THE ISOLATION AND CHARACTERISATION OF ANTIBIOTIC RESISTANT COLIFORM BACTERIA FROM ST. ANDREWS BAY

By

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A thesis presented for the Master of Science degree at the University of St. Andrews.

June, 1978.

C E R T I F I C A T E

This is to certify that JOHN OKWUCHUKWU CHIKWEM has done his research work under my supervision, and that he has fulfilled the conditions of Ordinance 51 of the University of St. Andrews, so that he is qualified to submit the following thesis in application for the degree of Master of Science.

Lecturer in Biochemistry.

D E C L A R A T I O N

I hereby declare that I am the author of this thesis; that unless otherwise stated, all the references cited in the text have been consulted by me; that the work of which this thesis is a record has been done by myself, and that it has not been previously accepted for a higher degree.

D E D I C A T I O N.

This thesis is dedicated to Nature, the inexhaustible reservoir of all knowledge.

S U M M A R Y

A study is presented of the isolation of *Escherichia coli* from the St. Andrews coastal bathing area in general, and plasmid mediated antibiotic resistant strains in particular.

Some aspects of their conjugative properties with particular reference to the influence of growth phase, anaerobiosis, use of same bacterial serotype as donor and recipient, levels of resistance acquired by infected cells have been studied.

Plasmid classification by incompatibility and characterisation by molecular weight determination in agarose gel have also been studied for epidemiological reasons. A few, yet very important limitations in the classification of wild plasmids by incompatibility and characterisation of plasmid deoxyribonucleic acid by molecular weight determination in agarose gel have been observed, and suggestions have been made as to methods of containing them.

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I am greatly indebted to my supervisor, Dr. M.G. Burdon of the Department of Biochemistry for his indispensable advice, encouragement and guidance throughout the entire duration of this work.

My appreciation also go to my Head of Department, Professor G.R. Tristram and the acting Head of Department, Dr. S. Bayne for providing me with the opportunity to undertake this work and for the use of the equipments in the department.

I thank the Government of the Federal Republic of Nigeria for their financial support throughout the course of my study. I also thank the technicians in my department, particularly, Mr. J. Crawford, the Chief Technician and Mr. Ian Armitt the technician in charge of the laboratory where I did most of my research.

My sincere appreciation also go to Dr. D. B. Malcolm of the Department of Zoology for his interest and encouragement in the genetic aspect of this study, and to Dr. D. Thirkell of the Department of Biochemistry for reading through my text and making very useful corrections.

Most of all, I am grateful to God for the strength of mind, soundness of health and above all, love which He has always bestowed on me, particularly at this time.

1.1 Sources of bacteria found in sea water.

The sea is distinguished by its enormous size as compared with inland waters and also from most by its salt content which averages 3.5%. Hydrostatic pressure increases with the depth of the sea. Organic matter content is usually low except in heavily polluted waters; the sea temperature also varies in different parts of the world. As a result, it is usually inhabited by:-

(a) Free living organisms which are adapted to the conditions existing in the aquatic environment like mild halophiles, both aerobic and anaerobic. Apart from these genuine aquatic bacteria, a number of other organisms can also be recovered from sea water.

(b) Soil bacteria are washed into the sea from flowing waters and rainfall, and include most of the aerobic spore bearers. Their impact amongst the aquatic bacterial populations depends on the extent of the bacterial population in the soil from which they are leached.

(c) Fresh water bacteria can sometimes be recovered in sea water especially if these water sources empty into the sea, carrying with them their bacterial population.

(d) Air borne bacteria can also pollute the sea and other waters when they fall from the air on to water surfaces.

(e) Sewage pollution of sea waters appears to constitute the most important source of sea water bacterial contamination. Most of the organisms found from this source are normal intestinal flora of humans, animals and birds. Others live chiefly on decomposing organic matter of either animal or vegetable origin. Occasionally, pathogenic organisms may be included although, their numbers are often too small and searching for them directly, is impracticable for routine control purposes.

1.2 Faecal bacteria.

Many micro-organisms find their way into the sea water with domestic sewage which also has great quantities of organic and inorganic

nutrients. These organisms include the coliform bacilli, Clostridia and faecal streptococci. Sometimes, human pathogenic bacteria like the Salmonella typhi and Salmonella paratyphi can be isolated. Faecal streptococci, examples of which are Streptococcus faecalis and Streptococcus faecium, originate from human and animal sources and do not multiply in water though they may survive for a fairly long time.

The Clostridia found in water, include Clostridium septicum, Clostridium perfringens and Clostridium novyi. These anaerobic spore bearers remain alive for a fairly long time especially as they can go into sporulation and these spores can be found in fairly anaerobic environs of the water, notably, the muddy deposits.

Other normal intestinal flora include members of the Enterobacteriaceae, lactobacilli, bifidobacteria, Bacteroides spp; micrococci, Pseudomonas spp., and corynebacteria. These also pollute sea water although variations in the relative number of these bacterial types occur according to race, age, climate and diet (Draser and Hill, 1974). But the coliform bacteria, and in particular, Escherichia coli, faecal streptococci and Clostridium perfringens are used in water bacteriology as indicators of sewage pollution. Although, Escherichia coli is often the most predominant of coliform bacteria present in the human and animal intestine, it cannot proliferate in sea water and its presence therefore, is indicative of a recent faecal pollution (Report on public health and medical subjects, No. 71, 1969).

Streptococcus faecalis occurs in human faeces in numbers usually smaller than those of Escherichia coli, but this organism may sometimes, outnumber E. coli in animal faeces (Gieldreich and Kenner, 1969). In water, Streptococcus faecalis, dies at a slower rate than Escherichia coli but usually more rapidly than other coliform bacteria (Report on public health and medical subjects, No. 71, 1969). In the absence of E. coli,

the finding of faecal streptococci therefore is important confirmatory evidence that the pollution is of a faecal origin.

The anaerobic spore bearer, Clostridium perfringens, is present in faeces in much smaller numbers than E. coli, but the spores of this organism can survive in water longer than the non-sporing bacteria. Their presence in sea water therefore, suggests that faecal pollution has taken place, but in the absence of coliform organisms, this evidence indicates that the source of contamination was remote (Report, 71, 1969).

It is not very certain that coliform bacteria fulfil all the criteria for an indicator organism (Bonde, 1963, 1966, 1969). However, it is unlikely that any organism will be found to fulfil exactly the requirements laid down by this worker. It appears reasonable to doubt the suitability of E. coli especially when Bonde, (1969), repeatedly isolated Salmonella species without encountering the indicator bacterium, E. coli. Also, confusion with the aeromonads is frequent. Above all, E. coli appears to die more rapidly in water than either Streptococcus faecalis or Clostridium perfringens, (Report, 71, 1969), and the occurrence of enteropathogenic varieties of this organism is well documented (Cruickshank, Duguid, Marmion and Swain, 1973, 1975). Nevertheless, Escherichia coli appears best suited for the role of an indicator of faecal pollution of water.

1.3. The coliform bacteria.

There is no general agreement on the definition and membership of the group. Wilson and Miles, (1964), defines coliform bacteria as those Enterobacteria other than the Salmonella, Shigella and Proteus that generally though by no means always ferment lactose. Cruickshank et al.,(1973, 1975) use the term coliform bacteria to refer to those members of the Enterobacteria that are urinary tract, wound and opportunistic pathogens. In the latter definition, Pseudomonas pyocyanea which is not an Entero-

bacterium,is sometimes regarded as a coliform bacillus. The coliform group of bacteria has also been defined as Gram-negative, oxidase-negative, non-sporing rods capable of growing aerobically on an agar medium containing bile salts and able to ferment lactose within 48 hours at 37° with the production of both acid and gas (Report, 71, 1969). American Public Health Association (1971), classified coliform organisms as all the aerobic and facultatively anaerobic Gram-negative, non-sporing rod shaped bacteria which ferment lactose with gas formation within 48 hours at 35°. Since the issue at hand involves water pollution, bacteria conforming to the definition in either Report, 71, (1969) or, American Public Health Association, (1971), shall be regarded as coliform bacteria in this context.

Because of the abundance of Escherichia coli in faeces, this organism is referred to as a faecal coliform. In spite of the objections by Gallagher and Spino, (1968), no specific differentiation methods for discriminating between coliforms of human origin and those of other animals exist. It is therefore necessary that all faecal coliforms be considered as indicators of dangerous contamination, (Report, 71, 1969).

1.4 Differentiation of faecal and other coliforms.

MacConkey, (1905, 1909), introduced bile salts for the cultivation and differentiation of bacteria of intestinal origin.

Eijkman, (1904), proposed the elevated temperature test of 44° for the differentiation of faecal from non-faecal coliform bacteria.

Voges and Proskauer (1898), divided the Enterobacteria into two groups depending on whether acetyl-methyl-carbinol was produced or not from pyruvate.

Clarks and Lubs, (1915), described the methyl-red test, while Rogers, Clarks and Evans, (1914, 1915) differentiated coliforms from

faeces of warm blooded animals from those isolated from other sources on the basis of differences in gas ratios.

Koser, (1924), demonstrated that "Bacterium coli" isolated from faeces could not utilise citrate as a sole source of carbon in contrast to "Bacterium aerogenes".

Although the methyl-red, Voges-Proskauer and citrate tests made notable contributions to the classification of the coliform group, none was found entirely satisfactory when used alone in relating a specific group or an individual strain to its source.

Parr, (1938), studied the results of the previous workers and chose the indole, methyl-red, Voges-Proskauer and citrate tests as a combination of tests that would yield the best classification.

In the United Kingdom, differentiation employs either brilliant green bile lactose broth or lactose ricinoleate broth at $44^{\circ} \pm 0.25$, (Report, 71, 1969), while the same media are used in the United States at 44.5°, (American Public Health Association, 1971). Some coliforms produce gas at this temperature but only Escherichia coli and few others in addition produce indole at 44°.

1.5 Factors affecting the viability of coliform bacteria in sea water.

The population of bacteria in sea water under constant conditions diminishes roughly logarithmically and the time taken for a bacterial population to decrease by 90% is referred to as the T90 value. Naturally, certain factors affect the T90 value. In bright sunlight and shallow clear water, the T90 value will be shorter because of the bactericidal action of the ultraviolet rays of the sun. In the dark, the T90 value will be longer. Exposure time of the organisms is also reduced by water movements, and low temperature favours bacterial survival, though multiplication may not take place. Rainfall and high winds also improve survival rates as a result of oxygenation.

Nevertheless, the population of bacteria, particularly the coliforms found in sea water remains fairly constant in any given condition if the source of pollution is persistent. As a result, sudden changes in coliform bacteria count can be utilised for meaningful interpretation of results especially as to the intensity of the pollution. However, under fairly constant conditions, coliform bacteria population can increase or decrease without significant increase or decrease in the level of pollution.

(a) The amount of available food supply is most important in determining the number of bacteria in sea water. To this end, the concentration and composition of these food substances play a very vital role. However, organic compounds have other important roles, as activating and inhibiting factors.

(b) The effect of temperature on bacterial population varies with the amount of organic matter present in the water. High temperature in the absence of organic matter is lethal though in its presence, rapid multiplication of bacteria occurs. On the other hand, low temperature without organic matter ensures survival but not multiplication.

(c) Strong radiation inhibits life processes of bacteria. For example, Micrococcus denitrificans are inactivated and killed by light (Rheinheimer, 1974). Pigmented bacteria like Sarcina lutea on the other hand, are light tolerant. However, in turbid waters, inhibition will occur only in a few metres while in clear waters, the distance penetrated will be more. But opacity limits the distance penetrated in clear waters to 5 feet (Wilson and Miles, 1964).

(d) Most seas have a salt content of about 3.5% (Rheinheimer, 1974). Salts have an antiseptic action on many bacteria, and most coliforms will not survive in very salty waters. A salt content of more than 10% has been reported in the Dead sea and Black sea (ZoBell, 1946).

(e) Dissolved oxygen favours survival of the coliform bacteria and this condition is improved by tides and high winds. In heavily polluted

waters, anaerobic conditions may exist which will kill most coliforms.

(f) Rainfall and temperature influence the variation in bacterial content of waters. Collins, (1957), reported a higher coliform count in winter as a result of low temperature and high rainfall.

Other factors have also been reported. Rheinheimer, (1974), reported the contribution of flagellates to the extermination of bacteria, and Spencer, (1963), asserted that bacteriophages active against a variety of Enterobacteria can readily be demonstrated in polluted waters but not in waters remote from terrestrial contamination. Rheinheimer, (1974), thinks that they help in rapid decrease in bacterial numbers of sewage loaded waters. Metazoa, mussels and oysters have also been shown to feed on bacteria.

1.6 Technique of sampling and examination of water for coliform organisms.

Although coliform organisms are found in very small numbers in water, these bacteria are very common in the intestinal tract of man and animals. Any accepted procedure for sampling and examination must therefore guarantee that all steps involved are carried out aseptically.

Direct incubation techniques on solid agar are not normally useful except of course when the water sample is very heavily polluted. Two methods adopted by most laborarories all over the world are the multiple tube method and the membrane filtration technique.

In the multiple tube method, measured volumes of the water or dilutions of it are added to the tubes containing liquid differential medium. Provided negative results occur in some tubes, the most probable number of organisms in the original sample may be estimated from the number of tubes giving positive reaction; statistical tables of probability are used for this purpose (Report, 71, 1969).

Unfortunately, the sampling error of the multiple tube method is very high and most workers resort to the more accurate technique of membrane filtration, which has a confidence limit of about 95%, if the

bacterial count is above twenty. The membrane filtration technique also has other advantages. For example, colonies form on the membrane surface, and conditions of incubation can be varied to encourage growth of slow growers. Direct presumptive count can be obtained within eighteen hours incubation time.

1.7. Clinical uses and side effects of the antibiotics used in the study.

Ampicillin: This antibiotic was first described by Rolinson and Stevens (1961), and is administered orally like most of the other penicillins. It is active against most Gram-positive bacteria including Streptococcus faecalis and Listeria monocytogenes. But its outstanding advantage above benzyl-penicillin lies in its activity against Gram-negative bacteria. Its effect is bactericidal and unlike tetracycline, the same proportion of a large dose is absorbed as of a small one. Although excretion is mainly renal, fairly high concentrations are attained in the bile. It accumulates and persists in the amniotic fluid as a result of renal excretion by the foetus. High concentrations are attained in the cerebro-spinal fluid which makes it an effective treatment in cases of meningitis. It is also very effective for the treatment of urinary tract infections.

In spite of the broad clinical uses of this antibiotic, some undesirable side effects have been reported following its usage. As an example, if renal function is impaired, the rate of excretion is also reduced. Occasional gastric intolerance has been observed, but the most significant side effect has been rashes (Shapiro, Slone, Siskind, Lewis and Jick, 1969).

Streptomycin: This antibiotic is very readily soluble in water and its sulphate is the most important preparation as it causes least pain at injection site. Streptomycin is bactericidal and particularly active against Mycobacteria, Gram-negative bacilli and some strains of Staphylococci but inactive against Streptococci, pneumococci and Clostridia.

However, many side effects prevent its general use in the treatment of bacterial infections. It causes pain and irritation at the site of injection, headaches, lassitude and muzziness in the head, but aside from these, the most important complications appear to be its effect on the eighth nerve, deafness, hypersensitivity and neuromuscular blockade (Garrod, Lambert and O'Grady, 1973). Streptomycin is therefore no longer used therapeutically except in cases where the risk involved in its use is minimal as compared to the condition of the patient.

Tetracycline: The major advantage of tetracycline over streptomycin and penicillin is that it has a wider range of activity. It is also administered by mouth. Susceptible species not only include Gram-positive bacteria sensitive to penicillin, but many Gram-negatives which are not, and in addition, Mycoplasmas, Rickettsiae and Chlamydia. The tetracyclines are also active against tubercle bacilli but are very rarely used nowadays in the treatment of tuberculosis.

Many side effects associated with the use of tetracycline have been reported. As a result of its broad spectrum of activity, most normal flora of the lower bowel are killed together with the pathogenic organisms, resulting sometimes in invasion by opportunist organisms like the Candida albicans, Proteus and Pseudomonas species. Cook, Elliot, Elliot-Smith, Frisby and Gardner, (1957), also described cases of staphylococcal enterocolitis. Teeth staining occurs in infants (Garrod et al., 1973). Renal damage in patients with impaired renal function has been observed. In addition, liver damage can occur if tetracycline is given in excessive doses. Intracranial hypertension in children also occurs although the symptoms clear when the drug administration is discontinued (Garrod et al., 1973).

Chloramphenicol: This drug was the first broad spectrum antibiotic discovered, and is active against a wide variety of Gram-positive and Gram-negative bacteria, Chlamydia, Salmonella typhi, Haemophilus influenzae,

and Bordetella pertussis.

But chloramphenicol has also many side effects like soreness of the mouth resulting from overgrowth of the opportunist Candida albicans. There have been cases of granulocytopaenia and aplastic anaemia and also encephalopathy following treatment with chloramphenicol. The drug also causes hypothermia in infants, optic neuritis in children and its inhibition of protein synthesis is a cause of concern especially in antibody synthesis and wound healing (Garrod et al. 1973).

As a result of these side effects, chloramphenicol is not as a routine prescribed for minor infections. In fact, its use is almost confined to treatment of typhoid fever and salmonella infections, meningitis, severe respiratory tract infections due to Haemophilus influenzae and whooping cough. The daily dose and duration of course is also usually limited, especially in patients with impaired hepatic or renal functions and those at the very extremes of life.

1.8. Antibiotic resistance:

Studies on the mechanism of drug resistance in bacteria have revealed that resistance may be acquired by drug tolerance or destruction (Garrod et al. 1973).

(a) Drug tolerance:

Drug tolerant bacteria can grow in the presence of increased concentrations of a particular antibiotic indifferently or, although capable of multiplication in the presence of the drug, grow more luxuriantly in its absence. In yet some other cases, the organism may be drug dependent. The stability of drug resistance in these strains varies from one drug to the other. Cross resistance with chemically related drugs is also usual. The mode of acquisition of drug tolerance in laboratory experiments suggests spontaneous mutation, although Dean and Hinshelwood (1964), also suggested the possibility of a process of adaptation.

(b) Drug destroying bacteria:

Bacteria which acquire drug resistance by drug destruction, can do so by three known mechanisms:- transduction, transformation and conjugation.

(i) Transduction:

Transduction is effected by the agency of bacteriophages and as such, is governed by the phage type of the recipient. The transduced elements could be plasmids (Novick and Richmond, 1965) or chromosomal material (Asheshov, 1966). Jarolman, Bondi and Cowell, (1965) demonstrated transduction in vivo in mice and co-transduction with linked antibiotic resistance was also demonstrated by Novick and Richmond, (1965).

Transduction occurs in various Enterobacteria, but limited host ranges of phages expands the event to most members of same species. It appears that transduction plays a major part in antibiotic resistance of Staphylococcus aureus, (Cruickshank et al. 1973, 1975).

(ii) Transformation:

The mechanism of acquisition of resistance by transformation was first demonstrated in the pneumococci. However, it was after so many years of this discovery that it was shown that the transforming agent was deoxyribonucleic acid (DNA).

Transformation is not confined to any particular character but can be demonstrated for any character whose inheritance by the recipient bacteria is readily detectable, including drug resistance. Therefore, the mechanism offers an efficient tool for the direct investigation of the relationship between DNA structure and function.

(iii) Conjugation:

Acquisition of drug resistance by conjugation was first reported by Japanese workers who showed that multiple drug resistance can be transferred from resistant Escherichia coli to sensitive Shigella strains (Watanabe, 1963). This mechanism of transfer was demonstrated in vitro by Ochiai, (1959), and Akiba, Koyama, Ishiki, Kimura and Fukushima (1960).

Kagiwada, Kato, Rokugo, Hoshino and Nishiyama, (1960) and Akiba (1961) also showed this to be true with human volunteers, while Mitsuhashi (1960), demonstrated it in dogs and Akiba (1961), in mice.

Outside Japan, Datta, (1962, 1965), reported the incidence of infectious drug resistance in strains of Salmonella typhimurium in London. It is now obvious that transmissible drug resistance is common in several bacterial species and that resistance can be transferred widely among the Enterobacteriaceae (Harada, Suzuki, Kameda and Mitsuhashi, 1960), Vibrio cholerae (Baron and Falkow, 1961), Pasteurella pestis, (Ginoza and Matney, 1963), and Serratia marcenscens (Falkow, Marmur, Caret, Spilman and Baron, 1961). The infective agent is an extrachromosomal element known as plasmid.

Plasmid mediated antibiotic resistance normally depends on the inactivation of the antibiotic in which enzymes specified by the plasmid convert the antibiotic into an inactive form either by breakdown or substitution. Richmond and Sykes, (1973), demonstrated the destruction of penicillins and related antibiotics by plasmid specified β-lactamases. The attack on chloramphenicol is an example of inactivation by substitution catalysed by specific acetylases. The aminogycoside antibiotics are inactivated by acetylation, phosphorylation or adenylylation. Altered permeability of cells has been responsible for some plasmid mediated antibiotic resistance. Franklin and Higginson, (1969), showed this with tetracycline in which reduced internal concentration of the drug was demonstrated in resistant cells.

Plasmid mediated drug resistance occurs in both Gram-negative and Gram-positive bacteria but the control of the inactivating enzymes has been shown to be different. Richmond and Sykes, (1973), found that inactivating enzymes by Gram-negative bacteria are produced constitutively but inducibly in Gram-positive bacteria. Drug inactivation by plasmids

also resembles chromosomally determined antibiotic inactivation but the enzymes involved in the two systems differ in properties.

Plasmid determined drug resistance also depends on the synthesis of plasmid-specified iso-enzymes which replace host enzymes inactivated by the antibacterial agent. Wise and Abou-Donia (1975) demonstrated a plasmid encoded dihydropteroic acid synthase iso-enzyme insensitive to sulphonamides in Escherichia coli. Also Amyes and Smith (1974), found that resistance to trimethoprim in E. coli carrying plasmid was due to the production of a new form of dihydrofolate reductase which was unaffected by trimethoprim.

