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BACTERIOPHAGE FOR CYTOPHAGA SUCCINICANS (RL-8)

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Although bacteriophages have been recognized for a number of years, little attention has been given to phages infecting members of the order Myxobacterales. Since 1955 myxophages have been isolated for two genera of fruiting myxobacteria, Chondrococcus columnaris and Myxococcus xanthus. Phages have also been reported for Sporocytophaga cauliformis and the marine organism, Cytophaga marinoflava. The present investigation deals with the isolation of a bacteriophage infecting the freshwater myxobacterium Cytophaga succinicans.

The bacteriophage studied was isolated from a freshwater stream. This phage, ϕ RL-8R, has a hexagonal head, $1180 \overset{\circ}{\text{A}}$ in diameter, connected by a definite neck to a tail, $1900 \overset{\circ}{\text{A}}$ long by $250 \overset{\circ}{\text{A}}$ wide. No contractile sheath, tail plate or tail fibers have been found, although two prongs can be seen at the base of the tail.

Reproduction of the phage in the host culture was studied on overlay agar plates, as well as in broth cultures of C. succinicans (RL-8). In both growth situations, an effect of temperature on the replication of the phage was observed. On overlay plates, there was a marked decrease in plating efficiency at 25C, compared to that at 18C. Plaques produced at the higher temperature were also very small and uneven, while those which developed at 18C were clear and round, ranging from 0.25 to 1.0 mm in diameter. In broth cultures of the host cell, no phage development was detected at 25C, while increases in phage titer of four log units were observed over a period of several days at 18C.

Studies of broth replication of the phage at 18C also revealed the development of phage-resistant cells. The presence of these cells may provide a partial explanation of why a decrease in culture turbidity during phage production was never observed.

Phage replication was also studied under anaerobic conditions, since the host organism is capable of CO₂-dependent anaerobic growth. The phage was not able to replicate anaerobically under the conditions used, though excellent cell growth occurred.

A single cycle of phage reproduction was examined using the classical single-step growth experiment. ØRL-8R was found to have a latent period of 135 minutes and a rise period of 90 minutes. The burst size was calculated to be 112 phage progeny per infected cell.

A variety of other characteristics were examined for ØRL-8R. Properties such as pH stability, sensitivity to chemical agents, and stability and maintenance of phage stocks were found to be very similar to those of most other bacteriophages. The temperature sensitivity of ØRL-8R was observed to be somewhat lower than that of many other phage systems, since it became unstable at 37C and above. Like many other phage-host systems, studies showed that calcium at concentrations between 10^{-3} M and 10^{-4} M may act as a cofactor in adsorption. In some situations it was found that low concentrations of magnesium (10^{-4} M) could substitute for calcium.

Another aspect of study with ØRL-8R included an examination of the host range of the phage. It was found to be infective for one out of 34 freshwater cytophaga isolates tested. Two strains of Chondrococcus columnaris and seven genera of eubacteria demonstrated no sensitivity to the phage.

Isolation and Characterization of a Bacteriophage
for Cytophaga succinicans (RL-8)

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ISOLATION AND CHARACTERIZATION OF A
BACTERIOPHAGE FOR CYTOPHAGA SUCCINICANS (RL-8)

INTRODUCTION AND HISTORICAL REVIEW

Discovery of Bacterial Viruses
and the Origins of Phage Research

The existence of viruses capable of attacking bacteria was first suggested by Twort in 1915 (22). He described a disease of micrococci in which the colonies underwent a "glassy transformation" and could no longer be subcultured into fresh medium. This disease could be transmitted readily by transferring a portion of a glassy area to a normal colony. Among the possible explanations for this phenomenon, Twort suggested that the agent of the disease might be a virus capable of destroying the micrococci. Although Twort was the first to recognize that bacteria are subject to infection with filterable viruses, his report of this phenomenon went without notice until analogous discoveries were made by Felix D'Herelle in 1917 (22). D'Herelle gave the antibacterial virus its present name "bacteriophage" (now often shortened to "phage"). His theories on the nature and uses of this element caused much controversy in the ensuing decades. D'Herelle did a great deal of work on basic techniques used in the study of phage. In a lengthy book published in 1926 he described

the essential features of the phage phenomenon, explained methods of titering lysates, and discussed many aspects of the phage life cycle which have been verified by subsequent research.

During the first twenty years of phage research many controversies arose among investigators over the true nature of the bacteriolytic phenomenon. André Gratia and Jules Bordet were only two of numerous opponents of D'Herelle. Their efforts to prove their own theories retarded the progress of objective and scientific phage research for some time. (22)

A more scientific approach to the study of bacteriophages began to develop in the 1930's with the work of Burnet and Schlesinger. Their contributions to phage research became the foundations for the modern fields of bacterial genetics and molecular biology. Burnet (22) carried out extensive work on the physical and physiological properties of phage. His studies included investigations on the antigenic composition of phages and on the mechanism of adsorption of the phage to the host cell. Schlesinger (22) concentrated on the chemical and physicochemical methods of studying phage. He was the first to investigate the size and mass of the phage particle, as well as its chemical composition and the kinetics of its adsorption to the bacterial cell.

When the physicist Max Delbrück entered the field of phage research in 1938, the entire trend of bacteriophage research changed.

Delbrück considered phage to be ideal objects for the study of the mechanism of biological self-replication. Delbrück, with Emory Ellis (22), designed the one-step growth experiment to demonstrate the intracellular process of phage development. He initiated a new school of phage workers who, by concentrating their efforts on the T-phages of Escherichia coli, developed the phage system as a tool for the study of hereditary processes. The results of Delbrück's efforts and influence have made extensive contributions to the field of molecular biology. In the past thirty years, the study of bacteriophages has expanded past the phage systems of E. coli to include many other host-virus systems, and the applications, modifications, and extensions of this work have become almost limitless.

Characteristics of Bacteriophages

Bacteriophages are subcellular entities which are dependent upon bacterial cells for reproduction, and are very elementary in structure and composition. The phage particle consists of a nucleic acid core surrounded by a protein coat (7). Its composition is approximately 50 to 60% protein, 40 to 50% nucleic acid, and a trace of lipid (6). The nucleic acid core is in the form of a long filamentous molecule, consisting of ribonucleic acid (RNA), or either single- or double-stranded deoxyribonucleic acid (DNA). The protein coat is an assembly of identical morphological units which may be

composed of smaller subunits. Modern terminology defines the nucleic acid as the core, the whole protein coat as the capsid, and the morphological subunits as capsomeres. The complete infectious particle is termed the virion (7).

Bradley (7) has done extensive work on the ultrastructure of bacteriophages. He claims that all viruses are built on strict geometrical lines. Based on their structural morphology, phages can be classified into six morphological types. Three of these forms have a hexagonal head with a tail structure. They vary on the basis of their tail length and flexibility, as well as the presence or absence of a contractile sheath and/or tail appendages. Of the three remaining types, two consist of symmetrical hexagonal structures, one with large capsomeres at each apex of the hexagon, the other without these components. The sixth type is a long, flexible filament, with no additional structures.

Bradley (7) maintains that the fundamental structural differences described above represent differences in nucleic acid. Based on his classification of viral properties, the three head-tail groups contain double-stranded DNA; the hexagonal form with large capsomeres and the filamentous type contain single-stranded DNA; and, the simple hexagon has single-stranded RNA.

A survey of the types of phage particles active against various groups of bacteria can give some indication of the size range of

bacteriophages. Coliphages range from the T-even phages with heads 800 to 1,000 Å in diameter and tails 1,000 Å long, to the small icosahedral phages like ϕ X174 and f2 with diameters of about 200 Å. Some phages of Serratia and Aerobacter have irregular heads 1350 Å in diameter and massive tails 2350 Å by 275 Å. The filamentous phage of Pseudomonas is 16,000 Å in length, with a diameter of 75 Å. One Bacillus phage, $\phi_{\mu-4}$, is so small (100 Å) that no obvious structure has been discerned. (7)

The stability of the bacteriophage particle also has been used as a criterion in phage classification. Phage can be very stable under the appropriate conditions, but the environmental factors which affect this stability can act differently on different phage particles. In general, phage are inactivated by substances or conditions that denature proteins, or that react chemically with proteins or nucleic acids. (1)

Phages are generally stable in their own lysates, provided there are no specific inactivating agents present from the lysed cells, and there are suitable electrolytes present. Purified phage particles are usually stable in neutral, buffered solutions, using distilled water, with 0.1 M NaCl and 0.001 M $MgCl_2$. Low temperatures also favor phage stability, especially if other conditions are suboptimal. As an example, phages are usually stable over a pH range of 5 to 8, but at low temperatures this can be extended to 4 to 9 or 10. (1)

Chemical agents which may inactivate phages in varying degrees include detergents, chelating agents, urea and urethane, mustard gas and nitrogen mustard, some alcohol mixtures, and ether. Formaldehyde and oxidizing agents also inactivate phage particles, but mild reducing agents have no harmful effect. And some agents which are lethal to bacteria have little or no effect on phage activity, such as cyanide, fluoride, and thymol. (1)

There are also certain physical treatments which may inactivate phages under the appropriate conditions. These include sonic vibrations, surface denaturation, heat inactivation, and osmotic shock. Heat inactivation is of particular importance, since each individual phage has a characteristic inactivation temperature. This inactivation follows the pattern of first order kinetics, presumably because it involves protein denaturation. In chemically defined media the heat susceptibility of the phage depends on the chemical composition of the medium. (1)

Infection of a bacterium with a bacteriophage may lead to two different responses. One results in bacterial lysogeny and the other in cell lysis. The lysogenic response is the more complicated of the two and may result from the infection of a susceptible bacterium with a temperate phage. In this situation the phage genome is carried by the bacterial cell from generation to generation in a noninfectious form. Studies with the coliphages have shown that under these

conditions the phage genome may be integrated into the host chromosome. Occasionally this lysogenic relationship is destroyed, and infectious phage particles are synthesized and released by lysis of the host cell. Other lysogenic cells infected by the freed phage are immune to them, are not lysed, and continue to grow normally. To detect the free phage it is necessary to infect an indicator strain of the host organism which can permit phage production.

In the case of the lytic response, the phage does not establish a dormant state in the host cell. Instead, after a phage infects a cell, the cell undergoes lysis, liberating a burst of new phage progeny. Phage that enter the lytic cycle are termed virulent. There is also a special class of virulent phage (intermediate) which exhibits extreme virulence and does irreparable damage to the cell. It should be pointed out that all types of phage, either temperate or virulent, can follow the productive cycle in some host system and be detected by cell lysis. This process of phage reproduction has been termed vegetative multiplication.

Four major steps in vegetative phage multiplication are recognized. These are the adsorption of the phage to the host cell, the injection of its genetic material, intracellular multiplication, and the release of the progeny phage by cell lysis. The first of these steps, adsorption, involves the attachment of the phage to the surface of the cell or to some cellular appendage. Most phage adsorb to the cell

wall, but electron micrographs have shown that some infect via pili, or even attach to the flagella (7). Adsorption can be studied quantitatively by following the disappearance of free phage from a mixture with bacteria before phage replication occurs (18).

The rate of phage adsorption follows first order kinetics with respect to the concentration of both bacteria and phage. The ratio of phage to host cells is termed multiplicity of infection (MOI) and actually refers to the number of phage adsorbed per bacterium, though it is used even when the ratio is lower than one. (18) It has been proposed that adsorption is a two-step process, starting with a reversible attachment which, after a short while, is followed by irreversible attachment (1).

Adsorption is a complex process involving an interaction between specific phage components and specific bacterial receptor sites. The phage components are found on the protein coat of the infective particle. In the larger phage types, the tail or tail fibers mediate in attachment. For phages with no tail structures, the nature of the attachment structure has not been conclusively demonstrated. On the bacterial surface, the receptor sites seem to be areas of antigenic complementation with the phage structure, however, the exact nature of the interactions is still obscure. (18)

The second stage of vegetative multiplication is the injection of the phage genome. Unlike all the other aspects of this process, it

cannot be observed with the electron microscope (7). However, other methods have been employed to determine the events which occur during the injection step. Hershey and Chase (22) were the first to carry out experiments which showed that the entire phage particle does not infect the host cell. They first labeled the coliphage T2 with either one of two radioactive isotopes, P^{32} (in the nucleic acid), or S^{35} (in the proteins). Different aliquots of an E. coli culture were then infected with purified stocks of either P^{32} - or S^{35} -labeled T2 phage. After allowing a period for adsorption of the particles, infected cells were separated from free phage by low-speed centrifugation. These cells, resuspended in fresh medium, were agitated in a Waring blender to strip off any portion of the phage which may not have entered the cell. By measuring the type of radioactivity remaining after this treatment, Hershey and Chase found that 75 to 80% of the adsorbed phage protein, labeled with S^{35} , was sheared off of the cells. Only 20 to 35% of the adsorbed P^{32} could be removed from the bacteria. The results of these studies demonstrated that the phage nucleic acid enters the host at injection, but its protein coat remains outside the cell.

