PHAGE - MEDIATED TRANSFER OF GENETIC MATERIAL

BY

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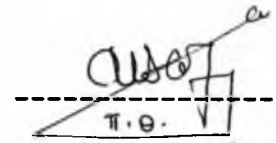
DECLARATION

I certify that this work has not been submitted to any other university for any degree. The experimental work was carried out by me. Due acknowledgement has been given for all the guidance received.



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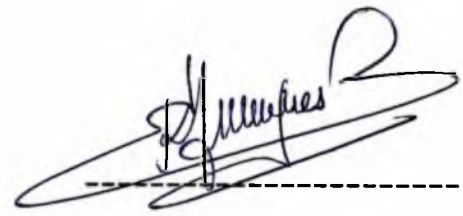

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DEDICATION

To my dear parents and Miss Comfort Naa Densua Djoleto.

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ABSTRACT

Ten phages were isolated randomly from sewage sources and disposal points on the University of Ghana,Legon campus, purified and characterised as delivery systems for the resistance marker genes, Str^r and Ben^r. The phages were found to have proteins of relative molecular weights ranging from 9,000 - 100,000. Eight phages were found to be morphologically related to the tailed phages. The other two were tailless phages. All the coliphages isolated were morphologically related to the T-even phages, whilst Sf RBCL 15 and Sd RBCL 5 were related to the P-phages. The tailless phages were found to be morphologically related to the ϕ phages (ϕ6 and ϕX174 phages). The coliphages were found to be closely related to each other. They did exhibit some detectable cross reactivity to Sd RBCL 5, Sd RBCL 23 and Sf RBCL 15. The coliphages were not related to the S. typhi phages.

Selected pathogenic bacteria from the Noguchi Memorial Institute for Medical Research and the Medical School of the university of Ghana were screened for the presence of the antibiotic resistance gene marker. All the bacteria, except three, were found to be highly resistant to the marker antibiotics used : Salmonella Group D was relatively more sensitive to all three antibiotics ; Staphylococcus aureus was sensitive to Benzylpenicillin and Salmonella typhi was sensitive to Tetracycline.

The isolated phages with the exception of St RBCL 20 exhibited the ability to transduce the resistance marker genes from one bacterium to sensitive bacteria at a high frequency.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.0 GENERAL INTRODUCTION

The discovery of the efficacy of antibacterial agents in the treatment of the "mirage" of bacterial infections was seemingly an answer to a problem of grave concern to both researchers and clinicians. But the effective use of these "miracle" drugs was short lived due to the emergence of drug resistance or tolerance by target cells/organisms.

This phenomenon of drug resistance is of considerable economic importance and often has grave consequences to the developing world. It also serves as a major challenge to the pharmaceutical industry because the development of resistance ensures that effective drugs are limited in their usefulness (Hayes and Wolf, 1990).

The current socio-economic situation caused by poverty and underdevelopment in developing countries leads to conditions of overcrowding and unhygienic environs thus creating a favourable environment for the interaction of many bacteria leading to the spread of genes among them. These factors coupled with the extensive administration of antibiotics in human and veterinary medicine and their use as supplement in animal feeds have led to the rapid spread of antibiotic resistance genes among bacteria. Such conditions constitute a powerful selective force for the evolution of virulence factors (eg. resistance factors) which are not only retained but also sorted out and transferred via delivery systems such as plasmids, transposons and bacteriophages

(Phages) (Phillips, 1986).

Phages are usually used as agents of transfer of gene markers because of the ease of isolation, purification and manipulation of their genes. They also provide an insight into the evolutionary processes and molecular aspects of host-parasite relationships.

1.1 BACTERIOPHAGES

1.1.1 General Features

Bacteriophages are bacterial viruses which may be viewed as DNA elements with an added extracellular phase to their life cycles. The replication of bacteriophage genetic material and the expression of its genes are reliant on the enzymes of the host cell. Phages are parasites of the cell they infect. They have developed mechanisms to ensure release of their progeny from the infected cell. Phages can afford to produce more copies of themselves per infection: some produce as many as 10,000. This number, the "burst size" per infection is a characteristic value, different for different phages (Freifelder, 1983). Phages have the ability to leave the host and this allows a more rapid spread to other cells than can be achieved by plasmids, whose spread is mediated primarily by conjugation. Phages have evolved mechanisms to protect their genetic material so as to survive exposure to the extracellular environment (Freifelder, 1983).

They attain extracellular stability by protecting their sensitive genetic material from potentially harmful environmental

agents such as nucleases. This is done by "wrapping" the viral genome with a protein coat, the capsid, composed of many tightly packed protein subunits. To achieve efficiency in packaging, phages have evolved three basic morphological types namely: icosahedral tailless, tailed icosahedral and filamentous phages. Some variations exist within the tailed phages based on their contractile nature and the length of the tail (Bradley, 1971)

Most phages have a single nucleic acid molecule per phage particle. Depending on the individual phage this may be DNA or RNA, single or double stranded, circular or linear, and range in size from 1.8 to 200 Megadaltons (Mdal) (Freifelder, 1983). Double-stranded DNA phages are more common. The presence of more than one nucleic acid per mature phage virion is rare, but not unheard of. For example, each particle of Ø6, which infects Pseudomonas phaseolica, contains three linear double-stranded RNAs (Freifelder, 1983).

Phages infect cells by recognition and attachment to specific receptor molecules, often proteins, on the cell surface. Due to this specificity phages exhibit a selectively limited host ranges.

Since infection is fatal, the presence of phage receptors on a cell can have a large negative selective value. Therefore, the receptor will be expected to be lost from a bacterial population. To counteract this phenomenon, most phages utilize as their receptors molecules of some positive value to the cell. For example, some phages bind to the pili synthesized by conjugative

F^+ cells; the receptor is a protein that normally functions in the uptake of maltose by the host. The receptor for the Escherichia coli phage TI is a protein engaged in iron uptake by the cell (Freifelder, 1983).

The specificity of the phage adsorption process imparts resistance to infection upon cells lacking the specific receptor required for binding. The bacterial restriction-modification system serve to further limit the host range (Arber and Linn, 1969).

1.1.2 Life Cycle

Different phages have evolved different solutions to the problems of infection, replication and release of progeny. Despite these differences, one may speak of a generalised life cycle with six component stages namely: adsorption, entry of the phage nucleic acid into the host cytoplasm, redirection of host metabolism for the production of phage, replication and expression of viral nucleic acid, assembly of mature phage and release from the host (Martins, 1978).

Adsorption

This is the initial phase of infection. It involves contact between the phage particles which collide randomly with bacteria. They recognize and bind to specific receptor sites on the surfaces or pili of susceptible cells. The environment is of importance at this stage of adsorption. The recognition may

involve protein–protein or ionic interactions involving inorganic salts. For example, some phages have specific cationic requirements, such as that of T4 and lambda(λ) for Ca^{2+} and Mg^{2+} respectively. Tryptophan appears to release the fibers from association with the sheath, so that they are free to interact with the bacteria surface. In phage T4, the fibres can be seen to form "a jacket" around the sheath in the absence of tryptophan and to extend in its presence (Brenner et. al. 1962).

In the tailed phages, it is often the free tips of the tail fibres which mediate recognition and binding to the surface of the host. Tailless phages adsorb by specific interactions between receptors on the host and components of the phage coat.

Entry of the Phage Nucleic Acid into the host cytoplasm

Adsorption is followed by insertion/injection of the phage nucleic acid. The tailed phages appear to possess a lysozyme-like activity which breaks some of the covalent bonds of the host's cell wall in preparation for injection (Wood and Edgar, 1967). Injection is achieved by a contraction of the tail which brings it to the cell surface driving its core through the cell wall and the membrane. The core of the tail is cylindrical and hollow, and the phage nucleic acid then passes from the phage head via this channel to the cytoplasm of the host (Martins, 1978). This mode of entry of nucleic acid in T-phages was demonstrated in the famous experiments of Hershey and Chase(1952). In some phages (eg. T4 of E. coli) this injection

process is rapid, being completed within 1 minute after adsorption. In others like phage T7 of E. coli injection requires 10 minutes or more and spans 30% of the infection cycle. Slow injection in this phage serves as a timing mechanism to coordinate replication of the phage DNA with the assembly of progeny genomes into mature particles. This prevents the undesirable and premature assembly of virions and packaging of the DNA before the replication process has produced a full complement of daughter phage DNAs (Freifelder, 1983).

In the tailless phages such as the single-stranded DNA phage ØX174 of E. coli, direct injection of the nucleic acid does not occur. The phage adsorbs reversibly to receptors on the host. The DNA enters the host with coat protein remaining on the cell surface. In the filamentous phage, the entire virion, protein as well as nucleic acid, enters the cell. The coat becomes located on the inner cell membrane and the nucleic acid exits into the cytoplasm. There are obvious advantages since the virus genomes may remain protected in an alien genosphere, awaiting appropriate conditions for replication (Martins, 1978).

Redirection of host metabolism for Phage Production

The ability of the invading virus to reprogramme the host cell ribosomes, redirect and control protein synthesis results in what is essentially a "biochemical coup d'etat". At this stage the strategy of infection becomes aggressive and offensive as compared to the relatively passive role of the two processes

discussed above. The invading phage is right at the center of metabolic control (Martins, 1978). Literary, the infected cell is converted to a "phage factory". By such diversity of mechanisms , phages achieve the shutdown of host DNA replication and the expression and preferential replication of their own genetic material.

Replication and expression of Viral Nucleic Acid

Phage DNA replication is a complex process using both phage and host encoded enzymes. The specific replication of the phage nucleic acid usually involves either alteration of the recognition specificity of host replication enzymes or the production of new enzymes. It leads to the synthesis of a string (concatemer) of phage genomes joined in tandem. Single genomes are cut off the concatemer and packaged into phage coats. Thus linear genomes with single-stranded ends are regenerated (Scaife and Goman, 1985).

Production of mature virions requires the assembly of phage protein coats from their monomer subunits and packaging of the newly produced daughter phage genomes into these shells. All coat proteins and additional proteins required for coat assembly and packaging of phage nucleic acid are encoded by the phage genomes. If only the original parental viral genome were expressed during infection it would take prohibitively long to produce sufficient amounts of these proteins to package all the daughter phage genomes. To overcome this limitation, both

parental and daughter phage nucleic acids are simultaneously replicated and expressed within the infected cell (Russel, 1973). This provides both ample progeny genomes and sufficient amounts of these proteins to package all the daughter phage genomes. It would be detrimental to produce phage coat proteins early in the infection cycle because they would begin to assemble and incorporate phage nucleic acid before these genomes had been replicated a sufficient number of times to give a high yield of progeny. Thus phages usually exhibit a temporary regulation of gene expression such that the synthesis of coat and maturation proteins, and many proteins involved in host lysis, is delayed until ample number of progeny genomes have been reproduced. Temporal regulation of phage protein production usually occurs at the transcriptional level. Some of the schemes employed in the regulation and expression of gene replication and expression as found in genes of T4, T7, λ ,ØX174, QB are thoroughly reviewed by Freifelder (1983).

Assembly of Mature Phage

Phages generally produce all the components required for the assembly of mature progeny. In the simplest systems, such as the small RNA phages QB and MS-2, assembly is simple and straight forward; coat proteins bind to each daughter RNA as it is synthesized. Those bound to the same molecule then interact with one another to form the mature icosahedral shell in which the RNA resides (Casjens and King, 1975).

In larger phages, the shell is comprised of more than one type of protein. Their assembly to form the coat and the incorporation of nucleic acid into it, are regulated by morphogenic enzymes encoded by the phage (Murialdo and Becker, 1978). In ØX174, seven proteins are involved in the assembly, but only four of these are present in the final mature virion. There is evidence to indicate that the host plays a direct role in the assembly of the larger phages. Some components of the phage may be chemically altered during the assembly process (Hendrix and Casjens, 1975).

In the large complex phages, such as T4, T7 and λ, head and tail structures assemble separately. Each is composed of multiple copies of many different proteins. Their association with another during the assembly requires the sequential action of several maturation enzymes (Dickson et. al., 1970). For the tailed phages, tail fibres assemble and mature separately and attach to the tailed heads at this stage (Katsura, 1989).

Because of the phage replication mechanics, progeny nucleic acids are often produced in long concatemers containing multiple linked genomes. During packaging, these are cut into genome length size for incorporation into the virion. In T4, a "headful" packaging mechanism functions to guarantee incorporation of a full phage genome: one end of the DNA enters the head and the strand continues entering until the head is full. A phage enzyme then cleaves the concatemeric DNA at the point of its entry into the virion. Lambda progeny are also

synthesized as concatemers. Each genome-worth of DNA within these concatemers ends in a 12bp sequence known as the "cos" (or cohesive) site (Campbell, 1981). A cos site separates each pair of adjacent phage DNAs in the concatemer. Upon packaging of the DNA into the phage a terminase (or ter) activity at the entrance to the phage head recognises the cos site and cleaves the DNA, ending the packaging effect. This ensures that a mature or genome-length molecule containing all the sequences is packaged (Katsura, 1989).

Release from the Host

There are three known mechanisms of release of progeny virions from the host. In the first of these, a lytic enzyme (lysozyme) produced late in the infection cycle weakens or destroys the cell wall and the host lyses, releasing the phages. This is the mechanism of progeny dispersal in the medium and large phages of E. coli (Wood and Edgar, 1967).

In the small icosahedral RNA coliphages (eg. MS-2, F2, R17, QB) no lytic enzyme is known to be produced. The great number of progeny produced by these phages, 5,000 to 10,000 per cell, form large crystalline arrays within the host. These damage the host cell membrane via unknown mechanisms and causes lysis of the host, releasing the phages (Freifelder, 1983).

A third method of release is exhibited by the filamentous phages of E. coli. The host neither dies nor lyses, rather, these phages are extruded intact through the cell wall and

membranes. Infection by these phages is therefore a chronic infection wherein the host cell remains alive but continues to excrete mature virions for the remainder of its existence (Freifelder, 1983).

1.1.3 Lysis:Lysogeny cycle

On injection into the cell two alternatives are available to the phage, the lytic or lysogenic cycle (Herskowitz and Hagen, 1980). For some phages the replication-assembly-release sequence follows immediately upon infection of the host. These are the lytic phages which follow the lytic cycle. For the majority of known phages, however, the production of progeny virions need not necessarily follow directly after infection (Scaife and Goman, 1985). Such phages are known as the temperate phages. Temperate phages are capable of assuming a dormant existence within the host. During this time the expression of most phage genes, such as those for nucleic acid replication and transcription, and virion structural protein, is totally repressed. In the majority of cases this state is accompanied by phage insertion and replication as part of the bacterial chromosome. This process of integration of phage nucleic acid into the host chromosome is termed "lysogenization". Cells in which this has occurred are called "lysogens". In such a situation, only a small region of the genome is transcribed. The protein synthesised from this mRNA is a repressor, which binds near the two promoters controlling the expression of the first genes in the lytic cycle.

Repressor binding at these sites prevents transcription of the early genes and thus prevents the entire cascade of gene expression that would result in phage replication and host lysis (Anderson et. al. 1981).

The lysogenic state is a stable one, and the infected cell metabolises and propagates normally. This state can be maintained nearly indefinitely under appropriate conditions until "induction" occurs. "Induction" is the process whereby lysogeny is broken down. The prophage excises from its site in the host chromosome and enters the lytic cycle. " Induction" is caused by a lot of factors or agents including UV light, exposure to carcinogenic agents such as mitomycin C, fluoropyrimidines and shifts in temperature (Berksdale and Arden, 1974). " Induction" leads to derepression of all repressed genes of the lytic cycle, allowing them to be transcribed. Among the first proteins synthesised after induction is the excisionase (Nash, 1981). This protein reverses the integration reaction, causing excision of the phage DNA from the host chromosome. Excision is followed by expression of the genes involved in phage reproduction and maturation. This leads to a switch over to the lytic cascade (Herskowitz and Hagen, 1980). Apart from the more common "integrative lysogenisation" is the less common type which involves no integrative mechanisms, rather the phage DNA circularises, replicates and is passed onto daughter progeny during host chromosomal replication as plasmids (Starlinger, 1977).

A lysogen cannot be reinfected by a phage of the type that first lysogenised it; this resistance to "superinfection" is called immunity (Freifelder, 1983). Often a lysogen obtains properties not present in the original bacterium. This leads to a change in phenotype known as phage conversion (Herskowitz and Hagen, 1980). Some affected traits include restriction systems, surface polysacharides and toxin production. Many of these acquired phenotypic traits are to help the lysogen to achieve immunity to superinfection (Campbell, 1981).

Most temperate phages integrate into the host chromosome at specific host sequences. A few phages show little or no sequence specificity for the integration site. A notable example is the "Mu" phage of *E. coli*, so named because its low insertion specificity causes it to integrate into any of a number of host genes, destroying their continuity and thereby causing insertional mutagenesis (Freifelder, 1983).

The DNAs of temperate phages reach a branchpoint in their life cycles after injection into the cell: they may follow either a lytic or lysogenic life cycle. In Lambda, lysogeny is favoured either when environmental nutrient levels are low or when there are numerous phages in the surrounding medium (Herskowitz and Hagen, 1980). The lysis: lysogeny decision is made soon after the phage DNA enters the host. In λ, entry into lysogeny is dependent upon the concentration of a phage-encoded protein, cII, which is made by transcription/translation of a small phage genome segment immediately after infection. At sufficiently high

concentrations, cII directs λ into the lysogenic pathway. cII activates genes whose protein products catalyse integration of phage DNA into the host chromosome and repress the genes for phage reproduction and lysis of the host. Sufficient cII concentrations are obtained at high phage densities or multiplicities of infection. This favours lysogeny (Herskowitz and Hagen, 1980). For example, it has been reported that infections of Salmonella typhimurium by wild type phage P22 generally results in a lytic response at low multiplicities of infection (MOI) and a lysogenic response at high MOI's (Steinberg and Gough, 1976). By lysogenising under such conditions, a phage can delay production and release of daughters until the concentration of competitors in the environment decreases, thereby enhancing the chances that its progeny will find susceptible hosts to infect (Herskowitz and Hagen, 1980).

1.1.4 **Bacteriophages as Antigens**

Bacteriophages are good antigens producing antisera with high inactivation constants. The sera of animals immunised with phages contain antibodies to all surface constituents of the virus. For the T-even phages, there are antibodies to the phage membranes, the tail fibres and the sheaths. If raptured phages are used as antigens, antisera may also contain antibodies to internal proteins and nucleic acids. The different kinds of antibodies evoked are dependent on the structural complexity of the phages used as antigens. The antibodies elicited by a virus

are not necessarily homogeneous with respect to their specificity. Some react only with the constituents of the virus used for immunization, whereas others combine also with those of serologically related phages (Jesaitis and Zinder, 1977).

When an antiphage serum is mixed with a phage suspension, the phage may be neutralised, agglutinated or it may acquire the ability to fix complement. Each of these reactions can be used for the identification and quantitation of phages or their constituents as well as for the detection of phage-specific antibodies in the antiserum. The most frequently employed serological reaction with phages is their neutralisation. The reaction is carried out by performing an antiphage-antiserum assay. The reaction is followed by measuring the decrease in the number of infective particles in the phage-antiserum mixture (Clausen, 1981). The number of infective particles can also decrease as a result of aggregation of virus by antibodies. However, as long as the viral concentration is less than 10^7 particles / ml, this process is very slow; therefore phage suspensions containing 10^3 to 10^6 particles are used in the antibody neutralisation assays (Clausen, 1981).

Since phages of various species differ in their serological specificity, the neutralisation reaction is used for identification and classification of bacterial reactions. The inactivation of a phage isolate by antiserum produced to another phage shows that the phage capsids contain same antigens and that the two isolates may be the same. Phages may be serologically

related but not chemically identical. Serological unrelatedness can indicate either a gross difference in the capsid such as the presence or absence of a tail, or may be due to a chemical difference which may be serologically undetectable. However, if two phages are serologically related, even slightly one can say that they are very similar save for a degree of difference in their antigenic structures which could have been produced by natural mutations of a common ancestor (Bradley, 1971).

The degree of neutralisation activity is subject to variable conditions such as temperature, time, pH and salt concentration. Other variables such as antigenic site, the relative proportion of reactants, the lineage of cells, and the participation of other substances such as complement may affect the neutralisation assays (Mandel, 1985).