Novick, (1969), demonstrated that resistance factors and related plasmids frequently determine resistance to more than one antibiotic and additionally may carry the determinants of resistance to heavy metals and to antibacterial agents other than antibiotics. In fact, resistance to inorganic ions in Staphylococcus aureus is specified by genes carried on the same plasmid as determine resistance to penicillin and erythromycin. Stoleru, Gerbaud Bouanchaud and LeMinor, (1972), also demonstrated that hydrogen sulphide production in the E. coli is linked to tetracycline resistance. Other plasmid dependent resistances have also been reported including determinants for haemolysin, enterotoxin and K88 antigen (Royer-Pokora and Goebel, 1976; Oskov and Oskov, 1966; Smith and Halls, 1967; Smith, 1968).

Since the discovery of bacterial conjugation, several workers have shown that genetic material can be transferred by this process. During conjugation, one cell acts as donor or male while the other acts as recipient or female, and the transfer of genetic material requires direct cell to cell contact achieved by means of the sex pili (Brinton, 1965, 1971). The genetic materials transferred may contain markers, some of which specify resistance to antibiotics. Recipient or female cells acquire male sexuality after conjugation and could now act as donors in subsequent matings. In

this way, whole populations of bacteria could be infected with antibiotic resistance genes.

The mechanism of bacterial conjugation has been studied by several workers, and although there is no general agreement on several issues, by all the workers, yet there appears to be a fair knowledge as to the process leading to the transfer of genetic material.

(a) Specific pair formation:

Specific donor and recipient cell unions were observed by Lederberg, (1956) with phase contrast microscopy and by Anderson, Wollman and Jacob, (1957) with electron microscopy. This union is stable and Brinton (1965,1971), demontrated that donor pili were essential for specific pair formation. It was thought that while the donor cells possess donor pili, recipient cells possess a cell surface component (Curtiss,1969). But while specific antigens were demonstrated for the pili, no antigenic relationship could be demonstrated with the recipient cell surface component. Besides, donor cells can also infect other donor cells (Tomoeda, Inuzuka and Date, 1975).

(b) Effective pair formation:

In this process, establishment of cellular contact between participating cells takes place such that transfer of genetic material can proceed. There is no doubt that sex pili are necessary for effective pair formation (Brinton, 1965, 1971). However, the role of the pili in the transfer of genetic material has been interpreted differently by several groups of workers. Meynell and Lawn, (1967), found that sex pili gpl, stabilised unions but were not necessary for Col. 1 transfer, whereas I pili were necessary for transfer. Brinton (1965) suggested that sex pili were used as conjugative tubes and Curtiss, (1969), postulated that transfer occurred with cells in wall to wall contact achieved by pilus retraction into the donor cells.

Although an axial hole about 1.5 to 3nm in diameter, capable of

accommodating a single strand DNA was demonstrated in the F pili (Lawn, 1966; Brinton, 1971) the I pili rarely show the presence of an axial hole. It seems likely that in many cases, wall to wall contact achieved by pilus retraction may be responsible for nucleic acid transfer (Jacobson, 1972; Bradley, 1972). This latter view has been supported by various workers who found that under certain conditions, sex pili can retract. Novotny and Fives-Taylor (1974), demonstrated this with cyanide treated cells while Bradley (1966, 1972), with phage treated cells.

(c) Chromosome and sex factor mobilisation:

Jacob and Wollman (1956), predicted the integration of the autonomous sex factor into the chromosome as a pre-requisite for subsequent chromosome transfer. But Clowes and Moody (1966), found that integration was not necessary for transfer of genetic material. Also, it is not certain if the initiation of plasmid replication occurs at origin or termination of chromosome replication. For example, while Cooper (1972), proposed that plasmid replication occurred at origin, Collins and Pritchard (1973), found that it occurred at termination of chromosome replication.

(d) Deoxyribonucleic acid transfer:

In this process, the genetic information is transferred from donor to recipient in a definite sequence. If the sex factor is integrated, then only part or very rarely all of the chromosome is transferred. Gross and Caro (1966) demonstrated the presence of a double stranded DNA in the recipient, one strand pre-existing while the other was synthesised after or during transfer. Curtiss (1969), resolved this by the use of minicells and the demonstration that donor cells only transferred single strand DNA, the other being synthesised in the recipient. In the sex factor, F, both newly transferred single strand DNA and that left in the donor are rapidly converted to covalently closed circular (CCC) double stranded DNA. Studies by several workers indicate that transfer specific DNA synthesis

occurred in both donor and recipient, and it has been suggested by some workers that transfer is dependent on the continued functioning of this process in the donor at least (Bressler, Lanzov and Lichachev, 1973). In spite of the conclusion by Barbour (1967), that replicative DNA synthesis in the recipient was not necessary for conjugal DNA transfer, Bressler et al. (1973) demonstrated that transfer-specific DNA synthesis occurred in the recipient.

(e) Recombinant formation:

Following conjugal transfer of genetic material from a donor to a recipient, synapsis between homologous segments of donor and recipient genome, formation of effective homologous pairing, reassortment of donor and recipient genetic information to yield new combinations of genetic information and the segregation of recombinant chromosomes from non-recombinant chromosomes occur (Curtiss, 1969). Curtiss, Charmella, Stallions and Mays (1968), showed that in E. coli crosses, there was a requirement for homology between the DNA of the donor and recipient. These workers therefore argued that this requirement for homology might be related to the integration of the fragment or the actual efficiency of transfer might be affected. It appears this is the case (Curtiss et al. 1968).

1.9 Factors affecting transfer efficiency in mating experiments:

Ippen and Valentine (1965) and Brinton and Beer (1967), demonstrated that sex pili synthesis increased most rapidly during the exponential phase of growth and reached a maximum during the late exponential or early stationary phase of growth. The latter workers also showed that in some bacterial strains, pili synthesis decreased as cultures entered the stationary phase of growth.

The mean number of pili per bacterial cell, the length and probably the formation of specific pairs is influenced by the growth medium (Tomoeda

et al. 1975). Starvation of male cells results in loss of donor pili. Also, donor cells grown in mild anaerobic conditions have been found to produce more sex pili resulting in higher mating ability and therefore of transferability of genetic material (Curtiss, Caro, Allison and Stallions, 1969).

Novotny and Lavin (1971) studied the influence of cultivation temperature on sex pili synthesis and hence of conjugation efficiency. These workers showed that the optimal growth temperature for sex pili synthesis was between 37° and 41° and that at 25°, no pili synthesis occurred.

In addition to the environmental factors mentioned above, the adverse effects of metabolic disorders on sex pili synthesis have been demonstrated by several workers. For example, Novotny, Taylor and Lavin, (1972), revealed that ultraviolet irradiation of bacterial cells resulted in fewer and shorter pili. Energy poisons such as sodium cyanide, 2,4-dinitrophenol and sodium azide prevent pili outgrowth or cause the disappearance of pili from cell surface (Brinton, 1971). Arsenate was also found to inhibit the synthesis of sex pili (O'Callaghan, Bundy, Bradley and Paranchych, 1973).

1.10 Methods for determination of antibiotic resistance in bacteria:

Several methods have been adopted for routine sensitivity testing in many laboratories. The most common is the diffusion test because it is quick, simple and reliable with skilled workers. Many variations of the method are used in different laboratories but the principle is the same.

(a) Disc diffusion method:

Antibiotic containing discs, usually of filter paper are used from which diffusion takes place of the antibiotic through the solid medium thereby inhibiting the growth of the organism to a distance which depends on the sensitivity of the organism to the particular drug contained in the disc.

Unfortunately, results of diffusion tests are affected by several factors. Some workers reported that certain constituents of test medium affect results of diffusion tests (Garrod et al. 1973). These constituents include blood, electrolytes, sugars and agar. Suggestions for a specially formulated test medium seemed reasonable but several variations were reported between different manufactured products (Garrod et al. 1973).

Other factors affecting this method include depth of the medium, size of inoculum, antibiotic content of the disc, and length of incubation.

In spite of the routine applicability of various modifications of the diffusion method, the results obtained are merely qualitative. The levels of resistance or sensitivity cannot be precisely determined by this method.

(b) Tube dilution method:

This is perhaps the most accurate method for the determination of minimum inhibitory concentration. However, the method is too time consuming for routine use and is therefore restricted to cases where dosage is dependent on the results of the test as in the treatment of bacterial endocarditis, tests of slowly growing organisms like the Mycobacterium tuberculosis, and the demonstration of small degrees of resistance. Sometimes it is difficult to differentiate the bactericidal level from the bacteriostatic level and in such cases tube contents may have to be sub-cultured on solid medium. Also, it involves the use of too many tubes and in a busy laboratory, this would be a major disadvantage.

(c) Velvet pad replica plate method:

The major disadvantage of this method is that the pad transfers only about 0.5% of the cells present (Garrod et al. 1973). The results

are also very variable. The sensitivity of the method also depends on the pressure applied on the pad and this results sometimes in very heavy inocula.

(d) Celophane transfer method:

Though this method is used by many workers, and Garrod et al. (1973) even described some of the results as revealing, the main disadvantage of the method is that some protein bound antibiotics are carried over by the celophane especially if the concentration is too high to prevent growth of surviving bacteria.

(e) Agar dilution method:

In this method dilutions of the antibiotic under test are carried out in the solid agar medium. Its great advantage lies in the fact that more than five different strains can be tested on the same plate, at the same time. Moreover, most of the factors which affect the disc diffusion method, have no bearing in the interpretation of results. It also reveals if the resistance affects the whole inoculum or just a handful of cells. However, plates used must be properly dried to prevent spreading.

1.11 Methods for demonstrating drug resistance transfer by conjugation:

In the laboratory, bacterial conjugation can be demonstrated either microscopically, (Lederberg, 1956), or on a selective medium. Whereas bacterial cells can be shown microscopically in conjugational events, the most reliable way of demonstrating that genetic material and in fact determinants for antibiotic resistance have been transferred is by selecting for resistance amongst otherwise sensitive recipient bacteria on the appropriate selective medium.

In order to ease the problem of differentiating between donors and recipients on the selective plate after mating in liquid medium, it is common practice to use selective agents which could be nutrients or antibiotics in order to obtain mutants of the recipient bacteria. Nalidixic acid and rifampicin are commonly used especially as it is simple to obtain

resistant mutants.

Spot matings on an agar plate could also be carried out with success. The great advantage of this method lies in the fact that as many as eight diploids can be prepared per plate. However, nalidixic acid is not recommended for plate matings because it inhibits DNA synthesis and therefore prevents transfer in plate matings. Also the broth applied to a spot can sometimes interfere with scoring of recombinants, especially if a nutritional marker is used for counterselection instead of a drug resistant marker (Miller, 1972).

A large number of strains can be mated efficiently by replica matings. Master plates are prepared by gridding freshly growing donor colonies on to rich plates and incubating for six to eight hours. Fresh overnight culture of recipient is then spread evenly over the selection plate surface. When dry, the master plate is replicated on to the selective plate. The advantage of this method is that about 50 colonies can be tested for their donor properties in one step.

Though the last two methods are convenient and have been used by several workers in many successful experiments, mating in liquid medium and selection of diploids on solid selective medium appears preferable as the efficiency of transfer can be more accurately determined, and mutants resistant to nalidixic acid and rifampicin are simple to obtain.

1.12 Classification of plasmids by incompatibility tests:

Plasmids possess specific compatibility properties which limit coexistence of closely related plasmids (Inselberg, 1974). By this phenomenon, closely related plasmids fail to be stably maintained together. This property has been exploited by Datta (1975b) in classifying plasmids. In this way conjugative plasmids have been classified into 20 groups by incompatibility in Escherichia coli. It is generally accepted that within

an incompatibility group, all plasmids determine the same type of sex pili, of which five distinct types have been identified and visualised by electron microscopy in E. coli K12 - F, I, RP1, W, and N.

The studies of Smith (1974) and Rodriguez-Lemoine, Jacob, Hedges and Datta (1975), show that for plasmids of group H and S, the production of the conjugal apparatus is temperature sensitive and this evidence which is true for all tested members of these groups reveals that within an incompatibility group, all plasmids have the same, or closely related transfer genes.

Incompatibility of plasmids must be differentiated from other similar phenomena, like surface exclusion. For example, incompatibility affects a plasmid after it has entered a cell already containing a related plasmid, whereas in surface exclusion, the incoming plasmid does not enter the cell because a stable union between donor and cell carrying the related plasmid is prevented. Incompatibility also differs from dislodgement (Coetzee, Datta and Hedges, 1972), which occurs between normally compatible plasmids at introduction, and resulting in the displacement of resident plasmid. With incompatibility, the unestablished plasmid is neither destroyed nor lost, but just fails to replicate (Dubnau and Maas, 1968).

Classification of plasmids by incompatibility is usually carried out by the methods prescribed by Datta (1975b), in which a second plasmid is transferred into a culture already carrying a resistance plasmid. Selection is carried out for the incoming plasmid only. Transconjugant clones are purified and tested for the presence of the resistance characters determined by each of the two plasmids and if the introduction of either plasmid always eliminates the other, the two plasmids are incompatible. Many plasmids can be allocated to incompatibility groups by this method (Datta, 1975b).

Because, incompatibility reflects the specificity of plasmid replication, (Datta, 1975b), and because DNA of different plasmids of the same compatibility group hybridizes extensively, whereas plasmids of different

groups have few polynucleotide sequences in common (Falkow, Guerry, Hedges and Datta, 1974; Grindley, Humphreys and Anderson, 1973), it appears justifiable to adopt this system of classification because it also seems to divide plasmids into phylogenetically distinct groups, and this could be used to great advantage for epidemiological and ecological studies.

1.13 Isolation of plasmid deoxyribonucleic acid:

The probability that plasmids are composed of DNA was first concluded from the studies of Silver and Ozeki (1962) and some Japanese workers (Watanabe, 1963). Later, the F' factor DNA was identified as a separate satellite peak from the chromosomal DNA (Falkow, Wollheiter, Citarella and Baron, 1964). When isolated extracellularly, the molecule takes the form of a "supercoil"; the DNA helix being twisted around itself. Sometimes, the DNA duplex is nicked or broken, the so called "open circular" DNA.

Many of the methods therefore used for the isolation of plasmid DNA, depend on the supercoiled, covalently, closed, circular structure within the bacterium. This property which is a result of the topological requirement for constancy in number of interstrand crossovers, facilitates their physical separation from other non-circular DNA species.

Thus Cohen and Miller (1969) isolated plasmid DNA from E. coli by exploiting the resistance of circular DNA to alkaline denaturation and the ability of nitrocellulose to bind denatured DNA selectively.

Freifelder and Freifelder (1968), applied the technique of specific labelling of plasmid DNA and agar gel chromatography in their successful isolation of F' lac DNA of Escherichia coli.

Many methods of gentle lysis of bacterial cells have been developed in which chromosomal DNA remains attached to a cellular component during low speed centrifugation. Clewell and Helinski (1969), Humphreys,

Willshaw and Anderson, (1975), used the non-ionic detergent, polyoxyethylene cetyl ether (Brij 58), in isolating plasmid DNA which appears to be present in the cytoplasm and as such does not sediment with the Brij resistant cellular fraction.

Hirt (1967), also described a method of separation of polyoma DNA by the preferential precipitation of the higher molecular weight cellular DNA in the presence of sodium lauryl sulphate and a high sodium chloride concentration. Modifications of this method have been used by Guerry, LeBlanc, and Falkow (1973), and Meyers, Sanchez, Elwell and Falkow (1976) for the separation of plasmid DNA.

Differences between plasmid and host DNA base ratios have been reported by several workers. Falkow and Citarella (1965), exploited these differences in the isolation of plasmid DNA which have a G+C content of between 45 and 55 percent.

Several E. coli mutants have been identified as having abnormal cell division that results in the segregation of progeny cells which do not contain any appreciable amounts of chromosomal DNA. Thus the DNA of segregant minicells is almost entirely plasmid DNA and therefore provides an efficient biological separation of plasmid from chromosomal DNA (Adler, Fisher, Cohen and Hardigree 1967).

Radloff, Bauer and Vinograd (1967), reported a method for the detection and isolation of closed circular DNA in the presence of the intercalating dye, ethidium bromide. The binding of the intercalating dye was shown to cause a partial unwinding of the duplex structure in closed circular DNA (Bauer and Vinograd, 1968). Such unwinding results in a change in the number of superhelical turns, so that the total number of turns in the molecule remains constant. A little, yet critical amount of dye binding reduces the number of superhelical turns to zero but further dye binding results in the formation of superhelices of the

opposite sign. These new superhelices bring about mechanical stresses in the duplex and a more ordered conformation in the molecule, thus increasing the free energy of formation of the dye-DNA complex. Consequently, the maximum amount of dye that can be bound by the closed molecule is smaller than that by the linear or nicked circular molecule. It therefore follows that since the buoyant density of the DNA-dye complex is inversely related to the amount of dye bound, the buoyant density of the closed circular DNA-dye complex at saturation, is greater than that of the linear or nicked circular DNA-dye complex. Bauer and Vinograd (1968), demonstrated that the effects already discussed results in a buoyant density difference of approximately 0.04gm/ml. in caesium chloride containing saturating amounts of ethidium bromide.

1.14 Characterisation of plasmid deoxribonucleic acid:

It was Lang, Wolf and Russel (1967), who observed from their studies that most plasmid DNA contained molecules of one size. Molecular weight determination of plasmid DNA therefore appears to be an easy and reproducible method for the preliminary characterisation of plasmid DNA.

(a) The molecular weights of plasmid DNA can be estimated from sedimentation coefficients in neutral sucrose gradients. Fiers and Sinsheimer (1962) found that the ratio of covalently closed circular DNA to open circular molecules was 1.33-1.7:1.

(b) A single nick in just one strand of the duplex is sufficient to convert covalently closed circular DNA into open circular molecule. Applying this principle, Freifelder (1968) obtained some earliest measurements of the molecular weights of covalently closed circular molecules by measuring the relative losses of CCC-DNA and corresponding gains in open circular DNA in a series of DNA samples exposed to increasing doses of X-irradiation.

(c) DNA molecules can be fragmented and heat denatured to separate the two strands of the double helix. On cooling, each single stranded fragment will reassociate with its homologous complementary strand to form a double stranded fragment. Thus, measurement of the rates of reassociation was studied and found to give a measure of genome size (Britten and Kohne, 1968).

(d) If DNA is allowed to diffuse to an air water interface at which there is a monolayer of protein, the thin DNA molecule is adsorbed to the liquid surface. Lang and Mitani (1970), employed this technique in studying the size of plasmid DNA using the electron microscope.

Lang et al. (1967) also traced contours of open circular molecules and succeeded in measuring their lengths accurately with the aid of a map measurer.

(e) Agarose gel electrophoresis has also been used by several workers for determining the size of plasmid DNA (Meyers et al. 1976). These workers reported that the logarithm of relative migration of standard CCC plasmid DNA through gel against logarithm of plasmid molecular weight gave a linear plot, in 0.7% agarose.

(f) Aber and Linn (1969), predicted that there are special sequences on foreign DNA which on entry into the cell are recognised by a restriction endonuclease and cleaved. In order to protect these sequences on its own DNA, the cell has a modification methylase which imparts methyl groups to nucleotides within these sites, making them resistant to the endonuclease. This prediction was found to be true for Haemophilus (Roy and Smith, 1973), and resistance transfer factor systems (Boyer, 1973), but only partially true for the E. coli system (Linn, Lautenberger, Eskin and Lackey, 1973). The genes controlling the structure of these enzymes are found on phage chromosomes, bacterial chromosomes and plasmid DNA molecules. The

specificity of a set of restriction and modification enzymes is determined by the sequence of nucleotide base pairs recognised by the enzymes. On the basis of structural and functional considerations, Boyer, (1971), divided these restriction and modification systems into two groups; types 1 and 2. The type 1 enzymes are structurally complex, limit digests are difficult or impossible to achieve and the 5'-terminal nucleotide produced by the endonucleolytic event is not available for phosphorylation, (Linn et al. 1973). With these properties, the analysis of the sequence cleaved by endonucleases of this class is impossible.

The type 2 restriction and modification enzymes, represented by several Haemophilus systems as well as E. coli systems genetically controlled by R-factors are smaller and require only magnesium ions for endonucleolytic activity on unmodified DNA. Also, all of the type 2 restriction endonucleases make double stranded breaks in unmodified DNA which can be analysed. Certainly, smaller DNA fragments resulting from cleavage with restriction enzymes would lighten the problem of analysis of large DNA molecules. This was proved possible by Allet, Jeppesen, Katagiri and Delius, (1973), who used the resistance transfer factor R1 endonuclease to digest lambda DNA and then separate the corresponding fragments by gel electrophoresis.

1.15 Aims of present investigation:

Since the discovery of transferable drug resistance in bacteria by Japanese workers, (Watanabe, 1963; Meynell, Meynell and Datta, 1968), resulting mainly from the widespread use of antibiotics (Anderson, 1965, 1968), several workers have demonstrated that resistance factors are common in non-pathogenic Escherichia coli of the alimentary tract of man (Watanabe, 1963; Moorhouse and McKay, 1968), and animals (Anderson and Lewis, 1965; Anderson, 1968).

Consequently, many organisms carrying resistance factors were

isolated from raw sewage (Sturtevant, Cassel and Feary, 1970), and it was expected that rivers and coastal waters would ultimately be contaminated with these organisms. This expectation was justified by the studies of Smith (1970), Smith, (1971) and Smith, Farrel and Dunican, (1974). Of course, the principal concern of these workers was Escherichia coli with transmissible drug resistance to chloramphenicol, considered to be potentially more dangerous because its resistance could be transmitted to Salmonella typhi and so render the treatment of typhoid fever more difficult. In the light of recent studies on the side effects of most antibiotics used for therapy, this exclusive concern no longer seems justifiable. Similar concern has therefore been shown to in fact, all the antibiotics (Garrod et al. 1973). The most commonly used of these drugs therefore have become the most commonly investigated.

Inselberg (1974) observed that closely related plasmids fail to be stably maintained together in the same cell. By this incompatibility of related plasmids, Datta (1975a, 1975b) classified plasmids into twenty groups.

