

STUDIES OF INTERACTIONS BETWEEN STANDARD
AND MUTATED ARTHOROBACTER SIMPLEX STRAINS
AND STANDARD AND MUTATED STRAINS OF ITS
BACTERIOPHAGE

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UNDER THE GUIDANCE
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BOMBAY

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in

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by

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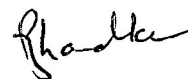
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C H A P T E R - I

GENERAL INTRODUCTION

Viruses are a unique class of infectious agents. They were originally distinguished because of their minute size and their obligatory intracellular multiplication. The truly distinctive features of viruses are now known to be their simple structural organisation and their mechanism of replication. A complete virus particle or virion consists of genetic material (either DNA or RNA) capable of autonomous replication and is surrounded by a protein coat called as capsid and is sometimes enclosed by an additional protective layer of an envelope. The viruses multiply in cells by synthesis of their separate components followed by their assembly.

Viruses are subdivided into three main classes : animal viruses, bacterial viruses (bacteriophages), and plant viruses. Within each class, each virus is able to infect only certain species of cells. The host range is determined by specificity of attachment to the cells which depends on properties of capsid/envelope and specific receptors on host cells.

Bacteriophages first attracted the attention of Max Delbruck in 1930⁽¹⁴⁾. For the next twenty-five to thirty years, study of phage-bacterium interaction provided model for investigation of process of infection and also provided knowledge of general importance to molecular biology.

The process of infection by a lytic bacteriophage takes place in five steps :

1. Attachment of phage to host surface.



2. Penetration of phage nucleic acid.
3. Synthesis of phage components.
4. Assembly of phage components.
5. Release of progeny phages.

Attachment of a phage depends on specific interaction between two complementary sites : attachment site on surface of phage and receptor site on host cell surface. Attachment of a phage is influenced by pH and salt composition of the medium. Phage infectivity is neutralised by antibodies that cover the attachment sites before the phage attaches itself to the host cells. The nucleic acid of the phage is released from the capsid after the phage becomes irreversibly adsorbed to the host cell. Even after penetration of viral nucleic acid, host may not release infectious phages due to either action of nucleases on phage nucleic acid or for want of appropriate machinery for synthesis of phage components. Phage nucleic acid then directs the synthesis of phage components which assemble to form mature infectious virions that are released by the infected host cells.

Bacteria are often used as starter cultures in several industrial processes which yield end products such as amino acids, vitamins, antibiotics, wines, vinegar, steroids, etc. Large scale cultivation of the starter culture becomes obligatory for such industrial processes and infection of the starter culture by a phage may lead to lysis of the culture, thereby the industry may suffer from a heavy economic loss. Study of process of infection by a phage may indicate a way to prevent the infection by a bacteriophage and may save the industry from resulting loss.

Bacteriophages are used for precise identification of strains of bacteria within a genus or species e.g. Salmonella, Staphylococcus and Pseudomonas and is of considerable value in the epidemiological study of outbreaks of infection due to these organisms. Most Pseudomonas and Staphylococcus phages are active against a number of different strains so that patterns of lytic activity are usually observed. These patterns are reproducible within narrow limits and determine the 'phage type' of the particular strain. When a bacterium gets mutated, mutation may change the structure of a receptor site present on its cell surface which may alter its susceptibility to infection by a particular bacteriophage resulting in an alteration of 'phage-type' of the mutated strain.

Importance of recombinant DNA technology opens up new possibilities for the synthesis of new products with the help of microorganisms.⁽⁶⁾ Until around 1970, microorganisms could only produce materials coded for by the cells own chromosome. Genetic selection could improve level of production but it could not yield a totally new product. Using recombinant DNA technology, it is theoretically possible to introduce any gene or a set of genes into microorganisms and cause it to synthesize the immediate gene products. Recombinant DNA technology utilizes ~~v~~ectors such as plasmid or a phage genome for transfer of the foreign gene(s) into a receipient microorganism.

Overall, phages are of great importance in industrial processes, in study of epidemiology of certain

diseases and in recombinant DNA technology. Study of interaction between host bacterium and its specific phage at the level of adsorption, penetration, multiplication, assembly or release will improve the utility of phage technology.

In this project, we have studied the interaction between host bacteria and its phage at the level of adsorption by employing a strain of Arthrobacter simplex and its specific phage.



C H A P T E R - I I

REVIEW O F L I T E R A T U R E

2.1 HISTORY OF BACTERIOPHAGES

In 1915, Twort⁽⁶⁸⁾ discovered that parasitic life of viruses is not confined only to higher eukaryotic cells but bacteria can also be utilized as their hosts by some viruses. Twort found a transmissible agent that was capable of passing through bacteriaproof filters and could destroy bacteria. In 1917, d'Herelle⁽⁶⁵⁾ made an independent discovery of an analogous filterable agent and called it a bacteriophage which was later shortened to just a 'phage'.

d'Herelle had described phages as 'particulate, invisible, filtrable, self reproducing' viruses that are obligate parasites of bacteria.⁽⁶⁵⁾ d'Herelle also suggested a method for enumerating phages which is in use even today. Viable phages can be counted by mixing a small volume of suitably diluted phage suspension with a few drops of young, actively growing host bacterial culture which is spread on a plate containing a suitable medium. Since bacterial cells are present in excess, each phage particle will infect a single bacterium. Surface of the thus spread plate will contain both infected and uninfected bacteria. Upon incubation at a suitable temperature, uninfected bacteria multiply to form confluent or matt growth on the medium but each infected bacterium is lysed after a short time period, liberates hundreds of progeny phages which again infect nearby bacterial cells which will also be lysed in due course. This cycle will continue and will yield an area of clearing in the confluent bacterial growth known as plaque.⁽¹⁾

By counting the number of plaques produced, the

number of plaque forming units in the original phage suspension can be calculated.⁽¹⁾

Although a method to count the bacteriophages was discovered earlier, actual observation of a phage was made possible by the development of an electron microscope. The first electron micrograph of a phage was taken by Ruska in 1940. Luria and Anderson first obtained electron micrographs of T phages.⁽⁶⁵⁾ It was observed that phage particles are tadpole like structures with a hexagonal head and a tail. Tail seemed to be an organ of adsorption to the host bacterium.

Phages vary widely in their size and shape, type of nucleic acid they possess and are classified accordingly. A number of very small phages are devoid of a 'tail' and thus resemble small animal viruses in morphology.⁽⁶¹⁾ Certain phages contain RNA as genetic material⁽⁷⁸⁾ and can be filamentous.⁽⁸⁰⁾

2.2 LIFE CYCLE OF A PHAGE

Phages can be broadly divided into two types : virulent or temperate.

Virulent phages are able to multiply to produce hundreds of progeny phages in an infected host cell, which is eventually lysed to liberate phages it contains. These phages are said to follow 'lytic cycle' of multiplication. Temperate phages either enter a lytic cycle like a virulent phage or establish a kind of symbiotic relation which results in continued multiplication of host bacteria, each of daughter

cell so produced will harbour a copy of the genome of the temperate phage. Bacteria which carry temperate phages are said to be 'lysogenic', the form of the carried phage is known as 'prophage'. Whether a temperate phage enters into lytic cycle or into lysogenic state is determined by the environmental conditions and by physiological state of bacteria.⁽³⁶⁾

Lysogenic phages may play a role in determining the phenotype of host bacteria. Freeman found in 1951, that the property of producing fatal diphtheria toxin is attributed by the genome of a temperate phage resident in toxigenic strains of Corynebacterium diphtheriae.⁽²¹⁾ The dependence of diphtheria bacilli on specific lysogenisation for their ability to produce toxin and therefore, for their virulence is a matter of considerable epidemiological interest. Zabriskie⁽²²⁾ discovered in 1965 that erythrogenic toxin responsible for specific skin rash of scarlet fever caused by group A streptococci is also produced by a lysogenic phage. Many more examples of such a type of phenomenon can be given.^(75, 69) Because of correlation between lysogenisation and toxin production, the phenomenon became known as 'lysogenic conversion or phage conversion'.⁽²⁾

2.3 STRUCTURE OF A PHAGE

When highly purified preparations of classical bacteriophages like T series of phages or temperate phages P22 and λ are analysed chemically, they are found to contain only two major substances : proteins and nucleic acid besides the minor quantities of lipids, carbohydrates.⁽²⁹⁾

