STUDIES ON THE ECOLOGY AND MOLECULAR BIOLOGY OF TRANSFERABLE DRUG RESISTANCE FACTORS IN COLIFORM BACTERIA.

Thesis

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ECOLOGY OF TRANSFERABLE AND NON-TRANSFERABLE RESISTANCE IN COLIFORM BACTERIA ISOLATED IN CISKEI AND TRANSKEI.

Introduction

It was as early as 1904 that Paul Ehrlich propounded the idea of a "magic bullet". This "magic bullet" or chemotherapeutic agent, as he also called it, had to meet certain requirements:

(a) a high activity against pathogenic micro-organisms

(b) easy absorption by the body

(c) activity in the presence of body fluids and tissue

(d) a low degree of toxicity

(e) must not allow the development of resistant micro-organisms.

The discovery of the sulphonamide derivative, Prontosil, by Domagk in 1935 was one of the initial steps in the search for this "magic bullet". This, together with the production and purification of the antibiotics penicillin, by Fleming, Florey and Chain in 1942 and streptomycin, by Waksman in 1943, heralded a new era in the fight against bacterial infections. The majority of modern antibacterial agents have to a large extent met the requirements of Ehrlich's "magic bullet". They have however failed to prevent the development of resistant bacterial strains. This has been particularly noticeable in the past twenty years since the sudden emergence of multiple-resistant bacteria,

many of which can transfer resistance to several drugs in one step by a process of conjugation. This phenomenon which has serious medical implications has prompted numerous studies on the origin, epidemiology, biochemistry and genetics of transferable drug resistance.

(A) Medical Aspects of R Factors

Abraham et al (1941) showed that it was easy to render a highly penicillin-sensitive strain of Staphylococcus resistant by sub-culturing the organism repeatedly in increasing sub-minimal inhibitory concentrations of penicillin. It was soon discovered however that there existed naturally penicillin-resistant strains of Staphylococci. These strains existed long before penicillin therapy was established (Kirkby, 1944). Naturally occurring penicillin-resistant strains of Staphylococci produced penicillinase, an enzyme which destroyed penicillin (Abraham and Chain, 1940). In the course of time, the naturally penicillin-resistant staphylococcal strains began to appear with increased frequency in hospital wards. A crisis situation was averted when penicillinase-resistant penicillins were discovered; the first of these to be clinically useful was dimethoxyphenyl penicillin (Rolinson et al, 1960).

In the early 1950's, new antibacterial agents were introduced and although drug-resistant strains of bacteria were obtained in vitro under experimental conditions, little interest was aroused in the clinical field. However the

sudden emergence of multiple-resistant Shigella strains in Japan soon attracted much attention from clinicians and geneticists. In 1955, a quadruple-resistant (cmp, str, sul, tet) Shigella strain was isolated and it was not long before similar reports were received from other workers in Japan (see Mitsuhashi, 1965). Mutational theories could not account for the rapid increase in multiple-resistance. Work by Ochiai et al, Akiba et al, Mitsuhushi et al and Watanabe et al (reviewed in Mitsuhashi, 1971) indicated how this took place; transmission of multiple-resistance occurred through the transfer of R factors by conjugation between bacteria. Reports of transferable drug resistance were soon received from several other countries, including France, Great Britain, Greece, Germany, Hungary, the Netherlands, South Africa and the U.S.A. (Smith, 1968). Transfer of resistance was however not just confined to Shigellae. It was in fact proved with human volunteers that multiple drug resistance could be transferred from multiple-resistant E. coli to Shigellae in the intestinal tract (in Watanabe, 1963). Subsequently it has been shown that resistance can be transferred widely among the Enterobacteriaceae (Harada et al, 1960) as well as in Vibrio cholerae (Baron and Falkow, 1961), Pasteurella pestis (Ginoza and Matney, 1965), Serratia marcescens (Falkow et al, 1961), Proteus (Nakaya et al, 1960) and Pseudomonas aeruginosa (Roe, 1971). Transferable resistance has also been shown to exist in Aeromonas salmonicida (Aoki et al, 1971), Chromobacterium violaceum, Rhizobium and

Agrobacterium tumefaciens (Datta et al, 1971).

Transfer of resistance by conjugation has so far only been shown to occur in Gram-negative bacteria although transduction of drug resistant plasmids has been shown in Staphylococci (Novick and Morse, 1971, Lacey, 1971). The fact that resistance transfer can take place between bacteria of different genera takes on added significance when one considers that several of these organisms are responsible for a number of serious diseases such as dysentery, urinary infections, typhoid fever, cholera and plague. The clinical significance of the spread of multiple resistance is well illustrated by epidemics of dysentery due to Shigellae in Japan and of gastro-enteritis due to Salmonella typhimurium in England. In 1956, 14 out of 4,399 strains of Shigella (0.3%) isolated in Japan were resistant to one or more of four antibiotics (chloramphenicol, streptomycin, sulphonamide, tetracycline) and of these, 7% showed multiple resistance. In 1966, 3,392 out of 4,292 strains of Shigella (79%) were resistant and of these 97% showed multiple resistance (Mitsuhashi, 1971). During an outbreak of gastro-enteritis at a London hospital, Datta (1962) isolated R factor-containing S. typhimurium. Between then and the winter of 1964-1965, the incidence of resistant strains of this species rose from about 3% to 61% (Anderson, 1968). A recent report tells of an outbreak of typhoid in India from which S. typhi strains carrying R factors to multiple antibiotics, including chloramphenicol, have been isolated (Paniker and Vimola, 1972). Chloramphenicol is

the drug of choice in the treatment of typhoid.

The high incidence of multiple resistance is not confined to hospitalized patients. For example Moorhouse (1969) reports that 81 out of 100 strains of Enterobacteria isolated from the faeces of normal healthy infants in Dublin were antibiotic-resistant. Sixty-eight of these strains were able to transfer their resistance to E. coli K12.

There are numerous reports of surveys such as described above, and most point to the increased frequency of multiple resistance and to the high percentage of strains harbouring R factors. There are however few which show in a concrete way how the problem can be overcome. Bulger et al (1970) report a decrease in the percentage of resistant E. coli and Klebsiella-Enterobacter isolated from patients at the University of Washington Hospital, Seattle, from mid-1959 through 1968. They attribute this decrease to careful planning by hospital staff; appropriate antimicrobial therapy was administered after standardized sensitivity testing while careful environmental hygiene and strict use of isolation procedures were adhered to. The procedure was supervised by a hospital epidemiologist with the help of an active hospital infections committee.

Richmond (1972) points out that while transfer is important in continually widening and topping up the range of organisms in which clinically important antibiotic resistance is encountered, it is selection that maintains the high incidence of these strains in the environment.

And since the selection imposed is likely to be proportional to antibiotic usage, there appears to be a need for some policy, national or world-wide, to regulate antibiotic usage.

(B) Classification of Transferable Plasmids

Plasmids are usually first classified according to the function which they perform in the cell. Thus colicinogenic factors are classified as such because of the colicins which they produce, R factors because they confer on their host resistance to antibacterial drugs, the Ent and Hly plasmids because of enterotoxin and haemolysin production respectively, the F sex factor for the conjugal activity which it confers on its host. Yet this primary classification is somewhat arbitrary in that it depends on observed characteristics but is liable to omit certain characteristics which although easily identifiable, were not looked for and hence remained undetected. For example, 35 per cent of fi^{-} R factors isolated by Meynell and Datta (1969) also determined the production of colicin I, but because they were originally detected by resistance to antibiotics, they were designated R factors and not colicin factors.

Since the discovery that bacterial cells harbouring transmissible plasmids can produce special types of pili, these plasmids have been classified by the type of pili whose production they govern. Many Enterobacteria produce various types of "common pili" (Brinton, 1965) but these are found regardless of whether the cell harbours transmissible plasmids or not. However Brinton et al (1964) reported that

cells containing the F sex factor whether in an autonomous or integrated state, produced a different type of pilus which was named the "sex pilus" to differentiate it from the "common pili". Two types of sex pili have been identified (Lawn et al, 1967): the F-type pilus which is typified by the sex pilus produced by the F sex factor and the I-type pilus which is typified by the sex pilus produced by the col I colicinogenic factor. The two types of sex pili can be differentiated in a number of ways (Meynell et al, 1968; Novick, 1969):

(a) Difference in length - F-pilus up to 20μ and I-pilus up to 2μ.

(b) Specificity as receptors to male specific phage - e.g. F-pili adsorb spherical RNA phages such as MS2, f2, QB, M13 and filamentous DNA phages such as f1 and fd. I-pili adsorb filamentous DNA phages such as If1 and If2.

(c) Antigenic response - F and I-pili differ antigenically from each other.

Most sex factors have determinants for repression of their own fertility. Only two wild-type sex factors are naturally derepressed and produce sex pili all the time; these are F and col V (which is presumably closely related to F since it is the only sex factor with which it shows superinfection immunity) (Meynell and Datta, 1969). The effect of this repression is to prevent or drastically reduce the formation of sex pili. The ability of an R factor to inhibit F was attributed by Egawa and Hirota (1962)

to a cytoplasmic repressor produced by the R factor . Watanabe et al (1964) initiated the practice of referring to R factors (and other sex factors) that repressed the fertility of F as fi^+ (for fertility inhibition) and those that did not as fi^-. Although fi^- sex factors do not suppress the function of F, they do nevertheless form a repressor which reduces their own conjugal activity. Since most sex factors are in a repressed state, they will not produce sex pili and transfer will only occur at low frequencies. Similarly it will not be possible for male-specific phages to infect the bacterial cell. The study of these sex factors has been greatly enhanced since the isolation of stable derepressed mutants (Meynell et al, 1968). The depressed fi^+ sex factors have been found to produce sex pili which closely resemble those produced by the F sex factor and which are also specific for the same male-specific phages (Meynell and Datta, 1967). Likewise some derepressed fi^- sex factors have been found to produce sex pili similar to the I-type sex pilus (Meynell and Lawn, 1967, Edwards and Meynell, 1968) and to act as receptors for I-specific phages (Meynell and Lawn, 1968).

One of the most significant criteria of classifying plasmids is superinfection immunity. Bacteria carrying one plasmid hinder the entry of others of the same group (surface exclusion). Also two plasmids of the same group will not co-exist stably in the same host (incompatibility) (Meynell et al, 1968). In bacteria, incompatibility appears to be a fundamental property of replicons in that

it is exhibited by all plasmids isogenic for autonomy functions (Novick, 1969). According to the "replicon model" (Jacobs et al, 1963), genes in order to replicate must be part of a genetic unit, the replicon, which occupies a unique intracellular specific site where replication can be initiated, probably on the cell membrane. Different kinds of plasmids have different sites and therefore do not interfere with each other's continued existence within the cell (Meynell et al, 1968). Most of the work on superinfection immunity has been done with the F sex factor, colicinogenic factors and R factors (fi^+ and fi^-). However it appears as though the same principles hold for most other plasmids (Meynell et al, 1968).

Scaife and Gross (1962) showed that two F sex factors could not co-exist stably in the same cell by transferring the F lac^+ factor to lac^-F^+ and lac^-Hfr recipients. However, apart from the col V factors, most plasmids appear to belong to different incompatibility groups to that of F (Kahn and Helinski, 1964). There is no cross-immunity between fi^+ and fi^- factors but immunity is evident with most pairs of wild type R factors when both members are fi^+ or fi^- (Mitsuhashi et al, 1962; Watanabe et al, 1964). Classification into compatibility classes has given rise to five classes among the fi^+ plasmids (Hedges and Datta, 1972) and four classes among the fi^- plasmids (Datta and Hedges, 1971 and 1972). The classes include the following plasmids:

fi$^+$ plasmids - class F I; includes F, col V2, col V3 and R386

class F II; includes many fi$^+$ R factors

class F III; includes col B-K98 and col B-K166

class F IV; R124

A fifth group includes R62, an fi$^+$ R factor which determines I-like pili and has the compatibility of a typical I-like plasmid.

fi$^-$ plasmids - those which determine I-pili constitute one class. The other classes have been named N, P and W.

Attempts have also been made to classify plasmids according to their physical properties (Falkow et al, 1969); for example G + C ratio, nucleotide sequence, density, size, etc.

More comprehensive studies are required before such a system of classification can be used generally. It should be noted that when Guerry and Falkow (1971) examined the degree of nucleotide similarity of several plasmids, they found the DNA of F-like plasmids to share a high degree of similarity to each other but very little with I-like plasmids.

(C) Origin of R Factors

One of the first questions that arise when seeking a solution to the origin of R factors is whether the presence of R factors confers on the host cell any advantages. It is undoubtedly true that in the presence of the drug to which the R factor confers resistance, the host cell will

have a decided advantage over sensitive cells. The replacement of predominantly sensitive bacterial populations by ones carrying R factors under the selective pressures of antibacterial drugs has been shown repeatedly *in vitro* as well as *in vivo*. The same however cannot be said in the absence of the selective action of antibiotics. Novick (in Chabbert *et al*, 1969, p. 239) states that wild sensitive strains have been shown clearly to have an advantage over R factor-carrying strains (the advantage seems to be that plasmid-free cells grow faster). Yet R factors have been found in strains isolated in "pre-antibiotic" communities (Gardner *et al*, 1969; Davis and Anandan, 1970). It was also reported by Smith (1967a) that an R factor was found in an *E. coli* strain isolated in the 1930's and lyophilized in 1946, suggesting the evolution of this R factor prior to the introduction of antibiotics. On the other hand, a fortuitous relation may result in a selective advantage for R^+ cells. For example, Smith (1967b) reports that some R factors alter the susceptibility of bacteria to N-methyl-N'-nitro-N-nitrosoguanidine (NTG), an organic chemical with strong mutagenic properties. Although this particular example may not have played a part in the evolution of R factors, there may be other fortuitous relationships which have influenced the evolution of R factors. It is also likely that certain R factors have been selected by natural exposure of bacteria to antibiotic-producing organisms such as *Streptomyces*, *Penicillium*, *Cephalosporium*, etc. (see discussion on Second Survey p. 41).

Gardner et al (1969) propose that studies of drug-free communities may reveal a pattern of R factors with limited genotype.

Mitsuhashi (1969) also points out that the enzymes produced by R factors which inactivate the drugs through acetylation, phosphorylation or adenylation require ATP or acetyl coenzyme A for activity. These requirements suggest that acetylating or phosphorylating enzymes may normally be functional in some strains of bacteria and that following spontaneous mutation, they may have acquired specificity for a particular drug. Although this argument appears plausible, particularly when it is considered that many antibacterials have chemical structures similar to substrates normally present in micro-organisms, it may not account for specificity to compounds synthesized in the laboratory which do not have their counter parts in nature.

Another aspect to the origin of R factors is the question as to whether resistance genes originated from chromosomal genes or from plasmid genes. There is considerable disagreement between Watanabe et al and Anderson et al on the genetic structure of R factors. Watanabe (1963) suggests that the resistance genes originated from some bacterial chromosome, not necessarilly from Enterobacteriaceae, and that the resistance transfer factor arose in much the same way as F-prime is formed. In other words, the factor is integrated into the chromosome and when it comes off, it carries with it chromosomal resistance

genes. This hypothesis is supported largely by transductional experiments on a number of R factors in which drug-resistance markers and their conjugal transferability were co-transduced by phage P1kc in *E. coli*. In cases where the transduced resistance genes lacked conjugal ability, the R factors were assumed to have become integrated into host chromosomes or to form heterogenotes with the help of the transducing phage genome (Watanabe *et al*, 1968). Watanabe and Fukasawa (1961) considered that unless the resistance genes which in some cases segregated from the transfer factor were integrated in the chromosome, they would be unable to replicate autonomously and would assume the state of abortive transductants (Staker, 1956). Such abortive transductants should fail to form colonies on drug-containing media but in fact resistant transductants inherited their drug resistance quite stably. Hence the conclusion that they were integrated in the chromosome.