Plasmids are supercoiled, covalently, closed, circular DNA molecules. This property has been the basis of its isolation from the bacterial cell by most workers (Cohen and Miller, 1969; Freifelder and Freifelder, 1968; Godson and Sinsheimer, 1967; Hirt, 1967; Falkow and Citarella, 1965; Adler et al. 1967; Radlof et al. 1967).

Molecular weight measurement appears to be a reliable method for the preliminary characterisation of plasmid DNA because of the observation that most plasmid DNA contained molecules of the same size (Lang et al. 1967). Agarose gel electrophoresis was employed by some workers for molecular weight determination because the logarithm of molecular weight and relative migration gave a linear plot (Meyers et al. 1976). But Allet et al. (1973) also observed that separation of large molecules in

agarose gel was difficult owing to the fact that large molecules migrated together in close bands. These workers also reported the use of RTF-R1 endonuclease to digest lambda DNA and separation of the fragments by gel electrophoresis, thus introducing not only a more efficient method for separating DNA molecules of different sizes, but also providing fragments of whole DNA which could be studied independently of one another.

It was therefore decided that an estimation of the number of E. coli in the St. Andrews coastal bathing area be carried out. From the population of Escherichia coli, the number resistant to some commonly used antibiotics: streptomycin, ampicillin, chloramphenicol and tetracycline should be studied with a view to finding out those whose resistances were carried on transferrable drug resistance plasmids.

Furthermore, the transfer properties of some of the plasmid mediated drug resistant strains were to be studied in conjugation experiments with recipient E. coli, K12, R-negative strain.

It was also decided to select some strains (5) on the basis of efficient transferability and to classify these by the incompatibility tests of Datta (1975b).

Finally it was decided to isolate plasmid DNA from some of the strains and to characterise them on the basis of molecular weight determination in agarose gel. It was thought that if a simple and yet reliable method of classification of plasmid DNA could be evolved from this study, the technique would be of immense value in the study and control of infectious drug resistance.

2. Materials and methods:

2.1 Media:

(a) MacConkey broth (Report 71, 1969). The Oxoid dehydrated base (CM 5A) was used. This had the following formula:

Peptone (Oxoid L 37)	20gms.
Lactose	10gms.
Bile salt (Oxoid L 55)	5gms.
Sodium chloride	5gms.
Bromo-cresol purple	0.01gms.
Distilled water	1000ml.
pH	7.4

Autoclaved at 121°for 15 minutes.

(b) Peptone water for indole reaction (Report 71, 1969).

(c) Nutrient broth. The Oxoid dehydrated base was used. The formula was as follows:

Lab lemco powder (Oxoid L29)	1gm.
Yeast extract (Oxoid L20)	2gms.
Peptone (Oxoid L37)	5gms.
Sodium chloride	5gms.
Distilled water	1000ml.
pH	7.4

Autoclaved at 121°for 15 minutes.

(d) Thioglycollate nutrient broth (Cruickshank *et al.* 1975). Concentrations of thioglycollic acid varying from 0.01 - 0.1% were added to the nutrient broth (c).

(e) Modified Koser's citrate medium (Report 71, 1969).

(f) Glucose phosphate medium for the methyl-red and Voges-Proskauer tests (Report 71, 1969).

(g) "L" broth. The following formula was used:

Tryptone T (Oxoid L43)	10gms.
Yeast extract(Oxoid L21)	5gms.
Sodium chloride	0.5gm.
Glucose	0.1gm.
Distilled water	1000ml.
pH	7.0

Autoclaved at 121° for 15 minutes.

(h) MacConkey agar (Report 71, 1969). The Oxoid dehydrated base (CM7) was used. The formula used was as follows:

Peptone	(Oxoid L37)	20gms.
Bile salts	(Oxoid L55)	5gms.
Sodium chloride		5gms.
Lactose		10gms.
Neutral red		0.075gm.
Agar no. 3	(Oxoid L13)	12gms.
Distilled water		1000ml.
pH		7.4

Autoclaved at 121° for 15 minutes.

(i) Nutrient agar. The Oxoid dehydrated base (CM3) was used. The formula was the same as for the nutrient borth (c), but for the addition of 15 grams of Oxoid L13 agar.

2.2 Reagents:

(a) Kovacs reagent (Report, 71, 1969).

(b) Methyl-red (Report, 71, 1969).A concentration of 0.04% in 40% ethanolic distilled water was prepared.

(c) Potassium hydroxide (Report, 71, 1969). A concentration of 40% (w/v) in distilled water was prepared for the Voges-Proskauer test.

(d) Creatine (Report, 71, 1969).

(e) Methyl-violet stain (Cruickshank et al., 1975). A concentration of 0.5% in distilled water was used.

(f) Iodine solution . The following formula was used:

Iodine	10 grams
Potassium iodide	20 grams
Distilled water	1000 ml.

The iodine was dissolved in 250 ml. of potassium iodide solution and then made up to 1000 ml.

(g) Neutral-red solution (Cruickshank et al., 1975)

Neutral-red	1 gram
1% acetic acid	2 ml.
Distilled water	1000 ml.

(h) 25% sucrose (w/v) in 0.05M Tris-HCl; pH 8.0 (Bauer and Vinograd, 1968).

(i) Lysozyme (Sigma Chemical Company Limited, St. Louis, U.S.A).

(j) Ethidium bromide (Sigma Chemical Company Limited, St. Louis, U.S.A.).

(k) Agarose A; obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

(l) Ribonuclease: type 1A bovine pancreas. Obtained from Sigma Chemical Company, St. Louis, U.S.A. Stock solution of 1mg/ml in 50mM sodium acetate, pH 5.0.

(m) TES buffer (Meyers et al. 1976).

(n) Restriction endonuclease Eco R1 dilution buffer:

Potassium phosphate	10mM
Sodium chloride	0.2M
EDTA	1mM
Mercaptoethanol	0.7mM
Bovine serum albumin	2mg/ml
Triton	0.2% (v/v)
pH	7.0

(o) EcoR1 enzyme solution:

Tris-HCl	1M
pH	7.5

(p) Electrophoresis buffer: (x10)

EDTA	0.1M
Tris-HCl	0.36M
NaH_2PO_4	0.3M
pH	8.2

2.3 Antibiotic solutions:

(a) Ampicillin: Antibiotic was obtained from Boots Chemists, South Street, St. Andrews. Stock solution of the antibiotic containing 5mg/ml was prepared in sterile distilled water, and stored at 4°.

(b) Chloramphenicol: Antibiotic was obtained from Boots Chemists, South Street, St. Andrews. Stock solution was prepared in ethanolic distilled water (0.625%) and contained 5mg/ml. Storage was at 4°.

(c) Nalidixic acid: Antibiotic was obtained from Sigma Chemical Company, St. Louis, U.S.A. Stock solution of the antibiotic containing 1mg/ml was prepared in a solution composed of 350 parts of acetone and 25 parts of chloroform. Storage was at 4°.

(d) Rifampicin: Antibiotic was obtained from Sigma Chemical Company, St. Louis, U.S.A. Stock solution of the antibiotic was prepared in methanol and contained 1mg/ml. Storage was at 4°.

(e) Streptomycin: Antibiotic was obtained from Boots Chemists, South Street, St. Andrews. Stock solution containing 5mg/ml was prepared in distilled water and stored at 4°.

(f) Tetracycline: Antibiotic was obtained from Sigma Chemical Company, St. Louis, U.S.A. Stock solution containing 5mg/ml was prepared in ethanolic distilled water (50%), and stored at 4°.

2.4. Organisms: (standard strains)

(a) The following standard cultures used for the incompatibility tests were obtained from Naomi Datta of the Royal Postgraduate Medical School, University of London. The incompatibility groups of the plasmids and their antibiotic resistance pattern are shown in the section on results:- RA1; R40a; R386; R1; R124; R27; R144; R621a; R391; R386; R471a; R446b; N3; R16; RP4; R478; Rtsl; S-a.

Host strains were E. coli J53 resistant to nalidixic acid.

(b) The following two cultures used for the conjugation experiments were supplied by Dr. S. Bayne of the Department of Biochemistry, University of St. Andrews:

(i) E. coli 9484:- an R negative lactose fermenting, non gas producer, used as recipient in the conjugation experiments, unless otherwise stated.

(ii) E. coli, ED 2111: a non-lactose fermenting mutant with plasmid mediated antibiotic resistance to tetracycline and chloramphenicol. Levels of antibiotic resistance are given in the section on results. Unless otherwise stated, this organism was used as standard donor strain in the conjugation experiments.

(c) Cultures were stored in nutrient agar slopes at 4°.

2.5 Examination procedure:

(a) Collection of water samples:

Water samples were collected in 250 ml. screw capped medical flats , previously sterilised by autoclaving. Samples were collected one hour before high tide at a sampling spot close to the coastal bathing area near the ruins of the St. Andrews castle.

All the aseptic precautions laid down in Report 71, (1969), were observed during sampling. Samples were tested in the laboratory within

one hour of collection.

(b) Membrane filtration:

The methods described in Report, 71, (1969), were used, with the following components of Millipore equipment:- Sterifil filtration unit (xx1104700), membrane filters (HAWG 04750), absorbent pads (AP 1004 S0) and plastic petri dishes (PD 10 047 00).

(c) Plate counts:

Surface plate counts on MacConkey agar and nutrient agar were carried out by the methods of Miles and Misra (1938). Nutrient broth was used as diluent in all the conjugation experiments.

(d) Incubation of cultures:

All plate cultures were incubated at 37°. Confirmatory biochemical tests for the E. coli isolates were carried out at the stipulated temperatures in thermostatically controlled hot water baths.

(e) Identification of E. coli isolates:

The purity of the colonies suspected to be E. coli was confirmed by results of Gram-reaction, and the recommendations of Report, 71, (1969). This involved testing the organisms for citrate utilisation, fermentation of lactose with the production of both acid and gas at 44°, production of indole at 44°, positive methyl-red test and negative Voges-Proskauer test.

(f) Determination of the minimum inhibitory concentration of isolates:

The agar dilution method (Garrod et al., 1973), was adopted. The following procedure was followed:-

(i) A range of dilutions of the antibiotic was prepared in sterile distilled water such that when added to agar, the final concentration required would be obtained.

(ii) The appropriate amount of the antibiotic was added to the sterile, molten agar after it had been cooled to about 47° in a water bath; and after mixing, it was poured into sterile petri dishes.

(iii) After setting, the plates were dried at 37° with lids tipped.

(iv) Broth cultures of organisms to be tested were diluted 1/100 and inoculated onto the agar with the aid of a standard wire loop.

(v) Cultures were incubated at 37° in an incubator for 18 to 24 hours. The minimum inhibitory concentration of the antibiotic inhibiting growth was then recorded.

(g) Isolation of nalidixic acid mutants:

The method used was as described by Miller, (1972). Several drops of overnight broth culture of the E. coli isolate were spread onto each of three MacConkey agar plates containing nalidixic acid (20µg/ml). Plates were incubated at 37° overnight. Resistant colonies were purified onto the same type of selective plate.

(h) Isolation of rifampicin resistant mutants:

The method used was as described by Miller (1972). Six drops of fresh overnight culture of the E. coli isolate were spread onto each of three MacConkey agar plates containing rifampicin (100µg/ml). Plates were incubated at 37° overnight, after which resistant colonies were purified onto the same rifampicin containing plates.

(i) Transfer of resistance by conjugation to sensitive E. coli:

The method described by Curtiss et al. (1969) were modified to suit our experimental conditions. Standardisation of tests were carried out before the test proper in order to determine the effects of:-

(a) different growth phases on the efficiency of resistance transfer.

(b) anaerobiosis on the efficiency of resistance transfer. Thioglychollic acid (0.01-0.1%), nutrient broth was used as growth medium in this experiment, and

(c) the same E. coli serotype used as both donor and recipient on efficiency of resistance transfer.

The general design in the conjugation experiment was as follows:-

Cultures of donor and recipient bacteria were grown to logarithmic phase in thioglycollic acid (0.02%), nutrient broth at 37° with gentle rotation. Equal numbers of donor and recipient cells (determined by optical density readings at 610nm with the SP 600 spectrophotometer), were mixed in a sterile container and incubated at 37° with gentle rotation for 60 minutes. Total counts of recipient cells were obtained by surface counts on MacConkey agar plates containing either nalidixic acid (20µg) or rifampicin (100µg/ml). Counts of infected recipient cells were also obtained on MacConkey agar plates containing the drugs to which donor cells are resistant to and either nalidixic acid or rifampicin. The counting technique used was that of Miles and Misra (1938). Plates were incubated at 37° for 18 to 24 hours and colony counts were obtained with the aid of a colony counter.

(j) Incompatibility test of five wild type isolates:

Five wild type plasmids transferred to E. coli 9484 as host strain were tested by the Methods of Datta (1975b). Standard strains used were the eighteen J53 strains obtained from Naomi Datta. All the standard strains used were nalidixic acid resistant mutants while the wild strains were rifampicin resistant mutants. The general method adopted was as follows:-

(i) A second plasmid was transferred as described in the conjugation experiments into a culture already carrying a resident plasmid. Equal number of cells of both cultures were used.

(ii) After incubation for 60 minutes, selection of incoming plasmid was carried out on appropriate selective plate.

(iii) Ten colonies of transconjugant clones were purified and tested for the presence of antibiotic resistances determined by each of the two plasmids. If the resident plasmid was eliminated from all the clones tested, the test was repeated in the opposite direction.

(iv) If the resident plasmid was not eliminated from all transconjugant clones, the clones with double plasmids were tested for stability by showing the continued presence of markers of both plasmids after many generations in a drug free medium, and separate replication by observing if the plasmids were separately transmissible when the clones with double plasmids were used as donor to an R-negative recipient. Plasmids which coexisted stably and transferred separately were regarded as compatible.

(k) Isolation of plasmid deoxyribonucleic acid:

The method used for the isolation of plasmid DNA was the sodium lauryl sulphate, sodium chloride precipitation method described by Meyers et al., (1976).

SDS-salt precipitation method (Meyers et al., 1976).

Plasmid containing strains were grown overnight in 30 ml. of broth and harvested by centrifugation. The cells were suspended in 1.5 ml. of 25% sucrose in 10 mM Tris, 1 mM EDTA, pH 8.0. 0.2 ml. of lysozyme was added (5mg/ml in 0.25M Tris, pH 8.0), and the suspension swirled at 37° for one minute in a hot water bath. The suspension was then placed in an ice bath for 5 minutes and then 0.4ml of EDTA (0.25M, pH 8.0) was added; the suspension was left for another 5 minutes on ice. Sodium lauryl sulphate was then added to a concentration of 1%, and after cell lysis, 5M sodium chloride was added to a final concetration of 1M.

Lysates were stored at 4° overnight after which they were centrifuged at 17,000g for 30 minutes at 4°. The volume of the cleared lysate was doubled by the addition of distilled water, 20 µg of ribonuclease (1mg/ml in 50mM sodium acetate, pH 5.0, heated for 10 minutes at 90°) per ml. was added and the lysate was incubated at 37° for 1hr. 1 volume of Tris (50mM)-saturated phenol was added and the tube inverted several times, then centrifuged at 12,000g for 30 minutes at 20° to obtain a clear aqueous phase. The clear aqueous phase was brought to 0.3M sodium

acetate and twice the volume of cold (-20°), 95% ethanol was added to precipitate the DNA. The tube was kept at -20° overnight. The precipitated DNA was recovered by centrifugation at 12000 x g at -10° for 20 minutes. The ethanol was thoroughly drained from the tube and DNA, suspended in 0.1ml of TES buffer.

(l) Eco R1 digestion procedure of lambda phage DNA:

1 µg of lambda DNA and 3 µl of diluted enzyme solution (1:30 in Tris-HCl buffer) was incubated in a final volume of 0.05 ml, for one hour at 37°. The reaction was stopped by heating at 70° for 10 minutes. 30 µl of this digest was used for agarose gel electrophoresis.

(m) Agarose gel electrophoresis of plasmid DNA:

0.7 gm of agarose was incubated for 40 minutes at 90° in 90 ml of distilled water. After cooling to 65°, 10 ml of 10-fold concentrated electrophoresis buffer and 10 µg/ml ethidium bromide were added.

Electrophoresis was carried out in a gel slab measuring 18 x 19 x 0.3 cm. 20 µl of plasmid DNA was mixed with 5 µl of a dye solution containing bromophenol blue (0.07%) and ficoll (2%) and applied to sample wells.

The concentrated electrophoresis buffer was diluted ten times with distilled water, and electrophoresis was carried out at 30 volts for 18 hours.

The gels were observed under long wave ultra-violet light immediately after electrophoresis.

Measurements of relative mobility of bands in gel were obtained manually with a ruler.

3. RESULTS:

3.1 Membrane filtration counts:

Samples of water collected as previously described were subjected to membrane filtration using the Millipore apparatus already mentioned. Membranes were incubated on MacConkey agar at 37° for 24 hours for the coliform count.

Suspected coliforms were tested by the IMVIC tests and Gram-reaction for the identification of Escherichia coli. Coliforms identified as Escherichia coli gave the following reactions:-

Test	Result
indole production at 44°	positive
methyl-red test at 30°	positive
Voges-Proskauer test at 30°	negative
citrate utilisation at 30°	negative
lactose fermentation at 37° and 44° with gas.	positive
Gram-reaction	Gram-negative bacilli

Table 1 shows the coliform count obtained from ten samples of water. Triplicate and mean counts from each sample are shown.

Table 2 also shows the number of Escherichia coli identified by the IMVIC test and Gram-reaction from the total coliform bacteria. Triplicate and mean counts from each sample are shown.

The results show a wide variation in the number of coliforms and Escherichia coli isolated on different days from the same sampling site. The results also show that the percentage of E. coli amongst the coliform organisms ranges from 22 to 27%. An exception was observed only in one sample with 38.1% of E. coli.

Table 1

Recovery of coliform bacteria per 10 ml at 37° by membrane filtration from 10 samples of water using MacConkey agar. Triplicate and mean counts on each sample are presented.

	WATER SAMPLES.									
	1	2	3	4	5	6	7	8	9	10
Count 1	50	66	42	68	42	48	76	41	30	69
2	59	57	39	73	34	39	67	42	28	68
3	52	62	36	64	35	38	69	38	29	66
Mean Count	54	62	39	68	37	42	71	40	29	67

Table 2

Recovery of Escherichia coli per 10 ml by membrane filtration from 10 water samples, confirmed by Gram-reaction, indole test at 44°, citrate utilization, methyl red, Voges-Proskauer, lactose fermentation with acid and gas at 37° and 44°. Triplicate, mean counts and percentage of total coliforms on each sample are presented.

	WATER SAMPLES									
	1	2	3	4	5	6	7	8	9	10
Count 1	10	14	11	16	11	18	19	11	8	15
2	15	18	7	21	8	15	17	11	7	17
3	11	13	8	14	10	14	18	9	7	18
Mean Count	12	15	9	17	10	16	18	10	7	17
% of total coliforms	22.2	24.2	23.1	25	27	38.1	25.3	25	24.1	25.4

3.2 Minimum inhibitory concentration of antibiotics on the isolates:

The minimum inhibitory concentration of antibiotics (ampicillin, chloramphenicol. tetracycline, streptomycin, nalidixic acid and rifampicin) on the wild isolates was determined on antibiotic containing nutrient agar plates as previously described. The E. coli standard 9484 was used as control for the test. Incubation during the test procedure was carried out at 37° in a hot air incubator.

The usual minimum inhibitory concentration (μg/ml) of a sensitive E. coli is given by Garrod et al. (1973) as follows:-

ampicillin	8 μg/ml.
chloramphenicol	2-8 μg/ml.
tetracycline	1 μg/ml.
streptomycin	2 μg/ml.
rifampicin	10-20 μg/ml.
nalidixic acid	3-7.5 μg/ml.

The control organism did not show any resistance outside this margin, but in the case of the wild isolates, resistance was confirmed if levels of resistance exceeded the following levels:-

ampicillin	10 μg/ml.
chloramphenicol	10 μg/ml.
tetracycline	5 μg/ml.
streptomycin	5 μg/ml.
rifampicin	20 μg/ml.
nalidixic acid	8 μg/ml.

Figure 3.1 shows the results obtained on 1236 E. coli isolates, details of which is given in appendix 3.1. The results show that all the isolates were sensitive to nalidixic acid and rifampicin; 8.09% of the organisms were resistant to streptomycin; 4.2% to ampicillin and tetracycline while 0.89% were resistant to chloramphenicol.

FIGURE 3.1. (top right)

Antibiotic resistance of 1236 wild Escherichia coli isolates to ampicillin, chloramphenicol, streptomycin, tetracycline, nalidixic acid and rifampicin. Percentage of isolates resistant to the different antibiotics are shown. The abbreviations used represent the following antibiotics:-

A = Ampicillin

C = Chloramphenicol

S = Streptomycin

T = Tetracycline

N = Nalidixic acid

R = Rifampicin

FIGURE 3.2. (bottom right)

Antibiotic resistance combinations of the 103 wild E. coli antibiotic resistant isolates. The antibiotic combinations and the percentage of resistant isolates identified with each combination are shown. The abbreviations used and the antibiotics they represent are shown below.