1.2 REARRANGEMENT AND REASSORTMENT OF GENETIC MATERIAL

Despite the requirements of structural and genetic stability for the stable functioning of DNA, and its transmission to daughter generations, there are evolutionary advantages to be gained from mechanisms that exchange DNA, either between two DNA molecules or between two organisms. Such interchange allows an organism to rearrange its own genetic material, producing new genes that encode advantageous traits. It also allows the incorporation of desirable traits from other cells (Freifelder, 1983). It must be emphasized that irrespective of the mode of gene transfer, the information must be incorporated into the new

host's hereditary apparatus if it is to be propagated as part of the apparatus when the cell divides.

Genetic rearrangements can have biological importance on two time scales: on an evolutionary scale, where the effects are seen after many generations, and on a developmental scale, where the effects are apparent within a single generation (Cohen and Shapiro, 1980). The following are several mechanisms by which bacteria obtain and rearrange genetic material.

1.2.1 Genetic Recombination

Genetic recombination refers to the reassortment of a series of nucleotides along nucleic acid molecules. It allows the exchange of DNA between two genomes. This feature allows for the incorporation of DNA sequences bearing desirable mutations. These reassortments can occur within one molecule to produce deletions, inversions, transpositions or duplications. It can also occur between two separate parental molecules to produce a recombinant (or two of them) derived in part from each parent (Freifelder, 1983).

One of the most useful parameters for distinguishing between modes of recombination is based on the base sequence homology, or lack of it between two DNA molecules (Nash, 1981). General (or homologous) recombination is dependent on the extent and degree of homology between parental molecules. In such situations recombination in most organisms is a fairly frequent event, occurring largely at random along regions where the two

homologous parental DNAs are brought into register. It is commonly dependent on physical breakage and reciprocal exchange of DNA sequences in a region of extensive homology (Cohen, 1976). This class of recombination includes varieties which differ based on certain distinguishing characters such as (a) whether or not one of the parental molecules is single-stranded and (b) whether the product is one intact DNA sequence or two and if two, whether or not they are reciprocal (West et. al., 1981). Homologous recombination is rec (for recombinant) A protein dependent. Another protein rec BC is also required(Hotchkiss, 1974).

Recombination can also occur between DNA molecules in which there is very little, if any, homology. When a recombination event occurs at highly preferred or specific position on one or both of the parental strands, it is referred to as site specific recombination. Site specific recombination in prokaryotes can be of two types: replicative and conservative recombination (Nash, 1981). Based on the involvement of one or both parental molecules the event is referred to as single or double site specific recombination (Kleckner, 1981). One of the most thoroughly characterized examples of site specific recombination is the integration and excision reaction of phage lambda (Landy and Ross, 1977). The range of phenomena associated with site specific recombination includes the generation and integration of episomal factors, the highly mobile antibiotic resistance elements, the influence on gene expression by small DNA sequences and the transition between vegetative genomes and host integrated

prophage (Freifelder, 1983).

Another kind of non homologous recombination occurs at apparently random sites between regions of DNA molecules. This is referred to as illegitimate recombination. Often chromosomal aberrations leading to illegitimate recombination occur in the vicinity of preformed genetic elements that are able to insert at different sites of DNA molecules (Starlinger, 1977) which is rec A independent. Illegitimate recombinations are limited to cases of apparent randomness or very low specificity such as duplications, transpositions, deletions (with the exception of the site specific transposon-mediated transpositions such as Mu insertions and deletions), excisions to produce transducing phage genomes, and fusions of non homologous ends of DNA (Weisberg and Adhya, 1977, Eisenstark, 1977). The acquisition of certain new segments of genetic material by plasmid and phage genomes can occur by illegitimate recombination of DNA sequences that have little or no ancestral relationship (Cohen and Shapiro, 1980).

To take advantage of the benefits of genetic exchange, bacteria have evolved several means of obtaining DNA from other cells. These normally occur via conjugation, transformation, transduction. Such transfers are mediated by phages, transposons and plasmids.

1.2.2 Conjugation

This involves the unidirectional transfer of genetic material among cells. Conjugation is directed by a series of proteins whose genes reside on plasmids. Transfer of genetic material during conjugation consists of three stages: exit of DNA from the donor, entry of DNA into the recipient and a transit between the two. The exit of DNA from the donor during conjugation does not require cell lysis, analogous to the exit of the viral DNA of filamentous phage such as M13 (Clark and Warren, 1979). It requires cellular contact, following which all or part of the bacterial chromosome is transferred (Levinthal, 1974). It is initiated by the formation of specific donor-recipient pairs. A most essential feature for a normal conjugation system is the presence of a sex factor in the donor strain. The transfer of chromosomal DNA and its detection after transfer, requires an interaction between the sex factor and the donor chromosome. It is also dependent on the ability of the recipient cell to integrate a portion of the transferred genetic information (Low and Porter, 1978).

In conjugation, the transfer factor with or without one or more other plasmids or part of the chromosome is transferred from the initiating cell to its partner, which can then pass on some or all of its newly acquired genetic material to its progeny and to other transfer negative cells (Clark and Warren, 1979). Conjugal transfer involves a replicative process where only a single strand is transferred, the other remains in the donor cell

and new complementary strands are synthesized in donor and recipient cells. This feature distinguishes such a transfer from transformation and transduction, which involve double strand transfer. Unlike transformation and transduction, transfer is not restricted to donor and recipient strands with close taxonomic relationships even though transfers occur readily between related strains. This has important medical implications; for example, antibiotic resistance can be conferred onto potentially pathogenic but previously antibiotic sensitive bacteria of other genera by plasmid transfer. This conjugal transmission of plasmids accounts for the spread of antibiotic resistance among bacteria and is a probable answer to the phenomenon of the evolution of multiple drug resistance species (Clark and Warren, 1979). Conjugation is one of the major modes of transfer of genetic material or markers such as drug resistance, sex and colicinogenic factors among bacteria(Novick,1980).

1.2.3 Transduction

Transduction is the interbacterial transfer of DNA via a bacteriophage (Cohen and Shapiro, 1980), and the transfer is followed by DNA integration or plasmid formation in the recipient cell. After integration, a DNA mechanism exists to prevent the rapid degradation by nucleases in the recipient cells. Bacterial genetic material is carried as an insertion, or substitution, within the phage genome (Low and Porter, 1978). The transducing

DNA that does not become intergrated into the recipient organisms persists for at least several hours as undegraded and unreplicated molecules which probably represent the basis for abortive transduction (Low and Porter, 1978). All transduction events are abnormal, in the sense that bacterial genes are transferred by a structure that evolved primarily to carry the viral genome that normally codes for its production and transport. During transduction a small minority of the recipient organisms acquire some property of the donor strain and transmit it as a stable genetic character to their progeny (Ebel-Tsipis et. al. 1972b). Bacteriophages have a narrow host range which implies that transduction is usually between closely related strains.

Transduction is classified into two groups, according to the range of bacterial genetic markers available for transmission in a phage-bacteria system. In generalised transduction almost any genetic marker of a donor strain can be transduced; whilst in specialised transduction only markers which are located in the region of the prophage attachment sites can be transduced (Ozeki and Ikeda, 1968). Specialised transducing particles are formed only after induction of a lysogen, while generalised transducing particles seem to be formed in all lytic infections (Ebel-Tsipis et. al. 1972a).

The production of a generalised transducing particle is thought to result from the accidental packaging of a piece of bacteria DNA inside a phage-like particle, which is

indistinguishable from the infectious particles of the associated phage with regard to external features such as size and shape, adsorption characteristics and antigenic properties (Ozeki and Ikeda, 1968). Generalised transducing particles are valuable experimental tools because they can be used to generate new bacterial strains and to map genes (Freifelder, 1983). Generalised transduction has also been used to modify the genotype of recipient plasmids and to transfer a variety of extrachromosomal factors. For example the P1 phage of E. coli has been used to transfer the F (plasmids that carry the sex determinants) , R (plasmids that carry antibiotic resistance determinants) and C (plasmids that carry colicinogenic determinants) factors (Low and Porter, 1978).

The creation of the hybrid phage-bacterial genomes, which are transferred during specialised transduction, most often occurs as a rare abnormal excision event starting with the normal integrated state of the prophage genome. If all the phage genes required for lytic growth are present, the phage can form plaques; otherwise it can only be propagated in the presence of coinfecting phage to supply the missing functions or as a prophage. The ability of specialised transducing phages to form genetically stable lines distinguishes them from generalised transducing phages whose DNA can neither persist as prophage nor be efficiently repackaged into progeny virions (Weisberg and Adhya, 1977). Specialised transduction has proven to be a useful working tool for genetic analysis and manipulation. By this

method it is possible to employ *in vitro* packaging technology which allows the incorporation of a DNA of interest into phage particles and its subsequent introduction into a bacterium using the phage as a vehicle for the delivery. This can be a powerful tool in gene manipulation and engineering (Freifelder, 1983). Its use in fine structure mapping is analogous to mapping by transformation although it does not suffer from the problems of spurious linkage since only a small proportion of the phage lysate is transduced contrasted to the potential activity of every DNA molecule in a transforming preparation. The degree of linkage of genetic markers can be inferred from the relative frequency with which they are co-transduced (Ely and Johnson, 1977). Transducing phages are useful workhorses of molecular biologists because they enable one to replicate specific DNA segments apart from the rest of the chromosome (Campbell, 1976).

1.2.4 **Transformation**

The uptake of DNA either chromosomal or plasmid by bacteria and its retention under appropriate physiological and environmental conditions is termed transformation (Smith *et. al.*, 1981). The overall process of transformation may be divided into several common steps, each of which can be experimentally studied. These are the development of competence, binding of DNA, uptake of DNA, formation of a preintegration complex and the integration of DNA into the recipient cell chromosome (Smith *et. al.*, 1981). When the DNA is that of a phage, the term

of a phage, the term "transfection" is applied.

Basically there are two types of transformation which are based on the mode of acquisition of competence, which is either physiologically or artificially attained (for example $CaCl_2$ mediation). However, individual species have unique requirements which must be defined before transformation can be successful (Low and Porter, 1978).

In transformation in Pneumococcus species, Bacillus subtilis and Haemophilus influenzae, donor DNA enter recipient chromosome by a displacement mechanism. In these systems, a single strand fragment of the donor DNA becomes integrated into the recipient chromosome at the site of the displacement event(s) (Ebel-Tsipis et. al. 1972b). If the DNA that is taken up is homologous, a substantial fraction becomes inserted into the continuity of the genome of the recipient bacterium, creating regions, several thousands nucleotides in length, in which one strand is donor DNA and the complementary strand is recipient DNA. The efficiency with which homologous single-stranded DNA is integrated into its appropriate genetic location in the bacterial genome provides support for a number of proposals that suggest a single-stranded DNA terminus in initiating exchange events between double-stranded DNA molecules (Fox, 1978).

Though transformation is usually attributed to a single-stranded chromosomal DNA uptake pathway, a double-stranded DNA uptake pathway has been proposed to account for transfection data in B. subtilis (Low and Porter, 1978). Plasmid DNAs are similar

in many respects to viral DNA and seem to be handled by cells in the same fashion during transformation. Plasmid, phage and chromosomal DNAs all seem to be taken up by the same mechanism in naturally competent cells (Smith et. al., 1981).

In the case of plasmid DNA, which has its own unique replication and maintenance genes, stable maintenance of the introduced DNA requires that the recipient cell successfully replicate the molecule. DNA fragments lacking replication origins will be successfully introduced by transformation only if (1) the host lacks a nuclease sufficiently active to destroy all incoming DNA and (2) the fragments taken into the cell recombine with the chromosome (Freifelder, 1983). Transformation can be used to study in vitro manipulation of bacterial genes by providing a method for the reintroduction of genetically functional DNA into a cell (Mandel and Higa, 1970). Plasmid transformation is extremely useful because plasmids can be used as cloning vectors to isolate, amplify and analyse genes from many sources. Artificial transformation has been developed because of its usefulness in genetic engineering and gene analysis (Smith et. al., 1981).

1.3 ANTIBIOTICS

Antibiotics are microbial products which in low concentrations(of the order of ug/ml) can inhibit or kill microorganisms. Antibiotics are used to mark genes for gene transfer studies.

Antibiotics generally act in a three step process involving penetration, binding and drug effect. The antibiotic must first traverse any bacterial envelopes or barriers which separate it from its site of action. It then goes on to bind to a target molecule with the production of an antibacterial effect (Bellido and Pechère, 1989). This basic mode of action is illustrated in Fig. 1.

On binding to the appropriate target molecules, the antibiotics act in one of four main ways, namely;

i. the inhibition of essential metabolic reactions,
ii. interference with the synthesis of the bacterial cell wall,
iii. disorientation of the structure of the plasma membrane,
iv. impairment of nucleic acid and protein biosynthesis (Bowman and Rand, 1984).

The antibiotics used for this study were (1) an inhibitor of cell wall synthesis, Benzylpenicillin-a B-lactam,(2) Streptomycin,an aminoglycoside and an inhibitor of protein biosynthesis and (3) Tetracycline,also an inhibitor of protein biosynthesis.

PENICILLINS

The Penicillins or Penams together with the Cephalosporins or Cephems belong to the B-lactams(Bellido and Pechère, 1989). The representative structures of some Penicillins are as shown in Fig. 2.

Fig. 1: Mode of action of an antibiotic. Antibiotic action of whatever kind on a given bacterium is a three step process: penetration, binding, effect.

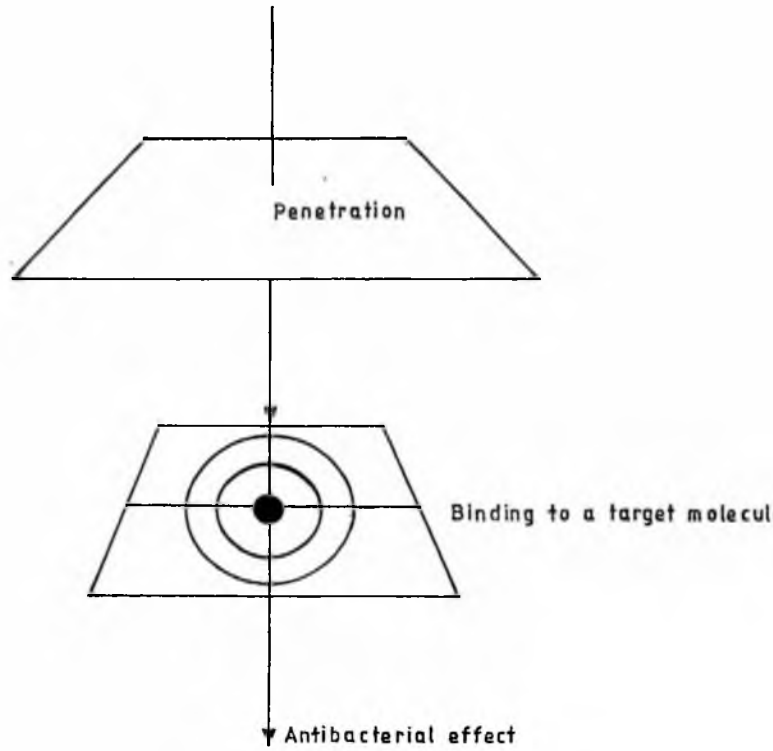


Fig. 2: Shows the basic structure of Penicillin with examples of the type of side chains

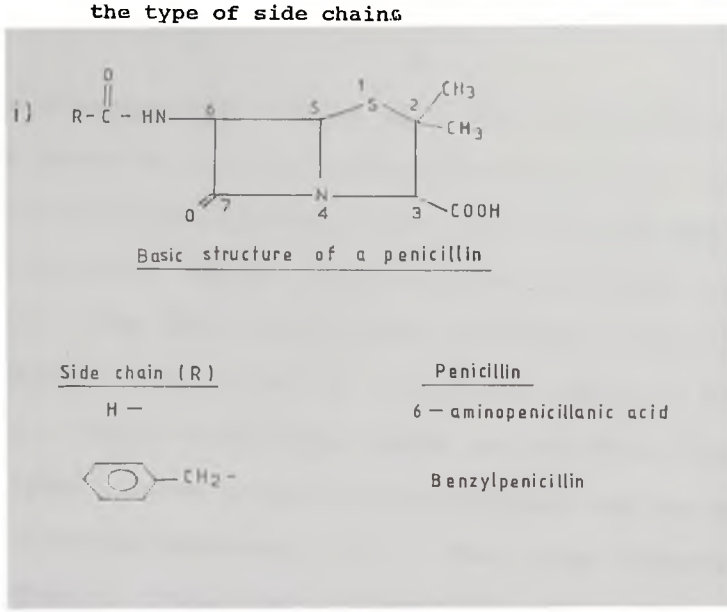


Fig. 3: Streptomycin

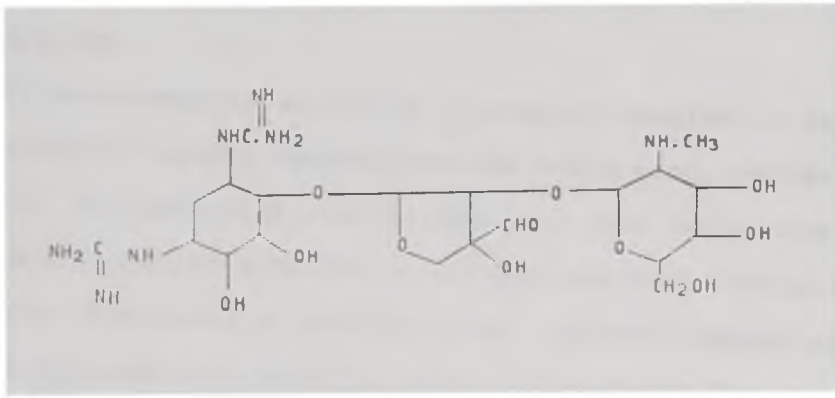
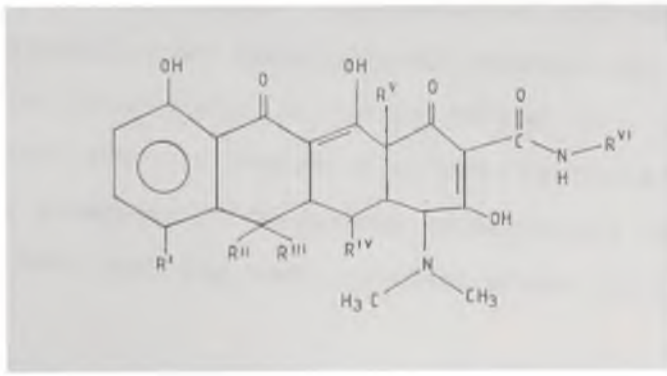


Fig. 4: Tetracycline



The B-lactam antibiotics with their characteristic -lactam ring are known to bind to a characteristic target molecule, the Penicillin Binding Proteins (PBPs) (Bellido and Pechere, 1989). On reaching their target molecules the antibiotic begins to exert its action. The PBPs are enzymes involved in the laying down of peptidoglycan which forms the structural skeleton of the bacterium. When the B-lactam binds to the PBPs, the peptidoglycan can no longer be synthesized and the microbial cell dies (Bellido and Pechere, 1989). They thus interfere with cell wall synthesis. This leads to swelling and lysis of the cell (Snow and Franklin, 1981).

STREPTOMYCINS

The Streptomycins belong to a group of chemically related therapeutically useful Aminoglycosides which also include Kanamycin, the Neomycins and Gentamicin. They are narrow spectrum antibiotics with bacteriostatic and bacteriocidal activities (depending on concentration) against Mycobacterium tuberculosis and gram negative bacteria including Escherichia coli, Pseudomonas aeruginosa, Neisseria gonorrhoeae, Pasteurella pestis, Haemophilus influenzae, Klebsiella sp. and some species of Proteus, Salmonella and Staphylococci (Duerden et. al., 1987). The structure of Streptomycin is shown in Fig. 3.

Streptomycin exerts a number of effects on bacterial cells. It affects the integrity of the plasma membrane and the metabolism of RNA. But its most important effect is the

inhibition of protein biosynthesis at the level of the 30S ribosomal subunit. The site of drug action has been localised to the smaller subunit of the ribosome which binds mRNA (Bowman and Rand, 1984).

TETRACYCLINES

Another group of antibiotics which inhibit protein biosynthesis are the Tetracyclines. The basic structure of Tetracycline is shown in Fig. 4.

