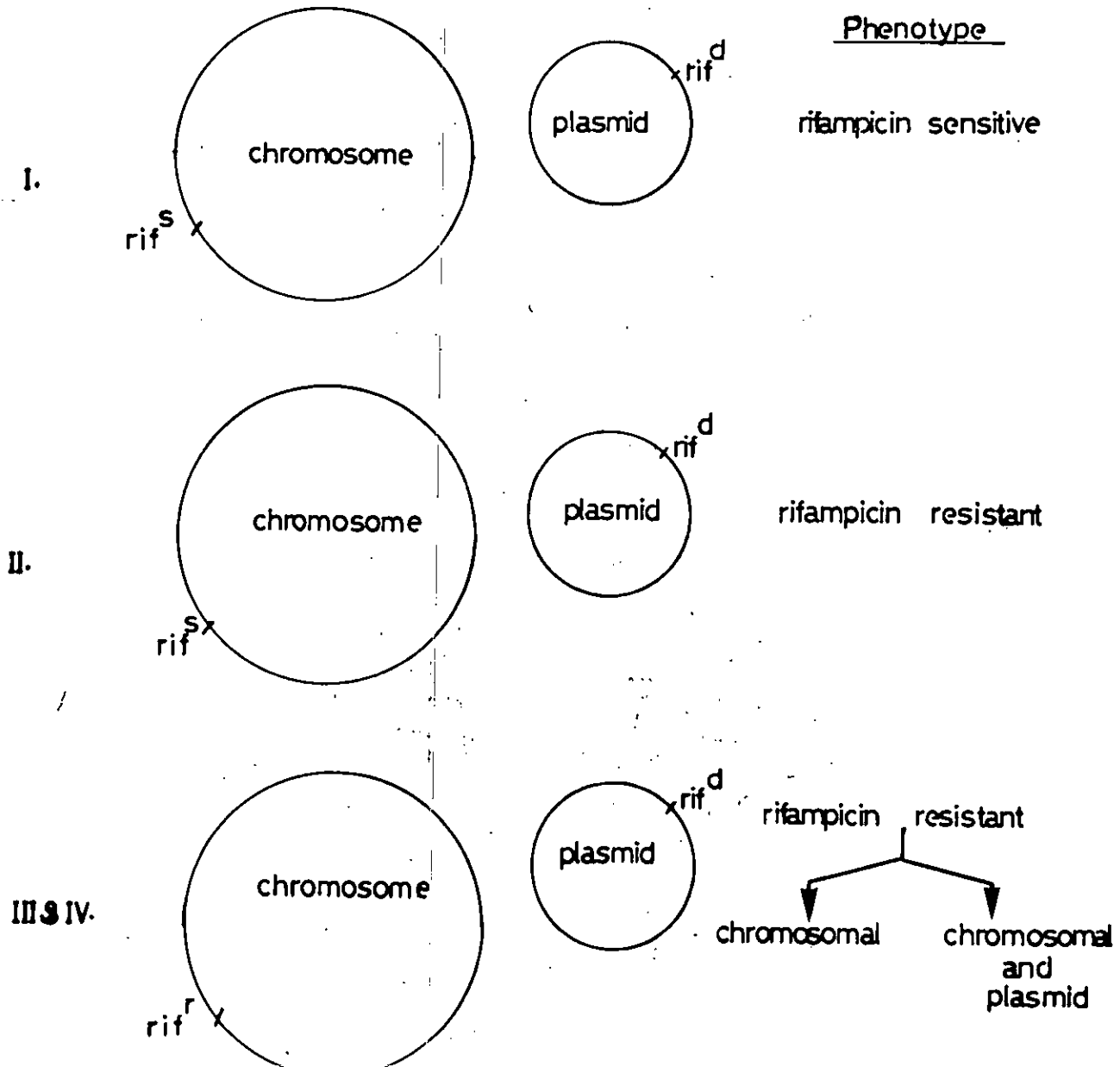
6. The distinction between the rifampicin resistant pZD23
hybrids and the spontaneous resistant ones

For the distinction between the various rifampicin
resistant hybrids which have received one of the plasmids,
pZD23, and between the spontaneous rifampicin resistant
mutants, the following incompatibility experiment was
conducted.   It was carried out to prove that the
rifampicin resistant bacterial species are illustrated
in Part II of Fig. 14 and not Part III.

Two matings were carried out for each of the bacterial
species which are listed in Table 19; the plasmid RP4 λatt
has been transferred from ZD28 (F⁻ his⁻ trpA9761 argE171
recA56/RP4 λatt) into the bacterial species and its
rifampicin resistant derivative which presumably harbours
the plasmid pZD23.   The reduction in the transfer of
RP4 λatt into the pZD23 host and the reduction in the
percentage of the rifampicin resistant transconjugants will
indicate, firstly, that the recipient does harbour pZD23
and secondly, that its rifampicin resistance is not due
to a chromosomal spontaneous rifampicin resistant mutation.

Liquid culture matings were carried out between the
donor ZD28 (F⁻ his⁻ trpA9761 argE171 recA56/RP4 λatt) and
the following recipients: Hafnia alvei møller, ZDH10
(Hafnia/pZD23), Klebsiella pneumoniae, ZDK105 (Klebsiella/
pZD23), AW1 (metB B₁⁻ recA56 str-s λ^S), ZD136 (metB B₁⁻
recA56 str-s λ^S/pZD23), Citrobacter freundii, ZDC104
(Citrobacter/pZD23), Serratia marcescens, ZDS104 (Serratia/
pZD23?).   The transfer of RP4 λatt from ZD28

(F$^-$ his$^-$ trpA9761 argE171 recA56/RP4 λatt) into Salmonella
typhimurium and ZST10 (Salmonella arg$^-$/pZD23) was carried
out by Millipore filter mating (see Methods) for 24 hours.
The incubation time for the matings of Hafnia, Klebsiella
and their derivatives was eight hours whereas the
incubation for the matings of Citrobacter, Serratia and
their derivatives was ten hours.   E.coli incubation time
of the mating mixtures was three hours.

Table 19 compares the frequencies of transfer (per
donor) of RP4 λatt into a particular bacterial species
with its transfer into a pZD23 hybrid of the same species.
It is shown (Table 19) that there is a reduction in the
frequency of RP4 λatt transfer to the rifampicin resistant
pZD23 host.   However, there is no difference between the
frequency of transfer of RP4 λatt into Citrobacter freundii
and ZD104 (Citrobacter/pZD23).

The transconjugants of all the matings of Table 19
were tested, as described earlier, for their resistance
to the rifampicin.   Only 8% of the transconjugants of
ZST10 (Salmonella arg$^-$/pZD23) and 6% of ZDC104 (Citrobacter/
pZD23) transconjugants were resistant to rifampicin.   The
test transconjugants of ZDK105 (Klebsiella/pZD23), ZDH10
(Hafnia/pZD23) and ZD136 (metB B$_1^-$ recA56 str-s λ^s/pZD23)
were rifampicin sensitive.

It is concluded that the transfer of the plasmid
RP4 λatt into the bacterial species harbouring pZD23 is
reduced in comparison with its transfer to the same species
lacking the plasmid pZD23.   In addition, the transfer of

Legend to Table 19

The incompatibility of pZD23 with RP4 λatt is shown by the comparison of the transfer of

RP4 λatt into the bacterial species with its transfer into the pZD23 hybrid of the same

species.   This experiment is to prove that the rifampicin resistant pZD23 hybrids of

the various bacteria are not spontaneous rifampicin resistant mutants;   the transfer of

RP4 λatt has resulted in the loss of rif^D phenotype from most of the transconjugants.

Although the table illustrates the reduction of the transfer of RP4 λatt to the rifampicin

resistant pZD23 carrier, C.freundii shows no such reduction;   however 6% of its

transconjugants are rifampicin resistant (*).

** the mating technique was Millipore filter mating (see Methods).

The incubation temperature for all the matings was 30°C except E.coli matings whose incubation

temperature was 37°C.   The selective plates for transconjugants were glucose minimal media

containing carbenicillin and the growth requirements for the auxotrophs such as methionine

and thiamine for E.coli transconjugants and arginine for Salmonella transconjugants.

The absence of growth of the donor and the recipients on the selective plates as well as

testing the antibiotic resistances of the donor and recipients (see Methods) served as

control.

Table 19. The distinction between the rifampicin resistant pZD23 hybrids and the spontaneous resistant ones.

Bacterial species	The frequencies of transfer of RP4 λatt per donor		
	Transfer to bacterial species	Transfer to rifampicin resistant pZD23 hybrids	Fold difference in the frequencies of the transfer to both recipients
Escherichia coli	89%	2.8×10^{-4}	3×10^{3}
Citrobacter freundii	5.8×10^{-3}	$7.1 \times 10^{-3}*$	0.8
Hafnia alvei møller	1.3×10^{-4}	2.1×10^{-6}	62
Klebsiella pneumoniae	4.8×10^{-5}	3.0×10^{-6}	16
Serratia marcescens	1.7×10^{-5}	6.5×10^{-6}	2.6
Salmonella typhimurium	$3.5 \times 10^{-4}**$	$9.4 \times 10^{-6}**$	37

RP4 λatt results in the loss of the rif^D phenotype from some if not all the transconjugants.

c. Discussion

I have chosen a total of twelve bacteria genera which belong to four families, the Enterobacteriaceae, Pseudomonadaceae, Rhizobiaceae and Azotobacteriaceae. The bacterial species are Escherichia coli, Enterobacter aerogenes, Citrobacter freundii, Hafnia alvei møller, Klebsiella pneumoniae, Proteus morganii, Proteus vulgaris, Salmonella typhimurium, Serratia marcescens, Rhizobium leguminosarum, Pseudomonas aeruginosa and Azotobacter vinelandii. They have a wide range of G + C content of their DNA and they represent widely divergent evolutionary branches of the bacterial phylogenic tree.

1. Representatives of the Enterobacteriaceae

    i. General view

E.coli rpoB3 (rif^D) was transferred into these bacterial species via pZD23 and pZD100. All members of the Enterobacteriaceae (except Serratia) have become resistant to the rifampicin after receiving E.coli rpoB3 gene; whereas none of the bacterial species belonging to the other families has changed their rifampicin sensitive phenotype.

The morphology of the colonies of the bacterial hybrids particularly the size, seems to be similar to E.coli but H.alvei møller cells which received pZD23 have grown at 37°C into very small sized colonies. However, they grow into normal size when transferred to 30°C. This

cannot be due to the presence of pZD23 in the cell because
Hafnia itself doesn't grow into normal size on glucose
minimal medium at 37°C whereas on LB it does.

However, the acquisition of the rifampicin resistance
phenotype of these bacterial species reflects their close
relatedness to E.coli.   The linkage maps of Klebsiella
pneumoniae, Citrobacter freundii and Salmonella
typhimurium though they differ in the number of the mapped
genes, they are strikingly similar to E.coli;   for this
reason it has been concluded that the linkage map is a
conserved character that is unaltered in the
Enterobacteriaceae family (Sanderson, 1976).

Klebsiella is the only member of the Enterobacteriaceae
that contains the nitrogen fixation gene, nif (Riley and
Anilionis, 1978).   When the nif gene and the linked genes of
ribitol and arabitol metabolism of Klebsiella are
introduced via plasmids or transduction they integrate
into their analogous positions near his (Riley and
Anilionis, 1978).

ii.   Serratia marcescens

Among the tested members of the Enterobacteriaceae,
only Serratia marcescens was sensitive to rifampicin after
introducing pZD100 or pZD23 into it.   It
has been claimed that the divergence of Serratia from E.coli,
in the evolutionary process, is much more than Enterobacter,
Klebsiella or Citrobacter (Grimont and Grimont, 1978) and
that it is more distantly related to E.coli than Citrobacter,
Salmonella, Klebsiella pneumoniae and Enterobacter aerogenes

(Grimont and Grimont, 1978). It is not clear that pZD100 and pZD23 exist as independent extrachromosomal elements because neither of them was able to transfer back from Serratia to E.coli; if they are integrated into the chromosome then this integration will also reflect a close relatedness to E.coli as the integrated recombinants are obtained in crosses of closely related genera (Sanderson, 1976). However, it has been pointed out that plasmid transfer from Serratia into E.coli is often inefficient (Grimont and Grimont, 1978) and that the P plasmids are transferred with difficulty, though the data are not shown (Grimont and Grimont, 1978). In fact, another derivative of RP4 namely RP4::Mu was introduced into Serratia marcescens (Faelen et al, 1977) but there is no available information as to whether it can be transferred out of it.

There exists the possibility that the presence of extrachromosomal elements in Serratia such as R factors belonging to S and L compatibility groups (Hedges et al, 1975; Grimont and Grimont, 1978) code for the production of the bacteriocin marcescin A (Prinsloo, 1966; Timmis and Winkler, 1973). One cannot exclude the possibility of the existence of an R factor in the Serratia species used for this study as it is naturally resistant to ampicillin and tetracycline. R factors conferring the resistance to ampicillin and to tetracycline are found in Serratia marcescens (Hedges et al, 1975). The presence of the antibiotic resistance determinants on the residing R factor and the E.coli rpoB3 plasmid may allow the

formation of a hybrid non-transmissible plasmid; a hybrid as a result of molecular interaction with the residing bacteriocin plasmid which codes for the marcescin A may also be formed. However, the marcescin A plasmid is <u>tra</u>⁻ and all attempts to transfer it into another <u>S.marcescens</u> or into <u>E.coli</u> have failed in the presence or the absence of the "helper" derepressed R1-19 R factor (Timmis and Winkler, 1973). In this case the failure of the transfer back of both pZD100 and pZD23 is not surprising. Finally, <u>tra</u> genes of these plasmids may not be expressed in this species.