A = Ampicillin

C = Chloramphenicol

S = Streptomycin

T = Tetracycline

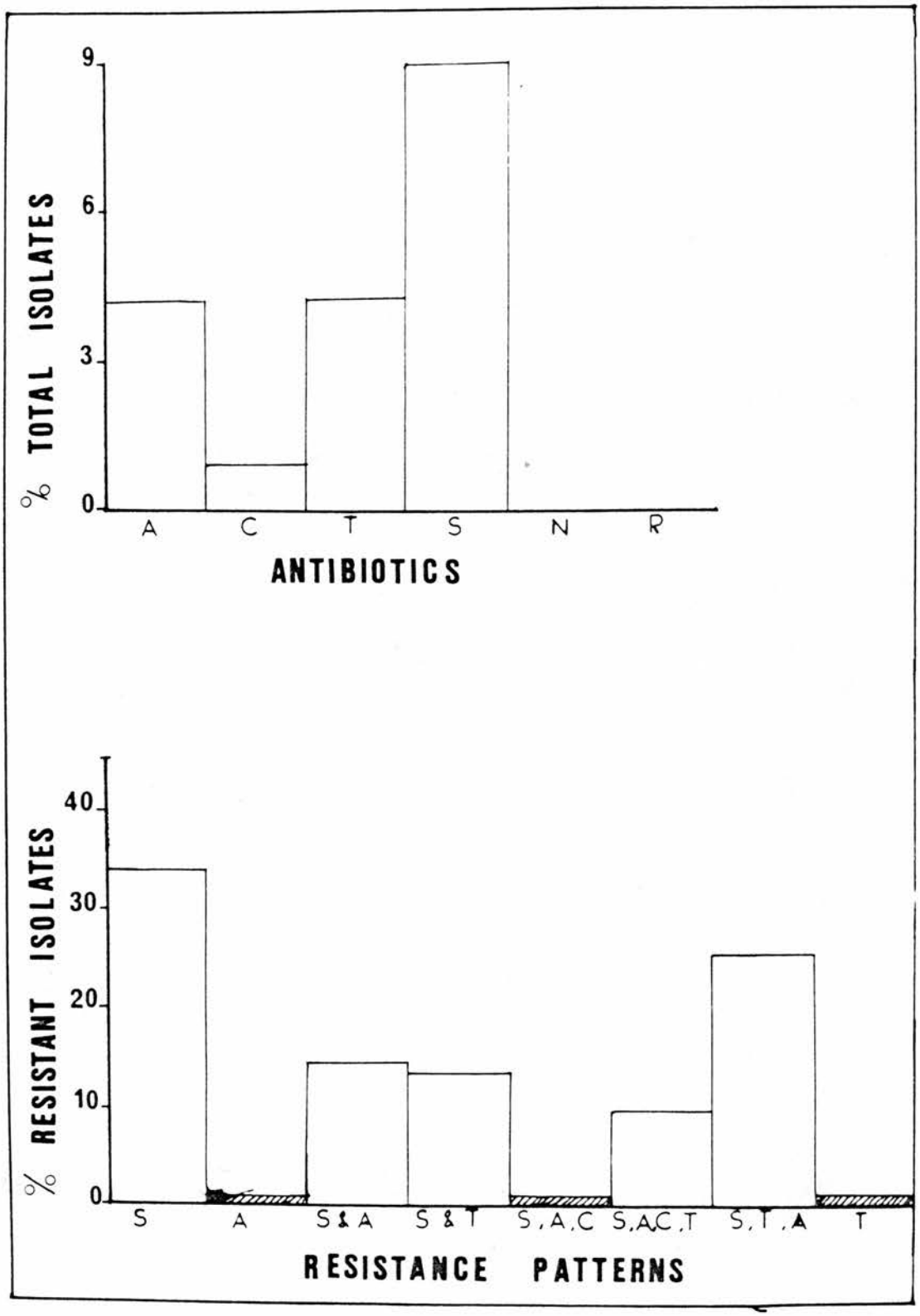

% TOTAL ISOLATES
9
6
3
0
A
C
T
S
N
R
ANTIBIOTICS
% RESISTANT ISOLATES
40
30
20
10
0
S
A
S & A
S & T
S, A, C
S, A, C, T
S, T, A
T
RESISTANCE PATTERNS

FIGURE 3.3. (top right)

Antibiotic resistance levels of 52 ampicillin resistant wild Escherichia coli isolates. The levels of resistance in μg/ml of ampicillin and the percentage of the ampicillin resistant isolates are shown.

FIGURE 3.4. (bottom right)

Antibiotic resistance levels of 100 streptomycin resistant wild E. coli isolates. The levels of resistance in μg/ml of streptomycin and the percentage of streptomycin resistant isolates are shown.

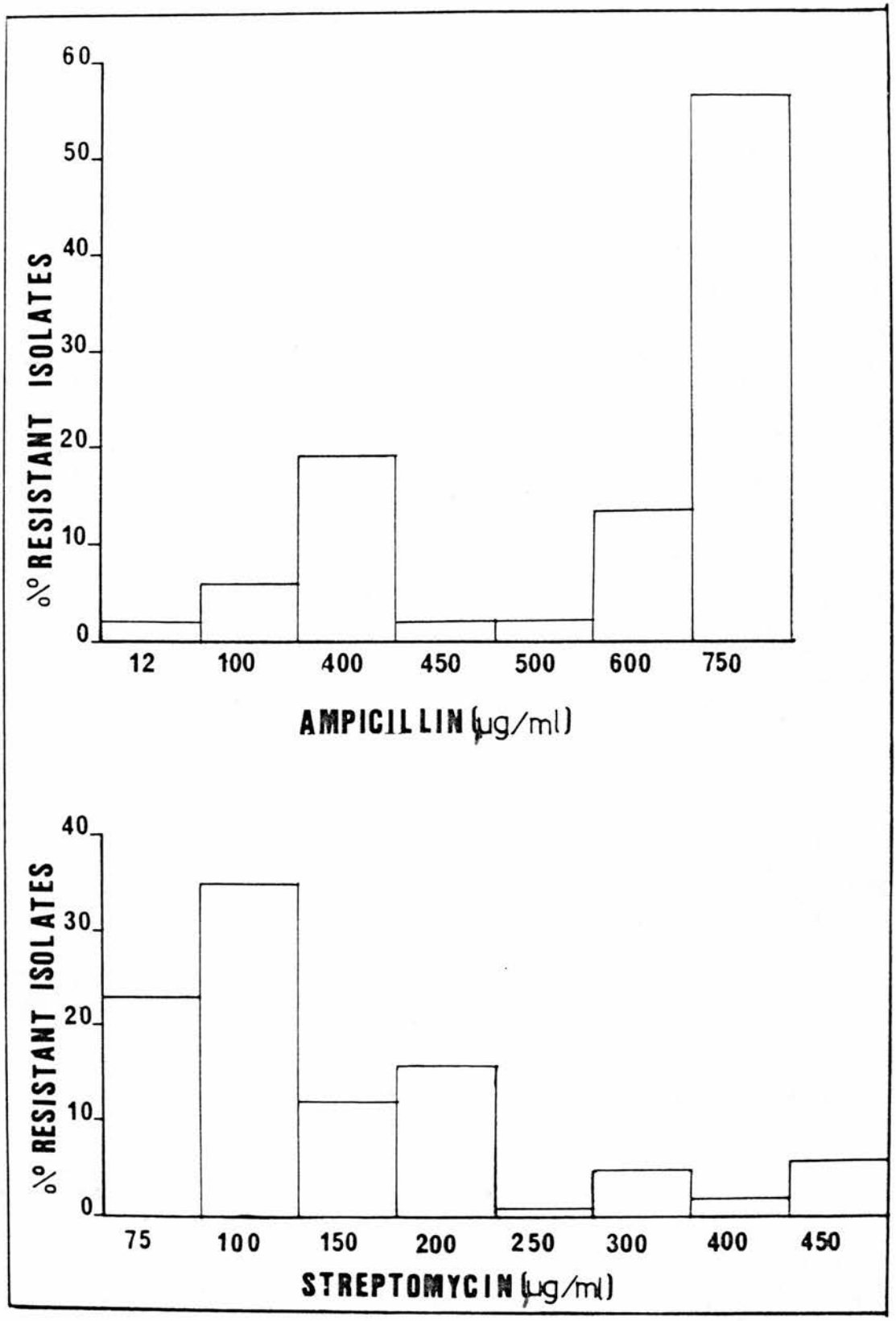

60
50
40
30
20
10
0
% RESISTANT ISOLATES
12
100
400
450
500
600
750
AMPICILLIN (µg/ml)
40
30
20
10
0
% RESISTANT ISOLATES
75
100
150
200
250
300
400
450
STREPTOMYCIN (µg/ml)

Figure 3.2 shows the multiple drug resistance pattern of the isolates. This result shows that only 103 of the 1236 isolates were resistant to the antibiotics used for the test, which means that resistant organisms accounted for 8.33% of the isolates. There were eight combinations in the resistance pattern of the isolates. Streptomycin alone accounted for 34%; streptomycin + ampicillin combined accounted for 14.56%; streptomycin + tetracycline combined accounted for 23.59%; streptomycin + ampicillin + chloramphenicol accounted for 0.97%; streptomycin + ampicillin + chloramphenicol + tetracycline combined accounted for 9.7%; streptomycin + tetracycline + ampicillin combined accounted for 25.24%; tetracycline alone accounted for 0.97%; ampicillin alone accounted for 0.97%. The only antibiotic which did not show resistance alone was chloramphenicol. The results also show that multiple resistance was associated with all chloramphenicol resistant strains. The results are summarised in appendix 3.2.

Figure 3.3 shows the levels of resistance acquired by ampicillin resistant isolates. The result demonstrates that about 56% of the ampicillin resistant isolates acquired a very high level of resistance (750 μg/ml); 19.2% were resistant to 400 μg/ml; very few organisms showed resistance to 12 μg/ml, 450 μg/ml, and 500 μg/ml. The result is summed up in appendix 3.3.

Figure 3.4 shows the levels of resistance acquired by the streptomycin resistant isolates. 35% were resistant to 100 μg/ml; 23% to 75 μg/ml; very few organisms were found resistant to more than 250 μg/ml of streptomycin and none was found resistant to more than 450 μg/ml. This result suggests that the level of resistance of isolates to streptomycin is low. The result is summed up in appendix 3.4.

Figure 3.5 shows the levels of resistance acquired by the tetracycline resistant isolates. The bulk of the isolates resistant to tetracycline showed a very high level of resistance (1000 μg/ml). 90.4% of the

FIGURE 3.5. (top right)

Antibiotic resistance levels of 52 tetracycline resistant wild Escherichia coli isolates. The levels of resistance in μg/ml of tetracycline and the percentage of tetracycline resistant isolates are shown.

FIGURE 3.6. (bottom right)

Antibiotic resistance levels of 11 chloramphenicol resistant wild E. coli isolates. The levels of resistance in μg/ml of chloramphenicol and the percentage of chloramphenicol resistant isolates are shown.

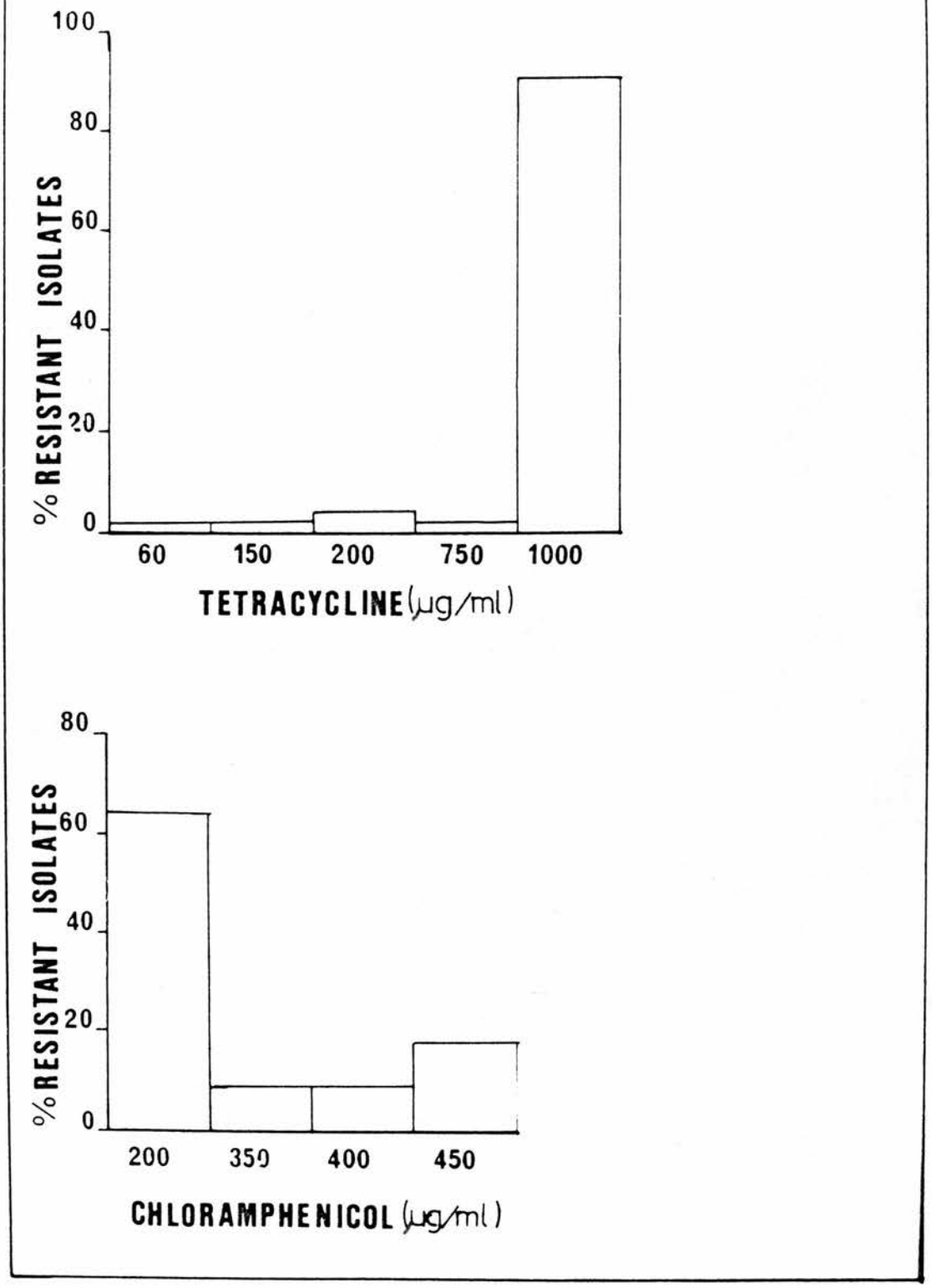

100
80
60
40
20
0
% RESISTANT ISOLATES
60
150
200
750
1000
TETRACYCLINE (µg/ml)
80
60
40
20
0
% RESISTANT ISOLATES
200
350
400
450
CHLORAMPHENICOL (µg/ml)

FIGURE 3.7. (right)

Growth curve of optical density readings against growth time of Escherichia coli 9484 monitored every hour for 10 hours at 610nm with the SP 600 spectrophotometer.

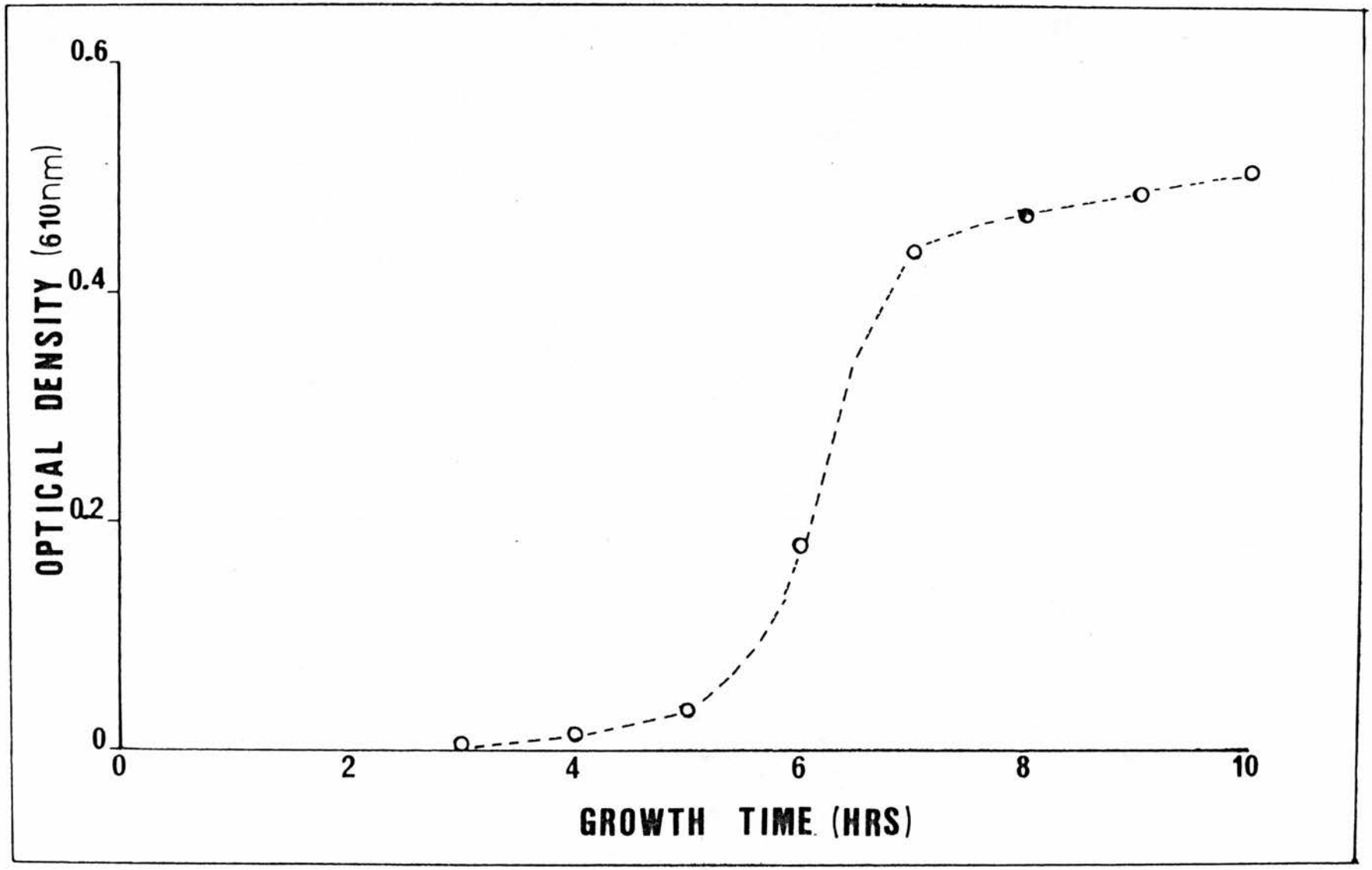
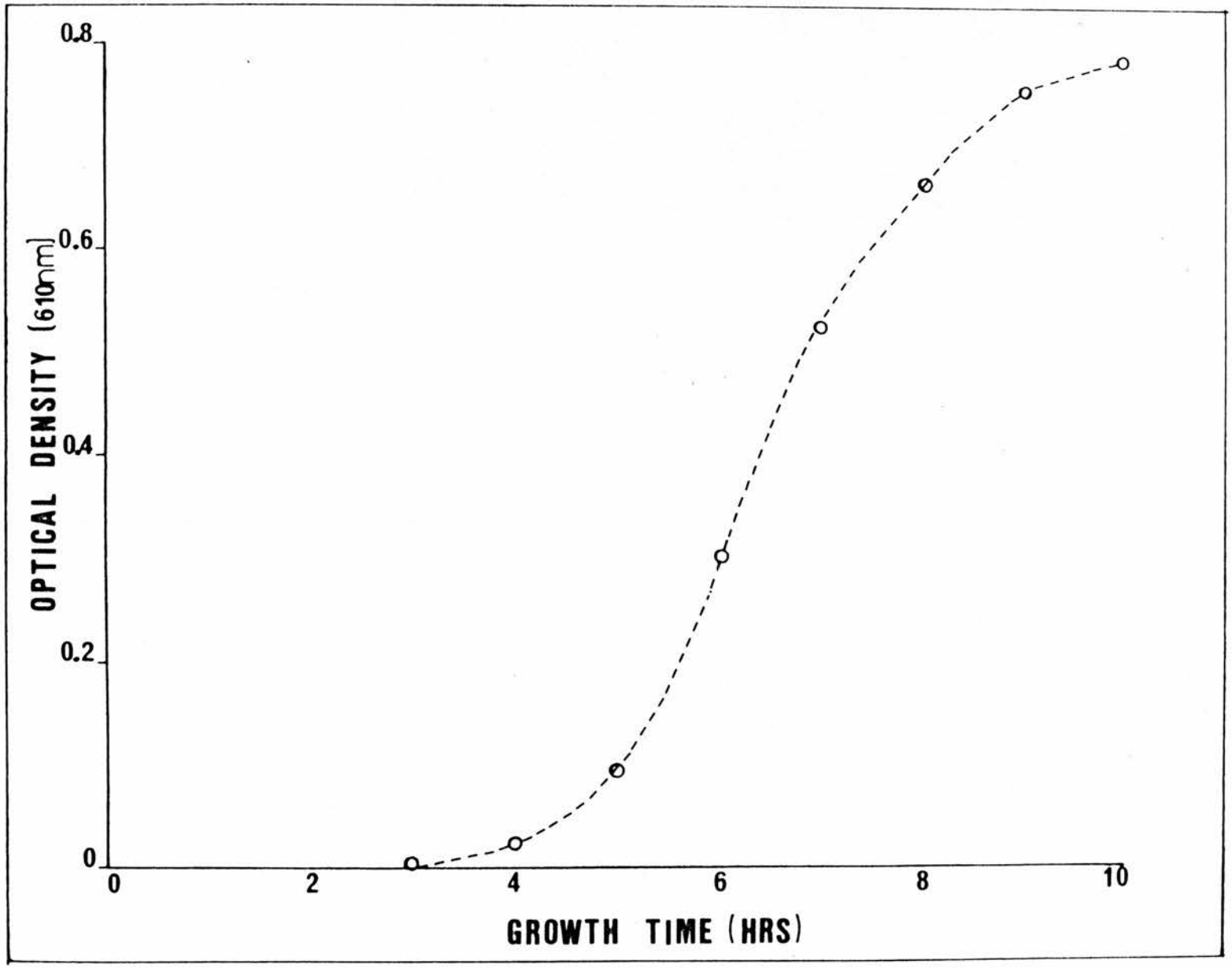

0.6
0.4
0.2
0
OPTICAL DENSITY (610nm)
0
2
4
6
8
10
GROWTH TIME (HRS)

FIGURE 3.8 (right)

Growth curve of optical density readings against growth time of ED 2111 monitored every hour for 10 hours at 610nm with the SP 600 spectrophotometer.


0.8
0.6
0.4
0.2
0
OPTICAL DENSITY (610nm)
0
2
4
6
8
10
GROWTH TIME (HRS)

isolates were resistant to this level of antibiotic. Below that level the proportion was rather random. The result is summed up in appendix 3.5.