Numerous studies have been carried out on the third phase of the lytic cycle, intracellular multiplication. By use of the electron microscope ultrathin sections of infected host cells can be observed for intracellular changes (7). Uninfected cells normally have distinct

dense areas representing their chromatin, or nuclear material. A few minutes after T-phage infection, E. coli cells show no organized nuclear bodies. After a longer time interval variable amounts of granular material can be observed. In some studies, morphological elements of the phage become visible as head components. Eventually, intact phage can be observed within the cytoplasm, and cell lysis occurs shortly thereafter. These electron microscopic observations have been carried out in conjunction with chemical analyses of infected cells. It has been shown that the phage nucleic acid breaks down the host DNA and takes over the metabolic mechanisms of the cell. The nucleic acid of the phage particle directs the replication of more of its own nucleic acid, and then uses cell constituents to construct protein shells for each new phage. When all possible progeny have been assembled, the cell is lysed and cycles of reinfection can occur within the bacterial culture. (18)

Quantitative studies of intracellular multiplication have been made possible by the application of the one-step growth experiment of Ellis and Delbrück (18). This experiment involves mixing phage with a concentrated suspension of sensitive bacteria under standard cultural conditions. The phage are allowed to adsorb to the cells for a brief period, then the mixture is diluted to an extent that drastically reduces the chances of phage-cell encounters, effectively stopping adsorption. Samples of this diluted mixture are taken at intervals

and assayed for the number of infectious particles present. From this type of experiment a characteristic growth curve emerges, with several distinct phases of development. First there is a low plateau of infective centers which represents the latent period. Here the phage counts are constant because each infected cell, when plated, produces only one plaque, regardless of its intracellular phage content at the moment of plating. The next phase is a rise in the number of infectious centers which represents the lysis of the infected cells and the release of progeny phage. This rise ends in a final plateau, due to the completion of the lysis of the cell population. Because the mixture was highly diluted, there should be no new reinfection of bacteria by the phage progeny.

The one-step growth experiment is a very useful tool in the study of bacteriophages because it allows the observation of a single cycle of phage reproduction, and also provides some characteristic data which is distinctive for each phage-host system. One of these properties is the burst size, or average yield of phage per infected bacterial cell. This is determined by dividing the total number of liberated phage by the number of initially infected bacteria. Other distinctive characteristics of a phage-host system which can be observed in one-step growth experiments include the length of the latent period and the rise period. (18)

Adams (1) discusses a number of phage properties which are

commonly used as taxonomic criteria for bacteriophages. One of these is the serological relationship between different strains of phage. Other criteria include the size and morphology of the phage particle, its chemical composition, the length of its latent period, and its susceptibility to inactivation by various chemical agents and physical treatments. Distinctive physiological properties, such as ionic requirements for adsorption, also contribute to the classification of phage. All these properties can demonstrate resemblances and differences between any two phage strains. If two phages seem to be closely related, based on the above criteria, Adams proposes the use of mixed infection experiments with a common sensitive host strain. If genetic recombination is possible in the development of two phages within the same host, the two strains may be classified in the same species. Mutual exclusion (where only one of the two phages reproduces) would indicate that the phages are not the same species.

Myxobacterial Phages

Since Delbrück initiated research on the T-phages of E. coli, the study of bacteriophages has expanded to very nearly all groups of bacteria. This is particularly true for the members of the Eubacteriales, the true bacteria. In many of the groups of higher bacteria, bacteriophages have not yet been discovered, primarily because very little is known about the microorganisms themselves. According to

Stent (22), phages have not been found for the spirochetes, the budding bacteria, the pleuropneumonia group, or the rickettsias. Luria (18) maintains that no phages have yet been found for the iron, sulfur, or nitrifying bacteria. And both authors state that no phages have been reported for the gliding bacteria, classified in Bergey's Manual of Determinative Bacteriology (8) as the order Myxobacterales. However, in 1955 Anacker and Ordal (2) reported the first phage isolated against a member of the myxobacteria. The sensitive organism was Chondrococcus columnaris, an aquatic fruiting myxobacterium, and a fish pathogen. Since then a number of reports have appeared in the literature regarding the isolation of myxobacterial viruses.

It was of particular interest to find bacteriophages for the myxobacteria because they represent a group of microorganisms very different from the eubacteria. The order Myxobacterales consists of organisms whose vegetative cells are flexible, gram negative rods of low refractility, with the capacity to glide over solid surfaces. The colonies produced by these organisms are very characteristic, having a thin, flat, rapidly extending edge in a monolayer of cells. This extending colony edge may leave a trail of slime as it moves. (8)

The higher myxobacteria, which include the genera Chondrococcus and Myxococcus, develop fruiting structures which contain microcysts. These structures are resting forms of the vegetative cells. The lower myxobacteria, primarily the genus Cytophaga,

produce no fruiting structures and form no resting cells, though they can remain viable over long periods of time under the proper conditions. The genus Sporocytophaga includes a group of myxobacteria which are intermediate between the higher and lower myxobacteria. These organisms produce microcysts, but do not form fruiting structures. (8)

Since the initial report on the bacteriophage isolated against Chondrococcus columnaris, further studies have been carried out to characterize additional phage isolates for this organism. These studies were undertaken, in part, to compare myxobacterial phages with those of the eubacteria. Kingsbury and Pacha in 1964 (17) and Kingsbury and Ordal in 1966 (16) reported the major characteristics of a selected phage isolate. Morphologically it resembled the coliphage T2, having a similar head, 600 \AA in diameter, and tail, $1,000 \text{ \AA}$ long and 200 \AA wide. This phage, C2, also had a well defined neck joining the head and tail. Most properties reported for this phage were very much like those of similar phages for eubacteria. It did have a longer latent period (100 minutes); however, this is probably due to the slower generation time of the host organism. The rise period for phage C2 was 90 minutes, also longer than that of eubacterial phages, and its burst size was 23. It was found to require 4×10^{-3} M calcium for adsorption.

In 1964 (10) and 1966 (11) Burchard and Dworkin reported the

isolation of a bacteriophage (MX-1) for Myxococcus xanthus from cow dung. It too had a polyhedral head with a tail, including a sheath and tail plate. The head was 750 \AA in diameter; the tail was $1,000 \text{ \AA}$ long. Like the Chondrococcus phages, its nucleic acid was DNA, and it had a lengthy latent period of 120 minutes. The rise period for phage MX-1 was also 120 minutes, culminating in a burst size of 100 phage per cell. Like phage C2, MX-1 required calcium for adsorption (10^{-3} M), as well as 10^{-2} M monovalent cation.

Spencer (21) in 1960 isolated several bacteriophages from sea water samples, one of which was subsequently found to be specific for a marine cytophaga, Cytophaga marinoflava. Extensive electron microscopic work has been done on this phage by Valentine, et al. (25) and Valentine and Chapman (24). Both negative stained preparations of the phage alone and ultrathin sections of the infected cell have been studied. This phage isolate also contained DNA and had a conventional head, 600 \AA in diameter, and tail, 860 to $1,000 \text{ \AA}$ in length. Chen et al. (12) has made preliminary studies of the chemical and physical properties of this cytophaga phage. He reported that the phage, NCMB 385, had a latent period of 180 minutes and a burst size of 20.

Stürzenhofecker (23) reported preliminary findings of bacteriophage isolates for another freshwater myxobacterium, Sporocytophaga cauliformis. A number of phages were isolated from freshwater,

plankton, and mud samples of surface streams. These phage isolates exhibited typical bacteriophage morphology (spherical head and tail) and physiology. As for all the other myxobacterial phages, the latent period was much longer than that of similar eubacterial viruses. The data given for a representative phage included: a head diameter of 850 to 1,000 Å, a short tail of undetermined length, a latent period of 150 minutes, a rise period of 90 minutes, and a burst size of 81. It was also reported that calcium (10^{-3} M) enhanced phage production in broth cultures. In many respects, the Sporocytophaga phages were very similar to the phages described for eubacteria, as well as those isolated for the myxobacteria.

The present investigation was initiated in an attempt to isolate and subsequently characterize a phage infective for another myxobacterium, Cytophaga succinicans (RL-8). This bacterium, which was isolated and characterized by Anderson and Ordal (3, 4), is a non-pathogenic, facultatively anaerobic, freshwater myxobacterium which exhibits a CO_2 -dependent requirement for anaerobic glucose fermentation. Fermentative myxobacteria have rarely been encountered, and Cytophaga succinicans was selected as the host strain in this study, in part because of its unique metabolism. This bacterium was also chosen because it is a freshwater cytophaga, a group of organisms for which no phage have previously been reported. It is the hope of the author that the present investigation will contribute

to the further characterization of Cytophaga succinicans, as well as assisting in future studies of similar organisms and their viruses.

MATERIALS AND METHODS

The bacteriophage host, Cytophaga succinicans (RL-8) was obtained from E. J. Ordal, University of Washington. Stock cultures of the organism were maintained in deeps of cytophaga agar consisting of 0.05% tryptone, 0.05% yeast extract, 0.02% beef extract, 0.02% sodium acetate, and 0.4% agar. The cultures were grown at 25C for several days, checked for purity by plating, and stored in the refrigerator at 4C. These cultures were transferred monthly. When it was necessary to streak out the culture, cytophaga medium containing 1.5% agar was used. Cytophaga plates were dried for one to two days before using, to restrict the spreading of the colonies.

Liquid cultures of C. succinicans were grown in tryptone broth. This medium contained 0.5% tryptone and 0.002% CaCl_2 at pH 7.2. Log phase cultures were used for all phage work. These cultures were obtained by transferring an overnight tryptone broth culture to fresh medium and incubating for three to four hours at 25C.

A streptomycin-resistant mutant of C. succinicans was obtained by selection using the gradient plate technique (9). The gradient plates were prepared by pouring a bottom layer of cytophaga agar containing 10 $\mu\text{g}/\text{ml}$ streptomycin (Nutritional Biochemicals Corp., Ohio) into each plate, and allowing the agar to harden at an angle so that one edge of the plates contained no agar. When solid, the plates

were placed flat and cytophaga agar with no added streptomycin was poured over the bottom layer to provide an even agar surface which contained varying concentrations of the antibiotic. These plates were dried overnight before 0.1 ml of a broth culture of C. succinicans was spread over the surface of the agar.

A number of colonies which exhibited some resistance on the gradient plates were selected and inoculated into cytophaga deeps with 10 $\mu\text{g}/\text{ml}$ streptomycin. The cultures which grew on this medium, after ten days incubation at 25C, were subsequently transferred into cytophaga deeps containing increasing amounts of streptomycin. The mutant selected using this method was resistant to 50 $\mu\text{g}/\text{ml}$ streptomycin and was maintained in cytophaga deeps with that level of antibiotic. Liquid cultures of the organism were grown in tryptone broth containing 50 $\mu\text{g}/\text{ml}$ streptomycin.

Isolation of the bacteriophage followed the general procedure outlined in Adams (1). Thirty mls of a water sample was combined with 3 mls of 10X concentrated tryptone broth and inoculated with 1 ml of the host strain. This culture was incubated at 18C for 24 hours, then refrigerated overnight. The cells were removed by centrifugation at 10,000 RPM for ten minutes and the supernatant was retained and treated to eliminate viable bacterial cells by addition of 3 mls of chloroform. This sample was stored in the refrigerator at 4C and tested for the presence of phage using the overlay plating

technique described by Adams (1).

Media used for the myxobacterial-myxophage system were altered to provide nutrient concentrations suitable for the growth of C. succinicans. The soft agar overlay consisted of 2.5 mls of a medium containing 0.5% tryptone, 0.002% CaCl₂, and 0.7% agar. The same medium was used to prepare base plates except that the agar concentration was increased to 1.1%. When plating streptomycin-resistant cells, the base agar was supplemented with 10 µg/ml streptomycin. Four to eight drops of a log phase culture (approximately 1×10^7 cells/ml) were used for a background inoculum. The plates were incubated at 18C and plaques were counted between 24 and 48 hours after plating.

