THEOS

This is to certify that the

dissertation entitled

ISOLATION, CHARACTERIZATION AND FUNCTIONAL SIGNIFICANCE OF BACTERIOPHAGES IN THE RUMEN MICROBIAL ECOSYSTEM.

presented by

THEODROS TADESE

has been accepted towards fulfillment
of the requirements for

PhD degree in Animal Science

Major professor

Date October 11, 1993

O-12771

ISOLATION, CHARACTERIZATION AND FUNCTIONAL SIGNIFICANCE OF BACTERIOPHAGES IN THE RUMEN MICROBIAL ECOSYSTEM

By

THEODROS TADESE

A DISSERTATION

submitted to
Michigan State University
in partial fulfilment of the requirement
for the degree of

DOCTOR OF PHILOSOPHY

Department of Animal Science

1993

ABSTRACT

There are limited reports in the literature concerning the occurrence of bacteriophages in the rumen. No particular attempts have been made to study the interactions of ruminal bacteriophages and predominant cellulolytic rumen bacteria. Furthermore, there is no information available regarding their host range or physiochemical characteristics. In this study, wild type cellulolytic bacteria and a ruminal spirochete, *Treponema saccharophilum*, were isolated by anaerobic roll tube technique. The morphology of these bacteria were determined by Gram staining followed by microscopic examination and their fermentation by-products determined by gas chromatography (GC) and high performance liquid chromatography (HPLC) respectively. Isolation of ruminal phages were carried out by the double agar overlay method. To monitor cell lysis and regrowth of infected cells, optical density readings (600 nm) were determined with a spectrophotometer. Morphologic features of the free phages and those attached or within bacteria were identified by transmission electron microscopy after negative staining or thin sectioning. Five morphologically distinct bacteriophages were identified in ruminal fluid from a cow fed alfalfa hay. A phage possessing an icosahedral head symmetry (100 x100 nm) and a short rigid tail (150 x 20 nm); a phage with a cuboidal head (110 nm) and a short flexible tail (290 nm); a cluster of phage particles with distinctly hexagonal heads (50 x 50 nm) and no tail; a phage with an icosahedral head (40 nm) and sheathed tail (120

nm); and filamentous like-phage particles, variable in length (100 to 400 nm) were observed. A bacteriophage associated with an identified ruminal spirochete, *Treponema saccharophilum*, was also identified. Of particular importance was the isolation of a bacteriophage, resembling the lambda phage for *E. coli* in its morphologic and physiologic characteristics. This phage was temperate for a ruminal cellulolytic anaerobe, *Ruminococcus albus*, but obligately lytic to another ruminal anaerobe, *Butyrivibrio fibrisolvens*, strains D1 or 49. The phage appeared to have a wide host range specificity, and infected other ruminal strains including: *Fibrobacter succinogenes*, S85; *Bacteroides amylophilus*, H18; *Selenomonas ruminantium*, HD4; *Ruminococcus flavefaciens*, FD1; and *Ruminococcus albus*, 7 and 8. The total genome of the phage as determined by pulsed field gel electrophoresis (PFGE) technique was found to be 48.5 Kb. The temperate nature of the phage was studied by hybridization techniques. Restriction fragments (smaI) of the phage were shown to hybridize with restriction fragments of *R. albus* DNA, indicating that the phage was indeed harbored in the cellulolytic anaerobe.

ACKNOWLEDGMENTS

I would first of all like to extend my deepest gratitude to Dr. Melvin T. Yokoyama for being such a great advisor, committee chair, and guide, for offering me an opportunity to do research of my interest. Your advice has always been pertinent and seasoned with experience. My graduate committee members, Dr. Steven Rust, Dr. Margaret Benson, Dr. Werner Bergen, Dr. Robert Cook and Dr. Roger Maes, I thank each of you for the valuable input and guidance in the program.

My thanks goes to Dr. Susan Hengemuehle, for her continual help in providing laboratory supplies whenever needed.

I wish to extend sincere thanks to Dr. John Garlach, Dept of Medical Technology, for allowing me to use their pulsed field gel electrophoresis technique equipment.

I thank Dr. Paul Coussens, Dept. of Animal Science, for allowing me to use some of his laboratory equipment and his time for discussion.

My thanks would not be complete without acknowledging Dr. Karen Klomparens, Center for Electron Optics, for her assistance in electron microscopy.

The author is indebted to the Rackham Foundation and the Department of Animal science for supporting this project.

TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

List of Tables

List of Figures

List of Figures

Terminology

Indicator	A bacterial strain sensitive to a specified kind of phage, used to identify phage.
Induction	Experimental elicitation of phage development in lysogen.
Integration	The insertion of one genetic element into another to form a single chromosome.
Lysate	Disrupted bacteria. Phage strains are usually preserved and used in the form of lysates of infected cultures.
Lysogen	A bacterium that possesses and transmits to its progeny the power to produce phage particles and is therefore prone to lysis.
Lysogenization	The process following infection of bacteria with phage and leading to establishment of lysogeny.
Lysogeny	A stable coexistence of a phage within DNA of the host bacterium.
Pseudolysogeny	Short term coexistence of host and phage, as opposed to true lysogeny which allows the viral genome to remain for an indefinite time inside the infected cell without killing it or producing virions.
Plaque	A circular clearing caused by local growth of phage and lysis of bacteria on the surface of a nutrient agar plate.
Temperate	Term applied to a phage able to lysogenize its host.
Transduction	Transfer of bacterial markers from one cell to another by phage particles.

Transfection	Introduction of cloned viral DNA into cells treated by a calcium/phosphate procedure which favors the uptake of viral DNA by the cell.
Virulency	A virulent or intemperate phage species is one unable to lysogenize its host.
Superinfection	Reinfection by phage of a cell that carries a prophage, or is otherwise already infected.
Prophage	Integrated phage DNA within a bacterium chromosome.
Host Restriction	A phenomenon in which a bacterium type x is able to distinguish a phage that has been grown in a type x bacterium from one grown in a different type such as Y and is able to prevent the phage grown in Y from carrying out a successful infection.
Multiplicity of Infection	The ratio in the number of phage to that of the bacteria in one particular infection assay.
Generalized Transducing Phage	A phage which can produce particles containing only bacterial DNA.
Specialized Transducing Phage	A phage which occasionally produce particles containing both phage and bacterial DNA sequences.
Virion	A complete virus, that is the virus DNA is completely enclosed within protein coat.

INTRODUCTION

Ruminal microorganisms play an essential role in the digestive processes of the ruminant. These microorganisms consisting primarily of bacteria, protozoa and fungi occuring in ecological balance, will efficiently degrade and ferment food material ingested by the animal. The fermentation end products along with the microbial protein synthesized in the rumen are used by ruminants to meet their nutritional requirements for growth and other energy requiring processes. Ruminal microorganisms have been intensively studied and the information from such studies have provided a better understanding of these microorganisms in the efficient degradation and fermentation of feed stuffs. Although a great deal is known about the predominant bacteria and protozoa, there is a major gap in our knowledge of the ruminal bacteriophages within the complex ruminal ecosystem. Bacteriophages are viruses which are capable of infecting only bacteria. A typical bacteriophage contains only a few different types of molecules, usually several hundred protein molecules of one to ten types (depending on the complexity of the phage) and one nucleic acid molecule (Freifelder, 1983). There are large numbers and many different types of bacteriophages in the rumen (Paynter, 1969; Ritchie, 1970; Klieve,

1988). Their occurrence in the rumen indicates that they may be a constant feature of the microbial population. Since their discovery, bacteriophages have been regarded as being important in the ecology of their host bacteria. For many years, their influence on bacteria was thought to be solely on the elimination of sensitive bacteria by the process we now call virulency. However, recent work has identified other significant functions of bacteriophages, such as control of toxigenicity in bacterial species and their capacity to transfer genetic information from one host to another. A bacteriophage may influence a bacterial population in the following ways:

1. By the elimination of sensitive bacteria, a virulent phage may cause the replacement of such a bacteria, by a phage resistant mutant differing in ecological function from the original bacteria.

2. A temperate phage may lyse a certain proportion of an original bacterial population and lysogenize the remainder. Apart from conferring protection against the phage concerned, lysogenization may cause specific structural changes in the host's surface membrane as a result of prophage formation.

3. Transduction (Terminology, xi) of genetic properties may occur with or without lysogenization and may be effected by either virulent or temperate phages.

All three of the above properties of a given bacteriophage may offer some bacterial hosts ecological advantages in which to operate. This may be particularly true in the complex ruminal ecosystem, in which bacteriophage activity may be important in determining bacterial relationships, and in influencing the numbers and kinds of bacteria in the rumen.

Although the occurrence of bacteriophages in the rumen is documented, virtually nothing is known about their origin, host range and specificity, virulence, induction and effects on fermentation efficiency. The lysogenic state of microorganisms is widely distributed and is the normal condition of microorganisms in a given stage of their evolution. The majority of bacteriophages found in the rumen may exist in harmony with their hosts in a state of lysogeny or pseudolysogeny (Klieve, 1989), and are probably being induced periodically. The periodic loss of important fiber degrading bacterial species from the rumen and the high turnover of the bacterial population in the rumen of forage fed ruminants (Hungate, 1966) might also be explained by the activities of the bacteriophages. Major 'blooms' of bacteriophages have been observed in the rumen (Klieve, personal communications). Any factor which affects the balance of the ruminal microbial population and their rate of metabolism of ingested feed may have an important effect

on the nutrition of the host animal. At present the range of types of bacteriophages isolated from the rumen is limited and appears unrepresentative to the numerous bacterial species occurring in the rumen.

The objectives of this thesis research were:

1. **To demonstrate the occurrence of bacteriophages in the rumen ecosystem and to identify phages which infect specific ruminal bacterial species.**
2. **To determine the host specificity and range, induction, and effects of the isolated ruminal bacteriophages on their bacterial hosts.**
3. **To determine the effects of the bacteriophage on the interactions between ruminal bacterial species in mixed and cocultures.**

The knowledge acquired from these studies will provide a better understanding of host-parasite relationships in the rumen ecosystem. The knowledge which would be gained from this research could also be used to develop new approaches for genetically modifying rumen bacterial species by using isolated bacteriophages for genomic libraries and as cloning vectors. The use of bacteriophages in genetic engineering is likely to

increase, as they exist everywhere in the bacterial world and transformation by plasmid DNA is not practical in all bacteria. In general, the potential of ruminal phages have not been fully exploited. Phages in the rumen have never been assayed for transducing ability, which can possibly be used to modify particular bacterial species.