An argument which has been used against a chromosomal origin for transferable resistance determinants is the fact that the mechanisms of extra-chromosomal resistance are often different from those of chromosomal resistance. For example, chromosomal resistance to streptomycin in *E. coli* is due to a single mutation to a high level of resistance and results from alteration to the structure of the ribosomes (Brock, 1966). Streptomycin resistance carried by R factors is low level and results from inactivation of streptomycin by adenylation (Yamada *et al*, 1968). Similarly, differences

between chromosomal and extrachromosomal resistances have been shown for chloramphenicol (Ramsey, 1958; Okamoto and Suzuki, 1965) and kanamycin (Takana et al, 1964; Okamoto and Suzuki, 1965). It should nevertheless be noted that certain penicillinases produced by R factors have been found to be similar to those produced by chromosomal genes of A. aerogenes (Datta and Kontomichalou, 1965) indicating that chromosomal and plasmid resistance genes may have a common origin in some cases.

Anderson (1965a) provides evidence for a different pathway in the evolution of R factors. He maintains that the R-determinants and the transfer factors behave as basically independent elements which become associated with each other to form R factors when they are present in the same cell. Anderson and Lewis (1965) devised an experiment to show the presence of the R-determinants (non-transferable) and transfer factors as separate entities. The experiment consists of mixing a wild-type drug-sensitive strain (donor containing transfer factor) with a strain carrying an R-determinant but no transfer factor (intermediate recipient), and adding to the mixture a fully drug-sensitive culture (final recipient which has an additional marker) which carries neither a transfer factor nor an R-determinant. The transfer factor in the donor strain migrates into the intermediate recipient where it combines with the R-determinant to form an R factor which is then transferred into the final recipient. This can be annotated as follows:

$$A(t) + B(r) + C \longrightarrow C(tr)$$

The method has been used to screen bacterial strains for sex factors or non-transferable plasmids (Anderson, 1965b) Strong support for Anderson's model comes from biochemical and biophysical studies of R factors in *E. coli* and *P. mirabilis* by Silver and Falkow (1970). They examined a derepressed R factor in *E. coli* by labelling and density gradient centrifugation techniques and obtained a single plasmid peak at density 1.711 g/cm^3. The same R factor in *Proteus* yielded three components of density 1.709, 1.711 and 1.716 g/cm^3. Working on a variant of the derepressed R factor which did not contain any of the resistance markers but which did confer conjugal activity to its *E. coli* host, they obtained a component of density 1.709 g/cm^3. They came to the conclusion that the 1.711 component consists of a composite molecule made up of the 1.709 and 1.716 components which represent the sex factor and R-determinants respectively. The fact that these two components can exist autonomously and independently in *Proteus* indicates that the R factor consists of a combination of separate and independent determinants (resistance and transfer) each of which controls its own replication. However when the composite molecule is formed, one of the replicon systems is presumably repressed.

The evidence at this stage tends to favour Anderson's model but this does not discount the possibility that both models may account for the formation of different R factors.

Hayes (1968) argues that if sexuality were a real

asset, one might anticipate that it would have become a more common property of bacteria in general. However one might argue that under particular selective pressures, sexuality does assume a highly significant role as has been shown by the role R factors have played in the survival of resistant populations following antibacterial therapy. The elucidation of the role played by cryptic plasmids which have been isolated (Novick, 1969) may provide some answers to these questions. For all we know, the isolated transfer factors which occur may be all that remains of a promiscuous era in bacteria which abated when selective forces which favoured their presence were withdrawn. Such a cycle has already been shown with the reduction but not the complete disappearance of R factors upon withdrawal of a particular antibiotic (Richmond, 1972).

C H A P T E R I

SURVEY OF TRANSFERABLE AND NON-TRANSFERABLE DRUG RESISTANCE.

Summary

Coliform bacteria from a number of different sources were isolated and tested for transferable and non-transferable drug resistance. In the first survey, strains were isolated from urban white, urban Xhosa and rural Xhosa communities. The percentage of drug resistance and resistance transfer was approximately the same in the urban white and rural Xhosa populations but was higher in the urban Xhosa population. In the second survey, strains were isolated from urban and remote Xhosa populations and their environments. The selective pressures of anti-bacterials in the urban Xhosa population was clearly demonstrated. The environment of the urban Xhosa population (a crowded township) was shown to act as a reservoir for R factors. This was in contrast to the low incidence of R factors in the remote Xhosa population and its environment.

(A) Materials and Methods:

(a) Media, Drugs and Chemicals:

Preparation of all media, drug and chemical solutions are described in Appendix A.

(b) First Survey:

(1) Origin of strains: Strains were obtained from the following sources:

(i) Urban Whites - Strains were isolated by the East London Clinical Laboratory from stool or urine samples from white patients whose homes were either in Bunker's Hill or Beacon's Bay, two suburbs of East London.

(ii) Urban Xhosa - Strains were isolated by the East London Pathological Laboratory from stool or urine samples from Xhosa patients at the Frere Hospital, East London, whose homes were either in Duncan Village or Mdantsane, two townships on the outskirts of East Londor.

(iii) Rural Xhosa - Strains were isolated from stool or urine samples from patients at the Butterworth Hospital which is situated in Butterworth, a small rural town in the Transkei (East Coast of South Africa). Swab samples were inoculated into brilliant green bile broths which were then dispatched to the East London Pathological Laboratory.

(2) Isolation of strains: Strains were received and stored on nutrient agar slopes. Upon arrival at our laboratory, each strain was streaked onto a MacConkey Agar plate. A single non-mucoid red colony was selected from each plate for further tests. Each colony was inoculated

into a test tube containing 10ml. of brilliant green bile lactose broth and a Durham tube. The test tubes were incubated at 37°C for 48 hours at the end of which production of gas was observed. Only those strains which were able to produce gas were regarded as coliform bacteria and were retained.

(3) Determination of drug resistance spectrum: Each strain was grown overnight in 5ml. nutrient broth. A 0.1ml. aliquot of the culture was spread on a nutrient agar or wellcotest agar plate and sensitivity discs placed on the surface. Discs were arranged in such a manner as to avoid possible interactions between drugs (see plates I & II). Where possible, more than one disc per drug was used. The plates were left to stand at room temperature for 30 minutes to allow the drugs to diffuse into the agar. The plates were then incubated for 24 hours and examined for zones of inhibition. Where at least one disc per drug produced a zone of inhibition, the bacterial strain was taken as being sensitive. The recommendations made by Waterworth (1971) regarding the application of discs for the determination of drug resistance were taken into account. In the case of drugs where discs were not available (cephalexin, trimethoprim and vioform) the drug was incorporated in the medium and broth cultures of the test strains were streaked onto the medium.

(4) Isolation of a Naladixic Acid Resistant E. coli: For conjugation experiments to determine whether the resistant strains could transfer their resistance, a suitable

PLATE I - Sensitivity discs on nutrient agar:

AM 25 (ampicillin 25 µg); CR 15 (cephaloridine 15 µg);

C 10 (chloramphenicol 10 µg); C 30 (chloramphenicol 30 µg);

K 30 (kanamycin 30 µg); TE 30 (tetracycline 5 µg);

S10 (streptomycin 10 µg); TE 10 (tetracycline 10 µg).

Strain was resistant to ampicillin, chloramphenicol, streptomycin and tetracycline.

PLATE II - Sensitivity discs on Wellcotest agar:

SXT 25 (sulphamethoxazole 23.5 µg/trimethoprim 1.5 µg); PN 25 (ampicillin 25 µg); NA 30 (naladixic acid 30 µg); F 200 (nitrofurantoin 200 µg); S300 (triple sulphonamide 300 µg); S 25 (streptomycin 25 µg); TE (tetracycline 50 µg).

Strain was resistant to ampicillin, sulphonamides, streptomycin and tetracycline.

recipient with a known marker was required. As very few bacteria showed resistance to naladixic acid, and as resistance to this drug is usually associated with a non-transferable mutation on the chromosome (Chabbert et al, 1969) it was decided to use naladixic acid resistance as a marker.

A wild type Escherichia coli strain sensitive to all the drugs tested was chosen as a recipient. This strain was isolated from a stool sample from a patient at the East London Clinical Laboratory. Two nutrient agar plates containing naladixic acid (30 µg/ml.) were overlaid with 0.5ml. of an overnight broth culture of the E. coli strain. They were incubated for 24 hours and examined for growth. Four colonies were obtained and one of these was rechecked for naladixic acid resistance. The strain was sensitive to all the drugs except naladixic acid. This strain was labelled 25R and used as the recipient in all subsequent mating experiments. The stability of the mutation has been adequately shown in that after numerous generations, the strain has consistently shown itself to be resistant to naladixic acid.

(5) Test for Transfer of Drug Resistance: One millilitre of an overnight broth culture of a resistant strain and 1ml. of an overnight broth culture of the recipient strain were both inoculated into 5ml. nutrient broth. The mixed culture was incubated overnight and then streaked onto selective media containing naladixic acid and one of the drugs (i.e. the one for which resistance transfer was

being tested). Where there was no growth after 24 hours incubation, the resistant strain was regarded as being unable to transfer resistance to that drug. Where growth was sparse (less than 5 colonies per streak) the experiment was repeated to determine whether the colonies were due to mutation or low frequency transfer. If there were again less than 5 colonies the strain was assumed to be unable to transfer resistance to that drug. However if there were five or more colonies, the strain was assumed to transfer resistance at low frequencies and the resistance was classified as being transferable. As controls, the donor and recipient strains were tested individually for mutation to resistance by streaking onto selective media.

(c) Second Survey:

As a result of the pattern of resistance obtained in the first survey, a second survey was initiated for reasons discussed on p. 34. A comparison was made between strains isolated from rural and urban communities and their environments.

(1) Origin of strains: Strains were isolated from the following sources:

(i) Rural community: stool samples were obtained from Xhosa "first time" out-patients at the Madwaleni Mission Hospital with no record of previous contact with a doctor or hospital. The mission hospital is situated in a very remote area of the Transkei.

Environmental samples were obtained from the soil, water, rivers, huts, cooking areas and cattle pens in an

area within a radius of 10 - 15 km from the Madwaleni Mission Hospital.

(ii) Urban community: stool samples were obtained from Xhosa hospitalized patients at the Frere Hospital in East London. The patients lived either in Duncan Village or in Mdantsane townships.

Environmental samples were obtained from soil, water, sewage and drains in the above two townships.

(2) Isolation of Strains: Sterile cotton wool swabs were used to collect the stool samples. These were placed in sterile brilliant green bile broth and posted to our laboratory.

Soil, water, sewer, drain and other environmental samples were collected in sterile standard containers. Within 12 hours, aliquots of these samples were transferred to sterile brilliant green bile broth. Upon arrival at our laboratory, a similar procedure for the isolation of coliform bacteria was employed as has already been described (p.18). However a number of modifications were made to the procedure.

(i) It was found to be necessary to use MacConkey Purple Agar instead of MacConkey Agar as some difficulty had been previously experienced with the colour reaction of bacterial cells on the latter medium. Thus a non-mucoid yellow clone (see plates III & IV) was selected from each plate for further tests.

(ii) Each coliform strain was also tested for the production of indole. Coliforms which gave a positive

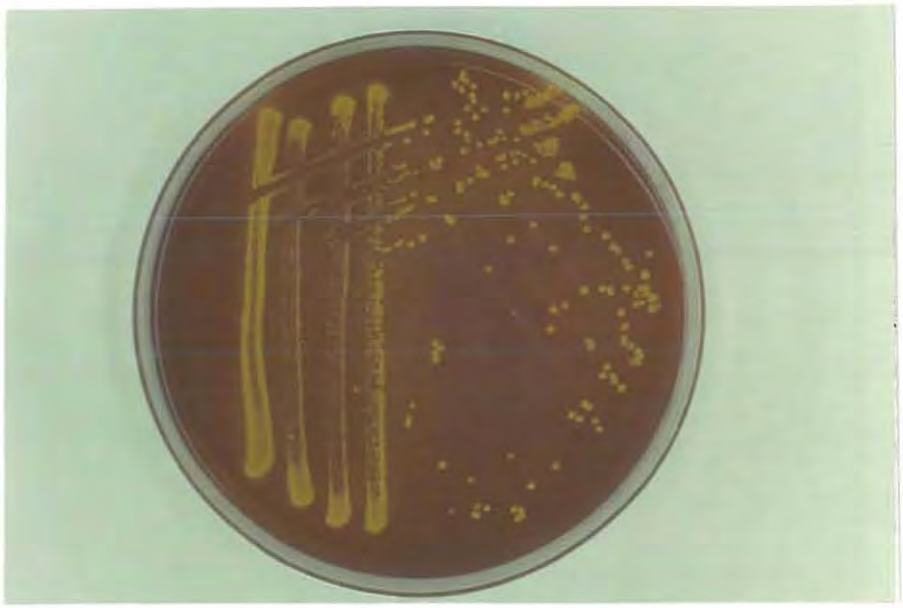


PLATE III - Yellow non-mucoid colonies on MacConkey Purple Agar.

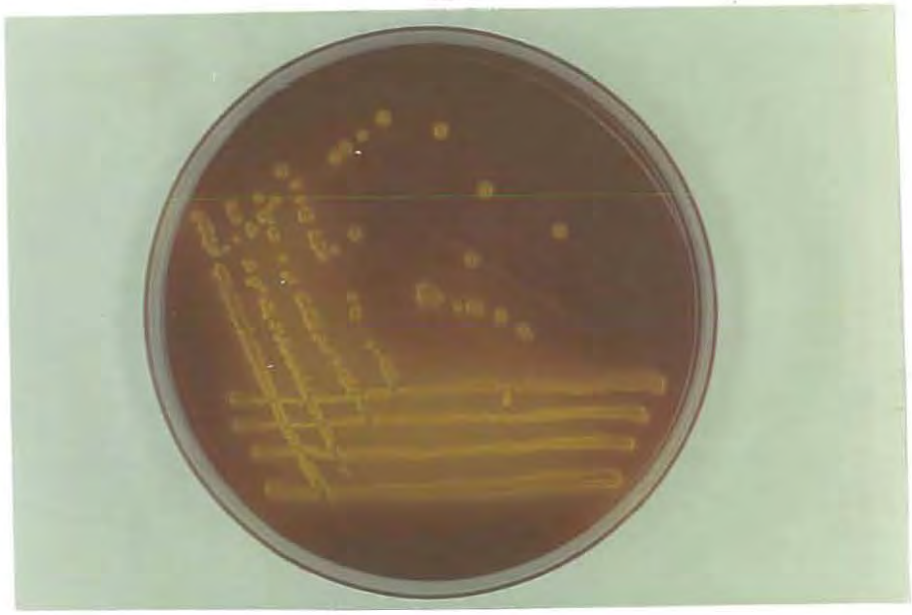


PLATE IV - Yellow mucoid colonies on MacConkey Purple Agar.

indole reaction were classified as Escherichia coli and those which gave a negative indole reaction were classified as "other coliforms" (Cowan and Steel, 1961). Strains to be tested were inoculated into 10ml. of tryptone broth and incubated for 48 hours. Diethyl ether (1ml.) was added, the mixture shaken and 0.5ml. of Ehrlich's solution (para-dimethyl-amino-benzaldehyde, ethanol and conc. HCl) gently added down the side of the tube. A red colour appearing in the upper layer was taken as indicative of the presence of indole.

Determination of drug resistance and transferable resistance was carried out as previously described (p.19).

(d) Additional Minor Surveys:

A number of surveys with limited numbers of samples were also carried out. The isolation of strains from these samples and the tests carried out on them were as described in the Second Survey.

(1) School children :- stool samples were obtained from a number of children at the Nkondwane Farm School near Butterworth. Children in their early teens were selected who had never consulted a doctor and hence presumably had not taken any of the drugs for which resistance was being tested. These children were taken to a playing field and asked to pass a stool. It is interesting to note that each child was able to produce a faecal sample on demand. A sterile cotton wool swab was dipped into the stool and placed in sterile brilliant green bile broth.

(2) Madwaleni Hospital Wards - a limited number of samples were obtained from patients in the General Ward and in the Tuberculosis Isolation Ward. The samples were processed as previously described.

(B) Results and Discussion:

Abbreviations used for drugs and chemicals are listed in Appendix B.