Broth replication in an aerated static culture was used to prepare phage stocks. For this purpose a 2500-ml Fernbach flask containing 200 mls of standard tryptone broth was inoculated with phage and log phase cells at an input ratio of about 0.1 phage/cell. The flask was incubated at 18C for three days, and the phage harvested by centrifugation. The supernatant was freed of viable bacterial cells by either Millipore filtration or addition of chloroform. All phage stocks were stored under refrigeration at 4C.

Broth replication was also studied under anaerobic conditions. For this work the fermentation medium developed by Anderson and Ordal (4) was used. This medium contained a basal solution of

0.18% peptone, 0.09% yeast extract, and 0.09% beef extract, adjusted to pH 7.0 before autoclaving. To this solution was added filter-sterilized 0.18% sodium bicarbonate and 0.9% glucose. Anaerobic conditions were obtained with a BBL #06-200 GASPAK anaerobic jar.

Adsorption studies were carried out using a differential plating procedure on streptomycin as described by Bertani (5). This method involved adsorbing the phage to normal, streptomycin-sensitive host cells under the desired conditions. Samples taken at various intervals were plated on normal cells using standard tryptone medium to determine total phage and on streptomycin-resistant cells to detect free, unadsorbed phage. Streptomycin-sensitive cells infected with phage cannot grow when plated on streptomycin agar, and no plaques are produced by these cells. The free phage, however, can adsorb to the streptomycin-resistant host strain, and as a result all plaques on the streptomycin plates represent only unadsorbed phage.

The single-step growth experiment was performed by the method of Delbrück and Luria (13). Essentially, the procedure involved allowing adsorption to occur for 30 minutes with an initial ratio of about 0.05 phage/cell. At the end of the adsorption period, the amount of free phage remaining was determined from a 1:100 dilution of the sample, using the streptomycin plating technique described above. An aliquot was taken from the diluted adsorption sample and further diluted 1:20 into the first growth flask. From this first

growth flask, a sample was diluted 1:10 into a second growth flask. Periodic samples were taken from both flasks and plated on normal cells for total phage present. The number of phage released per infected cell was calculated by dividing the total number of phage produced by the number of initially infected cells, after first subtracting the amount of unadsorbed phage from both values.

Phage prepared for observation with the electron microscope were concentrated by centrifugation of a 20 ml aliquot of 6×10^8 phage/ml in the Spinco L-2 ultracentrifuge at 20,000 RPM for three hours. The viscous, soft pellet obtained upon decanting the supernatant was resuspended in 0.2 ml of 0.5% sucrose, allowed to stand overnight, without agitation, and was titered prior to use. A drop of this suspension, containing 4×10^{10} infective particles/ml, was added to a 400 mesh, flat copper grid previously coated with a formvar film, and allowed to stand for ten minutes. The excess was removed and the suspension allowed to dry. This process was repeated on the same grid, to insure that sufficient numbers of phage were present. When the grid was dry, a drop of 1% phosphotungstic acid, pH 6.8, was added to the surface and immediately removed with a piece of filter paper. Grids prepared in this way were observed for phage particles using a Philips EM 300 electron microscope.

RESULTS

Isolation of a Phage Infecting
Cytophaga succinicans (RL-8)

The natural habitat of *C. succinicans* is freshwater. This organism has been isolated from various aquatic habitats, including fish aquaria and fish from natural waters. For this reason it was thought that a bacteriophage infective against this organism might be found in a freshwater environment. Oak Creek, a small stream near Corvallis, Oregon, was arbitrarily chosen as the source of the water samples used in the isolation procedure, described in the section on Materials and Methods. Both filter-sterilized and unfiltered water samples were incubated with the cells in a tryptone medium. In both cultures dense growth was obtained in 24 hours at 18C. Although lysis of the cultures was not observed, samples were clarified and the supernatants tested for the presence of phage by the plaque assay procedure (1). Plaques characteristic of those produced by a phage were found to arise only from the unfiltered water sample. The phage was obtained by picking from the center of an isolated plaque and transferring into 1 ml of standard tryptone broth. A ten-fold series of dilutions of this sample were plated and observed for the transmissibility of the phage. Plaques were observed on the plates prepared, and a proportional decrease in titer was noted upon

dilution. From these findings it was concluded that the lytic agent was a bacteriophage. The isolate was designated ØRL-8R.

Phage stocks were initially prepared by plating a series of dilutions of the 1 ml suspension. Those plates with near or complete lysis were harvested by adding a small volume of broth to the plates and scraping off the overlay agar with a bent glass rod. The agar and cells were removed by centrifugation and the supernatant was retained as the phage stock.

Development of the Plaque Assay Procedure

On the original isolation plates it was noted that the cell lawns were very granular and gave rise to uneven and poorly defined plaques. Smooth lawns with the clearest plaques possible are desirable for accurate phage assays. For this reason an investigation was made to detect the cause of the poor lawns. Factors studied included the growth phase of the seed cells, the method of mixing the overlay agar tubes (by hand or with a Vortex mixer), and the length of time the cells remained in the waterbath at 46C before plating. Results of these studies showed that cells of all different growth phases produced smooth lawns, as did cells mixed into the agar either by hand or machine. The factor which had the greatest effect on the smoothness of the lawns was the length of time the cells were left in the waterbath. After five minutes of incubation in the waterbath the

lawns obtained were very poor. This could suggest that the cells do not remain viable at 46C for more than a few minutes. The plating method was subsequently altered by adding the cell inoculum to each tube immediately prior to plating the sample.

Once smooth cell lawns were obtained, it was of interest to determine the optimum conditions for plaque formation, as well as the efficiency of plating (EOP). Plaque formation was studied at 25C because this is the optimum growth temperature of the host organism. It was also studied at 18C because this is near the maximum temperature of the environment from which the phage was isolated. Initial observations of plates incubated at both temperatures showed that the cell lawns were very smooth under both conditions. However, plaques produced on plates incubated at 25C were much smaller and more difficult to see. A higher plating efficiency also was observed on plates incubated at 18C, as shown in Table 1. Subsequently, all phage plates were incubated at the lower temperature.

Table 1. Efficiency of Plating of ØRL-8R on Cytophaga succinicans at 18C and 25C.

Temperature	Phage Titer
18C	2.5×10^9
25C	$\sim 1 \times 10^5$

The results of these initial temperature studies seemed to indicate some relationship between the size of the plaques and the density of the cell lawn. The lawns on the 25C plates were observed to be much more dense, while those which developed at 18C were lighter, probably due to the slower growth of the host cells at the lower temperature. To test the effect of cell density on plaque size, a culture of cells was grown to an OD₅₂₅ of 0.80, and a series of dilutions was prepared and plated with the phage. The EOP remained the same for all cell concentrations tested, however, a definite change was noted in plaque size, as can be seen in Figure 1. The plaques were relatively large at the lowest optical density plated, and very small at the highest optical density plated. On the basis of these studies of plaque formation, all phage assays were made using a host culture of an OD₅₂₅ in the range of 0.05. An aliquot of the phage and a few drops of the cell inoculum were added to a tube of overlay agar. This mixture was immediately poured onto the basal agar plate and allowed to solidify. This was done in order to avoid prolonged exposure of the cells to a temperature of 46C. Plates were incubated at 18C to allow the most efficient production of plaques.

Plaque Morphology

The plaques which developed on plates prepared in the manner

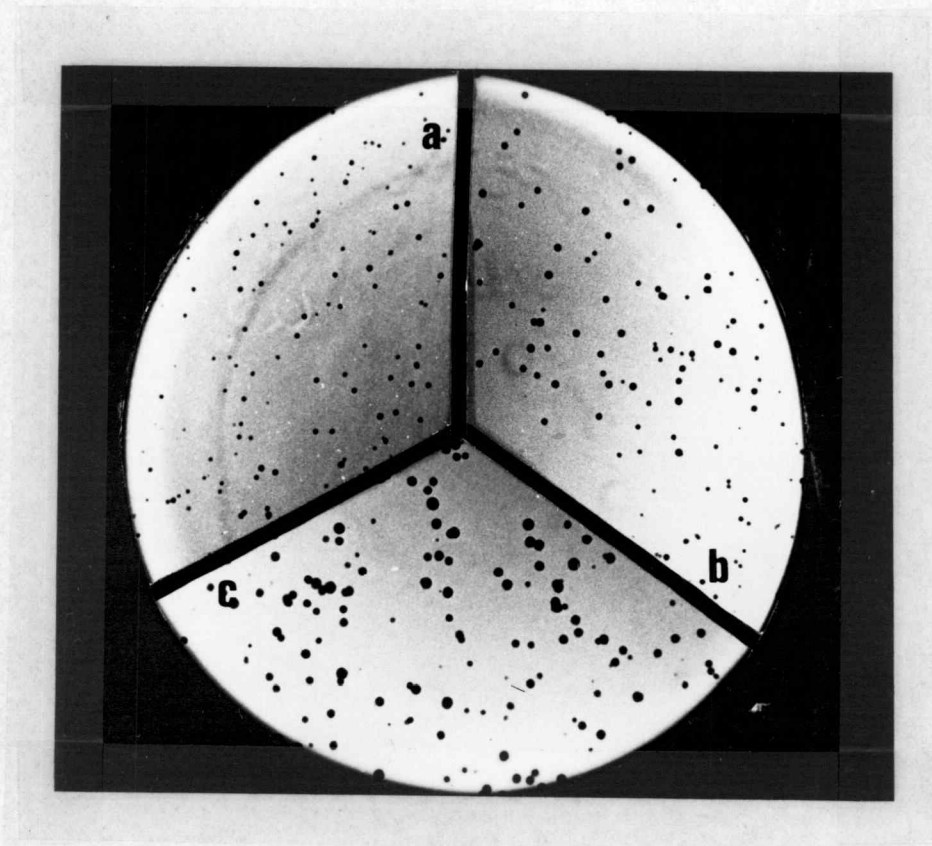


Figure 1. Variation in plaque size with the density of the cell inoculum on overlay plates. OD_{525} of (a) was 0.80, (b) 0.29, (c) 0.06.

described above were clear and round with well defined edges (see Figure 1). They normally ranged in size from 0.25 to 1.0 mm in diameter. Under all conditions studied, the plaques developed between 24 to 48 hours, depending on how long it took the host cells to grow to a visible layer. Once the plaques developed, however, they did not increase in size and maintained their appearance for several weeks. Resistant colonies sometimes appeared in the center of the plaques only after the plates were over a week old.

Particle Morphology

While the detection and enumeration of bacteriophage is normally carried out using the overlay plating method (1), actual observations of the phage particle require more specialized techniques. Details of phage morphology are best studied with the use of the electron microscope. In this investigation, initial observations of the phage isolate were made with an RCA EMU 2D electron microscope. The resulting images were very small and indistinct due to the low resolving power of this type of electron microscope. Very few phages were found in preliminary observations; consequently, efforts were made to increase the titer of the phage suspension before attempting further electron microscopic work.

Initial procedures used to increase phage titer included centrifugation at different speeds and times, as well as dialysis of phage

suspensions against 40% polyethylene glycol. Although the latter method succeeded in increasing the phage titer, cells, debris, and tryptone were also concentrated, and the preparation was unsuitable for examination. Low speed centrifugation to remove cells and debris, followed by high speed centrifugation to pellet the phage, provided the most satisfactory material for study. However, many cells and other particulate materials were still retained with the pelleted phage in these preparations. Considerable improvement in the purification procedure will be necessary before high quality preparations of the phage can be observed.