They are broad spectrum antibiotics with a wide range of bacteriostatic activity against both gram-negative and gram-positive bacteria. Pseudomonas aeruginosa and Proteus vulgaris are among the few bacteria that are not affected by Tetracyclines (Duerden et. al.,1987). They appear to act by inhibiting the binding of aminoacyl-t-RNA to the mRNA-ribosome complex. The Tetracyclines bind to both mRNA and to the 30S subunit of the ribosomes, the latter site being the most important in their prime function of inhibition of protein biosynthesis. They prevent the enzymatic binding of the aminoacyl-t-RNA into the ribosomal aminoacyl receptor or A site, thereby preventing codon-anticodon interaction, a vital step in protein synthesis. The concentration of the drug required to inhibit mammalian protein biosynthesis is far higher than that for inhibiting growth of microorganisms (Bowman and Rand, 1984). They are useful as therapeutic agents both in human and veterinary medicine.

1.4 DRUG SENSITIVITIES AMONG BACTERIA

Drug sensitivity is the response or susceptibility to a particular drug by a disease-causing organism. Areas to be reviewed will include the factors responsible for the variation in dose response among bacteria to antibiotics and some current trends to forestall these problems.

The effectiveness of a drug is due to the ability to intervene in the 'struggle' between the host's defenses and the invading organisms. Some of these organisms are highly susceptible to particular drugs and are termed **'sensitive'**. At the other end of the 'sensitivity scale', is the least susceptible which is usually referred to as resistant strain. In the clinical context, an organism is said to be resistant if it is not killed or inhibited by drug concentrations readily attainable in the patient. Usually the response of a given organism to the drug is seldom absolute and it can be overcome by increasing the drug concentration *in vitro*. But such a high drug concentration leads to problems of increased blood levels with possible toxic side effects on vital organs of the host such as the liver and kidney.

Some of the antibiotics have useful levels of activity against specific parts of the "spectrum" of bacteria. These are referred to as narrow spectrum antibiotics. Examples of such drugs include Cloxacillin, Benzylpenicillin, the Macrolides and the Polymyxins. Others are effective against at least some members of most genera and are often referred to as broad

spectrum antibiotics. Examples include the Aminoglycosides, Chloramphenicol and the Tetracyclines. Some antibiotics tend to prevent the bacteria from multiplying and are termed bacteriostatic drugs while others known as bacteriocidal drugs tend to kill the bacteria. These are used when the patient is immunocompromised or when the infection is overwhelming (Duerden, et. al., 1987). Antibiotic drug sensitivities are determined either by some form of disc diffusion method or by the agar dilution method (Bryant, 1972).

Variations in drug sensitivities may arise due to variations in the response of permeability barriers of bacteria to drugs, lack of a suitable intracellular target, the ability to switch to alternative metabolic pathways and the development of detoxification mechanisms (Hayes and Wolf, 1990). Resistance to antimicrobial agents may occur by chromosomal mutation or be acquired by one of the classical gene transfer mechanisms, or possibly by phenotypic adaptation in a hostile environment, that is, under selective pressure of antibiotics as occurs in prolong exposure to antibiotics or "training" of bacteria, resistant species thrive better than the sensitive species (Wiedemann, 1986).

Bacteria exhibit varying responses in the permeability of their cell walls to drugs. This leads to difficulties in drug accessibility to the target site. The gram-negative bacteria with a more complex cell wall are able to provide a more formidable barrier to antibiotics such as the B-lactams. A

familiar example of loss of cell permeability is the situation where the drug is converted to a derivative that does not bind its membrane receptor in the cell wall. This gives a 'false impression' of changed permeability. Loss of cell permeability may also be due to the synthesis of an additional permeability layer, mutation of the permeation mechanism and specific antagonism to antibiotic transport. It is believed that some mutants of E. coli and S. typhi with enhanced resistance to Ampicillin result from the synthesis of an additional permeability barrier. The reduced uptake of Streptomycin and Erythromycin may be due to the development of a permeability barrier. It has also been reported that altered drug penetration by the reduction or loss of the outer membrane porin molecules, which function as channels for the drug permeation, may be responsible for Cephalosporin and Tetracycline resistance (Snow and Franklin, 1981; Jaffe et. al., 1983).

Another important factor in the variation of drug sensitivities among bacteria is the variation in/or lack of affinity between the drug and the appropriate bacterial receptors. For the PBPs, the level of inhibition of growth correlates with the level of saturation of the receptor protein sites by the B-lactams or any other drug; that is, the lower the affinity the greater the amount of drug needed and the least sensitive the bacteria is to the drug. There is also a chance for the modification of the receptor proteins which may lead to loss of affinity for the drug and hence a diminished uptake of

the drug (Wiedemann, 1986).

Some bacteria exhibit a variation in their response to a suitable intracellular target. In Streptomycin resistance, there is lack of a ribosomal protein, due to modification of the 30S subunit, to which it can bind. Thus the drug is unable to exert its characteristic effect on protein biosynthesis (Snow and Franklin, 1981). In drug responses by bacteria the number of lethal and non-lethal targets is important. A balance favouring the availability of more lethal targets leads to a high sensitivity whereas an increase in non-lethal targets reduces the amount of drug available. This mechanism of resistance is exemplified by Streptococcus faecalis (Wiedemann, 1986).

The ability of some bacteria to switch to alternative metabolic pathways leads to variations in drug sensitivity. In organisms resistant to sulfonamides, there is the production of an inhibitor which competes with the normal metabolite. Some drugs depend on essential biosynthetic processes where they are able to inhibit some essential end product to the organism. In such a situation, cellular resistance may develop if the bacteria utilize an alternative means of acquiring the desired compounds from exogenous sources. An example is resistance to Cotrimoxazole (Duerden et. al., 1987).

Another mechanism which accounts for differences in drug sensitivities is the utilization of detoxification or hydrolytic processes to inactivate drugs. Notable enzymes involved in such processes include the B-lactamases which inactivate the B-lactams

by a hydrolytic ring cleavage and the acetyl transferases which acetylate and inactivate Chloramphenicol (Snow and Franklin, 1981). The detoxification of Penicillin is illustrated in Fig. 5.

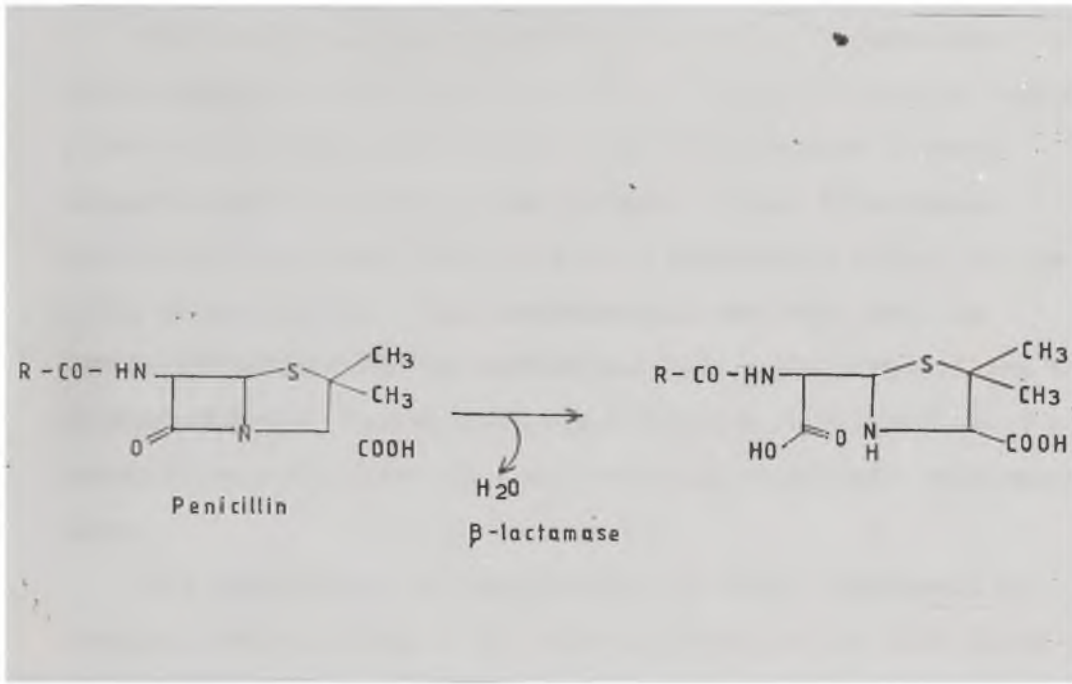
Some bacteria elaborate enzymes that acetylate, adenylate, or phosphorylate the Aminoglycosides (Wiedemann, 1986).

Bacterial cells employ a host of mechanisms in resisting drug action, some of which has been outlined above, but the final result - emergence of resistant strains occurs by an interplay of several mechanisms involving many genes. For example, amongst the B-lactams, the mechanism of drug resistance in gram-negative bacteria is due to a complex interaction involving drug affinity for the target site, the lactamase activity, amount of drug in the periplasmic space, and the number of lethal target sites (Wiedemann, 1986).

Currently the following methods are being employed to enhance drug sensitivity; multidrug therapy, enzyme inhibition and chemical modification of the existing antibiotics.

The multidrug therapy is utilized in three basic ways; the use of one agent to inhibit enzymes that would normally destroy the partner drug; the use of an antibiotic to promote the entry of another to an inaccessible intracellular target site and the use of two or more antibiotics to mutually suppress the resistant strains within the bacterial population. This approach has been highly successful especially in antituberculosis therapy. An

Fig. 5: Drug inactivation or hydrolytic cleavage (action of B-lactamases on B-lactam)



example is the use of a combination of Isoniazid, Streptomycin and p-Aminosalicylic acid in the treatment of tuberculosis (Duerden et. al., 1987).

The use of enzyme inhibitors has been most successful with the B-lactams. The use of naturally occurring B-lactam compounds with a high inhibitory effect on the B-lactamases of most enterobacteria has shown some success. These B-lactamase inhibitors have been found to have a protective effect on the other B-lactams with high antibacterial activity but low inhibitory effect on the lactamases such as the Penicillins and Cephalosporins. Commercial preparations of Clavulanic acid with Amoxycillin and Ticarcillin are currently available (Wiedemann, 1986).

The development of therapeutically useful compounds by chemical modification of the existing antibiotics also shows an improvement in the efficacy of antibiotics. These semi-synthetic compounds tend to exhibit stability to the B-lactamases. Examples include the new Penicillins such as Temocillin and Mezlocillin, the new generation Cephalosporins such as Cefoxitin and Ceftriaxone, the Monobactams such as Aztreonam and the Carbapenems such as Imipenem (Wiedemann, 1986). The Carbapenem exhibits an extraordinarily broad spectrum of antibacterial activity encompassing most gram positive and gram negative bacteria. However, by a strange irony, this compound otherwise enzymically stable, is readily hydrolysed by a dehydropeptidase located in the brush border of the proximal renal tubule of the

nephron, so that in therapeutic use the drug is formulated with a dehydropeptidase inhibitor, Cilastatin (Kahan et. al., 1983).

Though new advances have been made to combat the threat of drug resistance, a new development is the emergence of multidrug resistance (Wiedemann, 1986). Though gene transfer has already been discussed to be an advantageous trait,the rapid spread of antibiotic resistance gene markers (using a diversity of delivery systems such as phages, transposons and plasmids) among bacteria has shown it to be an undesirable feature in the treatment of bacterial infections. The overall objective therefore will be the investigation of the possible role of phages as delivery systems for the transfer of the antibiotic resistance gene markers among bacteria.

The specific aims of the study were:

- the screening of some selected pathogenic bacteria for the presence of the antibiotic resistance gene markers.
- the isolation and purification of phages as delivery systems for the antibiotic resistance gene markers
- characterization of the purified phages, using their protein profile by SDS-PAGE, morphological features by electron microscopy, their relatedness using the antibody neutralisation assay.
- a study of mediated transfer of the antibiotic resistance gene markers by the characterised phages.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

BACTERIA

Two sources of bacteria were used. The first group was obtained from the Bacteriology Unit of the Noguchi Memorial Institute for Medical research, University of Ghana, Legon. The bacteria were Pseudomonas aeruginosa (Ps. aeruginosa), isolated from pus, Staphylococcus aureus (S. aureus) from a wound, Shigella dysenteriae (Sh. dysenteriae) Salmonella typhi (S. typhi) and Shigella flexneri (Sh. flexneri) were isolated from faecal samples.

The other group was obtained from the Microbiology Department of the University of Ghana Medical School. They were Klebsiella species (Kleb. sp.), Escherichia coli (E. coli), Proteus species (Pro. sp.), Pseudomonas aeruginosa (Ps. aeruginosa), Shigella flexneri) and Salmonella Group D (Sal. Group D). The bacteria were kept on agar slants at 4°C.

MEDIA

Nutrient broth and nutrient agar were used for all the microbiological assays. They were obtained from Fluka Chemie AG, CH-9470 Buchs, Switzerland and Difco Laboratories, Detroit, Michigan, USA. Purified phages were stored in phage storage medium (SM) (Appendix 1) at 4°C.

ANTIBIOTICS

Streptomycin Sulfate, Benzylpenicillin potassium salt and Tetracycline were used. They were obtained from Fluka Chemie AG, CH - 9470, Buchs, Switzerland. 2 mg/ml of antibiotic stock solution was used for all the assays.

All other reagents used were obtained from Fluka Chemie AG, CH-9470, Buchs, Switzerland unless otherwise stated. They were of analytical grade where possible.

Freund's complete adjuvant was obtained from Difco Laboratories, Detroit, Michigan, USA whilst Folin-Ciocalteau's reagent and Polyethylene glycol 6000 were from Hopkins and Williams, Chadwell, England. Glycine and Sodium azide were obtained from BDH Chemicals Limited, Poole, England. Standard Protein molecular weight markers were obtained from two sources: Carbonic anhydrase, a low molecular weight marker from Sigma and a high protein marker mixture of molecular weight ranging from 18,500 to 330,000 from Pharmacia.

Table 1: SOURCE OF SAMPLE SUSPECTED TO CONTAIN BACTERIOPHAGE

SAMPLE NO.	SOURCE
1	Stagnant Water, Vaughan Dam, Botanical Gardens, University of Ghana, Legon.
2	Wet soil, tap at Botanical Gardens, University of Ghana, Legon.

Table 1: SOURCE OF SAMPLE SUSPECTED TO CONTAIN BACTERIOPHAGE (Cont'd)

SAMPLE NO.	SOURCE
3	Gutter Water, infront of Legon Hall, University of Ghana, Legon.
4	Stagnant Water, tap at Annex B, Legon Hall, University of Ghana, Legon.
5	Water from an air conditioner, G.W.S.C. Treatment plant near Legon.
6	Stagnant Water, G.W.S.C. Treatment plant near Legon.
7	Pond Water, Balme Library, University of Ghana, Legon.
8	Pond Water, Akuafo Hall, University of Ghana, Legon.
9	Pond Water taken at one site, Main Pond at University Gate, University of Ghana, Legon.
10	Pond Water taken at another site, Main Pond at University Gate, University of Ghana, Legon.
11	Wet soil, tap behind Botany Department, Legon.
12	Refuse, Refuse Dump, Lower Hill, University of Ghana, Legon.

Table 1: SOURCE OF SAMPLE SUSPECTED TO CONTAIN BACTERIOPHAGE (Cont'd)

SAMPLE NO.	SOURCE
13	Refuse, Refuse Dump, Kitchen, Legon Hall, University of Ghana, Legon.
14	Marshy soil, Botanical Gardens, University of Ghana, Legon.
15	Sewage, Meat Processing Laboratory, Animal Science Department, University of Ghana, Legon.
16	Gutter Water, behind Botany Department, University of Ghana, Legon.
17	Sewage, Mensah Sarbah Hall, University of Ghana, Legon.
18	Gutter Water, infront of Akuafo Hall, University of Ghana, Legon.
19	Animal droppings, Number 5, Legon Hill, University of Ghana, Legon.
20	Sewage, Annex C, Legon Hall, University of Ghana, Legon.
21	Soil, Coffee Plantation, Botanical Gardens, University of Ghana, Legon.
22	Sinna Dura's Garden, Faculty of Agriculture, University of Ghana, Legon.
23	Gutter Water, infront of Drama Studio, University of Ghana, Legon.

Table 1: SOURCE OF SAMPLE SUSPECTED TO CONTAIN BACTERIOPHAGE (Cont'd)

SAMPLE NO.	SOURCE
24	Discarded agar plates, Department of Biochemistry, University of Ghana, Legon.
25	Spill from sewage, Annex B, Legon Hall, University of Ghana, Legon.
26	Gutter Water, infront of Sanitation Department, University of Ghana, Legon.

2.2 METHODS

2.2.1 Isolation of Phages

Samples were randomly collected from various locations on the University of Ghana campus (Table 1). They included liquid (eg. sewage, stagnant water) and solid samples (eg. soil, droppings from poultry).

The liquid samples were first filtered through a Whatman number one filter paper to remove debris and then through an 0.22 um bacteriological filter to remove bacterial contaminants into sterile bottles.

The solid samples were mixed with nutrient broth, filtered through a Whatman number one filter paper and then through an 0.22 um bacteriological filter. The filtrates were stored over chloroform at 4°C.

ASSAYING FOR PHAGE INFECTIVITY:

The suspected phage samples were tested for phage activity using the plaque assay technique. 500ul of each sample was added to 1 ml of an overnight culture of bacterial cells which serves as indicator cells. 5 ml of molten overlay agar (1.0% w/v Nutrient broth and 1.2% w/v Nutrient agar, 1mM $CaCl_2$, 1mM $MgSO_4$) was added. The mixture was then poured on an underlay agar (1.0% w/v Nutrient broth, 1.5% w/v Nutrient agar, 1mM $CaCl_2$, 1mM $MgSO_4$). The test was done in duplicate with a control which had the indicator cells without the phage sample.

The plates were incubated overnight at 37°C. After the incubation period, the plates were examined for the presence of lesions or plaques on the lawn of bacterial cells grown on the agar plates. The presence of plaques was taken as an indication of phage activity and scored as (x) for suspected phage activity.

The detected plaques were repeatedly picked into sterile nutrient broth (1% w/v) with a sterile cocktail stick. A few drops of chloroform were added. The phage samples were replated several times on the same indicator bacteria to confirm the activity of the plaques picked. The confirmed phage activity was scored as (+). The isolated phage samples were stored as phage stocks at 4°C.

2.2.2 Phage Titration and Propagation

The phage stocks were titred to determine the appropriate concentration to use in preparing large phage material for

subsequent work.

A ten fold serial dilution of the concentrated stock was prepared. 50 ul of the diluted samples were added to 1 ml of an overnight indicator bacteria culture. 5 ml of an overlay agar was added and the mixture poured over an underlay agar. A duplicate was made. Plates were incubated overnight at 37°C. Plaque counts were made and the titre values determined (from the plaque counts and the dilution factor) and recorded.

Based on the information obtained from the phage titration, the phages were propagated on their appropriate indicators to obtain large volumes of phage lysates for subsequent biochemical studies.

For the large scale propagation, 200 ul of the appropriate concentration of the phage stocks were used to obtain very high plaque count which gave confluent lysis. For each phage sample, 50 plates were prepared and incubated overnight at 37°C.

After the incubation, the overlay agar were scraped into a sterile beaker with a sterile spatula. Sterile nutrient broth (500 ml) and chloroform were added. The mixture was homogenised in a waring blender (HGB 200) for 15 minutes. The "foamy" suspension was then centrifuged at 18,000g for 30 minutes at 4°C using a Tomy's High Speed centrifuge. The supernatant (phage Iysate) was decanted into sterile flasks and stored over chloroform at 4°C.

2.2.3 Purification of Phage

A modified scheme of Yamamoto was used in the purification of the isolated phages (Maniatis et. al., 1989). Two 50 ml portions of a phage stock culture were made up to a final concentration of 1M (2.92g/50 ml) with sodium chloride. The mixture was swirled to dissolve the salt and kept on ice for an hour. Bacterial debris were then removed by centrifugation at 11,000g for 10 minutes at 4°C. The clear supernatants were collected in a clean beaker, solid polyethylene glycol (PEG 6,000) was added to a final concentration of 10% (w/v). The PEG was dissolved by slow stirring on a magnetic stirrer at room temperature. The resulting solution was cooled in ice water for 1 hour to allow the phage particles to precipitate. The precipitated phage particles were recovered by centrifugation at 11,000g for 10 minutes at 4°C. The supernatant was discarded and the centrifuge tubes tilted to allow for any fluid to drain away from the pellet. The pellet was resuspended gently in 1 ml of storage media (SM, appendix.) using a wide bored sterile pipette equipped with a rubber bulb. The sides of the centrifuge tubes were thoroughly washed with SM to rinse off any phage particles that might be sticking on the sides of the tubes. An equal volume of chloroform was added to the phage suspension and vortexed for 30 seconds using the Stuart's autovortex mixer. The organic phase was separated from the aqueous phase by centrifugation at 1,600g for 15 minutes at 4°C. The aqueous phase which contains the bacteriophage was recovered.