In addition to the bacteriocins, <u>S.marcescens</u> is known to produce exocellular nucleases (Winkler and Timmis, 1973) which may kill the <u>E.coli</u> recipient cells and prevent the selection of the transferred back transconjugants; this should not be the case because it was possible to conduct the mating experiments between the <u>E.coli</u> donors and the <u>Serratia</u> recipient earlier.

failure of the transfer back of pZD100 into E.coli is due to residing plasmid

<u>Serratia</u> <u>marcescens</u> differs from <u>E.coli</u> in many other respects, in addition to the previously mentioned ones (see Table 16), for example the G + C content of the <u>Serratia</u> is 60% which is higher than the 50% range of the G + C content of <u>E.coli</u>. Differences are occuring between the <u>Serratia</u> and <u>E.coli</u> in the amino acid sequences, molecular weight and regulation of several enzymes such as asparaginase, homoserine dehydrogenase and the enzymes

involved in the tryptophan biosynthesis (Konze-Thomas and Rüger, 1976). There are some differences in the gene order between E.coli and S.marcescens for example the four structural genes argE, argC, argB and argH map in this order in E.coli, whereas in S.marcescens, argG is located between argB and argH (Sanderson, 1976; Grimont and Grimont, 1978). Furthermore, Dekio et al (1970) found that at least nine 30S and nine 50S ribosomal proteins can be distinguished between E.coli and Serratia on columns of carboxymethyl-cellulose.

However, the comparative studies, carried out in vitro, between the RNA polymerases of Serratia and E.coli has revealed the similarity in their molecular structure (Fukuda et al, 1977; Konze-Thomas and Rüger, 1976). The in vitro analysis of the subunits of RNA polymerase of S.marcescens has revealed that they have the same migration as the subunits of the E.coli RNA polymerase during acrylamide electrophoresis indicating the similarity of their molecular weights (Konze-Thomas and Rüger, 1976); they found that the replacement of the $\beta'_{Serratia}$ by the $\beta'_{E.coli}$ in the reconstitution experiment has yielded 85% of the activity of the homologous Serratia reconstituates. In addition, β subunit of RNA polymerase of E.coli and Serratia can fully substitute each other. The replacement of $\beta_{Serratia}$ with $\beta_{E.coli}$ has yielded 96% of the activity of the homologous Serratia reconstituates and the replacement of $\beta_{E.coli}$ with $\beta_{Serratia}$ has yielded 92% of the activity of the E.coli homologous reconstituates.

Konze-Thomas and Rüger (1976) have concluded that there is no decisive difference, in vitro, between the RNA polymerase of E.coli and Serratia marcescens.

In an earlier work on the genetics of the ribosomal proteins, Sypherd and Osawa (1974) have introduced an E.coli episome JCH13, which carries the genes for the 30S and 50S ribosomal proteins, into Serratia marcescens and by applying the chromatographic analysis they have observed the presence of the ribosomal proteins of both species in the hybrid cells.

The phenotypic differences between Serratia marcescens, ZDS100 (Serratia/RP4 λatt), ZDS103 (Serratia/pZD100) and ZDS104 (Serratia/pZD23) are the resistance of ZDS100 and ZDS103 to carbenicillin (100 µg/ml) and kanamycin (25 µg/ml) and the resistance of ZDS104 to kanamycin (25 µg/ml). The decision as to whether ZDS104 (Serratia/pZD23) is a spontaneous kanamycin resistant mutant will depend on how reproducible the results of the incompatibility experiment are (see Table 19). However, the lack of reduction in the efficiency of transfer of RP4 λatt into a Citrobacter hybrid carrying pZD23 indicates that the RP4 inc gene(s) are not expressed in Citrobacter. In addition, Sakanyan et al (1978) has shown that these genes map in the region between 2.1-9.8 kb either between Ap^r and EcoRI site or between Ap^r and Tc^r. Since pZD23 expressed incompatibility then it is possible to conclude that inc gene(s) is located between Ap^r and Tc^r genes of RP4. Furthermore ZDS103 and ZDS104 have overproduced the red pigment, prodigiosin

at low concentrations of rifampicin which can be visualised
by the appearance of a red ring around the rifampicin
disc of the Antibiotic Multodisk; the position of this
ring is at lower concentration of the rifampicin than
the inhibitory concentration.

The red ring had not appeared in the case of
S.marcescens or ZDS100 (Serratia/RP4 λatt) implying that
the rifampicin does not induce the synthesis of the
prodigiosin by itself. It is possible to explain this
phenomenon on the basis that the presence of the E.coli
β and β' subunits results in overproduction of the
prodigiosin in the presence of low concentration of
rifampicin.

The rate of prodigiosin synthesis is increased in the
presence of the alanine, proline and histidine amino acids
(Williams et al, 1976) as the proline is incorporated
intact, all the alanine (except the carboxyl group)
whereas the histidine does not enter the prodigiosin
directly (Lim et al, 1977). The rate of the protein
synthesis is also increased (Williams et al, 1976);
whereas in the presence of the amino acids which does not
stimulate the prodigiosin synthesis, the rate of
protein synthesis has not increased.

It is known, in E.coli, that the rate of β and β'
synthesis is increased in the presence of low effective
concentrations of rifampicin (Hayward et al, 1973; Bass
et al, 1979); it is possible that it is increased also
in Serratia marcescens and in this case either rif^D is not
dominant over Serratia rif^S allele or it is not possible

for E.coli $\beta\beta'$ subunits to assemble with the Serratia RNA polymerase subunits.

The other alternative that the ZDS103 (Serratia/ pZD100) and ZDS104 (Serratia/pZD23) are spontaneous antibiotic resistant mutants cannot be considered because ZDS103 was selected in the presence of the carbenicillin (100 µg/ml) and the kanamycin (25 µg/ml) and the frequency of the spontaneous mutation to carbenicillin and kanamycin should be much lower than the frequency of the transfer of RP4 or its derivatives into it; ZDS104 was selected in the presence of kanamycin (25 µg/ml) only, however the frequency of transfer of pZD23 into it, is similar to the transfer of pZD100 (Table 17); the minimal inhibitory concentration of the kanamycin is 10 µg/ml (Winkler et al, 1978).

It is $\overbrace{\text{Possible}}$ to attribute the inexpression of E.coli rpoB3 (rif^D) in Serratia to be due to one of the two main reasons: First, that E.coli gene is subjected to the attack of the restriction mechanism of Serratia; that is not possible because there is a clear phenotypic difference between the Serratia harbouring pZD100 or pZD23 and the Serratia with or without RP4 λatt. Second, that the rif^D allele of E.coli is not $\bigcirc$ dominant over the rif^S allele of the Serratia. It has been suggested that may be rif^D is a complex mutation as it is unstable in a rec$^+$ background segregating rif^r alleles that are no longer dominant over the rif^S allele (Scaife and Linn, personal communication).

Nevertheless, there is no good agreement between the current taxonomy (Buchanan et al, 1974) and my results on Serratia. Although Buchanan et al (1974) have pointed out that there are many suggestions to combine one or more of the genera Klebsiella, Enterobacter, Hafnia and Serratia in the near future, I belive that Serratia stands quite distinctly from the others.

iii. Salmonella typhimurium

Salmonella species is genetically very similar to E.coli and it has been found that the gene arrangement on its chromosome is remarkably similar to that of E.coli (Sanderson and Demereck, 1965; Sanderson, 1972; Sanderson and Hartman, 1978). In fact, the linkage maps of Salmonella typhimurium, Citrobacter freundii and Klebsiella pneumoniae are very similar to E.coli and to each other (Sanderson, 1976). Despite the similarity between E.coli and S.typhimurium in linkage maps there are some differences that have emerged from the comparison between the two maps (Riley and Anilionis, 1978); the comparison has revealed the existence of twelve loops ranging between 0.6-1.5 map units in length and reflecting the changes between the two species whether it is an addition or deletion of genetic material (Riley and Anilionis, 1978). However, it has been shown that at least four 30S and six 50S ribosomal proteins of S.typhimurium are distinguishable from E.coli by carboxymethyl-cellulose chromatography (Dekio et al, 1970).

There was an apparent difference in the behaviour of

the Salmonella receiving pZD100 and those which receive

pZD23. It has been found that ZST10 (Salmonella arg⁻/

pZD23) are resistant to ⬭ rifampicin (100 µg/ml)

whereas ZST11 (Salmonella arg⁻/pZD100) has not been

converted to the rifampicin resistant phenotype on

receiving pZD100; this cannot be attributed to the

restriction of the rpoB3 of pZD100 in Salmonella as it

was possible to transfer back rif^D into E.coli (see

Table 18). The most important fact is that the problem

doesn't seem to be in the recognition of the E.coli rpoB3

by Salmonella host neither in the dominance of the rif^D

allele over the rif^S allele of Salmonella. The major

structural difference between pZD23 and pZD100 is the

presence of the λ genome on the latter. The bacteriophage

λ when introduced into Salmonella is established as a

multicopy plasmid (Falkow and Baron, 1970; Falkow, 1975);

another difference in the behaviour of λ in Salmonella

typhimurium is the absence of zygotic induction of the

bacteriophage λ when transferred from E.coli into it

(Baron et al, 1970); it seems that this is not true in

the case of S.typhimurium. Thus the anomaly of the

expression of E.coli rpoB3 carried by pZD100 in Salmonella

could be explained to be due to the anomaly of the

behaviour of the bacteriophage λ. Nevertheless,

Salmonella typhimurium was able to express E.coli rpoB3

which was carried by pZD23 and this ⟨means⟩ that

Salmonella is closely related to E.coli as it seems to be

able to accommodate a foreign β subunit in its RNA

polymerase holoenzyme which may replace its own subunit without affecting its functioning.

. iv Proteus spp.

Proteus vulgaris is distinguished from Proteus morganii by its swarming ability; the swarming phenomenon of P.vulgaris has been investigated (Jones and Park, 1967; Williams and Schwarzhoff, 1978) and various methods for its prevention has been proposed (Hayward and Miles,, 1943; Sandys, 1960; Cruickshank et al, 1975; Hayward et al, 1978; Williams and Schwarzhoff, 1978).   The two species of Proteus, namely P.vulgaris and P.morganii vary with respect to the guanine + cytosine percentage of their DNA which is found to be about 40% and about 50% respectively (Hill, 1966; Buchanan et al, 1974).   Despite the differences between the two species of Proteus they behave in a rather similar way on receiving the plasmid pZD23. They become resistant to the rifampicin (100 µg/ml) due to the presence of the E.coli rif^D β subunit irrespective of the difference in their G + C content.   Two colonial sizes of the Proteus hybrids were observed in the presence
growing
of the rifampicin.   The majority are poorly colonies (i.e. small size colonies) and can be ascribed to a number of causes such as poor synthesis of β subunit, breakdown of β subunit, poor assembly and poor function of RNA polymerase.   The minority exhibited the normal colony size. In fact, RNA polymerase of P.mirabilis seems to have
the
similar structure of $\alpha_2\beta\beta'\sigma$ to E.coli but σ subunit is larger in size than the σ subunit of E.coli and more

acidic in charge and _the_ α subunit is more basic than the

corresponding E.coli subunit (Fukuda et al, 1977). This

variation in the RNA polymerase subunits of P.mirabilis

may be true for the P.morganii and P.vulgaris RNA

polymerases and may explain the low expression of the

E.coli rpoB3 gene in Proteus

However, this is not the first attempt _at_ investigating

E.coli gene expression in Proteus _and_ there are many

examples which demonstrate the low expression of

E.coli genes in this bacterium. lac genes of E.coli were

introduced into P.mirabilis by an episome F-lac (Falkow

et al, 1964) and a reduction in the differential rate of

the β-galactosidase synthesis was noticed;   it is

interesting that Falkow et al (1964) have also observed

two colonial sizes among the lac$^+$ exconjugants.   More

recently, Roberts and Baumberg (1978) have introduced the

E.coli lac genes via the plasmid pGC91.14 which carries

the transposon Tn951 into P.mirabilis and compared the

rate of the β-galactosidase synthesis with that of the

Proteus harbouring F-lac;   they found that the β-galactosidase

and the induction ratio of both F-lac and pGC91.14 are much

lower in Proteus than in E.coli.   This had led to the

conclusion that the transcription and translation

machineries recognise the E.coli exogenous system with a

varying degree of inefficiency.   However, this conclusion

is in good agreement with the earlier hypothesis of Dale

and Smith (1971) that the inefficient expression of the

foreign DNA in Proteus is due to the inability of its

sigma subunit to recognise the initiation site on

foreign DNA such as F-lac. It is possible to accept this

hypothesis because, as mentioned earlier, it has been

shown that the σ subunit of Proteus is different from

that of E.coli as it is larger in size and much more

acidic in charge (Fukuda et al, 1977).