Figure 2 shows a negatively stained representative phage particle at a final magnification of 364,320X, observed with a Philips EM 300 electron microscope. The phage head appeared to be six-sided, or hexagonal in shape. Calculations from several similar micrographs showed the head diameter to be approximately 1180 \AA . The tail was found to be 1900 \AA long, and 250 \AA wide. There was evidence in all of the micrographs examined of a definite neck at the base of the head, where the tail was attached. It should also be noted that there appeared to be two prongs or protrusions at the end of the tail. These were also evident on the tails of several other particles observed, and could possibly function in the attachment of the phage to the cell. None of the electron micrographs examined provided evidence for the presence of a contractile sheath or tail

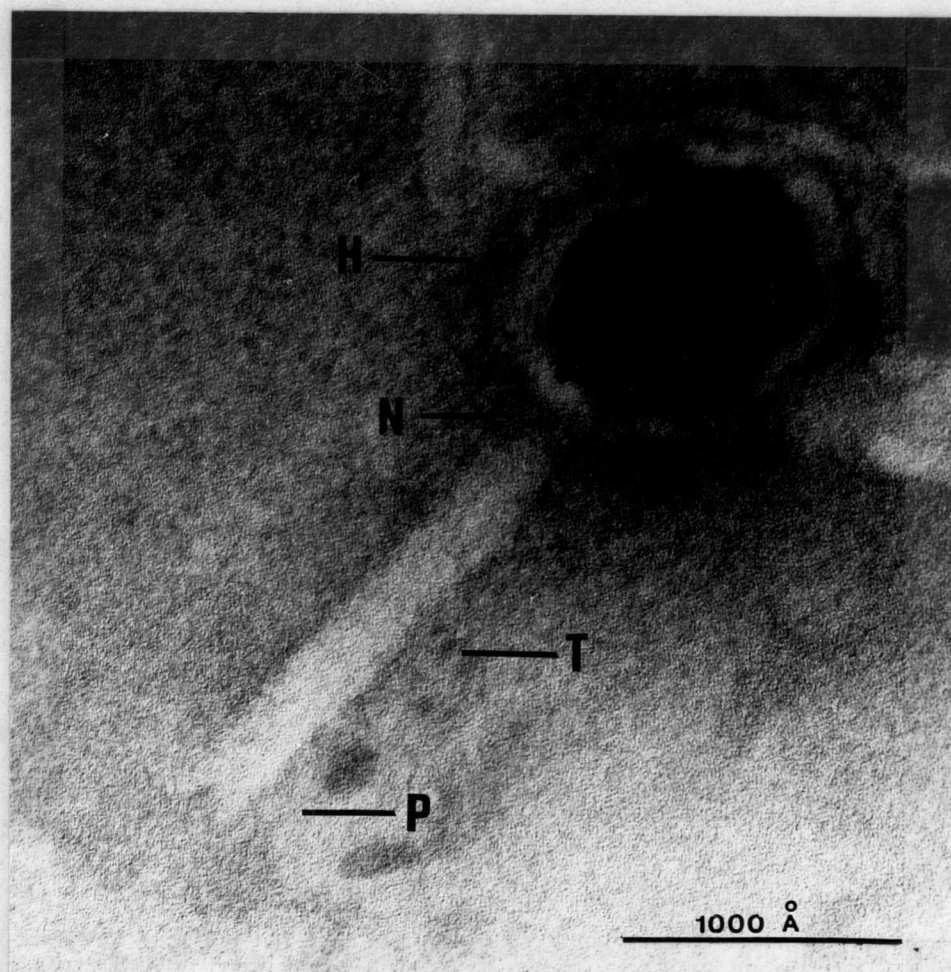


Figure 2. Electron micrograph of ϕ RL-8R for *Cytophaga succinicans* (RL-8). Negative stain with phosphotungstic acid. Magnification 364,320X. H indicates the phage head; N, the neck area of the tail; T, the tail; P, the prongs at the base of the tail.

fibers, however, the quality of the micrographs may have obscured these structures. It should be pointed out that the dark inner region of the head (Figure 2) indicated that it was empty. In future electron microscopic work with this particle, more careful procedures must be developed to keep the head component intact.

Phage Stability

In the study of a bacteriophage, the stability of the particle to various physical and chemical treatments can aid in its characterization. A knowledge of the conditions which increase phage stability is also of importance in determining mechanisms for preserving phage stocks for extended periods of time. As with most phages, ϕ RL-8R was found to be relatively stable in its own lysate. Lysates stored at 4C showed no decrease in titer over periods of at least three months, as shown by Table 2. Other methods tested for increasing the stability of ϕ RL-8R were lyophilization and freezing. Upon lyophilization in skim milk all infectivity was lost, hence this procedure could not be used to maintain the phage. Freezing suspensions of the phage was found to be more successful. Two ml samples of the phage were frozen in small screw cap vials by placing them at -15C. Defrosting was done by allowing the vials to stand at room temperature for about an hour. Gradual inactivation of the phage was detected, as shown in Table 3, although after the initial

decrease in titer which occurred the first week, the phage stock was relatively stable up to six months.

Table 2. Stability of ϕ RL-8R in a Stock Lysate at 4C.

Time (in months)	Phage Titer
0	1.73×10^8
1	1.82×10^8
2	2.1×10^8
3	1.9×10^8

Table 3. Inactivation of ϕ RL-8R by Freezing.

Freezing Time	Phage Titer
0	2.0×10^9
1 day	6.3×10^8
1 week	2.5×10^7
1 month	2.0×10^6
6 months	8.8×10^6

The sensitivity of the phage to heat also was studied. In initial experiments, the inactivation of the phage in tryptone broth was measured at 46C. This temperature was tested to determine how long the phage samples would remain viable prior to plating after being added to overlay agar held at that temperature. Subsequently, thermal stability was determined by subjecting the phage to temperatures ranging from 2 to 50C. Results of these studies are shown in

Figure 3. At 46C, or above, inactivation of the particles was very rapid. At 37C the phage was relatively stable for one hour, however, continued incubation at this temperature caused a gradual loss in titer. At 25C, or below, there was no loss in titer after eight hours of incubation. These results emphasized the importance of plating the phage as rapidly as possible after adding it to overlay agar.

Another physical characteristic which is distinctive for each phage is stability to variations in pH. To test the effect of pH on ØRL-8R, aliquots were suspended in tryptone broth having pH values ranging from 2 to 10. The phage suspensions were incubated at 4C and samples were taken periodically for titration. As shown in Figure 4, the phage was stable over a pH range from 6 to 9. At a pH of 4 or below a rapid inactivation of the particles occurred, whereas under alkaline conditions (pH 10) inactivation occurred at a slower rate.

Sensitivity to chloroform and ether was examined by adding excess quantities of these chemicals to tryptone broth lysates of the phage. The mixtures were shaken and held at 4C. Samples were taken at intervals and assayed for viable phage. The results (Table 4) showed that the phage titer dropped by 38% after four days in the presence of ether. When chloroform was tested, the titer decreased about 84% in eight hours. The results of this study clearly show that ØRL-8R was sensitive to chloroform. For this reason chloroform

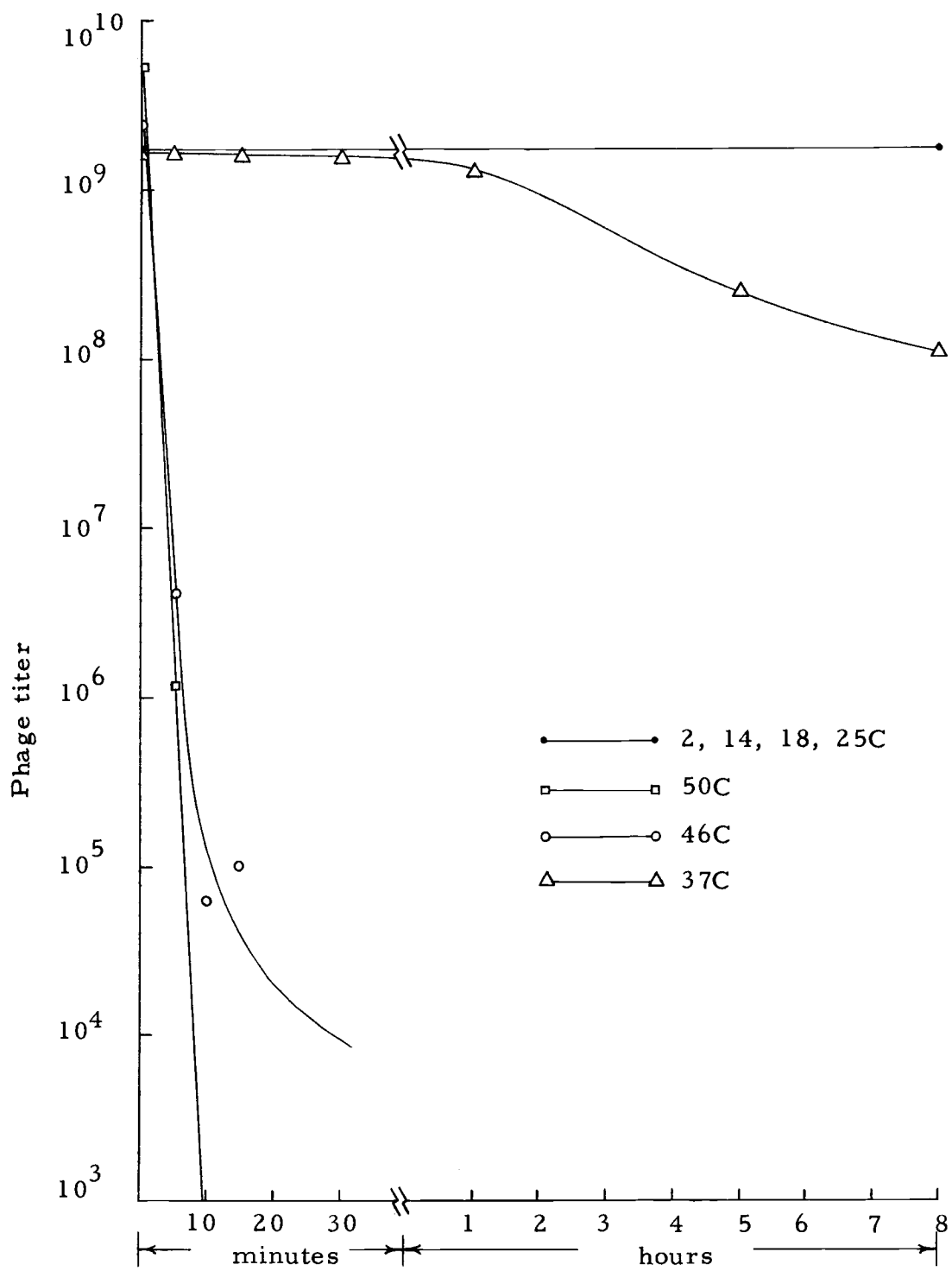


Figure 3. Heat sensitivity of ØRL-8R.

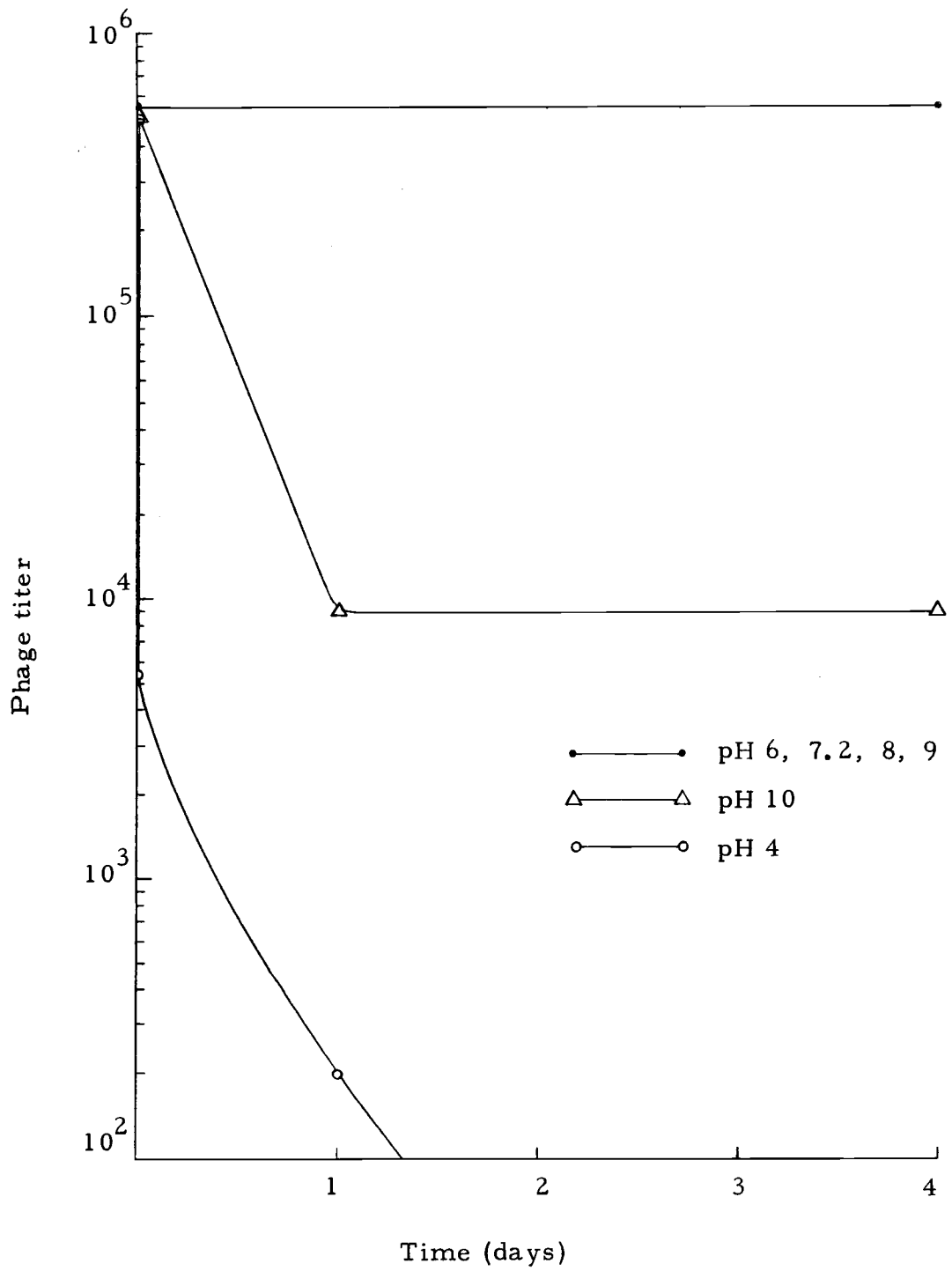


Figure 4. pH sensitivity at ØRL-8R at 4C.

was not used in the latter parts of this study to preserve lysates.