The phage particles were repelleted by centrifugation at 18,000 rpm for 3 hours at 4°C using a Tomy centrifuge with rotor 4N. The supernatant was poured off and the "glassy" pellet dissolved in 0.5 ml of SM. The phage suspension was stored at 4°C.

2.2.4 Partial Characterisation of Phage

2.2.4.1 Phage Protein Profile by SDS - PAGE

Analysis of the proteins of the purified phages was performed by SDS - PAGE using the Laemmli's discontinuous system (1970). The phage proteins were separated on disc gels of the following composition:

Main (or separating) Gel - 10%

3M Tris - HCl, pH 8.0	3.75 ml
Acrylamide: NN-methylene bisacrylamide (30:0.8)	10.00 ml
2% (w/v) Ammonium persulphate	0.70 ml
Distilled water	15.25 ml
10% (w/v) Sodium Dodecyl Sulphate (SDS)	0.40 ml
TEMED	5.00 ul

Stacking Gel

0.47M Tris-HCl, pH 6.8	5.00 ml
Acrylamide: NN'-methylene bisacrylamide (30:0.8)	7.50 ml
2% (w/v) Ammonium persulphate	1.20 ml
Distilled water	25.70 ml
10% (w/v) SDS	0.40 ml
TEMED	20.00 ul

Polymerised gels were formed (10cm long separating gel plus 1.5cm stacking gel) in siliconised glass tubes.

The phage samples for the electrophoresis were solubilised in solubilising buffer (50mM Tris-HCl pH 7.0, 2% (w/v) SDS, 5% (v/v), 2-mercaptoethanol, 0.05mg bromophenol blue as tracking dye) by heating in boiling water for 5 minutes. The solubilised phage proteins (50 ul) and 10 ul of marker proteins were electrophoresed at a constant current of 3mA per tube until the tracking dye was a centimeter from the bottom of the gel.

The gels were stained overnight for proteins with Commassie blue (appendix). The gels were destained in three changes of destaining solution (appendix) and then placed on a light box for visualisation of polypeptide bands. The mobilities of the bands were measured and a representative electrophoretogram made. The relative molecular weights of the phage proteins were determined from a calibration curve of the marker proteins.

2.2.4.2 Electron Microscopy

A drop of the purified phage suspension was placed on coated copper grids from a finely drawn glass pipette. Excess phage solution was blotted away by placing a piece of filter paper by the edge of the copper grids. The grids were air dried slowly in a petri dish and stained with uranyl acetate stain at pH 7.2 by the drop method (Bishop et. al., 1974). Excess stain was blotted away by placing a piece of filter paper by the edge of the grids. The stained grids were immediately observed with a Hitachi H-600

transmission electron microscope at an acceleration voltage of 75 KV. The images were photographed at a magnification of 160,000 by the low dose method and the images documented.

2.2.4.3 Phage Relatedness by the Antibody Neutralization Assay

RAISING OF ANTISERA

Based on similar morphological features and the presence of two common polypeptide bands amongst the coliphages, Ec RBCL 26, was selected to raise antisera for the neutralisation assay (Mandel, 1985).

A purified sample of Ec RBCL 26 of known protein content was separated by running an analytical SDS polyacrylamide slab gel electrophoresis. The sample was electrophoresed for 2 hours at 150V. The polypeptide bands were electrophoretically transferred onto nitrocellulose paper by the method of Bittner et. al., (1980) as follows: the polyacrylamide gels, porous pads and nitrocellulose paper were rinsed with distilled water and equilibrated with the transfer buffer (25mM Tris, 192mM glycine, 20%(v/v) methanol pH 8.3) for 30 minutes. The separated proteins were transferred from the gels unto the nitrocellulose paper for 1 hour at 100V using BioRad's Mini trans-blot kit. A thin strip of the nitrocellulose paper was cut, washed in four changes of PBS-Tween buffer (pH 7.4) and stained overnight with Indian ink (see appendix) on a shaker bath for visualisation of the transferred bands. The location of two of the protein bands which were common to most of the phages were noted and the

corresponding positions on the unstained paper marked. The bands were cut and mashed in a mortar with saline to extract the protein. This was done in duplicate to obtain enough antigenic material for raising of antisera. The protein content of the bands was determined by the method of Folin and Lowry (Plummer, 1987).

The immunization protocol as described by Armah et. al., (1990) was used:

C57BL mice were obtained from the Noguchi Memorial Institute for Medical Research, University of Ghana, Legon. The mice (2 per group) were immunised intraperitoneally with 50 ug of antigenic material in 0.1 ml Freund's complete adjuvant. The mice were batched into four groups; mice in group A were injected with polypeptide band of molecular weight 9,600, mice in group B with a polypeptide band of molecular weight 10,700, those in group C with a purified sample of phage Ec RBCL 26 and group D mice with a saline control.

Booster immunization of 50 ug antigenic material in saline were administered intraperitoneally on days 21 and 34 after the first injection. Sera were collected from mice before each immunization step from the tail to test for the activity of antisera raised. Sera from the experimental animals were collected by cardiac puncture on day 37 after the first injection. The blood samples were collected and centrifuged in a Beckmann microfuge B and the sera collected into sterile tubes. The antipolypeptide and antiphage sera obtained were preserved with 0.1% (w/v) sodium azide and stored at -70°C for the neutralisation assays.

ANTIBODY NEUTRALIZATION ASSAYS:

The antisera were serially diluted in broth. 100 ul portions of each dilution were mixed with equal volumes of phage suspension diluted from the stock to contain ~ 2 x 10^5 particles /ml. Each mixture was thoroughly mixed and incubated at 37°C for 30 minutes in a water bath. 1 ml portions of an overnight culture of the indicator cells were added to the incubated mixture and assayed for phage infectivity by the plaque assay technique. From the plaque count, the appropriate dilution of antisera to neutralise phage infectivity was determined.

The antisera were diluted to the desired concentration, mixed with appropriate phage concentration (for an 'easy' plaque count) and incubated over a time range of 0 - 60 minutes. After the incubation period, 1 ml of indicator cells was added to each tube and phage infectivity assayed for by the plaque assay technique. The data obtained was used to plot the antibody neutralisation curves as described by the method of Mandel(1985). The coefficient of neutralisation was determined from the slope of the curves and the dilution factor.

2.2.5 **Sensitivity Test**

The Minimum Inhibitory Concentration(MIC) of the test antibiotics on the bacteria was determined by the agar dilution method. Agar plates (1% w/v Nutrient broth in 1.5% w/v Nutrient agar) containing a known amount of antibiotic (Tetracycline, Streptomycin or Benzylpenicillin) were prepared. An overnight

nutrient broth. A 100 ml aliquot of the diluted culture was then added to the agar plates and the cells spread evenly on the plate with a sterile glass spreader. The plates were incubated overnight at 37°C in an Eyela Soft incubator (SL1-600, Tokyo Rikakikai Co., Limited, Japan). For each experiment a control plate without antibiotic was also prepared.

Inhibition or dose response curves were plotted from the data obtained. The MIC's were taken as the lowest antibiotic concentration at which there is no visible growth of bacterial cells during the period of incubation.

2.2.6 Induction of Resistance

Antibiotic resistant bacteria strains for transduction studies were obtained by the method of "training" and "patching" (Rodrigues, 1991b). Antibiotics used for the induction studies were Tetracycline (Tet), Streptomycin (Str) and Benzylpenicillin (Ben). The resistant strains were depicted Tet^r, Str^r and Ben^r based on the particular antibiotic used.

"TRAINING"

An overnight culture was diluted and plated on solid medium containing an antibiotic concentration lower than the amount determined for the MIC's (Table 7) by the agar dilution method. Isolated colonies were then transferred and streaked on agar plates containing an increasing concentration of antibiotics (0 - 500 ug / ml).

"PATCHING"

An overnight bacteria culture was centrifuged at 5,000 rpm with a Denley BR401 refrigerated centrifuge (10 x 15 ml angle rotor) for 30 minutes. The supernatant was decanted and the bacteria pellet 'scoped' unto a plate containing a higher amount of antibiotic than the determined MIC's. After ten days of incubation at 37°C the cells were streaked on fresh antibiotic plates to obtain single colonies.

The resistant strains were selected based on their ability to grow and were kept on agar slopes containing the new antibiotic concentrations at which the bacteria grew.

2.2.7 **Transduction**

A modified procedure of the method of transduction described by Ohsumi, Vovis and Zinder (1974) was used.

Antibiotic - containing agar plates were prepared using Streptomycin and Benzylpenicillin based on information obtained from Table 8a (Bacteria which showed a high degree of drug tolerance and which could be sustained at the final MIC's were selected for transduction studies).

Dilutions of the purified phages were grown on an overnight culture of donor resistant indicator cells (Table 8b) by the plaque assay technique. The phages which showed plaqueing activity were harvested and titred. 100 ul of various dilutions of the phage stock were used to infect recipient sensitive bacteria strains and grown at antibiotic concentrations at which

the resistant strains grew by the plaque assay technique. The plates were incubated at 37°C for a week and scored for transductants. Two control experiments were performed, one assay had sensitive bacteria without phage and the other phage without bacteria.

The transduction frequencies were determined from the relation: the number of antibiotic resistant transductants obtained from a unit volume of phage lysate divided by the number of plaque forming units present in that volume.

CHAPTER 3

RESULTS

3.1 PRELIMINARY SEARCH FOR PHAGES

The plaque assay technique is dependent on the ability of viruses to multiply generally at a very rapid rate leading to bacterial lysis with the formation of lesions or plaques (Scaife and Goman, 1985). This local lesion response by bacteriophages on bacterial lawns was used as an indicator of phage infectivity. The number of such lesions is a reflection of the number of infective particles applied to the test bacteria.

As shown in Tables 2a and 2b, twenty-six samples were tested for phage activity. Suspected phage activity was scored as X , phage activity as + and a blank space indicates absence of phage activity (Tables 2a, 2b).

Of the twenty-six samples tested, ten of them plaqued on the indicator strains. They were two on Shigella dysenteriae, two on Salmonella typhi, one on Shigella flexneri (Table 2b) and five on E. coli. The isolated phages were given identification names and numbers for easy reference in subsequent work; such as Ec RBCL 26 (Table 3). The first two letters of the designation refer to the indicator strain, RBC refers to the code name of the investigators (R=Rodrigues, B=Biney, C=Cato), L to the location of the sample (Legon) and the numbers to the source of the samples. The ten phages were referred to as Sd RBCL 5, Sd RBCL 23, St RBCL 4, St RBCL 20, Sf RBCL 15, Ec RBCL 7, Ec RBCL 8, Ec RBCL 10, Ec RBCL 25 and Ec RBCL 26.

Tables 2a & 2b show results of the preliminary screening of the 26 suspected phage samples for their infectivity on the two groups of indicator bacteria. Samples which infected the indicator bacteria were replated to confirm their plaqueing activity.

Table 2a: Preliminary Search for Phages

SAMPLE	INDICATOR STRAIN *				
	Ps. aer.	S. aur.	S. typhi	Sh. dys.	Sh. flex.
1					
2			X		
3				X	
4			X +	X	
5		X	X	X +	
6				X	
7			X		
8			X	X	
9					
10					
11					
12				X	
13					
14					
15			X		
16			X	X	
17				X	
18			X	X	
19		X		X	
20		X	X +		
21		X		x	
22		X	X	X	
23		X	X	X +	
24					
25					
26					

FOOTNOTE:
* - Isolates from NMIMR, Legon
X - Suspected phage activity
\+ - Presence of " "
Blank space - Absence of phage activity

Table 2b: Preliminary Search for Phages

SAMPLE	INDICATOR STRAIN * *				
	Ps. aer.	Sh. flex.	Sal. Gp.D	Kleb. sp.	E. Coli
1					
2					
3					
4					X
5					X
6					X
7					X +
8	X				X +
9					
10					X +
11					
12					
13					X
14					
15		X +			
16	X				
17					
18	X				
19	X				
20					
21	X				
22					
23					X
24					
25					X +
26					X +

KEY:
** - Isolates from UGMS, Legon
X - Suspected phage activity
\+ - Presence of " "
Blank space - Absence of phage activity

NOTE: None of the suspected phage samples could plaque on Pro. sp., Kleb. sp. and Sal. Gp. D

Two distinctive plaque types were observed of the isolated phages on their indicator strains. These were very tiny plaques on *E. coli* and large plaques on the *Shigella* sp. and *Salmonella* sp. Fig. 6 shows representative photographs of these plaques.

The four indicator strains *Sh. dysenteriae*, *S. typhi* from the NMIMR group and *Sh. flexneri* and *E. coli* from the UGMS group were selected as indicators for all assays involving the plaque assay technique.

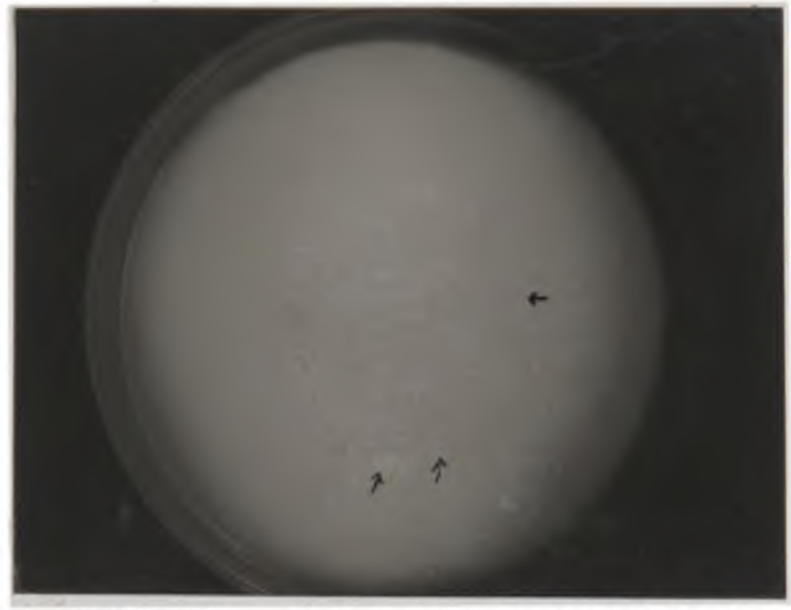
3.2 PHAGE TITRATION AND ASSAY

It has been shown that at high phage dilutions a single plaque is initiated by one infective particle and hence the total number of plaque forming units in the original suspension can be determined (Martins, 1978). This is the underlying principle of the phage titration assay. Information gained from the titration is useful in phage propagation and for preparing large phage material.

The phages were titred and the results presented in Table 3. The coliphages had the highest titre values, followed by Sf RBCL 15, Sd RBCL 5, Sd RBCL 23, St RBCL 4 and St RBCL 20.

Fig. 6 shows photographs of phage plaques on indicator bacterial lawn when phage activity was tested by the plaque assay technique.

Fig. 6: Photographs of Phages Plaqueing on Indicator Strains.



(a) Ec RBCL 10 - showing tiny Phage plaques (arrowed)

(b) Sd RBCL 5 - showing large Phage plaques (arrowed)

Table 3 shows results of titration of the isolated phages on the indicator strain by the plaque assay technique. The selected indicator strains were infected with serial dilutions of the isolated phages which showed plaqueing activity on them. The titre values were calculated from the plaque counts or plaque forming units (pfu) and dilution factor.

Table 3: Titration of Phages

DESIGNATION	TITRE VALUE pfu/ml
Sd RBCL 5	5.0×10^{9}
Sd RBCL 23	2.5×10^{9}
St RBCL 4	4.2×10^{8}
St RBCL 20	2.0×10^{7}
Sf RBCL 15	3.0×10^{11}
Ec RBCL 7	3.2×10^{13}
Ec RBCL 8	4.5×10^{12}
Ec RBCL 10	1.2×10^{15}
Ec RBCL 25	1.8×10^{13}
Ec RBCL 26	1.7×10^{12}

3.3 PARTIAL CHARACTERISATION OF PHAGE

3.3.1 Electron Microscopy

The morphological features of the isolated phages were studied by electron microscopy. Figs. 7 show electron micrographs of the ten isolated phages. Eight of the samples were found to be tailed and two tailless.

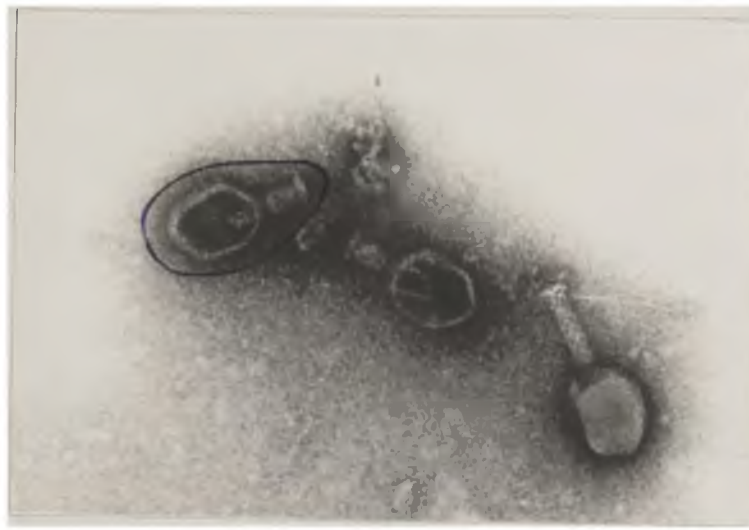
The tailed phages were Ec RBCL 7, Ec RBCL 8, Ec RBCL 10, Ec RBCL 25, Ec RBCL 26, Sf RBCL 15, Sd RBCL 5, St RBCL 20 and the tailless St RBCL 4 and Sd RBCL 23.

Sf RBCL 15* and Ec RBCL 25* show two distinctive morphological conformations, phage with an uncontracted sheath and a tailed phage with a contracted sheath (Figs. 7d, e, g, h). The electron micrograph of Ec RBCL 7 shows two distinctive morphological conformations, one with a contracted sheath (circled) and an uncontracted sheath (Fig. 7a). In addition to the contracted or uncontracted sheaths, the coliphages show base plates with spikes (Figs. 7a - f) whilst Sf RBCL 15 shows a flat base plate (Figs. 7g, h). Sd RBCL 5 shows base plate (Fig.7j).

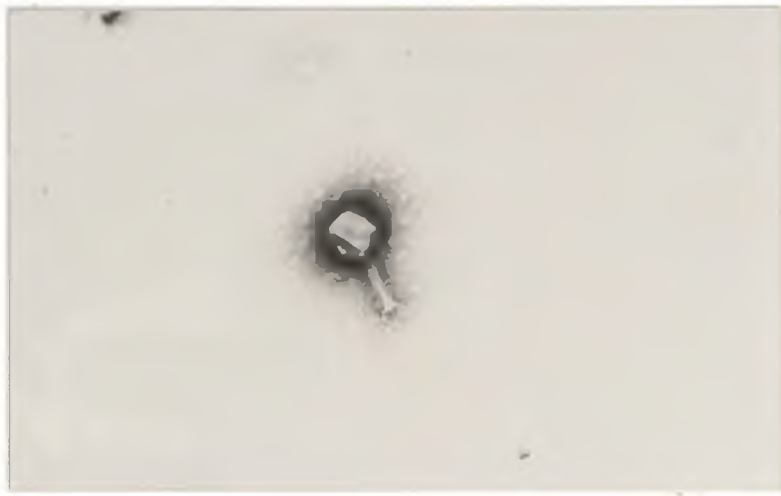
Fig. 7 Shows Electron micrographs of negatively stained phages.
(a) Ec RBCL 7* (b) Ec RBCL 8 (c) Ec RBCL 10 (d) Ec RBCL 25
(e) Ec RBCL 25* (f) Ec RBCL 26** (g) Sf RBCL 15 (h) Sf RBCL 15*
(i) St RBCL 20 (j) Sd RBCL 5 (k) St RBCL 4 (l) Sd RBCL 23

Purified suspensions of phages were negatively stained with uranyl acetate (2% w/v). The grids were observed on a Hitachi H - 600 transmission electron microscope at an acceleration voltage of 75KV and a magnification of 160,000.