There are four possible types of phage nucleic acids. Single stranded DNA is present rarely in bacteriophages. Examples of phages containing such DNA are ϕ X 174⁽⁶¹⁾ and filamentous phages.⁽⁸⁰⁾ Double stranded DNA is most common in bacteriophages. The most wellknown example is T series of phages.⁽⁷²⁾ Single stranded RNA is found in male specific coliphages⁽¹³⁾ where as double stranded RNA is extremely rare, only detected so far in cystoviruses.⁽⁵⁷⁾ The type of nucleic acid present in phages can be determined by staining with acridine orange.⁽⁵⁷⁾ The phage nucleic acid can also be extracted to determine its base composition, nuclease sensitivity and buoyant density. Single stranded nucleic acids are detected by absence of sharp melting profile upon heating and the non equivalence of adenine and thymine or guanine and cytosine.⁽⁶²⁾

When phage particles are subjected to osmotic shock by rapid transfer from high salt concentrations to distilled water, the DNA dissociates from proteins and becomes sensitive to the action of deoxyribonuclease. If such osmotically shocked phages are observed under electron microscope, phage heads appear to be 'empty' giving ghost-like appearance. The phage 'ghosts' retain all the original proteins but lose the property of infectiousness.⁽²⁸⁾

The famous experiment designed by Hershey and Chase (1952) to demonstrate that infection of bacteria by a coliphage results from injection of phage nucleic acid into bacterial cells. He used radioisotopes (^{35}S and ^{32}P) to tag proteins and nucleic acid components of phage T_2 respectively. It was

shown that around 80% of phage sulphur which is in phage 'coat' remains outside the infected cells and can be removed without altering the course of infection but the bulk of radioactive phosphorous present in the phage DNA remains associated with infected bacteria and can be removed from phage progeny as a result of infection.⁽²⁹⁾

Though DNA is injected by a phage to a host cell during the process of infection, purified nucleic acids of all coliphages are unable to infect Escherchia coli. E.coli are gram negative bacteria possessing thick, multilayered cell wall. If cell wall is partly digested with the help of lysozyme and EDTA, spheroplasts are formed. Spheroplasts are susceptible to infection by purified nucleic acids from all coliphages.⁽⁴¹⁾ Since spheroplasts lack phage attachment sites, intact phages are incapable to cause infection in spheroplasts.

The DNA from certain phages is known to contain unusual bases. In Bacteriophage PBS₁, thymine is replaced by uracil or hydroxymethyl uracil or cytosine is replaced by hydroxymethyl cytosine in T even phage series.⁽⁴⁷⁾

Along with nucleic acids, the other major component of phages is proteins. The simplest of all phages are the RNA phages consisting of one predominant nucleocapsid protein of about 15×10^3 daltons. The amino acid sequencing of two members of group I phages have been established.⁽⁴⁵⁾ Both contain 129 amino acid residues, differ by 23 amino acid replacements but are overall similar in structure.

Phage QB is serologically related to this group.⁽⁶⁷⁾ The protein of QB consists of 131 amino acids and is similar in structure to group I phages.⁽⁴²⁾ RNA phages also contain one molecule each of another protein called as protein A or maturation factor⁽²³⁾ which helps the phage in process of adsorption.

QX 174 phage shows greater protein complexity. It consists of three major and one minor component. The 12 spikes present on the surface of the capsid may serve as organs of adsorption.⁽⁷⁾

Phage λ consists of three major proteins assigned to head, tail and the internal of capsid.⁽³³⁾

The large T even phages show the greatest protein complexity.

The major head protein representing 85%, consists of peptide chains having molecular weight of 42×10^3 daltons with N terminal alanine and C terminal glycine.⁽⁴⁾ It appears to be identical in T_2 and T_4 . Three small proteins differ among strains. The contractile tail sheath consists of 144 molecules of proteins associated with carbohydrates at terminal amino group. Contraction seems to result from conformational change of this protein.⁽²⁰⁾ The roles of the bound Ca^{2+} and ATP released during process of infection are not very clear. The tail plates consist of three proteins, the tail tubes of one predominant protein⁽¹¹⁾ and the tail fibres consist of two main structural proteins along with minor components. The lysozymes associated with T_2 and T_4 phages are completely sequenced.⁽³⁵⁾

Thus all phages contain in addition to nucleic acid, one or several proteins. Usually one of the viral proteins is in close association with its nucleic acid. This combination represents the nucleocapsid. Phage proteins determine the structure of phage capsid, specificity of attachment to cell receptor sites and serological specificity of a phage.⁽³⁷⁾ A few of the proteins may be enzymatically active. Phage proteins also provide protection to phage nucleic acids.⁽⁴⁴⁾

Besides nucleic acid and proteins bacteriophages may contain a small amount of lipids and carbohydrates. Lipids or lipoproteins may be required for attachment of virus to cells in the initial step of infection.⁽⁴⁴⁾

All viruses contain carbohydrates since nucleic acid is an indispensable constituent of viruses and nucleic acid is composed in part of either deoxyribose or ribose. In addition to nucleic acid carbohydrates, bacteriophages such as T_2 , T_4 and T_6 phages have glucose attached by an O glycosidic link to 5 hydroxy methyl cytosine. Presence of carbohydrates is also detected in two Bacillus subtilis phages : PBS_1 and SP8. The glucosyl units in these phages appear to be attached to the cytosine and guanine rings.⁽⁴⁷⁾ Precise function of glucosyl units is not known, but the presence of glucosyl units appears to confer resistance to degradation of nucleic acids by diesterases.

2.4 PHAGE MULTIPLICATION

The process of phage multiplication in an infected cell was studied initially by the 'phage group' which was formed

around 1940 and included scientists such as Delbruck, Hershey, Luria and Demerac. They studied the phage-host interaction employing E.coli as the host cells. Delbruck and Ellis designed the 'one step growth' experiment which is the basic procedure for studying phage multiplication. It showed clearly that progeny of infecting phage particle appears after a period of constant titer. Equally important was Doermann's experiment in 1952 which studied the intracellular events that follow phage infection.⁽¹⁷⁾

2.4.1 GENERAL PROCESS OF MULTIPLICATION

The process of infection by a phage on a molecular level consists of the interaction between the host cell and its specific phage. The whole process takes place as follows : Phage attaches to a susceptible cell at specific sites. Either whole phage or phage nucleic acid penetrates within the host cell. If whole virus penetrates, then multiplication is preceded by rupture of the phage capsid to release the nucleic acid. The genetic information present within nucleic acid of the phage then becomes functional. Viral DNA is copied into mRNA which is then translated into proteins. If phage nucleic acid is RNA, it can act as mRNA directly or the viral RNA is transcribed to provide mRNA. Proteins resulting from translation of mRNA may be the enzymes needed for the synthesis of phage components. Synthesis of phage proteins requires functional host cell machinery. Amino acids and nucleotides synthesized by the host cell are incorporated into phage proteins and nucleic acids respectively. Ribosomes, tRNA, translational factors from the host cell are utilized in

the synthesis of phage components. Phage protein and nucleic acid components are produced separately which accumulate in cell cytoplasm until a critical concentration is reached. Then assembly of progeny phage particles begins. In the final step, mature phage particles are released from the infected cell.

2.4.2 PHAGE ADSORPTION & PENETRATION

In systems in which bacteriophages are in a liquid medium containing susceptible cells, phage particles are brought into contact with the host cells by diffusion and brownian motion. Attachment leading to infection occurs upon collision of the phage with host cell in response to structural and electrostatic complementarity between cell surface and phage particles.⁽²⁴⁾ Phage attachment sites and cell receptor sites are mainly made up of proteins. These proteins may be conjugated such as glycoproteins or lipoproteins.⁽⁵⁸⁾ Infective phages are initially reversibly bound to the cells. Irreversible phage adsorption is influenced by the concentration of certain ions in the medium, particularly Mg^{2+} and Ca^{2+} .⁽³⁹⁾

Gram positive bacteria seem to be susceptible to entry by large and small phages but gram negative bacteria have very impermeable cell walls and their phages have developed specific structures to enable them to pass through multilayered walls. The tails of T even phages are highly complex structures consisting of hinged fibres for reversible attachment, base plate with pins for irreversible adsorption and a tail core inside the contractile sheath for piercing the

wall so that phage DNA can enter within the bacterial cell.⁽³⁾

Small DNA phages consist of 12 spikes containing pentamers of two proteins forming at the base and one molecule of another protein at the apex.⁽⁷⁾ Infection organ of RNA phages is the molecule of maturation factor.⁽⁶⁴⁾ The fibrous DNA phages have a special protein located only at one end of the particle. This protein accompanies the DNA to its site of intracellular attachment.⁽²⁷⁾ The phages of this group may be the only ones to enter E.coli cells intact.⁽³⁴⁾ Certain small DNA and RNA containing phages attach themselves to the F pili. RNA phages attach to their sides whereas DNA phages attach to the tips of F pili.⁽⁸⁾

The experiment by Hershey and Chase⁽²⁹⁾ indicated that only DNA entered the cell during phage T₂ infection. In the infection caused by filamentous DNA phages which attach to the F pili, both DNA and coat protein penetrates within the infected cell whereas in case of male RNA phages which also attach to sex pili, only RNA and not phage proteins enters the cells.⁽¹⁹⁾

2.4.3 PHAGE REPLICATION

Since in majority of the phages, only nucleic acid enters the cells, it directs the synthesis of phage proteins to provide the phage components and also achieves several hundred fold replication of its own to provide the genetic material for the progeny phages.