The low activity of the β-lactamase which is produced

by R1818 in Proteus mirabilis when compared with E.coli

offers another example of the anomalous expression of the

foreign DNA in Proteus (Smith, 1969). The repression

of β-galactosidase synthesis in E.coli by galactose

becomes induction in P.mirabilis F-lac$^+$ (Colby et al, 1968).

The anomalous synthesis of the β-galactosidase in

P.mirabilis was also observed by Colby and Hu (1968);

the relative inefficiencies of the various inducers was

found to be different from those in E.coli. In addition

to the lactose system and the β-lactamase, a third example

of anomalous expression of foreign DNA in Proteus has been

described (Baumberg and Dennison, 1975). It is concerned

with the expression of the F-like R factors R1 and its

derepressed derivatives R1-16 (o^c) and R1-19 (i$^-$) in

Proteus mirabilis; piliation of Proteus cells harbouring

the derepressed plasmids is expressed at lower level in

E.coli and no repression in R1 harbouring cells.

Nevertheless, an intermediate level of repression was

found in R1 carrying cells of P.morganii (Baumberg and

Dennison, 1975).

The failure of the transfer of the I-like R factor,

ColI, into the Proteus group was explained to be possibly
due to the inability of the Proteus to recognise perhaps
one or more operons of the plasmid (Datta and Hedges, 1972).

The genetics of Proteus has been reviewed (Coetzee,
1972) and the chromosomal map is established (Coetzee,
1978; Coetzee, 1979). Although Proteus differs from
other members of the Enterobacteriaceae in gene arrange-
ment (Sanderson, 1976; Coetzee, 1979) there are other
criteria which seems to be conserved in Proteus such as
the 16S ribosomal RNA (Fellner, 1974) which showed the
strongest heterologous binding to the S4 ribosomal protein
of E.coli (Daya-Grosjean et al 1973) and the tryptophan
synthetase system (McQuade and Creighton 1970; Reyes and
Rocha, 1977).

2. Representiaves of the non-Enterobacteriaceae

It is demonstrated (Table 17) that all the bacterial
species belonging to families other than the Entero-
bacteriaceae have failed to express E.coli rpoB3 (rif^D)
gene and these species are Rhizobium leguminosarum,
Pseudomonas aeruginosa and Azotobacter vinelandii.
However, these species are different in that some of them
were unable to transfer rpoB3 back to E.coli such as
Rhizobium (see Table 18). The species under this category
will be discussed separately as follows.

i. Rhizobium leguminosarum

Rhizobium leguminosarum does not resemble Serratia
with respect to its inability to be donor for the plasmids;
it was possible to transfer the RP4 part of the plasmids out

of Rhizobium into E.coli but not out of Serratia.   However,
the growth curve experiment (Fig. 13) does not distinguish
between the Rhizobium harbouring or lacking E.coli rpoB3.
Therefore the question here is stressed to the presence
or the absence of the E.coli gene in Rhizobium host before
asking whether the gene is expressed or not.

Recent studies provided physical evidence for the
presence of endogenous plasmids in R.leguminosarum
and other Rhizobia spp. which range in molecular weight
between 70-400 x 10^6 daltons (Nuti et al, 1977; Casse
et al, 1979).   Some of these plasmids determine the
production of the bacteriocin by the R.leguminosarum
(Hirsch, 1979) and the ability to nodulate peas (Johnston
et al, 1978).   Most interestingly, the plasmid RP4 has
been transferred into R.leguminosarum (Beringer, 1974)
and R.lupini (Tucher, 1974) and was found to interact with
the resident plasmids of Rhizobia (Johnston, A. 1980,
personal communication).   RP4 was found to be highly
unstable in R.lupini (Pühler and Burkardt, 1978) as
shortened RP4 has been detected in R.lupini minicells
(Tucher, 1974).   It is plausible to explain the loss of
the E.coli rpoB3 to be due to the interaction with the
various extrachromosomal elements which are harboured
by R.leguminosarum.   At the moment it is not easy to
decide whether the rpoB3 gene is restricted by the Rhizobium
restriction enzyme or whether it is not restricted but its
product is not more dominant over the rif^S allele.   The
distinction between the two cases could be clearly resolved

after the isolation of both plasmids pZD23 and pZD100

from E.coli which have received them from Rhizobia and

after comparing their structures with the unmodified

plasmids.

My attempt to transfer an E.coli piece of DNA into

Rhizobium was not the first; it has been reported

earlier that R.lupini can receive F'lac (Datta and

Hedges, 1972) although no further description has been

given of whether the E.coli lac genes are expressed

in Rhizobium or not or even how the selection for the

F'lac was carried out in Rhizobium. Further recent

investigation was carried out by Nagahari et al (1979)

as they have transferred a hybrid plasmid, RP4-trp,

which carries the whole E.coli tryptophan operon into

R.leguminosarum strains carrying mutations in different

trp genes; they have found that these trp mutations

were complemented by the presence of this plasmid. This

is a prominent example of heterospecific gene

expression of E.coli trp genes in Rhizobium where its

RNA polymerase's ability to recognise the E.coli promoters

is demonstrated and where the products are used by the

cell to complement the chromosomal trp mutations. However,

this is the answer of a different question from the one

that I have asked; this is a question of whether the

whole operon of one system can operate in an alien cell

but the demand in my experiment is higher as I have

asked that in addition to the recognition of the E.coli rpoBC genes

by the host RNA polymerase, I have asked for the

interaction of the foreign β subunit with the native

subunits of the host RNA polymerase and for the functioning

of the hybrid enzyme in the host.   Any block in one of

these steps could lead to the failure of the detection of

heterospecific gene expression.

ii.   Pseudomonas aeruginosa

The detection of the expression of the E.coli rpoB3

(rif^D) gene has failed in another species, outside the

Enterobacteriaceae, and that is Pseudomonas aeruginosa.

All the tested pZD100 hybrids were rifampicin sensitive

(5-100 μg/ml) but they were able to transfer rif^D pheno-

type back to E.coli (see Table 18).   The sensitivity of

pZD100 hybrids of P.aeruginosa to rifampicin could

therefore be due to the failure of its transcriptional

or translational machineries in the recognition of the

E.coli rpoB3 gene.   However, versatile carriers have been

used for the transfer of the E.coli trp operon into

P.aeruginosa trp mutants such as RP4-trp (Nagahari et al,

1977) and RSF1010-trp (Nagahari, 1978), on transfer of

these plasmids, the trp^- phenotype of Pseudomonas was

converted into the trp^+ phenotype and the expression of

E.coli trp was constitutive as the level of the tryptophan

synthesising enzymes was much higher than the wild type

(Nagahari et al, 1977; Nagahari, 1978).   This situation

is similar to the previously discussed Rhizobium example

to which the E.coli trp operon has been transferred

(Nagahari et al, 1979).

In P.aeruginosa the trp operon of E.coli was transferred

and tested for its expression and E.coli trp genes were
able to suppress Pseudomonas trp mutation but it was out
of the control of the Pseudomonas repression system.
In other words, P.aeruginosa RNA polymerase was able to
recognise the promoter for the E.coli trp operon and there
was no transcriptional or translational barrier.

In my experiment if the E.coli $\beta\beta'$ subunits of RNA
polymerase have been formed in Pseudomonas they may not
have been as stable as when they are produced in E.coli
but rather degrade rapidly. However, even if the $\beta\beta'$
subunits of E.coli were stable in Pseudomonas aeruginosa
the problem of the reassembly with the other subunits of
the enzyme RNA polymerase is probably not easily passed
over by the $\beta\beta'$ of E.coli in Pseudomonas.

The plasmid F110 (Low, 1972) mistakenly quoted to
carry the rpoA and the rpoB genes (Mergeay and Gerits, 1978)
harbours the chromosomal region between 85'-90' including
the rpoB and the rpoC genes (Low, 1972; Bachmann et al,
1976).   It was transferred from E.coli into the rifampicin
resistant Pseudomonas fluorescens mutants and the mero-
diploids for the rpoB gene became sensitive to the
rifampicin due to the dominance of the E.coli $rpoB^+$ (rif^S)
allele over the Pseudomonas rif^r allele (Mergeay and
Gerits, 1978).   However, this dominance was temporary
and the reappearance of the rifampicin resistant clones
was noticed.   The authors have interpreted the loss of
the dominance of the rif^S allele to be due to many reasons
such as the loss of the rif^S allele by deletion, the

reassociation of the Pseudomonas subunits conferring the resistance to the rifampicin or the modification in the regulation of the RNA polymerase; but no further comments about the stability of the newly synthesised β subunit of E.coli RNA polymerase in the alien cell. It is possible that the inability of the E.coli β subunit has allowed the reassociation of the rif^r allele and consequently the conversion of the rifampicin sensitive phenotype into the rifampicin resistant one.

However, Pseudomonas fluorescens and Pseudomonas aeruginosa are different in many respects. P.aeruginosa is pathogenic to man and can grow at high temperature of $41^\circ C$ whereas P.fluorescens is non-pathogenic and cannot grow at $41^\circ C$ (Cruickshank et al, 1975). P.aeruginosa genome size is larger (7×10^9 daltons) than P.fluorescens genome (5×10^9) (Holloway, 1975). Two tests can distinguish between P.aeruginosa and P.fluorescens to which the first is positive and the second is negative and these are the ethanol utilisation in ammonium salt sugars (ten days) and the Tween 80 hydrolysis (five days) (Cruickshank et al, 1975). The utilisation of Trehalose or mesoinositol as a sole carbon source is characteristic of P.fluorescens and not of P.aeruginosa. In contrast, Geraniol can be utilised by P.aeruginosa and not by P.fluorescens as a sole carbon source (Cruickshank et al, 1975). The genetics of P.aeruginosa has been extensively reviewed (Holloway, 1969; Holloway, 1975; Holloway and Krishnapillai, 1975; Holloway et al, 1979) but no

detailed information is available about the genetical structure of P.fluorescens; for this reason the genetical comparison of both species is not available. However, the different responses to the various microbiological tests of both species reflect their genetic variation.

Johnston et al (1971) have purified RNA polymerase of P.putida and found that the molecular weight of α (44,000) and σ (98,000) are higher than that of E.coli (Burgess, 1976) whereas β and β' subunits have the similar molecular weights of 155,000 and 165,000 respectively. If the molecular weights of these subunits corresponds to the molecular weights of P.aeruginosa subunits of RNA polymerase then this difference in the size of the α and the σ subunits may stand as barrier for the assembly of the β subunit of E.coli in the hybrid P.aeruginosa.

iii. Azotobacter vinelandii

The behaviour of Azotobacter vinelandii which received the plasmids pZD100 and pZD23 was similar to the behaviour of Pseudomonas in that it did not recognise E.coli rpoB3 gene and that it has transferred the plasmids pZD100 and pZD23 back into E.coli. However, Azotobacter trans-conjugants harbouring pZD23 like ZDH10 (Hafnia/pZD23) produced very small sized colonies at 37°C but not at 30°C; the possibility that this temperature sensitivity is due to the interaction of the foreign subunits of the RNA polymerase (see Scaife, 1976) cannot be ruled out. The transfer of E.coli DNA into A.vinelandii has not been reported previously. However, it has been reported that

the molecular weights of the subunits of A.vinelandii RNA
polymerase are exactly the same as E.coli (Burgess, 1976)
but apparently the similarity, here, of the molecular
weights of the subunits of the enzyme RNA polymerase does
not necessarily imply the close relatedness between two
bacterial species. Moreover, A.vinelandii RNA polymerase
is able to recognise the same T7 promoter and terminator
sites that are recognised by E.coli RNA polymerase (Wiggs
et al, 1979); although this investigation reflects something
of the relatedness between the two bacterial species,
it was carried out in an in vitro system which is different
from the in vivo environment.

In an earlier investigation on the reconstitution of
30S ribosomal particles, Nomura et al (1968) found that
the 16S rRNA from A.vinelandii can interact with the
ribosomal protein of E.coli to form biologically active
30S ribosomes and for this reason they have suggested that
certain regions of the 16S rRNA which are involved in the
specific interaction with the ribosomal proteins are
conserved in evolution. The molecular weights and the
number of the ribosomal proteins of A.vinelandii are also
conserved as they are similar to those of E.coli (Sun et al,
1972). Despite these similarities between the systems
of Azotobacter and E.coli I have classified it with the
distantly related bacterial species to E.coli because of
the failure of the detection of the expression of the
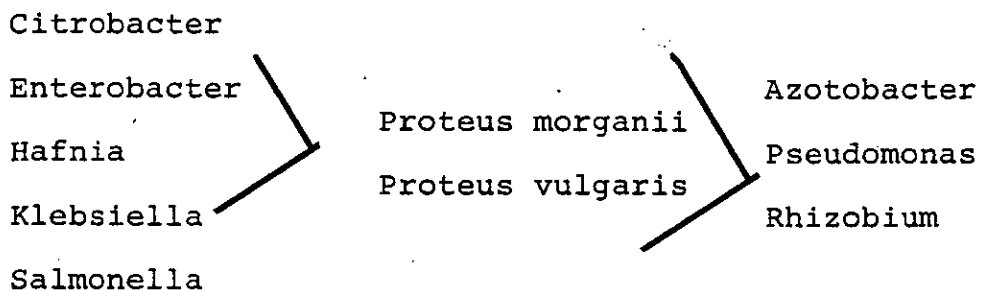E.coli rpoB3 in it.