Figure 3.6 shows the levels of resistance acquired by the chloramphenicol resistant isolates. Most of the isolates (63.6%) were resistant to 200 µg/ml of the antibiotic. None was resistant to more than 450 µg/ml of the antibiotic as was also observed with streptomycin. The result is summarised in appendix 3.6.

3.3. Growth curve of the E. coli 9484 and ED 2111:

Figure 3.7 demonstrates the growth curve obtained with the standard E. coli 9484. Cultures were grown in nutrient broth and optical density readings were taken at 610nm using the SP 600 spectrophotometer. Cultures were grown in triplicate . Results show the mean of the three readings obtained. The result shows that the organism was in the lag phase in the first four hours after which it entered the exponential phase of growth. Stationary phase set in from the 8th hour. The result also shows that the organism did not grow heavily before stationary phase. A summary of the result is given in appendix 3.7.

Figure 3.8 illustrates the growth curve obtained from the standard organism ED 2111. The cultures were grown and readings obtained as previously described for E. coli 9484. The results show that the lag phase of growth dominated the first three hours after which logarithmic phase set in. Stationary phase set in from the 9th hour. This result showed that the ED 2111 grew more luxuriantly than the E. coli 9484, and attained a maximum optical density reading of 0.78. It also showed that ED 2111 spent exactly $1\frac{1}{2}$ times the number of hours spent by the E. coli 9484 in the logarithmic phase. It is interesting that the maximum optical density readings of the two organisms also differ by $1\frac{1}{2}$ times. The result is summarised in appendix 3.8.

FIGURE 3.9. (right)

Plot of bacterial count against growth time of E. coli 9484 performed by plate count and optical density readings at 610nm using the SP 600 spectrophotometer.

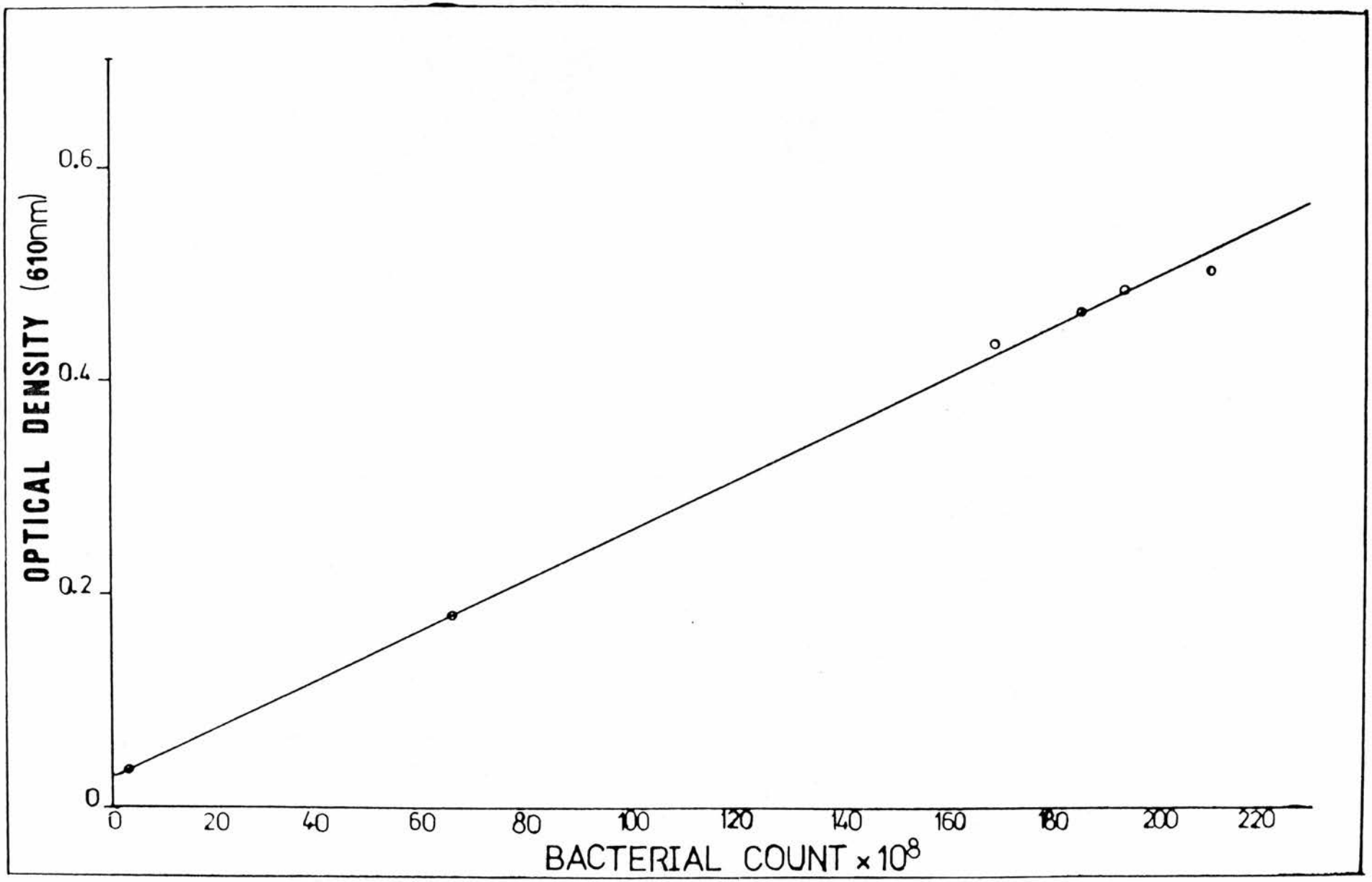

OPTICAL DENSITY (610nm)
0.6
0.4
0.2
0
0
20
40
60
80
100
120
140
160
180
200
220
BACTERIAL COUNT × 10^8

FIGURE 3.10. (right)

Plot of antibiotic resistance transfer efficiency at different growth phases using the ED 2111 as donor to E. coli 9484. Total counts were obtained on plates containing nalidixic acid (20 μg/ml), and infected recipients were selected on plates containing 50 μg/ml each of chloramphenicol and tetracycline and 20 μg/ml of nalidixic acid.

$$\text{Efficiency} = \frac{\text{total count of infected cells}}{\text{total count of recipient cells.}}$$

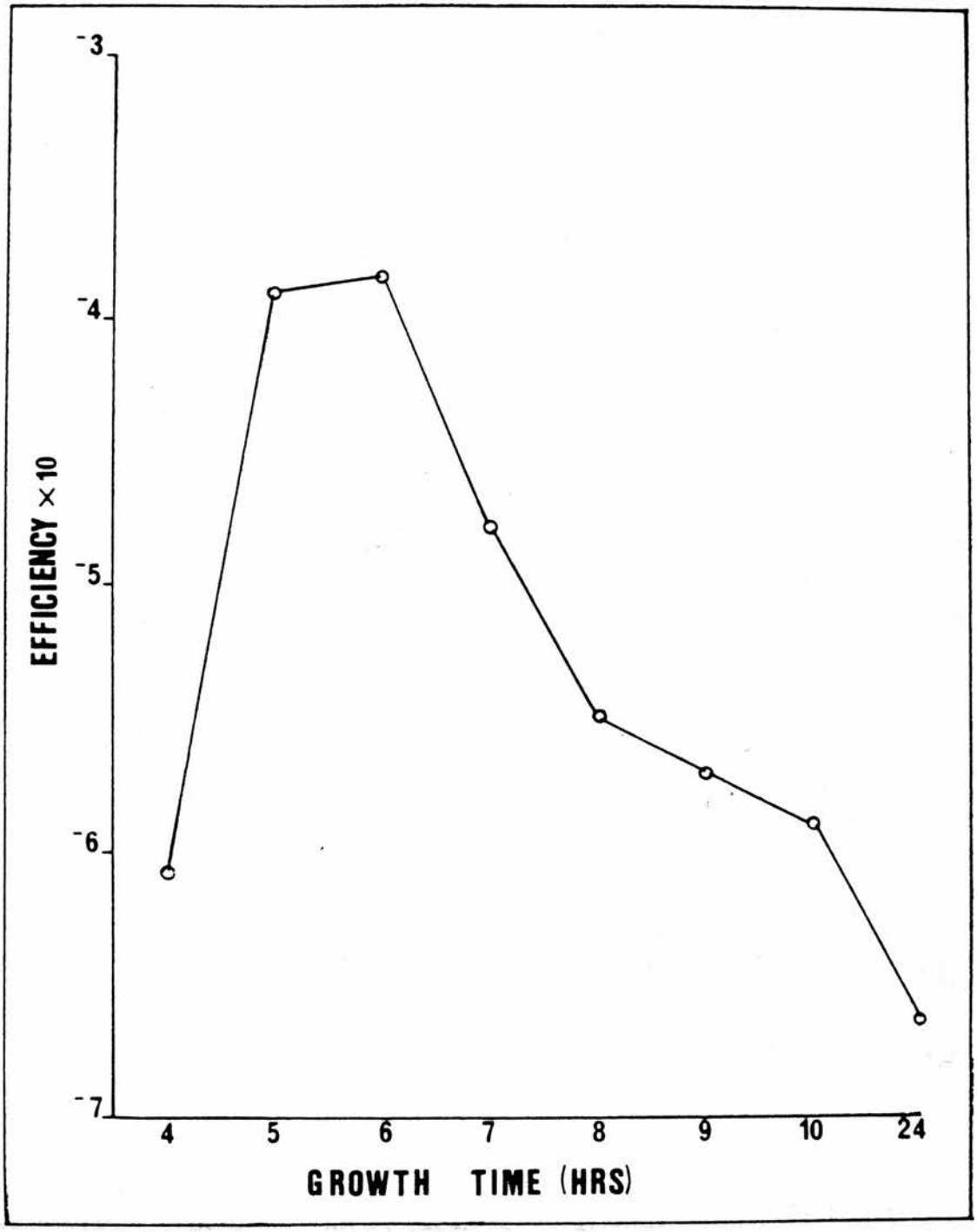

-3
-4
-5
-6
-7
EFFICIENCY × 10
4
5
6
7
8
9
10
24
GROWTH TIME (HRS)

FIGURE 3.11. (right)

Plot of antibiotic resistance transfer efficiency against varying concentrations of thioglycollic acid carried out to determine the effect of anaerobiosis on efficiency of resistance transfer. ED2111 was used as donor to *E. coli* 9484. Organisms were grown in varying concentrations of thioglycollate broth to mid log. phase, and mated for 1 hour. Total recipients were selected on plates containing 20 μg/ml of nalidixic acid, while infected cells were selected on plates containing 20 μg/ml nalidixic acid and 50 μg/ml each of chloramphenicol and tetracycline,

$$\text{Efficiency} = \frac{\text{total count of infected cells}}{\text{total count of recipient cells.}}$$

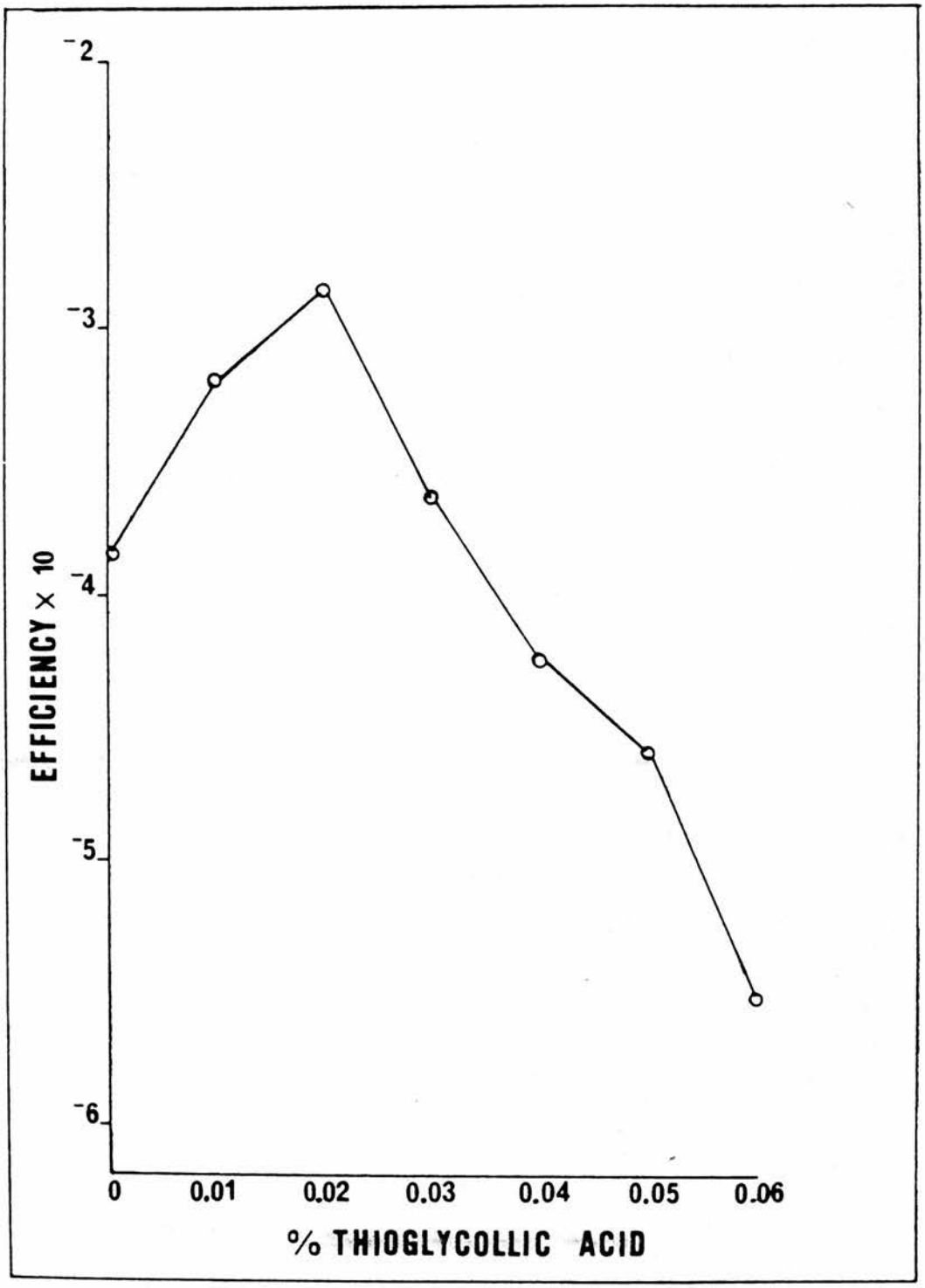

-2
-3
-4
-5
-6
EFFICIENCY × 10
0
0.01
0.02
0.03
0.04
0.05
0.06
% THIOGLYCOLLIC ACID

Figure 3.9 shows a plot of optical density against bacterial count. The test organism was the E. coli 9484. The result shows that there were about 32 x 10^8 E. coli bacteria per optical density reading of 0.1. The result is summarised in appendix 3.9.

3.4. Effect of growth phase on transfer efficiency:

The efficiency of resistance transfer using ED 2111 as donor and E. coli 9484 as recipient was monitored throughout the different growth phases. Conjugation experiments were performed every hour as previously described.

Figure 3.10 shows a plot of the transfer efficiency during the different conjugation periods. Compared with figure 3.8, the maximum efficiency was recorded after 6 hours of growth which represents a mid-point in the logarithmic phase. In the early and late stages of the logarithmic phase, the organisms were not very efficient in transferring resistance. In the stationary phase as well, there was a great decline in the efficiency of transfer. The results are summarised in appendix 3.10.

3.5. Effect of anaerobiosis on the efficiency of resistance transfer:

The effect of anaerobiosis on the efficiency of resistance transfer was studied using the two standard strains. Anaerobiosis was achieved by the use of thioglycollate nutrient broth.

Figure 3.11 shows the effect of anaerobiosis on the efficiency of resistance transfer. The result shows that mild anaerobiosis favours higher efficiency of resistance transfer. The maximum efficiency observed was with 0.02% thioglycollic acid. The organism did not grow at 0.07% thioglycollic acid. The result is summed up in appendix 3.11.

3.6. Effect of using same organism as donor and recipient on resistance transfer efficiency:

A comparative study was made using ED 2111 and E. coli 9484 as

donors to E. coli 9484. The E. coli 9484 used as donor in this study was previously infected with plasmid from ED 2111.

Table 3 shows the result of this experiment. The result shows that the efficiency of transfer was improved when the same organism was used as both donor and recipient than when different organism was used as donor. The result shows a 5.7 times improvement.

Table 3.

Effect of using the same organism as both donor and recipient on antibiotic resistance transfer efficiency, compared with use of different organism as donor. Donors were ED 2111 and E. coli 9484 infected with plasmid from ED 2111, and recipient was E. coli 9484. Triplicate and mean efficiencies are presented.

		Donors	
		ED 2111	E coli 9484
Efficiency	1	1.8×10^{-4}	9.62×10^{-4}
	2	1.64×10^{-4}	1.01×10^{-3}
	3	1.66×10^{-4}	9.84×10^{-4}
Mean efficiency		1.7×10^{-4}	9.84×10^{-4}

Table 4.

Antibiotic resistance levels of infected E. coli 9484 after conjugation experiments with donor ED 2111 and five wild isolates. Wild isolates were JC 4, JC 14, JC 82, JC 109 and JC 165. Resistance levels of donors and infected recipients are shown. The presented level of resistance of infected recipients is the mean of estimation on ten colonies.

	Ampicillin	Streptomycin	Tetracycline	Chlor-amphenico
Resistance (µg/ml) ED 2111	-	-	1000	450
E. coli 9484	-	-	450	300
JC 4	-	250	-	-
E. coli 9484	-	75	-	-
JC 14	600	200	-	-
E. coli 9484	150	25	-	-
JC 82	500	100	-	450
E. coli 9484	250	50	-	100
JC 109	-	100	-	-
E. coli 9484	-	25	-	-
JC 165	750	200	1000	-
E. coli 9484	150	75	200	-

3.7 Resistance level of infected cells:

Ten colonies each from E. coli 9484 infected with plasmids from ED 2111 and wild strains JC 4, JC 14, JC 82, JC 109, JC 165, were tested for their levels of resistance by the methods already described. Table 4 shows the results obtained from the tests. The original resistance level of the donors are also shown. The results shown are the mean of the resistance level from the ten tested colonies.

The results show that in all the infected recipients tested, the

FIGURE 3.12. (top right)

Proportion of different antibiotics transferred by wild *E. coli* isolates to *E. coli* 9484 during conjugation. Each antibiotic transferred together with the percentage of resistant wild isolates transferring them are shown.

FIGURE 3.13. (bottom right)

Proportion of different antibiotic combinations transferred by wild *E. coli* isolates to *E. coli* 9484 during conjugation. Each combination together with the percentage of resistant wild isolates transferring them are shown.

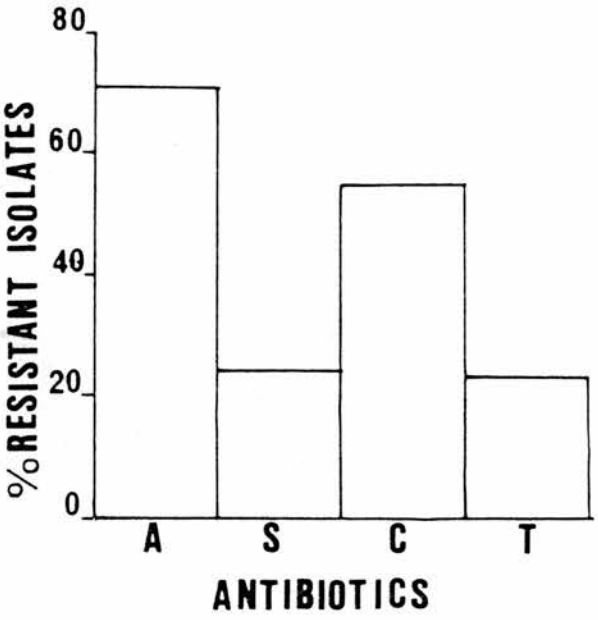
Note: The abbreviations used in the two figures represent the following antibiotics:-

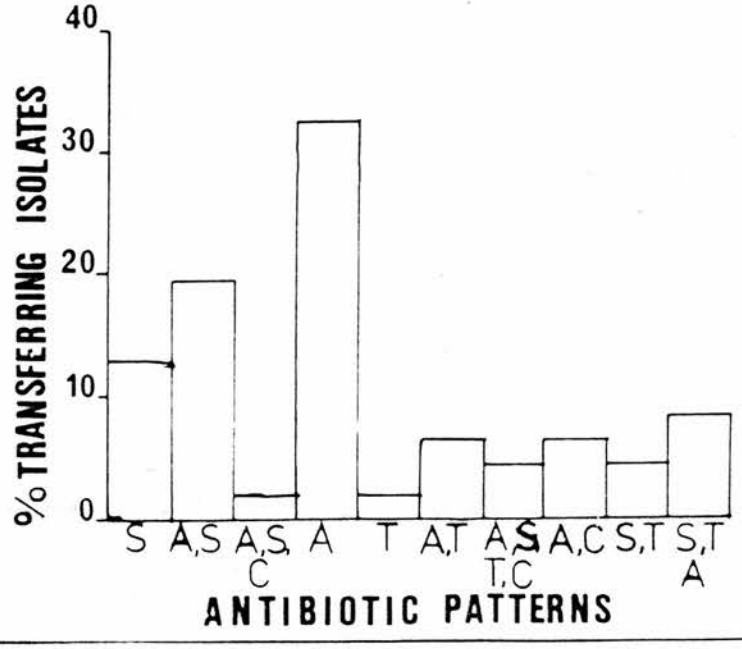
A = ampicillin

C = chloramphenicol

S = streptomycin

T = tetracycline


80
60
40
20
0
% RESISTANT ISOLATES
A
S
C
T
ANTIBIOTICS


40
30
20
10
0
% TRANSFERRING ISOLATES
S
A,S
A,S, C
A
T
A,T
A,S T,C
A,C
S,T
S,T A
ANTIBIOTIC PATTERNS

levels of resistance were much lower than in the donor bacteria. With ampicillin, 20-50% of donor resistance level was expressed. With streptomycin, levels of resistance acquired ranged from 12.5 to 50%. With tetracycline, 20 to 45% of donor resistance level was acquired by the recipient. And with chloramphenicol, 22 to 67% of donor resistance level was acquired by the infected recipient cells.