In order to study these functions, several

experiments were designed in the 1950s in which T even phage infected bacteria were lysed at various times during their latent period and lysates were analysed for detection of phage precursors.

Since T even phages are good antigens and can induce formation of antibodies upon injection into an animal such as a rabbit and since these antibodies combine specifically with phage proteins, it was shown that significant amount of antibody precipitable phage protein appears in phage infected cells several minutes before the formation of infective phage progeny.⁽⁷¹⁾

When premature lysates of normal and phage infected bacteria were observed under electron microscope, it was noted that empty phage heads and isolated phage tails appear about 3 minutes before the end of eclipse.⁽⁴⁰⁾

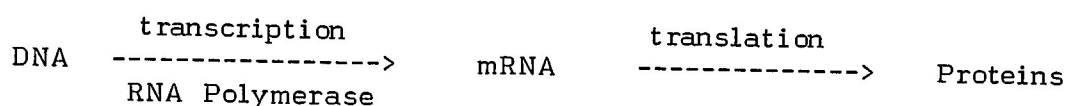
Hershey et al observed that proteins synthesized very early in the latent period do not get incorporated into phage particles but can carry out enzymatic function which seems to be required for phage DNA and protein synthesis. Many such enzymes were later discovered.^(38, 50)

The fact that T even bacteriophages unlike their E.coli host contain 5 hydroxy methyl cytosine in place of cytosine was utilized in the search for an intracellular precursor DNA molecule. The analysis of infected E.coli cells showed that synthesis of progeny phage DNA begins early in the eclipse period ⁽⁴³⁾ in a semiconservative manner.

In 1959, Sinsheimer started studying ØX 174 phage containing single stranded DNA as its genome. He discovered that the single stranded DNA of the parental phage enters into double stranded replicative form in the course of its life cycle.

Replication of RNA in RNA viruses employs RNA dependent RNA polymerase which catalyses the incorporation of nucleoside triphosphates into RNA.⁽⁵¹⁾ Replication of single stranded RNA involved formation of a double stranded replicative form.⁽⁷³⁾

Another major function of viral genomes is coding for necessary proteins which include both, enzymes necessary for synthesis of viral constituents and viral structural proteins. The process of protein synthesis follows 'central dogma' of molecular biology.



If the phage RNA is double stranded, RNA substitutes for DNA in the central dogma. If it is single stranded, it often acts directly as mRNA.⁽⁷⁰⁾

2.4.4 ASSEMBLY & RELEASE

Thus in an infected cell, the phage specified proteins and nucleic acid are synthesized separately. Therefore, they must be brought together to form infectious phages in the process of multiplication. This process is

called as assembly and it may take place in two ways. Either the phages can self assemble spontaneously as in case of structurally simple phages⁽³¹⁾ or the phage genome may code for certain morphogenetic factors that are not structural proteins of the phage but are required for the normal phage assembly to take place as in case of tailed bacteriophages.⁽⁴⁶⁾

When sufficient number of infective phages are assembled within an infected cell, the cell may burst releasing phages and the cellular content in the surrounding medium as in case of lytic infection caused by virulent phages. Rupture is usually aided by two phage coded enzymes : a lipase that attacks cell membrane and a lysozyme that hydrolyzes the cell wall.⁽¹⁴⁾

Budding is another release mechanism exhibited by phages. It is observed in fibrous DNA containing phages of E.coli such as fi, fd and MB.⁽³⁰⁾

The yield of phage particles per cell varies with the phage, the host and growth conditions. The burst size for phages may vary from 10 to 1000.⁽⁴⁴⁾

2.5 SIGNIFICANCE OF PHAGES

Phage research was mostly carried out in the 1950s and 1960s. In 1950s, the experiments performed were mainly centered on genetics of bacteriophages and later the attention was focused on replication and regulation of phage nucleic acid. Lately the scientists have been showing less interest in bacteriophages due to the growing realisation that animal viruses could be cultivated and manipulated as easily as

bacteriophages and due to the direct significance of animal viruses in the field of medicine.

Even then, phages are of great significance in genetics even today. Transduction is a widely studied gene transfer mechanism in bacteria which requires phages as vectors of foreign DNA.⁽⁷⁹⁾ In transduction, fragments of genetic material of host bacterium are occasionally inserted into the genetic material of progeny virus particles which serve as a vector in the process of transduction. Transduction is a very widely spread phenomenon that occurs in E.coli,⁽⁵²⁾ Shigella,⁽⁴⁸⁾ Pseudomonas,⁽⁴⁹⁾ Staphylococcus,⁽⁵³⁾ Proteus⁽¹⁰⁾ and B.subtilis.⁽⁶⁶⁾

Phage genetics is also important in manipulating biological systems for scientific or economic reasons. Genetic engineering has made possible the creation of organisms having new genotypes or phenotypes. Gene manipulation was carried out earlier with the help of techniques such as mutagenesis,⁽²⁶⁾ gene transfer,⁽²⁵⁾ or genetic recombination.⁽⁷⁷⁾ But these techniques are of a random nature and may involve complex procedure for selection of the recombinant. Genetic engineering or recombinant DNA technology is a directed way to achieve gene manipulation. It involves isolation of desirable gene fragments and joining them in a desired combination. Recombinant DNA molecules can then be introduced in a recipient cell by CaCl_2 transformation process.⁽⁵⁴⁾

Recombinant DNA technology involves use of a vector or a cloning vehicle which accepts the donor fragment.⁽⁷⁴⁾ To

be useful, the vector must have three properties :

1. Ability to replicate.
2. Ability to introduce vector DNA into a cell.
3. Ability to grow on a selective medium for detection of the recombinant DNA in the recipient cell.

The three most common types of vectors in use are plasmids, phages and viruses. All three have a replication origin, carry genes that can be identified easily and can be introduced into cells by the CaCl_2 transformation technique. Only a small number of phage vectors are in common use and their properties are listed below.⁽⁶³⁾

<u>Phages as cloning vehicle</u>	<u>Genotype or characteristics</u>
gt4 B	can lysogenize, thermally inducible
λ - Charon	lac 2^+
λ Δ Z1	insertion occurs in lac z gene .
M13 mp7	contains lac P, lac O, lac z; single stranded DNA.

A single bacterial species with a particular set of pathogenic activities may include strains of different types that are distinguishable in minor characters. Recognition of a type of a strain isolated from a patient may be of epidemiological value in tracing sources or modes of spread of infection in a community.⁽¹⁸⁾ The typing of strains may depend upon the susceptibility of the culture to lysis by each of a set of type specific lytic bacteriophages, the 'phage type' of the culture is then determined on the basis of its pattern of susceptibility to different phages.

The absence of a suitable method for serological typing of S. aureus led to the development of phage typing as a means to investigate staphylococcal disease in man.⁽⁵⁵⁾ Phage typing has now been used for this purpose for over 25 years and the method is continuously modified in response to changes in the epidemiological situation.⁽⁵⁹⁾ Phage typing allows subdivision of a serological entity as in the case of S. typhi which is divisible into 80 different phage types.

Although typing with the basic set of phages revealed numerous patterns of lysis, it was possible to divide the organisms in a small number of subdivisions. Certain combinations frequently occurred in patterns and the other combinations were rare. For example, lysis of a strain of S. aureus by phage 52 was associated with lysis by phage 29 or phage 52A but less often with lysis by phages 6 or 7 and very rarely by phages 3A or 3B. Thus it appeared that Staphylococci belonged to a series of phage groups which included strains lysed by one or more of a restricted set of phages.⁽⁵⁵⁾ Similar phage groups are also noticeable in Salmonella species.

Besides the importance of phages in gene technology and medicine, phages are also of great concern in industrial microbiology. Transmission by dust in the air enables them to infect microorganisms employed in industrial fermentations. There is not much information available on this subject but actual amount of damage to a fermentation caused by a phage attack will depend upon particular fermentation and on phase of the fermentation at the time at which phage attack occurs.

If contaminating phage is able to infect rapidly growing sensitive host microorganisms employed in an industrial fermentation, it will lyse the infected cells leaving the phage resistant clones as survivors. Phage infection of a fermenting microorganism may not pose a problem if the phage is not infectious to the starter culture.