There are other sytems, besides RNA polymerase, which

have been thoroughly investigated in the various members
of the Enterobacteriaceae. The tryptophan operon is one
of the well characterised systems in E.coli (Yanofsky,
1960; Platt, 1978). Furthermore, various studies were
carried out comparing this system in E.coli and in other
members of the Enterobacteriaceae in order to gauge the
evolutionary distance between the various bacterial species
and E.coli. Various comparative studies of the tryptophan
synthetase α chains of E.coli, Salmonella typhimurium,
Aerobacter aerogenes and Serratia marcescens were carried
out (Creighton et al, 1966; Li and Yanofsky, 1972a;
Li and Yanofsky, 1972b; Li et al, 1973b). The α subunit
of tryptophan synthetase of E.coli, S.typhimurium and
A.aerogenes are very similar in size and appear to be truly
homologous as has been revealed by the comparison of the
peptide patterns of its trypsin plus chemotrypsin digests
(Creighton et al, 1966). The similarity of the α chain
in these bacteria was concluded following the comparison
of its amino acid sequences (Li and Yanofsky, 1972a), the
amino acid sequences of fifty residues from the amino acid
termini (Li and Yanofsky, 1972b), the nucleotide sequence
of the α chain structural gene (Li et al, 1973a) and the
amine terminal sequence (Li et al, 1973b). From these
studies it was concluded that the similarity of the α
chains of the following bacterial species to that of E.coli
decreases in the following order: E.coli > Shigella >
Aerobacter > Salmonella > Serratia > Pseudomonas.

However, the comparative studies have proceeded with

the β2 subunit of the tryptophan synthetase of the various

members of the Enterobacteriaceae and E.coli (Rocha et al,

1972; Rocha et al, 1975; Rocha et al, 1978; Brennan and

Rocha, 1978; Rocha and Brennan, 1978). All these

studies support the conclusion that the general surface

structure and the α subunit binding site of the β2 subunit

from the Enterobacteriaceae have been strongly conserved;

in other words, the trpB cistron of the tryptophan is

relatively conserved in this family. Furthermore,

trpC gene products in the Enterobacteriaceae have been

compared immunochemically and have been found to be a

homologous group of proteins (McQuade et al, 1970; Reyes

and Rocha, 1977); it is indicated that the similarity

of the trpC gene product (phosphoribosylanthranilate

isomerase-indolglycerol phosphate synthetase) to E.coli

among the bacterial species examined is in the following

order: Aerobacter > Salmonella > Proteus > Serratia.

The order of the relatedness of these bacterial species

to E.coli seems to be similar to that which came from the

studies of the α subunit of the tryptophan synthetase

(Li and Yanofsky, 1972b; Li et al, 1973b). Nevertheless,

Denny and Yanofsky (1972) have reached the same conclusion

although they have looked from a different angle: the

addition of the tryptophan analogue 3-indolylacrilic acid to

cultures of Shigella, Salmonella, Aerobacter, Serratia and

Proteus stimulates the synthesis of the RNA which anneals

to the bacteriophage DNA carrying the trp operon of E.coli;

the stability of the hybrids at higher temperatures

decreased in the following order: E.coli > Shigella >

Salmonella > Aerobacter > Serratia.   In summary, as far

as the tryptophan cistron is concerned, the members of

the Enterobacteriaceae seems to be closely related to

E.coli.   My data shows that as far as the E.coli rpoB

expression in the different bacterial species is concerned

their relatedness to E.coli decreased in the following order:

Citrobacter

Enterobacter   Azotobacter

Proteus morganii

Hafnia   Pseudomonas

Proteus vulgaris

Klebsiella   Rhizobium

Salmonella

and this order is proportional to the taxonomic distance

separating these bacteria.

The ribosomes provide another good system for the

study of molecular evolution as they occur in all

stages of evolution, in prokaryotic and eukaryotic

organisms (Stöffler, 1974).   The genetic organisation of

the ribosomal RNA and the ribosomal proteins in E.coli has

been recently reviewed (Nomura et al, 1977; Nierlich, 1978).

The molecular weights and the number of the ribosomal

proteins of Proteus vulgaris, Salmonella typhimurium and

Azotobacter vinelandii are similar to E.coli (Sun et al,

1972) though the conservation in their size and number

does not reflect the homology of their primary structure.

A comparative study was carried out for the ribosomal

proteins of many members of the Enterobacteriaceae such as

E.coli, A.aerogenes, C.freundii, E.carotovora, S.typhimurium,

S.marcescens and Klebsiella (Geisser et al, 1973; Schaad, 1974). These bacterial species are included in my study except Erwinia carotovora. Geisser et al (1973) have observed the similarity in molecular weight of the ribosomal proteins in all the tested members of the Enterobacteriaceae; their ribosomes possess electrophoretically identical proteins S7, L7/L12, six of the proteins of the 30S subunit S3, S4, S11, S14, S19, S21 and four proteins of the 50S subunit L2, L14, L24 and L28. These results (Geisser et al, 1973) are in agreement with my data and confirm the close relatedness between the bacterial species of the Enterobacteriaceae. Moreover, the immunological studies prove to be more sensitive than the electrophoretic tests and its data were consistent (Schaad, 1974).

In addition, some of the ribosomal proteins such as L7/L12 of the Enterobacteriaceae species are functionally
So
conserved that they can be reconstituted in the E.coli
S
sytem and produce active ribosomes (see Daya-Grosjean, 1973). Sequencing analysis of the 16S rRNA showed that its structure is highly conserved in the Enterobacteriaceae (Fellner, 1974). However, the conservation of the 16S rRNA was confirmed by the heterologous binding of the E.coli ribosomal protein S4 to the 16S RNA's of various genera of the Enterobacteriaceae family (Daya-Grosjean, 1973). As a result of these comparative studies it became possible to conclude the taxonomical relationship of the bacterial species and the degree of conservation in the structure

of their ribosomal proteins (Wittmann and Wittmann-Liebold, 1974). Recently, a phylogenic tree of the Entero-bacteriaceae genera has been constructed as a result of the study of the ribosomal protein compositions in these bacteria (Hori and Osawa, 1978); the relatedness of the bacteria was measured by cochromatographing ⌒the differentially labelled ribosomal proteins of two organisms on a column of carboxymethyl cellulose. The proximity of the bacterial species to E.coli decreases in the following order: E.coli > Salmonella > Citrobacter > Enterobacter > Klebsiella and this order is similar to that established as a result of the earlier described studies of the tryptophan synthetase system.

The rpoBC plasmids pZD100 and pZD23 belong to the P1-group of incompatibility and are of great interest because of their widespread transferability and because they harbour the E.coli rpoBC genes; in addition, they carry the ribosomal genes of the translation machinery. Thus they provide a good opportunity for the investigation of the expression of these genes in the alien cell; in other words, their transfer to another bacterial species could allow the formation of hybrid ribosomes and thus reveal the various evolutionary aspects among the bacterial species.

My estimation of the G + C contents of the DNA of E.aerogenes (see Table 16) falls within the known range of 52-59 moles % (Buchanan et al, 1974). However, S.marcescens's G + C % is slightly higher than the range of 53-59 moles % (see Table 16; Buchanan et al, 1974),

but it is similar to the published value of Grimont and Grimont (1978). Surprisingly, the G + C % value for Hafnia alvei møller is quite different from the range 52-57 moles % (see Table 16; Buchanan et al, 1974); however, my estimation of the Hafnia G + C content falls within the range of 47.2-49.5 moles % (Izard et al, 1978). The estimation of Izard et al (1978) was based on five strains of Hafnia alvei møller which is the same species that has been used in this work.

It seems that the G + C content of the DNA is not a major criterion that affects the heterospecific gene expression because the results obtained in this work have emerged indpendently of the wide range of the GC content of the bacterial species. On the basis of my results I can conclude that promoters are highly conserved parts of the DNA and this is consistent with the independent in vitro results of Wiggs et al (1979).

METHODS AND MATERIALS

Chapter 3 - Methods and Materials

Methods

1. Mating Techniques

a. Liquid culture mating (Miller, 1972)

This mating technique was employed for all E.coli matings as well as most enteric bacteria. E.coli donor and recipient overnight L-broth cultures were diluted in fresh L-broth (see Media) and were incubated in a shaking water bath at the appropriate temperature for about two hours. The bacterial counts for each culture was estimated by using a Petroff-Hausser counting chamber. Equal volumes (usually one ml) of the log-phase cultures of the donor and the recipient were mixed in a 25 ml sterile volumetric flask and were incubated in a waterbath shaker-incubator for two hours unless otherwise stated. The mating mixture was then serially diluted in buffer (see Media) and an aliquot of 0.1 ml of each dilution was mixed with 2 mls of molten top agar at 48°C and poured over the freshly prepared selective plate for transconjugants which was subsequently incubated after the solidification of the top agar layer.

b. Spot mating (Miller, 1972)

This mating technique was employed mainly where many donors for the same mating experiment were used for the transfer of the plasmid into the recipient cell. The donor and the recipient cultures were prepared for the matings as described above (Section 1a). A small volume of 10-15 μl of one of the parents was spotted over a fresh

nutrient plate appropriate for the bacterial species involved in the mating. An equal volume of the other parent culture was spotted over the first one. The spots were left to dry and finally the plates were incubated at a suitable temperature for 24 hours. A loopful sample of cells was streaked out of the centre of the confluent growth of each spot onto the selective plate which is specific for the particular mating. As a control, the donor and the recipient were streaked onto the same plate.

c.   Millipore filter mating (Towner and Vivian, 1976)

The donor and the recipient cultures were prepared for the mating as described earlier (Section 1a). Two mls of the culture of each parent were mixed in a sterile 25 ml flask. Three mls of the mating mixture were aseptically filtered through a 0.45 μm Millipore filter which was subsequently transferred to the surface of a freshly prepared nutrient plate using sterile forceps; the plate was incubated at the appropriate temperature.

At the end of the mating time, the loaded Millipore filter was transferred aseptically into a wide-mouth sterile flask into which three mls of buffer were added. The bacteria were resuspended by their vigorous whirli-mixing for two minutes using Fisons Whirlimixer. Finally, the mating mixture was serially diluted in buffer and plated on the appropriate selective plates as described earlier (Section 1a).

The controls for the mating experiment

For every mating experiment, the following basic controls were done:

a) The absence of growth of the donor cell on the selective plate for transconjugants.

b) The inability of the recipient cell to grow on the selective plate for transconjugants.

c) The sensitivity of both parents to the antibiotics using the Oxoid Antibiotic Multodisk.

d) The estimation of the frequency of spontaneous resistance of the recipient cell to the antibiotic(s) used for the selection of the transconjugants. This was done by plating a known volume of the recipient culture onto the selective plate. The cells which survived were the spontaneous resistant mutants.

2. The Oxoid Antibiotic Multodisk Test (Cruickshank et al, 1975)

A sample of 0.1 ml of log-phase nutrient bacterial culture was gently mixed with 2 mls of molten top agar at $48^{o}C$ and was poured over a freshly prepared nutrient plate such as L-B or Ty. The Multodisk was layered over the set bacterial lawn with sterile forceps. The sensitivity to a particular concentration of any antibiotic on the Multodisk is recognised by the appearance of a clear zone around that disc. Resistant cells grow confluently independent of the presence of the disc. The Oxoid Multodisk carries one disc for each of the following

antibiotics:

    PN - Ampicillin (50 µg/ml)

    TE - Tetracycline (10 µg/ml)

    Py - Carbenicillin (100 µg/ml)

    K - Kanamycin (25 µg/ml)

    RD - Rifampicin (100 µg/ml)

3. Microbiological Tests (Cruickshank et al, 1975)

a. Methyl red test

Five mls of glucose phosphate peptone water (see Media) was inoculated with one colony from an LB-plate of each bacterial species as well as E.coli for 48 hrs. Five drops of methyl red indicator solution [Methyl red (0.1 g), Ethanol (300 ml), distilled water (2oo ml)] were added, mixed and immediate reading was taken.   Red colour indicates positive tests (the production of sufficient acid during the fermentation of glucose) and yellow colour was observed for the negative tests.

b. Voges-Proskauer test

Glucose phosphate peptone water cultures were prepared as described in (a).   One ml of KoH (40%) and three mls of α-naphthol (5% in absolute ethanol) were added.   The appearance of purple colour in 2-5 minutes indicates a positive test.   (Later, the change of the colour to crimson was observed.)

This colorimetric test is used for the detection of the production of acetyl methyl carbinol ($CH_3.CO.CHOH.CH_3$) or its reduced product 2,3 butylene glycol ($CH_3.CHOH.CHOH.CH_3$)

from the fermentation of carbohydrates.   The colour

appears as a result of the reaction between diacetyl

$(CH_3.CO.CO.CH_3)$ and a guanidino group under alkaline

conditions.   Diacetyl $(CH_3.CO.CO.CH_3)$ is the oxidation

product of acetyl methyl carbinol or 2,3 butylene glycol.

c.   Combined test of hydrogen sulphide production and

   gelatin liquefaction

   The medium containing the gelatin was firstly prepared

as follows:

Nutrient broth	20 g
Peptone	12.5 g
NaCl	5 g
Gelatin	120 g
Distilled water	1 L
Ferric chloride (10% aqueous solution)	5 ml

All constituents were dissolved in the distilled water

except ferric chloride.   The pH was adjusted to 7.6

using "PYE model 74" pH meter.   The medium was steamed

and filtered through a Buchner funnel;   it was then

sterilised by autoclaving at 121^OC for 10 min.   The

freshly prepared solution of ferric chloride was

sterilised through a 0.45 μm Millipore filter and was

added to the cooled autoclaved medium (about 50^OC).