3.8. Antibiotic resistance transfer using wild isolates:

As already indicated, the E. coli 9484 was used as recipient in all the tests. The rest of the test was carried out as already described for the conjugation experiments.

Figure 3.12 shows the individual antibiotics used for the test and the percentage of the resistant organisms transferring their resistance. The results show that ampicillin was transferred more than any other antibiotic (71.15%), followed by chloramphenicol with 54.55%, streptomycin with 24% and tetracycline with 23.08%. The result is summarised in appendix 3.12.

Figure 3.13 shows the different combinations of the antibiotics transferred by the isolates. The result shows that there were 10 combinations of the antibiotics transferred. Ampicillin took part in seven of these. The result also shows that chloramphenicol was never transferred alone but in combination with other antibiotics. In most cases, the pattern of transfer did not reflect the same pattern of resistance in the isolates. The total proportion of organisms that transferred resistance accounted for 44.66% of the resistant isolates. Most of the isolates only transferred part of their resistance and this may have accounted for the different pattern of transfer. The result is summarised in appendix 3.13.

3.9. Degree of efficiency of resistance transfer with different drugs:

The efficiency of transfer with each antibiotic was estimated by comparing the number of infected recipients with the total number of recipient bacteria in the culture.

FIGURE 3.14. (top right)

Plot of varying efficiencies of transfer exhibited by wild isolates transferring ampicillin resistance to E. coli 9484. Total recipient cells were obtained on nalidixic acid (20 μg/ml) containing plates while infected cells were selected on plates containing 25 μg/ml of ampicillin and 20 μg/ml of nalidixic acid.

$$\text{Efficiency} = \frac{\text{total count of infected cells}}{\text{total count of recipient cells.}}$$

FIGURE 3.15. (bottom right)

Plot of varying efficiencies of transfer exhibited by wild isolates transferring chloramphenicol resistance to E. coli 9484. Total recipients were obtained on nalidixic acid (20 μg/ml) containing plates while infected cells were selected on plates containing 25 μg/ml of chloramphenicol and 20 μg/ml of nalidixic acid.

$$\text{Efficiency} = \frac{\text{total count of infected cells}}{\text{total count of recipient cells.}}$$

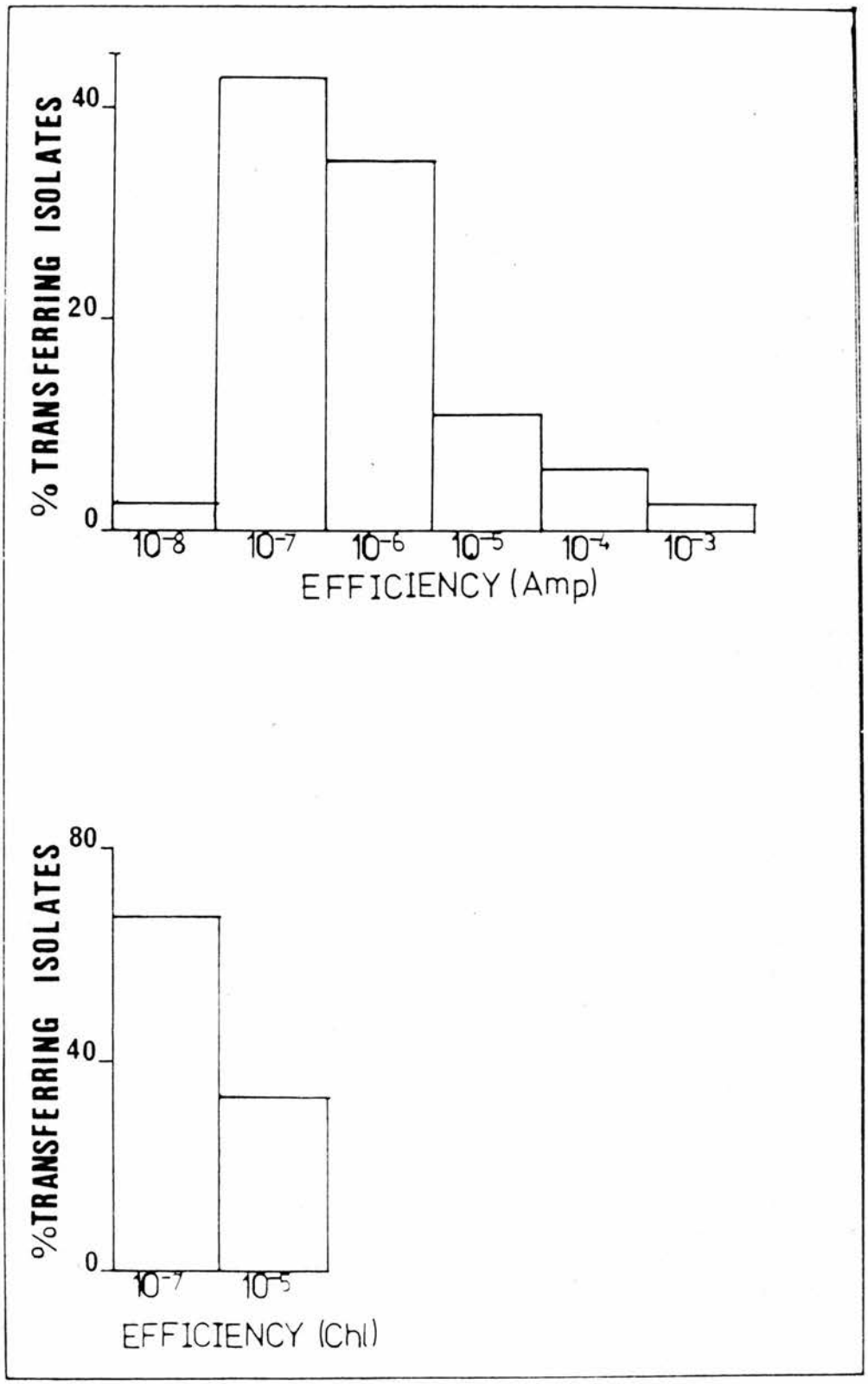

% TRANSFERRING ISOLATES
40
20
0
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
EFFICIENCY (Amp)
%TRANSFERRING ISOLATES
80
40
0
10^{-7}
10^{-5}
EFFICIENCY (Chl)

FIGURE 3.16. (top right)

Plot of varying efficiencies of transfer exhibited by wild isolates transferring tetracycline resistance to *E. coli* 9484. Total recipient cells were obtained on nalidixic acid (20 μg/ml) containing plates while infected cells were selected on plates containing 25 μg/ml of tetracycline and 20 μg/ml of nalidixic acid.

$$\text{Efficiency} = \frac{\text{total count of infected cells}}{\text{total count of recipient cells.}}$$

FIGURE 3.17. (bottom right)

Plot of varying efficiencies of transfer exhibited by wild isolates transferring streptomycin resistance to *E. coli* 9484. Total recipient cells were obtained on nalidixic acid (20 μg/ml) containing plates while infected cells were selected on plates containing 25 μg/ml of streptomycin and 20 μg/ml of nalidixic acid.

$$\text{Efficiency} = \frac{\text{total count of infected cells}}{\text{total count of recipient cells.}}$$

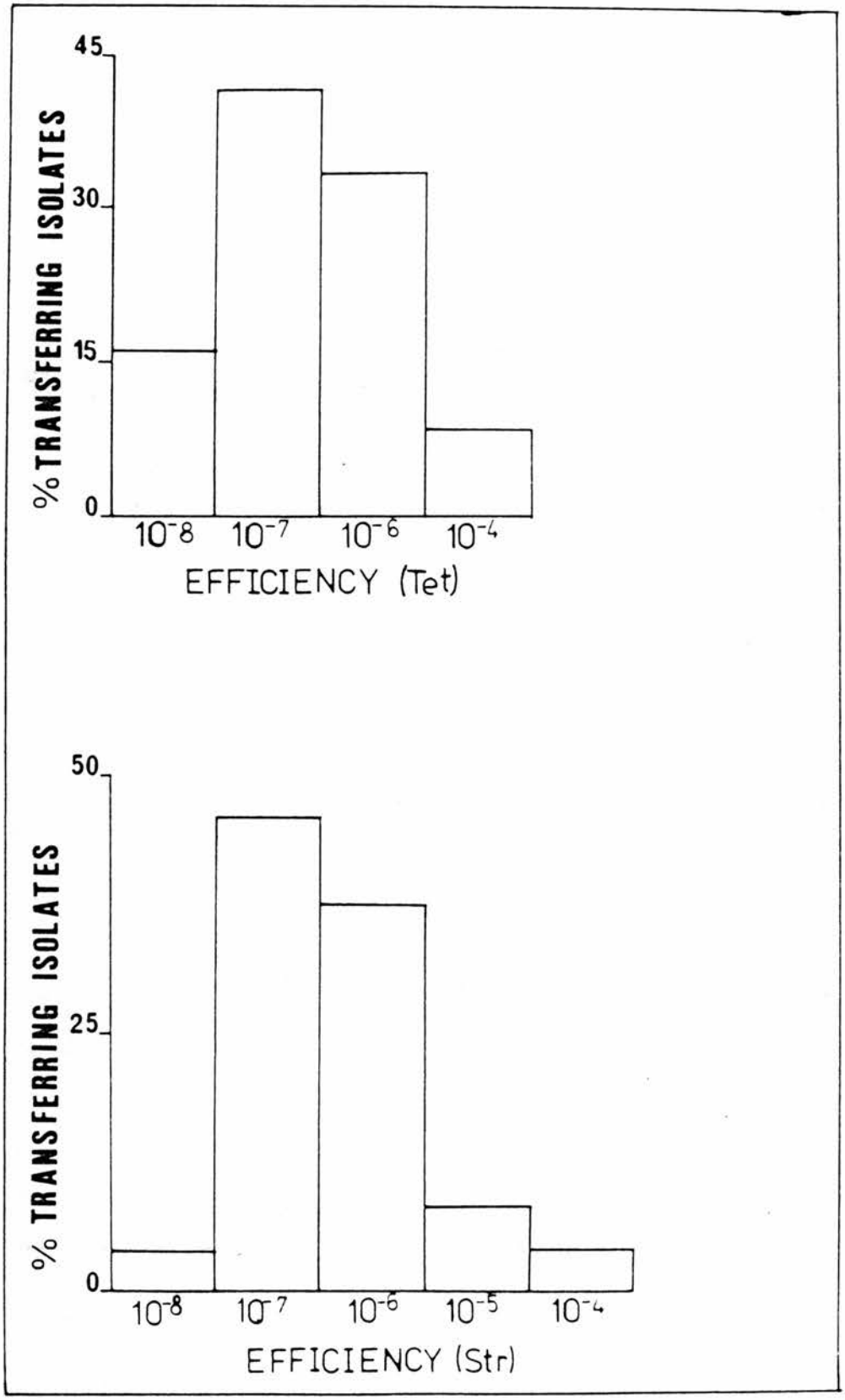

45
30
15
0
% TRANSFERRING ISOLATES
10-8
10-7
10-6
10-4
EFFICIENCY (Tet)
50
25
0
% TRANSFERRING ISOLATES
10-8
10-7
10-6
10-5
10-4
EFFICIENCY (Str)

Figure 3.14 shows the efficiency of transfer of the ampicillin resistant isolates. The result shows that about 43% of the ampicillin transferring isolates had an efficiency of 10^{-7}, and 35% had efficiency of 10^{-6}. Few of the organisms transferred with better efficiency. The results are summarised in appendix 3.14.

Figure 3.15 shows the results obtained with the chloramphenicol transferring strains. 66.7% of the isolates transferred chloramphenicol at an efficiency of 10^{-7}. The rest of the isolates transferred at an efficiency of 10^{-5}. Details of the result are shown in appendix 3.15.

Figure 3.16 shows the results obtained with the tetracycline transferring strains. 41.6% of the isolates transferred at an efficiency of 10^{-7}, while 33.3% of them transferred at an efficiency of 10^{-6}. Details of the result are shown in appendix 3.16.

Figure 3.17 shows the results obtained with the streptomycin transferring isolates. 46% of the isolates transferred at an efficiency of 10^{-7}, while 37.5% transferred at 10^{-6}. The details of this result are shown in appendix 3.17.

3.10. Classification of plasmids by incompatibility:

Five wild strains, JC 4, JC 14, JC 82, JC 109, and JC 165 in host strain E. coli 9484 were classified by incompatibility, the methods of which have been previously described.

Table 5 shows the antibiotic resistance pattern of the test strains together with the standard ones.

Table 6 shows the results of the incompatibility test using wild type plasmid from JC 4. The result shows that the wild type plasmid is incompatible with R-27 (Inc. H group). The test shows compatibility with the rest of the standard strains. But in the case of R-16 (O group), tetracycline resistance was lost in the cells with double plasmids (hereafter referred to as "doubles") after overnight incubation.

Table 7 shows the results with wild type plasmid from JC 14. The result shows that the wild plasmid was incompatible with three standard types; R-40a, R-27, and R-16. With R-27 and R-16, transfer into the second culture occurred but tetracycline resistance was lost in the "doubles" after overnight incubation, at 37°. With R-40a, no transfer into the second plasmid took place even when the test was carried out in the opposite direction.

Table 8 shows the results of incompatibility with JC 82 wild plasmid. The result shows that the wild plasmid is incompatible with R-27 and therefore belongs to group H.

Table 9 shows the results of incompatibility test using JC 109 wild plasmid. The result shows that the wild plasmid is incompatible with R-478 (Inc. S group). With the rest of the standard strains used, compatibility is shown. However, in the case of R-16 (Inc. O group), ampicillin resistance was lost in the "doubles" after incubation at 37° overnight.

Table 10 shows the results of incompatibility test using JC 165 wild plasmid. The results show that the wild plasmid is incompatible with R-27, and therefore belongs to group H.

The overall results show that in two cases out of five, complete incompatibility was shown with R-27. In yet another two cases out of five, the wild plasmids shared incompatibility with R-27 and R-16. In the fifth case the wild plasmid shared incompatibility with R-16 and R-478.

In the cases where incompatibility was complete and specific with one strain, no correlation exists between antibiotic resistance pattern of standard strain and wild strain.

Table 5.

Antibiotic resistance pattern of the standard and wild strains used for plasmid classification by incompatibility tests. Methods for sensitivity tests have already been described.

E coli strain	Plasmid group	Antibiotic resistance (μg/ml). Chloramphenicol	Tetracycline	Ampicillin	Streptomycin
R 40a	Inc C	-	-	100	-
N3	Inc N	-	50	-	75
R386	Inc fI	-	50	-	-
R391	Inc J	-	-	-	-
R1	Inc FII	100	-	100	-
R387	Inc K	100	-	-	75
R27	Inc H	-	50	-	-
R471a	Inc L	-	-	100	-
R16	Inc O	-	50	100	10
R 446b	Inc M	-	50	-	75
RA1	Inc A	-	50	-	-
RP4	Inc P	-	50	100	-
Rtsl	Inc T	-	-	-	-
R124	Inc fIV	-	50	-	-
R478	Inc S	25	25	-	-
R621	Inc 1	-	50	-	-
R144	Inc 1	-	5	-	-
S-a	Inc W	100	-	-	25
JC4	Wild type	-	-	-	250
JC14	Wild type	-	-	600	200
JC82	Wild type	450	-	500	100
JC109	Wild type	-	-	-	100
JC165	Wild type	-	1000	750	200

TABLE 6

Results of incompatibility tests of JC4 wild strain with eighteen standard strains. Methods of tests have already been described. Amp = ampicillin; tet = tetracycline; chl = chloramphenicol; str = streptomycin; rif = rifampicin; nal = nalidixic acid.

Standard E. coli strain	Selective Plates	Transfer efficiency	Stability of 10 colonies	Independent replication
R40a	Amp, rif.	1.2×10^{-4}	8 out of 10	Independent
N3	Tet, rif.	9.8×10^{-5}	10 out of 10	Independent
R386	Str, nal.	8.2×10^{-5}	10 out of 10	Independent
R391	Str, nal.	1.4×10^{-6}	10 out of 10	Independent
R387	Chl, rif.	9.0×10^{-5}	10 out of 10	Independent
R27	Str, nal.	1.2×10^{-7}	NIL	
R471a	Str, nal.	8.8×10^{-6}	10 out of 10	Independent
R16	Str, nal.	1.02×10^{-5}	10 out of 10 A,S No Tet.	Independent
R446b	Tet, rif.	9.0×10^{-7}	10 out of 10	Independent
RA1	Str, nal.	8.2×10^{-6}	10 out of 10	Independent
RP4	Str, nal.	8.0×10^{-7}	10 out of 10	Independent
Rtsl	Str, nal.	1.1×10^{-7}	10 out of 10	Independent
R124	Str, nal.	9.8×10^{-7}	8 out of 10	Independent
R478	Str, nal.	7.8×10^{-6}	10 out of 10	Independent
R621	Str, nal.	9.2×10^{-6}	10 out of 10	Independent
R1	Str, nal.	9.0×10^{-6}	8 out of 10	Independent
R144	Str, nal.	1.2×10^{-6}	10 out of 10	Independent
S-a	Chl, rif.	8.2×10^{-6}	10 out of 10	Independent

Table 7

Results of incompatibility tests of JC14 wild strain with eighteen standard strains. Methods of tests have already been outlined. Amp = ampicillin; tet = tetracycline; Chl = chloramphenicol; Str = streptomycin; nal = nalidixic acid; rif = rifampicin.

Standard E. coli strains	Selective Plates	Transfer efficiency	Stability out of 10 colonies	Independent replication
R40a	Str; nal	NIL	-	-
N3	Amp, nal	1.1×10^{-4}	10	Independent
R386	Tet, rif	9.2×10^{-5}	10	Independent
R391	Amp, str, nal	9.8×10^{-8}	10	Independent
R1	Str, nal	1.2×10^{-4}	10	Independent
R387	Amp, nal	1.1×10^{-4}	10	Independent
R27	Amp, str, nal	1.1×10^{-4}	Amp, str. 10 Tet - nil	
R471a	Str, nal	1.1×10^{-7}	10	Independent
R16	Str, nal	9.2×10^{-8}	Amp, str - 10 Tet - nil	
R446b	Tet, rif	1.16×10^{-4}	10	Independent
RA1	Amp, str, nal	1.02×10^{-4}	10	Independent
RP4	Str, nal	9.1×10^{-7}	10	Independent
Rtsl	Amp, str, nal	1.1×10^{-4}	10	Independent
R124	Tet, rif	1.2×10^{-5}	10	Independent
R478	Amp, str, nal	9.0×10^{-7}	10	Independent
Rb21	Amp, str, nal	9.0×10^{-8}	10	Independent
R144	Amp, str, nal	9.2×10^{-7}	10	Independent
S-a	Amp, nal	9.8×10^{-6}	10	Independent

TABLE 8

Results of incompatibility tests of JC82 wild strain with eighteen standard strains. Methods of tests have already been outlined. Amp = ampicillin; str = streptomycin; tet = tetracycline; chl = chloramphenicol; rif = rifampicin; nal = nalidixic acid.

Standard E coli strain	Selective plates	Transfer efficiency	Stability out of 10 colonies	Independent replication
R409	Str, chl, nal	1.02×10^{-6}	10	Independent
N3	Amp, chl, nal	7.8×10^{-6}	10	Independent
R386	Amp, str, chl, nal	1.2×10^{-4}	10	Independent
R391	Amp, str, chl, nal	1.01×10^{-6}	10	Independent
R1	Str, nal	9.7×10^{-5}	10	Independent
R387	Amp, nal	1.02×10^{-4}	10	Independent
R27	Amp, str, chl, nal	8.6×10^{-5}	Amp, str, chl - 10 Tet - nil	
R471a	Str, chl, nal	7.9×10^{-4}	10	Independent
R16	Tet, rif	1.2×10^{-5}	10	Independent
R 446b	Tet, rif	8.6×10^{-4}	10	Independent
RA1	Tet, rif	8.7×10^{-5}	9	Independent
RP4	Tet, rif	1.1×10^{-4}	10	Independent
Rtsl	Amp, str, chl, nal	8.8×10^{-5}	10	Independent
R124	Tet, rif	1.1×10^{-4}	10	Independent
R478	Tet, rif	8.1×10^{-7}	7	Independent
R621	Tet, rif	9.7×10^{-6}	10	Independent
R144	Amp, str, chl, nal	8.8×10^{-5}	10	Independent
S-a	Amp, nal	9.2×10^{-6}	10	Independent

TABLE 9

Results of incompatibility tests of JC109 wild strain with eighteen standard strains. Methods employed in the tests have already been described. Amp = ampicillin; Chl = chloramphenicol; Str = streptomycin; tet = tetracycline; rif = rifampicin; nal = nalidixic acid.

Standard E coli strains	Selective plates	Transfer efficiency	Stability out of 10 colonies	Independent replication
R40a	Str, nal	9.2×10^{-6}	10	Independent
N3	Tet, rif	9.8×10^{-5}	10	Independent
R386	Str, nal	8.2×10^{-6}	5	Independent
R391	Str, nal	8.7×10^{-6}	10	Independent
R1	Str, nal	8.4×10^{-6}	7	Independent
R387	Chl, rif	1.2×10^{-4}	10	Independent
R27	Str, nal	8.8×10^{-7}	10	Independent
R471a	Str, nal	9.0×10^{-7}	8	Independent
R16	Str, nal	1.02×10^{-6}	Str, Tet - 6 Amp - nil	Independent
R446b	Tet, rif	1.2×10^{-7}	10	Independent
RA1	Str, nal	9.0×10^{-6}	10	Independent
RP4	Str, nal	8.8×10^{-5}	10	Independent
Rtsl	Str, nal	1.02×10^{-7}	10	Independent
R124	Str, nal	8.8×10^{-7}	10	Independent
R478	Tet, chl, rif	9.2×10^{-8}	Tet, chl, - 10 Str - nil	
R621	Str, nal	7.9×10^{-6}	10	Independent
R144	Str, nal	8.4×10^{-7}	10	Independent
S-a	Chl, rif	8.7×10^{-7}	10	Independent

TABLE 10

Results of incompatibility tests of JC265 wild strain with eighteen standard strains. Methods of tests have already been outlined. Amp = ampicillin; str = streptomycin; tet = tetracycline; chl = chloramphenicol; rif = rifampicin; nal = nalidixic acid.