Table 4. Sensitivity of ØRL-8R to Ether and Chloroform.

Agent	Time	Phage Titer	Percent Loss
Ether	0	1.6×10^9	0
	4 days	9.8×10^8	38
Chloroform	0	2.5×10^9	0
	10 minutes	1.3×10^8	48
	1 hour	7.7×10^8	69
	8 hours	3.9×10^8	84

The stability of the phage was measured at 18C in solutions other than tryptone broth, since other suspending media were used in selected experiments. The phage was found to be stable for four hours in the starvation buffer of Denhardt and Sinsheimer (14), pH 7.2. Phage added to casein hydrolysate broth was stable during a 30 minute period. Stability for longer periods of time was not tested in this medium. In distilled water, the phage titer decreased by 74% over a period of 52 hours. When distilled water was supplemented with 10^{-2} M calcium, however, this instability was not evident. The phage was found to be unstable in the fermentation medium of Anderson and Ordal (4), which was used for anaerobic growth of C. succinicans.

Host Range of the Phage

In order to characterize ØRL-8R further, a host range study was done. Ability of phage to infect more than one host organism may indicate some antigenic similarity between the host cells. It may also suggest some genetic affinity between the sensitive organisms (19). In this study, the phage isolate was plated with 34 other cytophagas isolated from freshwater. Only one of these isolates, designated 253 and described in a taxonomic study by Peggy K. Nitsos (20), was sensitive to this phage. This culture was tested with antiserum prepared against the original RL-8 host strain and produced some agglutination, though less than that observed with the normal host.

In addition to the cytophaga isolates tested, two strains of Chondrococcus columnaris were plated with the phage. Neither culture produced plaques with the phage dilutions plated. Several different eubacteria were also tested for sensitivity to the C. succinicans phage. Plaque formation was never observed when the following organisms were used as the host strain: Proteus vulgaris, Serratia marcescens, Bacillus cereus, Escherichia coli, Aerobacter aerogenes, Pseudomonas aeruginosa, and Staphylococcus aureus.

Phage Replication in Broth

In most phage-host systems development can occur within the host in broth cell cultures. Production of phage under these conditions can often be observed by total rupture of the host culture, visible as a lessening in the density of cell suspensions. During the initial isolation procedure in this investigation, no such phenomenon was observed, although phage were produced. It was postulated that the culture conditions used were not optimal for efficient phage production which would result in complete lysis of the broth culture. Because of this possibility, studies were carried out in an effort to enhance phage production in broth by varying procedures and environmental conditions.

In the initial experiments, phage were added to host cells in 50 mls of tryptone broth and incubated at 25C, in both static and shake cultures. After one day of incubation, the static culture was put on a shaker and incubation was continued. No visible culture lysis was observed in either flask. In a second set of experiments, carried out at the same temperature, phage and cells were combined and allowed to stand for 30 minutes and then added to each of four 250-ml culture flasks containing 50 mls of tryptone broth. One of these flasks also contained 0.1% yeast extract to provide a possible growth factor; another included a 0.1% agar suspension with the

tryptone to determine if trace nutrients in agar were necessary for replication; and the third contained 0.003 M NaNO_2 as an inducing agent. The fourth flask had no special additives, but was aerated by bubbling filtered air through the culture. None of these attempts produced clearing of the host culture, though the cells grew well under all conditions. In a subsequent experiment, the incubation temperature was varied. The phage-cell system was allowed to stand for 30 minutes at 18C, and then was added to 50 mls of tryptone broth, and incubated as a static culture at the same temperature. Again, no clearing of the growing host culture was observed.

In order to determine whether culture lysis was dependent on the availability of surface aeration, a new approach was attempted simulating the agar overlay plate method. Preliminary experiments were initiated in which approximately 1/4 inch basal agar was poured into each of three 250-ml flasks and allowed to solidify. Ten mls of tryptone broth was then added to each flask, covering the surface of the agar with a thin layer of liquid. The flasks were inoculated with phage and cells in the proportions used for overlay plating. Of the three flasks prepared, two were incubated as static cultures, one at 18C, the other at 25C. The third flask was incubated on a shaker at 25C. In addition to observing these flasks for evidence of lysis, samples were assayed for phage at zero time, 18 hours, and after 42 hours of incubation. As shown in Table 5, both static and shake

cultures incubated at 25C decreased in phage titer by 99% in 42 hours. This was an unexpected finding, since the host cells grow readily at this temperature. The phage titer of the culture incubated at 18C, however, increased from 4.5×10^6 to 1.9×10^{10} during the same time interval, although no clearing of the culture was observed. An aliquot of the phage-cell suspension was taken from the 18C flask and added to a thin layer of fresh broth, in an attempt to obtain further phage production. After 24 hours at 18C the cell culture was dense, but no increase in phage was detected upon plating. The results of these experiments indicate that, under the conditions employed, phage replication in broth occurs without visible evidence of lysis. Furthermore, these findings suggest that broth replication is temperature sensitive. Replication occurred at 18C, but not at 25C.

Table 5. Effect of Temperature and Aeration on Broth Replication of ØRL-8R.

Time (hours)	Phage titer under conditions of incubation		
	Static, 18C	Static, 25C	Shake, 25C
0	4.5×10^6	3.8×10^6	3.1×10^6
18	1.8×10^{10}	1.4×10^5	1.8×10^6
42	1.9×10^{10}	2.8×10^4	6.2×10^4

To determine if a layer of basal agar was required for phage

production, a modification of the above experiment was carried out at 18C. One culture flask contained ten mls of broth alone, the other held the same volume of broth over a thin layer of basal agar. Phage and cells were added to each flask, and the contents assayed for phage over a period of 24 hours. In both flasks, the phage titer increased from 3×10^6 to 4×10^{10} . No clearing of either culture was noted. From this experiment it is evident that agar was not essential for broth replication of the phage. Replication occurred equally well in tryptone broth alone.

The efficiency of phage production in thin layers of broth suggested that surface aeration might have some influence on the final titer. To test this hypothesis, a 250-ml culture flask containing 100 mls of broth was inoculated with phage and cells. At the same time, 200 mls of broth were added to a 2500-ml Fernbach flask to provide a thin layer of medium, and inoculated with the same proportions of phage and cells. Both flasks were incubated at 18C for four days. Initially the phage titer in each flask was 9×10^4 . At the end of the experiment, the titer in the small culture flask was 2×10^7 , and in the larger Fernbach flask was 6×10^8 . Although it is evident that replication occurred in both flasks, the higher titer obtained in the flask containing a shallow layer of medium suggests that aeration indeed enhances phage reproduction in broth. As a result of the information obtained from these experiments, phage stocks were

routinely prepared by adding approximately 2×10^7 phage and 1×10^8 cells in 200 mls of broth in a Fernbach flask. The flask was always incubated as a static culture at 18C, since no broth reproduction of the phage was ever obtained at higher temperatures. The phage progeny were harvested after three to four days of incubation.

Once it was possible to acquire phage stocks from broth cultures, variations in the harvesting procedure were studied. Removal of cells was carried out by centrifugation. Sterilization of phage-containing supernatants was tried by filtration using both membrane and Seitz filters. Addition of chloroform was also tested as a means of inhibiting bacterial growth. The results of these experiments are shown in Table 6. It will be noted that some phage were lost during centrifugation. Presumably the lost fraction was spun down with the cells. The results presented in Table 6 also show that a reduction in numbers of phage occurred upon filtration. Seitz filtration resulted in virtually a total loss of infective units, whereas membrane filtration (Millipore HA) allowed the recovery of phage, though a definite drop in titer was always noted. Chloroform treatment tended to yield varying drops in the initial titer, and was considered a less reliable method of sterilization than membrane filtration. Although all of the harvesting procedures tested resulted in loss of phage, the final system adopted for harvesting phage stocks consisted of centrifuging the cells at 8,000 RPM for 15 minutes in

250-ml centrifuge bottles. The phage supernatant was then sterilized by membrane (Millipore HA) filtration, though occasionally chloroform was used. Titters were always checked at each step in the harvesting process.

Table 6. Effects of Various Steps in the Harvesting Process on the Titer of Phage Stocks.

Phage Titer	Process			
	Removal of Cells 7,000 RPM, 10 minutes	Sterilization		
		Chloroform	Seitz filter	Membrane filter
Initial	5×10^9	8.5×10^8	2×10^9	8.5×10^8
Final	4×10^9	2.4×10^7	$< 10^2$	1.7×10^8

Although the above procedures were satisfactory for the preparation of phage stocks, the reason or reasons for the absence of lysis and clearing of the culture during this process remained unknown. During one experiment in phage production, when the titer had reached 10^{10} , samples were taken, diluted, and plated on cytophaga agar to isolate colonies from cells present in the phage-host system. Six such colonies were selected at random, inoculated into deeps, and subsequently tested for sensitivity to the phage by overlay plating. All six cultures were found to be resistant to the phage. These cultures were tested with RL-8 antiserum and found to react

as well as the original phage-sensitive strain.

To determine whether phage-resistant cells were present in the normal host culture, the stock culture was plated and five colonies were selected. These isolates were tested for phage sensitivity and four of the five were found to be susceptible. The host stock was plated out again, and 40 colonies were isolated and tested for phage sensitivity. One of the previously isolated sensitive cultures was also plated out, and 20 colony isolates were tested. All 60 cultures were sensitive to the phage, producing normal plaques with the same efficiency as the original host stock. This seemed to indicate that the resistant cells did not arise spontaneously in the host culture, unless exposed to the phage.

These results provided a possible explanation of the inability of the phage to totally lyse host cell cultures. Phage-resistant cells would continue to proliferate and maintain turbidity in the culture, thus obscuring total lysis. These data might also explain why titers higher than 10^{10} were never reached during broth replication of the phage. Phage normally continue to reinfect the culture until no more viable cells remain; if resistant cells arise before this can occur, maximum phage production might be inhibited. Further experiments should be carried out to determine at which point during phage production resistant cells arise, and whether this resistance was due to inability of the cells to adsorb phage, or whether

intracellular phage production was inhibited.

Phage Replication under Anaerobic Conditions

Once phage reproduction in liquid medium had been established, it was possible to investigate other properties of the phage which involve broth replication. Of particular interest in this phage-host system was the possibility of anaerobic production of phage by the host cells. Since C. succinicans is capable of CO₂-dependent anaerobic glucose fermentation (4), it was possible to study phage replication under anaerobic conditions. A preliminary experiment was carried out using the anaerobic medium of Anderson and Ordal (4) to determine if the phage could develop within the host in this medium under aerobic conditions. Ten mls of the fermentation broth were added to each of two 250-ml culture flasks and one was inoculated with both phage and cells at an input ratio of about one phage per cell. The control flask contained the same amount of phage without cells. These flasks were incubated at 18C, and assays were carried out periodically to determine the numbers of phage present. Table 7 shows the results obtained over a period of three days. It is evident from the results obtained in the experimental flask that the phage was capable of reproducing aerobically in this medium. As shown in Table 7, the titer in this flask increased nearly two log units in one day. However, in the control flask, a significant decrease in phage

titer occurred, indicating that the phage was unstable in the fermentation broth.

Table 7. Aerobic Production of ϕ RL-8R by Cytophaga succinicans at 18C in Fermentation Medium.