Two mls of the medium was transferred aseptically with a

warm pipette into small sterile test tubes which were

immediately inoculated, individually, with a single colony

from a young LB-plate of the bacterial species to be

tested.   The inoculated test tubes were sealed with parafilm and were incubated at $20^{o}C$.   Although it is recommended to incubate the growing cultures for at least seven days, it was possible to see the results of my experiments after only two days incubation;   the continuous inspection of the cultures for seven days has not revealed any alteration in the results.   The production of H_2S is indicated by the blackening of the culture (production of ferric sulphide) whereas the liquefaction of gelatin is noticed by the change of the state of the culture from the solid state to the liquid state at $20^{o}C$.

d.   Citrate utilisation test

This test is employed to distinguish most enteric bacteria from E.coli which is unable to utilise citrate as a sole carbon and energy source for growth.

The medium employed for this test is Simmon's citrate medium (see Media).   It is green in colour (pH 6.8) due to the presence of bromothymol blue indicator which is yellow at pH 6.0 and blue at pH 7.6.

Single colonies were streaked onto freshly prepared Simmon's citrate medium and were incubated at $37^{o}C$ (except Proteus sp. which was incubated at $30^{o}C$) for 48 hrs. Positive results are indicated by the appearance of the bacterial growth and the change of the green colour of the medium into blue.

For testing the bacterial strains harbouring the plasmid pZD100, pZD23 or RP4 λatt, the medium contained all the antibiotics, resistance to which are conferred by that plasmid.

e.   Sorbitol fermentation test (Cruickshank et al, 1965).

Medium-2 was prepared (see Media) and was dispensed

in 3 ml volumes into small test tubes.   Durham tubes

(size 30 x 6 mm) were inverted in these tubes so that

air bubbles are avoided.   The tubes were then autoclaved

at 121°C for 15 min, 0.3 ml of sterile sorbitol (5%) was

added to each tube.   The sorbitol was sterilised by

filtration through a 0.45 μm Millipore filter.   The tubes

were inoculated with single colonies of the tested

bacterial species and they were incubated at 37°C for

24 hrs.   The colour of Medium-2 is green due to the

presence of bromothymol blue Bromothymol blue [(1 g),

0.1 N NaOH (25 ml), distilled water (475 ml)] . Positive

results of the production of acid and gas from the

fermentation of sorbitol is indicated by the change of the

colour of the culture from green to yellow and by the

displacement of the culture in Durham tube with the

liberated gas.

f.   The phenyl pyruvic acid test (Cruickshank et al, 1965)

This colorimetric test was employed to distinguish

Proteus sp. from other enteric bacteria, as it is able to

transform phenylalanine into phenyl pyruvic acid.   5 mls

of L-broth cultures of Proteus morganii and Proteus

vulgaris were prepared in ½ oz bottles by the inoculation

of fresh L-broth with a single colony of each Proteus sp.

from MacConkey's plate.   The cells were harvested by

centrifugation at room temperature using a bench centrifuge

and they were resuspended in 0.5 ml L-broth.   0.5 ml of

DL-phenylalanine (0.2% in saline)[*] was added followed by one drop of phenol red (0.01%) and the alkalination with 0.1 M Na_2CO_3. The mixture was vigorously shaken and was incubated in an almost horizontal position for four hours at $30^\circ C$. H_2SO_4 (10%) was added until the colour changed from pink to yellow . $(NH_4)_2 SO_4$ was added until saturation.   Half-saturated solution of $FeNH_4 (SO_4)_2$ was prepared and five drops of it was added to the yellow solution which was shaken thoroughly. Immediate change of the colour into dark green was observed.   As a control, the same procedure was applied using E.coli which showed no change in the yellow colour after the addition of $FeNH_4 (SO_4)_2$.

4.   In vitro DNA Manipulation Techniques

a.   The isolation of ⟨‿⟩ plasmid DNA (Hansen and Olsen,
       1978;   Humphreys et al, 1975)

       This procedure was employed for the isolation of pZD100, pZD23, pZD135, pZD44, RP4 and RP4 λatt DNA.

       One litre of L-broth culture of E.coli that harbours the plasmid was prepared in a two-litre capacity sterile conical flask.   The cells were grown in the presence of all the antibiotics (whose resistance determinants are carried by the plasmid) at the final concentration: ampicillin (50 µg/ml), tetracycline (10 µg/ml), kanamycin (25 µg/ml) and rifampicin (100 µg/ml).   The overnight

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[*]   Saline is an NaCl solution in water (0.85%).

cultures were harvested by centrifugation in MSE centrifuge model High Speed 18 at 10 Krpm and 4°C for 10 min. The pellets were resuspended in 20 mls of 50 mM Tris, pH 7.0 and were mixed in one polypropylene centrifuge bottle. The cells were repelleted under the same conditions of centrifugation. The washed pellet was resuspended in 34 mls of 25% sucrose solution by high speed vortexing at room temperature. A volume of 2.5 ml of lysozyme (10 mg/ml in 0.25 M Tris, pH 8.0) was added and four gentle inversions of the centrifuge bottle at a frequency of about 20 inversions per minute were applied. The bottle was immediately placed in an ice bath for five minutes. This was followed by the addition of 12.5 mls of 0.25 M EDTA, pH 8.0 and the application of five gentle inversions for mixing. The bottle was placed in an ice-bath for another five minutes. The next step was the addition of 12.5 mls of Sodium dodecyl sulphate, SDS (20% w/v in TE) and this was followed by eight cycles of heat pulse and mixing. This was achieved by incubating the bottle for 15 seconds in a 55°C water bath then removing it out of the water bath and applying five inversions during 15 seconds.

The alkaline denaturation and neutralisation steps followed the lysis of the cells and were carried out at room temperature. This was done by the addition of 12.5 mls of 3 N NaOH (freshly prepared solution) which was mixed immediately by continuous inversions for 3 minutes (at 20 inversions/min). Fifteen mls of 4 M Tris-HCl,

pH 7.0 were added and were mixed by many inversions for
2 minutes at the same frequency.   Sixteen mls of 20% SDS
(w/v) in TE [Tris (10 mM), EDTA (1 mM), pH 7.2] were
added and immediately followed by the addition of 32 mls
of 5 M NaCl which was mixed by 20 inversions at
20 inversions/min.   The polypropylene centrifugation
bottle was refrigerated at 4°C for 6 hrs, or overnight.

The removal of the salt precipitated chromosome -
membrane complexes was done by the centrifugation of the
overnight solution at 12 Krpm for 30 minutes at 4°C using
the MSE centrifuge model High Speed 18.   The mucoid
supernatant was collected in a chilled graduated plastic
beaker to measure its volume.   In my preparations, the
average volume of the supernatant was about 112 mls.   To
this supernatant, 0.25 volumes of 50% polyethylene glycol
(PEG 6000) was added and stirred with a plastic pipette.
The clear mucoid solution became turbid and was
refrigerated at 4°C for 6 hrs, or overnight.

The precipitated DNA was pelleted by centrifugation
for 10 min at 1 Krpm and 4°C using type SS34 rotor of
the Sorvall RC2-B centrifuge.   The pellets were resuspended
in 5 mls of cold TES [Tris (0.005 M), EDTA (0.005 M),
NaCl (0.8 M), pH 7.2] and were kept in an ice bath.
Caesium chloride was added at 0.95 g for each ml of the
cold solution.   The refractive index of the CsCl solution
was adjusted to a final reading of 1.392 using ABBE "60"
refractometer, model B.   Ethidium bromide was added to a
final concentration of 200 µg/ml.

The solution was ultracentrifuged at 38 Krpm for 40 hrs at 15°C in a Beckman Ultracentrifuge, model L2-65B using 50-Ti rotor.

The tubes were examined under an ultraviolet source. Two bands appeared; the upper was chromosomal and the lower was plasmid DNA band which was collected with a syringe. The colour of the collected DNA was purple at this stage due to the presence of ethidium bromide; it was extracted three times with equilibriated isopropyl alcohol (propan 2-ol). This was done by adding equal volumes of alcohol and plasmid preparation in a conical polyethylene tube leaving it on a rotor wheel for 15 minutes. Isopropanol was removed (now purple in colour) with a pasteur pipette. The process was repeated three times.

Traces of caesium chloride and ethidium bromide were removed by dialysis against TES - Dowex (50 W-X8) buffer, pH 7.2 for 24 hrs which was followed by two successive dialyses in TE buffer. The optical density was measured in a Carl Zeiss spectrophotometer PMQ11 at 260nm.

The concentration of the plasmid DNA that has been prepared in my experiment ranges between 220-330 µg/ml.

b. The chromosomal DNA preparation Muller et al, (1975) with slight variation.

This method was used for the preparation of E.coli, E.aerogenes, H.alvei møller and S.marcescens chromosomal DNAs. One litre of overnight L-broth cultures of E.coli and H.alvei møller and Simmon citrate cultures of E.aerogenes

and S.marcescens were centrifuged at 10 Krpm for about

30 min using MSE centrifuge model, High Speed 18.

Each pellet was suspended in 80 mls of standard

saline citrate [sodium citrate (0.015 M), NaCl (0.15 M),

pH 7.0] and the cells were repelleted under the same

conditions.   Pellets were resuspended in 90 mls of

standard saline citrate.   Lysozyme (50 mg) was added to

lyse the cells at $37^\circ C$ for 5 min.   Sodium dodecyl

sulphate, SDS, was added to a final concentration of 1%

and the solution was incubated in a $70^\circ C$ water bath for

10 min and was subsequently cooled to $50^\circ C$.   Pronase was

added at 500 µg/ml (Pronase solution 10 mg/ml in water,

was incubated at $37^\circ C$ for one hour before use);   the solution

was stirred for 15 min at $50^\circ C$ and immediately cooled to

$20^\circ C$.   5 M $NaCLO_4$ (25 ml) was added and followed by

vigorous shaking of the mixture for 20 min with equal

volume of chloroform-isoamyl alcohol (24:1).   Corex

repelcoted tubes were used instead of plastic tubes as

the latter dissolve in chloroform.

The mixture was centrifuged using a Sorvall RC2-B

centrifuge.   The solution is divided into three layers,

the upper is the nucleic acids, the interphase is the

protein and membrane layer and the lower is the chloroform.

The supernatant upper layer was collected into chilled

graduated plastic beakers to measure its volume.   An equal

volume of ethanol was added to precipitate the nucleic

acids and was left overnight at $-20^\circ C$ (Duggleby, C., 1979,

personal communication).   The precipitate was collected

by centrifugation of the repelcoted 30 ml Corex tubes in
Sorvall RC2-B at 3 Krpm and at $4^{o}C$ for 10 min only. The
precipitate was dissolved in 5 ml phosphate buffer
[Na_2HPO_4 (7 mM), NaH_2PO4 (2 mM), EDTA (1 mM), pH 7.0],
CsCl (6.65 g to 7 ml solution). Ethidium bromide was
added at 200 µg/ml final concentration. The solution
was ultracentrifuged for 40 hrs at 40 Krpm and $15^{o}C$ using
5o-Ti rotor and L2-50 Beckman ultracentrifuge. The
chromosomal DNA band was collected under the ultraviolet
source as it appeared as a pink fluorescent band.

Ethidium bromide was extracted three times with
equilibriated isopropanol and in the case of <u>E.coli</u>, the
ethidium bromide extraction process was repeated five times.

The DNA preparation was dialysed against saline
phosphate buffer for 24 hrs [Na_2HPO_4 (7 mM), NaH_2PO_4 (2 mM),
EDTA (1 mM), NaCl (180 mM), pH 7.0] and two subsequent
dialyses against phosphate buffer. The density of the
DNA preparation was measured in a Carl Zeiss spectro-
photometer at 260nm. The concentration of the DNA in the
various preparations ranged from about 200-700 µg/ml.

c.   Restriction analysis of plasmid DNA

Two endonuclease restriction enzymes were utilised
in the restriction analysis of the plasmids, pZD23 and pZD44.
These enzymes are endoR.<u>Hind</u>III (Boehringer) and
endoR.<u>Bam</u>HI (prepared by Mrs K. Mileham, Laboratory of
Professor K. Murray). Both enzymes require the same
buffer for their storage and activity.