Standard E coli strains	Selective plates	Transfer efficiency	Stability out of 10 colonies	Independent replication
R40a	Str, tet, nal	9.8×10^{-6}	6	Independent
N3	Amp, nal	8.7×10^{-8}	Str, tet - 10 Amp - 2	Independent
R386	Amp, str, nal	7.9×10^{-6}	10	Independent
R391	Amp, str, tet, nal	1.2×10^{-8}	10	Independent
R1	Str, tet, nal	1.1×10^{-6}	8	Independent
R387	Amp, tet, nal	8.2×10^{-6}	8	Independent
R27	Amp, str, nal	7.8×10^{-7}	Amp, tet - 10 Str - nil	
R471a	Str, tet, nal	9.0×10^{-8}	10	Independent
R16	Str, nal	8.2×10^{-6}	10	Independent
R446b	Amp, nal	9.3×10^{-6}	7	Independent
RA1	Amp, str, nal	9.8×10^{-6}	8	Independent
RP4	Str, nal	8.6×10^{-7}	10	Independent
Rtsl	Amp, str, tet, nal	9.4×10^{-7}	6	Independent
R124	Amp, str, nal	9.2×10^{-7}	10	Independent
R478	Amp, str, nal	7.4×10^{-7}	8	Independent
R621	Amp, str, nal	9.4×10^{-6}	7	Independent
R144	Amp, str, tet, nal	8.9×10^{-8}	10	Independent
S-a	Amp, tet, nal	8.7×10^{-6}	8	Independent

PLATE 1 (top right)

0.7% agarose gel electrophoresis of S-a and R1 plasmid DNA obtained by the sodium lauryl sulphate-sodium chloride precipitation method and subjected to electrophoresis at 30 volts for 18 hours. From left to right shows S-a plasmid DNA with molecular weight of 25 mega-daltons in the first three wells, and R1 plasmid DNA with molecular weight of 65 mega-daltons in the last three wells.

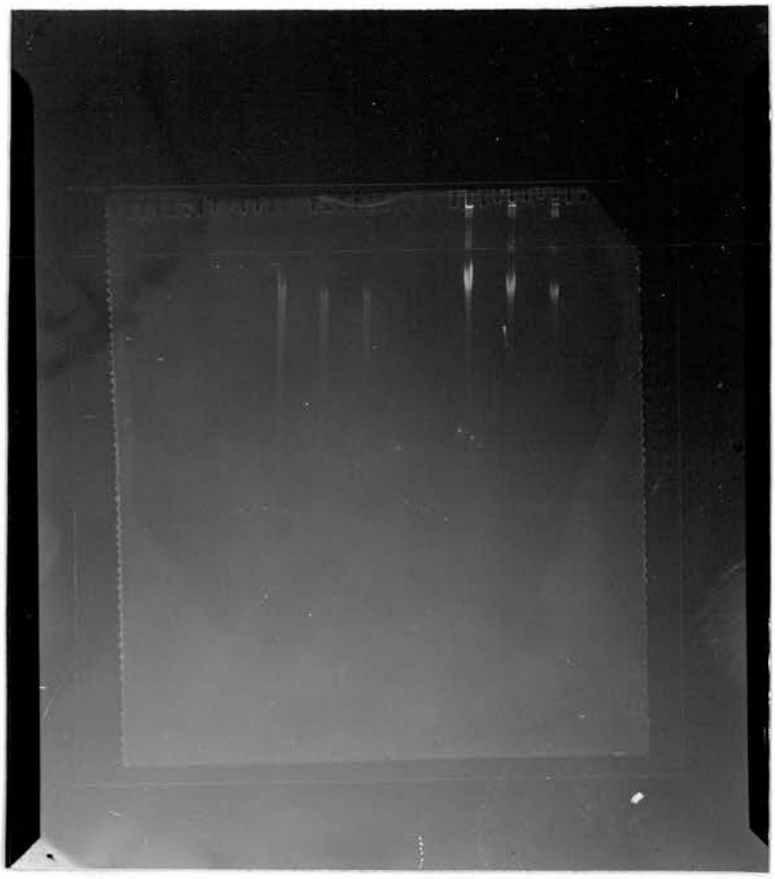
Note the smearing effect caused by long storage and the closeness of bands of very different molecular weights.

PLATE 2 (bottom right)

0.7% agarose gel electrophoresis of plasmid DNA. All but the lambda DNA supplied by Dr. D.B. Malcolm of the Department of Zoology, were prepared by the sodium lauryl sulphate-sodium chloride precipitation method. Electrophoresis was carried out at 30 volts for 18 hours.

From left to right, JC4, R16 (48 md), lambda (30 md), JC82, JC165, S-a (25 md).

Note that there is closeness of bands from all the samples, and that JC165 shows three plasmid bands.



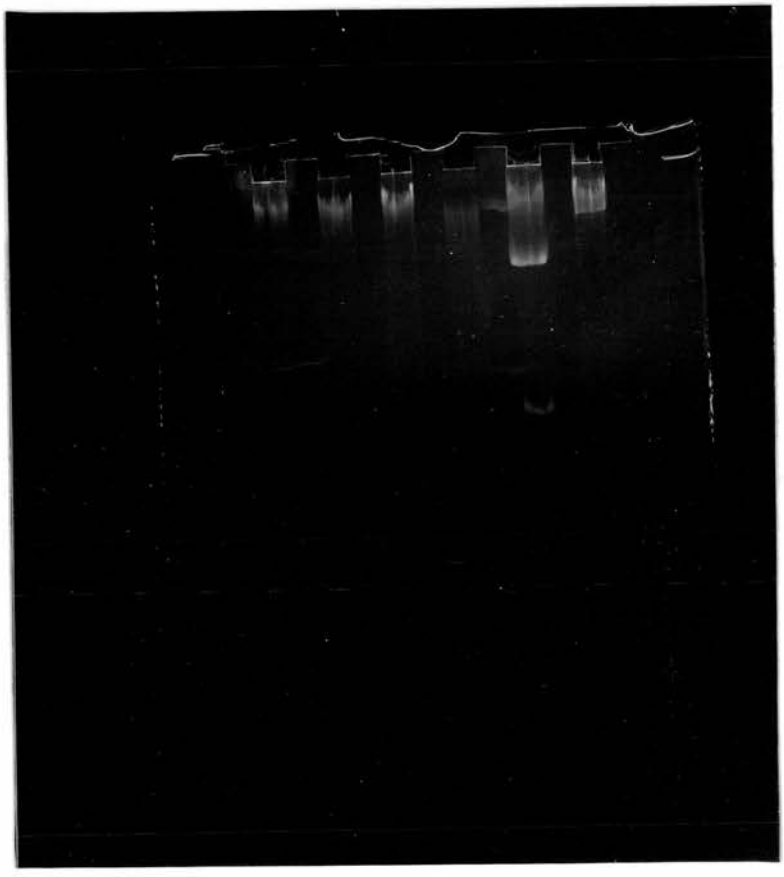


PLATE 3 (right)

0.7% agarose gel electrophoresis of EcoR1 digest of lambda phage DNA, supplied by Dr. D.B. Malcolm of the Department of Zoology, and carried out at 30 volts for 18 hours. 1 µg of DNA was digested with 5 µl (1:30) enzyme solution.

Note that there are six bands. Mobility of each band with the molecular weights are presented in Table 11.

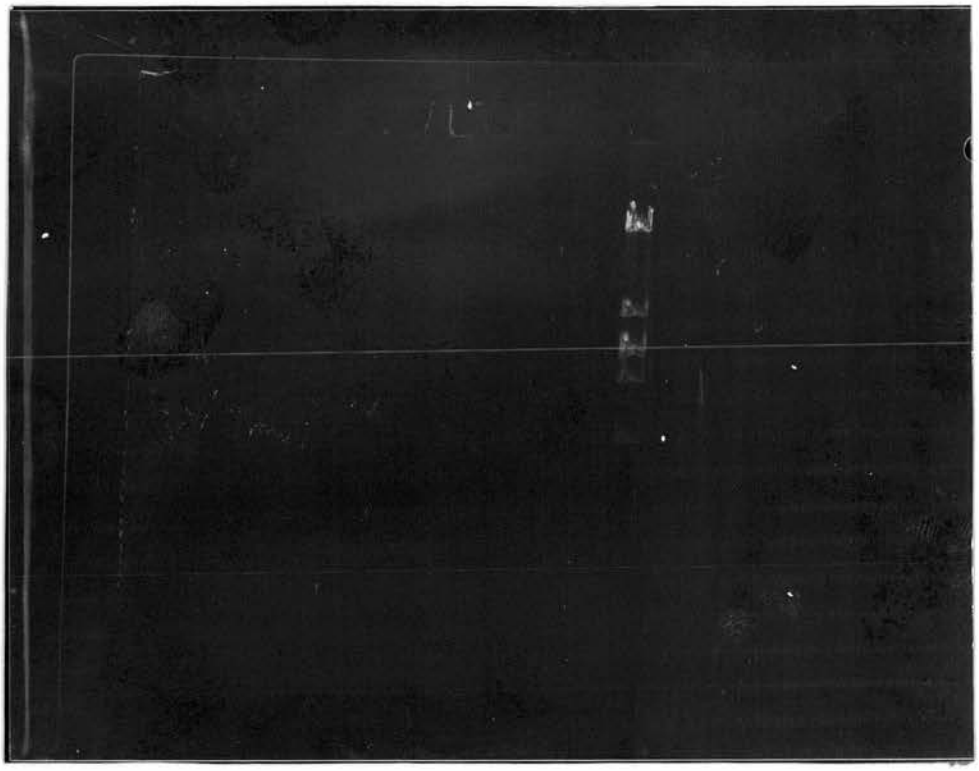
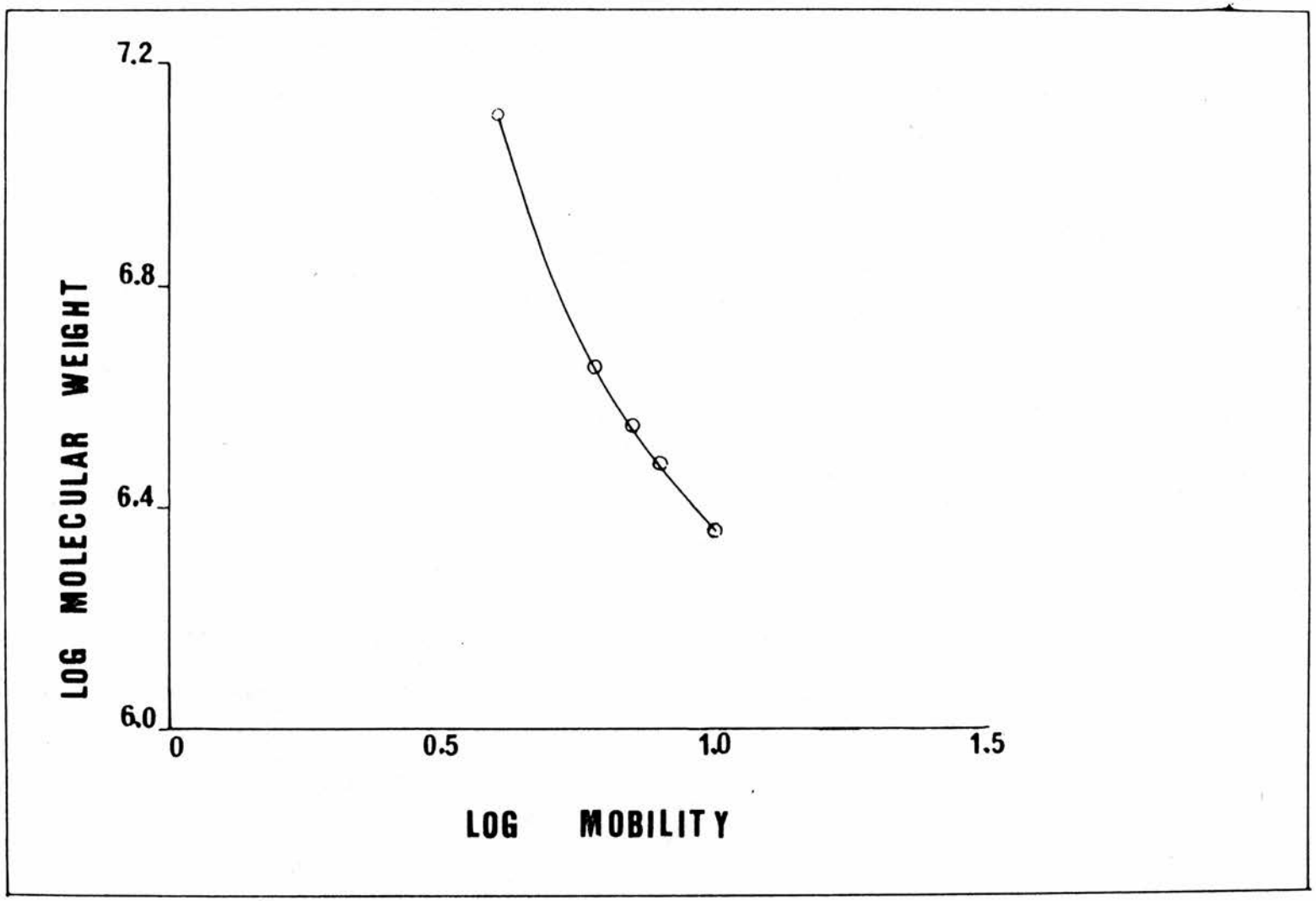


FIGURE 3.18. (right)

Plot of logarithm of molecular weight against logarithm of relative mobility in 0.7% agarose of phage lambda DNA (1 µg) digested with 5 µl of EcoR1 (1:30) enzyme solution at 37°for 1 hour, and electrophoresis of digest carried out at 30 volts for 18 hours. Measurements of mobility of fragments were carried out under ultra-violet light with a ruler, while molecular weight of respective fragments were obtained from the studies of Allet et al. (1973).


7.2
6.8
6.4
6.0
LOG MOLECULAR WEIGHT
0
0.5
1.0
1.5
LOG MOBILITY

3.11. Characterisation of plasmid DNA by molecular weight determination in agarose gel:

Plates 1-3 show the photographs of agarose gel electrophoresis of plasmid DNA.

In plate 1, the separation of the plasmid DNA is very poor. The preparation had been stored at -20° for a week prior to electrophoresis. The photograph also shows that most of the plasmid bands have migrated about the same distance through the gel, even though their molecular weights as shown are different.

In plate 2, all the specimens share a common band which has migrated to the same extent in the gel, the difference in the molecular weights of the standards as shown notwithstanding. One of the specimens shows three plasmid bands.

In plate 3, an Eco-R1 digest of lambda phage DNA is shown. Six bands are visible. The molecular weights of each fragment and their relative mobility in the gel is also shown in Table 11. A plot of logarithm of molecular weight against logarithm of relative migration of the separated fragments is shown in figure 3.18. The plot shows a linear curve.

TABLE 11

Mobility of Eco R1 lambda DNA digest in 0.7% agarose gel. Molecular weights and relative mobility of fragments are presented.

	LAMBDA PHAGE DNA FRAGMENTS					
	1	2	3	4	5	6
Molecular weight x 10^6	13.69	4.49	3.54	3.54	3.0	2.31
Relative mobility (mm)	40	60	70	72	80	110

4. Discussion

4.1 Assessment of the methods used to enumerate bacteria in water:

A sucessful practice in the enumeration of bacteria in water depends on all the plates possessing the same chance of being inoculated by the organisms in the suspension, the independent and random distribution of the organisms on the plate, the same possibility for the development of the colonies on all plates, and the fact that each colony develops independently of each other (Bonde, 1963).

With good technique, most of these problems can be avoided. However, it is almost impossible to achieve total success with some of them. Nevertheless, it should be feasible if suitable volumes and/or dilutions are used.

Aside from the above, some drawbacks are specifically associated with the use of membrane filtration for the enumeration of water bacteria. Membranes are unsuitable for use with waters of high turbidity with few coliform organisms, because the membranes become blocked before sufficient water can be filtered (Report 71, 1969). Membranes are also unsuitable if non-coliforms outnumber the coliform bacteria. Carefully chosen dilutions and selective media should help to overcome these problems. There are also reports of the selective adsorbtion of certain substances by membranes (Report 71, 1969). Burman (1960,1967), found that aniline dyes and other toxic substances from media or from water samples are adsorbed by membranes in sufficient amounts to inhibit the growth of some bacteria. With certain makes of membranes colonies tend to spread (Burman, 1960). But of course, the greatest setback of the membrane filtration method is its use of solid media which tends to dry out at elevated temperatures. Some workers minimise this by wrapping plates in polythene bags and incubatinng in a

water bath. In this investigation, cultures were incubated at 37^{o} in a hot air incubator, hence suspected colonies were confirmed by the IMVIC tests. Also the use of membrane filtration eliminates one of the basic criteria for the identification of coliform organisms; gas production. This causes delay in the confirmatory tests, which usually takes from 2-3 days.

In the present study it was imperative that the membrane filtration technique be adopted, primarily because of the need to isolate each individual colony and also because it is a more reliable method for the quantitative estimation of the bacterial content of water.

As shown in the results, even-though, there were variations in the counts obtained from the same sample, it appears that most of the triplicate counts fell within ten percent of the mean, but none was outside fifteen percent. Also, there appears to be a wide variation in the results obtained from different samples from the same sampling site on different sampling days. The highest counts were obtained from the fourth and seventh samples which were taken on days when the sea was very rough, while the lowest count was obtained from the 9th sample, taken on a hot summer day. There may be no general rule to this observation, but if this were true of all times, then the risk to users of the coastal bathing area should be reduced, since such areas are usually out of use when the sea is very rough.

4.2. Antibiotic resistance patterns of the Escherichia coli isolates:

The investigations of Smith (1970), incriminates human beings through sewage as the main source of resistant Escherichia coli in river and sea water. This worker showed that in water flowing through rural areas, the incidence of drug-resistant E. coli was low, while the same water flowing through urban areas yielded a high count of drug-resistant E. coli. The significance of using E. coli as an indicator

of water pollution has already been discussed. If these two explanations be true, then one must explain the patterns of resistance amongst the isolates as representative of the patterns of resistance in the local sewage discharged into the sea at any given time. Since all the specimens used in this study were obtained within a period of thirty days, it is not possible to comment on the constancy or variability of this pattern with time or season. The pattern of drug resistance shown in the results of this study only reflect the resistance of the antibiotics used. It is possible that a higher proportion of the bacteria would have been resistant had more antibiotics been included in the tests.

Smith (1970, 1971) and Falkow (1975) observed that chloramphenicol resistance was not expressed alone but in combination with one or more antibiotics. The same observation was made in this investigation. The reason for this is not clear, but the implication is obvious. Chloramphenicol is the best drug for the treatment of typhoid fever and any resistance to this antibiotic would be very undesirable, particularly as it appears from this study and those of Smith (1970, 1971), that multiple drug resistance is common with chloramphenicol. Fortunately, if the results of this study should represent a general trend, the incidence of chloramphenicol resistance is lowest amongst the antibiotics tested. It is also possible that the mechanism of drug resistance in the isolates may be different. For example, some of the resistances may be chromosomally determined while others may be plasmid specified. On the other hand, multiple resistance in the isolates, such as was observed with some of the isolates in this investigation, even if accounted for by one mechanism, could have been extended as a result of cross resistance (Falkow, 1975). This worker reported that a single locus may specify resistance to a number of drugs and that this was brought about by modifying enzymes with different substrate specificity. It is therefore

possible that this is the case with chloramphenicol resistance. This, however does not explain why chloramphenicol was the only drug whose resistance was not expressed alone. Probably, the same phenomenon may be true for the other antibiotics if the test were extended to include all other resistance determinants.

It is not very clear how levels of resistance to antibiotics are determined. But the mechanism may be different for each drug and type of factor specifying resistance. For example, Chang and Flaks (1970), reported that high level streptomycin resistance results from a single step mutation, while low level resistance was reported as due to decreased antibiotic uptake (Gunderson, 1967). But Hubacek, Brana and Cejka (1975) showed that high level streptomycin resistance was as a result of a synergistic action of two low level resistance determinants; one present in the resistance plasmid which determines the synthesis of streptomycin adenylylating enzyme and the other, a new chromosomal mutation which occurs in R+ cells in the presence of a higher concentration of streptomycin.

4.3. Limitations of the methods of monitoring bacterial growth by plate counts and optical density readings:

Some workers had observed that the optical density of a suspension of live bacteria was greater in dilute salt suspension than in distilled water, though it is now known that this observation was caused by the construction of the apparatus used, (Postgate, 1967). Sensitivity of the measurement increases with light of shorter wavelength although, the lower limit of sensitivity is reached with bacterial suspensions containing about 10×10^6 per mililiter (Stanier, Adelberg and Ingram 1976). Also the method only measures bacterial density and gives no clue as to the number of bacteria actually viable in the suspension.

On the other hand, even though plate counts record the number of bacteria in a known volume able to form colonies on the medium, the

standard deviation is the square root of the number counted; and no gain is made by counting more than 300 colonies (Postgate, 1967). It is also possible to have more than one colony on the same spot, though this could be minimised by the proper mixing of the suspension and by reducing the number of bacteria on the plates. Another source of error, is that in a growing culture, the cells continue to multiply even while the dilution process is taking place (Stanier, Adelberg and Ingram, 1976), and cell growth cannot be effectively checked without affecting viability. It was not considered necessary in this study to apply cold shock treatment during dilution for fear that some of the cells may be killed.

The limitations listed above notwithstanding, it was not considered that the accuracy of the growth monitoring would bear any appreciable impact on the results of later experiments, as the exercise was only intended as a guide to the most suitable time for conjugation.