LITERATURE REVIEW

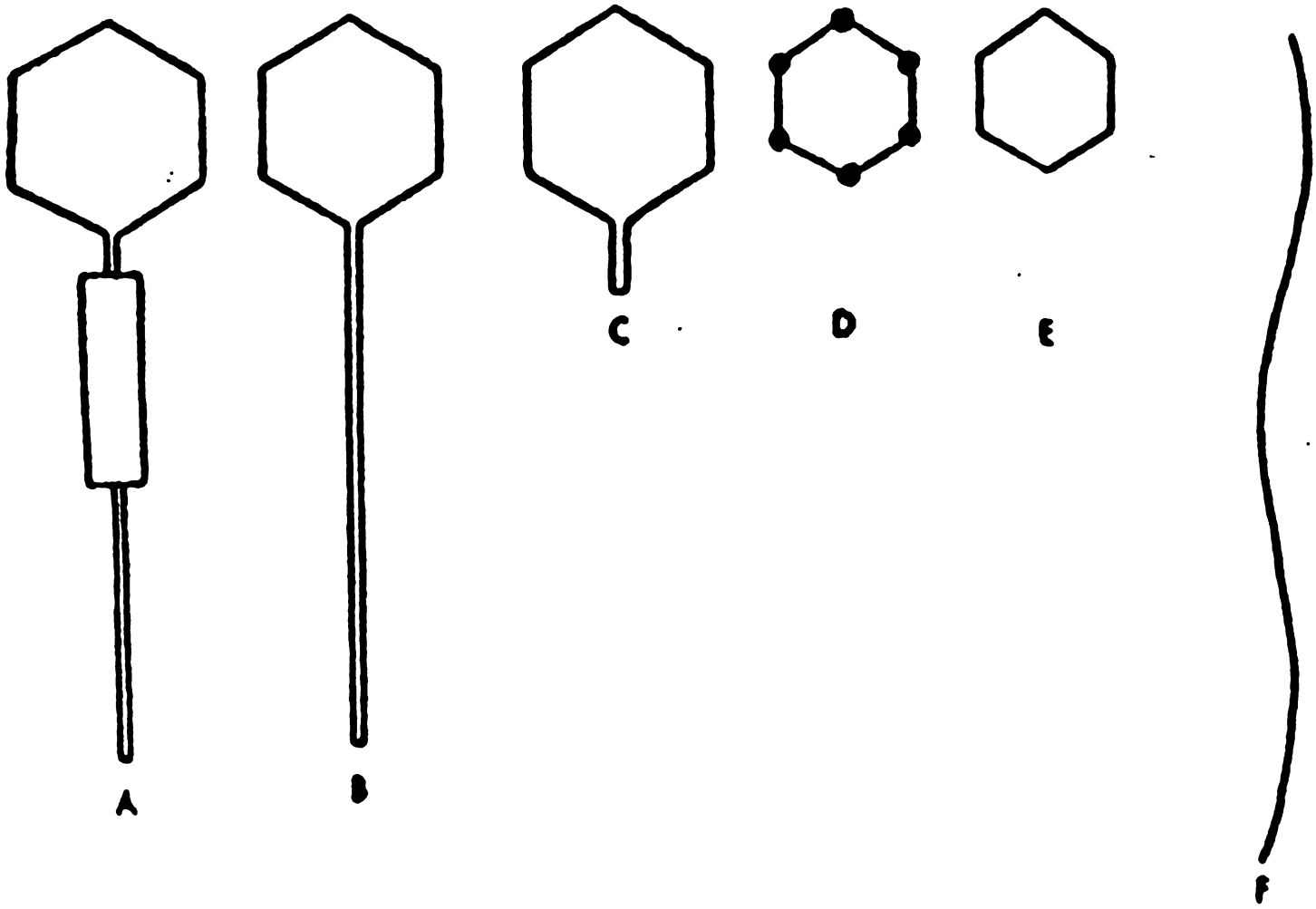
Bacteriophages seem to occur everywhere in the bacterial world. In order to perpetuate their progeny, bacteriophages multiply in either of two ways. In productive growth, a bacteriophage undergoes four main steps: adsorption, infection, multiplication proper and release. Once a bacteriophage adsorbs to the host receptor, it injects its DNA molecule into the bacterium and directs the synthesis of numerous gene products, which promote autonomous replication of the phage DNA, its packaging into phage particles, and lysis of the cell to release about one hundred progeny particles, all within 50 min. This sequence of events is called either the " lytic", " virulent", " vegetative", or " productive" cycle. A phage which initiates lytic cycles only is called " virulent" (xii). A bacteriophage can also replicate as part of the host bacterial chromosome. The injected DNA first directs the synthesis of a gene product called integrase, which promotes insertion of the phage chromosome into the DNA of the bacterial host, and then express other genes that act promptly to repress autonomous DNA replication and most phage function. When these processes take place, the phage DNA is inserted into the bacterial chromosome at a characteristic insertion site. The inserted phage DNA is called prophage (xii). Its replication contributes to viral

growth indirectly, because every cell carrying a prophage is a potential source for production of phage particles. The phage that undergoes a stable symbiosis with the bacterium is known as temperate. The bacterium that harbors the prophage is called lysogenic, because it has acquired the potential to produce phages and thus to lyse. Moreover, it has become immune against superinfection by homologous phages and sometimes has new metabolic or antigenic properties that persist as long as the prophage is present. It may even reproduce more rapidly than its non-lysogenic parent. This phage-host equilibrium may break down spontaneously or upon induction by some stimuli, usually ultraviolet light or mitomycin C. The lysogenic state is beneficial to the phage and its host: it ensures perpetuation of the phage and confers at least one new property to the bacterium, anti-phage immunity, which has an evident survival value. Lysogeny occurs in many, if not all branches of Eubacteria. The frequency with which an infecting bacteriophage initiates lysogenization rather than productive phage growth depends on the state of the bacteria, the temperature, and the genotypes of the phage and the host. Many bacteriophages carry mutant phages which cannot replicate, but may be visualized in lysate of induced cells. They are variable in morphology, including complete phages with full or empty heads,

headless tails or tailless heads, and polyheads and polysheaths. Some phages are also known to undergo pseudolysogeny or carrier state. This refers to the simultaneous presence of phages and bacterial cells in the same culture. Phage DNA is not integrated and only some of the cells of the bacterial culture are infected. Any phage, virulent or temperate, is theoretically, capable of generalized transduction (xii). This means that in the course of phage multiplication a 'headful' of bacterial DNA is randomly packaged into the capsid of a phage. The transducing particle, which has none of the normal phage genes, is nonviable. By contrast, the transducing phage in specialized transduction (xii), is always temperate, usually requires induction, and contains both phage and host DNA.

The bacterial viruses exhibit a greater diversity in form than any other group. There are six basic morphological types of bacteriophages. The most complex of these, Group A, has a head with a hexagonal outline which may or may not be elongated. A tail with a contractile sheath is attached to the head. The second group in order of structural complexity, Group B, also has a six sided head and a tail. However, the tail is relatively flexible; it may or may not have terminal appendages. The tail is longer than the head diameter and has no contractile apparatus. The third type, Group

C, also has a tail and a six sided head, but the tail is shorter than the head diameter or maximal dimension, and may also have appendages attached to it, but, again it is non-contractile. The fourth type, Group D, has no tail, but is still six sided in outline though of symmetrical appearance. Each apex of the hexagon has a knob or large capsomere on it. In the fifth group, Group E, the large capsomeres are absent and the virion shows a simple regular hexagonal outline. The sixth group, Group F, is quite unlike the others. The virion has the form of a long flexible filament with no additional structures of any kind attached to it. Bacteriophages have been isolated from a large proportion of bacterial genera. The present overall picture of the distribution of the six basic morphological forms may be a distorted one. One or two fundamental facts are, however, apparent. First, a basic morphological type of phage is not restricted to any one bacterial genus or species; that is to say, contractile phages have been isolated for both G (-) and G (+) bacteria of widely separated genera. Furthermore, a single phage is highly specific, its activity being usually restricted to closely related species of a single genus (Bradley, 1967).



Basic morphological types of bacteriophages.

1. Ruminal Bacteriophage- Host Interaction

High numbers of morphologically different types of bacteriophages have been detected in the rumen of sheep (Hoogenraad, 1967) and cattle (Paynter et al., 1969; Ritchie et al., 1970). Paynter et al. (1969) examined the ruminal contents for a period of one year from a cow fed alfalfa hay and detected bacteriophages, both attached and unattached to bacteria, at concentrations of about $5x10^7$ per ml in all samples. Similarly, Ritchie et al.(1970) characterized bacteriophages in the rumen of cattle and sheep, and found more than 125 morphologically distinct types, with 65 different bacterial species showing intracellular phage infections. In a recent study by Klieve and Bauchop (1988), 26 distinct types of bacteriophages were identified and large numbers of phages, 2×10^7 to 1×10^8 were present in ruminal fluid from sheep and cattle. Although, it is obvious from the literature that indigenous rumen bacterial species are infected with bacteriophages, the detection of bacteriophages associated with specifically identified rumen bacterial species has been very limited. Hoogenraad et al.(1967) showed that ruminal spirochetes were heavily infected with bacteriophages. Orpin and Munn (1974) reported that a bacteriophage was associated with *Eadie's Oval* and also with an unidentified, obligately anaerobic Gm (-) rod which effected the

biohydrogenation of unsaturated fatty acids. Bacteriophages have also been shown to infect transient *serratia* species (Adams et al., 1966), and *Fusobacterium necrophorum* (Tamada et al., 1985). Bacteriophages have also been detected for temporal species such as *Bifidobacterium ruminale* (Matteuzzi and Sozzi, 1971), which is found in the rumen of young preruminant calves on a milk diet. The only rumen bacteria which has been studied in detail with regard to bacteriophage is *Streptococcus bovis*, which is found in high numbers when a high starch diet is fed (Iverson and Millis, 1976a; Tarakanov, 1974). Lysogeny of the ruminal *Streptococcus* species has been well established (Iverson and Millis, 1976a, Tarakanov, 1974). A majority of streptococcal strains harbor temperate phages that can be readily induced by ultraviolet light and mitomycin C. In some cases, induction can also occur spontaneously. For most of these studies, lysis of the culture was observed following induction, and temperate phages were identified by lysis of the appropriate indicator strain or, more often, by detection of phage particles using electron microscopy. In a series of studies, Iverson and Millis (1976a, 1976b, 1977) isolated and characterized bacteriophages which infect *S. bovis*, and reported that only one strain of *S. bovis* was lysogenic. Of the 23 strains of *S. bovis* tested, only 4 percent were inducible

by mitomycin C. In contrast, Tarakanov (1974, 1976) reported that lysogeny was common in *S. bovis* strains, which were isolated from the rumen of sheep and cattle. Fifty five percent (10/18) of *S.bovis* strains from sheep were lysogenic; 39 percent were induced by UV - light irradiation, while 27 percent (5/18) were spontaneously induced. Of the 48 *S. bovis* cultures obtained from cows, 50 percent were lysogenic, and about 84 percent were spontaneously induced and, only 13 percent were induced using chloroform. The differences in the lysogenic characteristics of isolated *S.bovis* strains in both studies have been attributed to variation in geographical area and variability of individual animals (Iverson, 1976a). However, as lysogeny is normally a stable characteristic in microorganisms, the difference in lysogenic cultures due to geographic location seems remote. In spite of the differences, lysogeny is believed to be the source of bacteriophages in the rumen of cattle and sheep (Tarakanov, 1974). The attention focused on the lysogenic nature of *S. bovis* is based on the possibility that lytic phages occurring in ruminal fluid originate from phages harbored by the lysogen. Recently, similar observations have been made by Lockington et al. (1988), who reported on the isolation and characterization of a ruminal temperate bacteriophage from *Selenomonas ruminantium*, an important propionic acid

producing ruminal bacteria. Klieve et al. (1989) reported the induction of a temperate bacteriophage in a wide range of ruminal bacteria by mitomycin C. Of 38 ruminal bacterial species investigated, 9 (23.7%), namely, *Streptococcus intermedius AR36*, *Streptococcus bovis AR3.*, *Eubacteria ruminantium AR2*, *AR35.*, *Bacteroides ruminicola subsp.brevis*, *AR29*, *AR30*, *AR32.*, *Butyrivibrio fibrisolvens AR14.*, and *Ruminococcus flavefaciens*, produced phage- like particles. All these accumulated evidence clearly demonstrate the ubiquity of temperate phages which are carried as lysogens by ruminal microorganisms.

Apart from these studies, no research has yet been conducted to determine whether bacteriophages infect other predominant rumen bacterial species, especially those species responsible for plant polymer degradation.

2. Morphologic Diversity of Rumen Bacteriophages

Rumen bacteriophages interacting with ruminal bacterial species have been subjected to extensive morphologic characterizations. Structural identification and measurement of phage heads, tails, collars, and base plates by electron microscopy has demonstrated the presence of a diverse number of morphologic types in the rumen. Paynter et al. (1969) examined bacteriophages isolated from cattle ruminal fluid. The dimensions of

the phages found in the cow were different from any of those found in sheep (Hoogenraad et al., 1967) or from bovine phages active against *S.durans* (Brailsford, 1968). Nevertheless, each phage contained a tail and a polyhedral shaped head. Base plate-like structures were evident only on two of the six bovin phages seen. The dimensions in all the phages varied and ranged from 103 by 103 to 137 by 128 nm for the head and from 666 by 34 to 134 by 29 nm for the tail. Similarly, Ritchie et al.(1970) examined the morphologically different phage-like entities in sheep and cattle which were fed normal diets. The most prevalent types of phages had contractile tails and cuboidal heads. Their overall length ranged from less than 70 nm to greater than 80 nm, with head diameters varying from less than 30 nm to greater than 15 nm. The next numerous types were those with noncontractile tails with no apparent tail sheath. Matteuzzi and Sozzi (1971) identified bacteriophages specific for *Bifidobacterium thermophilum*, from rumen of a cow fed high carbohydrate diet. These phages had octahedral heads and long curved tails with base plates. The morphology of *S. bovis* phages isolated from bovine rumen, lysogenic cultures of *S. bovis*, and abbatoir ruminal wastes was examined and compared by Iverson and Millis (1976b). Phages isolated from the rumen had hexagonal heads with a diameter of 65 nm, and

noncontractile tails of 175 nm in length, terminating into a knob with short spikes. However , phages from lysogenic cultures of *S. bovis*, and 50 percent of the phages isolated from abbatoir wastes had hexagonal heads measuring 50 nm in diameter, and tails with short noncontractile spikes which were 32 nm long. Klieve et al. (1988) identified 26 morphologically distinct types of bacteriophages, from ruminal fluids of cattle and sheep, collected over one year. On the basis of the scheme of Bradley (1967), they were classified as follows: Group A has long contractile tails; Group B has long, noncontractile tails; and Group C has short, noncontractile tails. The remaining phages were newly classified (Klieve et al., 1989) as: Group D, which are tailless, icosahedral and have a large capsomere; and Group F, which are filamentous phages.