(a) First Survey:

The number of coliform strains isolated from each source in the first survey was: urban whites 112, urban Xhosa 47, and rural Xhosa 93. The overall results (Tables I & II) indicate that there is little difference between the incidence of resistant strains and R factors among coliform strains from urban white and rural Xhosa populations. The only exception is the high degree of streptomycin resistance (27.9%) in the rural Xhosa population. Streptomycin is extensively used to combat the high incidence of tuberculosis among the Transkei Xhosa. According to a member of a member of a mobile TB unit, great difficulty is experienced in obtaining continual co-operation from patients who require treatment. Hence therapy is often haphazard and irregular and this may contribute to the high incidence of streptomycin resistance.

The high degree of resistance (82.9%) and R factors (61.5%) in strains from urban Xhosa is striking (Table I). This could be the result of the use of drugs among an overcrowded, poor and uneducated population with inadequate domestic hygiene facilities and sewage treatment. The unhygienic neighbourhood would serve as a reservoir for resistant strains. Due to the low level of education and unfavourable economic position, the situation would be aggravated by patients failing to complete a course of drugs

TABLE I

FIRST SURVEY - INCIDENCE OF DRUG RESISTANCE
AND TRANSFERABLE RESISTANCE
IN COLIFORM STRAINS.

Origin of strains	Total No. of strains	Resistant strains	Strains showing transferable resistance
Urban Whites	112	58 (51.8%)	26 (44.8%)
Urban Xhosa	47	39 (82.9%)	24 (61.5%)
Remote Xhosa	93	54 (58.1%)	26 (48.1%)

TABLE II

FIRST SURVEY - RESISTANCE AND TRANSFER PATTERNS FOR INDIVIDUAL DRUGS.

Drug	Origin of coliform strains								
	Urban Whites			Urban Xhosa			Remote Xhosa		
	Resistant strains		Transferable resistance	Resistant strains		Transferable resistance	Resistant strains		Transferable resistance
	No.	%	No.	No.	%	No.	No.	%	No.
Ampicillin	50	44.6	25	36	76.6	22	47	50.5	25
Cephaloridine	12	10.7	4	5	10.6	1	14	15.0	3
Chloramphenicol	23	20.5	14	12	25.5	6	20	21.5	12
Gentamicin	0	0	0	0	0	0	0	0	0
Nalidixic acid	0	0	0	0	0	0	0	0	0
Nitrofurantoin	8	7.1	2	2	4.3	2	1	1.1	1
Streptomycin	9	8.0	0	4	8.5	0	26	27.9	2
Tetracycline	28	25.0	18	12	25.5	9	20	21.5	5

on discharge from hospital or on feeling better. It should be noted that the higher incidence of resistance in the urban Xhosa as compared to the urban white and rural Xhosa populations, is mainly due to the high incidence of ampicillin resistance. The incidence of resistance and R factors is of the same order for the other drugs (Table II). Maré and Coetzee (1966) examined the incidence of resistance and R factors in strains of E. coli isolated in the Pretoria area. They also found a high incidence of resistance (78%) but a much lower incidence of R factors (19%). This could be attributed in part to the fact that a Salmonella typhimurium strain was used as a recipient. The incidence of R factors in E. coli in Japan (Mitsuhashi, 1971) was 66.3% which is of the same order as that encountered in the urban Xhosa (61.5%).

The patterns for multiple resistance and multiple transfer are shown in Tables III and IV. In all three population groups, ampicillin and, to a lesser extent, chloramphenicol and tetracycline resistance determinants were found in most multiple resistance transfers (Table IV). Similar results were obtained in the overall multiple resistance pattern (Table III). A breakdown shows that out of 102 strains which showed multiple resistance, 99 strains (97%) carried the ampicillin resistance determinant, 59 strains (58%) carried the chloramphenicol resistance determinant, 60 strains (59%) carried the tetracycline resistance determinant and 29 strains (28.5%) carried the streptomycin resistance determinant. In the multiple

TABLE III

FIRST SURVEY - MULTIPLE RESISTANCE PATTERN.

Resistance Pattern	No. of strains		
	Urban Whites	Urban Xhosa	Rural Xhosa
amp	12	12	10
cpd	4	-	-
cmp	-	1	-
nft	1	-	-
str	-	-	6
tet	2	1	-
1 resistance - total	18	14	16
amp, cpd	4	1	4
amp, cmp	5	2	4
amp, nft	1	1	-
amp, str	2	1	1
amp, tet	7	3	2
cmp, nft	1	-	-
str, tet	-	-	1
2 resistances - total	20	8	12
amp, cpd, cmp	-	-	2
amp, cpd, nft	1	-	-
amp, cpd, str	-	-	5
amp, cpd, tet	-	1	-
amp, cmp, nft	-	1	-
amp, cmp, str	-	-	2
amp, cmp, tet	14	11	3
amp, str, tet	1	-	4
3 resistances - total	16	13	16
amp, cpd, cmp, nft	1	-	-
amp, cpd, cmp, str	-	1	2
amp, cpd, cmp, tet	1	-	2
amp, cpd, str, tet	-	1	1
amp, cmp, str, tet	-	-	3
4 resistances - total	2	2	8
amp, cpd, cmp, nft, tet	-	-	1
amp, cpd, cmp, str, tet	1	1	1
cpd, cpm, nft, str, tet	1	-	-
5 resistances - total	2	1	2

TABLE IV

FIRST SURVEY - RESISTANCE TRANSFER PATTERN.

Resistance	No. of strains		
	Urban Whites	Urban Xhosa	Remote Xhosa
amp	4	10	7
cpd	2	-	-
cmp	-	1	-
str	-	-	1
1 resistance - total	6	11	8
amp, cpd	-	-	2
amp, cmp	-	2	9
amp, str	-	-	1
amp, tet	7	5	2
2 resistances - total	7	7	14
amp, cpd, cmp	1	-	-
amp, cpd, nft	-	1	1
amp, cmp, tet	10	3	3
amp, nft, tet	-	1	-
3 resistances - total	11	5	4
amp, cpd, cmp, nft	1	-	-
amp, cpd, cmp, tet	1	-	-
amp, cmp, nft, str	1	-	-
4 resistances - total	3	-	-

transfer pattern, 100% carried ampicillin resistance, 61% chloramphenicol resistance, 63% tetracycline resistance but only 4% carried streptomycin resistance.

The higher incidence of resistance (82.9%) and R factors (61.5%) in the urban Xhosa (Table I) prompted the initiation of a second survey. It was decided to compare the incidence of resistance and R factors in patients and their environment from an urban and a remote population. This would possibly show any existing relationship between the incidence of R factors in the population and in the environment which it inhabits, and whether such an environment does act as a reservoir for R factors.

(b) Second Survey:

As explained above, it was decided to compare the incidence of resistance and R factors in urban and remote populations and their respective environments. The same urban Xhosa population as had been used in the first survey was chosen. The samples from the remote population were obtained from the Madwaleni Mission Hospital as it provided easy access to a Xhosa population which lived in a remote region and which had not been exposed to antibacterial treatment.

Once again, the incidence of resistance (68.6%) and R factors (47.3%) was high in the urban Xhosa population. The resistance pattern for the urban Xhosa patients in the second survey (Table VI) was essentially the same as far as the urban Xhosa population in the first survey. There were however two major differences; the incidence of

TABLE V

SECOND SURVEY - INCIDENCE OF RESISTANCE AND R FACTORS IN COLIFORM BACTERIA FROM URBAN AND REMOTE COMMUNITIES.

	No. of strains tested	R*	TR⊕
Remote population	90	18.9	11.8
Remote environment	51	25.5	0
Urban population	67	68.6	47.8
Urban environment	83	66.3	23.1

R* = % resistant strains

TR⊕ = % resistant strains which carry R factors

TABLE V1

SECOND SURVEY - RESISTANCE AND TRANSFER PATTERNS FOR INDIVIDUAL DRUGS

DFUG	Remote Patients (50 strains)		Remote Environment (51 strains)		Urban Patients (67 strains)		Urban Environment (83 strains)	
	R*	TR°	R	TR	R	TR	R	TR
Ampicillin	6.7	16.7	3.9	-	49.3	45.4	20.5	29.4
Cephalorexin	8.9	-	15.7	-	5.9	-	41.0	15.6
Cephaloridine	5.5	-	15.7	-	7.3	-	31.3	16.0
Chloramphenicol	1.1	-	2.0	-	19.4	30.7	3.6	66.7
Kanamycin	-+	-	-	-	18.0	67.0	3.6	-
Naladixic acid	-	-	-	-	1.5	-	-	-
Nitrofurantoin	-	-	-	-	-	-	-	-
Streptomycin	11.0	-	6.0	-	46.3	9.7	24.1	-
Tetracycline	1.1	-	-	-	22.4	26.7	7.2	16.7
Trimethoprim	-	-	6.0	-	8.9	-	4.8	-
Septrin	-	-	-	-	8.9	-	1.2	-
Vioform	13.3	-	19.6	-	18.0	-	43.4	-

R* = percentage resistant strains

TR° = percentage resistance strains which transfer resistance

-+ = 0%

TABLE VII

SECOND SURVEY - MULTIPLE RESISTANCE PATTERN.

Resistances	No. of strains			
	Urban Xhosa		Remote Xhosa	
	Patients	Environment	Patients	Environment
amp	-	1	-	-
cpx	-	2	1	-
cmp	-	1	-	1
str	7	8	5	1
tet	2	-	-	-
trm	-	-	-	2
vfm	2	7	6	3
1 resistance - total	11	19	12	6
amp, cpd	1	-	-	-
amp, cmp	2	-	-	-
amp, str	4	2	1	-
amp, vfm	-	2	-	-
cpx, cpd	1	4	1	3
cpx, str	-	1	2	-
cpx, vfm	-	4	-	-
cpd, vfm	-	1	-	-
cmp, str	-	-	1	-
cmp, vfm	-	1	-	-
str, vfm	1	1	-	-
2 resistances - total	9	16	5	3
amp, cpx, cpd	-	1	1	-
amp, cpm, str	1	-	-	-
amp, cpm, tet	3	1	-	-
amp, cpm, vfm	1	-	-	-
amp, kan, str	4	1	-	1
amp, kan, tet	2	1	-	-
amp, kan, vfm	1	-	-	-
amp, str, tet	2	1	1	-
amp, str, vfm	2	-	1	-
cpx, cpd, str	-	1	-	1
cpx, cpd, vfm	1	13	1	3
cpd, trm, vfm	-	1	-	-
3 resistances - total	17	20	4	5
amp, cpx, cpd, vfm	-	1	2	1
amp, cpx, trm, bac	-	1	-	-
amp, cpm, kan, tet	1	-	-	-
amp, kan, str, tet	1	-	-	-
amp, str, tet, vfm	-	2	-	-
amp, str, trm, bac	3	-	-	-
cpx, cpd, str, vfm	1	2	-	-
4 resistances - total	6	6	2	1
amp,cpx,cpd,str,trm	-	1	-	-
amp,cpx,cpd,trm,vfm	-	1	-	-
amp,cpd,kan,str,tet	-	1	-	-
amp,cpd,kan,str,tet	-	1	-	-
amp,cpm,kan,str,tet	2	-	-	-
5 resistances - total	2	3	-	-
Also				
amp,cpm,kan,str,tet, trim,bac	1	-	-	-
amp,cpm,kan,str,tet, trim,bac,vfm	1	-	-	-
amp,cpx,cpd,kan,str, trim,bac,vfm	1	-	-	-
	3	-	-	-

TABLE VIII

SECOND SURVEY - RESISTANCE TRANSFER PATTERN.

Resistance	No. of strains			
	Urban Patients	Urban Environment	Remote Patients	Remote Environment
amp	7	4	2	-
cpx	-	3	-	-
cpd	-	2	-	-
cmp	1	-	-	-
kan	1	-	-	-
str	1	-	-	-
tet	1	-	-	-
1 resistance - total	11	10	2	-
amp, cmp	3	-	-	-
amp, kan	3	-	-	-
amp, tet	1	-	-	-
cpx, cpd	-	2	-	-
2 resistances - total	7	2	-	-
amp, cmp, kan	1	-	-	-
amp, cmp, tet	-	1	-	-
amp, kan, tet	2	-	-	-
3 resistances - total	3	1	-	-
Also amp, cmp, kan, str, tet	1	-	-	-

ampicillin resistance was lower (76% in 1st survey, 49% in 2nd survey) and that for streptomycin was much higher (8% in 1st survey, 46% in 2nd survey). Due to lack of suitable data, no reason could be assigned to this change of resistance pattern. Kanamycin resistance which was not determined in the first survey, was also high, particularly the number of strains which could transfer this resistance (8 out of 12 strains). Kanamycin, because of possible side-effects, is usually reserved for TB infections which do not respond to streptomycin therapy and also for serious bacillary infections. Cohen (1969) suggested that resistance to kanamycin tends to occur as a component of "large" R factors (i.e. multiple-resistant). Out of the 8 strains which transferred kanamycin resistance, the associated resistance pattern was: 1 single, 3 double, 3 triple, 1 quintuple (Table VIII). There does appear to be a tendency for kanamycin resistance to be associated with R factors carrying multiple resistance determinants. Therefore chemotherapeutic agents which are likely to select "large" R factors should be avoided where possible. From the urban Xhosa and their environment, a number of strains were isolated which carried resistances (not necessarily transferable) to more than three drugs (Table VII). Thus 12 strains were resistant to four drugs, 5 to five drugs, 1 to seven drugs and 2 to eight drugs. Although this trend is at present relatively limited, it could result in serious medical implications, particularly if it were to develop among pathogenic bacteria. The arsenal of antibacterial agents available to medical practitioners

would be vastly reduced and they would find themselves almost as helpless as their colleagues in the pre-antibiotic era.

The high incidence (23.1%) of R factors in the environment of the urban community is noteworthy (Table V). Firstly it indicates that R factors can exist in bacteria from the environment. Secondly it confirms our suggestion in the first survey that the crowded urban Xhosa township studied is serving as a reservoir for R factors. This is highlighted by the findings of Grabow and Prozesky (1972). These workers studied the incidence of coliform bacteria carrying R factors in hospital waste water and city sewage. The incidence of resistant strains in the hospital waste water was considerably higher than in city sewage and of the resistant strains from the hospital waste water, 20% carried R factors. Yet the township which we studied is approximately 12 km from the nearest hospital and 23.1% of the resistant strains carried R factors.

Stutevant et al (1971) who studied the incidence of R factors among faecal coliforms isolated from raw sewage suggested that the source of antibiotic resistant coliforms was probably faecal. Similarly, since most samples isolated in the urban Xhosa environment came from sewers, one would expect the source of R factors to be faecal and that it would be related to the incidence of R factors in the urban Xhosa population. However it should be noted that the patterns of multiple resistance and of multiple transfer are different in the patients and in their

environment (Tables VII & VIII).

The resistance and transfer patterns for cephalosporins are interesting. A higher percentage of resistance to cephalosporins was obtained in bacteria isolated from the environment than from stool specimens (Table VI). Only bacteria from the urban environment were found to transfer resistance to cephalosporins. The cephalosporins are at present not used extensively in either this urban community or in the mission hospital studied. Cephalosporin was first obtained from the fungus Cephalosporium isolated from a sewer in Sardinia (Abraham, 1967). If this fungus is present in the environment, it may have resulted in the selection of cephalosporin resistant strains. The use of two different cephalosporins was motivated to determine whether coliforms which were resistant to a cephalosporin administered orally (cephalexin) would also be resistant to one administered parenterally (cephaloridine). Table VII shows that out of 57 strains which were resistant to either drug, 41 were resistant to both, 12 to cephalexin only and 4 to cephaloridine only. This may be due to a slight difference in activity between the two drugs. However it should be noted that different methods were used to test for sensitivity to each drug. Cephalexin was incorporated in a selective medium whereas cephaloridine sensitivity discs were used and this may also account for the difference.