Fig. 7: Electron Micrographs of the Isolated Phages (Magnification x160,000)

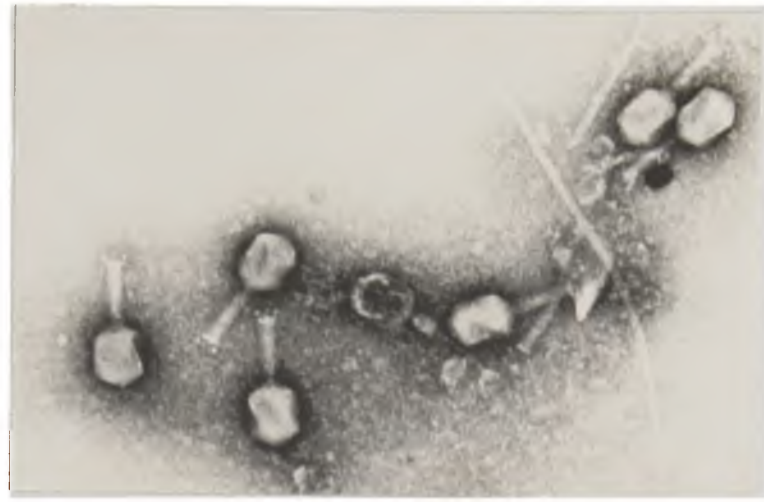


(a) Ec RBCL 7*

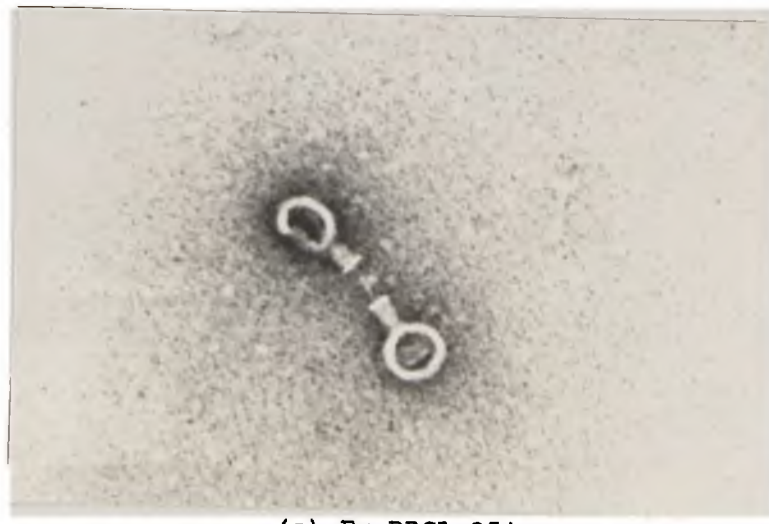


(b) Ec RBCL 8

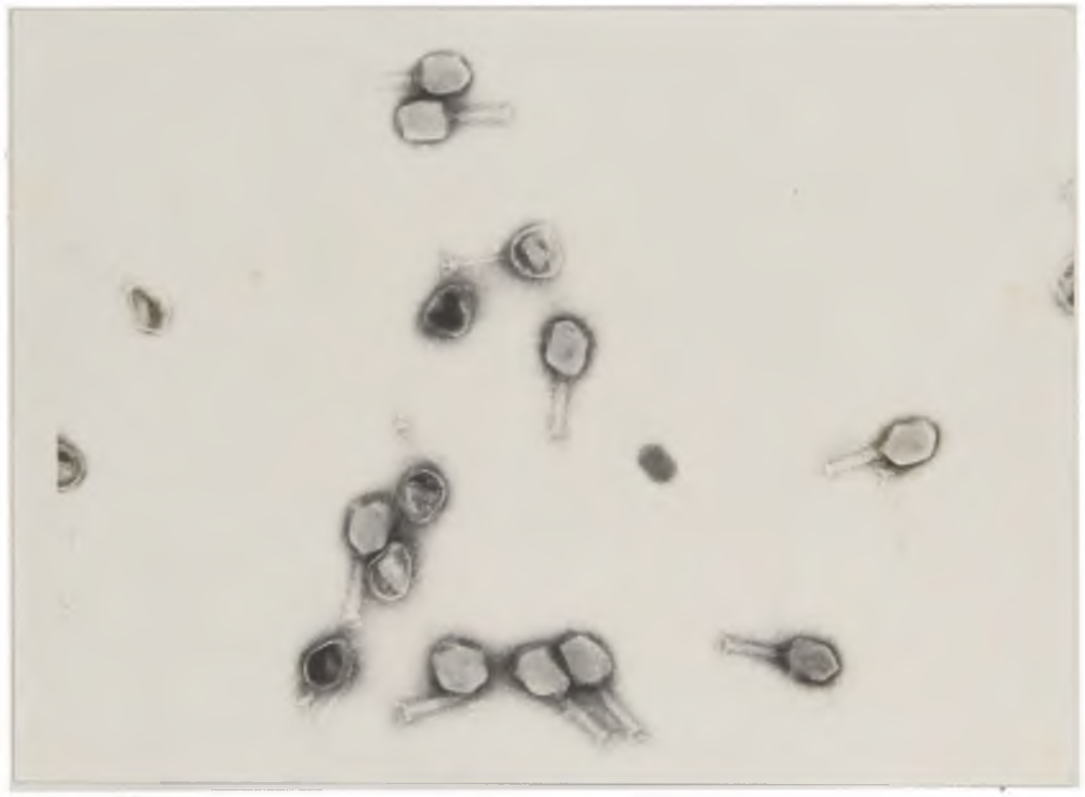
(c) Ec RBCL 10



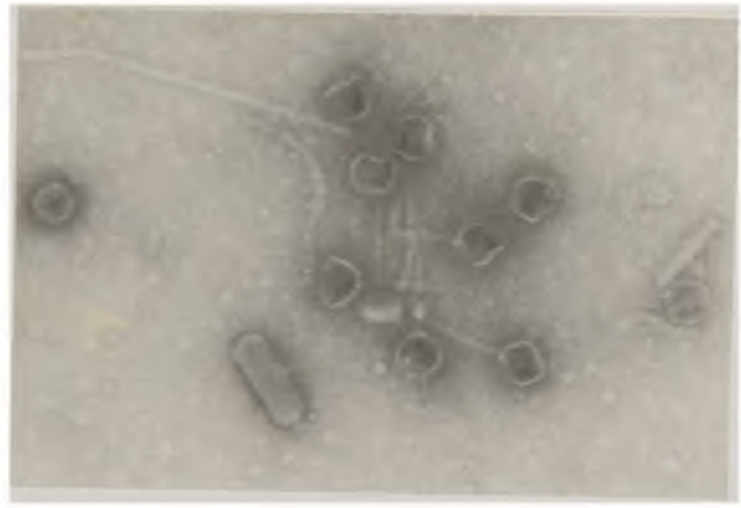
(d) Ec RBCL 25*



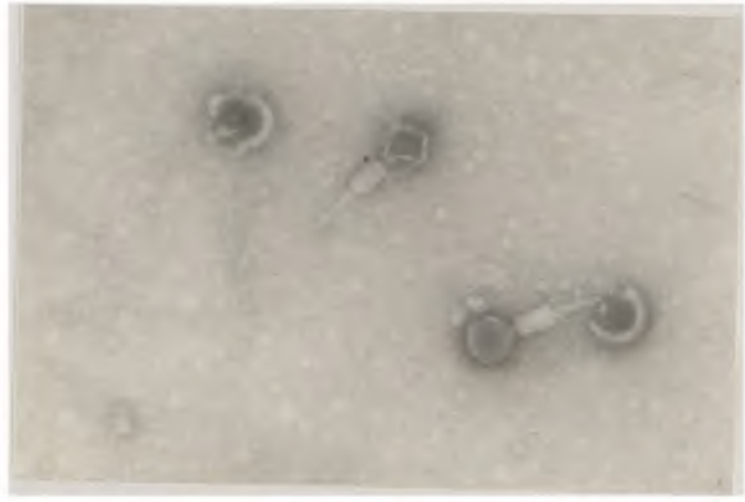
(e) Ec RBCL 25*



(f) Ec RBCL 26**

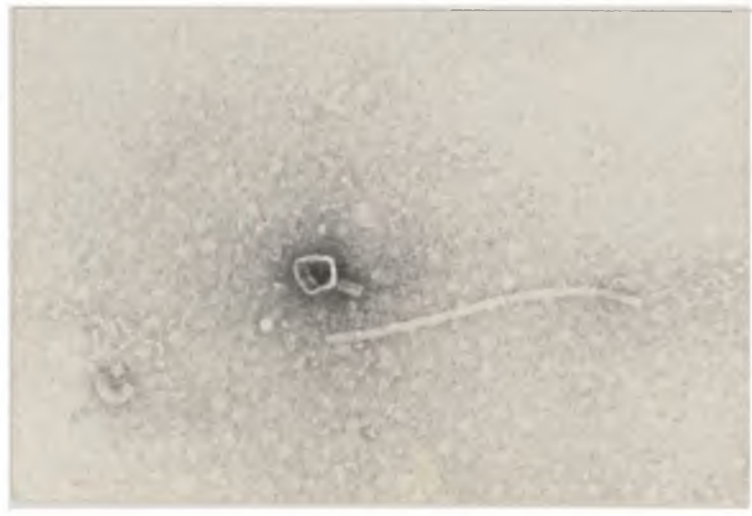


(g) Sf RBCL 15*

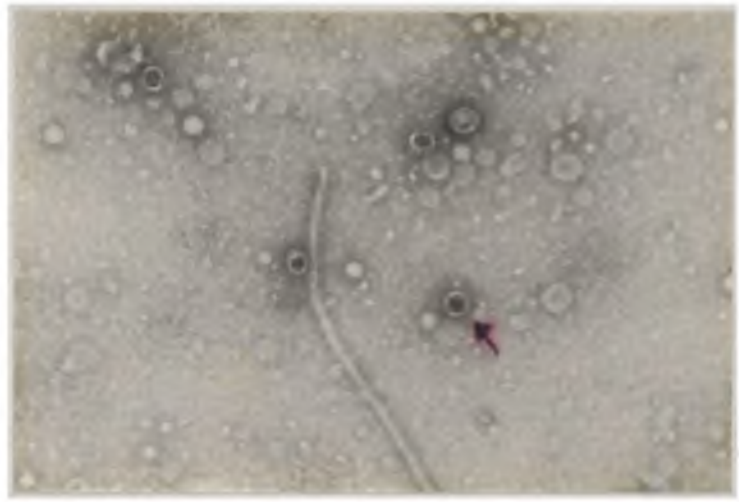


(h) Sf RBCL 15*

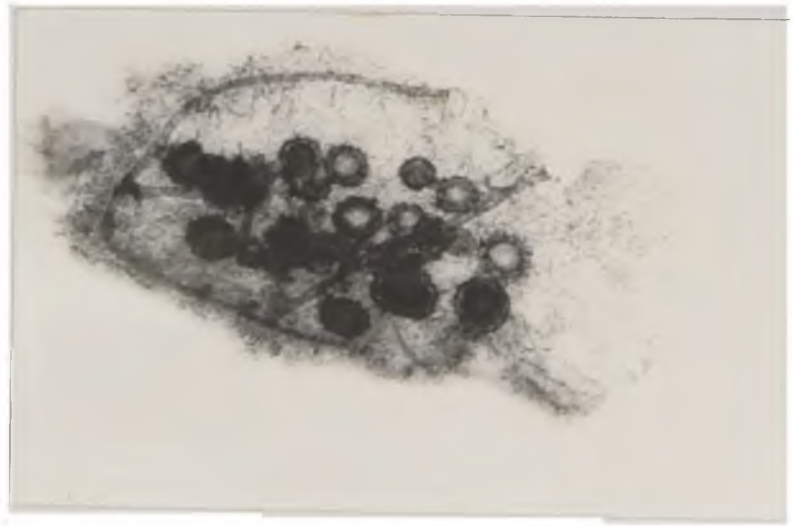
(i) St RBCL 20



(j) Sd RBCL 5



(k) St RBCL 4



(l) Sd RBCL 23

Table 4: Head and Tail Measurements from the Electron Micrographs in Fig. 7

PHAGE	HEAD (nm)	TAIL (nm)	
Ec RBCL 7	81	81	
Ec RBCL 8	56	63	
Ec RBCL 10	56	63	
Ec RBCL 25	56	63	
Ec RBCL 26	56	63	
Sf RBCL 15	44	88	
Sf RBCL 15*	44,50	88	
Sd RBCL 5	55	88	
Sd RBCL 23	44,56	-	
St RBCL 4	20	-	
St RBCL 20	38	31	
T4	63	63	from Hayes (1968)
T2	75	88	

*Sf RBCL 15 (Fig. 7h)

3.3.2 **Protein Profile**

Table 5a shows a representation of the range of molecular weights of proteins from the phages. The phages were found to have proteins of relative molecular weight ranging from 9,000 - 100,000.

Marker proteins were run under the same electrophoretic conditions with the phage proteins. From the data obtained (Fig.

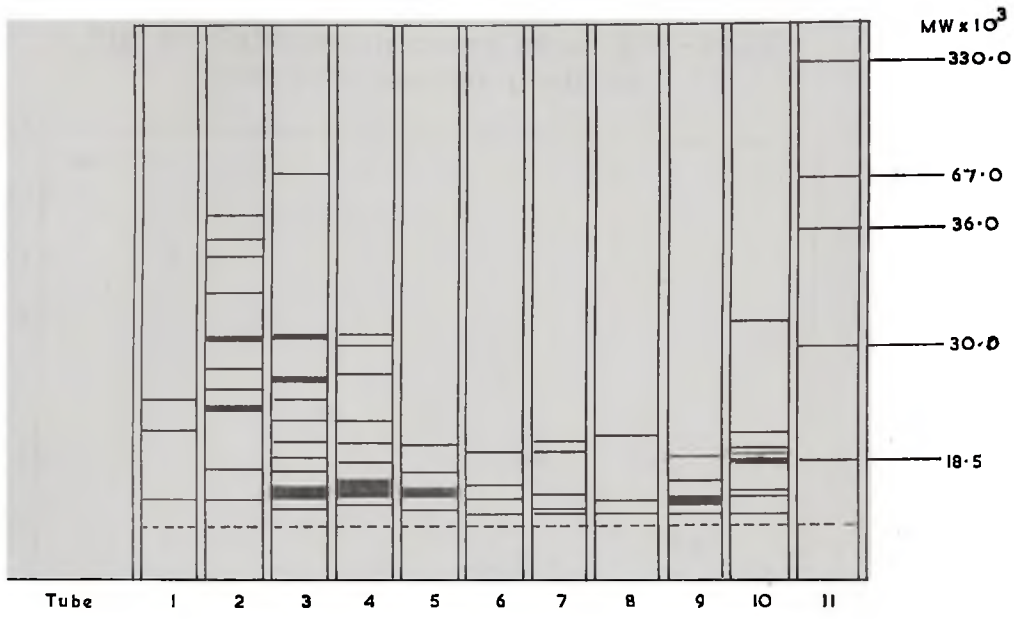
8) a calibration curve was plotted. The relative molecular weights of the phage proteins were determined from the curve. The range of molecular weights for each phage was recorded in Table 5a. Significant bands common to the phages were also determined from Figs. 8 & 9 and tabulated (Table 5b).

Sd RBCL 23 and St RBCL 4 {tailless phage Figs. 7(k & l) and Fig. 8} had a wider range of protein bands of different molecular weights than the tailed phages (Fig. 8). The tailed phages had protein bands with low molecular weights. A protein of molecular weight (Mol. wt.) 9,600 was found to be common to all the five coliphages whilst 10,700 was common to all but Ec RBCL 8.

Proteins of molecular weight 15,600, 17,900, 31,500, were found to be common to St RBCL 4 and St RBCL 20. The tailless phages St RBCL 4 and St RBCL 23 had two common protein bands of molecular weight 10,600 and 31,500.

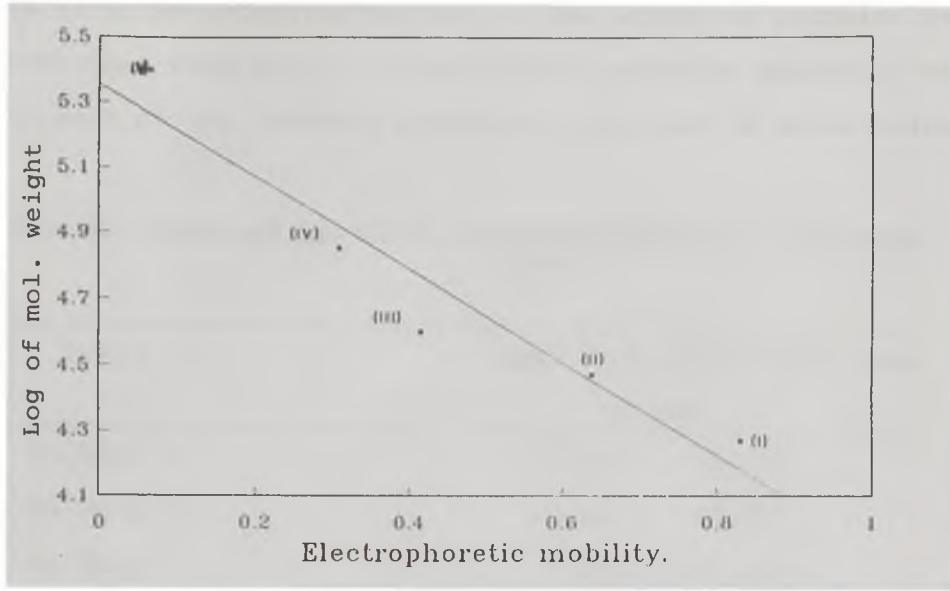
No protein band was found to be common to all the ten isolated phages, though four of the coliphages had two common protein bands of molecular weight 9,600 and 10,700. The presence of these two protein bands in the coliphages coupled with the high protein content and the distinctive morphological features of Ec RBCL 26**(Fig. 7f) forms the basis for its selection as the antigenic material used in raising antiphage sera for the antibody neutralisation assay (Table 5b).

Fig. 8 SDS-PAGE electrophorotogram of purified phage proteins run with marker proteins on 10 % acrylamide gel



TUBE	PHAGE	HOST
1	Sd RBCL 5	Shigella dysenteriae
2	Sd RBCL 23	Shigella dysenteriae
3	St RBCL 4	Salmonella typhi
4	St RBCL 20	Salmonella typhi
5	Sf RBCL 15	Shigella flexneri
6	Ec RBCL 7	Escherichia coli
7	Ec RBCL 8	Escherichia coli
8	Ec RBCL 10	Escherichia coli
9	Ec RBCL 25	Escherichia coli
10	Ec RBCL 26	Escherichia coli
11	MOLECULAR WEIGHT MARKER PROTEINS	

Fig 9: Calibration curve of an SDS-PAGE run with marker proteins.



	Marker Protein	Subunit Mol. Weight
I	- Ferritin	18,500
III	- Lactate Dehydrogenase	36,000
IV	- Catalase	67,000
V	- Thyroglobulin	330,000
		Approx. Mol. Weight
II	- Carbonic Anhydrase	30,000

The protein profile of the phages was determined by SDS-PAGE. From the relative mobilities as determined from Fig. 8, the relative molecular weights of the separated proteins were determined from Fig. 9. The range of relative molecular weight for each of the isolated phages was recorded as shown below.

Table 5a: Range of Relative Molecular Weights of Isolated Phages

PHAGE	RANGE OF RELATIVE MOLECULAR WEIGHT
Sd RBCL 5	10,600 - 20,600
Sd RBCL 23	10,600 - 68,500
St RBCL 4	9,800 - 100,000
St RBCL 20	10,200 - 31,500
Sf RBCL 15	9,900 - 15,000
Ec RBCL 7	9,600 - 14,500
Ec RBCL 8	9,600 - 15,000
Ec RBCL 10	9,600 - 16,200
Ec RBCL 25	9,600 - 13,900
Ec RBCL 26	9,600 - 34,400

The common polypeptide bands were determined from Figs. 8 & 9 for each phage and recorded as shown below.

Table 5b: Common Polypeptide Bands.

PHAGE	Relative Molecular weight of common Polypeptide Bands (x 1000)									
	9.6	9.9	10.6	10.7	11.0	14.5	15.6	17.9	20.0	31.5
Sd RBCL 5			+						+	
Sd RBCL 23			+							+
St RBCL 4			+				+	+	+	+
St RBCL 20							+	+		+
Sf RBCL 15		+			+					
Ec RBCL 7	+			+						
Ec RBCL 8	+	+			+	+	+			
Ec RBCL 10	+			+						
Ec RBCL 25	+	+		+						
Ec RBCL 26	+			+	+	+				

3.3.3 Antibody Neutralisation Assay

The coefficient of neutralisation (K) was determined by the method of Mandel, 1985 using the following relationship:

$$Pt = Poe^{-Kt/D} \quad \text{(Mandel, 1985)}$$

where Po = The original phage concentration in pfu/ml.