<u>Storage Buffer</u> (Mannheim Boehringer, Cat.No.: 220523)

Tris-HCl	0.01 M
NaCl	0.25 M
EDTA	0.1 mM
Dithiothreitol	1 mM
Glycerol	50% (v/v)
Bovine Serum Albumin	5 mg/ml
pH 7.4	

<u>Dilution Buffer (restriction buffer)</u>

Tris-HCl	0.01 M
NaCl	0.05 M
$MgCl_2$	0.01 M
Dithiothreitol	0.014 M
pH 7.6	

<u>The Restriction Procedure:</u>

<u>1. EndoR.HindIII</u>

This enzyme (in storage buffer) was diluted to 1 unit/µl
in dilution buffer (see above). 1 µl of the enzyme was
added to each 1 µg of plasmid DNA and 3.5 µl (10x)
restriction buffer and the volume was made up to 35 µl
with distilled water. The handling of these small volumes
of enzyme and plasmid DNA was with the help of the
Micropet disposable pipettes (Clay Adams) and Sarstedt
(size 46 x 5.7 mm, with attached stopper) polyethylene tubes.
The solution was thoroughly mixed by a very short spin
using Micro-centrifuge and was incubated for 1 hr at 37°C.

The restriction process was stopped by heating the
incubation mixture at 70°C for 10 min (Murray and Murray, 1974).

The digests were cooled immediately at 0°C.   8 μl of loading

buffer (tris-acetate, pH 8.2;   0.04% Bromophenol blue;

20% PEG 6000) was added to the digests.   The solution

was transferred by Hamilton-Bonaduz Microlitre syringe

into the well of the prepared horizontal gel for the

electrophoretic fractionation of the restriction fragments

(as described later).

2.   EndoR.BamHI

The procedure is exactly the same as described in

(1) but the incubation temperature is 30°C.

3.   The double restriction with endoR.HindIII and BamHI

The plasmid DNA was digested with HindIII (as

described in (1)) for 2 hrs at 30°C and BamHI was added to

the digest (as described in (2)) and the incubation continued

for another two hours.   The reaction was stopped by

heating at 70°C as described earlier.

4.   Agarose gel electrophoresis

I have used the horizontal system of agarose gel

electrophoresis (McDonell et al, 1977).   It consists of

two tanks (each with 400 ml capacity), one perspex slab

table (inner size 14 x 28 cm), two end spacers, one comb

with 13 teeth (size 0.6 x 1 cm) and a Shandon Southern

SAE 2761 power pack.   The slab table was supported by

the reservoir tanks and the spacers were held in position

by bulldog clips.   The comb was placed with perspex mounts

so that it is about 1 mm off the bottom of the perspex

table.   It was positioned at about 2 cm from one end.

One litre of Tris-acetate, TAE [Tris (0.04 M),

Na-acetate (0.02 M), EDTA (0.01 M)] was prepared and adjusted to pH 8.3 with glacial acetic acid. 400 ml of TAE was placed in each reservoir tank. To the remaining 200 ml, Agarose (Sigma (A-6013 type I) was added at 0.7% final concentration in a 500 ml conical flask and the mixture was stirred with a magnetic bar on BTL magnetic hot plate until the agarose was completely dissolved.

The boiling agarose was cooled to 50°C. Ethidium bromide (20 mg/ml) was added to agarose to a final concentration of 3 μg/ml. The cooled agarose-ethidium bromide solution was poured carefully over the horizontally set perspex table, so that the position of the comb is not disturbed and bubbles are not formed. The solution was allowed to set. The bulldog clips were removed and the comb was gently removed in a perpendicular upward lifting movement after pouring a few mls of the TAE buffer around it. The wells were filled with TAE buffer.

Two folds of filter paper were placed across the ends of the perspex table so that they overlap the gel from one end and they hand in the tank from the other end; they were saturated with TAE buffer. The samples were loaded into wells by Hamilton-Bonaduz Microlitre syringe which was rinsed with distilled water after every loading. Leads were connected so that the anode is far from the wells. The buffer is circulated by a Perspex Peristaltic Pump 10200 (LKB) at 5 ml/hr.

Electrophoresis was started at 120 V for 1.5 hrs and then lowered to 70 V for 21 hrs. The wells should not dry.

After the movement of the DNAs from the wells, they were sealed with 0.7% agarose in TAE. The gel was covered with Saran-Wrap.

At the end of the electrophoresis, I found that the bromophenol blue marker had migrated 15.5-16.5 cm. The horizontal gel was visualised under the long UV source. No further staining was needed as the electrophoretically separated restriction fragments appeared as fluorescent bands. The gel is now ready for photography.

5. The gel photography

The gel was gently slid onto the moistened UV box. White light of a nearly angle-poise table lamp was on. A ruler, which was lined with white lassotape, was placed along the gel. 4x red filter (Hoya 40.5 Ø R (25 A)) of Kodak-Specialist 3 camera (set above the UV box) was removed, the shutter was opened and the gel was focussed to that the numbers of the ruler could be read clearly; the shutter was closed and the red filter was returned to its position. Laboratory goggles were put on as well as disposable gloves. Ilford FP4 filmbox, filmpack and film-rack were prepared. The white light was turned off. The filter pack was loaded by removing one protective plate and Ilford FP4 film was placed so that the notch was against the right index finger, the flap was then closed and the protective plate replaced. The filmpack was placed in Kodak-Specialist 3 camera so that the film side was down. The UV light was turned on and the protective plate of the film-pack was pulled out. The shutter was

opened for 45 seconds, closed and the UV was turned off. The developing process was continued in the dark. The photographed film was removed and was clipped into the rack for developing.

The lids were removed from the three tanks of the development procedure. The film was immersed in the developer tank (Ilford Microphen) for 9 min with occasional shaking and was then transferred into the second tank (stop bath) for 1 min to stop the action of the developer and finally it was placed in the fixer tank (Ilford Hypam - rapid fixer with hardener) for 4 min. The lights could be turned on at this stage. When the film was removed from the fixer, it had a slightly purple background; it was put under running tap water until the purple colour disappeared (at least half an hour).

5. Sensitivity to Bacteriophages

a. The sensitivity of RP4 containing strains to PRR1

(Olsen and Shipley, 1973; Schmidt, personal communication, 1976).

LC agar plates were prepared by adding 6.5 ml of Multimix [0.5 M $CaCl_2$ (20 ml), 20% glucose (25 ml), 0.25% thymidine (20 ml)] to the molten sterile 500 ml LC bottom agar (see Media) and pouring into sterile 90 mm Sterilin petri dishes (type single vent) under aseptic conditions.

The bacterial lawn was prepared by adding 0.2 ml of the L-broth bacterial culture to 2.5 ml of the molten LC

top agar (to 100 ml LC top agar add 1 ml of 0.5 M $CaCl_2$)
and the mixture was poured over the freshly prepared LC plate.

PRR1 lysate (10^{10} pfu/ml) was serially diluted in
phage buffer at -0, 10^{-2} and 10^{-4};   15 μl of the various
dilutions was spotted over the bacterial lawn by the use
of sterile Eppendorf micropipettes.   PRR1 was also
spotted on LC agar plate with no bacterial lawn as a
control.   For every tested culture, one lawn on an LC
plate was prepared and was incubated without spotting the
phage lysate.   All the plates were incubated after
drying of the phage spots.

b.   λ immunity test (Miller, 1972)

The bacterial lawns were prepared by plating the
bacterial culture (0.2 ml) with molten BBL top agar over a
BBL plate.   Two bacteriphage λ lysates were used, namely
λv and λcI, which were spotted as described in section a.

6.   The Heat Induction of λcI857S7 Lysogens (Kirschbaum, 1973)

The 5 ml L-broth culture (in a 100 ml flask) of the
temperature sensitive λcI857S7 lysogen such as ZD100
(metB Bl⁻recA56 λcI857S7/pZD100) was grown at 30°C to
log-phase in a water bath-shaker.   The culture was shifted
to a 42°C water-bath shaker for 20 minutes and was
subsequently incubated at 37°C for 3 hrs with vigorous
aeration.   Chloroform was added (0.2 ml) and the culture
was incubated for a further 15 mins.   The lysate was
cleared by removing the cellular debris by centrifugation
at 5000 Xg for 15 min using the MSE bench centrifuge.   The
supernatant was collected and stored at 4°C.

7. The Estimation of the Standard Error of the Mean

(Bailey, 1959)

The statistical method was employed for finding the standard error of the mean for the measured molecules of the various plasmids. The mean is often written as:

$\bar{x} \mp \dfrac{S}{\sqrt{N}}$ where, $\dfrac{S}{\sqrt{N}}$ is the standard error

S, is the standard deviation

N, is the number of measured molecules.

The standard deviation is the square root of the variance and is calculated as follows:

$$S = \sqrt{\frac{\Sigma(x-\bar{x})^2}{N-1}}$$

where x is the contour length of one molecule

$\bar{x}$ is the mean $\left(\dfrac{\Sigma x}{N}\right)$

N is the number of the measured molecules.

Media

1. __BBL Bottom Agar__ (Parkinson, 1968)

BBL stands for Baltimore Biological Laboratories

NaCl   5 g

BBL Trypticase 10 g

Difco Agar   10 g

Distilled Water 1 L

2. __BBL Top Agar__ (Parkinson, 1968)

NaCl   5 g

BBL Trypticase 10 g

Difco Agar   6.5 g

Distilled Water 1 L

3. <u>Gelatin Medium</u> (Cruickshank <u>et al</u>, 1965)

Difco nutrient broth	20 g
Peptone	12.5 g
NaCl	5 g
Gelatin	120 g
Distilled Water	1 L

4. <u>Glucose Minimal Medium</u> (Clowes and Hayes, 1968)

Sterile 100 mls of Meynell salts (4x) is added to 300 mls of autoclaved water agar.   Glucose solution (20%) is added aseptically to a final concentration of 0.2%. Amino acids are supplied at 20 µg/ml.

5. <u>Glucose Phosphate Peptone Water</u> (Cruickshank <u>et al</u>, 1975)

Peptone	5 g
Dipotassium hydrogen phosphate, K_2HPO_4	.5 g
10% glucose	50 ml
Distilled Water	1 L

pH 7.6

6. <u>Grabow and Smit Minimal Medium</u> (Grabow and Smit, 1967)

This medium is used to grow <u>Proteus sp.</u>. It is a modification of the medium described by Davies and Mingioli, 1950.

K_2HPO_4	10.5 g
KH_2PO_4	4.5 g
Na_3-citrate $2H_2O$	0.47 g
$(NH_4)_2 SO_4$	1.0 g
$MgSO_4 \cdot 7H_2O$	0.102 g
Distilled Water	1 L
Agar	17 gL

After autoclaving the medium, the following supplements were added:

glucose (25 µg/ml), Bl (10 µg/ml), nicotinic acid (7.5 µg/ml), pantothenic acid (1 µg/ml), and methionine (20 µg/ml).

7. Hammersmith Stabs (Clowes and Hayes, 1968)

Difco nutrient broth	9 g
Difco agar	7.5 g
NaCl	5 g
Thymine	0.1 g
Distilled Water	1 L

8. L-Broth Agar (Lennox, 1955)

Difco tryptone	10 g
Difco yeast extract	5 g
NaCl	10 g
Difco agar	15 g
Distilled H_2O	1 L
pH 7.2	

9. L-Broth (Lennox, 1955)

Difco tryptone	10 g
Difco yeast extract	5 g
NaCl	10 g
Distilled Water	1 L
pH 7.2	

10. <u>LC Top Agar</u> (Masters, 1970; Goldberg ~~et al~~ 1974)

Difco tryptone	10 g
Difco yeast extract	5 g
NaCl	5 g
Difco agar	7 g
Distilled Water	1 L

11. <u>LC Bottom Agar</u> (Masters, 1970; Goldberg, 1974)

Difco tryptone	10 g
Difco yeast extract	5 g
NaCl	5 g
Difco agar	10 g
Distilled Water	1 L

12. <u>MacConkey's Agar</u> (Cruickshank <u>et al</u>, 1975)

Peptone	20 g
Sodium taurocholate, commercial (bile salt)	5 g
Distilled Water	1 L
Agar	20 g
Neutral red solution (2% in 50% ethanol)	3.5 ml
Lactose (10% aqueous solution	100 ml

pH 7.5

13. <u>Sugar Medium 2. Broth base</u> (Cruickshank et al, 1965)

Meat extract	5 g
Peptone	10 g
Sodium chloride, NaCl	3 g
Disodium hydrogen phosphate, Na_2HPO_4	2 g
* Bromothymol blue indicator solution	12 ml
Distilled Water	1 L

* Bromothymol blue solution consists of:

Bromothymol blue	1 g
0.1 N NaOH	25 ml
Distilled Water	475 ml

pH 7.3

14. <u>Meynell Minimal Salts</u> (4x) Clowes and Hayes, 1968)

Ammonium chloride	20 g
Ammonium nitrate	4 g
Sodium sulphate (anhydrous)	8 g
Dipotassium orthophosphate	12 g
Potassium dihydrogen orthophosphate	4 g
$MgSO_4$	0.4 g
Distilled Water	1 L

pH 7.2

15. <u>Simmon's Citrate Agar</u> (Cruickshank <u>et al</u>, 1975)

Sodium chloride, NaCl	5 g
Magnesium sulphate, $MgSO_4$	0.2 g
Ammonium dihydrogen phosphate, $NH_4H_2PO_4$	1 g
Potassium dihydrogen phosphate, KH_2PO_4	1 g
Sodium citrate $Na_3C_6H_5O_7.2H_2O$	5 g
Distilled Water	1 L
Agar	20 g
Bromothymol blue (0.2%)	40 ml

pH 6.8

16. <u>SY Minimal Medium</u> (Sherwood, 1970; Beringer, 1974)

Sodium succinate	1.35 g
$Na_2HPO_4.12H_2O$	0.45 g
$MgSO_4.7H_2O$	0.1 g
$FeCl_3.6H_2O$	0.02 g
$CaCl_2.2H_2O$	0.04 g
Distilled water	1 L

Before plating add thiamine hydrochloride, Biotin (vitamin H) and calcium pantothenate at a final concentration of 1 µg/ml each. Tryptophan and phenylalanine are added at 20 µg/ml. Streptomycin was added (500 µg/ml) when the selection for its resistance marker was required.