4.4 Effects of growth phase and anaerobiosis on resistance transfer efficiency:

The studies of Novotny and Lavin (1971), showed that the maximum number of pili occurred between 37^{o} and 41^{o}. Ippen and Valentine (1965) observed that pili synthesis increased most rapidly during the exponential phase of cell growth, reaching a maximum during the late exponential or early stationary phase. Also the mean number of donor pili per donor cell is higher in cultures grown in mild anaerobiosis than in conditions of vigorous aeration (Curtiss, 1969). It was thought necessary to investigate the effects of growth phase and anaerobiosis as a prelude to the conjugation experiments in order that the highest efficiency of transfer be obtained during the test.

As shown in the result, the highest efficiency was recorded in the mid-exponential phase. The reason for this is not very clear. However,

it appears that high density cultures mate less efficiently (Miller, 1972). It is also possible that the results are relevant to the two test strains only.

It appears from the result of this study that mild anaerobiosis improves the efficiency of drug resistance transfer. It is not clear why this is so. But there is reason to think that mild anaerobiosis apart from improving sex pili production also retards the growth rate of bacteria without affecting their transferability. Richmond and Moillo-Batt (1975) showed that phenotypically, R+ colonies and their transferability do not increase as fast as the growth rate of the bacteria. In which case, if mild anaerobiosis affects growth rate to a higher degree than resistance transfer, then one can explain this effect in this way. The level at which efficiency of transfer begins to fall may just represent a critical point at which transfer of resistance begins to be affected.

4.5. Effect of using the same bacterium as donor and recipient on resistance transfer efficiency:

Curtiss et al. (1968) showed that there was a requirement for homology between DNA of the donor and the recipient in the Escherichia coli crosses. In the present investigation, it was observed, as will be discussed later that the efficiency of transfer of resistance differed from one isolate to the other. In order to fully appreciate what mechanisms were involved, the role of the host cell was investigated using the same organism, E. coli 9484 as both donor and recipient.

It is not certain why a marked improvement in efficiency was observed when same organism was used as both donor and recipient. But it is likely that the host plays an important role in this. Curtiss et al. (1968) think that the requirement for homology may be related to integration of the transferred fragment or the actual efficiency of transfer. The involvement of restriction enzyme process is another possible

explanation, in which case, this could account for lowered efficiency between different organisms or even the inability to transfer to different strains. On the other hand, if recipient cells actually had surface receptors as was proposed by Curtiss (1969), then these sites would be more easily recognised when the same cells were involved in conjugation.

4.6. Resistance level of donor and infected recipient cells:

In the course of the present study, it was observed that no infected cells were isolated when the selective plates contained the same concentration of antibiotic as the resistance level of the donor. Initially, it was thought that resistance transfer did not take place. But following the observation of Falkow (1975) that infected recipients did not inherit same level of resistance as the donor, a lower antibiotic concentration was used in the selective plates to isolate the infected cells. When the infected recipients were tested for their levels of resistance, it was confirmed that infected cells did not inherit same level of resistance as the donor. There was no indication from the result if this only affected a particular antibiotic, or a particular level of resistance. And there does not appear to be any one particular explanation for this.

It could be possible that the antibiotic resistance level of donor is accounted for by two mechanisms; one plasmid specified while the other is chromosomally determined. In this case, recipient cells can only acquire plasmid specified antibiotic resistance. But, if this were true, it still does not explain why recipient cells did not acquire the same level of resistance as ED 2111 whose resistance, it is known, is plasmid specified. Also the reliance on coincidence would be too much to explain the same effect on all the antibiotics. Aside from the last two objections, it would have been possible to attempt an explanation on donors

harbouring two plasmids, one of which is non-transferable.

On the other hand, it is possible that newly infected cells acquire low level resistance which is increased when cells are exposed to higher concentrations of the antibiotic. Anderson (1968) reported a similar incident in which transferable drug resistance level was raised by exposing host strain to higher concentration of the respective drug. Hubacek et al. (1975) also showed this to be the result of a synergistic action of two low level resistance determinants; one plasmid specified and the other by chromosomal mutation in the plasmid carrying cells when exposed to the same antibiotic.

It is also possible that in some cases, neither of these explanations may be responsible for the lowered resistance level in infected cells. It may be that the level of resistance is not directly a property of the plasmid but depends on the level of expression of the inactivating enzymes specified by these plasmids. In that case, the potency of these enzymes may account for the levels of resistance shown by the organism.

4.7. Antibiotic resistance transfer characteristics of wild isolates:

During the conjugation experiments using the wild isolates as donors, attention was focussed on how many of the wild isolates transferred their resistance, in what combinations resistance transfer took place, and the involvement of each antibiotic in the resistance transfer process. Also the efficiency of transfer of each antibiotic was studied.

The finding that chloramphenicol and ampicillin were the most transferred of the four antibiotics is not important except for therapeutic considerations. Ampicillin is a broad spectrum antibiotic, commonly used because of its limited side effects. The significance of this result in the context of this study is that it would be easier for ampicillin

resistance to be acquired by conjugation, thereby reducing the efficacy of the drug for therapy. The usual concern over the acquisition of resistance against chloramphenicol has already been dealt with.

It is not clear if the same opportunities of resistance transfer in laboratory experiments do exist in the sea, or following ingestion of the bacteria in the bowels. Certainly, the frequency of the mating event may be lowered by environmental factors, but this does not rule out the possibility of the occurrence of transfer in the sea and in the bowels. Caution therefore, no matter how excessive cannot be overplayed in this case.

The proportion of resistant isolates transferring resistance is only relevant with the antibiotics tested. This number may increase if other antibiotics are selected for. Apart from this, the most specific natural barrier to fertility is the DNA restriction and modification systems (Glover, 1975). Anderson (1968), Anderson and Lewis (1965) also cited the possibility of non-transferable plasmids in their studies. These workers showed that resistance factors consist of two components; the resistance transfer factor and the determinants for drug resistance. Without the resistance determinants, the transfer factors though communicable have no drug resistance to transfer, and without the transfer factors, the resistance determinants cannot be transferred to new hosts. Bacteria also carry resistance factors that have lost their transferability

and it was suggested by Anderson (1968) that this could occur by the following mechanisms:-

(a) segregation of the resistance factors during transfer so that only resistance determinants enter the recipient,

(b) segregation during growth of the host strain, so that some of the progeny receive resistance determinants only,

(c) transduction to a fresh host in which the resistance determinant only is carried over by the transducing phage and

(d) destruction of the transfer factor by mutagens without damage to the resistance determinant.

It is also possible to explain the transfer deficient strains in the light of studies by Achtman et al. (1972) and Willetts and Achtman (1972). These workers studied three classes of mutants depending on the cistrons of the transfer operon altered. In one class of mutants, deficiency in surface exclusion and pili formation was observed. In another class, pili was not formed but normal surface exclusion was observed. In the third class, pili was made but deficiency of transfer was observed.

Most plasmids showing relaxed replication do not contain enough DNA to specify a transfer operon if the plasmids are small. Helmuth and Achtman (1975) disclosed that the largest of these small plasmids is only 17-18 mega-daltons in size, whereas the transfer operon of the F sex factor probably contains 15-20 mega-daltons of DNA.

If on the other hand the non-transferability of resistance by the wild isolates cannot be explained in any other way, then another possibility is that the resistance determinants are chromosomally located.

The results also showed a very poor efficiency of transfer for most of the wild strains. Perhaps, this can be explained in terms of fertility repression which is typical of most wild plasmids (Meynell et al. 1968; Finnigan and Willetts 1973).

4.8. Classification of wild plasmids by incompatibility:

As shown in the results, two of the wild plasmids were classified by this method, two others had doubtful results while the fifth could not be placed into one specific group.

Datta (1975b) observed some inherent problems in the classification of plasmids by incompatibility. These problems are as follows:-

(a) lack of distinguishable markers,

(b) very strong or entirely lacking surface exclusion,

(c) multiple plasmids and non-conjugative plasmids and,

(d) dislodgement.

Whereas the lack of distinguishable markers can be overcome, by the use of variants of multiple resistant plasmids lacking one or more resistant genes or by the use of nalidixic acid and rifampicin resistant mutants, the correlation between incompatibility and surface exclusion is not usually complete. For example, Datta and Barth (1975) reported that where there is a strong exclusion, it may be difficult to demonstrate compatibility. On the other hand, when incompatible plasmids do not exclude one another, the test for stability of coexistence is complicated by repeated re-infection. It is possible that this is the case with JC14 wild plasmid and the R40a standard where no doubles were isolated in the selective plates. Also, bacterial strains carrying several plasmids which are efficiently transferred together in conjugation present a peculiar kind of problem in the study of incompatibility especially if some of them are non-conjugative. In the case of dislodgement, a plasmid may be eliminated by the introduction of another plasmid, yet in other clones of the same bacterial strain, the two may prove, to be compatible.

It would be noted from the results of this test that the H plasmid group featured in four of the five tests, even though the antibiotic pattern of this plasmid and the wild strains are very different.

Perhaps the evolution of plasmids in relation to their transfer genes is different from that in relation to antibiotic resistance (Datta, 1975a).

4.9. Characterisation of plasmid DNA by molecular weight determination in agarose gel:

Meyers et al. reported the preliminary characterisation of plasmid DNA by molecular weight estimation in agarose gel. These workers also reported the successful deployment of this method for the molecular weight measurements of plasmids ranging from 0.6×10^6 to 95×10^6. Because it was not possible to completely classify the wild type plasmids by incompatibility and more so because the method reported by these workers appeared simple enough for this study, it was adopted in this investigation for the preliminary characterisation of plasmid deoxyribonucleic acid.

As already shown in the results, it appeared that all the plasmids ranging from 25 to 65 mega-daltons used in this study had the same migration rate in agarose gel. But the fragmentation of lambda phage DNA showed that the fragments could be separated on agarose gel and that a linear plot of log. molecular weight against log. relative mobility was possible. It appears from the results of this study that whereas plasmid DNA can be separated on agarose gels and whereas the logarithm of molecular weight against logarithm of relative migration gave a linear plot, this method cannot be employed for the molecular weight determination of larger molecules. This must have been responsible for the identical migration in gels shown in the results. This observation was also made in the studies of Allet et al. (1973). Gels of lower concentration than 0.7% could have been tried to improve resolution, but at such low concentrations gels become almost impossible to handle especially in the big gel slabs used in this study.

It also appears from plate 1 that excessive storage of plasmid DNA preparations even at temperatures of -20° produces a tailing effect, which may be due to the action of non-specific nucleases (Willetts, personal communication).

5. Conclusions.

An attempt has been made in this study to isolate and characterise plasmids carried by Escherichia coli contaminating the St. Andrews coastal bathing area for epidemiological reasons.

The techniques of water sampling, isolation and identification of Escherichia coli, antibiotic sensitivity testing and cojugation experiments appear relatively easy once standardisation of the adopted method has been carried out. However, the classification of plasmids and the characterisation of plasmid DNA proved to be problematic.

Most of these problems have been discussed earlier in the text, but some of them are presented here for the appraisal of future workers in this field. For example, even when a plasmid carrying strain fits into one group, the understanding is that the strain probably carries one plasmid. When therefore more than one plasmid is isolated from the same strain by any other means the full meaning of the classification is lost. From the studies presented in this text this method of classification does not appear to be very reliable and above all, it is time consuming.

The estimation of molecular weight of plasmid DNA in agarose gel from this study appears to be reliable and reproducible for the preliminary characterisation of plasmid DNA. But as already shown in the results and discussions, the resolution is very poor, or in fact non-existent for large plasmids. It would be necessary for a continuation of this study, to fragment the plasmid DNA by the use of restriction enzymes. However, this would impose the use of very pure plasmid DNA preparations. On the other hand if it is not possible to obtain very pure fractions, then plasmid bands from crude preparations can be eluted from gels after preliminary separation, and digested. The only problem here is that when bands are very faint, recovery may be difficult.

If the above mentioned problems are solved before a study of this kind is commenced, periodic sampling from a particular source, and characterisation of plasmids isolated from Escherichia coli from that source may provide valuable information on the mechanism of infectious drug resistance in the locality, particularly. Also, it should be possible to use DNA fragments from such studies in transformation experiments in order to fully understand the function of each DNA fragment.

Appendix 3.1

Resistance of *E. coli* isolates to different antibiotics. 1236 isolates were tested on antibiotic containing nutrient agar incubated at 37° for 18-24 hours. Number resistant to different antibiotics and percentage of total isolates are presented.

A = Ampicillin

C = Chloramphenicol

T = Tetracycline

S = Streptomycin

N = Nalidixic acid

R = Rifampicin.

	Antibiotics					
	A	C	T	S	N	R
Number resistant	52	11	52	100	0	0
% of total E. coli	4.2	0.89	4.2	8.09	0	0

Appendix 3.2

Antibiotic resistance patterns of the drug resistant isolates. 103 drug resistant isolates fitted into eight patterns. The patterns, number of isolates and percentage of total resistant isolates are presented. Interpretations of abbreviations are given below.

	Antibiotic resistance patterns.							
	S	A	A + S	S + T	S + A + C	S + A + C + T	S + T + A	T
Number of isolates	35	1	15	14	1	10	26	1
% of resistant isolates	34	0.97	14.56	13.59	0.97	9.7	25.24	0.97

S = Streptomycin
A = Ampicillin
C = Chloramphenicol
T = Tetracycline.

Appendix 3.3

Antibiotic resistance levels of ampicillin resistant isolates. Results were obtained from tests on 52 ampicillin resistant isolates. The number of isolates resistant to various antibiotic concentrations and the percentage of the resistant isolates are presented.

	Ampicillin concentration in µg/ml.						
	12	200	400	450	500	600	750
Number of isolates	1	3	10	1	1	7	29
% of resistant bacteria	1.9	5.8	19.2	1.9	1.9	18.5	55.8

Appendix 3.4

Antibiotic resistance levels of streptomycin resistant isolates. Results were obtained from tests on 100 streptomycin resistant isolates. The number of isolates resistant to various antibiotic concentrations and the percentage of the resistant isolates are presented.

	Streptomycin concentrations in µg/ml.							
	75	100	150	200	250	300	400	450
Number of isolates	23	35	12	16	1	5	2	6
% of resistant bacteria	23	35	12	16	1	5	2	6

Appendix 3.5

Antibiotic resistance levels of tetracycline resistant isolates. Results were obtained from tests on 52 tetracycline resistant isolates. The number of isolates resistant to various antibiotic concentrations and the percentage of the resistant isolates are presented.

	Tetracycline concentrations in µg/ml.				
	60	150	200	750	1000
Number of isolates	1	1	2	1	47
% of resistant bacteria	1.9	1.9	3.85	1.9	90.4

Appendix 3.6

Antibiotic resistance levels of chloramphenicol resistant isolates. Results were obtained from test on 11 chloramphenicol resistant isolates. The number of isolates resistant to various antibiotic concentrations and the percentage of the resistant isolates are presented.

	Chloramphenicol concentrations in µg/ml.			
	200	350	400	450
Number of isolates	7	1	1	2
% of resistant isolates	63.6	9.1	9.1	18.2

Appendix 3.7

Optical density readings of growth rate of E. coli 9484 monitored every hour for 10 hours, at 610 nm using the SP 600 spectrophotometer. Readings were taken in triplicate. Mean of the three readings is also shown.

	Growth time in hours									
	1	2	3	4	5	6	7	8	9	10
Reading 1	0.00	0.00	0.00	0.014	0.036	0.182	0.434	0.466	0.485	0.50
2	0.00	0.00	0.00	0.0141	0.045	0.180	0.436	0.466	0.486	0.50
3	0.00	0.00	0.00	0.014	0.034	0.179	0.435	0.464	0.488	0.51
Mean reading	0.00	0.00	0.00	0.014	0.035	0.181	0.435	0.465	0.486	0.50

Appendix 3.8

Optical density readings of growth rate of ED 2111 monitored every hour for 10 hours, at 610 nm using the SP 600 spectrophotometer. Triplicate and mean readings are presented.

	Growth time in hours									
	1	2	3	4	5	6	7	8	9	10
Reading 1	0.00	0.00	0.00	0.023	0.093	0.302	0.518	0.660	0.752	0.78
2	0.00	0.00	0.00	0.023	0.094	0.301	0.516	0.660	0.751	0.78
3	0.00	0.00	0.00	0.024	0.093	0.302	0.519	0.661	0.750	0.78
Mean reading	0.00	0.00	0.00	0.023	0.093	0.302	0.518	0.660	0.751	0.78

Appendix 3.9

Bacterial count of E. coli 9484 carried out every hour during the growth rate measurement (see appendix 3.7). Optical density readings were obtained every hour with the SP 600 spectrophotometer at 610 nm, while bacterial count was performed on nutrient agar plates by the technique of Miles and Misra (1938). Optical density readings and time with triplicate and mean bacterial counts are presented.

		Growth time in hours.						
		4	5	6	7	8	9	10
O.D. at 610 nm		0.014	0.035	0.181	0.435	0.465	0.486	0.505
Count x 10^8	1	0.361	3.46	65.8	168.0	184.6	192.6	209.7
	2	0.368	3.44	66.2	167.2	186.1	192.9	210.2
	3	0.349	3.43	64.8	169.0	184.3	193.9	210.1
Mean count		0.366	3.44	65.6	168.0	185.0	193.0	210.0

Appendix 3.10

Effect of growth phase on resistance transfer efficiency using ED 2111 as donor and E. coli 9484 as recipient. Conjugation was performed in nutrient broth at 37° and infected recipients were counter-selected on antibiotic containing plates. Bacterial counts were obtained by the technique of Miles and Misra. Triplicate and mean counts are presented.

	Growth time in hours.							
	4	5	6	7	8	9	10	24
O.D. 610nm	0.023	0.093	0.302	0.518	0.660	0.751	0.782	0.792
Efficiency 1	9.6×10^{-7}	1.0×10^{-4}	1.72×10^{-4}	2.1×10^{-5}	5.2×10^{-6}	3.0×10^{-6}	1.11×10^{-6}	3.8×10^{-7}
2	9.0×10^{-7}	0.98×10^{-4}	1.58×10^{-4}	2.2×10^{-5}	5.0×10^{-6}	3.0×10^{-6}	1.21×10^{-6}	3.62×10^{-7}
3	9.3×10^{-7}	1.2×10^{-4}	1.5×10^{-4}	2.48×10^{-5}	5.1×10^{-6}	2.88×10^{-6}	1.1×10^{-6}	3.38×10^{-7}
Mean Efficiency	9.3×10^{-7}	1.06×10^{-4}	1.6×10^{-4}	2.26×10^{-5}	5.1×10^{-6}	2.96×10^{-6}	1.14×10^{-6}	3.6×10^{-7}

Appendix 3.11

Effect of anaerobiosis on resistance transfer efficiency determined in conjugation experiments by use of thioglycollic acid nutrient broth. Donor strain was ED 2111 and recipient was *E. coli* 9484. Triplicate and mean efficiency are presented.

	Percentage of thioglycollic acid in nutrient broth.						
	0.0	0.01	0.02	0.03	0.04	0.05	0.06
Efficiency 1	1.30×10^{-4}	8.37×10^{-4}	1.42×10^{-3}	3.57×10^{-4}	7.67×10^{-5}	4.07×10^{-5}	4.77×10^{-6}
2	1.37×10^{-4}	8.25×10^{-4}	1.52×10^{-3}	3.65×10^{-4}	7.78×10^{-5}	3.65×10^{-5}	4.8×10^{-6}
3	1.38×10^{-4}	7.6×10^{-4}	1.41×10^{-3}	3.8×10^{-4}	7.5×10^{-5}	3.98×10^{-5}	4.68×10^{-6}
Mean efficiency	1.35×10^{-4}	8.07×10^{-4}	1.45×10^{-3}	3.67×10^{-4}	7.65×10^{-5}	4.0×10^{-5}	4.75×10^{-6}

Appendix 3.12

Proportion of wild isolates transferring resistance to *E. coli* 9484, in conjugation experiments. The antibiotics whose resistances were transferred, the number of isolates transferring and the percentage of resistant organisms are presented.

	Antibiotics			
	Ampicillin	Streptomycin	Chloramphenicol	Tetracycline
Number resistant	52	100	11	52
Number transferring	37	24	6	12
Percentage	71.15	24	54.55	23.08

Appendix 3.13

Antibiotic combinations transferred by wild isolates during conjugation to E. coli 9484. The number of bacteria transferring resistance, the number transferring with the various combinations and the percentage of transferring isolates are presented. Results were obtained from the 46 E. coli isolates transferring resistance.

	S	A + S	C + A + S	A	T	T + A	T + C + A + S	C + A	T + S	A + S + T
Number transferring	6	9	1	15	1	3	2	3	2	4
Percentage	13.04	19.56	2.17	32.61	2.17	6.52	4.34	6.52	4.34	8.69

S = Streptomycin

A = Ampicillin

C = Chloramphenicol

T = Tetracycline.

Appendix 3.14

Efficiency of transfer of ampicillin resistance transferring isolates. Number of isolates transferring at various efficiencies and percentage of isolates transferring resistance are presented.

	Resistance transfer efficiency (ampicillin).					
	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}
Number of isolates	1	16	13	4	2	1
Percentage	2.7	43.24	35.13	10.81	5.4	2.7

Appendix 3.15

Efficiency of transfer of chloramphenicol resistance transferring isolates. Number of isolates transferring at various efficiencies and percentage of isolates transferring resistance are presented.

	Resistance transfer efficiency (chloramphenicol)	
	10^{-7}	10^{-5}
Number of isolates	4	2
Percentage	66.67	33.33

Appendix 3.16

Efficiency of transfer of tetracycline resistance transferring isolates. Number of isolates transferring at various efficiencies and percentage of isolates transferring resistance are presented.

	Resistance transfer efficiency (tetracycline)			
	10^{-8}	10^{-7}	10^{-6}	10^{-4}
Number of isolates	2	5	4	1
Percentage	16.66	41.66	33.33	8.33

Appendix 3.17

Efficiency of transfer of streptomycin resistance transferring isolates. Number of isolates transferring at various efficiencies and percentage of isolates transferring resistance are presented.

	Resistance transfer efficiency (streptomycin)				
	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
Number of isolates	1	11	9	2	1
Percentage	4.16	45.83	37.5	8.33	4.16

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