An example of phage attack on a bacterial fermentation is the infection of Clostridium acetobutylicum employed in acetone-butanol fermentation. Evolution of fermentation gases, carbon dioxide and hydrogen ceases as the cells are lysed by the phage. Clostridium acetobutylicum produces inducible enzymes for conversion of acetic and butyric acids to acetone and butanol. If a severe phage infection occurs before these inducible enzymes are synthesized, fermentation products can never be recovered after the incubation period. (9, 56)

It is therefore highly beneficial to use a phage resistant variety of the industrial microorganism in which the production capacity is not affected.

In summary, since phages are involved in application of fields such as medicine, biotechnology and industrial microbiology, study of interaction between a bacterium and its specific phage will expand our knowledge of both utility and prevention of a phage in these areas.



AIMS AND OBJECTIVES

1. To obtain bacteriophage resistant mutants of Arthrobacter simplex by natural selection, physical mutagenesis and chemical mutagenesis.
2. To generate mutated strains of bacteriophages by natural selection, physical mutagenesis and chemical mutagenesis.
3. To study adhesion/adsorption interactions of mutant and original hosts and phages.



C H A P T E R - I I I

MATERIAL AND METHODS

3.1 Host bacterium - Arthrobacter simplex was supplied by Haffkine Institute, Parel, Bombay.

3.2 Bacteriophage specific for A.simplex was also provided by Haffkine Institute, Parel, Bombay.

3.3.1 MAINTENANCE OF A. SIMPLEX

The culture was maintained on Arthrobacter simplex agar (AS agar) slant kept at 4° - 8°C and subcultured every month.

3.3.2. PREPARATION OF THE HOST BACTERIAL CULTURE FOR PHAGE ASSAY

A loopful of the A.simplex from AS agar slant was inoculated into 5.0ml of AS broth, incubated at 34°C on a shaker overnight. 0.5ml of the overnight culture was inoculated into fresh AS broth, incubated at 34°C on a shaker for 4 hours. The purity of the culture was determined by plating on an AS agar plate to check for contamination by colony variation.

3.4 MAINTENANCE OF PHAGES

Phage lysate was prepared by inoculating 0.5ml of phage suspension containing 10^7 plaque forming units (pfu)/ml to 10ml of 4 hour old A.simplex culture in AS broth, was incubated at 34°C for 24 hours. Phage multiplication and bacterial lysis resulted into decrease in the turbidity of the broth culture. 2-3 drops of chloroform were added to the broth to prevent bacterial contamination. The lysate was maintained at 4 °C for 3 months, after which fresh lysate was prepared again using the same method.

3.5 PREPARATION OF HIGH TITER PHAGE SUSPENSION

Three AS agar plates were evenly overlayed with 4ml of molten AS agar at 50°C containing 0.2ml of 4 hour old A.simplex culture containing 10^{10} cells/ml and 0.1ml of phage suspension containing 10^{12} pfu/ml. The plate showed confluent lysis on overnight incubation at 34°C. The upper layer of the medium containing phages was scraped off, homogenised in 5.0ml of AS broth. The lysate was sedimented at 2800g in a centrifuge for 15 minutes. The pellet was discarded and the phage suspension was stored at 4°C for 3 months. The titer of phages obtained by this method was 10^{12} pfu/ml.

3.6 ENUMERATION OF PHAGES

The phage titer was determined by Adam's overlay technique.⁽¹⁾ Tenfold series dilutions of the phage suspension were made in phosphate buffered saline. AS agar plates were overlayed with 4.0ml of molten AS agar at 55°C containing 0.1ml of diluted phage suspension and 0.2ml of 4 hour old A.simplex culture containing 10^8 cells/ml. The plates were allowed to solidify, incubated overnight at 34°C to allow plaque formation. The plates showing discrete plaques were selected. The exact number of plaques on each of these plates was counted and average pfu/ml was calculated. Each dilution of phage suspension was plated in triplicate.

3.7 ENUMERATION OF A. SIMPLEX

Tenfold serial dilutions of A. simplex culture were made in physiological saline and 0.1ml of each dilution was plated

on AS agar plates in triplicate. The plates were incubated at 34°C overnight. Plates showing discrete colonies were selected, exact number of colonies on these plates was determined and colony forming units (cfu)/ml was calculated. Absorbance of each dilution of A. simplex was read at 480nm and a standard graph of cfu/ml vs. absorbance was obtained.

3.8 UV SURVIVAL CURVE OF A. SIMPLEX

The method described by Clowes and Hayes was employed.⁽¹⁰⁾

100ml overnight broth culture of A. simplex was centrifuged at 2800g for 15 minutes. The pellet was resuspended in 100ml of saline. Culture density was adjusted to 10^8 cfu/ml. 7ml aliquots of the suspension were delivered in 100mm petriplates and UV irradiated for 0, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200 seconds at a distance of 50cms from the UV lamp. Each of the irradiated aliquots and the unexposed controls were serially diluted and 0.1ml of the dilutions were spread on AS agar plates in duplicates. The plates were incubated at 34°C for 24 hours and number of colonies on each of the plates were counted. A graph of log of surviving bacteria vs. UV dose (in seconds) was plotted.

3.9 PREPARATION OF ANTIBODIES AGAINST THE PHAGES

Parental phage suspension and three plaque type mutants were selected for production of antisera. The titer of phage suspensions used for the experiment was 10^{12} pfu/ml. 0.5ml of each phage suspension was intravenously injected in

the marginal vein of a rabbit weighing 3.0kgs. Five booster immunisations of the same dose were given intravenously at an interval of 10 days. 20-25 ml of blood was collected from marginal vein of the rabbit 10 days following the last immunisation. The serum was separated, heated at 56°C for 30 minutes to inactivate the complement. Control serum was collected from a healthy, uninoculated rabbit.

3.10 SCREENING TEST FOR DETECTION OF ANTIPHAGE ANTIBODIES

The phage suspension was diluted with PBS to obtain 10^3 pfu/ml. 0.1ml of the diluted phage suspension was mixed with 4ml of molten AS agar containing 0.2 ml of 4 hour old A.simplex culture, was poured evenly on a AS agar plate. When the top layer of this plate solidified, a drop of specific antiserum was added to the surface of the plate. In a control plate, normal rabbit serum replaced the specific antiserum. Plates were incubated at 34°C overnight to show inhibition of plaque formation only in the area covered by the specific antiserum in the test plate.

3.11 DETERMINATION OF K VALUE

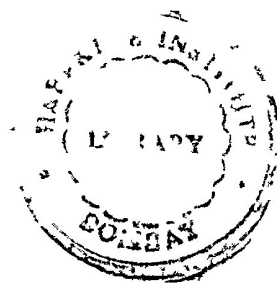
The reactivity constant of phage, host bacterium and an antiserum i.e. the K value was determined by the method of Kay.⁽³⁷⁾

Antiserum in the test was serially diluted in AS broth. 0.1ml of the phage suspension containing 10^3 pfu/ml was added to each tube and the tubes were incubated at room

temperature (RT) for 10 minutes. Four ml of molten AS agar at 55°C containing 0.2ml of 4 hour old host culture was added to each tube, mixed and overlayed on an AS agar plate in triplicate. Plates were incubated at 34°C overnight and number of plaques formed on each plate were counted. 0.1ml of parental phage suspension was overlayed with 0.2ml of host as a control. For calculation of K value, the following formula was employed :

$$\log Po/P = K \times 1/D$$

where Po = number of plaques in the absence of antiserum, P = number of plaques in the presence of antiserum and D = dilution of the antiserum. K value, i.e., the slope was calculated by regression analysis⁽¹²⁾ employing 1/D as the independent (x) and log Po/P as dependent (y) variables respectively. The variation in the experimentally determined value of slope was assumed to be normally distributed and the 95% confidence limit was calculated employing the method described by Daniel.⁽¹²⁾ Differences in the K values were analysed employing student's t test where t was calculated by the formula $t = (K_w - K_m) / \sqrt{S^2_{K_w} + S^2_{K_m}}$ where K_w and K_m are K values of wild type and mutant phage strains, $S^2_{K_w}$ and $S^2_{K_m}$ are their variances.



C H A P T E R - I V

R E S U L T S

4.1 DEVELOPMENT OF PHAGE RESISTANT MUTANTS OF

A SIMPLEX :

4.1.1 BY NATURAL SELECTION

Broth cultures of A. simplex were inoculated with the phage at a high multiplicity of infection ($\text{moi} = 10^2 - 10^3$) and incubated overnight at 34°C . Complete lysis was obtained in all the tubes. When the tubes were incubated further at 34°C for 3-4 days, 10 tubes out of 25 showed slight turbidity. Phage resistant mutants were obtained by streaking a loopful from these tubes on AS agar plates. Since all the colonies developed on a single plate had identical colony characteristics, a single colony was picked up at random, and checked for its phage resistance. The method employed does not allow for calculation of incidence of spontaneous mutation. The mutant strains were compared with wild type A. simplex on the basis of their morphological and biochemical characteristics which were found to be identical.