Another interesting feature of cephalosporin resistance (Table X) is that the majority (61 out of 76) of the

resistant strains fell within the "other coliforms" classification (i.e. not *E. coli*). Although it might be argued that the activity spectrum of the cephalosporins may not extend to the "other coliform" strains, this does not seem very likely if it is considered that 8 of these strains were able to transfer their resistance to one or both cephalosporins. In the case of vioform resistance, it is also found that the majority of "other coliforms" (42 out of 60) are resistant to vioform while only 33 out of 231 *E. coli* are resistant to this drug (Table X). Information on the spectrum of vioform is scarce, probably because of the difficulties encountered in preparing suitable selective media (vioform is almost insoluble in aqueous solutions) and also because it has been mainly used as an amoebicide. A report by Diding and Ström (1957) however showed *E. coli* to be the only strain among a number of Gram-negative strains tested, to be inhibited by vioform. Another interesting feature about the "other coliform" strains is that although they show a higher incidence of resistance than *E. coli* strains, they show a lower incidence of resistance transfer (Table IX). However it should be remembered that an *E. coli* strain was used as recipient in transfer experiments and the "other coliforms" may not have been able to mate as efficiently with *E. coli* as with a strain of their own genus. Watanabe (1963) pointed out that the frequencies of transfer depended to a large extent on the type of donor and recipient used.

TABLE IX

SECOND SURVEY - INCIDENCE OF RESISTANCE AND R-FACTORS IN *E. COLI* AND OTHER COLIFORMS FROM URBAN AND REMOTE COMMUNITIES.

AREA	*E. coli*			Other coliforms		
	No.	R*	TR⊕	No.	R	TR
Urban	106	60.4	43.7	44	84.1	19
Remote	125	14.4	11.1	16	75.0	0

*R = % resistant strains

⊕TR = % of resistant strains with R factors

TABLE X

SECOND SURVEY - INCIDENCE OF RESISTANCE TO VIOFORM AND CEPHALOSPORINS IN *E. COLI* AND "OTHER COLIFORMS".

	E. coli	Other coliforms
Total No. isolated	231	60
No. resistant to vioform	33 (14.3%)	42 (70.0%)
No. resistant to cephalorexin	9 (3.9%)	33 (55%)
No. resistant to cephaloridine	6 (2.6%)	28 (46.7%)

The detection of only two R factors in 141 specimens from the remote community suggests that R factors have a low incidence in drug-free communities. This agrees with the results of Maré (1968) and Gardner et al (1969). Maré investigated the incidence of R factors among bacteria isolated from 47 Kalahari bushmen and 535 wild animals in drug-free communities and no R factors were detected. Gardner et al investigated an antibiotic virgin population in the Solomon Islands. Out of 40 specimens processed, R factors were recovered from only 2, one originating from a soil sample and the other from the stool sample of one of the inhabitants. The striking difference in the incidence of R factors between the urban and remote communities provides strong evidence for the selective pressures of antimicrobial drugs.

(c) Additional Minor Surveys:

Out of 14 strains isolated from the school children, 2 were resistant to vioform and 1 to the cephalosporins and to vioform (this strain being the only "other coliform" whereas all the others were E. coli strains). The children from whom these strains were isolated all lived on remote rural farms and the lack of R factors substantiate the results of the second survey, i.e. the low incidence of R factors in the remote communities.

The results of the limited surveys carried out in the TB and General Wards of the Madwaleni Hospital are given in Tables XI and XII. Out of 31 strains, only one, which was isolated in the TB ward, was able to transfer resistance

TABLE XI

ADDITIONAL SURVEYS - RESISTANCE PATTERN FOR INDIVIDUAL DRUGS.

Drug	General Ward (16 strains)	T.B. Ward (15 strains)	School children (14)
Ampicillin	-	3	-
Cephalorexin	2	-	1
Cephaloridine	1	1	1
Chloramphenicol	-	1	-
Kanamycin	-	-	-
Naladixic acid	-	-	-
Nitrofurantoin	-	-	-
Streptomycin	7(45%)	13(86.7%)	-
Tetracycline	-	2	-
Trimethoprim	-	-	-
Septrin	-	-	-
Vioform	1	1	3

TABLE XII

ADDITIONAL SURVEYS - MULTIPLE RESISTANCE PATTERN.

Resistance Pattern	No. of strains		
	General Ward	T.B. Ward	School children
cpx	1	-	-
str	6	7	-
amp, str	-	1	-
cpx, cpd	1	-	-
cpd, str	-	1	-
str, tet	-	1	-
str, vfm	1	1	-
amp, cmp, str, tet	-	1	-

(ampicillin and chloramphenicol). The high number of strains from the TB ward which showed resistance to streptomycin (13 out of 15) indicates once again the strong selective pressures which ensue from drug therapy. These patients provide a hazard since upon release from hospital they will return to live in the remote community where they can possibly disseminate resistant strains.

(d) Conclusions:

The surveys carried out demonstrated the strong selective pressures of antibacterial drug therapy and the low incidence of R factors in communities where such pressures are absent. There are few regions which have not yet been influenced by modern antibacterial therapy but which are readily accessible to research laboratories. The Transkei is such a region and the rapid developments within it from a rural to a modern industrial comples will no doubt cause major ecological changes. Constant monitoring of the pattern of drug resistance in the bacterial flora in the inhabitants and the environment may thus prove useful in analysing the development of resistance in bacterial populations. Investigations could then determine whether careful control over the distribution and prescribing of antibiotics can prevent the increasing pattern of resistance which has occurred in other industrialized communities. A simple computer programme (Appendix C) has been prepared which in large surveys can help to determine rapidly the various combinations of resistances for up to four resistances per strain.

C H A P T E R II

SURVEY OF SEX FACTORS.

Summary

A survey of F-type sex factors was carried out using lysis by MS 2 phage as indicator for the presence of such factors. No lysis was detected in 588 strains tested (of which 283 were drug-sensitive strains).

R^+ cells were treated with the mutagen, nitrosoguanidine, in order to obtain a derepressed mutant of an R factor. No derepressed mutant was isolated. Attempts to isolate non-transferable drug resistance plasmids by curing with acriflavine or with ethidium bromide were unsuccessful.

(A) Materials and Methods:

The presence of sex factors was determined by plaque formation using male-specific bacteriophages. Since these phages enter the bacterial cell by attachment to sex pili, it is essential that the sex factor be able to produce such pili. Cells harbouring the F sex factor produce sex pili to which phage can attach. However most R factors produce a repressor which inhibits the formation of sex pili. Thus plaques will only be formed where the sex factor is in a derepressed state.

(a) Media, Drugs and Chemicals:

Preparation of all media, drug and chemical solutions are described in Appendix A.

(b) Phages:

Two male-specific phages were used:

(1) Phage MS 2 - a RNA phage which is specific for E. coli containing the F sex factor or any other sex factors which when in the derepressed state produce sex pili similar to those produced by the F sex factor (e.g. certain R factors and colicin factors). This phage was obtained from Prof. W. Hayes.

(2) Phage fil I - a DNA phage which is specific for E. coli containing sex factors which produce pili similar to those produced by the col I colicin factor. This phage was obtained from Prof. A. van Rensburg.

(c) Phage Techniques:

High titre lysates were prepared and assayed by the modification (Adams, 1959) of the double-agar-layer method

of Hershey, Kalmanson and Bronfenbrenner (1943). Phage sample (0.1ml) was added to 3ml. molten sloppy agar seeded with 0.1ml. overnight E.coli Hfr culture and the mixture poured over a nutrient agar plate. For the preparation of high titre phage lysates, 3 top agar layers showing total lysis were scraped off and added to 5ml. nutrient broth. Agar débris and bacterial cells were removed by centrifugation at 2,000g. and 10,000g. respectively. The cleared phage suspension was stores over chloroform (5-10% v/v) at 4°C.

The same techniques were used in an attempt to prepare high titre lysates of the fil 1 phage using E. coli SfI strain (which contains a col I factor) as host.

(d) Survey of F-type Sex Factors:

In order to determine the incidence of F-type sex factors in both sensitive and resistant bacteria, all the coliform strains were tested.

MS2 phage suspension was spotted on the surface of double-agar plates seeded with the bacterial test strain. The plates were observed for areas of lysis after overnight incubation. As the number of plates required for this experiment was high, a modification was developed which allowed 4 strains to be tested on each plate. Glass rods (5mm. diam.) were used to make crosses which fitted exactly into plastic petri dishes (8.6cm. diam.). The glass crosses were sterilized by autoclaving, stored in alcohol and flamed immediately prior to being used. The crosses were placed in the petri dishes before the lower nutrient agar layer had set. In this way four different strains could

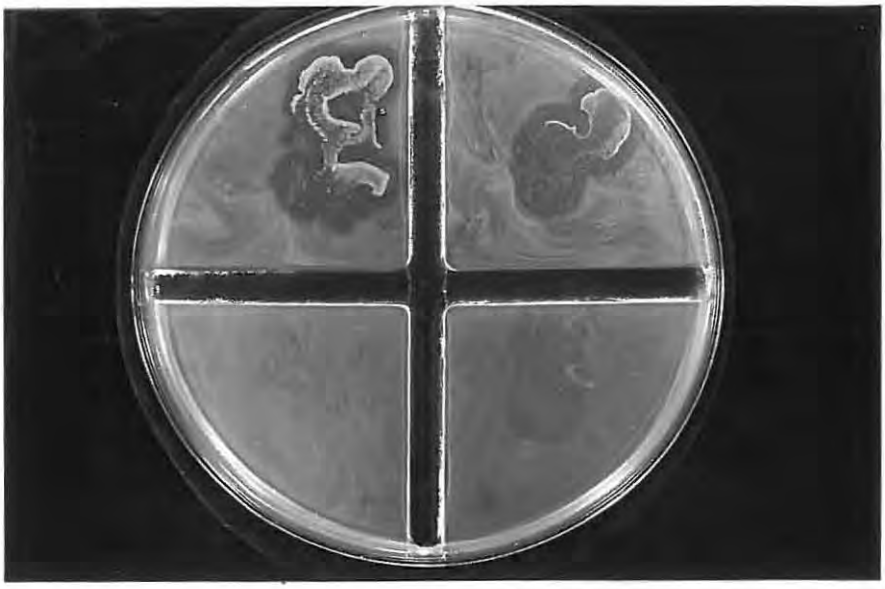


PLATE V - Lysis with phage MS2.

Controls with E. coli HfrH, E. coli HfrC, E. coli F-prime and E. coli F^- (no lysis; bottom left hand corner).

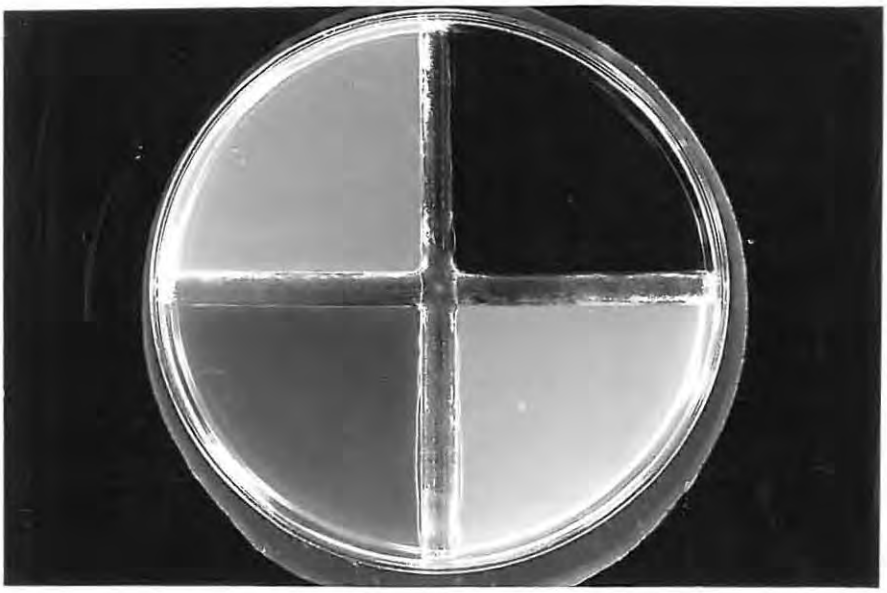


PLATE VI - Sectored agar plate.

Each sloppy agar sector contains a dye of different colour. No seepage between sectors is observed.

be tested, each in a different quarter demarcated by the glass cross (see plate V). The amount of sloppy agar used per strain had to be reduced from 3.0ml. to 1.5ml. so as not to overflow into adjacent sectors. To test whether there was any seepage into adjacent sectors, sloppy agar containing dyes of different colours were used. No such seepage was observed (see plate VI).

(e) Isolation of mutant de-repressed R factors:

An attempt was made to find a rapid method for the isolation of mutant de-repressed R factors so that the percentage of F-type R factors could be established readily in large batches of bacterial samples. The mutagen N-methyl-N'-nitro-N-nitrosoguanidine (NTG) was tested for its ability to yield de-repressed mutants. The strain used in this experiment was a wild-type E. coli which was resistant to ampicillin, chloramphenicol and streptomycin and which transferred resistance to the first two drugs.

(1) Method using NTG:

To 4.5ml. of prewarmed (37°C) fresh nutrient broth in a McCartney bottle, 0.5ml. of an overnight broth culture of the E. coli strain was added. The 5ml. culture was incubated for 90 minutes (to obtain a log phase culture) and was then centrifuged (2,000g. for 10 minutes). The pellet was resuspended in 5ml. of fresh prewarmed nutrient broth and 0.1ml. of NTG solution (1.5mg/ml.) added. The NTG-treated cells were incubated for 15 minutes, centrifuged and washed twice with 0.85% saline to remove the NTG. An aliquot (0.1ml.) of the washed cell suspension was transferred

to 5ml. of nutrient broth and incubated overnight. Dilutions of the overnight culture were plated on nutrient agar and single colonies were tested in either of two ways:

(i) One hundred and fifty colonies were selected and tested for lysis with MS2 phage as described in the previous paragraph (Survey of F Sex Factors).

(ii) Plates containing between 100-200 colonies were replica-plated onto a layer of sloppy agar seeded with the E. coli recipient strain (25 R, nal--resistant) and containing ampicillin (30 µg/ml) and naladixic acid (30 µg/ml). The idea behind this method was that if one of the colonies being replica-plated contained a de-repressed R factor, it would possibly transfer its R factor at high frequency to the recipient and ampicillin + naladixic resistant recombinants would arise and grow on the selective medium.

(f) Survey of Non-transferable Resistance Plasmids:

Hirota (1960) showed that the F sex factor in E. coli could be "cured" (eliminated) by growing the bacterium in nutrient broth containing acridine dyes. Similar curing has been reported with R factors in E. coli grown in the presence of acridine dyes (Watanabe and Fukasawa, 1961) or ethidium bromide (Bouanchaud et al, 1968).

It was decided to try both methods in an attempt to isolate non-transferable plasmids which carried drug resistance genes. Strains harbouring these plasmids could be used to detect sex factors in other bacteria by the recombination method of Anderson (1965).

(1) Elimination of plasmids with acriflavine.

This experiment was carried out on all strains isolated in the first survey which showed resistance to one or more drugs but which did not transfer resistance in mating experiments. The strain to be tested was grown overnight in 1ml. nutrient broth containing ampicillin (30 µg/ml). One loopful of the overnight culture was diluted in 1ml. sterile saline. A loopful of this suspension was then inoculated into

(i) 1ml. of nutrient broth

(ii) 1ml. of nutrient broth + acriflavine (50 µg/ml)

These cultures were incubated overnight. Suitable dilutions of the overnight cultures were prepared in sterile saline and plated on nutrient agar. After incubation for 24 hours, isolated single clones were transferred by stabbing or replica-plating onto nutrient agar plates containing ampicillin (30 µg). Although more tedious, stabbing was found to be more reliable than replica-plating. For each strain, 50 colonies originating from the nutrient broth culture and 50 colonies originating from the nutrient broth culture + acriflavine were transferred by stabbing to the nutrient agar + ampicillin plates. In the case of there being no drug resistance plasmid (or there being no effective elimination of plasmids), all 50 colonies from each culture were expected to grow on the nutrient agar containing ampicillin. In the case of curing, it was expected that a certain percentage of the colonies grown in nutrient broth containing acriflavine would be unable to grow on nutrient

agar containing ampicillin.

Sixty one strains were subjected to the above tests. Another 8 strains which were sensitive to ampicillin were treated the same way but ampicillin was omitted from the media. Instead, one of the drugs to which these strains showed resistance was used. It should be noted that a number of strains were inhibited by 50 µg/ml. of acriflavine. Thus with these strains the above procedure had to be repeated using a lower concentration of acriflavine (10 µg/ml.).