Pt = The final phage concentration after t minutes of incubation with antisera (pfu/ml)

t = period of incubation in minutes

K = Coefficient of neutralisation ($K\ min^{-1}$)

D = Dilution factor for the antiserum.

The above relationship can be reduced to $\log \frac{Pt}{Po} = \frac{-Kt}{2.3D}$

From the equation, a plot of log Pt/Po versus time can be plotted (Figs. 10a - c). The K value can be determined from the slope of the curve and the amount of dilution of the antiserum used.

For comparison between heterologous and homologous (or test) phages, the K'/K ratio can be calculated where

K' = the neutralisation constant for the heterologous phage

K = " " " " " homologous phage

at the same antiserum dilution. With the homologous phage this will be 1 (K'/K = 1), a ratio of less than 1 is usually obtained for related phages and zero for unrelated phages. It has been suggested that a value of 0.25 or less indicates a difference in the antigenic structures of the two phages (Bradley, 1971).

The K values were determined for each phage with a particular antiserum as described under methods. The protein content of the antigenic material were as follows:

Ec RBCL 26 - 875.0 ug/ml

Polypeptide band A (mol. wt. 9,600) - 200.5 ug/ml

" B (mol. wt. 10,700) - 212.5 ug/ml

The results are presented in Table 6a.

The K (min^{-1}) values for the homologous phage, Ec RBCL 26, were 11.27, 15.87 and 623.30 for A, B and C respectively. The corresponding values were determined for the other nine phages. The K'/K ratios were also determined (Table 6b). It can be inferred from Table 6b that seven phages showed some detectable cross reactivity to anti Ec RBCL 26. But Sd RBCL 5, Sd RBCL 23 and Sf RBCL 15 gave a K'/K ratio of less than 0.25 (Table 6b). This indicates a possibility of difference in their antigenic

structures to Ec RBCL 26.

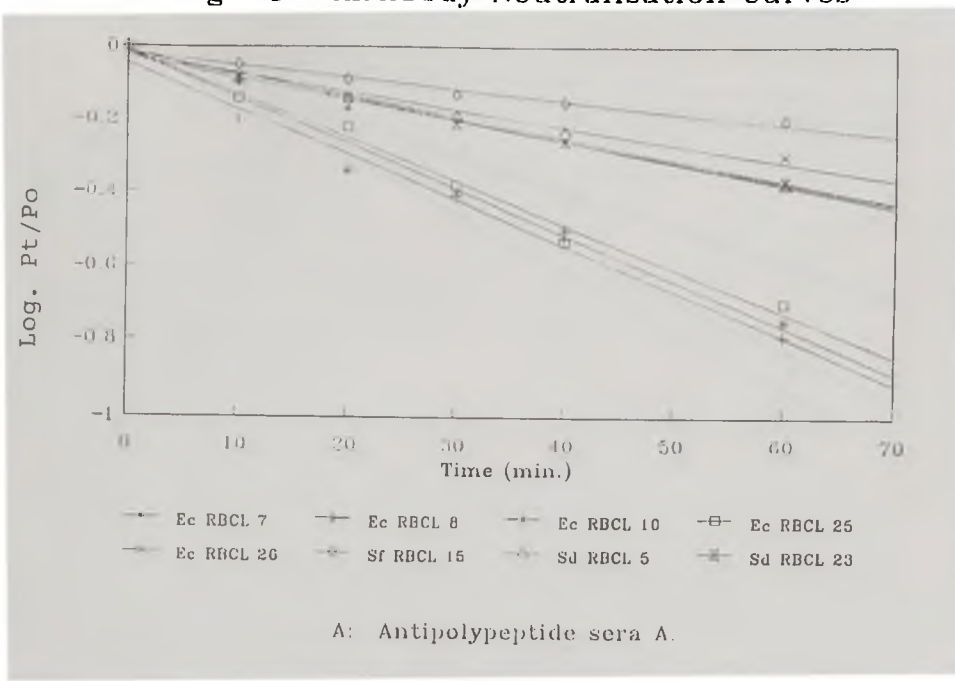
Comparably the coliphages are closely related to Ec RBCL 26. From Table 6b, the order of relatedness in decreasing magnitude is Ec RBCL 10, Ec RBCL 8, Ec RBCL 25 and Ec RBCL 7; followed by Sd RBCL 5, Sd RBCL 23 and Sf RBCL 15.

Antipolypeptide sera A and B seem not to play a very significant role in phage infectivity. Sd RBCL 23 and Ec RBCL 10 show an exceptionally high K'/K ratios (2.28 and 2.68) for antipolypeptide B respectively. These may play some role in their antigenic structures and hence their infectivity. Sf RBCL 15 shows a comparably low K'/K ratios for antipolypeptide sera A and B (0.67 and 0.57 respectively). This may probably account for the low degree of relatedness to Ec RBCL 26. Sf RBCL 15 though a tailed phage seems to be the least related to Ec RBCL 26 phage.

St RBCL 4 and St RBCL 20 may not be related to the test phage as deduced from Table 6a (K'/K = 0.00).

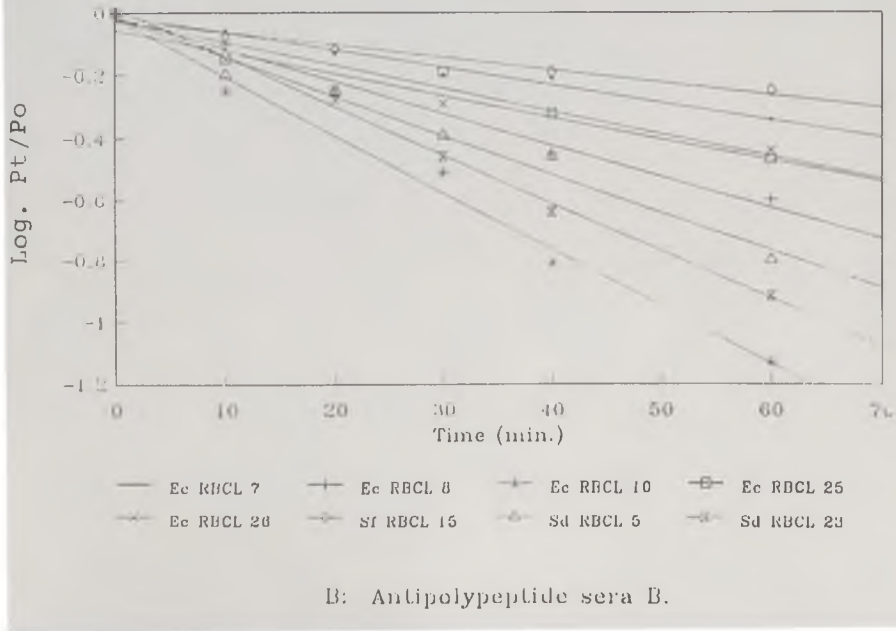
Figs. 10a - c show antibody neutralisation curves for Antipolypeptide sera A and B, and Anti Ec RBCL 26.

Fig. 10a. Antibody Neutralisation curves



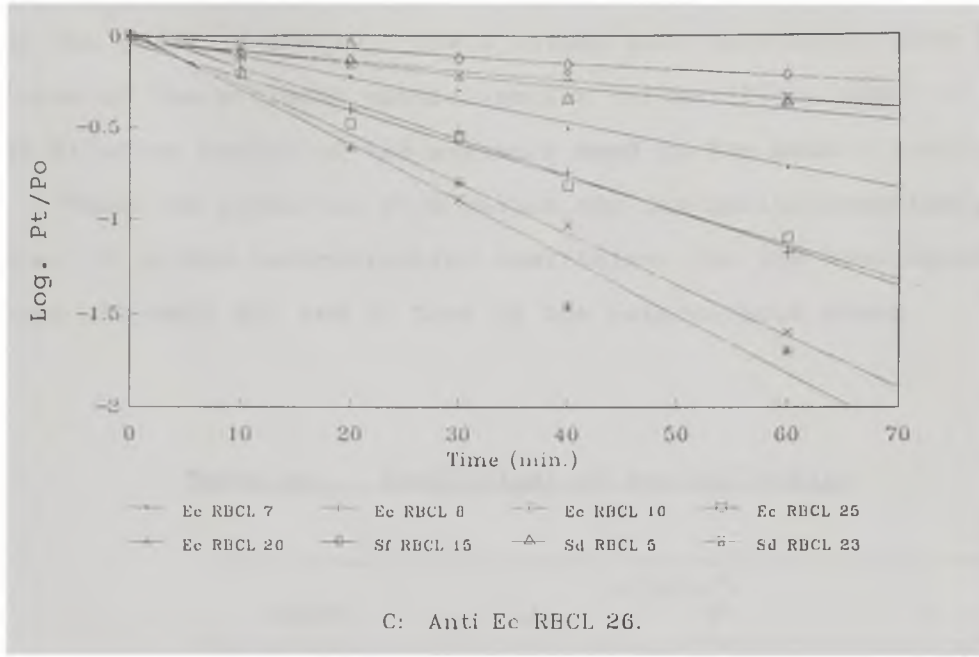
A: Antipolypeptide sera A.

Fig. 10b. Antibody neutralisation curves



B: Antipolypeptide sera B.

Fig. 10c. Antibody Neutralisation curves



C: Anti Ec RBCL 26.

Table 6a shows the coefficient of neutralisation (K) values for the isolated phages. The K values were calculated from the slopes of the antibody neutralisation curves (Figs. 10a - c) and the dilution factor of the antisera used in the neutralisation.

Table 6b shows the K′/K values for the antipolypeptide/phage sera. K is the neutralisation coefficient for the homologous phage (Ec RBCL 26) and K′ that of the heterologous phage.

Table 6a: Coefficient of Neutralisation

PHAGE	K (min^{-1}) A	B	C
Sd RBCL 5	13.80	28.75	147.20
Sd RBCL 23	14.03	36.11	119.60
Sf RBCL 15	7.59	8.97	82.88
Ec RBCL 7	13.34	12.65	280.60
Ec RBCL 8	28.52	23.46	455.40
Ec RBCL 10	28.75	42.55	703.80
Ec RBCL 25	27.37	16.79	423.20
Ec RBCL 26	11.27	15.87	623.30
St RBCL 4	0.00	0.00	0.00
St RBCL 20	0.00	0.00	0.00

FOOTNOTE:

A - Antipolypeptide Sera A (mol. wt 9,600 protein antigen used)

B - " " B (" " 10,700 protein antigen used)

C - Anti Ec RBCL 26

Table 6b. THE K'/K VALUES FOR ANTIPOLYPEPTIDE/PHAGE SERA OF Ec RBCL 26

PHAGE	A	B	C
Sd RBCL 5	1.22	1.81	0.24
Sd RBCL 23	1.24	2.28	0.19
Sf RBCL 15	0.67	0.57	0.13
Ec RBCL 7	1.18	0.80	0.45
Ec RBCL 8	2.53	1.48	0.73
Ec RBCL 10	2.55	2.68	1.13
Ec RBCL 25	2.43	1.06	0.68
Ec RBCL 26	1.00	1.00	1.00
St RBCL 4	0.00	0.00	0.00
St RBCL 20	0.00	0.00	0.00

K' = neutralisation constant for the heterologous phage
K = " " " " homologous "

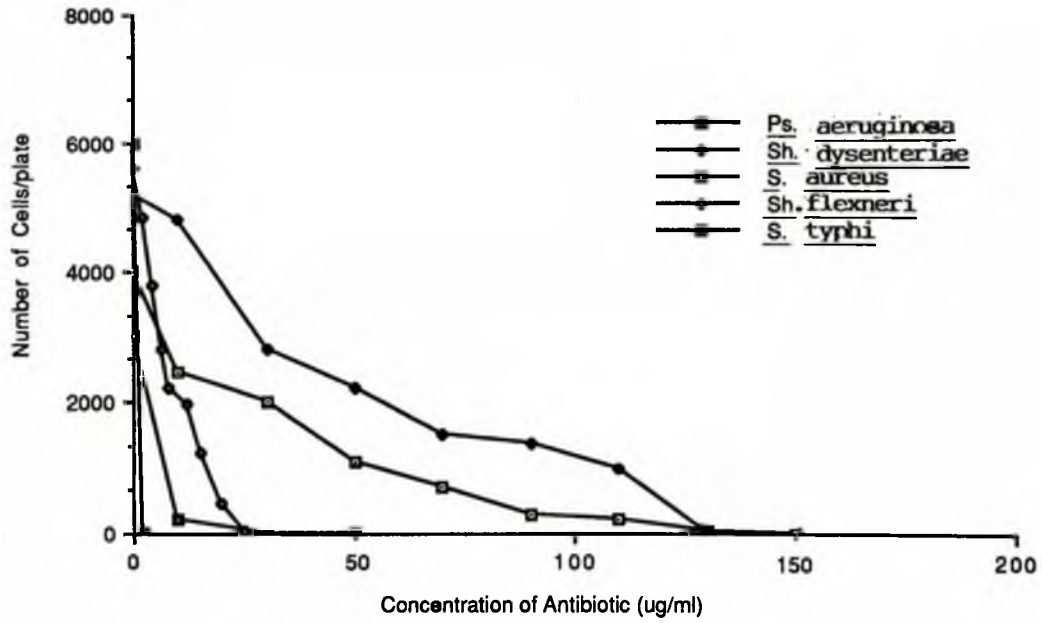
3.4 SENSITIVITY TEST

The selected pathogenic bacteria were screened for the presence of the antibiotic resistance gene marker by the agar dilution method (see methods). Unlike the disc sensitivity tests this method gives a more quantitative reflection of the response of the bacteria to the marker antibiotics. The dose response curves (Figs. 11-13) obtained show a decrease in bacterial cell counts with increasing drug concentration - which reflected the expected trend for an inhibition assay. The MIC's were taken as the lowest antibiotic concentration at which no bacteria growth occurred. These were determined from the curves and recorded as shown in Table 7.

All the bacteria showed a high tolerance to the antibiotics except Sal.Gp D. which was relatively more sensitive to all the

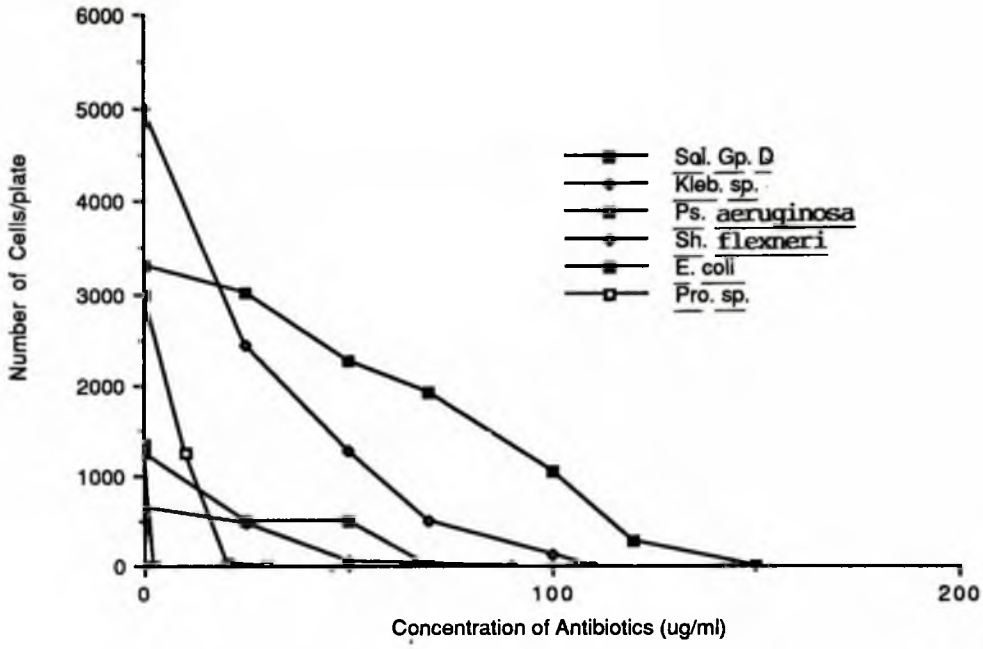
three antibiotics (2 ug/ml, 10 ug/ml and 5 ug/ml for Tetracycline{Tet}, Benzylpenicillin{Ben} and Streptomycin{Str} respectively). S. aureus was sensitive to Ben (4 ug/ml) and S. typhi to tet (2 ug/ml). On the average the bacteria from the NMIMR group (refer to materials) were more tolerant to Benzylpenicillin and Streptomycin than the UGMS group. The UGMS group was more tolerant to Tetracycline than the NMIMR group. Irrespective of the source, Ps. aeruginosa was found to be highly resistant to the marker antibiotics. Ps. aeruginosa and Sh. flexneri from the NMIMR group were more tolerant to the marker antibiotic than the UGMS group. Figs. 11-13 show dose response curves for the selected pathogenic bacteria tested for their antibiotic sensitivity by the agar dilution method.

Fig. 11a: Dose Response Curve of Isolates to Tetracycline (#)



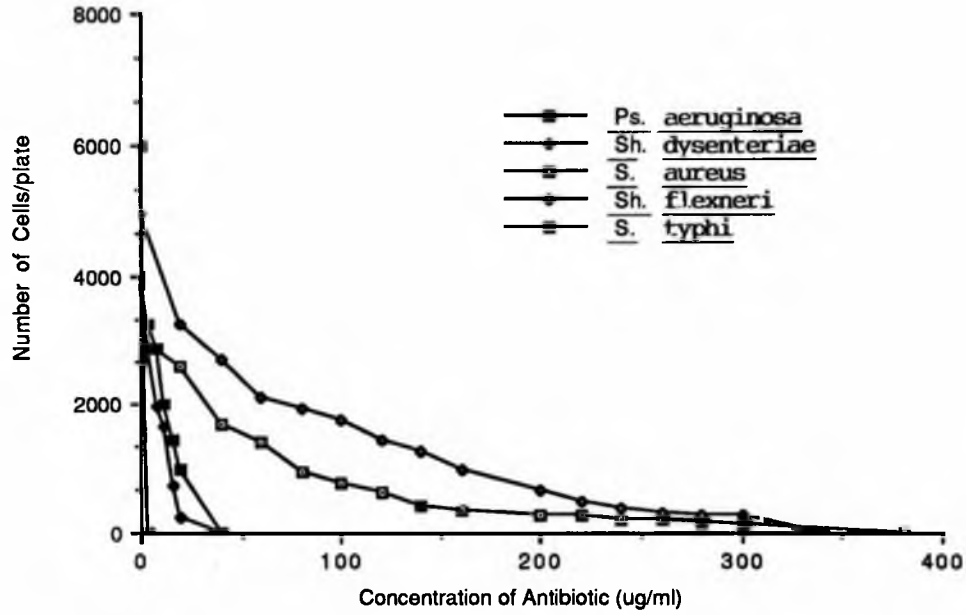
 # Isolates from the Bacteriology Unit, NMIMR

Fig. 11b: Dose Response Curve of Isolates to Tetracycline (#)