3. Properties of Rumen Bacteriophages

Brailsford et al. (1968) elucidated the properties of a transient ruminal species, *S. durans* phage isolate, from bovine rumen, fecal samples and lysogenic strains of *S. durans*. All phages appeared to be similar in their lytic activity, and produced plaques or a zone of lysis whose sizes ranged between 1 and 5 mm in diameter. All phages were sensitive to temperatures between 10 C and 70 C. They were completely inactivated at 70 C for 1 hr.

The viability of the phage was not affected by being in medium between a pH of 5 and 9 for 24 h. In a detailled study by Tarakanov (1976) to characterize *S. bovis* phages isolated from sheep rumen and lysogenic cultures of *S bovis* isolated from cows, all phages produced round, transparent, smooth plaques with a fairly distinct edge and diameters of 0.5 to 2 mm. Sixty eight percent of the phages were inactivated after three hours of incubation in ruminal contents. Their degree of inactivation increased with the increase in contact time, that is 85.5 to 97 percent inactivation took place in 30 hour incubation period. However, the phages reacted differently to treatment with different concentrations of acetic acid. Increasing the concentration of acetic acid to 0.1065 mmole per ml, which is the upper possible limit of this acid in the ruminal fluid, led to greater but still incomplete inactivation of these phages. Because of this phenomenon, acetic acid was assumed to be one of the links in the chemical regulation of the number of *S. bovis* phages in the rumen. The effect of urea on these phages was also studied, since urea has a denaturing effect on the protein structure of phage particles and urea is consumed by ruminants. Ninety percent of the phages were inactivated within 15 min of exposure to urea (8M) in culture. The effect of trisodium citrate (0.01%) on the phages of *S. bovis* also resulted in a high degree

of phage growth inhibition. Inhibition in the presence of citrate was believed to be a factor limiting the normal development of the infectious processes. The isolated phages for *S. bovis* were also shown to have different sensitivity to phenol and chloroform.

The functional role of phages in the rumen ecosystem is yet to be determined, but because phages are present in all ruminal fluid samples examined and there are several morphologically different types in rumen fluid at one time suggests that phage activity is important in the dynamics of the microbial population, and could be responsible for preventing wide shifts in the types of bacteria in the rumen, by keeping particular species in check when their numbers exceed the ecological balance. The only in vivo studies by Orpin and Munn (1974) to determine the role of ruminal bacteriophages showed that *Eadie's oval*, strain EO2, normally present at 10^6-10^7per ml in the rumen of sheep, decreased to zero in 10 days when the rumen was inoculated with an isolated bacteriophage which infected the EO2 strain. Control inoculation of the uninfected strain in other sheep resulted in no decrease in the numbers of this ruminal bacterium under similar conditions. In contrast, Iverson and Millis (1977) reported that the rapid succession of *S.bovis* strains with differing sensitivities to bacteriophages prevented their attempts to eliminate

S.bovis from the rumen with their isolated virulent bacteriophages. Clearly, there is a need to better understand the relationship of bacterial strain sensitivity to bacteriophage infection and the temperate and obligatory lytic nature of the bacteriophage in the rumen.

MATERIALS AND METHODS

1. Bacterial Cultures

All bacterial species used in this study have been shown to be involved in either the ruminal degradation of plant polymers, in nutritional cross-feeding, interspecies hydrogen transfer, growth factor requirements or biohydrogenation of polyunsaturated fatty acids. Although, most of the bacteria used in this study were laboratory strains which had been kept in culture collections for many years, it was also important to use new isolates of the major cellulose degrading bacterial species to avoid any possible mutational and physiologic changes after years of cultivation. Once selectively isolated and presumptively identified, these wild bacterial strains were used to screen for virulent and temperate bacteriophages in pure cultures. The ruminal bacterial species used in the research were: *R. albus*, strain 7, strain 8, wild type; *R. flavefaciens*, strain FD1; *B. fibrisolvens*, strain D1, strain 49, strain A38; *Fibrobacter succinogenes*, strain S85; *B. amylophilus*, strain H18; *T. saccharophilum,* wild type; *T. bryantii*, strain $B_2$5. *Butyrivibrio fibrisolvens* strains D1 and A38 were from the culture collection of M.P. Bryant, Univ. of Illinois, Urbana, Il and strain 49 was obtained from R.B. Hespell, USDA, Peoria, Il. All working cultures were maintained on 30% rumen fluid containing glucose-

cellobiose- starch (GCS-RF) medium (Bryant, 1962) agar slants held at 4C. In cultivation of bacterial strains, a loopful of the fluid of syneresis from agar slant cultures is inoculated into GCF-RF liquid medium, under anaerobic condition, and incubated at 39C for 24 h. About 0.1 ml of the bacterial culture is then subcultured every 24 h. into fresh liquid medium (4.0 ml). The composition of the medium consists of (g/l): glucose, 0.6; cellobiose, 0.6; starch, 0.6; yeast extract, 2.0; trypticase, 5.0; resazurin, 1.0; Mineral 1, 75 ml/l; mineral 2, 75 ml/l; cysteine-HCl, 20 ml/l; $NaCO_3$ (8%), 50 ml/l. Mineral I consisted of (g/l): $CaCl_2.2H_2O$, 1.6; KH_2PO_4, 6.0; $(NH_4)_2\ SO_4$, 6.0; $MgSO_4.7H_2O$, 2.5; NaCl, 12.0. Mineral II consisted of (g/l): K_2HPO_4, 6.0.

1.1. Isolation Of Cellulolytic Bacteria

Ruminal cellulolytic bacterial species used in the study were isolated from the rumen using the Hungate anaerobic technique (1950, 1970) as modified by Bryant (1972). Ruminal fluid was obtained from a rumen fistulated cow maintained on an alfalfa hay diet. Ruminal fluid was strained through four layers of cheesecloth, and then centrifuged at 1075 x g at 4 C twice to remove large debris. The ruminal fluid supernatant was serially diluted (10^{-1} - 10^{-10}) into an anaerobic dilution medium containing (ml/ 300 ml) mineral

I, 22.5; mineral II, 22.5; resazurin, 0.3, Na_2CO_3, 15.0; and cysteine-HCl, 6.0. The highest four dilutions (10^6 - 10^{10}) were inoculated into anaerobic roll tubes containing ground cellulose (2mg/ml; Whatman filter paper, Balston Ltd.) and agar medium. After 24 h. of incubation at 39 C, well isolated bacterial colonies were picked from the highest dilutions and transferred into liquid medium with cellulose (0.7 mg/ml) as substrate and then subcultured back into liquid medium containing cellobiose (0.7mg/ml) Bacterial species were characterized between transfers on the basis of their morphology by Gram stain. Once pure isolates were obtained, cells were identified on the basis of cellular morphology, culture characteristics, and fermentation products. Colonies of *Ruminococcus albus* were white. The cells were spherical, with adjoining sides of diplococci flattened. Cells occurred predominantly as pairs, with a few numbers occurring singly and only occasionally chains of up to four cells. The cells were Gm (-). Volatile fatty acids were determined by the method of Playne (1985). All bacterial isolates were maintained in 30% GCS- RF agar (2 %) medium and maintained at 4 C.

1.1.2. **Isolation Of Ruminal Spirochete**

The ruminal spirochete, *Treponema saccharophilum,* was isolated as described for cellulolytic bacterial

isolates, except the medium used contained pectin (0.7mg/ml) and rifampin (1 mg/ml) as selective media (Paster, 1985). On the bases of size, morphological features, and substrate degrading ability, the ruminal spirochete was identified as *T. saccharophilum* as previously isolated and characterized by Paster and Canale-Parola (1985).

1.2. **Bacterial Cell Count**

The method of choice for measuring the cell number of the microorganisms was both an optical one in a spectrophotometer (Bausch and Lomb, spectronic 70 colorimeter) and bacterial colony count in a roll tube containing agar media.

1.2.1. **Spectrophotometer Analysis**

Optical density (OD) of all bacterial cultures and bacterial cultures infected with bacteriophages were determined by spectrophotometry readings at 600 nm. In all experiments of bacterial cultures, an optical density of culture was measured. An OD value of 0.3 to 0.5 represented approximately 10^7 - 10^8 cells per ml.

1.3. **Fermentation Product Analysis**

Bacterial fermentation by-products were determined by gas-liquid chromatography using a Hewlett-Packard 5890

A gas chromatograph (Mt View. CA.) equipped with a 3 m (SS) column packed with GP 15% SP-1220. Nitrogen was the carrier gas. Temperature conditions were: oven, 125 C., injector, 180 C., FID, 180 C. *Ruminococcus albus* was identified by its fermentation products profile as determined by gas-liquid chromatography (Salanitro, 1975). A pure culture of *R. albus* was inoculated into only cellulose (0.7 mg/ml) or cellobiose (0.7 mg/ml) containing liquid media under anaerobic condition. Following incubation at 39 C, aliquot samples were taken at 24, 36 and 48 h. period and were centrifuged at 1075 x g twice for 20 min. The supernatant was filtered through a Millipore membrane filter (0.2 um pore size). One ml of the filtrate was placed in a vial with the addition of 75 ul of 1M sulfuric acid. All samples were kept at 4 C until analyzed by gas liquid chromatography. Fermentation by-products obtained from *Ruminococcus albus* treated cultures were analyzed by high performance liquid chromatography (HPLC). Each ruminal bacterial species was initially grown in 30% GCS-RF media for 24 h at 39 C. After centrifugation at 4 C for 20 min, pellets were pooled into fresh GCS-RF media and were incubated for 24 h at 39 C. The mixed culture (consisting of *B. fibrisolvens*, strain D1, and strain 49; *R. albus*, strain 7 and strain 8 and wild type; *R. flavefaciens*, strain FD1; *Fibrobacter succinogenes*, strain S85) was

centrifuged at 4 C for 20 min. The pellet was suspended in dilution media and transfer immediately into media containing xylan (0.5%) or glucose-cellobiose (GC) (0.5%) as substrate. Since some ruminal bacteria do not grow well without rumen fluid, branched chain volatile fatty acids (isobutyric,1 mM; N-valeric, 0.9 mM; isovaleric, 0.9mM; 2- methylbutyric, 0.9 mM; and phenylacetic, 0.9 mM) were added. The cultures were allowed to grow for 3 h at 39 C and were inoculated with the *R. albus* phage at a multiplicity of infection (M.O.I.) of 1:100. The infected cultures were incubated at 39 C and aliquots in 3 ml quantities were taken at 16 h and 36 h. Samples were centrifuged at 4 C for 20 min and the supernatant were filtered through a Millipore filter membrane (20 um-pore-size). The filtrates were transferred into small vials with the addition of 75 ul of 1 M sulfuric acid. The samples were kept at 4 C until analyzed.

1.3.1 Statistical analysis

The molar concentration of succinate, lactate, formate, acetate, propionate and butyrate was determined from 10 ul of each sample. Analysis of mean was performed using the Bonferonni t test procedure of SAS (1987).

2. Bacteriophage Isolation

Ruminal fluid (1L) was obtained from a rumen fistulated cow maintained on an alfalfa hay diet, strained through four layers of cheesecloth and centrifuged twice at 1075 x g for 20 min. The supernatant was passed through a sterile Millipore membrane filter (0.45 um pore size), and concentrated as described by Tamada et al. (1985). Ten ml of filtrate was added to 1.2 ml. of 5M NaCl solution, containing 0.6 gm of polyethylene glycol (PEG 6000). The mixture was held at 4 C overnight, then centrifuged at 1075 x g for 20 min. The pellet containing the concentrated bacteriophage was resuspended in GCS - RF medium and used for infection of ruminal bacteria.

2.1. Bacteriophage Plaque Assays

Virulence of the bacteriophage was determined by plaque diameter size and rate of plaque formation (Cluzel, 1987), using the double agar overlay technique of Gratia (1936) as described by Adams (1959). Motile bacteria, which do not produce confluent lawns, were examined in liquid medium inoculated with known concentrations of bacteriophages, with optical density readings (600 nm) of inoculated cultures measured against control cultures.

2.1.2. Double Agar Layer Method

All bacterial species except for *T. saccharophilum* were tested for bacteriophage infection by the technique of Gratia (1936) as described by Adams (1959). About 0.1 ml of the concentrated bacteriophage suspension was inoculated into exponentially growing cultures (0.4 ml) of ruminal bacterial species and incubated at 39 C for 20 min to allow for phage adsorption. The inoculated culture was added into a tube containing soft agar (0.7%) media kept at 47 C, gently vortexed and poured directly onto a sterile petri dish containing a bottom agar layer (2%) media. Plates were inverted and placed in a desiccator and the desiccator was flushed with carbon dioxide (100 %) for 20 to 30 min. The desiccator was then sealed and incubated at 39 C for 24 h. Plates were examined for plaque formation after 24 and 48 h. When plaques were observed, their diameter was measured. The plates were also photographed immediately.