Four strains (all of which grew in 50 µg acriflavine) were also tested using a range of acriflavine concentrations (5, 25, 50, 75 and 100 µg/ml.) to determine whether a minimal concentration of acriflavine was required to induce curing.

(2) Elimination of plasmids with ethidium bromide.

Bouanchaud et al (1968) reported that ethidium bromide was more effective in curing R factors than acridine dyes. It was thus decided to try eliminating R factors using ethidium bromide and should the method prove successful, it could then be used to isolate strains carrying non-transferable resistance plasmids.

Ten strains from the Second Survey carrying R factors were tested. The procedure adopted was essentially similar to that described using acriflavine. However the acriflavine was replaced by ethidium bromide (10^{-4}M).

(B) Results and Discussion:

The survey of sex factors was limited to F sex factors as no high titre preparation of phage fil I could be obtained. This was because no suitable derepressed E. coli strain could be isolated on which to grow the phage. The only strain available (E. coli SfI provided by Prof. van Rensburg) proved to be unsuitable for the following reasons:

(a) The strain did not grow well on nutrient agar or in nutrient broth.

(b) The col I factor harboured by the strain was supposed to be in a derepressed state when received. However when phage growth was attempted, the col I factor had already reverted to the repressed state and hence phage could not infect the E. coli strain. The strain possibly reverted to the repressed state prior to being sent to our laboratory since it had been stored for some time on nutrient agar without being used.

No such problems were encountered in building up high titre preparations of phage MS2.

Most surveys of transferable plasmids have been carried out on strains harbouring R factors. However, literature on the incidence of sex factors in sensitive bacterial strains is scarce. It was decided to investigate the incidence of F-type sex factors in all the strains isolated during the surveys. Since most R factors are usually in the repressed state, there was little hope of obtaining an indication of the preponderance of F-type R factors. Likewise, the presence of any other F-type sex factor

would probably be repressed by the repressor protein produced by F-type R factors. However it was hoped that the presence of de-repressed F-type sex factors (e.g. the E. coli F sex factor) would be detected in sensitive cells or in cells harbouring R factors whose repressor does not affect the F-type sex factors (e.g. I-type R factors). Controls with E. coli Hfr and F-prime strains showed that the method of lysis with MS2 phage which was employed, was adequate (see plate V). In all, 588 strains were tested, 283 of them drug-sensitive strains. None of the strains showed lysis with MS2 phage. These results indicate the low incidence of F-type sex factors in sensitive strains.

Mitsuhashi et al (1969) investigated the distribution of genetic elements among clinical isolates capable of conferring conjugal ability on non-infectious drug-resistant determinants. Out of 105 strains tested, 42 carried such genetic elements which were labelled "T factors". However, although they suggested that one of these T factors appeared to be different to the F factor, they did not provide conclusive evidence as to the real nature of these factors. The method which they used was a modification of the method of Anderson and Lewis (1965; see p. 14) which involves the use of a non-transferable drug-resistant plasmid. To establish a similar system, an attempt was made to isolate a non-transferable plasmid. All the strains in the first survey which showed drug resistance but which could not transfer this resistance were treated with acriflavine. Acridine dyes had been reported to "cure"

plasmids by Watanabe and Fukasawa (1961). No curing was obtained with any of the 29 strains tested. This could either be due to the absence of non-transferable plasmids or to the low efficiency of curing by acriflavine. Doubt has however been expressed as to the real curing ability of acridine dyes (Watanabe, 1969) and it has been suggested that the so-called curing may be due to selective accumulation of spontaneous plasmidless segregants in the presence of acridine dyes. It was reported by Bouanchaud et al (1968) that ethidium bromide was a more effective agent than acridine dyes in curing R factors. The method which they employed was tried on 10 strains isolated in the second survey which harboured R factors. Again no curing was obtained and no further strains were tested. Chabbert et al (1969) reported that curing of R factors with ethidium bromide varied greatly with different R factors.

An attempt was also made to isolate a de-repressed mutant of an R factor. Essentially, what was required was a method which would rapidly and easily yield de-repressed R factor mutants so that large numbers of bacterial strains could be screened for the type of R factors which they harbour. Adelberg et al (1965) showed that under certain conditions over 40 per cent of the survivors of nitrosoguanide (NTG) treatment may be mutants. Therefore NTG was used as a mutagen to try and induce mutations to obtain a de-repressed R factor. However no de-repressed R^+ cells were obtained. Meynell et al (1968) have developed other methods for the isolation of de-repressed R factors but these methods do

not lend themselves to rapid screening of large numbers of strains. Clearly, other methods will have to be devised for the rapid identification of the type of R factor present in a bacterial cell.

PART II

MOLECULAR BIOLOGY STUDIES OF A *PSEUDOMONAS AERUGINOSA* R FACTOR IN *ESCHERICHIA COLI*.

Introduction

The study of the physical and biochemical properties of R factors have helped in explaining some of their genetic and physiological properties. The autonomous replication and the conjugal transfer of R factors would indicate that they probably consist of the same genetic material as chromosomes, i.e. DNA. This has been shown to be the case, for example, by labeling techniques. Watanabe and Takano (in Watanabe, 1963) studied the effect of decay of ^{32}P on R factors in *E. coli* K-12. Their results indicated that resistance transfer factors are composed of DNA with a molecular weight close to those of F′ and phage lamda (Stent *et al*, 1957).

Schildkraut *et al* (1962) have shown that members of the *Enterobacteriaceae* differed significantly in their DNA base composition. Nakaya *et al* (1960) reported that R factors were freely transmissible to many members of the *Enterobacteriaceae*. Taking advantage of these features, Rownd *et al* (1966) examined R factors in a number of hosts in an attempt to obtain information on their base composition. An R factor from *E. coli* was transferred to *Proteus mirabilis*, *E. coli* and *Serratia marcescens*. DNA was isolated from the recombinant strains and analyzed by CsCl density-gradient centrifugation. Satellite DNA bands were obtained

by analytical density centrifugation which showed the R factor to consist of two species of DNA, the base composition of which was 58% and 52% G + C respectively. Treatment of the bacterial cells with acridine dyes or U.V. light resulted in the elimination of the R factor, and satellite DNA peaks could not be isolated from these "cured" cells. Using similar techniques, Falkow et al (1966) showed that different determinants of R factors consisted of different G + C ratios. For example, they found the determinant for chloramphenicol resistance to have a 56% G + C ratio (density 1.716 g/cm^3), the kanamycin, streptomycin and sulphonamide resistance determinants lay between 1.711 and 1.714 g/cm^3, while the tetracycline resistance determinant and the transfer factor DNA had a density of 1.709 g/cm^3, corresponding to 50% G + C ratio.

Marmur and Doty (1959) have shown that the melting temperature (Tm) of DNA is related to its G + C ratio; the higher the G + C ratio, the higher the Tm. Guerry and Falkow (1971) have used this relationship to determine the degree of polynucleotide sequence similarity between various R factors. Although F-type factors showed a high degree of similarity with each other, they were not closely related to I-type factors.

Another method used in isolating R factor DNA so that it may be studied relatively free of chromosomal DNA has been the isolation of minicells (Adler et al, 1967). These are mutants of E. coli cells which bud off at their extremities. In doing so, the portions which bud off

often contain plasmid DNA but no chromosomal DNA. Inselburg (1971) has analysed R factor DNA in minicells and shown it to exist as double-stranded linear, open circular and twisted circular monomers. In the minicell, the R-DNA can replicate although the extent of the replication appears to be limited. Levy (1971), also working with R factor DNA in minicells, has shown this system to offer a simple means of isolating large quantities of episomal DNA.

Because the density of most R factors corresponds closely to that of E. coli, Salmonellae and Shigellae chromosomal DNA, it has been difficult to study R factors in these species of bacteria. Since the DNA of Proteus has a density sufficiently different from that of R factor DNA, several R factors have been transferred to Proteus strains prior to being studied. During early work on the biochemical composition of R factors, it was noticed that the amount of R factor DNA in Proteus differed markedly from that in other hosts. Rownd et al (1966) observed that there was an average of one R factor copy per chromosome in E. coli and S. marcescens, but 12 copies per chromosome in P. mirabilis. Further work by Falkow et al (1969) has shown that the amount of R factor DNA in P. mirabilis varies at different growth phases. Throughout the exponential phase, the percentage R-DNA (% relative to host chromosomal DNA) remains constant. After entry into the stationary phase, the percentage R-DNA increases with time and levels off at a value of 48% after about 6 hours in stationary phase.

This corresponds to an increase in the number of R factors per host chromosome of 30 to 40 (Rownd et al, in Mitsuhashi, 1971, p. 123).

Silver and Falkow (1970) have confirmed that in E. coli, the R factor R1 exists as a single composite unit, of which there is only one per chromosome. This same R factor when studied in P. mirabilis resulted in a number of copies of three distinctly different units (Falkow et al, 1969). The first unit was shown to be the transfer factor, the second unit the resistance determinants and the third was a composite structure formed by recombination of the transfer factor and the resistance determinants. Van Rensburg (1972) has studied the replication of the various components of an R factor in P. mirabilis. In stationary phase cultures, this R factor DNA comprised 24% of the total amount of DNA and was represented by 10 copies of 50% G + C DNA and 36 copies of 58% G + C DNA. Replication of the different R factor components was studied under conditions which inhibited either protein or DNA synthesis. Inhibition of protein synthesis increased the R factor DNA fraction to 68% with a concomitant increase in the 58%/50% G + C ratios to 8:1. A change to minimal medium or cooling of the medium caused selective inhibition of the 50% G + C component. Van Rensburg suggested that a possible explanation for these findings was that the 50% G + C component (the transfer factor) of the R factor was membrane-bound and replicated in unison with the genome while the 58% G + C component replicated under relaxed control.

Rownd (1969) has proposed a model for replication of

episomes under relaxed control. The model predicted that individual copies of the R factor are selected at random for replication without discrimination as to whether any particular copy had been duplicated one or more times during that generation. The mechanism of doubling of the multicopy pool of R factors in P. mirabilis was investigated by transfering an exponential culture from ^{14}N-labeled medium to ^{15}N-labeled medium. The observed distribution of light, hybrid and heavy R factor DNA during the first generation in ^{15}N-labeled medium led to the following conclusions regarding the replication of individual R factors within the population of episomes:

(a) The initiation of replication of all the episomes within the pool could not have occurred simultaneously.

(b) The initiation of replication of at least some R factor copies could not have occurred simultaneously with the initiation of the host chromosome.

(c) The replication of any one R factor could not have occupied more than one fifth of the bacterial division cycle.

Rownd describes his model of random replication as follows: "The n copies of the episome exist in a pool from which individual copies are withdrawn at random for replication. After the first round of episome replication during any particular generation, the two replicas are returned to the pool and the pool is thoroughly mixed. For the next round of replication, another episome is selected from the pool without discriminating as to whether it has already

participated in replication during that generation. At any time during the bacterial division cycle, the probability of selecting a parental (unreplicated) episome or an episome which has already replicated during that generation depends on the weight fraction of the two classes of episomes in the pool. This process of random selection is repeated n times during each generation, that is, until there has been a doubling of the number of episomes. In an ^{14}N-^{15}N transfer experiment, this model predicts that approximately 25% of the episome DNA will be light (unreplicated), 50% will be hybrid in character (replicated once) and 25% will be heavy (replicated more than once) after one generation of ^{15}N-labeled medium."

Rownd also discusses his model in relation to the "replicon" model of Jacob et al (1963) and finds both models to be compatible but points out that the notion of a cytoplasmic pool of R factors does suggest that the replicon is not permanently attached to a membrane replication site when harboured in P. mirabilis.

Replication of R factors in P. mirabilis has also been likened to phage replication, and lysis due to multiple copies of R factors has been reported (Falkow et al, 1969; Van Rensburg, 1972).

The size of R factors has been determined by a number of methods (e.g. ^{32}P-incorporation, sedimentation, etc...). However, perhaps the most satisfactory results have been obtained with electron micrographs of R factor DNA. The size of the molecules varies in the literature but this is

usually because different R factors are being studied in different laboratories. Nisioka et al (1969) studied R factor 222/R3 in P. mirabilis and, using density-gradient centrifugation techniques, obtained a satellite DNA band with three peaks at densities 1.708, 1.711 and 1.717 g/cm^3. Electron micrographs revealed circular structures from each band with contour lengths, respectively of 29 μm (54×10^6 daltons), 36 μm (68×10^6 daltons) and 6 μm (12×10^6 daltons). It was suggested that the 36 μm molecule consisted of a recombination of the 29 and 6 μm components.

An E. coli strain harbouring an R factor originally isolated in a Pseudomonas aeruginosa strain was sent to us by Prof. van Rensburg. No work had yet been published on the molecular biology of an R factor isolated in Pseudomonas. It was decided to try and characterize this R factor as far as possible with the techniques and apparatus available in our laboratory.

C H A P T E R I

ISOLATION OF AN *ECHERICHIA COLI* THYMINELESS MUTANT.

Summary

A thymineless mutant of a naladixic acid resistant *Escherichia coli* strain was isolated. An R factor originating from a *Pseudomonas aeruginosa* strain was transferred to the *E. coli* thymineless mutant, thus facilitating the isolation of the R factor DNA.

(A) Materials and Methods.

(a) Media, Drugs and Chemicals:

Preparation of all media, drug and chemical solutions are described in Appendix A.

(b) Source of R Factor and Bacterial Strains:

The R factor studied was isolated in a Pseudomonas aeruginosa strain from a patient in Bloemfontein. When received by us, the R factor had been transferred by Prof. A. van Rensburg to an E. coli strain. This strain was received stored on nutrient agar. Although when originally isolated the R factor carried resistance determinants to ampicillin, streptomycin, sulphonamide and tetracycline, only the ampicillin resistance determinant remained when the E. coli strain was tested for its resistance spectrum in our laboratory.

Since one of the methods to be used in the study of this R factor involved labeling with tritiated thymidine, it was decided to isolate a thymineless mutant which could act as host strain for the R factor. The naladixic acid resistant E. coli used as the recipient strain in previous mating experiments was chosen as the host strain. This strain had been named 25 R (see p. 22). A thymineless mutant of 25 R was isolated as described below and the Pseudomonas R factor transferred to it.

(c) Isolation of Thymineless Mutant:

Two methods were used to try and isolate a thymineless mutant. The first method tried was a modification of the method for the isolation of auxotrophic mutants

developed by Davis (1948) and Lederberg and Zinder (1948). The method is based on the fact that penicillin kills only growing bacteria. Hence in a minimal medium, only prototrophs will grow and be killed whereas auxotrophs can be rescued.

The second method used was a modification of the procedure described by Stacey (1968). This method is based on the fact that E. coli mutants which require thymine can grow in the presence of aminopterin and thymine much better than thymine-independent strains, despite the ample supply of thymine. Thus, by prolonged incubation in a medium containing aminopterin, any thymineless mutant will outgrow other cells and can then be isolated.

(1) Penicillin method.

Mutation was induced in the E. coli strain (25 R) with nitrosoguanidine (NTG) by the method previously described (p. 53). The NTG-treated cells were washed twice with saline, re-suspended in 10ml. saline and 0.3ml. of this suspension transferred to 10ml. nutrient broth (NB). The culture was incubated overnight with aeration. The broth culture was diluted 10^{-1} in saline and 0.1ml. of the dilution transferred to 10ml. minimal medium (MM) containing penicillin (200 µg/ml). The culture was incubated for 6 hours with aeration. Dilutions of this culture were plated on slightly enriched MM agar (2.5 ml NB/100ml. MM). The plates were incubated for 24 hours. Small colonies (presumptive auxotrophic mutants) were transferred by stabbing to MM agar and MM agar + thymine

(50 µg/ml). The plates were incubated for 48 hours and then compared. Growth on MM + thymine but not on MM would indicate a thymineless mutant. (Note that MM was not supplemented with other bases, amino acids or vitamins as only thy^- mutants were required.)

(2) Aminopterin method.