Time	Experimental Flask	Control Flask
0	1.7×10^7	3.2×10^7
1 day	2.8×10^9	1.1×10^6
2 days	9.0×10^8	3.2×10^5
3 days	1.3×10^8	2.2×10^5

To determine if phage replication was possible under anaerobic conditions, a series of experiments was performed in which the cultures were incubated in a GASPAK anaerobic jar. Since only original and final samples could be taken using the anaerobic jar, four separate experiments were carried out. They were identical in procedure, varying only in the time of the final sample. For each experiment, two experimental tubes and two control tubes were prepared. The experimental tubes contained two mls of fermentation medium, 0.1 ml of phage (6×10^6 phage), and 0.1 ml of cells (6×10^6 cells). The control tubes were identical, lacking only the cell inoculum. One set of tubes, experimental and control, was incubated under anaerobic conditions at 18C, the other aerobically at the same temperature. Sets of nutrient broth tubes inoculated with either

Bacillus megatherium (aerobe) or Clostridium welchii (anaerobe) were incubated with each set of phage tubes, to verify the presence or absence of oxygen. The results of these experiments, as shown in Figure 5, indicated that the phage could not reproduce within the cell under anaerobic conditions. This was not due to an absence of host cell growth, since excellent cell growth, as evidenced by an increase in turbidity, occurred under both aerobic and anaerobic conditions. Both aerobic and anaerobic phage controls confirmed the results of the preliminary experiment, that the phage alone was unstable in the medium used.

Adsorption Studies

One of the characteristic aspects of a phage-host system involves the development of phage progeny within the host cell. The first step in this process is the adsorption of the phage particle to the host. In order to make quantitative studies of intracellular phage development, it is necessary to measure the number of phage which adsorb to, and thus presumably infect, host cells. Consequently, an investigation of the adsorption properties of a phage usually precedes studies of its growth cycle.

Preliminary adsorption experiments were carried out using a method described by Adams (1). This procedure involved adding phage to cells in a broth medium and assaying for the initial amount

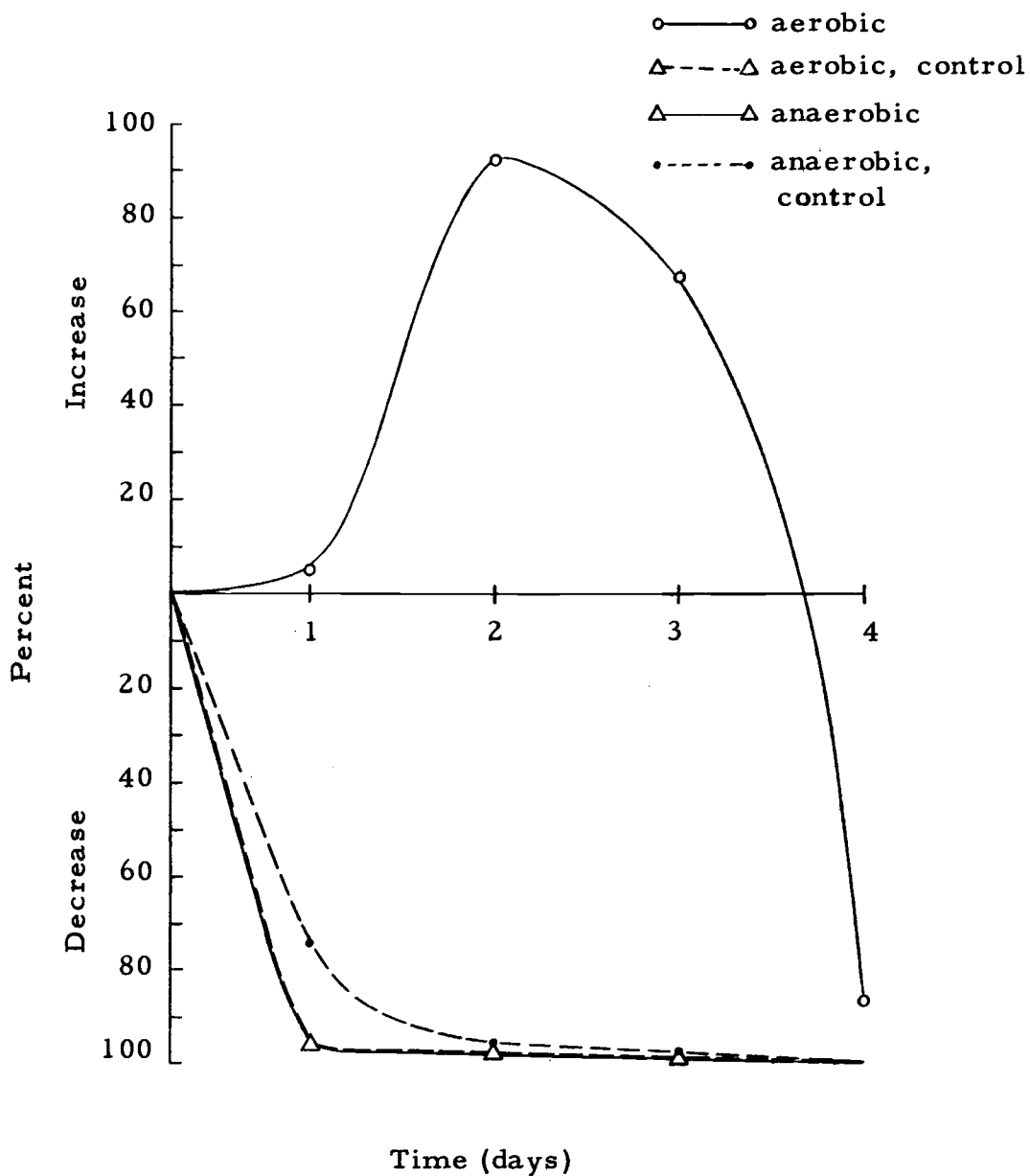


Figure 5. Production of ØRL-8R by Cytophaga succinicans in fermentation medium, under aerobic and anaerobic conditions at 18C.

of phage present. At the end of the adsorption period, a second sample was taken, diluted 1:100 to stop adsorption, and centrifuged to remove cells and any phage adsorbed to them. The supernatant was assayed for free phage, and from this value the percent of adsorption calculated. This type of experiment was used to study the effects of cations, temperature, and degree of aeration on the rate and amount of phage adsorption. In a number of experiments, conflicting results were obtained, and in some cases no adsorption was observed. An example of such findings is shown in the results of three identical experiments (Table 8) where percent adsorption was measured as a function of the age of the host cell culture. It can be seen that even when cells of the same age were tested, the percent of adsorption varied considerably from experiment to experiment.

Table 8. Effect of the Age of Host Cells on Adsorption of ϕ RL-8R to Cytophaga succinicans in Three Identical Experiments.

Age of Cells (hours)	Percent Adsorption		
	Experiment 1	Experiment 2	Experiment 3
0	23	32	37
2	37	0	64
4	55	27	
5			38
6	68	37	
8		30	51
10		23	17

A possible explanation for these inconsistent results was suggested when a control flask in one of these experiments showed a 45% decrease in phage, in the absence of cells. A subsequent experiment was carried out to determine if the centrifugation step was contributing to these results. Quantities of phage normally used in the adsorption experiments were centrifuged under standard conditions. Both Nalgene and glass centrifuge tubes were used to see if one type might be retaining phage more than the other. The decrease in phage titer was found to be 30% with Nalgene tubes and 18% with glass tubes. Because of these variations in phage titer when the suspensions were centrifuged it was evident that the centrifugation technique would not provide reliable results, and another method of measuring adsorption was developed.

As described in Materials and Methods, a streptomycin-resistant host mutant was selected using the gradient plate technique (9). This mutant was used to detect a decrease in free phage by differential plating of the phage-host culture on streptomycin agar. Only phage not adsorbed to cells would produce plaques with the streptomycin-resistant mutant on this agar, since the normal streptomycin-sensitive host was unable to grow under these conditions. In performing adsorption experiments using differential plating on streptomycin agar, several types of control plates were used. Antibiotic-sensitive cells were always plated on streptomycin plates

to insure that they could not grow. For samples where no difference in total and free phage was expected (i. e. initial samples and control samples without cells) dilutions were plated on both host systems, with and without streptomycin, to compare the efficiency of plating with both types of cells.

Adsorption experiments carried out using the streptomycin plating method were all identical in procedure. Phage and early log phase cells were combined in a ratio of approximately 0.03 phage/cell in a 250-ml culture flask containing ten mls of adsorption medium. The phage were allowed to adsorb for 30 minutes at 18C without shaking, since preliminary experiments indicated that agitation did not enhance adsorption. An initial sample was taken from the adsorption flask immediately after addition of the phage, to determine the original amount of phage present. At the end of the 30 minute period, another sample was taken and diluted 1:100 into iced tryptone broth to stop adsorption. All samples were plated on streptomycin-sensitive cells with standard tryptone agar, and on the resistant mutant culture with streptomycin in the basal medium.

The results of typical adsorption experiments are shown in Table 9. Where adsorption was studied in tryptone, the broth was prepared without any added CaCl_2 , and the appropriate concentrations of calcium or magnesium were added to each adsorption flask. Though the percent of adsorption in tryptone alone varied somewhat

from experiment to experiment, calcium concentrations between 10^{-3} M and 10^{-4} M always gave higher amounts of adsorption. From these experiments it appears that CaCl_2 in the proper concentrations is an effective adsorption cofactor. It should also be noted that in the tryptone medium, 10^{-4} M magnesium seemed capable of substituting for calcium.

Table 9. Effects of Various Ions on the Adsorption of ϕ RL-8R to Cytophaga succinicans in Tryptone Broth.

Ionic Environment	Percent Adsorption
0.01 M CaCl_2	33
0.001 M CaCl_2	43
0.0005 M CaCl_2	40
0.0001 M CaCl_2	45
0.01 M MgSO_4	36
0.0001 M MgSO_4	45
Tryptone alone	27

Because a significant amount of adsorption occurred in tryptone alone, attempts were made to study adsorption requirements in other types of basal media. Early log phase cells were washed twice and resuspended in distilled water, with and without cations. Control cells were also prepared by washing in distilled water and resuspending in the original culture supernatant. When these cells were used

in typical adsorption experiments, as described previously, no adsorption could be demonstrated. Previous studies had indicated a rapid loss in viability when host cells were washed with distilled water, and it is possible that the failure to obtain adsorption was due to a lack of viable cells. Perhaps actively metabolizing cells are required for adsorption of ϕ RL-8R. Similar studies carried out with cells suspended in the starvation buffer of Denhardt and Sinsheimer (14), as well as in tryptone broth with 10 μ g/ml streptomycin, seemed to support this theory. In neither situation were the host cells able to grow, and in neither type of experiment was any adsorption observed.

Adsorption was also studied in broth consisting of 0.04% vitamin-free casein hydrolysate and 0.0003% Na_2HPO_4 , pH 7.2. The results are shown in Table 10. Using the procedure described for the previous experiments, much less adsorption was found in the broth alone. The host cells were quite capable of growing in the casein hydrolysate broth and, as with tryptone broth, adsorption increased with the addition of 10^{-3} M calcium. In contrast to the adsorption studies carried out in tryptone broth, MgSO_4 did not enhance adsorption in the casein hydrolysate medium. However, it should be noted that only 10^{-3} M magnesium was tested. It would be desirable to study adsorption in the casein hydrolysate medium using a wider range of cation concentrations.

Table 10. Effects of Various Ions on the Adsorption of ϕ RL-8R to Cytophaga succinicans in Casein Hydrolysate Broth.

Ionic Environment	Percent Adsorption
0.001 M CaCl_2	16.5
0.001 M MgSO_4	4.5
Casein hydrolysate broth alone	3

The results of the adsorption experiments described above indicate CaCl_2 at a concentration of 10^{-3} M to 10^{-4} M enhances the adsorption of ϕ RL-8R to the host cell. Whether or not MgSO_4 can substitute for CaCl_2 as an adsorption cofactor is not clear. It would be desirable to carry out more extensive studies in an attempt to improve the rate and amount of adsorption obtained, as well as to define the adsorption cofactors for ϕ RL-8R more completely. Conditions which could be varied and examined include a wider range of ions, as well as the effect of tryptophane. Temperature effects should also be more carefully examined, since broth replication of the phage was not detected at temperatures above 18C. The casein hydrolysate medium should be satisfactory for these studies, since very little adsorption occurred in the basal medium alone.

It should also be noted that the existence of phage-resistant cells which arise in phage cultures may interfere with these studies,

and may account for much of the variation observed in results of similar experiments. The rate at which these resistant cells arise, as well as whether or not they are capable of adsorbing the phage, should be determined.