Ten mutants isolated by natural selection were subcultured every month on AS agar slant and checked for phage resistance. Only one out of the ten mutants retained its phage resistance after 10 passages.

4.1.2 BY PHYSICAL MUTAGENESIS

Physical mutagenesis was brought about by using the method described in (10).

50ml of the overnight broth culture of A. simplex was centrifuged at 2800g for 15 minutes, the pellet was

washed twice in PBS and resuspended in PBS to obtain 10^8 cfu/ml. 2 x 7 ml aliquots were taken in 100mm petriplates and were exposed to UV light for 150 and 180 seconds respectively. The time of exposure was determined from UV survival curve of A. simplex. The petriplates were kept in dark for 30 minutes. The UV exposed suspension was then centrifuged at 2800g for 20 minutes, suspended in fresh broth and incubated on shaker overnight to allow segregation of mutants. The suspension was diluted with fresh AS broth and 0.1ml of dilutions were spread on AS agar plates. Plates were incubated at 34°C overnight. The plates showing discreet colonies were selected and replicated on phage coated agar plates, incubated at 34°C for 24 hours. The colonies appearing on phage coated agar after incubation were the phage resistant mutants. Phage resistant mutants were subcultured on AS agar slants, maintained at 4°C. Fresh subcultures were made every month. Morphological and biochemical properties of the mutants were studied and found to be similar to those of the wild type A. simplex.

Since incidence of mutation was low, enrichment of mutants in AS broth was carried out. Therefore, the number of phage resistant mutants obtained could not be used to calculate the incidence of mutation.

Exposure time of 150 seconds did not yield any mutants. 2 phage resistant mutants were isolated when the cells were irradiated for 180 seconds. One of the mutants retained its phage resistance after 10 passages.

4.1.3 BY CHEMICAL MUTAGENESIS

4.1.3.1 Using N-methyl - N nitro-N-nitrosoguanidine (NTG)

Chemical mutagenesis using NTG was carried out by employing the method described in (32).

A 1mg/ml stock solution of NTG was made in PBS and autoclaved. 2 x 5ml of 4 hour old A. simplex culture containing 10^8 cfu/ml was centrifuged at 2800g for 20 minutes, resuspended in 5ml of fresh AS broth containing 150 and 200 ugm/ml of NTG respectively. The cells were incubated on shaker for 30 minutes, centrifuged and washed twice with PBS to remove any traces of NTG. The cells were suspended in fresh broth, incubated overnight to allow phenotypic expression. After incubation, the culture underwent the same treatment as in case of UV treated cells.

14 mutants were isolated by using 150 ugm/ml concentration of NTG, whereas 200 ugm/ml concentration yielded 46 resistant mutants. Though all these mutants showed coccoidal morphology in stationary phase and elongated rods in various arrangements in log phase which were identical to those of the wild type strain under similar conditions.

Out of the 60 mutants, 56 strains showed strong fermentation of glucose whereas 4 strains retained the original trait of the host bacterium namely weak or no fermentation of glucose.⁽⁵⁾ Since the latter trait is the hallmark of the genus Arthrobacter,⁽⁵⁾ this was considered as a major mutation. The remaining 4 mutants were employed in further studies. The biochemical characters are detailed in table number 1.

Table No. 1

BIOCHEMICAL CHARACTERISTICS OF ISOLATED PHAGE RESISTANT MUTANTS OF A. SIMPLEX

Mutagen used & its dosage	Strain No.	Glucose	Catalase	Nitratase	Gelatinase	Cellulase	Amylase	Growth at pH		
								5.4	7.0	8.2
Spontaneous	Sp 7	+	+	+	-	-	-	++	+++	+++
5BU (50)	M 6	+	+	+	-	-	-	-	++	+
5BU (50)	M 10	+	+	+	-	-	-	++	+++	+++
5BU (100)	M 8	+	+	+	-	-	-	+	++	++
5BU (100)	M 16	+	+	+	-	-	-	+	++	++
5BU (150)	M 11	+	+	+	-	-	+	+	++	+
5BU (200)	M 1	+	+	+	-	-	-	++	+++	++
5BU (200)	M 4	+	+	+	-	-	-	+	+++	+++
NTG (150)	M 3	+	+	+	-	-	-	+	+	+
NTG (200)	M 2	+	+	-	-	-	-	+	++	+
NTG (200)	M 5	+	+	-	-	-	+	+	+	+
NTG (200)	M 9	+	+	+	-	-	-	+	++	++
UV (180 sec)	M 12	+	+	-	-	-	-	+	++	++
UV (180 sec)	M 13	+	+	+	-	-	-	++	++	++
-	Wild type A.simplex	+	+	+	-	-	-	++	+++	++

Note : For sugar fermentation :- + : weak acid production, +++ : maximum acid production, - : no fermentation
 For enzymatic activity :- + : activity observed, - : absence of enzyme

Isolated phage resistant mutants were subjected to ten serial transfers at a monthly interval on AS agar slants and phage resistance of mutants was confirmed at each transfer.

4.1.3.2 5 Bromouracil (5BU)

Chemical mutagenesis using 5BU was brought about by using a method described in (10).

4 x 0.5ml of overnight broth culture of A. simplex was added to 5ml of fresh AS broth containing 50, 100, 150 and 200 ugm/ml of 5BU. The tubes were incubated on shaker for 6 hours. The cells were centrifuged at 2800g for 20 minutes and washed twice with PBS to remove 5BU. The pellet was resuspended in fresh AS broth and left overnight for segregation. The phage resistant mutants were then isolated by the same method as in case of UV treated cells.

Four phage resistant mutants each were isolated from A. simplex cells treated with 50, 100 and 200 ugm/ml concentration of 5BU whereas 150 ugm/ml concentration of 5BU yielded 3 mutants. 7 of these mutants retained phage resistance after 10 serial passages. Isolated mutants were morphologically and biochemically similar to the wild type A. simplex population.

4.2 ISOLATION OF MUTANT PHAGES

4.2.1 BY NATURAL SELECTION

Plates containing discrete plaques were observed to select plaques with altered morphology. These plaques

were picked up carefully using sterile toothpicks, mixed with 0.5ml of PBS and inoculated in 2ml of 4 hour old broth culture of A. simplex. The tubes were incubated at 34°C overnight. A control tube having same volume of only the host culture was also incubated. Decrease in the turbidity of the host culture containing plaque suspension was observed. Lysate was prepared from these tubes and was assayed by Adam's overlay technique to confirm the altered morphology of the plaques.

One rapid lysis mutant and 10 minute mutants were isolated by natural selection but 8 of the minute mutants did not show the altered plaque size when the prepared lysate was assayed further.

4.2.2 BY PHYSICAL MUTAGENESIS

4.2.2.1 Using phage infected bacterial cells

UV exposed mutant phages were obtained by the method described by Winkler.⁽⁷⁶⁾

0.5ml of overnight host culture containing 10^{10} cells/ml was inoculated in 7ml of AS broth and was kept on shaker at 34°C for 2 hours. 0.5ml of phage suspension containing 10^{12} pfu/ml was inoculated in the tube and incubation continued for 2 more hours. Contents of the tube were centrifuged at 2800g for 15 minutes. Pellet was suspended in 7ml of PBS, washed twice, resuspended in 7ml of PBS. Suspension was transferred to a 100mm petriplate and was exposed to UV light for 4 minutes at a distance of 15 cms from the UV lamp. The lid was replaced at the end of

exposure, plate was kept in dark for 30 minutes. The UV exposed suspension was centrifuged at 2800g for 15 minutes. Pellet was suspended in 7ml of fresh AS broth, incubated at 34°C overnight. The broth was then used for preparation of lysate. Original and diluted lysate (10^{-2}) was mixed with 0.2ml of 4 hour old culture of A. simplex in 4ml of molten AS agar at 50°C and overlayed on AS agar plate to detect phage mutants with altered plaque morphology. Unexposed phage suspension was also overlayed to provide control. (Figure 1)

Two rapid lysis mutants and one minute mutant were obtained by UV exposure of phages. (Figures 2 & 3)

4.2.2.2 Using phage suspension

The method used is described by Winkler.⁽⁷⁶⁾

10ml of phage suspension having 5×10^7 pfu/ml in PBS was irradiated in a 100mm petriplate with UV rays at a distance of 30cms from the UV lamp, stirred occasionally. Irradiation was interrupted after 1, 2 and 3 minutes to collect 1ml samples and irradiation was continued without loss of more than a few seconds. Samples were diluted with PBS, mixed with 0.2ml of 4 hour old host culture, 4ml of molten AS agar at 50°C and plated by the agar layer technique. 0.1ml of diluted, non irradiated phage suspension was used as a control. Plates were incubated at 34°C for 24 hours and screened for the presence of plaque morphology mutants.