The following 4 tubes were set up:

	A	B	C	D
Minimal medium	5ml	5ml	5ml	5ml
Aminopterin (3.5 mg/ml 0.01 N NaOH)	-	-	1ml	1ml
0.1N HCl (to neutralize NaOH)	-	-	0.1ml	0.1ml
M9 Buffer	1ml	1ml	-	-
Thymine (0.5 mg/ml)	0.5ml	0.5ml	-	0.5ml
Bacterial culture	-	0.1ml	0.1ml	0.1ml

Tube A - test of sterility of medium

Tube B - test of normal growth of bacteria

Tube C - test of effect of aminopterin

Tube D - test of effect of thymine

Tubes B, C and D were inoculated with 0.1ml of an overnight MM culture of the wild-type E. coli strain (25 R). The tubes were incubated for 24 hours and examined for growth.

Tube A - no growth

Tube B - good growth

Tube C - little growth

Tube D - good growth

Dilutions of tube D were plated on nutrient agar and

incubated overnight. One hundred colonies were transferred by stabbing to MM agar and MM agar + thy (50 µg/ml). After incubation for 24 hours, the plates were examined and 10 colonies appeared to be thy$^-$ mutants. Four strains which did not grow well were discarded. Colonies of the remaining 6 strains were suspended in saline and streaked onto MM agar + thy plates. To test the concentration of thymine required to maintain growth of the thy$^-$ strains, each strain was grown overnight, with aeration, in MM + thy (2 µg/ml) and MM + thy (50 µg/ml). None of the strains grew in 2 µg/ml of thymine and 4 grew on 50 µg/ml thymine. A mutant was required which grew at a thymine concentration of less than 5 µg/ml. The four strains which grew were plated onto MM agar plates containing 0, 2, 4 and 6 µg/ml thymine. It was noticed that 3 of the 4 strains occasionally grew on MM (i.e. either they were reverting to wild types or they were leaky mutants). These 3 strains were rejected. After repeated subculturing of the remaining strain on low concentrations of thymine, a stable thy$^-$ mutant clone was isolated which was able to grow on 2 µg/ml thymine. This strain was named 25 Rt. To transfer the Pseudomonas R factor to 25 Rt, mating was carried out with the E. coli strain harbouring the R factor as previously described (p. 22). Mating had to be carried out in nutrient broth as the donor strain did not grow on MM. The mating mixture was centrifuged and the cells washed twice with saline. The cells were resuspended in saline and recombinants were isolated on MM + thy + amp + nal.

A single colony was picked and rechecked for its growth characteristics on MM and MM + thy (2 µg/ml) and for its resistance spectrum (amp and nal only).

This strain, named 25 Rtr, was stored on MM + thy (2 µg/ml).

(B) Results and Discussion.

It is unfortunate that 3 out of 4 resistant determinants were lost from the R factor prior to work being done on it. Had this segregation of determinants happened at a later stage, one might have been able to isolate each segregant and studied them separately as well as together. A similar phenomenon was reported by Grinsted et al (1972) who also worked with a Pseudomonas R factor carrying several resistance determinants, some of which segregated on storage. However they were able to isolate a clone in which all the determinants existed stably.

The penicillin method yielded no thy^- mutant. The aminopterin method yielded a number of potentially useful mutants and eventually one was isolated which only required 2 µg/ml thymine for growth. According to Stacey (1968), one mutation yields mutants which require 50 µg/ml thymine and a further mutation reduces this requirement to less than 5 µg/ml.

The two strains isolated, 25 Rt and 25 Rtr, were used in the experiments to extract labeled chromosomal and R factor DNA.

C H A P T E R II

ISOLATION OF LABELED CHROMOSOMAL DNA.

Summary.

Techniques were developed for labeling and isolating E. coli chromosomal DNA. Due to the small yield of bacteria in labeled minimal medium, only low concentrations of DNA could be isolated.

(A) Materials and Methods.

These experiments were carried out to develop for our laboratory techniques for labeling, extracting and purifying DNA as well as density-gradient centrifugation and isotope-counting techniques.

The method employed was a modification of the extraction procedure developed by Marmur (1961).

(a) Bacterial Strain:

E. coli 25 Rt (see p. 72).

(b) Preparation of Cells:

The E. coli strain was inoculated in 150ml of MM (supplemented with 2 µg/ml thymine and 0.75 ml H^3-thymidine) and incubated overnight with aeration. The cells were harvested by centrifugation (2,000g for 10 min.) and washed twice with saline EDTA (total volume, 30ml.).

(c) Lysis of Cells:

The washed cells were re-suspended in 9ml saline EDTA, 5mg lysozyme added and the mixture was incubated at 37°C for 40 minutes. Sodium dodecyl sulphate (1.0ml of 25% solution) was added and the mixture incubated at 60°C for 15 minutes. It was then allowed to cool to room temperature.

(d) Protein Precipitation:

Sodium perchlorate (2.5ml of 5M solution) and an equal volume ($\pm$ 12ml) of chloroform/iso-amyl alcohol (24:1) was added. The mixture was gently mixed on an angled turntable (33 rpm) for 30 min. and then centrifuged (2,000g for 10 min.). The bottom chloroform layer was removed with a Pasteur pipette and the aqueous layer gently transferred to a clean

container. The chloroform/iso-amyl alcohol extraction was repeated 2 - 4 times (i.e. until the aqueous layer was clear).

(e) Nucleic Acid Precipitation:

Two volumes of cold ethanol ($\pm$ 20ml) was added to the clear aqueous layer from the protein precipitation and the two layers mixed with a glass rod. Any spoolings on the rod were dissolved in dil. SSC. The mixture was centrifuged (2,000g for 30 min.), the precipitate dissolved in dil. SSC and pooled with the spoolings (total volume, 3 - 5ml).

(f) RNA Degradation:

Ribonuclease (0.5ml of a 0.5 mg/ml solution) was added to the nucleic acid solution and incubated at 37°C for 60 - 75 minutes. The RNAse solution was adjusted to pH 5.0 and incubated at 80°C for 10 min. to inactivate any DNAse in the RNAse. Tests were carried out to verify the purity of the RNAse and also whether the above treatment altered its activity. Three solutions were prepared:

(i) DNA (0.5 mg/ml) - sperm herring DNA in SSC.

(ii) RNA (0.5 mg/ml) - yeast RNA in SSC.

(iii) DNA/RNA (0.25 mg/ml of each).

Each of the above solutions was divided into 3 aliquots. To the first aliquot 0.5ml of SSC was added, to the second aliquot 0.5ml of untreated RNAse solution was added and to the third aliquot 0.5ml of treated RNAse solution was added. These solutions were incubated for 60 - 75 min. at 37°C, and then dialysed overnight against 500ml SSC. The spectrum of each aliquot was then determined spectrophotometrically.

(g) DNA Precipitation:

Acetate-EDTA solution (0.5ml) and cold isopropanol (3 - 4ml) was added to the cooled RNAse-treated solution while stirring with a glass rod. Any spoolings on the rod were dissolved in dilute SSC. The mixture was centrifuged (2,000g for 30 min.), the precipitate dissolved in dilute SSC and pooled with the spoolings (total volume, 2 - 5ml).

(h) Purification of DNA by Sucrose Gradient Density Centrifugation:

Samples (1.5ml) of the DNA solution were layered on 15 - 50% sucrose gradients (in TES). Centrifugation was carried out at $4^{o}C$ in the SW 25.1 rotor for 7 - 9 hours at 25,000 rpm. Tubes were punctured at the bottom and 25-drop fractions ($\pm$ 0.7ml) collected with a fraction collector. Radioactive counting was carried out by mixing 0.1ml of each fraction with 10ml of dioxane scintillation cocktail.

(B) Results and Discussion.

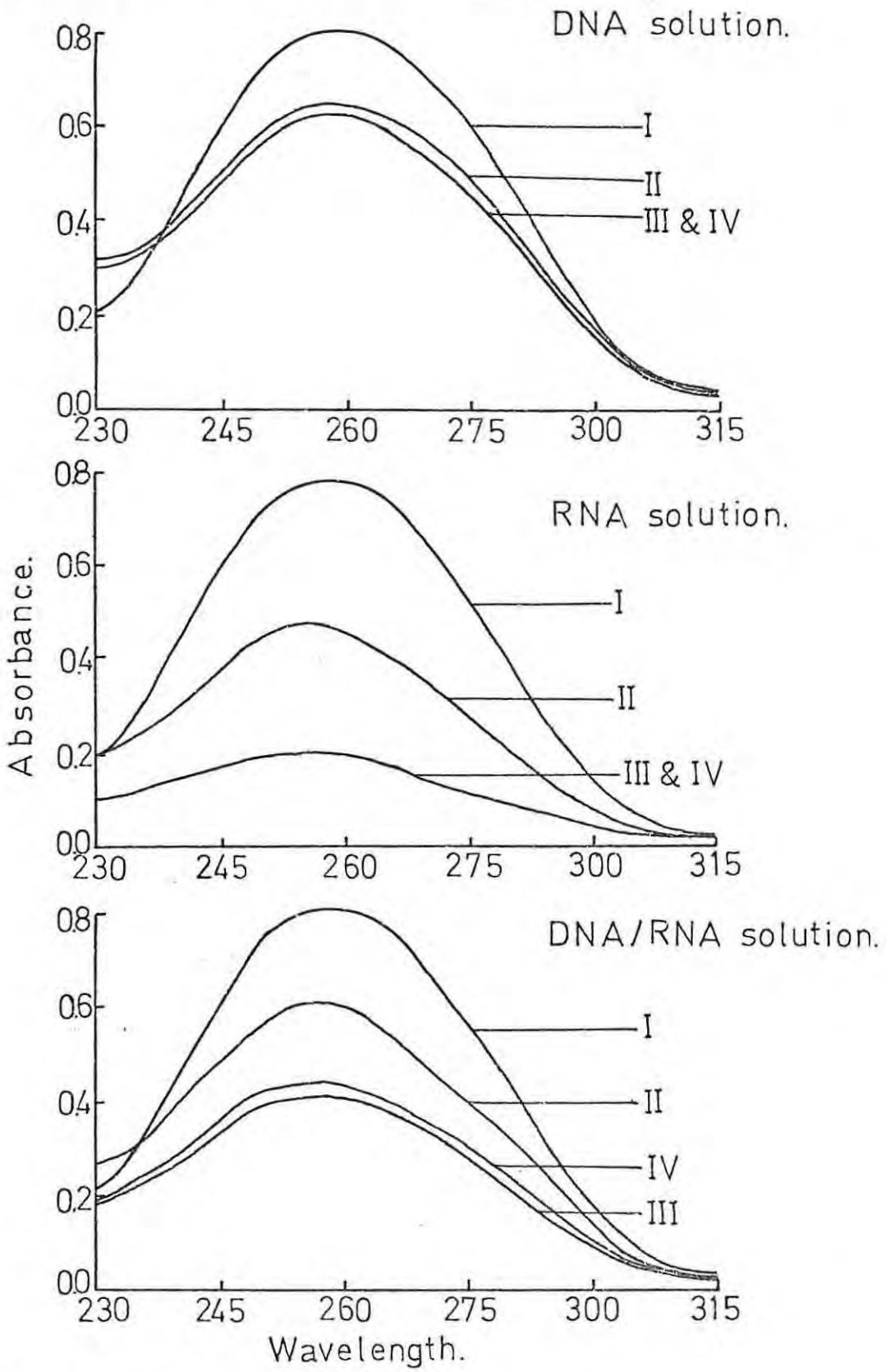
The amount of R factor DNA per E. coli cell is usually much less than chromosomal DNA. It is thus difficult to measure by spectrophotometric methods the amount of R factor DNA extracted from E. coli cells, particularly when the weight of cells is small. Only small weights of bacteria were available for each extraction owing to the cost of ^{3}H-thymidine used to label the DNA. Hence, it was decided to try and extract chromosomal DNA and to determine its concentration by spectrophotometric methods. Its radioactivity could be measured by scintillation counting and the amount of DNA emitting a given radioactive count could then be calculated. By measuring the radioactive count of R factor DNA, one could calculate the quantity of R factor DNA extracted (assuming chromosomal and R factor DNA to incorporate similar porportions of ^{3}H-thymidine). The experiment could also help in determining whether sufficient quantities of ^{3}H-thymidine were being incorporated in the DNA; i.e. this would provide information as to the efficiency of the labeling technique used.

Only small yields of bacterial cells were obtained as the E. coli thy^{-} mutant grew slowly in minimal medium. Bigger yields could not be obtained by growing the cells for extended periods of time as difficulty was experienced in lysing cells older than 22 hours. The average yield of wet-packed cells per 150ml of MM was $\pm$ 0.2g. Lysis of the cell suspension resulted in clearing of the suspension

and an increase in viscosity. Both these characteristics were affected by the age of the culture and the time of incubation. Very little nucleic acid was usually collected on the glass rod but centrifugation greatly increased the yield. The concentration measured spectrophotometrically ranged from 30 to 33 µg/ml. This concentration, however, dropped to a level almost undetectable ($\pm$ 1.7 µg/ml) on the spectrophotometer after treatment with RNAse. A scale expander reading (X5 - 10) usually had to be carried out to detect the presence of DNA. This meant that most of the nucleic acid extracted consisted of RNA. The possibility that the RNAse was contaminated with DNAse was ruled out by the tests carried out. No significant decrease in DNA concentration was noticed after incubating with RNAse (fig. 1). These tests also showed that treatment of the RNAse had no noticeable effect on its activity (fig. 1). The centrifugation to precipitate the DNA may have caused the strands to break up and some loss may have then occurred during dialysis. After any centrifugation, the supernatant was tested for tritiated thymidine to detect whether much DNA had remained in it. In the DNA precipitation with isopropanol, it was noticed that radioactive count was exhibited by the supernatant, indicating that a large amount of DNA remained unprecipitated in it. This DNA possibly consisted of small nucleotide chains too small to spool on the rod or to be precipitated by centrifugation.

Further purification of the DNA was carried out by

Fig. 1 - Tests for Purity and Activity of RNAse.



I - Solution before dialysis.

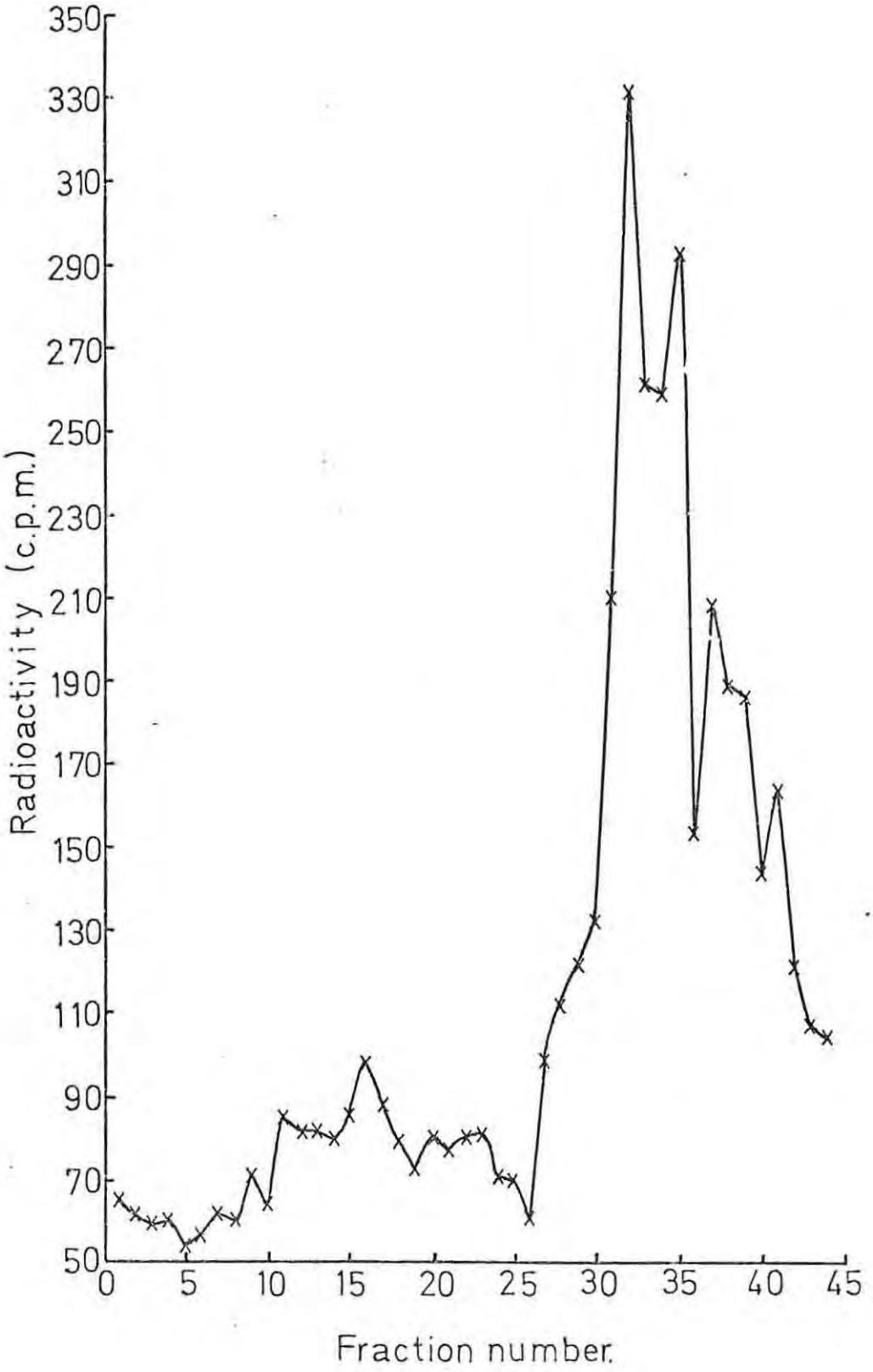
II - Solution after dialysis.