17. <u>Top Agar</u> (Clowes and Hayes, 1968)

Difco "Bacto" agar	6 g
Distilled Water	1 L

pH 7.0

18. <u>TY Broth</u> (Beringer, 1974)

Difco tryptone   5 g

Difco yeast extract   3 g

$CaCl_2.6H_2O$ 1.3 g

Distilled Water   1 L

pH 6.8-7

Agar (15 g) was added when TY Agar was prepared.

19. <u>Water Agar</u> (Clowes and Hayes, 1968)

Davis New Zealand Agar   20 g

Distilled Water   1 L

pH 7.2

20 <u>Buffers</u>

a. <u>Bacterial Buffer</u> (Clowes and Hayes, 1968)

KH_2PO_4 3 g

Na_2HPO_4 7 g

NaCl   4 g

$MgSO_4.7H_2O$ 0.2 g

Distilled Water   1 L

pH 7

b. <u>Phage Buffer</u> (Clowes and Hayes, 1968)

Na_2HPO_4 7 g

KH_2PO_4 3 g

NaCl   5 g

0.1 M $MgSO_4$ 10 ml

0.01 M $CaCl_2$ 10 ml

1% Gelatin solution   1 ml

Distilled Water   1 L

List of Strains

Strain	Genotype	Source
ZD100	metB B_1^- recA56 $\lambda\bar{c}$I857 S7/ pZD100	Z. AL-Doori
J62	pro trp his λ^+ str-r	J. Scaife
X240	metB B_1^- his strA lac	J. Scaife
ZD7	metB B_1^- recA56 Str^S λ^S/ RP4 λatt	Z. AL-Doori
TGL10	F^- his$^-$ trpA9761 argE171 recA56	T. Linn
ZD27	F^- his$^-$ trpA9761 argE171 recA56/ pZD100	Z. AL-Doori
Ymel	F^+ pro$^-$ SuIII$^+$ λ^S	J. Scaife
AW1	metB B_1^- recA56 str-s λ^S	J. Scaife
TGL6	metB B_1^- recA56 str-s λ^+ λ^R	T. Linn
ZD28	F^- his$^-$ trpA9761 argE171 recA56/RP4 λatt	Z. AL-Doori
ZD2723	F^- his$^-$ trpA9761 argE171 recA56/pZD23	Z. AL-Doori
X242	metB B_1^- his$^-$ recA56 strA lac rpoC4	J. Scaife
397C	argG B_1^- rpoCts	J. Scaife
ZD162	metB B_1^- recA56 str-r λ^+	Z. AL-Doori
Raf3	P.aeruginosa/pZD100	Z. AL-Doori
ZAV23	A.vinelandii rif^S/pZD100	Z. AL-Doori
ZAV21	A.vinelandii rif^r/pZD100	Z. AL-Doori
R.leguminosarum 897	phel trp-12 str-37	J. Beringer
ZDR19	phel trp-12 str-37/pZD100	Z. AL-Doori
ZDR21	phel trp-12 str-37/pZD23	Z. AL-Doori
ZAV24	A.vinelandii/pZD23	Z. AL-Doori
ZDH10	Hafnia/pZD23	Z. AL-Doori

Strain	Genotype	Source
ZDK105	K.pneumoniae/pZD23	Z. AL-Doori
ZD136	metB B_1^- recA56 Str^S λ^S/pZD23	Z. AL-Doori
ZDC104	C.freundii/pZD23	Z. AL-Doori
ZDS104	S.marcescens/pZD23	Z. AL-Doori
ZST10	S.typhimurium arg$^-$/pZD23	Z. AL-Doori
Raf4	P.aeruginosa/RP4 λatt	Z. AL-Doori
Raf8	P.aeruginosa rif^r/RP4 λatt	Z. AL-Doori
ZDR17	R.leguminosarum phe1 trp-12 str-37/RP4 λatt	Z. AL-Doori
ZDR18	R.leguminosarum phe1 trp-12 str-37 rif^r/RP4 λatt	Z. AL-Doori
ZDS100	S.marcescens/RP4 λatt	Z. AL-Doori
ZDS103	S.marcescens/pZD100	Z. AL-Doori
ZDS104	S.marcescens/pZD23	Z. AL-Doori
ZST11	S.typhimurium arg$^-$/pZD100	Z. AL-Doori
ZD6	metB B_1^- recA56 Str^S λ^S/RP4	Z. AL-Doori
ZD1	Thy$^-$/RP4	A. Jacob
5K	F$^-$ thr$^-$ leu$^-$ B_1^- suII strp^S rK$^-$/RP4 λatt	R. Pastrana
Enterobacter aerogenes	wild type	J. Fleming
Citrobacter freundii	wild type	J. Fleming
Hafnia alvei møller	wild type	J. Fleming
Klebsiella pneumoniae	wild type	J. Fleming
Proteus sp.	wild type	J. Fleming
Seratia marcescens	wild type	J. Fleming

Strain	Genotype	Source
Salmonella typhimurium ST1	argF111	J. Scaife
Azotobacter vinelandii AvM100	wild type	R. Olsen
Pseudomonas aeruginosa PAC143	wild type	P. Clarke

Phage

| Type	Source
λdrpoBC18	J. Scaife
λv	J. Cullum
λcI	J. Cullum
PRR1	L. Schmidt

Legend to strain list

All the strains included in this list are mentioned in
the text.   Some of them are derivatives of E.coli K12;
others are derivatives of the representatives of the
Enterobacteriaceas, Azotobacteriaceae, Pseudomonadaceae
and Rhizobiaceae families.   The genetic nomenclature is
in accordance with the recommendations of Demerec et al
(1966).   The explanation of the symbols can be found in
Bachmann and Low (1980).

APPENDIX

<u>Legend to Fig. 1</u> (Appendix)

This figure demonstrates the genetical and physical map of the plasmid RK2/RP4/RP1 (Thomas, C., D. Helinksi, 1980, personal communication). The targets for the restriction enzymes are shown outside the circle. The genetic markers carried by the plasmid are shown inside the circle. There is only one <u>Bam</u>H1 site and it is located within the ampicillin resistance determinants, $\underline{Ap}^r$; one <u>Hind</u>III site and it is located in the kanamycin resistance determinant, $\underline{Km}^r$. The estimated molecular weight is 56.4 kb; the inner numbers indicate the molecular weight in kilobases.
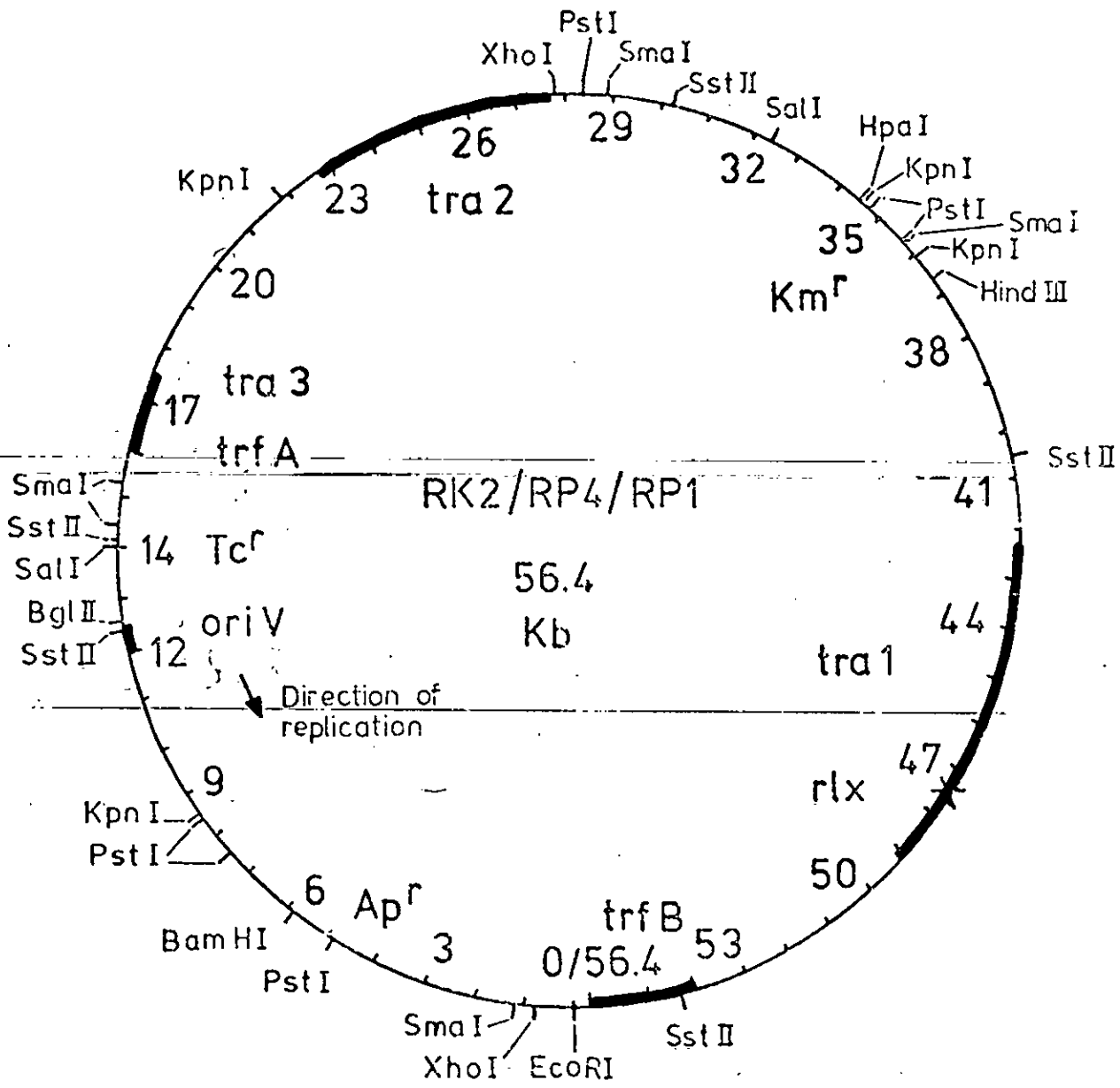
Legend to Fig. 2 (Appendix)

Electron micrograph showing one molecule of the temperature
sensitive plasmid pZD100.   It has a molecular weight of
123.9 kb which was measured relative to the nearest standard
molecule of the plasmid pSC101 (small circles).   Photography
by P. Beattie and J. Rennie.   The isolation of the plasmid
DNA is described in detail (see Methods).   Conditions of
spreading the plasmid DNA and photography are described
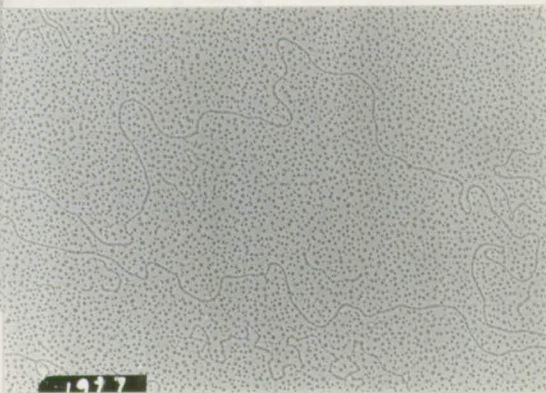earlier (see Legend to Table 11).
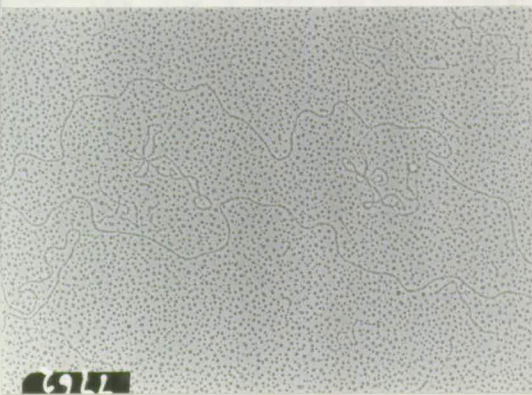
Fig. 3 (Appendix)