Plaque morphology mutants could not be isolated using this technique.

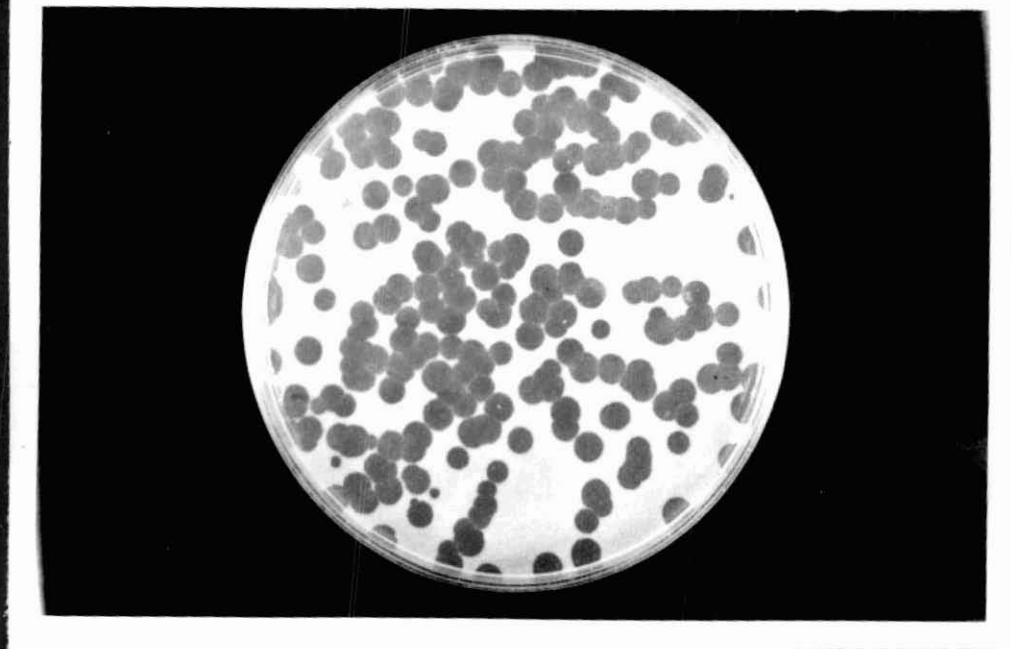


FIG 1:-NORMAL PLAQUE MORPHOLOGY OF STANDARD BACTERIOPHAGE ON HOST A. simplex.

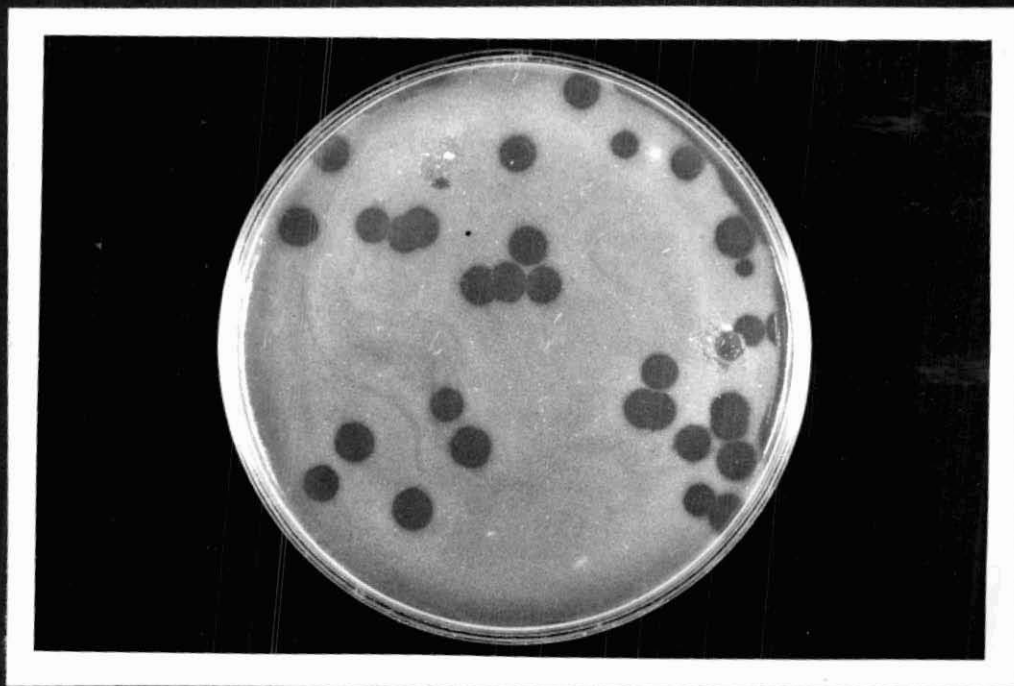


FIG 2:- PLAQUE MORPHOLOGY OF RAPID LYSIS MUTANT OF STANDARD BACTERIOPHAGE ON HOST A. simplex.

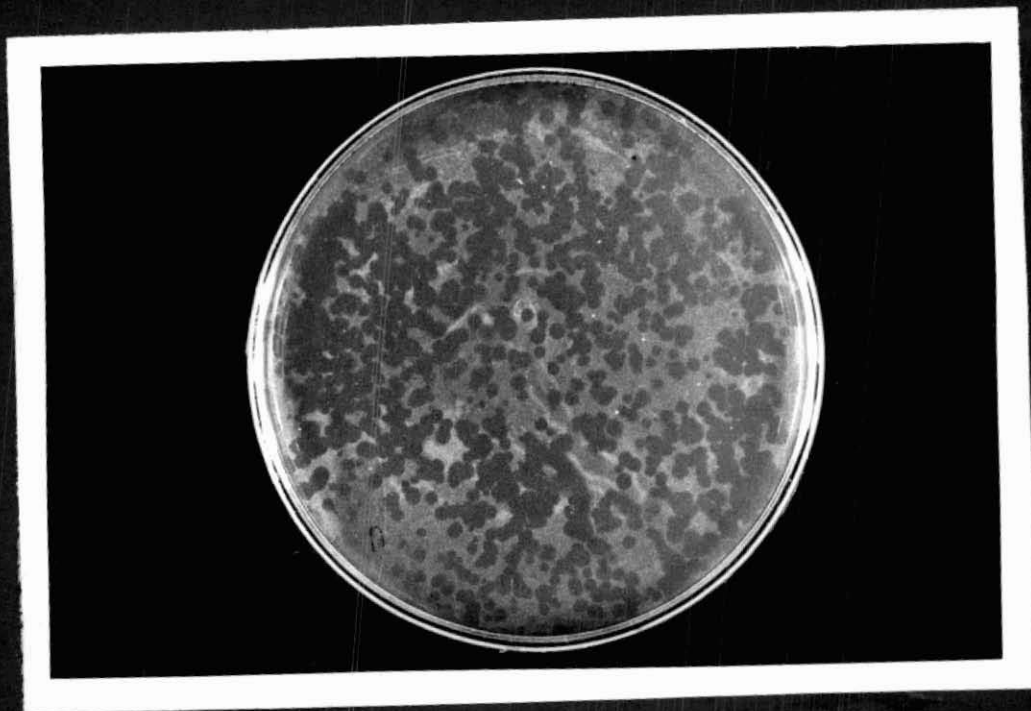


FIG 3:- PLAQUE MORPHOLOGY OF MINUTE MUTANT OF STANDARD
BACTERIOPHAGE ON HOST A. simplex

4.2.3 BY CHEMICAL MUTAGENESIS

Chemical mutagenesis of the phage was brought about using a method described in (10).

5ml of AS broth tubes containing 50, 100, 150 and 200 $\mu\text{g}/\text{ml}$ of 5BU were inoculated with 0.2ml of overnight broth culture of A. simplex containing 10^{10} cells/ml. The tubes were incubated at 34°C for 30 minutes without aeration and for further 2 hours with aeration. 0.05ml of phage suspension containing 10^{12} pfu/ml was added to each of the tubes and aeration was continued for 3 hours. Four drops of chloroform were added to each of the tubes, shaken gently for 30 seconds and kept at room temperature for 30 minutes. Plaque morphology mutants of the phage were detected by the same method mentioned earlier in physical mutagenesis of the phage.

Seven rapid lysis mutants and a minute mutant were obtained at 200 $\mu\text{g}/\text{ml}$ concentration of 5BU. 100 and 150 $\mu\text{g}/\text{ml}$ of 5BU yielded 4 rapid lysis mutants and 2 minute mutants each whereas 50 $\mu\text{g}/\text{ml}$ of 5BU yielded 2 rapid lysis mutants and 1 minute mutant.