III - Solution + untreated RNAse after dialysis.

IV - Solution + treated RNAse after dialysis.

centrifugation of the DNA solution on 15 - 50% sucrose gradients. A major DNA peak was obtained at fraction 32 while minor peaks were obtained at fractions 35, 37 and 41 (fig. 2). The major peak may possibly consist of the circular chromosomal DNA molecule. The minor peaks may be due to DNA molecules with one or more breaks in the DNA strands resulting in slower sedimenting forms of chromosomal DNA in various stages of unwinding.

Fig. 2 - Sucrose gradient (15 - 50%) purification of *E. coli* chromosomal DNA.



C H A P T E R III

ISOLATION OF LABELED R FACTOR DNA.

Summary.

Techniques were developed for the isolation of R factor DNA free of chromosomal DNA. The DNA of an R factor segregant originating from a *Pseudomonas aeruginosa* strain and harboured in an *E. coli* strain was isolated. This R factor segregant could not be eliminated by growth of its host in the presence of sodium dodecyl sulphate (1% w/v).

(A) Materials and Methods.

A modification of the method of Clewell and Helinski (1969) was utilised.

(a) Bacterial Strains:

E. coli 25 Rt and E. coli 25 Rtr (see p. 72).

(b) Preparation of Cells:

Cells were grown in MM containing 2 µg/ml thymine and 0.5 ml/100ml ^{3}H-thymidine. Cells were grown overnight in 5ml of medium, diluted ten-fold in fresh medium and incubated for 2 - 4 hours. Deoxyadenosine (250 µg/ml) was added to the medium as Boyce and Setlow (1962) had reported that the addition of certain nucleosides and deoxynucleosides enhanced the uptake by E. coli of exogenous thymidine. However the addition of deoxyadenosine did not appear to noticeably alter the amount of ^{3}H-thymidine taken up (as measured by the radioactive count of the medium before and after inoculation). The cell culture was centrifuged (2,000g for 10 min.) and the cells were re-suspended in 1ml of cold 25% sucrose (in 0.05M Tris, pH 8).

(c) Preparation of Lysates:

Lysozyme (0.2 ml of a 5 mg/ml solution in 0.025M Tris, pH 8.0) was added, and after the suspension had been maintained at 0°C for 5 min., 0.4 ml of EDTA (0.25M, pH 8.0) was added. The suspension was kept at 0°C (with occasional shaking) for another 5 min., after which lysis was brought about by adding 1.6ml of a detergent solution consisting of the following: 1% Brij 58, 0.4% sodium desoxycholate, 0.0625M EDTA and 0.05M Tris, pH 8.0. After 15 - 20 minutes,

the sample started to clear and the viscosity increased. The sample ($\pm$ 3ml) was placed in 5 ml cellulose nitrate tubes. In some cases the volume was made up to $\pm$ 5 ml with cold 0.05M Tris (pH 8.0). The sample was then centrifuged at 2°C for 25 min. in the SW 50L rotor at 25,000rpm. The supernatant was decantered and dialysed overnight against 1L TES (pH 8.0).

(d) Dye-buoyant Density Equilibrium Centrifugation:

Two ml of ethidium bromide solution (1 mg/ml in TES) was added to the dialysed sample and the volume made up to 10ml with TES. Where true density measurements were required, ethidium bromide was omitted. Caesium chloride (10g) was added and polyallomer tubes (13 ml) were filled with the solution. Density equilibrium centrifugation was then carried out in the fixed-angle 65 rotor at 44,000rpm for 44 - 48 hours at 15°C. At the end of the run, the bottom of the tubes were punctured and 20 drop fractions (0.25 - 0.30 ml) collected using a fraction collector. Aliquots of each fraction were used either for density determination, radioactivity counts or electron microscopy. The density was determined from the refractive index of fractions (Szybalski, 1968).

(e) Radioactive Counts:

A modification of the method of Bollum (1966) was used to remove CsCl which might have affected radioactive counts. Samples (0.1 ml) of each fraction were spotted on filter paper discs (Whatman No. 1, diam. 4.25 cm). The discs were successively immersed in 10% cold trichloroacetic acid

(TCA) (60 - 90 min.), 5% cold TCA (15 - 30 min.), cold 95% ethanol (15 - 30 min.) and diethyl ether (15 min.). The discs were then air-dried, folded, placed in vials and covered with 10 ml of toluene scintillation cocktail. Counts were performed in the Beckman β-mate II liquid scintillation counter.

(f) Electron Microscopy:

Samples to be used for electron microscopy were dialysed overnight against TES or 0.1M ammonium acetate. In some cases, the sample was treated with an equal volume of n-octanol to remove the ethidium bromide prior to dialysis.

The dialysed samples were then treated in either of two ways:

(1) The sample was spotted directly on electron microscope grids and coated.

(2) The sample was mixed with an equal volume of 0.04% cytochrome-C solution (in 1M ammonium acetate) and 0.2 - 0.4 ml of the mixture was then run down a clean wet ramp (glass slide) onto the surface of a 0.1M ammonium acetate solution in a Langmuir trough (80 cm x 10 cm x 1 cm). The movement of the surface layer was followed by sprinkling a small amount of talcum powder near the ramp. After 5 - 10 minutes, the surface was touched with a carbon-coated electron microscope grid. Excess water was removed by touching the surface of the grid on absolute ethanol for 30 seconds. The dried specimen was then coated (Kleinschmidt et al, 1963).

Coating was carried out by deposition of gold, chromium or palladium-gold at an angle of approximately 8^{o}. The metal used for shadowing was vapourized by heating on tungsten wire under a pressure of less than 10^{-4} mm Hg in a Hitachi HUS 3B Vacuum Evaporator.

(g) Elimination of R Factor:

Ingram et al (1972) reported that a Pseudomonas R factor could be eliminated from cells grown for 4 days in nutrient broth containing 1% (w/v) of sodium dodecyl sulphate (SDS). A similar procedure was used in an attempt to eliminate the Pseudomonas R factor harboured in E. coli 25 Rtr.

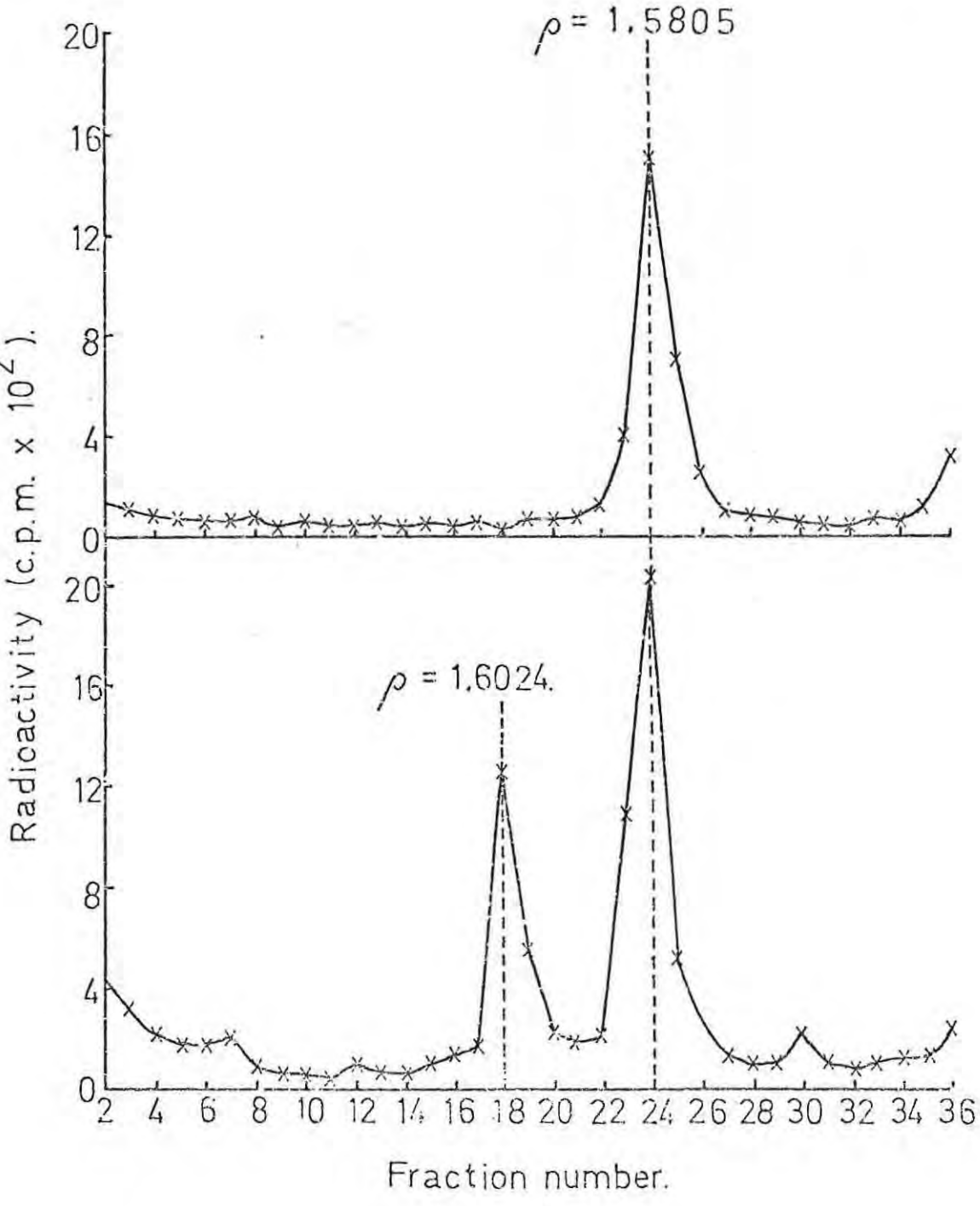
Nutrient broth (100ml) and nutrient broth + SDS (1% w/v) were inoculated with 0.1 ml of an exponentially growing culture of E. coli 25 Rtr. The cultures were incubated for 4 days without shaking. Dilutions of the cultures were plated on nutrient agar and nutrient agar + ampicillin (25 µg/ml). After 24 hours incubation, the plates were examined for growth.

(B) Results and Discussion.

Godson and Sinsheimer (1967) showed that treatment of E. coli cells with the neutral detergent Brij 58 seemed to affect the cell wall and membrane in such a way that it behaves as a molecular sieve. This method of lysis has since been used to isolate cytoplasmic nucleic acid molecules such as colicin factors (Clewell and Helinski, 1969), R factors (Cohen and Miller, 1970) and other plasmids (Olsen and Schoenhard, 1972) free of chromosomal DNA. A similar method was used in this experiment to isolate the Pseudomonas R factor which had been transferred to an E. coli strain. The E. coli thy$^-$ strains with and without the R factor were subjected to lysis and the lysate centrifuged to remove cell débris and most ($\pm$ 95%) of the chromosomal DNA (Clewell and Helinski, 1969).

Since the R factor DNA could not be separated directly from the chromosomal DNA by CsCl density-gradient centrifugation, it was necessary to add ethidium bromide. This dye has been shown by Radloff et al (1967) to have less affinity for closed circular DNA than for open or nicked DNA. This property has been used to isolate a wide range of closed circular DNA molecules; e.g. viral DNA (Bauer and Vinograd, 1967), R factor DNA (Cohen and Miller, 1970), colicin factor DNA (Clewell and Helinski, 1969). Dye-buoyant-density centrifugation of the E. coli strain without the R factor (25 Rt) produced only one peak at density 1.5805 (Fig. 3). The same strain harbouring the R factor resulted in an additional peak at density 1.6024 (Fig. 3).

Fig. 3 - Dye-buoyant Density Gradient Analysis of Cleared Lysates of Strains 25 Rt and 25 Rtr.



The fact that the R factor DNA could be separated in this way suggested that at least some of it existed as closed circular molecules. However, despite screening of a number of prepared grids, no electron micrographs were obtained which provided clearly demarcated molecules of R factor DNA. Further work was interrupted owing to the breakdown of the electron microscope which is expected to be out of use for 3 - 4 months. Of the various methods tried in the preparation of samples on electron microscope grids, the most successful was the one with cytochrome-C followed by shadowing with palladium-gold. It provided the clearest grids with a continuous background. The failure to obtain micrographs of R factor molecules was probably due to the extremely low concentration of DNA.

In a recent publication, Grinsted *et al* (1972) reported work of a similar nature on an R factor isolated in a strain of *P. aeruginosa*. The R factor caused resistance to carbenicillin, neomycin, kanamycin and tetracycline and was freely transmissible to *P. aeruginosa*, *E. coli* and *P. mirabilis*. However, a segregant of this R factor which caused resistance to carbenicillin only (Ingram *et al*, 1972), whilst freely transmissible to *P. aeruginosa*, was transferable to *E. coli* only at very low frequency (10^{-8}). Whereas the complete R factor could be isolated in the cytoplasm of its host, the carbenicillin resistance segregant could not and Ingram *et al* suggested that it had become inserted in the chromosome. This contrasts with the *Pseudomonas* R factor on which work was done in this laboratory. The

original R factor was resistant to ampicillin, streptomycin, sulphonamide and tetracycline and an ampicillin resistance segregant was obtained (see p. 69). Studies were only carried out on the ampicillin resistance segregant. This R factor segregant was freely transmissible between E. coli strains and as shown above was found to exist as a plasmid which could be isolated independently from the chromosomal DNA. Ingram et al also reported that they were unable to eliminate the R factor segregant by growing the cells in the presence of SDS (1% w/v). They provided this as further evidence that the R factor segregant was intergrated into the chromosome. However the R factor segregant studied in this laboratory was no eliminated by growth in the presence of SDS, although it could be isolated independently of the chromosome. Therefore failure to eliminate the R factor segregant by treatment with SDS cannot necessarily be used as a criterion for intergration into the chromosome.

APPENDIX A

Media and solutions were prepared as described below. Unless otherwise stated, sterilization by autoclaving was done at 15lb/sq.in. for 15 minutes (or longer for volumes greater than 250 ml).

Nutrient Broth: dissolve 8g Bacto-Nutrient Broth (Difco) in 1000 ml of distilled water. Autoclave.

Nutrient Agar: suspend 23g Bacto-Nutrient Agar (Difco) in 1000 ml of cold distilled water and heat to boiling to dissolve the medium completely. Autoclave.

Wellcotest Agar: one litre of deionised water is measured. Add 50 - 60 ml of this water to 32g of Wellcotest Sensitivity Test Agar (Burroughs Wellcome). Mix to a paste and gradually add the remaining water with mixing to form a suspension. Solubilise by heating at 100°C. Autoclave.

MacConkey Agar: suspend 50g of Bacto-MacConkey Agar (Difco) in 1000 ml of cold distilled water and heat to boiling to dissolve the medium completely. Autoclave.