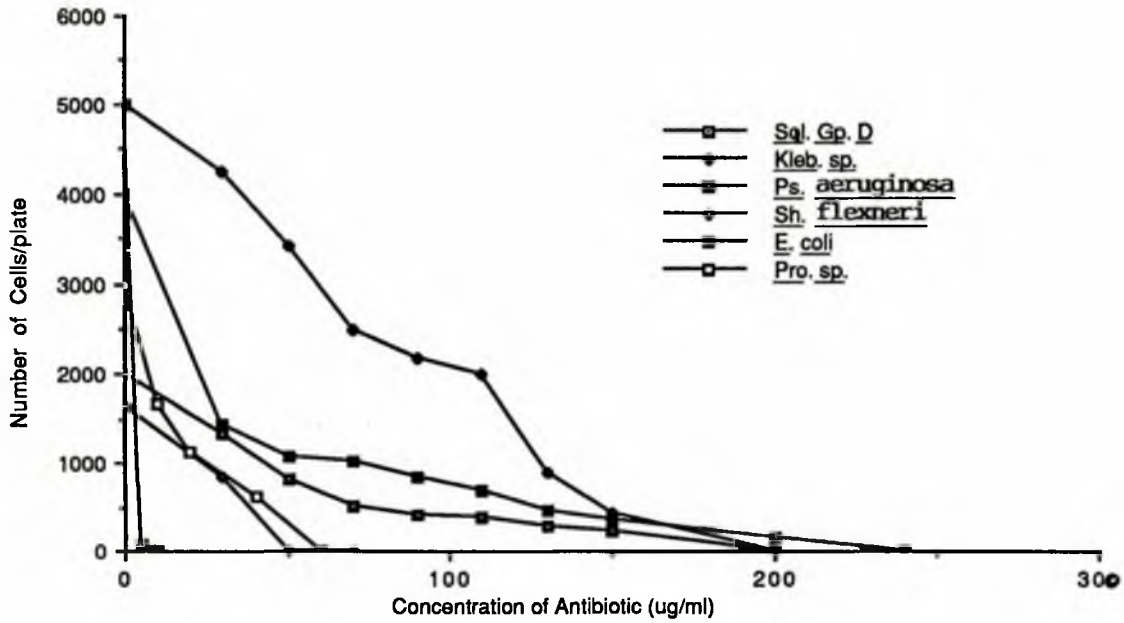
 # Isolates from the Microbiology Department, UGMS

Fig.12a: Dose Response Curve of Isolates to Benzylpenicillin (#)



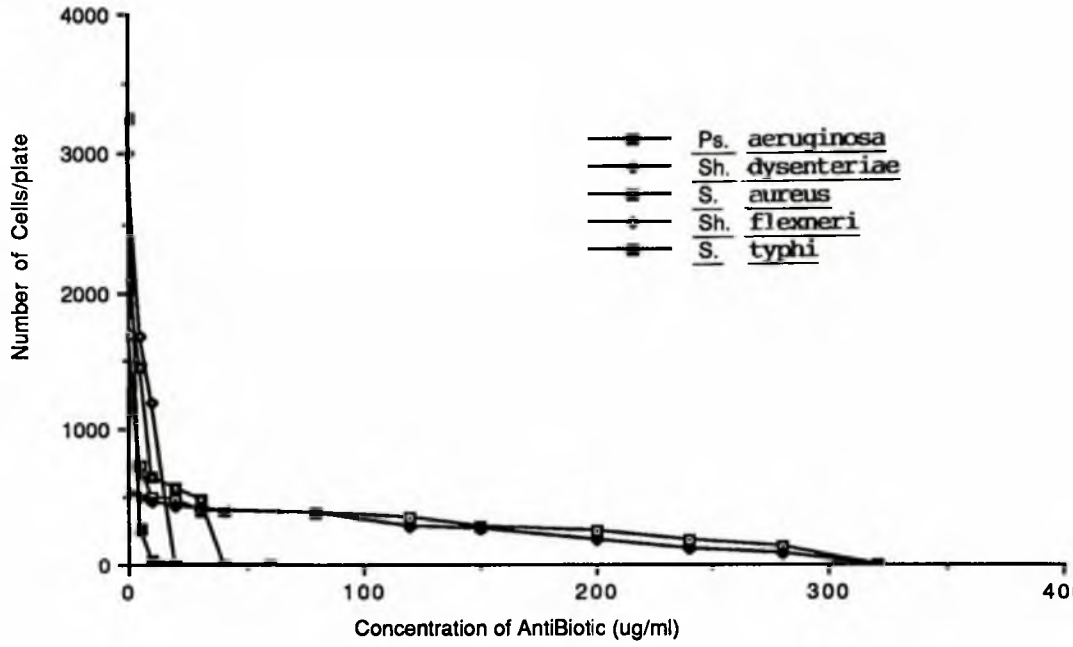
 # Isolates from the Bacteriology Unit, NMIMR

Fig. 4b: Dose Response Curve of Isolates to Benzylpenicillin (#)



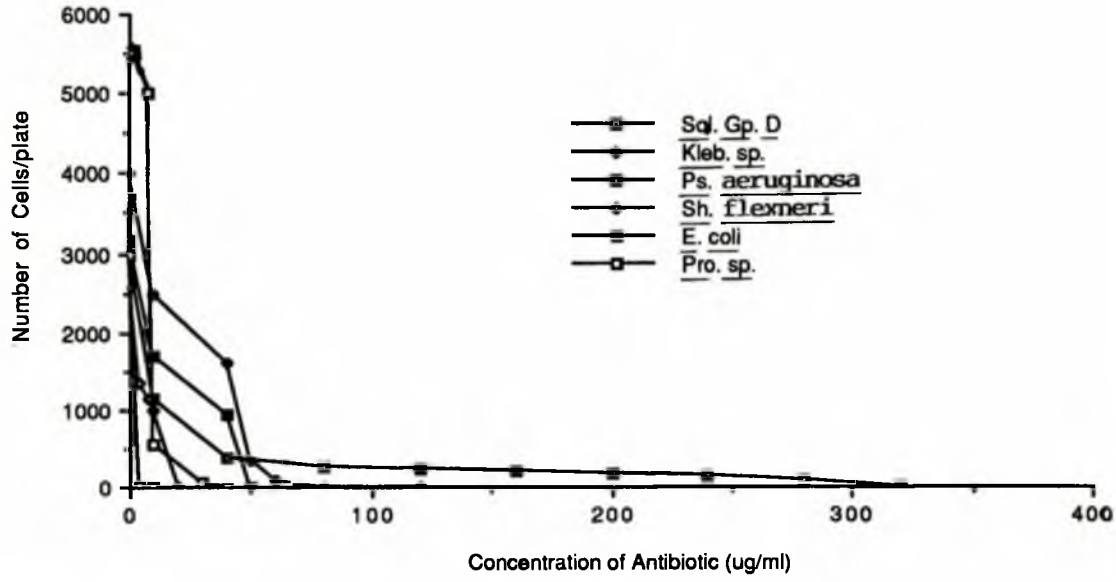
 # Isolates from the Microbiology Department, UGMS

Fig 13a: Dose Response Curve of Isolates to Streptomycin (#)



 # Isolates from the Bacteriology Unit, NMIMR.

Fig.13b: Dose Response Curve of Isolates to Streptomycin(#)



 # Isolates from the Microbiology Department, UGMS

The indicator strains were tested on the antibiotics and their response plotted as in Figs. 11-13. The minimum inhibitory concentration (MICs) were determined as the minimum antibiotic concentration at which the bacteria do not grow. The table below shows the MICs obtained.

Table 7: MIC's (ug/ml)

INDICATOR STRAIN	TETRACYCLINE	BENZYLPENICILLIN	STREPTOMYCIN	
Ps. aeruginosa	135	370	320	
Sh. dysenteriae	130	32	320	
S. aureus	34	4	40	*
Sh. flexneri	30	375	20	
S. typhi	2	34	20	
Sal. Gp.D	2	10	5	
Kleb. sp.	80	172	95	
Sh. flexneri	110	50	20	**
E. coli	142	240	50	
Pro. sp.	22	60	9	
Ps. aeruginosa	68	198	320	

* Isolates from the Bacteriology Unit, NMIMR, Legon
** Isolates from the Microbiology Department, UGMS, Legon.

3.5 INDUCTION OF RESISTANCE

The results obtained for the induction of resistance are shown in Tables 8a, b. The bacteria tolerated a higher percentage increase of Streptomycin and Benzylpenicillin (Table 8a, b). Mutant strains, Str^r and Ben^r were therefore selected for transduction studies. S. typhi grew at a higher concentration of 170 ug/ml from the initial MIC of 2 ug/ml on Tetracycline but the bacteria could not be sustained at this new concentration when restreaked. The indicator strains could be induced to a high degree of tolerance for the test antibiotics.

Table 8a shows Induction of resistance in the indicator strains when grown on Tetracycline, Benzylpenicillin and Streptomycin. From the induction studies, resistant strains were selected for transduction studies based on their colonial appearance as shown in Table 8b.

Table 8a: Training/Patching

INDICATOR Strain	TETRACYCLINE Initial MIC	TETRACYCLINE Final MIC	BENZYLPENICILLIN Initial MIC	BENZYLPENICILLIN Final MIC	STREPTOMYCIN Initial MIC	STREPTOMYCIN Final MIC
			(ug/ml)			
Sh. flexneri	110	130	50	500	20	240
E. coli.	142	220	240	500	50	240
Sh. dysenteriae	130	170	32	240	320	500
S. typhi	2	170	34	240	20	240

Table 8b: Selected strains for transduction studies.

Mutant Strain	Benzylpenicillin	Streptomycin
	Source of Mutant	
Sh. flexneri	Patching	Training
E. coli.	Patching	Training
Sh. dysenteriae	Training	Patching
S. typhi	Training	Training

3.6 TRANSDUCTION

The phages were screened for their ability to transduce the resistance markers Str^r and Ben^r All but St RBCL 4 and St RBCL 20 were capable of the transfer of the Str^r marker with a high frequency whilst nine phages excluding St RBCL 20 were capable of transducing Ben^r with a high frequency. On the average, the frequency of transfer of the marker genes were highest for the coliphages, followed by Sf RBCL 15, Sd RBCL 5 and Sd RBCL 23. St RBCL 4 showed a higher magnitude of transfer frequency for Ben^r than Sd RBCL 23. The markers were presumed to be chromosomal genetic markers and the phages were presumed to be capable of generalised transduction.

The average transduction frequencies of the two tested markers were approximately 0.96×10^{-4} transductants /pfu for Str^r and approximately 0.98×10^{-4} transductants /pfu for Ben^r.

Table 9 shows results of transduction frequencies when the antibiotic resistance gene markers were transferred to sensitive indicator strains using the isolated phages as delivery systems. The transduction frequencies were calculated from the relation: number of transductants obtained from a unit volume of phage lysate divided by the number of plaque forming units present in that volume (Wall and Harriman, 1974).

Table 9: Transduction Frequencies when antibiotic resistance gene markers were transferred to sensitive indicator strains

X 10^{-4} Transductants / pfu

PHAGE	MARKER Str^r	Ben^r
Sd RBCL 5	1.00	0.47
Sd RBCL 23	0.02	0.06
Sf RBCL 15	0.75	0.83
Ec RBCL 7	0.77	1.25
Ec RBCL 8	1.13	1.00
Ec RBCL 10	2.04	1.51
Ec RBCL 25	1.03	2.11
Ec RBCL 26	0.96	1.51
St RBCL 4	-	0.08

Str^r - Resistance marker gene for Streptomycin.

Ben^r - Resistance marker gene for Benzylpenicillin.

CHAPTER FOUR

DISCUSSION AND CONCLUSION

Phages have proven to be an admirable model for investigating the mechanisms involved in phage infection and as delivery systems for markers such as the antibiotic resistance genes. This was the basic system used in studying the transfer of the resistance marker genes induced in the selected indicator strains (Tables 8a & b). The plaque assay technique which is a common assay system for phage infectivity and many other diagnostic tests for genetic markers was used. The basic unit of this assay is the viable plaque count. The size and appearance of such a lesion resulting from an infection referred to as a plaque can give an important information about the virus responsible for it. The number of such lesions gives a reflection on the number of infective particles applied to the test object (Scaife and Goman, 1985).

Since virulent phages invariably destroy the bacteria they infect, the kind of environment best suited for their survival is one which is continually replenished with new and sensitive bacteria. Phages which are virulent for the intestinal bacteria have been found to be prevalent in nature and the prime source of isolation is the sewage system (Duerden *et. al.*, 1987). Samples were therefore screened from sewage sources and quite true to prediction ten phages were isolated. Apart from the source of bacterial host, plaque appearance is also influenced by the rate of growth of the bacterial lawns, the concentration of agar, the

freshness of the plates and the humidity of the incubator (Maniatis, Fritsch and Sambrook, 1989).

Phage plaques give much more information than the mere circumscribed zones of clearing seen on a lawn of bacterial growth. They show reproducible individualities of structure which are used to identify phage strains as well as different mutants of the same strain. From the plaques, two distinctive phage types were shown by the coliphages, shigella and salmonella phage And as shown in Fig. 6, small or tiny and large plaque types were observed. There were small plaques for the coliphages and relatively larger plaques for the shigella and salmonella phage • Hayes (1968) gave two reasons for the distinctive plaque sizes. Plaque sizes may vary due to mutation leading to the formation of plaque mutants. For example, plaques of phages T1, T3 and T7 are reported to be strikingly larger than those of the T-even phages. From the plaque sizes obtained and Hayes's assertion, the coliphages identified in this study may be T-even phages while the larger plaques may be due to the T-odd phages or some morphologically distinct phage types. Phages may also differ in their plaque morphology that is, their edges may be clear or fuzzy, abrupt or shelving, and may be circumscribed by halos of partial clearing or turbidity. All the plaques obtained had clear edges. Though these individualities reflect physiological differences in the phage-bacterium relationship which are usually determined by the phage genome and are phage specific, such indicators should be interpreted with caution because the plaque

size and morphology can change drastically when a particular phage is grown on different susceptible hosts or by mutation and even on the same host under different cultural conditions.

As already stated, out of the twenty-six samples screened only ten plaqued on the four indicator bacteria - E. coli, S. typhi, Sh. dysenteriae and Sh. flexneri. Of the ten phage isolates, five plaqued on E. coli, two on Sh. dysenteriae, two on S. typhi and a phage on Sh. flexneri. The phages did show a narrow host range. This goes to confirm the expectation that phage infection is limited to a single bacterial species and often to a few strains of that species. An interesting exception to this feature of narrow host range is the phage PRRI which is said to be able to infect any gram negative bacterium that carries the plasmid RP4 (Freifelder, 1983). A lot of factors contribute to the narrow host range specificity. The most prominent factors are the specificity of receptor site recognition, fidelity of the host restriction - modification system, a conducive environment for adsorption specific to each phage and the inability of the host cell to provide efficient complementary phage genes. For example Ca^{2+} and Mg^{2+} ions are usually added to enhance ionic interactions between phage and host. This provides a conducive environment for phage adsorption among certain phages like the T4 phages. Another limiting factor to the number of phage isolates obtained is the assay system used which was specific for lytic phages. Though some of the phage sample showed turbid plaques(an indicator of lysogenic phages) on

the indicator strains(eg. S. aureus) the phages could not be isolated due to the absence of an induction system to induce lysis.

The isolated phages were characterised as delivery system for transfer of the resistance markers induced in the selected indicator bacteria. Characters studied were their protein profile, phage morphology and immunogenic or antigenic relatedness.

From Fig. 8 and Table 5a, the protein profile of the isolated phages gave a molecular weight range of 9,000 - 100,000. Comparing the results obtained with that of Dickson et. al., (1970) for phage T4 indicates that probably more protein bands could have been obtained. The smaller number of protein bands obtained could be attributed to the fact that whilst Dickson et. al. (1970) used both high and low % gels, this study was restricted to a high % gel. The results obtained from their study were thus cumulated from all the gels making up for all the bands which could not be detected on a single gel. Also some of the bands could have co-electrophoresed or the concentration of some of the protein bands could have been lower than the detection limit of the test system.

Both tailless phages in this study, St RBCL 4 and Sd RBCL 23 had proteins with a wider range of molecular weights than the other phages. The wide range of protein units could play a role in their morphology and infectivity, and probably account for their tailless character (Figs. 7k & l). The two common bands of

molecular weight 10,600 and 31,500 may play a role in the assembly of the tailless phages and hence their morphology. They may also play a role in the initiation of phage infectivity. Tailless phages need some distinct proteins to account for their characteristic mode of infectivity and morphology. This point is buttressed by the presence of unique polypeptide bands which were not found in the tailed phages (Fig. 8). The extra polypeptide bands may account for the additional functions on the phage capsid in the absence of a tail. Despite the factors discussed, the number of polypeptide bands obtained may be due to subunits of one or a few proteins with specialised functions.

A study of the electrophoretogram in Fig. 8 brings out certain interesting features in the band pattern shown. The notable being the relation of protein bands to host range and phage morphology. The tailed phages (Figs. 7a - h, j) exhibit a protein band of molecular weight of ~11,000 which may play a role in their distinctive tailed phage morphology. The variations in tail size and conformation among them may probably be due to the slight variations in the molecular weight around 11,000 and the contribution of other proteins unique to each phage. This could also account for the variations in tail size among the coliphages as against the other tailed phages - Sf RBCL 15, Sd RBCL 5 and St RBCL 4.

The coliphages were found to have two common bands 9,600 and 10,700 (Table 5b). These bands, which seem to be host specific, may play a role in phage morphology and infectivity. This

possibility is substantiated by comparing with Dickson's (1973) finding that a 10,000 molecular weight protein in the T4 phage, though a non structural protein, was found to play a role in fiber assembly. Thus these common bands for the coliphages may play a role in fiber assembly in such a way as to contribute to host recognition during phage adsorption and infectivity. Osborn and Weber (1975) also reported of a 14,000 molecular weight capsid protein in coliphages such as QB and R17 (both tailless phages). An approximately 14,000 molecular weight protein band was also observed among almost all the isolated phages. This band may account for a capsid protein.

The shigella phages (Figs. 7j & l) though morphologically distinct, had a common band of molecular weight 10,600 which probably plays a role in a common host recognition. The morphological distinctiveness among the phages could be attributed to the presence of other unique polypeptide units of the isolated phages as shown in Fig. 8.

The isolated phages were found to be morphologically related to the tailed and tailless phages when examined using the electron microscope. Eight of them were tailed phage or phage - like particles and the remaining two were tailless phage particles (Fig. 7). Of the 8 tailed phages, the five coliphages were found to be morphologically comparable to the T-even phages. They all show contractile sheaths and base plates with spikes which are characteristic of the group A phages of Bradley (1971). Ec RBCL 7 and Ec RBCL 25 show two distinctive morphological

conformations, a contracted and uncontracted sheath (Figs. 7 a, d & e). These micrographs show a contractile phage with a contracted sheath as pertains to the period of DNA injection in the T-even phages. These phages may be either a T2 or T4 phages which are morphologically difficult to distinguish. But it is very evident that they are T-even phages. As stated by Bradley, structural variations do exist in the head sizes and tail assembly. The head and tail sizes of the isolated coliphages with the exception of Ec RBCL 7 were found to be comparable. Ec RBCL 7 had a larger head. These variations are clearly demonstrated in Figs. 7 a - f and Table 4. Comparing the head and tail sizes of the isolated coliphages with those of Hayes (1968) it can be inferred that Ec RBCL 7 may be a T2 phage and the other coliphages T4 phages.

Comparing Ec RBCL 8 and Ec RBCL 25 (Figs. 7b & d) with the other coliphages, their heads seem to be structurally distinct. They look more octahedral (more symmetrical) with edges of capsid absolutely straight compared to the slightly bent capsid edges of the other coliphages which are icosahedral (Figs. 7a, c - f). It was not possible to distinguish between the number of spikes on the base plate of the phages which gives a reflection of the radial symmetry of the tail, a characteristic feature for distinguishing variations within the group A phages.

Sf RBCL 15 though tailed shows flat base plates with and without contractile sheath (Figs. 7 h & g). This phage sample shows two phage types with distinct morphological features which

are more comparable to the P1 and P2 phages as described by Hayes(1968). The tailed phage with a flat base plate and no sheath may be a P1 phage whilst that with a contracted sheath and flat base plate may be a P2 phage. Figs. 7g & h show two particles with two different head sizes. This peculiarity may be a confirmation of Hayes' assertion that P1 phages from a single plaque show two different phage heads even from a fresh preparation. The noncontractile phage may be placed in group B of Bradley (1971).

Sd RBCL 5 was found to be a tailed phage with a contracted sheath, a flat base plate and an empty head (probably DNA is being injected into host as shown by the contracted tail sheath). This phage looks like a P2 phage with an empty head, a contracted sheath with a flat base plate as shown by Hayes. The head size is comparable to that of Sf RBCL 15 (Fig. 7h). It can be seen from Figs. 7g, h, j that variations exist in head shape and tail length among the P phages.

Fig. 71 obtained from Sd RBCL 23 show tailless phages with two distinct sizes, large and small particles. This phage may be a member of the C morphological group of Bradley which are distinguished by the lack of phage tails. Sd RBCL 23 looks like Ø6 phages as described by Freifelder (1983). Like the Ø6, Sd RBCL 23 show two particles, a larger enveloped and a relatively smaller particle. This information though helpful is inadequate without a confirmation of the nucleic acid types of the isolated phage.