4.3 INTERACTION OF MUTATED PHAGES WITH ORIGINAL AND MUTATED A. SIMPLEX STRAINS

Three phage mutants were selected at random to determine their K value. The interaction between standard and mutated phages and original and mutated A. simplex strains was studied. The wild type A. simplex strain was lysed by standard A. simplex phage, UV induced minute

mutant MCC, 5BU induced rapid lysis mutant 50L and spontaneous rapid lysis mutant LCA but the phage resistant mutants derived from wild type A. simplex by natural selection, UV exposure or by exposure to 5BU/NTG could not be lysed by standard A. simplex phage or its mutated strains : LCA, MCC or 50L.

4.4 DETERMINATION OF K VALUE

In order to determine K value of standard and mutated phages, antisera were raised against the standard A. simplex phage and LCA, 50L and MCC mutants. The antisera were checked for their capacity to neutralise the infectivity of phages by spot test.

Table number 2 shows the K values of different antisera against homologous phages. The table also shows K values of a few antisera in a heterologous combination. The kinetics of phage inhibition by the antiserum was always investigated employing the original A. simplex host.

K values for inhibition of infectivity of the standard phage and its rapid lysis mutants 50L and LCA, and the minute mutant MCC by the homologous antiphage antisera were determined. (Table number 2) Even though differences in the values were seen, they were not statistically significant.

K values for inhibition of infectivity of standard phage by the antiserum against the MCC, LCA and 50L mutants were also determined. Only the K value of inhibition by antibody against LCA mutant was of the same order as that of the antibody against the standard phage. In contrast, the K

values of inhibition by antisera against MCC and 50L mutants were significantly lower ($p < 0.05$) as compared to the K value of the antiserum against the original phage. These two phages namely MCC and 50L were mutants having exactly contradicting mutations, former being a minute mutant and the latter being rapid lysis mutant. These results are considered as highly significant.

Table No. 2

K VALUES OF INHIBITION OF INFECTIVITY OF WILD AND MUTANT

A. SIMPLEX PHAGES TO THE ORIGINAL A. SIMPLEX HOST

Sr.No.	Phage used	Serum against	K. value $\pm$ 95% C.L.
1.	Standard Phage	Standard phage	6521 $\pm$ 1097
2.	Phage mutant 50L	Phage mutant 50L	6189 $\pm$ 1863
3.	Phage mutant LCA	Phage mutant LCA	5582 $\pm$ 1255
4.	Phage mutant MCC	Phage mutant MCC	7144 $\pm$ 1303
5.	Standard phage	Phage mutant MCC	3763 $\pm$ 478
6.	Standard phage	Phage mutant LCA	5980 $\pm$ 1662
7.	Standard phage	Phage mutant 50L	4329 $\pm$ 598
C. L. : Confidence limits.			

C H A P T E R - V

D I S C U S S I O N

The project was designed to study interactions between standard and mutated A. simplex strains and standard and mutant strains of A. simplex specific phage.

Both host and phage mutants were obtained by natural selection, physical mutagenesis and chemical mutagenesis.

Natural selection yielded a total of 10 phage resistant mutants of A. simplex, but the characteristic of phage resistance seemed to be unstable and was lost within three to six passages. Only one of the mutants : Sp 7 retained the property after 10 passages. Unstable nature of phage resistance is also observed by several other workers.⁽⁶⁰⁾ Since susceptibility to a phage is determined by the two separately evolving genomes of the bacterium and the phage, chromosomal change in either of the entities may lead to an overall change in the pattern of phage resistance.

Phage resistant mutants of A. simplex were raised by using UV rays, 5BU and NTG. 2 UV induced mutants, 15 5BU induced mutants and 60 NTG induced mutants were obtained. Out of 15 5BU induced mutants, 8 reverted back to wild type within 10 passages. This could be due to the reversible nature of mutations induced by 5BU.⁽²⁴⁾ Out of 60 NTG induced phage resistant mutants of A. simplex, only 4 showed classical biochemical properties of the genus Arthrobacter. This can be explained by the ability of NTG to cause multiple mutations at multiple sites. These 4 mutants were collected for further studies.

All phage resistant mutants of Arthrobacter simplex

showed morphological characteristics in both stationary and logarithmic phases identical to that of wild type.

Plaque morphology mutants of standard phage specific to A. simplex were isolated using natural selection, physical mutagen, such as UV rays and chemical mutagen such as 5BU.

Natural selection yielded 10 minute mutants and 1 rapid lysis mutant. Of the 10 minute mutants, 8 reverted back to the wild type plaque morphology, indicating unstable nature of this characteristic.

To obtain UV induced plaque morphology mutants two approaches were employed. In one approach phage lysate was exposed directly to UV while in the other approach infected host cells were exposed. Only latter approach yielded 2 rapid lysis mutants and 1 minute mutant. Failure of former approach to yield any phage mutants may be due to inert nature of phage genome since UV is known to be more effective in actively replicating DNA.⁽²⁴⁾

It may also be due to lower exposure time.

23 plaque morphology phage mutants (17 rapid lysis mutants and 6 minute mutants) were isolated by treatment of 5BU.

From the plaque morphology mutants of phage obtained by various treatments, 3 mutants were selected randomly to study their K value. These mutants were (i) UV induced minute mutant MCC; (ii) 5BU induced rapid lysis

mutant 50L; and (iii) spontaneous rapid lysis mutant LCA.

In the study of the effect of phage specific antisera on the interaction between host and phage, the equation $\log P_o/P = K 1/D$ is expected to hold true. K, the slope of the above equation can be defined as a constant of reactivity between phage and its specific antiserum and it reflects the extent of interaction between a phage and its host at a given concentration of antibodies. Since the above equation is a straight line equation, experimental data is also expected to show linearity. The fact that such linear relationship existed in our experiments was proved by employing student's t test on calculated slope K. The null hypothesis for the student's t test was that there was no linear relationship ($K = 0$) and the t value was calculated by the formula $t = (K - 0) / \sqrt{S^2_K}$ where S^2_K is the variance of K and can be calculated by using the formula $S^2_K = S^2_{y/x} / \sum x_i^2 - (\sum x_i)^2/n$ where $S^2_{y/x}$ is the variance of y as a function of x. $S^2_{y/x}$ was calculated from the formula $S^2_{y/x} = (n-1/n-2) (S^2_y - K^2 S^2_x)$ where S^2_y is the variance of experimentally determined values of y and $K^2 S^2_x$ is the variance obtained by calculating the theoretical values of Y for every value of X employing the experimentally determined slope namely the K. K values were always investigated employing the wild type A. simplex strain. When the K values for inhibition of infectivity of the standard phage and its rapid lysis mutants 50L and LCA, and the minute mutant MCC by the homologous antiphage antisera were determined, differences observed in the values were not statistically significant. When K values for inhibition of infectivity of

standard phage by the antiserum against the MCC, 50L and LCA mutants were determined, the K value of inhibition by antibody against LCA mutant was of the same order as that of the antibody against the standard phage. In contrast, the K values of inhibition by antisera against MCC and 50L mutants were significantly lower ($p > 0.05$) as compared to the K value of the antiserum against the original phage. These two phages namely MCC and 50L were mutants having exactly contradicting plaque morphology, former being a minute mutant and the latter being a rapid lysis mutant. These results have to be evaluated in the light of our present concept of phage-bacterium interaction.

The infection of host bacterium by a phage consists of the wellknown steps : (1) adsorption, (2) penetration, (3) multiplication, (4) lysis and release. The antibodies are expected to block the first step, namely the adsorption of the phage to the bacterium. So the comparison of K values of different antisera against different mutant phages may show significant differences. Hence with this objective, K values of inhibition of plaque formation by different phages, by their respective antisera were experimentally determined and compared. And no significant differences were observed. However, these results need not totally rule out the importance of mutational events bringing about changes in the phages that can affect the adsorption rates. In order to investigate this facet, K values of inhibition of plaque formation by wild type phage, by antisera against 50L, LCA and MCC phage mutants were

experimentally determined. This approach was taken because 50L and LCA produced large plaques with sharp edges and MCC produced small plaques as compared to the plaques of the wild type phage. Theoretically these two mutations can be due to one/several events of which adhesion and adsorption would be a significant one. the two contrasting morphological mutants can therefore have surface interactions quite different from one another and from the wild strain. The rapid lysis mutants could have higher rates of adsorption and minute mutant a lower rate of adsorption in comparison with the standard phage. Hence kinetics of inhibition of plaque formation by the antisera against the two types of mutants when tested against the same wild type phage is expected to differ from each other and from that of antiserum against the wild type phage. The three antisera against mutant phages are expected to show contrasting inhibitory profiles, i.e., K values of 50L and LCA mutants antisera to be lower and MCC mutant antiserum higher than the K value of the antiserum against the wild type phage. However this was not the case. Of these, K value of the antiserum against LCA mutant was of the same order as that of the antiserum against the standard phage. K value of the antiserum against 50L and MCC mutants were both lower than the K value of antiserum against the standard phage. This data very clearly indicates that plaque mutants need not necessarily arise out of alterations in the surface receptors for adhesion. The latter could be one of the several changes induced by mutational events responsible for plaque morphology mutants.