Electron micrograph showing one molecule of the temperature resistant plasmid pZD23 which is derived as a result of a deletion in the original plasmid pZD100.   The molecular weight of this plasmid is 83 kb;   it was measured relative to the nearest pBR313 molecule which appears as a small circle.   For further details of electron microscopy see Legend to Fig. 2 (Appendix).
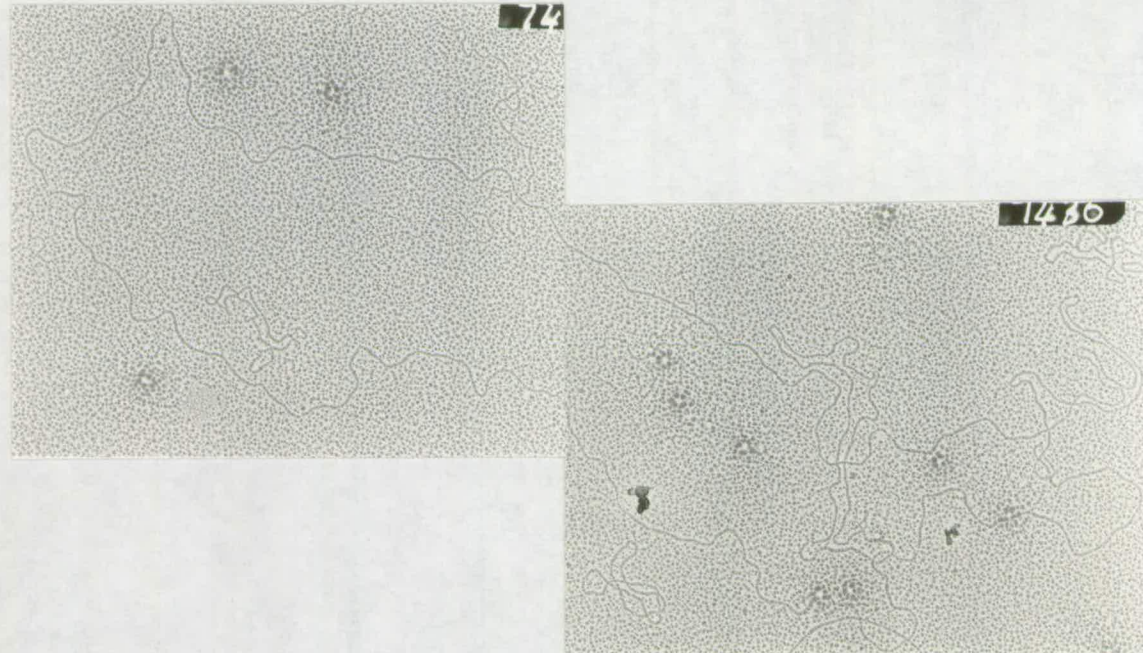
<u>Fig. 4</u> (Appendix)

This is the plasmid pZD44 molecule as seen under the electron microscope; the standard plasmid is pBR313. The plasmid pZD44 confers the resistances to the antibiotics ampicillin, kanamycin, tetracycline and rifampicin; it has a molecular weight of 88.6 kb.
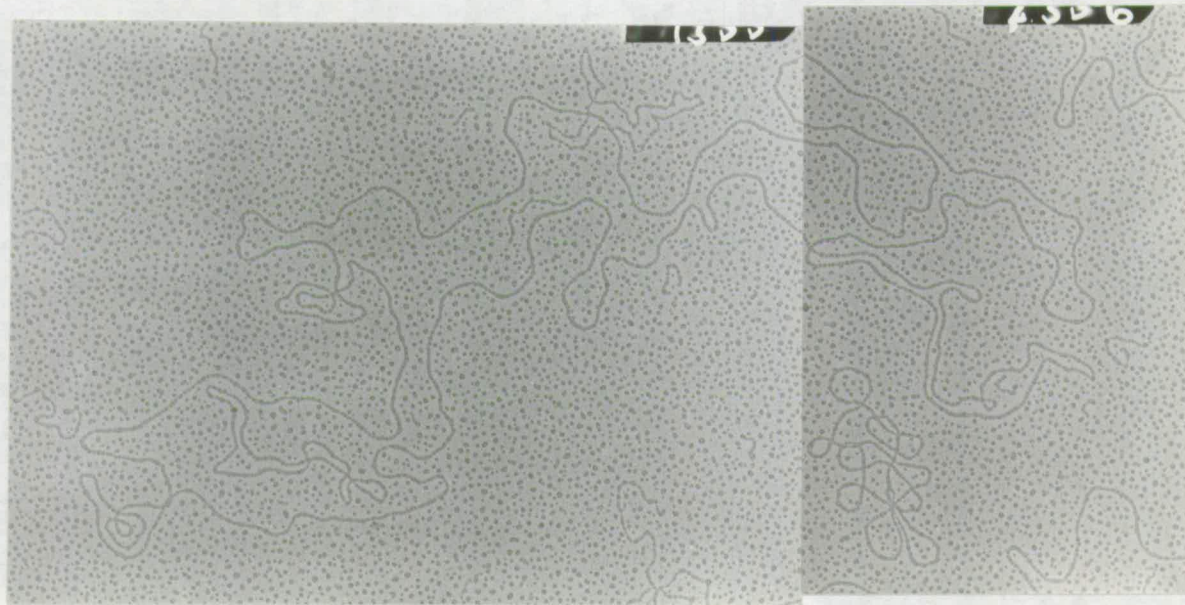
Legend to Fig. 5 (Appendix)

This is the linkage map of E.coli (Bachmann and Low, 1980). The structural genes for the subunits of the enzyme RNA polymerase are located as follows:

rpoA = 72 min

rpoBC = 89.5 min

rpoD = 66.5 min

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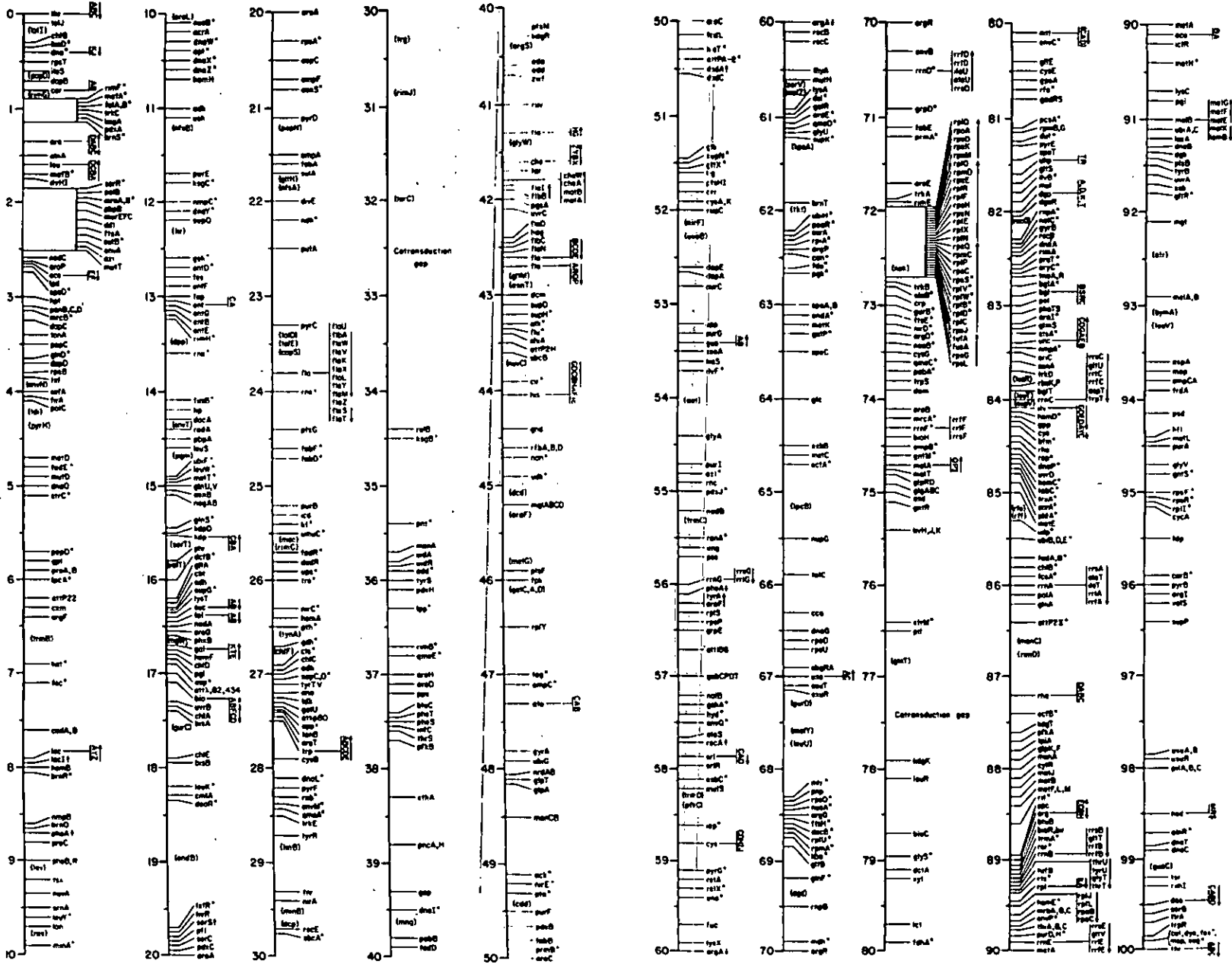   99 (2), 325-331

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LIST OF JOURNALS

Journal	Abbreviation
1. Annale De Pathologie	1. Ann.De.Path.
2. Annual Review of Biochemistry	2. Ann.Rev.Biochem.
3. Annual Review of Genetics	3. Ann.Rev.Genet.
4. Annual Review of Microbiology	4. Ann.Rev.Microbiol.
5. Antibiotiki	
6. Applied and Environmental Microbiology	6. Appl. and Env.Microbiol.
7. Archives of Microbiology	7. Arch.Microbiol.
8. Archives Romaines de Pathologie et Experimental Microbiologie	8. Arch.Romaines.Pathol. Exp.Microbiol.
9. Bacteriological Reviews	9. Bact.Rev.
10. Biochemical and Biophysical Research Communications	10. Biochem.Biophys.Res. Commun.
11. Biochemical Journal	11. Biochem.J.
12. Biochemistry	
13. Biochimica et Biophysica Acta	13. Biohim.Biophys.Acta.
14. British Medical Bulletin	14. Brit.Med.Bull.
15. Cell	
16. Comptes Rendus De L'Academie Des Sciences	16. C.R.Acad.Sci.(Paris)
17. Current Topics in Microbiology and Immunology	
18. European Journal of Biochemistry	18. Eur.J.Biochem.
19. FEBS Letters	19. FEBS Lett.
20. FEMS Microbiology Letters	20. FEMS Microbiol.Lett.
21. Gene	
22. Genetica	
23. Genetical Research	23. Genet.Res.
24. Genetics	
25. International Journal of Systematic Bacteriology	25. Int.J.Syst.Bact.
26. Journal of Antimicrobial Chemotherapy	26. J.Antimicrob.Chemoth.
27. Journal of Applied Bacteriology	27. J.Appl.Bact.
28. Journal of Biological Chemistry	28. J.Biol.Chem.
29. Journal of General Microbiology	29. J.Gen.Microbiol.
30. Journal of Medical Microbiology	30. J.Med.Microbiol.
31. Journal of Molecular Biology	31. J.Mol.Biol.
32. Journal of General Virology	32. J.Gen.Virol.
33. Journal of Virology	33. J.Virol.
34. Khimia	
35. Lancet	
36. Microbiological Reviews	36. Microbiol.Rev.
37. Microbiology and Immunology	
38. Molecular Biology Reports	38. Mol.Biol.Rep.

— Appendix: Further discussion on the various classes o
of the temperature resistant mutants (see p68-69).

The temperature resistant mutants are divided into

five groups. The first group (43%) comprises mutants which
are resistant to the antibiotics ampicillin,kanamycin,tetra-
cycline and rifampicin. Presumably these mutants harbour
plasmids that consist of most of the RP4 genome and at least
the bacterial segment of λdrpoBC18which contains the rpoB3
gene. The results of a λ sensitivity test and a zygotic in-
duction test will provide further information about the
presence of the λpart of λdrpoBC18 on these plasmids.
The second group of the temperature resistant mutants are
resistant only to ampicillin and they comprise 36%. The am-
picillin resistant determinant(s) of RP4 was reported to
transfer into the chromosome (Richmond and Sykes,1922) and
into various plasmids (Hedges and Jacob,1974) and has been
identified as the transposon tnA (Bukhari et al,1977). Thus,
the group of exconjugants which are resistant only to peni-
cillin may have arisen only by transposition of the ampi-
cillin resistance gene(s) to the chromosome with loss of
RP4 itself. To investigate this possibility one could
conduct some experiments where the P compatibility can
be tested. Secondly,Sakanyan et al (1978) has reported that
it is possible to cure pAS8(RP4-colEl) by treatment with
mitomycin C. One can thus ask whether treatment of this
group with mitomycin will result in the loss of the ampici-
llin resistance phenotype. In fact,the much simpler expe-
riment of detecting the transferability of ampicillin resis-
tance out of the cell will answer the question of whether the

the resistance determinant(s) of ampicillin is integrated into the chromosome or not.

The third group of 12.8% includes the temperature resistant mutants which have lost the resistance to rifampicin. This group may have arisen due to the excision of λdrpoBC18 from pZD1oo. As the structural gene for the β subunit rpoB3 (rif^d) is carried by the phage then its excision would result in the loss of rifampicin resistance in this group of the temperature resistant mutants.

The fourth group of these mutants are ampicillin sensitive but resistant to kanamycin,tetracycline and rifampicin. They comprise 3.5% of the temperature resistant mutants. This group may represent the mutants where the ampicillin resistance gene(s) has been lost during the process of transposition or from which it was deleted along with part of λ to create a plasmid,non-indicible by temperature shift.

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