MacConkey Purple Agar: consists of:

Bacto-Peptone (Difco)	10g
Bile Salts No. 3 (Difco)	9g
NaCl	10g
Distilled water	1000ml

The ingredients are dissolved by gentle heating and the pH adjusted to 7.4. Oxoid No. 3 Agar (15g) is added and the mixture steamed to dissolve the agar. Lactose (30g) and 12 ml of Bromocresol purple (1%

alcoholic solution) are dissolved in the medium. Autoclave.

Sloppy Agar: consists of:

Bacto-Nutrient Broth (Difco)	0.8g
NaCl	0.85g
Oxoid No. 3 Agar	0.7g
Distilled water	100ml

The medium is heated until all the ingredients have dissolved. It is then dispensed in 2.5 ml aliquots in capped sloppy agar tubes and autoclaved.

Brilliant Green Bile Lactose Broth: dissolve 40g of Bacto-Brilliant Green Bile 2% (Difco) in 1000 ml of distilled water. Autoclave.

Tryptone Broth: consists of:

Bacto-Tryptone (Difco)	10g
NaCl	5g
Distilled water	1000ml

Dispense 10 ml. aliquots in capped test tubes and autoclave.

Saline Solution: consists of:

NaCl	8.50g
Distilled water	1000ml

Minimal Salts: consists of:

NH_4Cl	20g
NH_4NO_3	4g
Na_2SO_4 anhydrous	8g
K_2HPO_4 anhydrous	12g
KH_2PO_4	4g
$MgSO_4.7H_2O$	0.4g
Distilled water to	1000ml

Dissolve each salt in cold water in the order indicated, waiting until previous salt is dissolved before adding next. Filter and autoclave. (pH 7.2).

20% Glucose Solution:

D-glucose	20g
Water to	100ml

Dissolve in warm water and autoclave at 5 lbs for 10 min.

Minimal Agar (MA):

Oxoid Ionagar No. 2	1.0g
Water	74ml
20% glucose sol. (sterile)	1ml
Minimal salts (sterile)	25ml

Melt the agar by autoclaving. Add the warmed, sterile salts and glucose. This medium is made up immediately before use.

Minimal Medium (MM):

Minimal salts (sterile)	25ml
20% glucose (sterile)	1ml
Sterile water	74ml

Mix the three components under aseptic conditions just before use.

TES Buffer:

NaCl	2.90g
EDTA	1.86g
Tris	3.63g
Water to	1000ml

Adjust to pH 8.0.

Selective Media:

Selective media containing one or more drugs were prepared by adding aliquots of solutions of the drugs to the molten agar (50°C) immediately prior to pouring plates. Nutrient agar was used in all selective media except where sulphonamides or trimethoprim were present in which case Wellcotest Agar was used. Each drug powder was assumed to be self-sterilizing and was dissolved in sterile distilled water. Fresh drug solutions were used prior to each experiment.

Drug	Conc. (µg/ml)	Amount	Vol. of water	Aliquot/ 100ml agar
amp	25	20mg	10ml	1.26ml
cpx	15	10mg	10ml	1.50ml
cpd	15	10mg	10ml	1.50ml
cmp	30	10mg	10ml	3.0ml
kan	30	10mg	10ml	3.0ml
nal[1]	30	2ml	7ml	0.30ml
nft[2]	200	20mg	-	20mg
str	25	10mg	10ml	2.50ml
tet	50	20mg	10ml	2.50ml
trm	1.5	15mg	10ml	0.10ml
vfm[3]	700	70mg	-	70mg

[1]To help dissolve the naladixic acid, 1ml of 1N NaOH (sterile) was added to the 7ml of water. The naladixic acid used was obtained as a suspension (Wintomylon Suspension).

[2]The nitrofurantoin powder was added directly to the

molten agar and shaken until dissolved. It was obtained in tablet form, 20mg of nitrofurantoin being equivalent to 54mg of tablet (Furadantin tablets).

[3]The Vioform powder was dissolved in 4ml of dimethyl sulfoxide (DMSO) and this solution added to the molten nutrient agar. The Vioform precipitated to form a fine dispersion.

SSC Buffer:

NaCl	8.8g
Na Citrate	4.4g
Water to	1000ml

Adjust to pH 7.0.

Conc. SSC Buffer = X10 conc. of SSC Buffer.

Dil. SSC Buffer = 1/10 conc. of SSC Buffer.

M9 Buffer:

Na_2HPO_4 anhydrous	7.0g
KH_2PO_4	3.0g
NaCl	4.0g
$MgSO_4.7H_2O$	0.2g
Water to	1000ml

Dissolve each salt in the order given before adding next.

Saline EDTA:

NaCl	0.84g
EDTA	3.72g
Water to	100ml

Dissolve the ingredients and adjust the pH to 8.0.

Acetate-EDTA:

EDTA sol. (0.1M)	0.1ml
Na acetate	4.08g
Water to	10ml

Dissolve the ingredients and adjust the pH to 7.0.

Dioxane Scintillation Cocktail:

2,5-Diphenyloxazole (PPO)	5g
Naphthalene	100g
Dioxane to	1000ml

The cocktail must be protected from daylight or neon light as it is markedly affected, resulting in erratic counts. This is found to occur particularly with old cocktail and hence scintillation cocktails are best prepared immediately prior to being used.

Toluene Scintillation Cocktail:

2,5-Diphenyloxazole (PPO)	6g
Toluene to	1000ml

Prepare as for Dioxane Scintillation Cocktail.

Source of Drugs and Chemicals:

Ampicillin: ampicillin trihydrate; a gift from Bristol Laboratories.

Acriflavine: British Drug Houses Ltd.

Brij 58: a gift from Honeywill-Atlas Ltd.

Cephalorexin: "Cephaloxin" capsules (500mg); a gift from Glaxo-Allenburys (S.A.)(Pty) Ltd.

Cephaloridine: "Ceporan" vials (1gm); a gift from Glaxo Allenburys (S.A.)(Pty) Ltd.

Chloramphenicol: "Chloromycetin" powder; a gift from Parke-Davis (Pty) Ltd.

Deoxyadenosine: 2' Deoxyadenosine monohydrate; British Drug Houses Ltd.

Deoxyribonucleic Acid: sperm herring DNA; Koch-Light Laboratories Ltd.

Dimethylsulphoxide: E. Merck A.G., Darmstadt.

Dioxane: 1,4-dioxan; Hopkins and Williams Ltd.

Ethidium Bromide: a gift from Boots Pure Drug Company (S.A.) (Pty) Ltd.

Kanamycin: kanamycin sulphate; a gift from Bristol Laboratories

Lysozyme: ex egg white, 40,000 units/mg; British Drug Houses Ltd.

Naladixic Acid: "Wintomylon" suspension; a gift from Winthrop, Sterling Drug (S.A.)(Pty) Ltd.

Naphthalene: Hopkin and Williams Ltd.

Nitrofurantoin: "Furadantin" tablets (100mg); a gift from S.K.F. Laboratories (Pty) Ltd.

n-Octanol: a gift from Chemistry Department.

PPO: 2,5-Diphenyloxazole; Beckman Instruments, Inc.

Ribonuclease: ex bovine pancreas; British Drug Houses Ltd.

Ribonucleic Acid: ex yeast RNA; British Drug Houses Ltd.

Sodium Dodecyl Sulphate: Hopkin and Williams Ltd.

Streptomycin: streptomycin sulphate B.P.; a gift from Glaxo-Allenburys (S.A.) (Pty) Ltd.

Tetracycline: tetracycline base; a gift from Bristol Laboratories.

Thymine: British Drug Houses Ltd.

Toluene: for scintillation; Beckman Instruments, Inc.

Trimethoprim: Burroughs Wellcome (Pty) Ltd.

Tritiated Thymidine: thymidine (methyl-H3), 1.0mCi/ml; The Radiochemical Centre, Amersham.

Vioform: iodochlorhydroxyquinoline; a gift from Ciba-Geigy (Pty) Ltd.

Apparatus.

Bench centrifuge: MSE centrifuge.

Electron Microscope: Hitachi HU IIB. Transmission E.M.

Refractometer: Carl Zeiss.

Scintillation Counter: Beckman β-mate II.

Spectrophotometer: Unicam SP 800A U.V. spectrophotometer.

Ultracentrifuge: Beckman L2-65B preparative ultracentrifuge.

A P P E N D I X B

The following nomenclature and abbreviations were used:

Autoclaving: - volumes up to 250 ml were autoclaved for 15 minutes at 15 lbs/sq.in. pressure.
- larger volumes were autoclaved for longer periods.

Incubation: - incubation was usually carried out at 37°C.

Overnight: - normally refers to a period 12 - 16 hours long.

Drugs: - the terms drugs, antibiotics and chemotherapeutic agents are used synonymously unless otherwise stated.

The following abbreviations have been used for the drugs:

ampicillin	-	amp
cephalexin	-	cpx
cephaloridine	-	cpd
chloramphenicol	-	cmp
kanamycin	-	kan
gentamycin	-	gen
naladixic acid	-	nal
nitrofurantoin	-	nft
streptomycin	-	str
tetracycline	-	tet
trimethoprim	-	trm
septrin	-	sep
vioform	-	vfm

DNA - deoxyribonucleic acid

DNAse - deoxyribonuclease

RNA - ribonucleic acid

RNAse - ribonuclease

A P P E N D I X C

A computer programme has been prepared to assist in the analysis of patterns of multiple resistance when large surveys are undertaken. The programme was written in ALGOL language and run on an ICL 1901A computer. The programme is only designed to analyse double, triple or quadruple resistance patterns. Because of the manner in which the results are produced, the data for double, triple and quadruple resistances must be fed in separately.

Analysis of Double-Resistant Strains.

An example of data and results (print-out) for a set of double resistances is given below:

Data

1	0	0	0	0	0	0	1
1	0	1	0	0	0	0	0
1	0	1	0	0	0	0	0
1	0	0	0	0	1	0	0
1	0	1	0	0	0	0	0
1	1	0	0	0	0	0	0
1	1	0	0	0	0	0	0
1	1	0	0	0	0	0	0
1	0	1	0	0	0	0	0
1	0	0	0	0	0	0	1
1	0	0	0	0	0	0	1
1	0	1	0	0	0	0	0
1	0	0	0	0	0	0	1
1	0	0	0	0	0	0	1
1	0	0	0	0	0	0	1
1	0	0	0	0	0	1	0
0	0	1	0	0	1	0	0
1	1	0	0	0	0	0	0
1	0	0	0	0	0	1	0
1	0	0	0	0	0	0	1

Results

19	4	6	0	0	2	2	7
4	5	0	0	1	2	7	
0	0	0	0	0	0		
0	0	1	0	0			
0	0	0	0				
0	0	0					
0	0						
0							

In the above example, 20 strains showed resistance to two out of a total of eight drugs. Resistance is denoted by a 1 and sensitivity by a 0. In the "Data" matrix, the first strain (1st line horizontally) was resistant to drug one and drug eight, the second strain to drugs one and three, the third strain to drugs one and three, etc. The first horizontal line in the "Results" matrix gives the total resistances to each individual drug; i.e. 19 strains were resistant to drug one, four to drug two, six to drug three, etc.

The first number in the second line is the beginning of the results of the double resistances. The results, reading from left to right, read as follows:

4 resistant strains to drugs 1 and 2

5 resistant strains to drugs 1 and 3

0 resistant strains to drugs 1 and 4, etc.

The last number of the second line reads;

7 resistant strains to drugs 1 and 8.

The third line then reads:

0 resistant strains to drugs 2 and 3

0 resistant strains to drugs 2 and 4, etc.

In this way, all the possible double combinations will be exhausted (the last being 7 and 8).

Analysis of Triple-Resistant Strains.

When the data for triple resistances is analysed, "Result" matrices for double and triple resistances will be printed out. However only that for triple resistances should be used as the one for double resistances gives irrelevant

results (e.g. a strain resistant to drugs 1, 2 and 3 will give the combinations 1 + 2, 1 + 2 and 2 + 3, none of which are relevant since the strain is resistant to three and not two drugs).

Similarly, when analysing the results for quadruple resistance patterns, double and triple resistance matrices should be disregarded.

Before each set of data is fed in, a card must be inserted giving the number of strains tested and the number of drugs to which resistance was tested. In the example given above, a card bearing the numbers 20 (i.e. 20 strains) and 8 (i.e. 8 drugs) was inserted before the data cards. This corresponds to step 4 in the programme which reads:

'COMMENT' N IS THE TOTAL NO. OF COLUMNS AND

M IS THE NO. OF ROWS.

The computer is then instructed to read N (20 in this example) and M (8 in this example).

COMPUTER PROGRAMME

```
00000000        15/09/72        COMPILED BY XALE MK. 5C
STATEMENT
     0          'PROGRAM'(SCAN)
     0          'INPUT'0=CR0
     0          'OUTPUT'0=LP0
     0          'BEGIN'
     1          'INTEGER' I,J,L,M,N;
     1          'INTEGER' K,TOT,P,Q,R;
     2          'INTEGER' 'ARRAY' A[1:150,1:15];
     3
     3          START:
     4               'COMMENT' N IS THE TOTAL NO. OF COLUMNS AND M THE NO. OF ROWS;
     4
     4               N←READ; M←READ;
     6
     6          INITIALISE:
     6               TOT←0;
     7
     7          READ DATA:
     7
     7               'COMMENT' DATA MUST BE PUNCHED AS EITHER 0 OR 1
     7               SEPARATED BY TWO SPACES;
     7
     7               'FOR' I←1 'STEP' 1 'UNTIL' M 'DO' 'BEGIN'
     8                    'FOR' J←1 'STEP' 1 'UNTIL' N 'DO' 'BEGIN'
    10                       A[I,J]←READ;
    12                       PRINT(A[I,J],1,0);
    13                       'END';
    14                  NEWLINE(1);
    15                  'END';
    16
    16          COMPUTATION:
    16               NEWLINE(10);
    17               'FOR' J←1 'STEP' 1 'UNTIL' N 'DO' 'BEGIN'
    18                    'FOR' I←1 'STEP' 1 'UNTIL' M 'DO'
    20               TOT←TOT+A[I,J];
    21               PRINT(TOT,3,0);TOT←0; 'END';
    24               NEWLINE(2);
    25               'FOR' K←1 'STEP' 1 'UNTIL' N-1 'DO' 'BEGIN'
    26                    'FOR' L←K+1 'STEP' 1 'UNTIL' N 'DO' 'BEGIN'
    28                       'FOR' I←1 'STEP' 1 'UNTIL' M 'DO'
    30                    'IF' A[I,K]=A[I,L] 'EQ' 2 'THEN' TOT←TOT+1;
    31               PRINT(TOT,3,0);TOT←0; 'END';
    34                    NEWLINE(1);'END';
    36               NEWLINE(2);
    37               'FOR' K←1 'STEP' 1 'UNTIL' N-2 'DO' 'BEGIN'
    38               'FOR' L←K+1 'STEP' 1 'UNTIL' N-1 'DO' 'BEGIN'
    40               'FOR' P←L+1 'STEP' 1 'UNTIL' N 'DO' 'BEGIN'
    42               'FOR' I←1 'STEP' 1 'UNTIL' M 'DO'
    44                    'IF' A[I,K]=A[I,L]=A[I,P] 'EQ' 3 'THEN' TOT←TOT+1;
    45               PRINT(TOT,3,0);TOT←0; 'END';
    48               'END';
    49               NEWLINE(1); 'END';
    51               NEWLINE(2);
    52               'FOR' K←1 'STEP' 1 'UNTIL' N-3 'DO' 'BEGIN'
    53               'FOR' L←K+1 'STEP' 1 'UNTIL' N-2 'DO' 'BEGIN'
    55               'FOR' P←L+1 'STEP' 1 'UNTIL' N-1 'DO' 'BEGIN'
    57               'FOR' Q←P+1 'STEP' 1 'UNTIL' N 'DO' 'BEGIN'
    59               'FOR' I←1 'STEP' 1 'UNTIL' M 'DO'
    61               'IF' A[I,K]=A[I,L]=A[I,P]=A[I,Q] 'EQ' 4 'THEN' TOT←TOT+1;
    62               PRINT(TOT,3,0);TOT←0; 'END';
    65          'END','END';
    67               NEWLINE(1); 'END';
    69          'END'
NO OF WORDS USED    12
COMPILED  PSCAN          ;F
```

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