C H A P T E R - V I

S U M M A R Y

The culture used in the present study was Arthrobacter simplex which is used industrially for steroid transformation.

A total of 88 phage resistant mutants of A. simplex were raised in this study, 18 of which lost the property of phage resistance within 10 passages and 56 mutants had significant biochemical differences as compared to the wild type. Remaining 14 mutants were used in the further study.

29 plaque morphology mutants of standard phage specific for A. simplex were isolated, three of which were used for raising antisera along with the standard phage.

The kinetics of interactions of phage and the host strain were studied in the presence of different antisera and the reactivity constants of the antisera, namely the K values were calculated employing the formula $\log P_0/P = K \times 1/D$ in which P_0 and P are number of plaques in the absence and presence of antiserum respectively and D is the dilution of antiserum employed. K values were calculated by regression analysis. The value of correlation coefficient for all the experiments was near +1 and the test t value was much higher than the expected value from the student's t test ($P < 0.05$) indicating a strong, direct and linear relation between $\log P_0/P$ and $1/D$ validating the fundamental assumption that $\log$ of fraction of phage surviving and antibody concentration ($1/D$) show a direct, linear relation.

It was observed that K values of inhibition of plaque formation by wild and mutant strains, by their

respective antisera were very similar. Some differences in the K values were observed when inhibition of plaque formation by the wild type phage was carried out employing antisera against the mutant strain. The inhibition kinetics did not show alteration in parallel with the plaque morphology of the mutant phages. This was interpreted to be in support of mutational events responsible for the changes in the plaque morphology of the various mutants to be multitargeted and not restricted to adsorption alone.



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A P P E N D I X

MEDIA COMPOSITION

1. Arthrobacter simplex broth :

Peptone	:	6	g
Yeast extract	:	3	g
Tryptone	:	4	g
Beef extract	:	1.5	g
Dextrose	:	1.0	g
Distilled water	:	1000ml	

All ingredients are dissolved in distilled water, pH is adjusted to 6.6 and medium is autoclaved at 121°C for 15 minutes.

To prepare a solid medium, all the ingredients are weighed and dissolved in distilled water, pH is adjusted to 6.6. 1.8g of Bacto agar is added per 100ml of the medium. The flask is kept in boiling water bath till agar is digested and the medium is autoclaved as above.

2. Cellulose broth :

$(\text{NH}_4)_2\text{SO}_4$	:	6.0	g
KH_2PO_4	:	1.0	g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	:	1.0	g
CaCl_2	:	0.1	g
Yeast extract	:	0.5	g
$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	:	0.0167	g
$2\text{nSO}_4 \cdot 7\text{H}_2\text{O}$	:	0.00018	g
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	:	0.00016	g
EDTA	:	0.02	g
Soluble cellulose	:	10	g

All the ingredients are weighed and dissolved in 975ml of distilled water, pH is adjusted to 7.0 with 0.1 N NaOH or 0.1 N HCl, the volume is made upto 1 liter and is sterilized by autoclaving.

Nitrate broth :

KNO ₃	:	0.02 g
Peptone	:	0.5 g
D/W	:	100 ml

Nutrient broth :

NaCl	:	0.5 g
Peptone	:	1.0 g
Meat extract	:	0.3 g

The ingredients are weighed, dissolved in 90ml of distilled water, pH is adjusted to 7.4 using 0.1 N NaOH or 0.1 N HCl, volume is made upto 100ml with distilled water and is sterilised by autoclaving.

Nutrient gelatin :

Nutrient broth	:	100 ml
Gelatin	:	12 gm

Peptone water base :

Peptone	:	1 g
NaCl	:	0.5 g

Ingredients are weighed and dissolved in 90ml of distilled water, pH of the medium is adjusted to 7.2 with 0.1 N NaOH or 0.1 N HCL, 1ml of Andrade's indicator is added, the volume is made upto 100ml with distilled water. The medium is tubed in 5ml amounts, Durham's tube is inserted and autoclaved at 121°C for 15 minutes. 10% solution of sugar in distilled water is sterilized by filtration, 0.25ml of 10% sugar is added to each of the previously sterilized peptone water base.

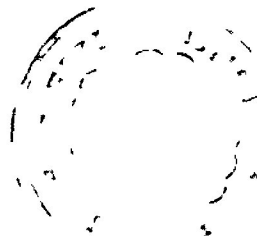
Phosphate buffer saline :

NaCl	:	0.8 g
K_2HPO_4	:	0.121 g
KH_2PO_4 :		0.034 g

Ingredients are weighed and dissolved in 90ml distilled water, pH is adjusted to 7.4 with 0.1 N NaOH or 0.1 N HCl, volume is made upto 100ml and sterilised by autoclaving.

Starch agar :

Nutrient broth	:	100 ml
Starch	:	1 g
Agar	:	2 g



R A W D A T A

1. Determination of K value of antiserum against standard phage and using the standard phage

X(1/D)	Y (log Po/P)	K Value	r	
.00005	0.115			
.00005	0.193			
.00005	0.141			
.0001	0.19	6521		P for
.0001	0.242	± 1097.1	0.963	linearity
.0001	0.29			< 0.005
.00025	0.44			
.00025	0.412			
.00025	0.502			
.0005	0.947			
.0005	1.218			
.0005	1.093			
.00075	1.792			
.00075	2.394			
.00075	2.394			

2. Determination of K value for phage mutant 50L and its specific antiserum

X (=1/D)	Y (> log Po/P)	K Value	r	
.00005	0.044			
.00005	0.134			
.00005	0.138			
.0001	0.18			
.0001	0.198	6189	0.894	P for
.0001	0.114	<u>+</u> 1863		linearity
.00025	1.217			< 0.005
.00025	0.756			
.00025	1.01			
.0005	1.057			
.0005	1.569			
.0005	2.171			
.00075	1.358			
.00075	1.87			
.00075	2.472			



3. Determination for K value for phage mutant LCA and its specific antiserum

X (=1/D)	Y (= log Po/P)	K Value	r	
.00005	0.089			
.00005	0.142			
.00005	0.156			
.0001	0.202			
.0001	0.271	5582	0.936	P for
.0001	0.271	<u>+ 1255.8</u>		linearity
.00025	0.467			< 0.005
.00025	0.433			
.00025	0.536			
.0005	1.069			
.0005	1.457			
.0005	1.37			
.00075	1.265			
.00075	1.633			
.00075	2.411			

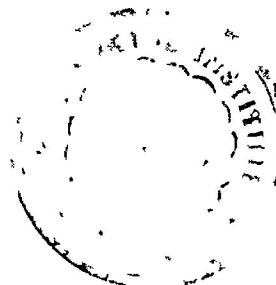


4. Determination for K value of phage mutant MCC and its specific antiserum

X (=1/D)	Y (= log Po/P)	K Value	r	
.00005	0.07			
.00005	0.11			
.00005	0.159			
.0001	0.365			
.0001	0.404	7144	0.957	P for
.0001	0.507	<u>+ 1303.1</u>		Linearity
.00025	0.486			< 0.005
.00025	0.577			
.00025	0.543			
.0005	0.932			
.0005	1.169			
.0005	1.131			
.00075	2.511			
.00075	2.511			
.00075	2.511			

5. Determination of K value of wild type phage and antiserum against MCC phage mutant

X (=1/D)	Y (= log Po/P)	K Value	r	
.00005	0.062			
.00005	0.04			
.0001	0.139			
.0001	0.184	3763	0.988	P for
.00025	0.345	$\pm$ 478		linearity
.00025	0.301			< 0.005
.0005	0.741			
.0005	0.947			
.00075	1.218			
.00075	1.139			



6. Determination of K value for antiserum against phage mutant LCA and standard phage

X (=1/D)	Y (= log Po/P)	K Value	r	
.00005	0.111	5980 ± 1662	0.946	P for linearity < 0.005
.00005	0.124			
.0001	0.212			
.0001	0.26			
.00025	0.412			
.00025	0.353			
.0005	0.947			
.0005	1.19			
.00075	1.549			
.00075	2.394			



7. Determination of K value of antiserum against phage mutant 50L and standard phage

X (=1/D)	Y (= log Po/P)	K Value	r	
.00005	0.072			
.00005	0.139			
.0001	0.148			
.0001	0.19	4329	0.986	P for
.00025	0.353	<u>+</u> 598		linearity
.00025	0.294			< 0.005
.0005	0.792			
.0005	0.979			
.00075	1.315			
.00075	1.491			

Detected