Single-Step Growth Cycle

With the development of a method for measuring adsorption, it was possible to extend the study of phage growth to intracellular development. This was carried out using the single-step growth experiment designed by Delbrück and Luria (13), as described in Materials and Methods. The adsorption flask was set up as for all previous adsorption experiments. At the end of the 30 minute adsorption period, a 1:100 dilution was made and plated to determine the amount of phage adsorbed to cells. This represents the number of infected cells. From this diluted sample a 1:20 dilution was made into a 250-ml culture flask to make the first growth flask. From the first growth flask, a 1:10 dilution was made into the second growth flask. All flasks were incubated at 18C. Samples were taken and plated from both growth flasks over a six hour period to determine the number of phage produced.

Figure 6 shows a single cycle of development for ϕ RL-8R. The latent period, during which the phage reproduced within the cell, was 135 minutes. The rise period, when phage were released through

cell lysis, was 90 minutes. The burst size was determined by the following formula:

$$\text{Burst size} = \frac{\text{Final titer}}{\text{Initial titer}} \cdot$$

For this calculation, compensation was made for unadsorbed phage remaining in the flasks. The burst size from this experiment was 112 phage/cell.

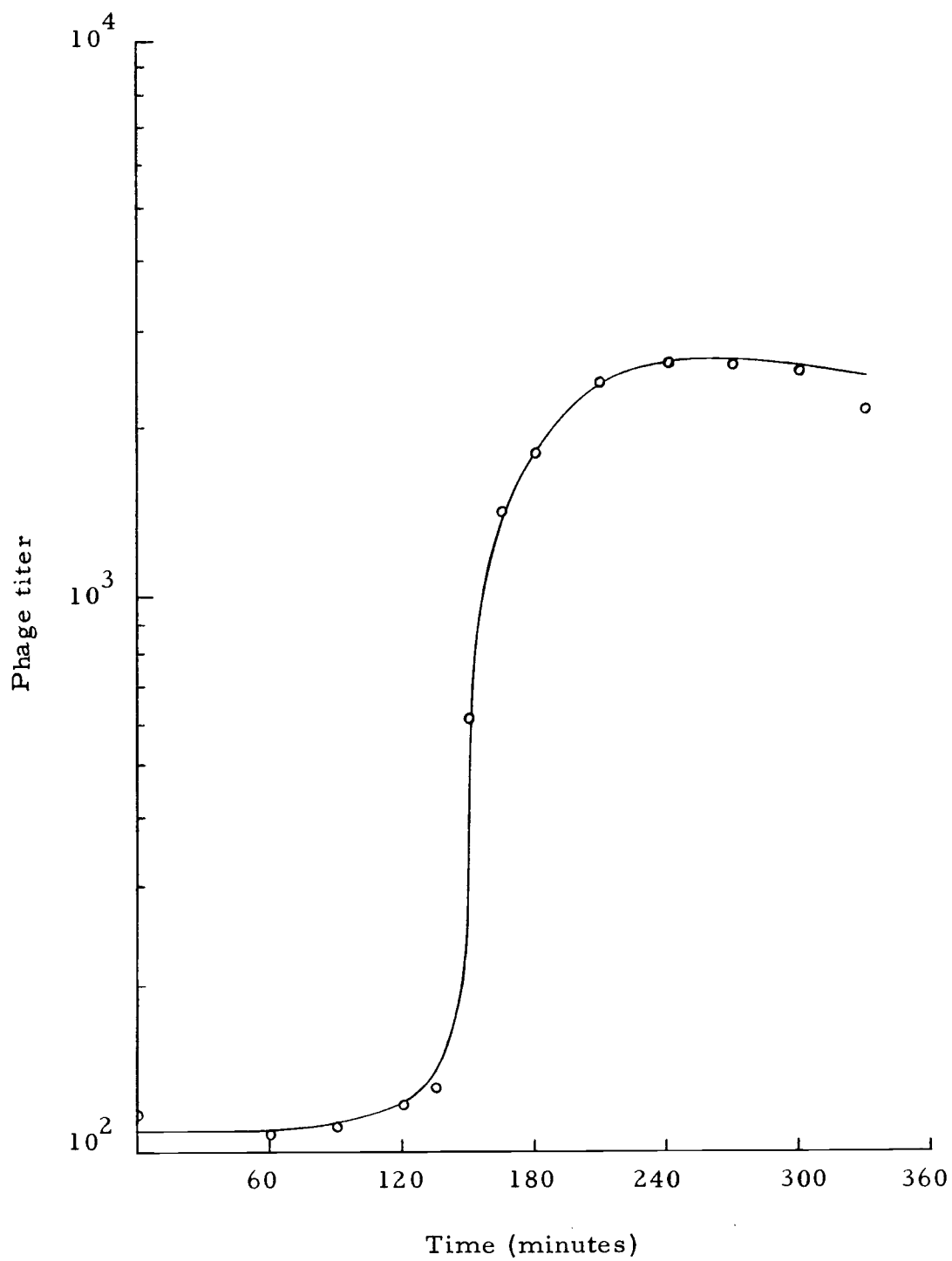


Figure 6. Single-step growth curve for ØRL-8R. Phage titer represents total phage, including unadsorbed phage.

DISCUSSION

This investigation was initiated in an attempt to isolate and characterize a bacteriophage for the freshwater myxobacterium Cytophaga succinicans (RL-8). The ease with which such a virus particle was obtained was not surprising since phages have been isolated for other myxobacters as well as for members of many other groups of bacteria. Bacteriophages have been isolated and studied for two fruiting myxobacteria, Chondrococcus columnaris (2, 16, 17) and Myxococcus xanthus (10, 11). Several phages have also been examined for Sporocytophaga cauliformis (23) and for the marine organism, Cytophaga marinoflava (12, 21, 24, 25).

In many respects all of these bacteriophages were found to be similar to each other and to ϕ RL-8R, the phage characterized in this study. All the myxophages have the same general morphology, consisting of a polyhedral head with some sort of tail. Table 11 provides a size comparison of these two major structural components for phages representative of each of the organisms mentioned above. The presence or absence of a neck, sheath and tail plate in these phages is also shown in Table 11. The data for the T-even coliphage were included as a comparison of a different phage-host system. All of the phages were fairly similar with respect to the size of their head and tail. It is of interest that only one other myxophage, phage

Table 11. Comparison of Size and Morphology of Several Bacteriophages with ØRL-8R.

Host and Phage	Size in Å			Presence or Absence of:		
	Head	Tail length	Tail width	Neck	Sheath	Tail Plate
<u>Cytophaga succinicans</u> (RL-8) ØRL-8R	1180	1900	250	Present	?	none observed, 2 prongs visible
<u>Chondrococcus columnaris</u> phage (2)	800	short?	?	--	--	--
<u>Chondrococcus columnaris</u> phage C2 (16)	600	1000	200	Present	Present	Present
<u>Myxococcus xanthus</u> phage MX-1 (11)	750	1000	--	--	Present	Present, plus spike
<u>Cytophaga marinoflava</u> phage NCMB 385 (25)	600	860-1000	--	--	Absent	Present, but enigmatic
<u>Sporocytophaga cauliformis</u> representative phage (23)	850-1000	present?	?	--	--	--
<u>Escherichia coli</u> T-even phage (7, 22)	650 x 950	950	200	--	Present	Present with 6 prongs

C2 for Chondrococcus columnaris, had a definite neck structure similar to that noted in the micrograph of \emptyset RL-8R. The myxophage varied with respect to the presence or absence of a contractile sheath, and a tail plate. The electron micrographs observed of \emptyset RL-8R did not provide any evidence for the presence of a contractile sheath. However, two prongs were observed at the tail base of several particles. Future studies may show these prongs to be the unextended forms of tail fibers which emerge when a sheath contracts, as is found in the T-even tail structures (7). It may also be that the prongs, as they appeared in the electron micrograph, function in attachment to the cell.

In order to study the morphology and structure of the \emptyset RL-8R particle in more detail, better preparations of the phage will be necessary. Initially, purification procedures must be improved to remove all whole cells and cell components. Differential centrifugation followed by density gradient separation or chromatography on an ion-exchange column may provide a more successful method of purifying the phage. If further concentration of the phage is necessary it could then be accomplished by high speed centrifugation for several hours. It may also be necessary to vary the solution used to resuspend the phage after pelleting. Sucrose was used as the suspending medium in the work carried out in the present study to avoid any possible clumping of the particles. Ammonium acetate or a buffer

in which the phage is very stable may be more suitable, and may provide more protection for the phage head, which was damaged in preparations used in this study. It is also possible that variations in the staining procedure may aid in electron microscopic observations of the phage particles. In some systems, uranyl acetate is used with phosphotungstic acid to selectively stain the nucleic acid. Trial and error will undoubtedly lead to the best results for this step in the staining process. Other methods such as shadow-casting with platinum might also reveal more structural detail.

Another aspect of the myxophages where similarities were encountered was their adsorption properties. The preliminary experiments completed with ϕ RL-8R indicated that between 10^{-3} M and 10^{-4} M calcium enhanced adsorption. Magnesium at a concentration of 10^{-4} M was found to be capable of substituting for calcium in one series of experiments carried out in this investigation, but additional studies are needed to verify these findings. Adsorption studies with the Chondrococcus columnaris phage C2 demonstrated that 4×10^{-3} M calcium was required, and magnesium would not substitute in that system. Similar work with the Myxococcus xanthus phage MX-1 showed that 10^{-3} M calcium was required for adsorption, as well as 10^{-2} M monovalent cation. For the Chondrococcus columnaris phage described by Anacker and Ordal, and the phages for Sporocytophaga cauliformis, no adsorption experiments were reported. However, in

both of these phage-host systems 10^{-3} M calcium was found to provide optimum phage production in broth cell cultures. Similar studies have not been described for phage NCMB 385 for Cytophaga marinoflava.

The characteristic properties of the single-step growth cycle also provided a basis for comparison of the myxophages. Table 12 shows the resulting values for each system, and again, the T-even coliphage data were included to demonstrate a similar cycle in a different phage-host system. Among the myxophages, including ϕ RL-8R, the data were quite similar. All the myxobacterial phages had a long latent period, as compared to the coliphage. This is probably due to the slower generation time of these organisms over that of E. coli. The rise period for the myxobacterial phages also covered a longer period of time. The greatest amount of variation was observed in the number of phage liberated per cell, which ranged from 20 for phage NCMB 385 of Cytophaga marinoflava, to 112 for ϕ RL-8R for Cytophaga succinicans. The burst size of a phage is a very distinctive property of each phage-host system, therefore these variations are not surprising.

The host range of ϕ RL-8R was found to be very narrow, as determined by testing the ability of the phage to produce plaques on 34 freshwater cytophagas, 2 strains of Chondrococcus columnaris, and 7 eubacteria. Of all the cultures tested, the phage was found to

Table 12. Comparison of Single Cycle Growth Characteristics of Several Bacteriophages with ϕ RL-8R.

Host and Phage	Length of time (minutes)		Burst Size (phage/cell)
	Latent Period	Rise Period	
<u>Cytophaga succinicans</u> (RL-8) ϕ RL-8R	135	90	112
<u>Chondrococcus columnaris</u> phage (2)	90	30	97 or more
<u>Chondrococcus columnaris</u> phage C2 (16)	100	90	23
<u>Myxococcus xanthus</u> phage MX-1 (11)	120-150	120	100
<u>Cytophaga marinoflava</u> phage NCMB 385 (12)	180	-	20
<u>Sporocytophaga cauliformis</u> representative phage (23)	150	90	81
<u>Escherichia coli</u> T-even phage (22)	24	10	100

produce plaques on only one of the cytophaga isolates. Based on a limited number of physiological characteristics, this organism, isolate 253, appears to be far removed from the natural host strain (20). However, there may be some genetic similarity between the two sensitive organisms.

Some of the other myxophages which have been tested for their ability to attack more than one host demonstrated an even narrower host range. Myxococcus xanthus phage MX-1 exhibited a host range which included only three laboratory strains of the original host. Another Myxococcus species, as well as a species of Cytophaga and Sporocytophaga, were not sensitive to phage MX-1. The host range of Chondrococcus columnaris phage C2 covered 67 strains of the host organism, including at least four serological groups. However, none of the Cytophaga species tested were found sensitive to this Chondrococcus phage.