2.2. Bacteriophage Extraction and Propagation

Plates that showed plaque formation were carefully scraped off the top agar and placed into tubes containing GCS - RF medium. The tubes were held for 24 h to allow diffusion of phage from the agar. After centrifugation at 4302 x g for 20 min, the supernatant was recovered and stored at 4 C for phage identification by electron

microscopy. Phage was usually grown in conditions that was optimal for its bacterial host. Propagation of the phage on solid medium was the most frequently used technique because it produced higher titers than propagation in liquid cultures. In liquid medium, the medium is inoculated with a known bacteria to phage ratio, and the mixture incubated until lysis occurred, which is not always visible. The ratio of bacteria to phage, or multiplicity of infection (M.O.I.),was 1:100. The bacteria inoculum was adjusted by optical density. Propagation on solid medium is as described in the section on the double agar method.

3. Induction of Lysogenic Bacteria

In screening for temperate bacteriophage, early logarithmic culture of the ruminal bacterial species was induced using mitomycin C (Cluzel, 1987) or by UV irradiation (Eisensark, 1967). Induction by UV-light or mitomycin C apparently involves inactivation of repressor. Ultraviolet light induction has been explained by assuming that some product ('inducing substance') either a DNA precursor or degradation product formed as a result of irradiation, complexes with and inactivates the phage repressor (Ben-Gurion, 1967; Hertman, 1967), as a result of which the prophage is excised from the host chromosome, followed by autonomous

DNA replication, phage production, and lysis. The inactivation may be indirect since it depends on a bacterial gene (Rec A) as well as on the structure of the repressor.

3.1. **Mitomycin C Induction**

Mid-log phase cultures of bacteria (1 ml) were inoculated into a 30 % GCRF medium containing, mitomycin C to give a final concentration of 5 and 10 ug per ml. Mitomycin C is an anti-tumor antibiotic complex($C_{15}H_{18}N_4O_5$) produced by a soil microorganism, *Streptomyces caespitosus* (Siuta, 1974). The mitomycin C treated cultures and the control cultures were incubated at 39 C for 15 to 30 min. The cultures were pelleted by centrifugation at 1075 x g for 20 min. The pellet was added into fresh GCSRF medium without mitomycin C, and further incubated in a shaking water bath (39C) for 6 h. All cultures were covered with aluminum foil as protection from exposure to light. The cultures were centrifuged at 1075 x g for 20 min and the supernatant was examined for presence of phage against an indicator strain by the double agar method, and by electron microscopy. Also, the OD of mitomycin C induced cultures were followed for their lytic activity at intervals of 20 min for about 24 h.

3.1.2. UV LIGHT INDUCTION

Mid- log phase growing cultures of ruminal bacterial species (1 ml) poured onto sterile petri dish, or confluent lawn of bacteria, were exposed to UV light for 15, 45, 60, 120 sec from a distance of 25 cm from a bactericidal UV lamp in a hood. The cultures were directly transferred into fresh GCSRF medium (4 ml) under anaerobic conditions. The cultures were covered with aluminum foil and incubated in a shaking water bath (39 C) for 6 h. The cultures were centrifuged at 1075 x g, for 20 min and the supernatant lysate were diluted 10 fold into GCS-RF broth and was tested for presence of bacteriophages against an indicator strain by the double agar layer method and by electron microscopy. Lysis of culture as may occur by UV irradiation was also followed by OD reading at 600 nm for 24 h.

4. Thin Sectioning

About 0.1 ml of concentrated phage filtrate suspension was inoculated into exponentially growing bacterial cultures and incubate at 39 C for 20 min. The cultures were centrifuged at 1075 x g for 20 min. Pellets were placed into warm agar (2%), allowed to solidify and fixed in cold 4 % glutaraldehyde in 0.1M sodium phosphate buffer (pH 7.2) for 1 h. Samples were washed three times in buffer, then post fixed in 1%

osmium tetraoxide in buffer for 1.5 h. at room temperature, and dehydrated by a graded series of ethanol. Embedding was done with a mixture of Epon - Araldite - spurs (Klomparens, 1986). Silver gold (70 - 80 nm) ultrathin sections were cut, stained with uranyl acetate, followed by lead citrate. Sections were examined with a JEOL 100 CX11 transmission electron microscopy at the M.S.U. Electron Optics Center.

4.1. **Negative Sectioning**

Formvar and carbon coated electron microscope grids were wetted with 5ul of the phage sample by the drop technique (Horne, 1965). The samples were negatively stained with 1% potassium phosphotungstic acid (pH 6.5), and grids were examined for phage particles by using a JEOL 100 CX11 transmission electron microscopy.

4.2. **Scanning Electron Microscopy**

A suspension of bacterial culture (1 ml) was mixed with 5% glutaraldehyde (1 ml) in 0.1M phosphate buffer (PH 7.2). Culture was placed in ice bucket for 30 min. A drop of 1% poly-L-lysine was dropped on a plastic petri dish and a glass coverslip layered on the drop. After standing for 5 min., the coverslip was removed and rinsed with drops of water. A drop of cells was placed on the coverslip to which poly-L-lysine had been exposed and the

suspension allowed to settle for 5 min. The coverslip was washed and placed in a coverslip holder and transfered through a series of ethanol washes, 25, 50, 75, 90 and 100 % with 5 min in each step. The sample was then critically dried, mounted to a stub and finally sputter coat the mounted coverslip. The specimen was examined by scanning electron microscopy at the M.S.U. Electron Optics Center.

5. **Genomic DNA Preparation**

Agarose blocks containing bacterial cells or supernatant phage lysate were prepared as described by McClelland et al. (1987) for separation of DNA by pulsed field gel electrophoresis (PFGE). *R. albus* culture infected with the phage and uninfected culture of *R. albus* were washed, twice, in L-buffer composed of, EDTA, 0.1 M; Tris-Cl, 0.01 M, NaCL, 0.85 M. The cultures were centrifuged at 1075 x g for 20 min at 4 C. A pellet (0.5 ml) was added into equal volume of 1% low melting temperature agarose (Sea-plaque) in L-buffer . These were dispensed into 8 x 3 x 2 mm wells in a mold plate and were allowed to solidify. The resulting blocks or 'inserts' were treated insitu with 1mg/ml of lysozyme (Sigma Co.) and 0.05% sarkosyl at 37 C for 24 h. Inserts were transferred in L-buffer with 1 mg/ml proteinase K and 1% SDS, and were incubated at 50 C for 48 h. The

inserts were again transferred into Tris- EDTA (TE, pH, 7.5) and stored at 4 C.

5.1. **Pulsed Field Gel Electrophoresis**

100 ml of 1% agarose (Bio-Rad's Molecular Biology Certified Grade) in 0.5 X Tris-Borate buffer (TBE) comprising, Tris-Base, 0.1 M; Boric acid, and EDTA, 0.1 M., was melted and poured on a casting stand. The agarose was allowed to cool at room temperature for 30 min. The comb was removed. Sample plugs or 'inserts' were cut into smaller pieces and were added to the wells using a spatula and pressed to the bottom of the wells. Each well was sealed with Low Temperature Melt Preparative Grade Agarose. The gel was placed on a clamped homogenous electric field (CHEF -DR 11, Bio-Rad Laboratories) chamber containing 2 liters of 0.5 X TBE buffer. Electrophoresis was run at 180 volts at 14 C with alternating pulse 40-80 sec. for 24 h. The gel was placed into ethidium bromide solution (o.5 ug/ml) and let stand for 30 min with constant shaking. After destaining for 1-3 h, the DNA band was photographed by placing the gel on a ultraviolet transilluminator (254-360 nm).

5.1.2. **Hybridization Technique**

DNA bands in gel were transferred onto nylon membrane (BIO-RAD) by the capillary transfer method

(Maniatis, 1989) as follows: The gel was placed in a dish and soaked in several volumes of 1.5 M NaCl and 0.5 N NaOH for 45 min.to denature the DNA. After the gel was rinsed in water, it was neutralized by soaking it in a solution of 1 M Tris (pH 7.4), 1.5 M NaCl at room temperature with constant agitation. The gel was removed and placed on a wetted nylon membrane (Bio-Rad) that was already mounted on a stack of glass plates to form a support in a dish containing transfer buffer (10 x SSC). A stack of paper towels was put on top of the gel onto which a glass plate was placed and press it down. The transfer of DNA from the gel to the nylon membrane was allowed to proceed for 24 h. The phage DNA was eluted and purified according to Maniatis (1989). Total or restriction fragments of the phage DNA was labelled by random priming using ^{32}p-dCTP (Boehringer Mannheim, Indianapolis, IN) as follows: Fifty nanogram of phage DNA in 2 ul was added to 7 ul of water in an Epindorff tube and boiled for 5 min. After it had been cooled immediately on ice, 3 ul AGT mixture (dATP, dGTP, dTTP); 2 ul reaction mixture ; 1 ul of klenow enzyme; and 5 ul of ^{32}p-dCTP (10 uci/ul or 3000 uci/mmole) were added. The mixture was incubated at 37 C for 30 min. The reaction was stopped with 5 ul of stop solution and the probe was boiled for 5 to 10 min. G-50 medium was placed in a column after which 100 ul of salmon sperm DNA (SSD)

was added for binding non specific DNA and the labelled probe was added. Elution was proceeded with 200 ul fraction with periodic addition of TE buffer. After determining the fraction of the labelled DNA fragments that has the highest count per minute, hybridization was carried out as follows: Hybridization solution containing, formamide, 50%; sodium dodecyl sulfate (SDS), 1%; NaCl, 1 M, dextran sulfate, 10%, salmon sperm DNA, 100 ug/ml; SSC salt solution, was placed into a tube and kept at 42 C water bath for 10 to 15 min. NaCl (0.58 g) was added to the solution and mixed, and the mixture was further incubated for 15 min. The tube was removed and the solution was poured into a bag containing nylon membrane, and the bag was sealed, and placed in water bath at 42 C for 15 min. with constant agitation. After incubation, denature SSD (100 ug/mL) and probe (1.2 ng/ml) was added, and the bag was sealed and incubated at 42 C overnight with constant shaking. Nylon membrane was washed, and exposed to a film for a week. The film was developed and examined for presence of hybridization.

RESULTS

Isolation of Ruminal Bacteriophages

Before an attempt was made to investigate any interaction between bacteriophages and bacteria of the rumen, we decided to first ascertain the presence of bacteriophages and identify as many bacteriophage morphotypes as possible in the ruminal contents obtained from a Holstein cow fed an all alfalfa hay diet. Such identification of ruminal bacteriophages, by negative staining and electron microscopy, may have provided clues as to which phage types would associate with which of the ruminal bacteria under study. Based on this study, five morphologically distinct bacteriophages were detected in ruminal fluid. The bacteriophages varied greatly in size, shape, and the nature of tail, as illustrated by the electron micrographs obtained. These included, a phage possessing an icosahedral shaped head (100 x 100 nm) and a short, non-contractile, rigid tail measuring 150 x 20 nm in length (fig.1a). The phage had no other peculiar features, such as a collar connecting the head to the tail nor a base plate at the tail terminus. According to the classification scheme of Bradley (1967), this phage should be assigned to group B. Another bacteriophage which was observed, had a cuboidal head (110 nm), and a short, noncontractile flexible tail, 290 nm in length (fig. 1b). Collar and base plate were not observed with

this phage. Based on its tail length to head diameter ratio, this phage would also be classified under group B. A cluster of phage like-particles with distinctly hexagonal shaped heads (50 x 50nm) was also observed (fig. 1c). Rather than a tail, these particles appear to have thread-like strands interconnecting the particles, with more than one particle appearing to branch off a common strand. These particles could be assigned to group E according to Bradley (1967), but this assignment is still tenuous because of the presence of the unusual interconnecting strands. A phage particle with a rather unique structure, an icosahedral head (40 nm) and a contractile sheathed tail (120 nm) was also seen in the rumen fluid (fig. 1d). This phage was classifiable under group A. The last type of phage like particles observed in ruminal fluid filtrate were large filamentous forms, varying in length (100 to 400 nm) and a diameter of about 50 nm (fig. 1e). These filamentous forms were numerous in the ruminal fluid filtrate, and were seen in every field examined. The shorter filamentous forms were either straight or slightly curved, while some of the larger forms showed sharp bends and right angle branching. BY their filamentous shape, these phage-like particles could be assigned to group F, according to Bradley (1967).