St RBCL 4 (Fig. 7k) shows a small phage of about 20nm in diameter (Table 4). It may be a member of ØX174 group of phages or small DNA viruses. Its size falls within the range of 20 - 30 nm for these phages. Like ØX174, it shows an enclosed icosahedral head and a spike region (arrowed in Fig. 7k). If the isolated phage is a ØX174, then at least four proteins will be expected (Freifelder, 1983). The observed polypeptide band pattern (eight bands) may be due to different subunits of a few proteins. For example ØX174 head protein (gene F) and spike protein (gene G) are said to be pentameric and it is thus possible some of the bands obtained may be subunits of one protein. St RBCL 4 may be a member of Bradley's C morphological group.

St RBCL 20 was found to be a tailed phage-like particle with plaqueing activity but no neutralising activity to the homologous phage or transducing activity for the test marker genes. The reason for this behaviour is unknown.

Results obtained in Tables 6a & b indicate some detectable cross-reactivity to the homologous phage for all the isolated phages with the exception of the S. typhi phages which did not show any change in plaque count after the antigen - antibody neutralisation assay.

For the T-even phages, there are antibodies to the phage membranes, the tail fibers and the sheath. The different kinds of antibodies are dependent on the structural complexity of the phages used as antigens (Jesaitis and Zinder, 1977). The

comparable isolated coliphages (Figs.7 a - f) were found to be closely related to the homologous phage (Ec RBCL 26) and hence to one another and are members of the same host range group. Based on their morphological features and host specificity they were expected to have a similar mode of infectivity and hence a closer degree of relatedness. The coefficient of neutralisation obtained confirms this expectation (Table 6a). The neutralising activity obtained is a reflection of the contribution of various polypeptide units found in the electrophoretogram in Fig. 8. For the coliphages it was found that apart from the two common bands, at least two other polypeptide bands were common to the phages or closely related to the homologous phage. This feature together with similarities in plaque morphology may account for the trend of phage relatedness obtained. Ec RBCL 10 was found to be closest of the phages related to the homologous phage. All the three detectable polypeptide bands in Ec RBCL 10 were found to be present in the homologous phage (bands of molecular weight 9,600, 10,700 and 16,200). Based on the information available coupled with phage morphology (Fig. 7), Ec RBCL 10 may be the same or very closely related to the homologous phage. For the other coliphages the trend in relatedness may be due to the contributions of unique polypeptide bands in addition to the effect of the common bands. The trend of relatedness obtained for the coliphages implies they are antigenically related save for differences in the antigenic structures which could have been produced by natural mutations from a common ancestors. The

finding that the coliphages are morphologically similar and more or less related to one another but were isolated from totally different samples of material collected from different locations suggest a common family or ancestry or a common mode of infectivity.

Sd RBCL 5, Sd RBCL 23 and Sf RBCL 15 gave a K'/K ratio of less than 0.25 (Table 6b). This, according to Bradley (1971) indicates a possibility of difference in their antigenic structure to that of the homologous phage. This implies there is no significant serological relationship. This result confirms the morphological differences established for this group by electron microscopy. These differences could be due to different amino acid sequences in the antigens or different spatial arrangement of the same antigens (Bradley, 1971). The detectable cross reactivity may be due to the presence of a few polypeptide units with molecular weights slightly close to that of the homologous phage leading to regions on the antigenic structure with similar or closely related amino acid sequences which probably contribute to the neutralisation activity.

St RBCL 4 and St RBCL 20 were found to be unrelated to the homologous phage (Table 6b). The unrelatedness indicates either a gross difference in the capsid such as the presence or absence of a tail, or equally well a chemical difference which may be morphologically undetectable such as differences in polypeptide band pattern from that of the homologous phage.

Antipolypeptide sera A and B seem to play some role in phage

infectivity as shown by a decrease in plaque count after the neutralisation assay. This effect was not too prominent without other polypeptide units as seen in the homologous phage. Its role may be similar to the contribution of the 10,000 molecular weight non-structural protein reported by Dickson (1973) to be involved in fiber assembly. On the average this may account for their effect on the neutralising activity of the coliphages. The exceptionally high K'/K ratio for Sd RBCL 23 by antipolypeptide sera B suggests that the 10,600 molecular weight protein band may be a prominent capsid protein with a role in phage infectivity.

Sf RBCL 15 though a tailed phage seems to be the least related to the homologous phage. This was expected for a phage with such morphologically distinct features (a long tail without a base plate) as shown in electron micrographs (Figs. 7g & h) and a distinct protein band pattern suggesting a different antigenic structure and mode of infectivity.

Bacteria respond in diverse ways to antibiotic administration. Their ability to be susceptible or tolerant to antibiotics is dependent on a complex interplay of factors such as permeability barriers, lack of affinity between the drug and its protein receptor, a suitable intracellular target, the ability to switch to alternative metabolic pathways and the production of detoxifying or hydrolytic enzymes (Wiedemann, 1986). The response shown by the sensitivity test may be due to any or an interplay of the above factors. Changes in antibiotic susceptibility among different species are a function of the

interaction of bacteria genomes (Chromosomes, plasmids and transposons) with specific host and environmental factors (Mayer, 1986).

Results from Figs. 11 - 13 and Table 7 indicate the presence of the marker genes for Tet., Ben. and Str. among the test bacteria. The MICs obtained were found to be higher than that of Bryant (1972). The determined MIC values (Table 7) were about 95% higher than that of Bryant for S. aureus, E. coli and Ps. aeruginosa for all three test antibiotics(see appendix). This may be attributed to improper drug administration by clinicians and individuals, introduction of new and well adapted species through infection, overcrowding and other environmental changes which lead to the selection of resistant mutants. Other factors which may account for the high MICs obtained are the effect of inoculum size, pH and type of media used. The source of organisms seems to also have an effect on the high MICs recorded. The bacteria from the NMIMR group seem on the average to be more tolerant than the UGMS group. This may probably be due to the fact that the NMIMR being a referral and research center, the highly resistant isolates of interest were stored for further work and as a bacteria bank for researchers while that of the UGMS group were kept purely for microbiological work without any bias for resistant species. Such cases of high resistance may arise due to prolonged exposure to antibiotics. Ps. aeruginosa showed a higher tolerance to all the test drugs. This confirms Duerden et. al., (1987) assertion of the development of resistance to

virtually all known antibiotics by Ps. aeruginosa. Sal. Gp D was relatively more sensitive to all three antibiotics, S. aureus was sensitive to Benzylpenicillin and S. typhi to Tetracycline. The sentivity shown by these organisms may be due to the presentation of a less formidable resistant mechanism (as described under literature review) to surmount the drug action.

The marker genes were selected by inducing resistance using the method of training and patching. These methods were used because the development of resistance to many drugs is said to resemble a "training" process - a process of selection of successively higher resistant mutants. Most mutants are reported to arise by spontaneous mutation and training (Stent and Calendar, 1978). The method of patching is dependent on spontaneous mutation. From Table 8a the markers were shown to have been induced to tolerate on the average higher concentrations of the test antibiotics. Str^r was found to be induced to a high tolerance of the drug. This confirms the assertion by Calendar and Stent , that Str^r character is controlled by three or more bacterial Str. genes of unequal potency. Each gene is said to be capable of mutating with roughly equal mutation frequency of 10^{-10} mutations per cell per generation to produce that level of resistance (slight, intermediate or high) which can be conferred on the bacteria. On the other hand, the Pen^r (eg. Ben^r) is said to be controlled by several equipotent bacterial genes, each of which can mutate spontaneously with a frequency of $\sim 10^{-7}$ per cell per generation

to confer a "one step" resistance on the bacterium. If only one Pen gene has mutated, bacteria manifest the lowest grade of Pen^r character. As the number of mutant genes increases the physiological effect stands out in a geometrical relation to the effect produced by either Pen mutant genes alone and the bacterium attains a much higher resistance (Stent and Calendar, 1978). Pen^r mutants confer penicillin resistance by several alternative routes, including the uptake of penicillin into the cell, altering the enzymatic assembly that governs cell wall synthesis and causing a chemical modification of penicillin itself.

Tet^r mutants just like Str^r mutants owe their resistance to a genetically controlled alteration of structural and catalytic members of the cellular engine for protein synthesis. S. typhi could not be sustained at 170 ug/ml. This might be due to the phenomenon of reverse mutation.

All the isolated phages with the exception of St RBCL 20 were found to be capable of transducing the Str^r, Ben^r marker genes at a high frequency though in terms of magnitude the transduction frequencies of Sd RBCL 23 and St RBCL 4 were quite low (Table 9). The average transduction frequencies for the markers were about 0.96×10^{-4} transductants/pfu and about 0.98×10^{-4} transductants/pfu for Str^r and Ben^r respectively. The number of transducing particles in a phage lysate can help one make a rough estimate of the phage particles carrying a transducing segment or bacterial DNA for any given genetic marker

(assuming that transducing particles package all genes with equal frequency). The difference between the average transduction frequencies is due to the fact that markers are not transduced at equal frequencies. The overall trend according to Wall and Harriman (1974) can be predicted by a gene dosage effect. These differences may be due to variations in the physical nature or mode of presentation and perhaps subsequently in the mechanism of integration of the transducing DNA fragments with different phages even in the same organism (Low and Porter, 1978). The variations may also be due to significant intrinsic differences among the transducing phages.

The transduction frequencies for transducing the markers were found to be higher for the coliphages than the other phages. This may be a reflection of variations in the probability of integration or recombination efficiency of the transferred bacterial DNA into host chromosome. The coliphages in this study transduced single markers at a higher magnitude than the P1 coliphage of Wall and Harriman (1974). But their P1 coliphage mutants showed a highly altered and enhanced transducing ability for *E. coli*.

Whether a transducing fragment is integrated to form a stable recombinant, or fails to be integrated, so that a heterogenote results, depends on the balance of two factors - the extent of homology between the fragment and the recipient chromosome and the degree of residual phage function which the transducing particle carries. The greater the former and the

less the latter, the higher will be the probability of an integration and vice versa (Hayes, 1968).

The frequency of transduction of a marker gene may vary with time during the latent period at which the transducing particles are harvested and with the location of the marker on the bacterial chromosome. Mutations in phage genes could increase or decrease transducing particles production. Wall and Harriman (1974) proposed that a phage nuclease with sequence specificity was needed to cut bacterial DNA to allow packaging. If the phage nuclease was altered in its specificity a great number of cuts would be made, more randomly, resulting in more transducing particles with less variation in the frequency of the markers present in them. This hypothesis is consistent or may account for the variation in transduction frequencies among Str^r and Ben^r. It has also been indicated that host mutations of an unknown nature can increase the level of transducing particles produced. Ultraviolet irradiation of transducing particles prior to infection increases the frequency of stable transduction, presumably by enhancing the chances of recombination while decreasing the frequency of abortive transduction. The high frequencies obtained may be due to an enhanced recombination efficiency caused by the use of induced resistance markers.

The transduction frequency is also a reflection of the effect of the ratio of stable to abortive transductants. The mechanism responsible for the early choice between integration and sequestration (as abortive transducing DNA) remains obscure

as does the mechanism that permits the insertion of duplex fragments. The difficulty in interpreting transduction frequency from biological data stems from the existence of abortive transductants, which constitute about 95% of all transductants and because individual markers manifest different transduction frequencies (Ebel-Tsipis et. al., 1972a).

Another important factor is due to the phenomenon of marker effects, or the seemingly abnormal and allele-specific changes in observed recombination frequency. The phenomenon of marker effects is said to have a strong influence on observable recombination frequency as seen in genetic studies of the Trp and Gal regions of E. coli by P1 transduction (Low and Porter, 1978). Differences in the average transduction frequencies may have been imposed by differences in the selective environment used in selecting transductants by the method of patching and training.

The transductants maintained their Ben^r and Str^r phenotype for several generations when used as indicator cells to phages that led to their transduction. The transductants supported plaque formation with a high efficiency. These factors taken together are typical of generalised transducing phages. This goes on to support the initial presumption of the capability of the phages of generalised transduction. In generalised transduction any genetic marker of a donor strain is carried within the lysate; that is, different parts of the genome are carried by different kinds of transducing particles.

For a better understanding of the transducing mechanisms of

the isolated phages further physical and chemical studies on density-labelled transducing particles and their DNA should be undertaken. This, coupled with the use of current analytical tools in molecular biology such as the polymerase chain reaction technology in studying induction of mutation ,transduction and genome and restriction site mapping may provide a better understanding of the mechanism of transduction by these phages. Information obtained could also be helpful in the use of these wild type phages as potential cloning vehicles of genetic material. This may also be helpful in the current investigations into the high incidence of drug resistance due to improper drug use. It may also help in gaining further insight into one possible mode of antibiotic resistance gene transfer among bacteria.

SUMMARY

(i) In order to transduce the antibiotic resistance gene markers, phages were isolated from a variety of sewage sources. Out of the twenty - six samples, ten phages were found to grow on E. coli, Sh. dysenteriae, Sh. flexneri and S. typhi. Five of the phages grew on E. coli, two on Sh. dysenteriae, two on S. typhi and one on Sh. flexneri. The phages exhibited a narrow host range specificity.

(ii) The isolated phages were purified and characterised as vectors for the transfer of the resistance markers, Str^r and Ben^r. The phages were found to have proteins of relative molecular weight ranging from 9,000 - 100,000. Eight of the phages were tailed and two were tailless . All the coliphages isolated were morphologically related to the T-even phages, whilst Sf RBCL 15 and Sd RBCL 5 were related to the P-phages. The tailless phages were morphologically related to the ϕ phages (ϕ6 and ϕX174).

(iii) Using antisera raised to one of the coliphages in a neutralisation assay ,the coliphages were found to be closely related serologically to each other. There was some detectable crossreactivity to the Sd RBCL 5, Sd

RBCL 23 and Sf RBCL 15. The phages that multiplied in S. typhi phages were unrelated to the coliphages.

(iv) St RBCL 20 was found to exhibit plaqueing activity but no neutralising activity to the homologous phage (Ec RBCL 26) nor transducing activity to any of the marker genes.

(v) Selected pathogenic bacteria were screened for the presence of the antibiotic resistance gene marker. They were found to be highly resistant to the marker antibiotics. The only exception being Salmonella Group D which was relatively more sensitive to all three antibiotics (2.0 ug/ml, 10.0 ug/ml and 5.0 ug/ml for Tetracycline, Benzylpenicillin and Streptomycin respectively) that were used in the study. S. aureus was sensitive to Benzylpenicillin (4.0 ug/ml) and S. typhi to Tetracycline (2.0 ug/ml). The results obtained indicate a high incidence of the antibiotic resistance gene marker among the test bacteria. It is therefore recommended that infectious disease states be kept under surveillance so as to have an up to date antibiogram. This will help formulate antibiotic policies for hospitals without good laboratory facilities. This knowledge would also be of help in the periodic formulation of the essential drug list.

The culture of public education on drug abuse and improper administration must be sustained.

(vi) The indicator bacteria were induced to a higher degree of drug resistance. The indicator strains could be induced to a higher degree of resistance for Streptomycin, followed by Benzylpenicillin and Tetracycline. Though S. typhi could be induced to grow in the presence of a higher concentration of 170 ug/ml from the initial MIC of 2 ug/ml, the bacteria could not be sustained at this new concentration of Tetracycline. This may be attributed to the phenomenon of reverse mutation.

(vii) All the phages with the exception of St RBCL 20, were found on the average to be capable of transducing the marker genes at a high frequency. The phages were presumed to be generalised transducing phages. Transduction may be one mode of transfer of antibiotic resistance among the select bacteria in nature. It is therefore recommended that the transducing phages be further studied as potential cloning vehicles for gene markers using current recombinant DNA technology.

APPENDIX

1. COMPOSITION OF MEDIA

NUTRIENT AGAR	g/l
Peptone from casein (C/ELB)	3.45
" " meat (F/PSB)	3.45
Sodium chloride	5.1
Agar agar	13.0
Final pH (37°C)	7.5

NUTRIENT BROTH	
Lab Lemco powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
pH	7.4 ± 0.2

STORAGE MEDIUM (SM)	
Sodium chloride	5.8
Magnesium sulphate heptahydrate	2.0
1M Tris-HCl (pH 7.5)	50.0 ml
2% (w/v) gelatin	5.0 ml

Sterilize (SM) by autoclaving at 121°C for 15 mins. Store in 50 ml aliquots. Sterile SM can be stored indefinitely at room temperature .

2. LITERATURE VALUES OF MICS BY BRYANT(1972)

Tetracycline

Proprietary name: Terramycin, Imperacin

Derived from: *Streptomyces rimosus*

Antibacterial spectrum: Broad

Action: Bacteriostatic

Peak blood concentration: Between 1.0 and 4.0 ug/ml (oral route)

Urine concentration: >200 ug/ml

Antibacterial activity: MIC(ug/ml)

S. aureus	0.12
S. pyogenes	0.06 to Resistant
S. pneumoniae	0.12
H. influenzae	0.12 -1.00
E. coli	1.00 -2.00

Benzylpenicillin

Proprietary name: Pebnemid, Solupen, Crystapen

Derived from: Penicillium chrysogenum 6 - (Phenylacetamido) penicillanic acid

Antibacterial spectrum: Narrow

Action: Bactericidal

Peak blood concentration: Between 1 and 5 IU/ml (intramuscular route)

Urine concentration: >500 IU/ml(1.0 IU = 0.6 ug)

Antibacterial activity: MIC(ug/ml)

S. aureus	0.03
S. pyogenes	0.015
S. pneumoniae	0.015
S. viridans	0.015
P. aeruginosya	Resistant

Streptomycin

Proprietary name: Strepolin

Derived from: Streptomyces griseus

Antibacterial spectrum: Broad

Action: Bactericidal

Peak blood concentration: Between 25 and 50 ug/ml(intramuscular route)

Urine concentration: >500 ug/ml

Antibacterial activity: MIC(ug/ml)

S. aureus	2.0
E. coli	4.0
P. aeruginosa	16.0
M. tuberculosis	1.0

3. **REAGENTS USED IN PHAGE CHARACTERISATION**

(a) SDS-PAGE
SOLUBILISING BUFFER

STOCK SOLUTIONS	VOLUME OF STOCK SOLUTION/ML	FINAL CONCENTRATION USED
50 mM Tris-HCl, pH 7.0	0.2	50 mM Tris-HCl, pH 7.0
4% (w/v) SDS	0.1	2% (w/v) SDS
β-mercaptoethanol (commercial reagent)	0.1	5% (v/v) β-mercaptoethanol
0.5 mg/ml Bromophenol blue	0.2	0.05 mg/ml Bromophenol blue
Distilled water	0.5	

REAGENTS FOR ELECTROPHORESIS

(i) 3M Tris-HCl, pH 8.8 (main gel buffer)

Dissolve 36.4g of Tris base in 60 ml of H_2O, adjust pH to 8.8 with HCl and make the volume to 100 ml with distilled water.

(ii) 0.47 M Tris-HCl, pH 6.8 (stacker gel buffer):

Dissolve 4.85g Tris base in 70 ml of H_2O, adjust to pH 6.8 with HCl and make up to 100 ml with distilled water.

(iii) Electrophoresis buffer: 0.025M Tris, 0.192 M glycine, 0.1%(w/v) SDS, pH 8.3 (30.3g Tris, 14.4g glycine, 1.0g SDS, add distilled water to 1,000 ml, pH 8.3).

(iv) Staining solution: Coomassie blue R - 250 0.18g, Glacial acetic acid 100 ml, Isopropanol 248 ml and make up to a litre with distilled water

(v) Destaining solution: 10% (v/v) glacial acetic acid 200 ml, 5% (v/v) Isopropanol 100 ml and make up to two litres with distilled water.

b) STAINING REAGENT FOR ELECTRON MICROSCOPY

2% (w/v) Uranyl acetate staining solution. Dissolve 2g of uranyl acetate salt and make up to 100 ml. Adjust pH to 7.4 if necessary.

(c) ELECTROTRANSFER OF PHAGE POLYPEPTIDE BANDS

(i) Transfer buffer: 25 mM Tris, 192 mM glycine, 20% (w/v) methanol pH 8.3 (30.3g Tris, 14.4g glycine, 200 ml methanol, distilled water to 1000 ml, pH 8.3)

(ii) PBS-Tween buffer: Sodium chloride 8.5g, Disodium hydrogen phosphate 1.096g, Sodium dihydrogen phosphate 0.315g, Tween 20 0.5 ml, Sodium azide 0.2g. Dissolve all in a litre of distilled water. The pH is adjusted to 7.4 with IM HCl.

(iii) Indian Ink staining solution: 0.1% (v/v) Pelikan fount india drawing ink in PBS-Tween buffer.

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