The myxophage with the widest host range was found to be ϕ -173-N, isolated by Nitsos (20) from a freshwater cytophaga. This phage, infective for the organism designated 173, was shown to have a host range which included nine other cytophaga isolates. All nine isolates could be distinguished on the basis of their cultural characteristics (20). Cytophaga succinicans was not among the organisms sensitive to ϕ -173-N.

The host range data discussed above for the two cytophaga

phages seem to support the theory of Meynell (19) that phage sensitivity can be interpreted at the genetic level. According to this theory, organisms which are susceptible to a given phage may have a high degree of genetic similarity. In the case of the two cytophaga phages described, the host range was found to include only a small number of organisms within this group. That some degree of genetic similarity may exist among members of the genus Cytophaga is suggested by the fact that the DNA base ratio is similar for the majority of species of the genus which have been studied (20).

Many of the characteristics of ϕ RL-8R which were studied are not as easily compared with the other myxophages, because analogous data were not always available. The range of pH stability of the phage was found to be very similar to that of most bacteriophages, as described in Adams (1). ϕ RL-8R was stable between pH values ranging from 6 to 9, while Adams maintains that phages are normally stable over a range of 5 to 8. That the phage was not stable at pH values below 6 did not seem unreasonable, since its natural environment would rarely be found to be very acidic.

The aquatic habitat of ϕ RL-8R may also account for the range of its temperature stability, which was found to be lower than that of phages for other types of bacteria. Adams (1) discusses the heat inactivation of many coliphages and several staphylococcal phages. For one of the coliphages the inactivation range started at 45C; for

all the others it was higher, increasing to as much as 65 to 75C. For ϕ RL-8R, a typical inactivation curve was obtained during a 30 minute exposure to 46C. The actual minimum inactivation temperature was not determined; however, over an eight hour period the same type of curve was observed at 37C. Since 37C is optimal for the growth of E. coli, most coliphages are very stable at that temperature. The optimum growth temperature for C. succinicans is 25C, and ϕ RL-8R was found to be stable at that temperature over an eight hour period. There is some question as to how long the phage would remain stable after the eight hour interval, since it could not reproduce in broth in the host cell at 25C. In fact, a 99% decrease in the phage titer of a broth cell culture was found at that temperature, over a period of 42 hours.

This inability of the phage to replicate in broth cell cultures at the optimum temperature of the host constitutes one area where further research should be carried out. The phage was able to replicate at 25C on overlay plates, though the EOP was considerably lower than that observed on plates incubated at 18C. The only explanations the author can suggest are that adsorption may be more difficult at the higher temperature, or that, due to an increase in growth rate, the cells may be able to overgrow the phage before the latter can replicate. If the frequency with which phage-resistant variants develop increases with the growth rate, this could easily prevent the phage

from replicating in a broth culture. On plates, adsorption may be facilitated by the close proximity of phage and cells. That plaques can form at 25C, though they are very small and faint, indicates that the phage can replicate once within the cell. To begin a study of this phenomenon, adsorption should be measured at both 18C and 25C. If adsorption is found to occur less effectively at the higher temperature, a search for special cofactors may be necessary. If adsorption is not inhibited at 25C, the possibility of the development of some form of lysogenic relationship might be considered.

Another point of interest with respect to broth replication of ϕ RL-8R was the development of phage-resistant cells in the culture when phage and cells were combined and incubated at 18C. Under these conditions phage were always produced up to a maximum titer of 10^{10} . Isolates of cells remaining in the culture were tested and found to be resistant to the phage. This phage immunity suggested the possibility that ϕ RL-8R was a temperate rather than a virulent phage. A temperate phage infecting the host cell causes the host to become lysogenic, and, consequently, immune to further infection by the same phage. The lysogenic cell does not lyse to produce phage progeny, but continues to propagate, carrying the phage in a non-virulent state and passing it along to cell progeny. This process produces a stable culture which is resistant to the phage.

While it is possible that ϕ RL-8R is temperate, the plaques

produced by this phage are not consistent with those formed by most temperate phages. Plaques formed by temperate phages are usually turbid because lysogenic cells have grown in their center. The plaques formed by ϕ RL-8R were always clear. To conclusively prove whether or not ϕ RL-8R is a temperate phage for C. succinicans, it would be desirable to cultivate the resistant host cells for several generations in specific phage antiserum and then induce the culture by some means and assay for the presence of phage.

When resistant cells were detected in phage-cell cultures, other possible explanations for this phenomenon were also sought. Hayes (15) discusses two types of pseudo-lysogeny which may provide some insight into the events of broth replication in this system. The first type involves the ability of some cells in a host culture to acquire a temporary phenotypic resistance to a phage at an early stage in the growth of the culture. Sensitive cells are present to allow phage reproduction, and the resistant phenocopies lose this property upon subculture. This type of pseudo-lysogeny apparently involves a temporary inability of the phage to adsorb to the phenotypic cell variants.

A second type of pseudo-lysogeny has also been discovered (15) which could actually be considered a form of abortive lysogeny. In this situation, the phage can be propagated for many generations within some of the infected cells. These cells give rise to a

proportion of uninfected daughter cells as well as to daughter cells which lyse and liberate phage. Stable lysogenic clones cannot develop, so phage are propagated by cell lysis. Hayes suggests that the phage can provoke some immunity in infected cells so that vegetative replication is partially repressed in a proportion of these cells.

These immune cells would then be responsible for the persistence of the phage, and would be considered to be in the carrier state.

At this point neither of these types of pseudo-lysogeny can definitely explain the resistant cells which arise during ϕ RL-8R broth replication. A number of experiments would be required to determine if either of these situations exist, or if the development of phage-resistant cells in this system is the result of an entirely different phenomenon. In extending this research, a much larger survey should be made for the existence of phage-resistant cells in the host culture, where no phage are present. The development of these resistant cells during broth replication of the phage should also be more carefully examined. Larger samples of cell isolates should be taken throughout the replication period, from zero time until maximum phage production is reached. These cultures could then be tested, first, for resistance to the phage, and second, if resistant, for the ability of the phage to adsorb to the cells. It would also be desirable to examine the incidence of phage-resistant cells at higher temperatures, since the degree of lysogeny in some phage-host

systems is influenced by temperature (1).

Results of the kinds of studies proposed above may lead to a more exact definition of what is occurring during broth replication of the phage. It is difficult to project the steps to be taken past these initial experiments, since the possibilities for investigation depend heavily on the immune nature of the phage-resistant cells and their development in phage cultures, as well as their absence in the stock culture. If the first type of pseudo-lysogeny actually exists in this system, then resistant cells should revert to phage sensitivity, which they did not do in preliminary tests. If the second type is responsible for the events occurring in broth replication, then immune cells should be found in the host culture. Studies revealed an extremely low incidence of resistant variants in the host stock, but larger samples may be necessary to confirm or change those observations. If it is found that immune variants develop with a much greater frequency at higher temperatures (25C), it may be that the phage is temperate, and can more efficiently enter the lysogenic relationship with the host at that temperature.

With a more complete characterization of the nature of phage-resistant cells, it may be possible to prevent their development in broth cultures. As a result of this, increased phage production may be possible. Other studies may also be extended to aid in increasing phage titers. At the termination of this investigation, the

development of a procedure for measuring phage adsorption had only recently been perfected. The continuation of adsorption experiments to determine the optimal requirements for this process may prove of value in the production of higher concentrations of phage. The advantage in obtaining high phage titers should become apparent when attempts are made to purify phage preparations, since some quantities of the infective particles are normally lost at each step. Factors which could be examined for their effect on phage adsorption include tryptophane, and different monovalent and divalent cations at varying concentrations.

The inability of C. succinicans to allow anaerobic phage production in the medium of Anderson and Ordal (4) was also of interest. The fact that under the same conditions, phage were able to replicate aerobically, indicates that some variation in anaerobic host metabolism may affect the production of phage. Perhaps some enzyme or intermediate compound required for phage synthesis is not produced by the cell during anaerobic growth. Another possibility is that end products of this metabolic process may somehow interfere with phage-requiring cell constituents. It is also possible that there may be an oxygen requirement for the adsorption of the phage to the host cell. This would be difficult to determine in the system used in these studies, since a decrease in free infective phage could represent either adsorption to the cells or inactivation by the medium.

However, it might be possible to test this hypothesis using radioisotopically labeled phage particles.

It could also prove of interest to study anaerobic phage production in other media where the phage may be more stable. Nitsos (20) has detected anaerobic growth of C. succinicans in several other media. If the host organism of ϕ RL-8R demonstrates the same growth capabilities, further investigations could involve testing phage stability in these media. If anaerobic phage development in other media was possible, the results of such work could provide many opportunities for studies of intracellular phage synthesis, as well as extending the characteristics of the adsorption properties of the phage.

There is one important area in the characterization of ϕ RL-8R where no work was attempted during the course of this project. This involves the identification of the phage nucleic acid. It is possible to postulate that the phage contains double-stranded DNA, since, according to Bradley's classification of bacteriophages (7), all phages with head and tail structures have this type of nucleic acid. This would be in agreement with the results of nucleic acid analyses carried out for all other myxophages examined. Highly purified preparations of phage will be required to determine the type of nucleic acid. A fairly simple procedure is described by Bradley (7) using acridine orange to differentiate double- and single-stranded molecules, and

DNase and RNase to determine the structure of the ribose component. In addition to this study, the melting point of the nucleic acid molecule, if it is double-stranded DNA, should be determined, to compare its base ratio with that of other myxophages, and with that of the host organism.

Though the characterization of the phage isolated in this study is in many respects incomplete, it has in part fulfilled the purpose of this project. The demonstration of the existence of the phage has, in itself, contributed to the characterization of the host organism. In addition, ϕ RL-8R may be used to indicate similarities between C. succinicans and other organisms, by a common sensitivity of the cells to the phage. Continuation of the studies described above may also provide information about the host cell which cannot be anticipated at this time. If ϕ RL-8R were found to be temperate, it could be utilized in transduction experiments to contribute to genetic studies of the myxobacteria. In some phage systems, characteristic adsorption properties can be used to study changes in the host cell wall. Eventually it may be possible to use myxophages for similar work, and this might contribute valuable information about the nature and composition of the flexible cell wall of myxobacteria.

SUMMARY

1. A bacteriophage was isolated which was infective for the freshwater myxobacterium, Cytophaga succinicans (RL-8). The phage possessed a hexagonal head 1180 \AA in diameter, attached to a tail by a definite neck. The tail was 1900 \AA long and 250 \AA wide. No evidence of a contractile sheath, tail plate, or tail fibers was found, though two prongs were observed at the base of the tail.
2. The production of plaques by ϕ RL-8R was studied using the agar overlay procedure. At 18C the phage produced clear, round plaques from 0.25 to 1.0 mm in diameter. However, at 25C the plaques observed were much smaller and uneven, and the EOP was considerably lower. A relationship also was noted between the size of the plaques and the density of the cell inoculum. The more dense the culture, the smaller the plaques formed.
3. The stability of the phage to various physical and chemical treatments was examined. For most aspects studied (pH stability, sensitivity to chemical agents, maintenance of phage stocks) ϕ RL-8R was similar to other bacteriophages. It was noted that the heat sensitivity of the phage started at a lower range than that for some other phages, since ϕ RL-8R was

- rapidly inactivated at 46C, and gradually at 37C.
4. Adsorption studies were carried out to determine if any special cofactors were required for the phage to attach to the host cell. Preliminary experiments indicated that calcium at concentrations from 10^{-3} M to 10^{-4} M enhanced adsorption. Magnesium may be able to substitute for calcium under some conditions.
 5. A single cycle of phage development within the host cell was studied using the one-step growth experiment. The latent period of ØRL-8R was found to be 135 minutes; the rise period was 90 minutes. The burst size was determined to be 112 phage/cell.
 6. Environmental studies were carried out to determine the optimal conditions for replication of the phage in broth cultures of the host organism. It was found that phage develop best at 18C under static conditions with maximum surface aeration. In such cultures no decrease in cell turbidity was noted with the increase in phage progeny. This absence of total lysis of the host culture may, in part, be attributed to the existence of phage-resistant cells which develop in such phage-host cultures. The nature of these resistant cells was not determined in this investigation. No phage production was detected in broth at 25C.
 7. The possibility of phage production under anaerobic conditions

was examined, since the host strain of Cytophaga succinicans (RL-8) was capable of CO₂-dependent anaerobic growth. No phage replication was detected anaerobically, although the host culture grew well.

8. The host range of ØRL-8R was investigated using 34 cytophaga isolates, 2 strains of Chondrococcus columnaris, and 7 eubacteria. The phage was found to be infective for only one of the cytophagas.

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