Figure 1. Transmission electron micrographs of negatively stained phage-like particles found in ruminal fluid filtrate.

a) Particle with icosahedral head, short, non-contractile rigid tail. Magnification, 72000 X

b) Particle with cuboidal head, short, non-contractile, flexible tail. Magnification, 108000 X

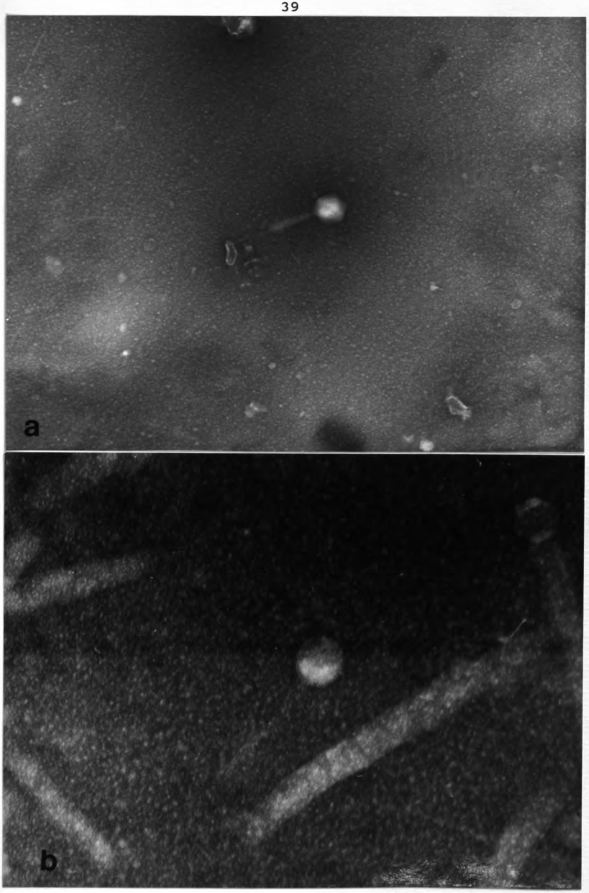
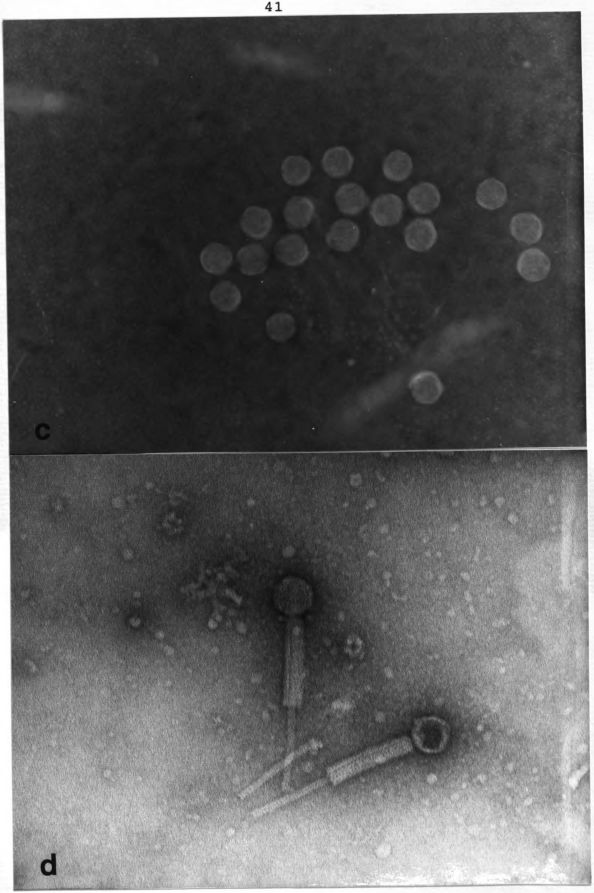

a
b

Figure 1. c) Cluster of particles with hexagonal heads and thin strands interconnecting the particles. Magnification, 150000 X

d) Particles with icosahedral head, short, contractile sheathed tail. Magnification, 210000 X


c
d

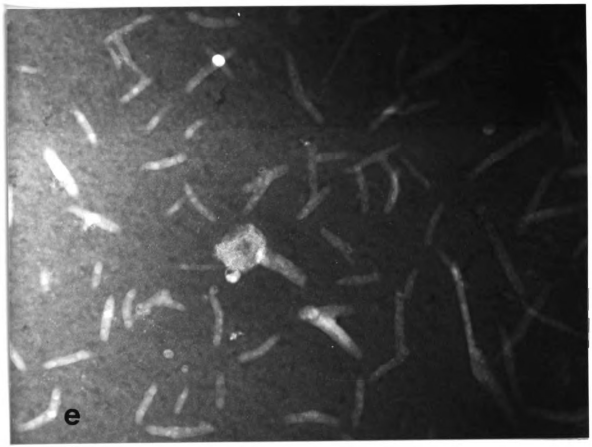


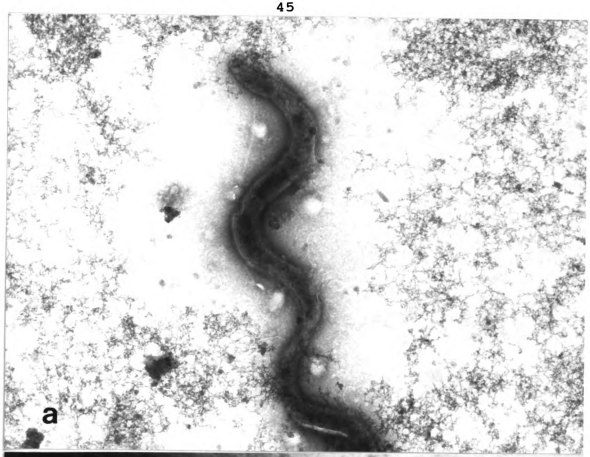
Figure 1. e) Filamentous like phage particles in various sizes. Magnification, 54000 X

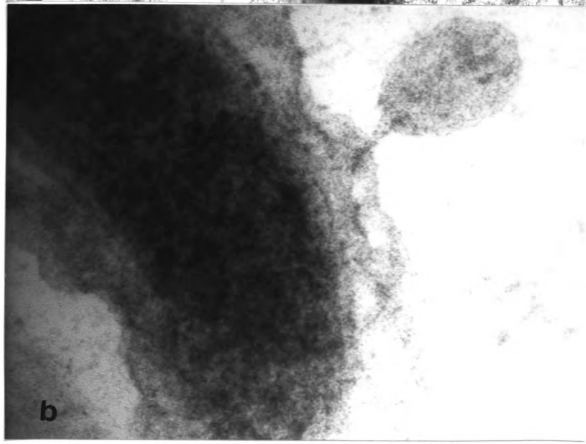
Bacteriophage - Spirochete Interaction

Once detection of various morphological types of phages in the rumen fluid was accopmlished, I decided to study the physical and physiological interactions between ruminal bacteriophages and those bacterial species selected for this study. Inoculation of concentrated ruminal fluid into an exponential culture of *Treponema saccharophilum* isolate with incubation for 20 min resulted in the attachment of numerous phage like particles to the cell surface of the ruminal spirochete (fig. 2a). The phage is distinguished by its cuboidal head symmetry and the presence of a short tail, which was observed to penetrate the outer sheath of the spirochete and terminate in the protoplasmic cylinder (fig 2b). The head of the phage is about 120 nm in length and 85 nm in width. The tail of the phage is non-contractile, measuring about 47 nm in length and 10 nm in width. There is neither a collar joining the head to the tail nor other apparent attachments. Because of the constant motility and swarming behavior of the spirochete, confluent lawns could not be developed for observing plaque formation by the phage, which could suggest possible physiological interaction between the bacteriophage and the spirochete.

Figure 2.a) Transmission electron micrograph of negatively stained *Treponema saccharophilum* showing the attachment of numerous bacteriophage to the cell surface. Magnification, 28500 X

b) Tansmission electron micrograph of ultrathin section of *Treponema sacharophilum* showing the penetration of a bacteriophage through the outer sheath into the protoplasmic cylinder. Magnification, 285000 X


a


b

Bacteriophage - *Butyrivibrio fibrisolvens* Interaction

Butyrivibrio fibrisolvens is a major hemicellulose degrading ruminal bacteria, and from the nutrition stand point is an important candidate to study in terms of its interaction with ruminal bacteriophages. Concentrated rumen fluid was inoculated into exponential cultures of *Butyrivibrio fibrisolvens*, strains D1, 49, and A38; and incubated for 30 min. The presence of numerous spheroidal phage-like particles were observed within the cytoplasmic space of both strains D1 and 49 (fig. 3a, 3b). However, no phage-like particles were found to be associated with strain A38. The detection of nearly mature phage-like heads in the cytoplasmic space of both strains D1 and 49, after 20 to 30 min incubation with ruminal fluid filtrate, might suggest that the maturation process is very rapid in this species. However, the length of exposure was much longer than 30 min since the time before and during processing of the culture must also be taken into account. Realistically the total time of exposure was more likely between 1 to 2 h. Attachment of phage-like particles on the outer cell surface of the *B. fibrisolvens* strains were not observed. Because a recognizable tail could not be clearly discerned within strain D1 or strain 49, it was unclear whether these were tailless phages or phages in the process of maturation. Examination of control cultures which had not been

inoculated with ruminal fluid, indicated that these laboratory strains of *B. fibrisolvens* had not been previously infected with the phage. The double agar overlay method for maintaining anaerobiosis was successful in developing confluent lawns of *B. fibrisolvens*. However, inoculation with ruminal fluid filtrate did not result in any plaque formation.

Bacteriophage - *Ruminococcus albus* Interaction

Ruminococcus albus is one of the three most predominant cellulolytic bacterial species in the rumen and therefore a logical ruminal species for study in terms of relationship with ruminal bacteriophage. When filter sterilized ruminal fluid was inoculated into a mid-log phase culture of a wild *Ruminococcus albus* strain, and then overlaid with soft agar onto solid agar media, plaques were produced after 24 h of incubation. The plaques were clear and smooth and measured 1 to 3 mm in diameter (fig. 4a). However, control plates, which had cells only grown in medium containing heat treated ruminal fluid, did not show plaque formation. When plates containing plaques were incubated longer, 48 h, a few of the plaques became turbid and were covered with surviving cells, suggesting growth of a few, preexisting bacterial cells which were resistant to infection with the bacteriophage or growth of cells which had already

Figure 3a) Transmission electron micrograph of ultrathin section of *Butyrivibrio fibrisolvens*, strain D1, showing intracellular phage like particle. Magnification, 150000 X

b) Transmission electron micrograph of ultrathin section of *Butyrivibrio fibrisolvens*, strain 49, showing intracellular phage like particles. Magnification, 150000 X

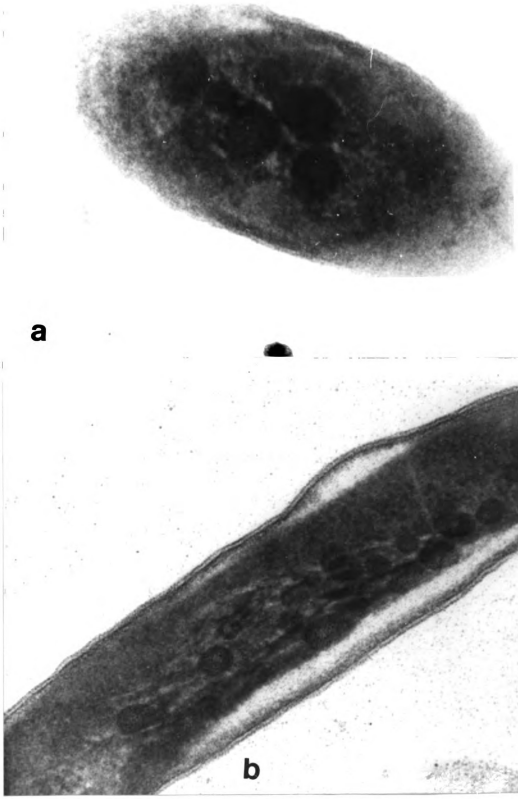
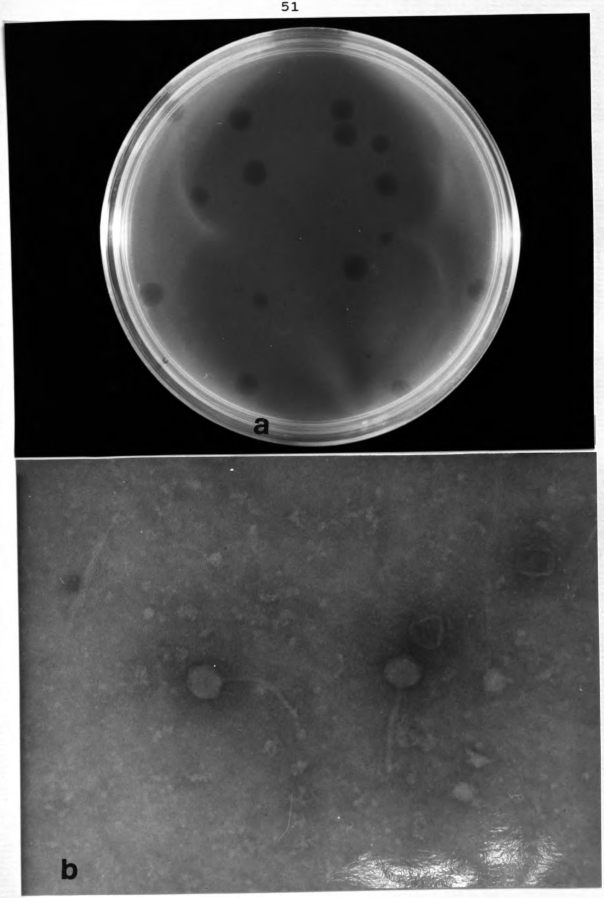

a
b

Figure 4a) Plate showing plaque formation by a ruminal bacteriophage on a lawn of wild isolate, *Ruminococcus albus*.

b) Transmission electron micrograph of negatively stained phage like particles found associated with *R. albus*. Magnification, 150000 X


a
b

been lysogenized by the phage. Clear plaques were removed from the plate by picking or by scrapping with a sterile Pasteur pipette, and crushed into a tube containing GCSRF medium, centrifuged, and the bacteriophage isolated from the supernatant. When the supernatant lysate was examined by electron microscopy, a small lambda-like phage particle was observed (fig. 4b). The phage had an icosahedral head, 50 nm in diameter and 60 nm in length. The tail was non-contractile and appeared to be flexible and measured 120 nm in length. Neither collar nor base plate was observed. As turning of clear plaques, which resulted from the lysis of sensitive bacterial cells, into turbid plaques is believed to be due to lysogenization of the bacteria by the phage that infected it, it was necessary to study the temperate nature of the phage in liquid culture. Accordingly, the growth and lytic ability of the phage in its host bacteria was determined by inoculating the phage, at a multiplicity of infection (MOI) of 1 to 100, into a mid-log phase culture of *R. albus*, and monitor growth by optical density reading (600 nm) at various periods of time. A synchronized step wise change in turbidity of the infected culture of *R. albus* was observed (fig. 5a). Five hours after incubation, a high number of bacteria were lysed and a considerable titer of phage, 1.6×10^6 pfu per ml were produced. On

longer incubation, that is 8 h later, the cells increased in their numbers and reached a cell density almost similar to early infection. Control culture did not show any lysis until much later, 48 h, of incubation time. Because of this phenomenon, the isolated phage was believed to be temperate for *R. albus*. Major hemicellulolytic laboratory strains of *Butyrivibrio fibrisolvens* D1 and 49 were found to be sensitive to the temperate phage. Contrary to the growth pattern observed in infected *R. albus*, *B. fibrisolvens*, strain D1 or 49 were significantly lysed by the phage and no characteristics of lysogenization of the infected D1 or 49 were seen on prolonged incubation (fig. 5b, 5c). Electron microscopic examination of supernatant lysate from infected broth culture of *R. albus* indicated a phage particle the same as the one identified in the plaque. Supernatant lysate from infected culture of D1 indicated the presence of both the lambda-like phage (fig. 4b) and a large number of filamentous-like phage particles (fig. 5d), previously not observed.

Figure 5a. Growth characteristics of wild *R. albus* in broth culture after infection by the *R. albus* bacteriophage.

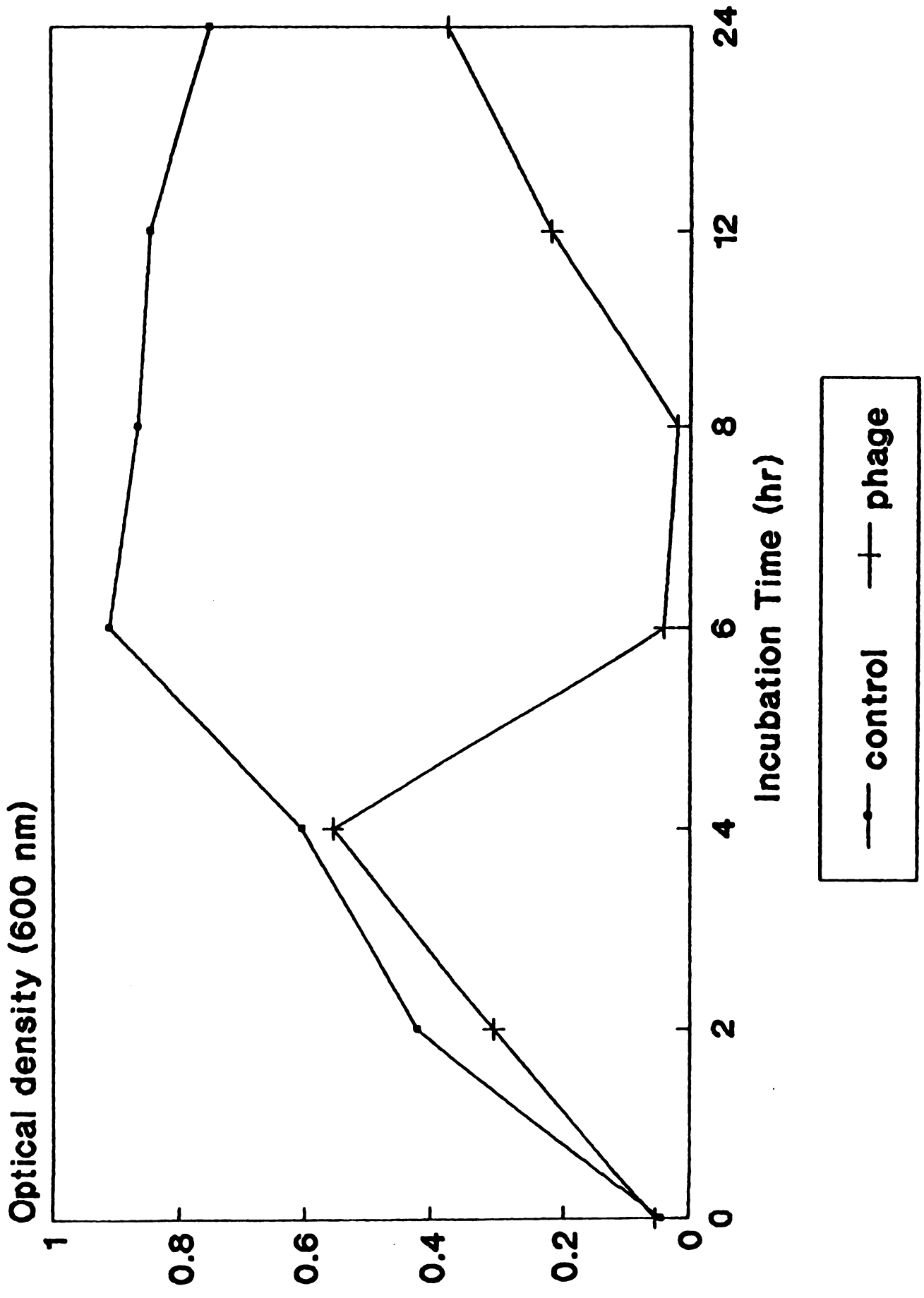

Optical density (600 nm)
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
12
24
Incubation Time (hr)
control
phage

Figure 5b. Growth characteristics of *B. fibrisolvens*, strain D1, after infection by the *R. albus* phage.

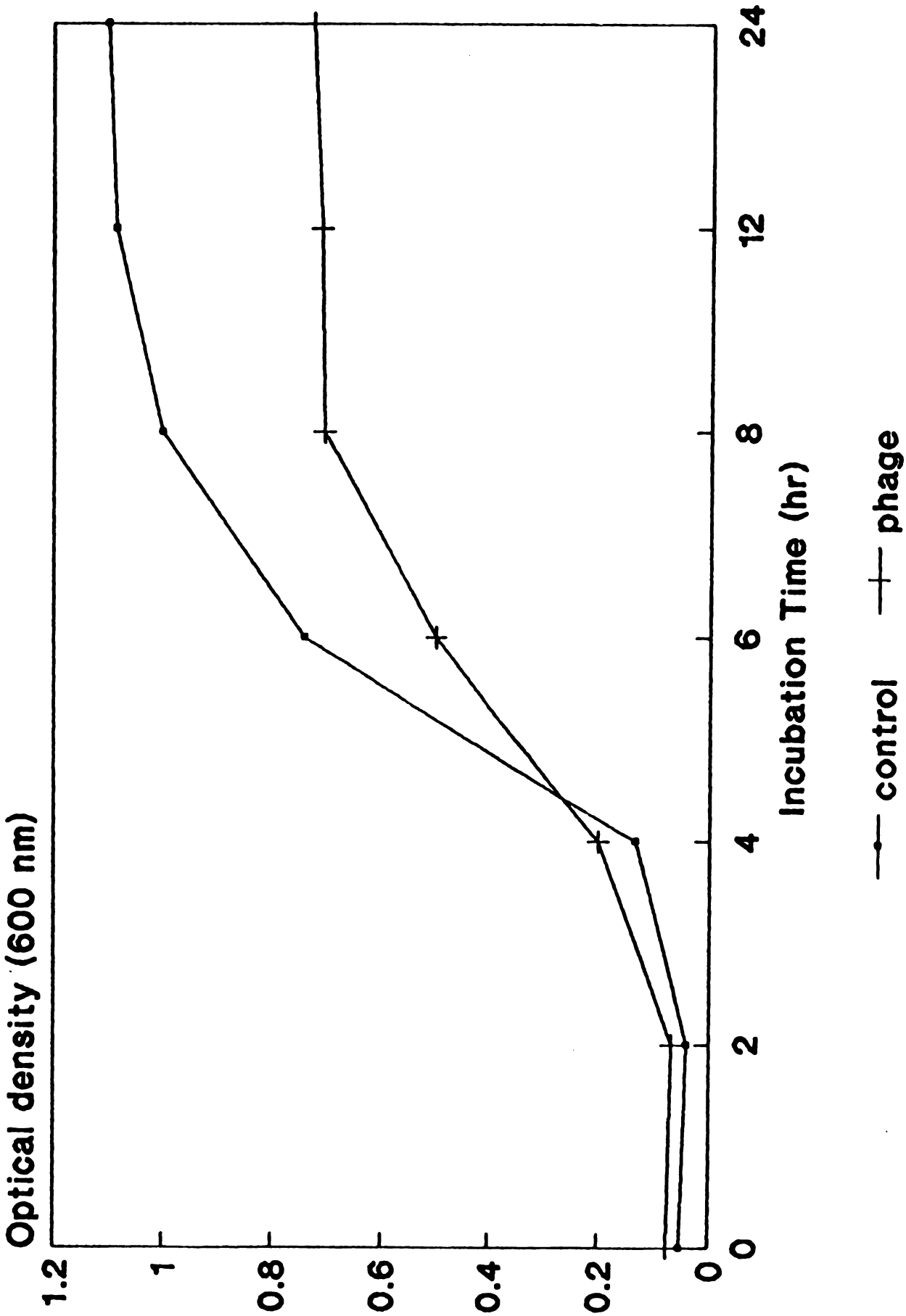
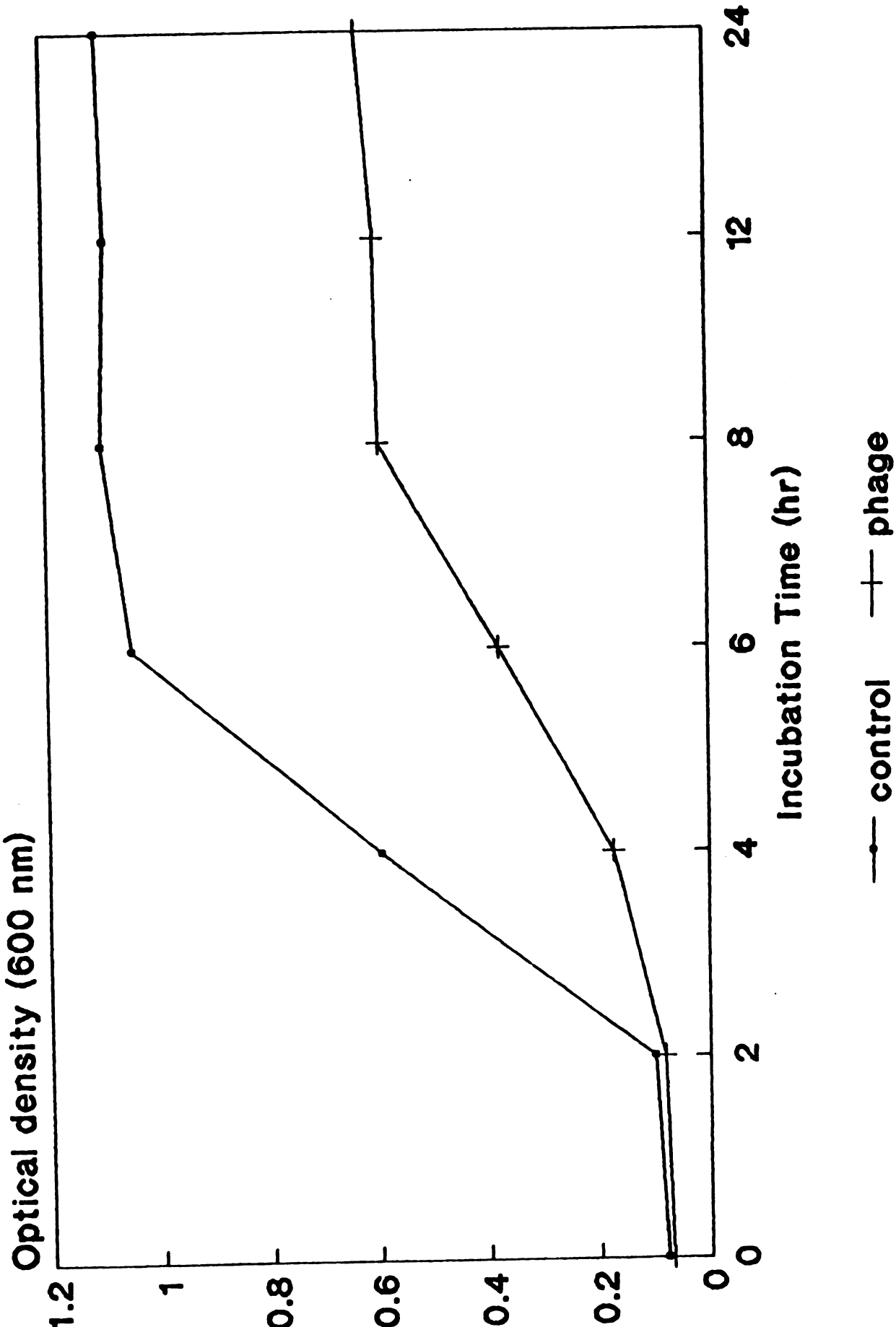

Optical density (600 nm)
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
12
24
Incubation Time (hr)
control
phage

Figure 5c. Growth characteristics of *B. fibrisolvens*, strain 49, after infection by *R. albus* phage.


Optical density (600 nm)
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
12
24
Incubation Time (hr)
control
phage

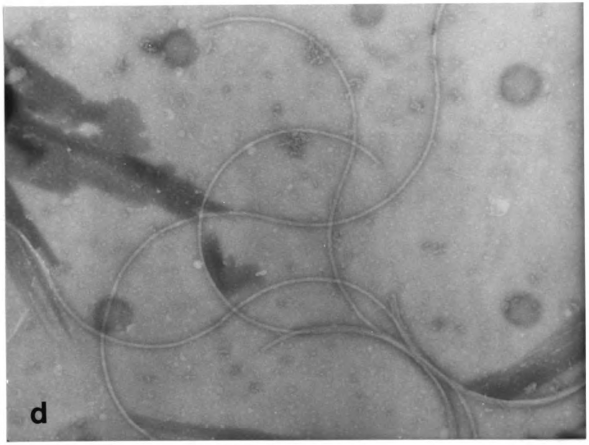


Figure 5d. Transmission electron micrograph of negatively stained filamentous phage like particles found in supernatant lysates of *B. fibrisolvens*. Magnification, 150000 X.

Host - Range Specificity of *R. albus* Phage

In studies to determine the optimal concentration of host bacteria, strain D1 or strain 49, required to support phage replication and hence plaque formation, it was determined that under normal conditions, an optical density of 0.3 to 0.5 was required. This represented about 10^7/ ml to 10^8/ ml of bacteria incubated with 10^5 to 10^6 pfu/ml of phage. At lower concentrations of host bacteria, the probability of a phage encountering a susceptible host were reduced and productive phage infection did not occur. The host range specificity of the temperate phage was, therefore, determined by placing 0.1 ml of the phage lysate containing 10^6 - 10^7 pfu/ ml on an agar overlay inoculated with the bacterial strain to be tested. Results of tests with ten ruminal cellulolytic and amylolytic species are shown in Table (1). The temperate phage was capable of lysing all the bacteria strains but with differing sensitivity. The degree of sensitivity, as determined by plaque forming unit (pfu) varied from one species of bacteria to another by several degree of magnitude. All these strains supported plaque formation that differed in numbers (10^3 to 10^7pfu/ml). *B. amylophilus*, H-18 and *S. ruminantium*, HD-4 were less sensitive and produced a lower titer of phage. *F. succinogenes*, S-85; *B. fibrisolvens*, D1 and 49; *R. flavefaciens*, FD1; and laboratory strains of

Table 1. Host Range Specificity of Phage Recoverd From *Ruminococcus albus*.

Bacterial Strains	Specificity (pfu/ml)
Ruminococcus albus, 7	10^6
Ruminococcus albus, 8	10^5
Ruminococcus albus, Wild	-
Ruminococcus flavefaciens, FD1	10^6
Ruminobacter amylophilus, H18	10^3
Fibrobacter succinogenes, S85	10^6
Selenomonas ruminantium, HD4	10^4
Butyrivibrio fibrisolvens, D1	10^6
Butyrivibrio fibrisolvens, 49	10^6

R. albus, 7and 8 produced a higher titer of phage. None of the plaques on any of these strains turned turbid when incubated longer than 24 h. Wild type *R. albus* did not produce plaques on its own confluent lawn, suggesting immunity conferred to superinfection by the same phage. Although, the phage appears to have a wide host range in its infectivity, no attempt was made to recover phage from every infected culture.

Temperature Sensitivity of *R. albus* Phage

The stability of the phage was determined at various storage conditions. It was found that about 20 % of the initial phage titer was lost after 6 months of storage at -135 C, and almost 50 % of the phage had disappeared when kept at 4 C for the same period of time. Phages are generally stable in their own lysate provided it contains suitable electrolytes and is free from specific inactivating agents derived from the lysed bacteria which are often difficult to remove by any means of filtration or otherwise (Freifelder, 1983). Because of this, supernatant phage lysate were treated with gelatin (1-2 %) before they were stored and to stabilize the titer.

In a study to determine the temperature tolerance of *R. albus* phage showed that the investigated phage differed in its sensitivity to a high range of temperatures (Table 2). Ninety seven percent of the

Table 2. Temperature Sensitivity of Phage Recovered From *Ruminococcus albus*.

Temperature (C)	Exposure (min)	pfu/ml (%)
39	30	100
39	60	100
50	30	100
50	60	100
70	30	3
70	60	0
90	30	0
90	60	0

Table 3. pH Sensitivity of Phage Recovered From *Ruminococcus albus*.

pH	Exposure (min)	pfu/ml(%)
3	120	20
6.8	120	100
10	120	100

phage were inactivated at 70 C for 30 min., but 100 percent of the phage were inactivated when exposed to 70 C for 1h. At 90 C for either 30 min or 1 h., no survival of phage was observed. However, the phage were resistant to 50 C for 30 or 60 min and no inactivation was seen.

PH Sensitivity of *R. albus* Phage

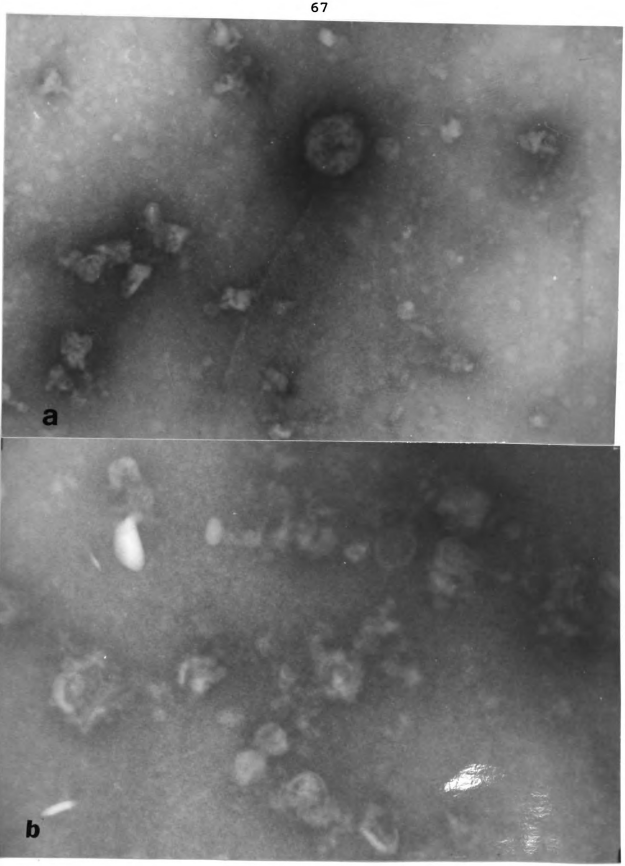
To investigate the resistance of the phage to a range of pH, phage was exposed to differing pH of GCS-RF media containing 30 percent ruminal fluid. The effect of pH on *R. albus* phage is indicated in Table (3). No phage were affected over a pH range between 6.8 and 10 for 2 h. However, at pH 3, 80 percent of phage were inactivated. Such a deleterious effect may not be reflected in the rumen since a decrease in pH does not usually take place much below pH 5 even during high intakes of concentrated diets by the ruminant animal.

Induction Of *R. albus* Culture

R. albus phage was inducible by ultra-violet light irradiation (fig. 6a). Irradiation for at least 45 to 60 sec of mid-log phase culture of *R. albus* was required to give inducible phage. Supernatant lysate from the induced host culture was plated with indicator cells, of *B. fibrisolvens* D1, and plaques were produced. Lower (20 sec) or higher (120 sec) UV exposures brought about no

Figure 6a) Transmission electron micrograph of negatively stained phage induced by UV irradiation from *R. albus*. Magnification, 72000 X

b) Transmission electron micrograph of negatively stained phage mutants induced by mitomycin C from *R. albus*. Magnification, 72000 X


a
b

phage induction, which might have resulted in less exposure of cells for those exposed for 20 sec and no induction, or high exposure which might have resulted in progressive lysis of cultures. When doses other than the optimal dose were used, that is either too low or too high, no plaques were obtained. Mitomycin C (5 ug/ml) also induced *R. albus*. Supernatant lysate from either mitomycin C or ultraviolet irradiation, examined by electron microscope, showed many incomplete particles, heads, and ghosts (fig 6b). In mitomycin C induced cultures, many more mutant phages were seen than intact phage particles. Lysate from this culture also did not produce plaques on indicator cells which suggest that mitomycin C, unlike UV irradiation, was not a specific inducing agent for *R. albus* phage despite the occurrence of mutant particles in the lysate. Spontaneous induction of *R. albus* phage has occasionally been observed in this study. Phages isolated from such cultures were identical in their morphology to the temperate phage of *R. albus* isolated from the concentrated ruminal fluid. Lysis of broth cultures of *R. albus* was also brought about by both mitomycin and UV-irradiation. When followed by optical density reading at 600 nm, mitomycin C exposed cultures of the either wild *R. albus*, or *B. fibrisolvens* strain D1 or strain 49, slowly for 20 min after which the cultures lysed gradually within 1 h (fig. 7a, 7b, 7c). Cultures

Figure 7a. Effect of UV irradiation on growth of *Ruminococcus albus* culture.

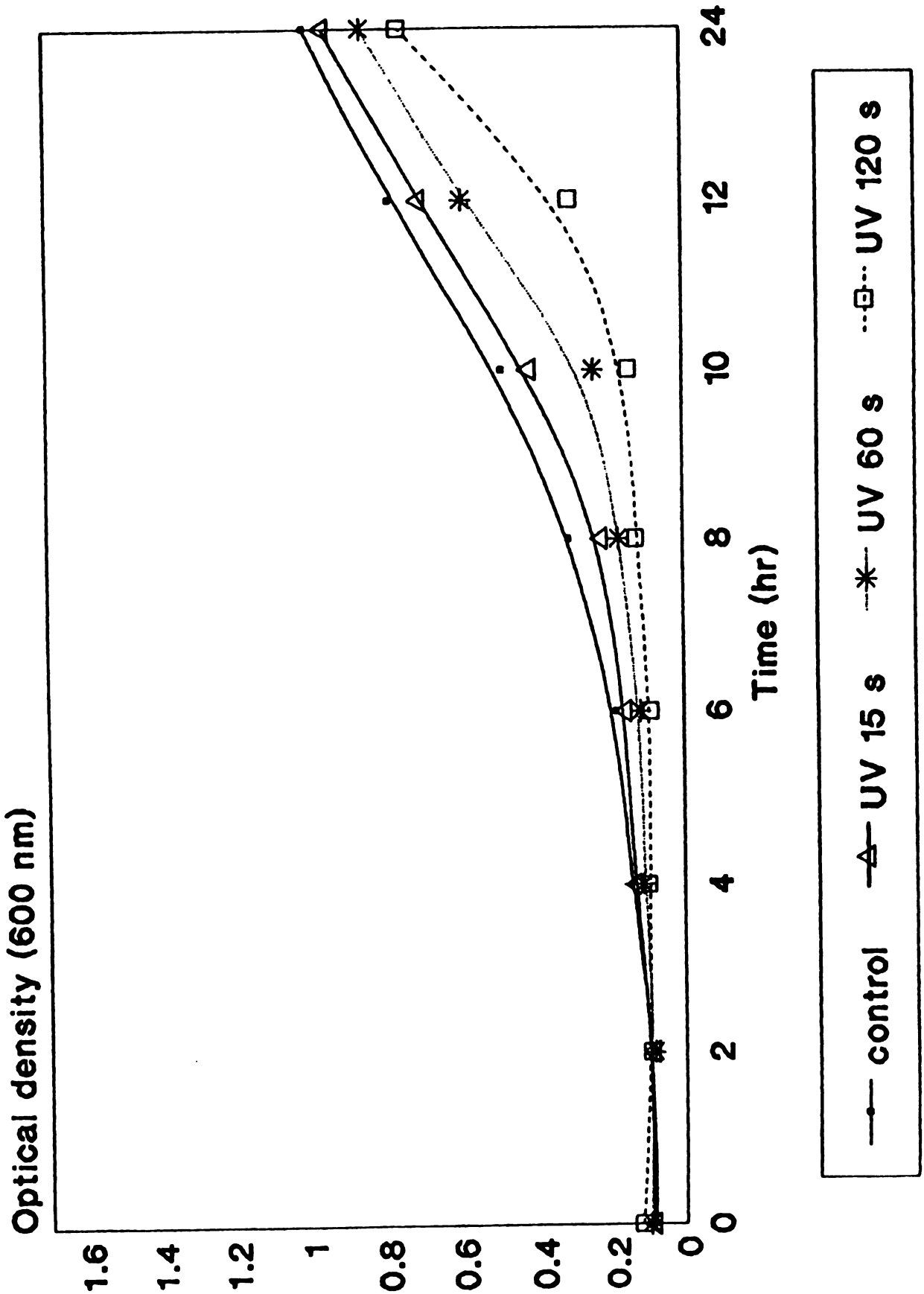

Optical density (600 nm)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
12
24
Time (hr)
control
UV 15 s
UV 60 s
UV 120 s

Figure 7b. Effect of mitomycin C or UV irradiation on growth of *Butyrivibrio fibrisolvens*, strain D1 culture.

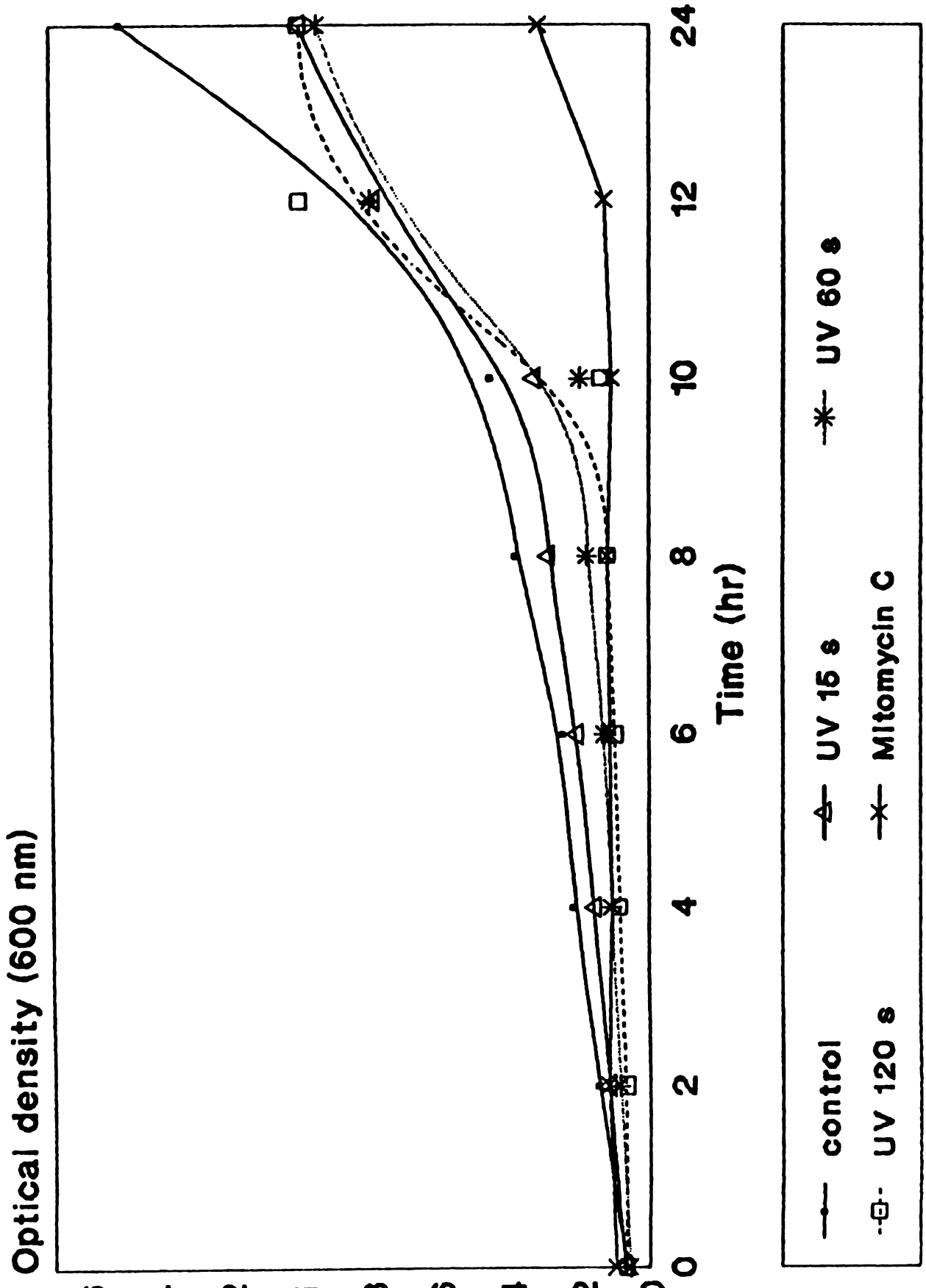
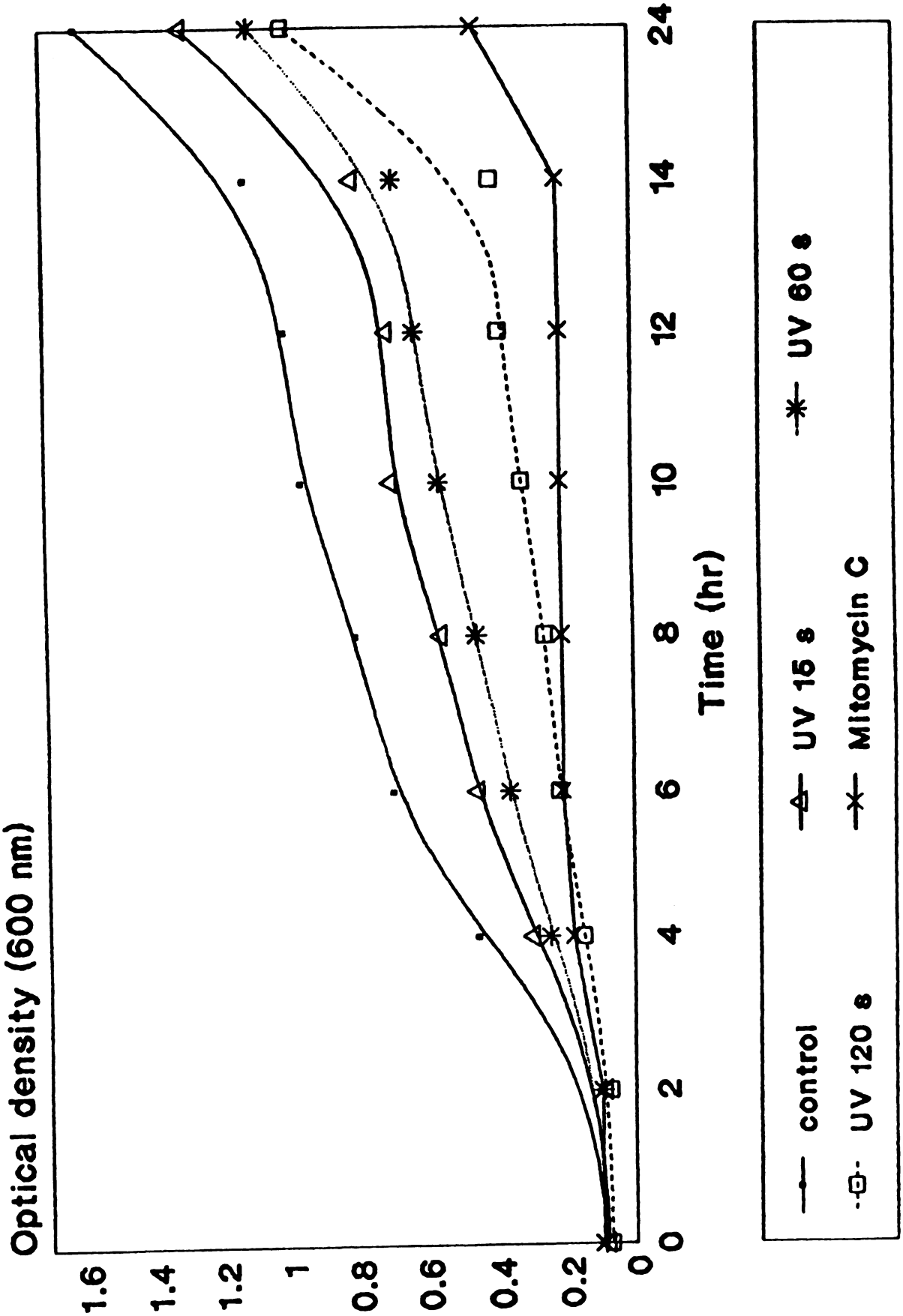

Optical density (600 nm)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
12
24
Time (hr)
control
UV 15 s
UV 60 s
UV 120 s
Mitomycin C

Figure 7c. Effect of mitomycin C or UV light irradiation on growth of *Butyrivibrio fibrisolvens*, strain 49 culture.


Optical density (600 nm)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
12
14
24
Time (hr)
control
UV 15 s
UV 60 s
UV 120 s
Mitomycin C

which were not irradiated or not exposed to mitomycin C showed a growth pattern similar to normal culture. These results suggest that mitomycin C or UV irradiation exert their effects on bacteria by induction which requires intact cells, which lyse eventually leading to disruption of some of the cells.

Genomic studies and hybridization of *R. albus* phage

To confirm the true temperate nature of the isolated *R. albus* phage, genomic and hybridization studies were carried out. DNA from both *R. albus* phage and host bacteria were isolated by pulsed field gel electrophoresis. Relative to a lambda phage DNA standard, the total genomic DNA of the phage was estimated to be about 48.5 Kb (fig. 8b), and the total genomic DNA extracted from the host, *R. albus* was approximately 970 Kb (fig. 8a). Total genome or restriction fragments (smaI) of infected and uninfected cultures of both *R. albus* and *B. fibrisolvens* were isolated and transferred to nylon membranes. The transfers were hybridized with a probe developed from restriction fragments (smaI) of the phage. The probe hybridized with five restriction fragments of DNA from the infected cultures of *R. albus*. About 15 percent homology in the DNA between the temperate phage and its host, *R. albus* was demonstrated. Hybridization occurred

Figure 8.a) Pulsed field gel electrophoresis of total and sma I digested DNA from uninfected and phage infected cultures of *R.albus* and *B. fibrisolvens*.

BI, Restriction fragment from infected *B. fibrisolvens D1*.
RN, Restriction fragment from uninfected *R. albus*.
RI, Restriction fragment from infected *R. albus*
BN, Total DNA from uninfected *B.fibrisolvens*,DI
BI, Total DNA from infected *B.fibrisolvens*,DI
RN, Total DNA from uninfected *R.albus*
RI, Total DNA from infected *R.albus*

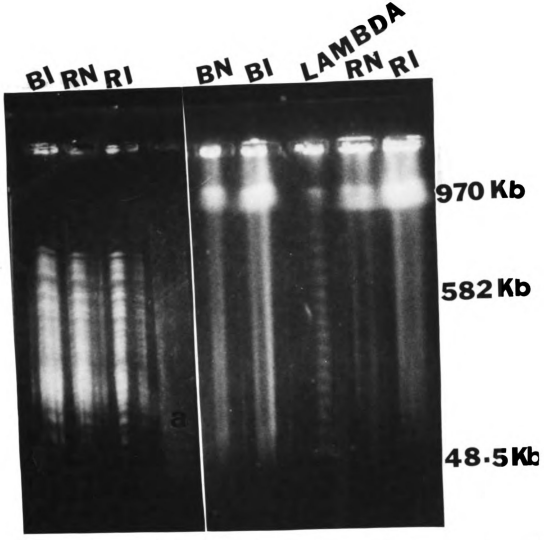
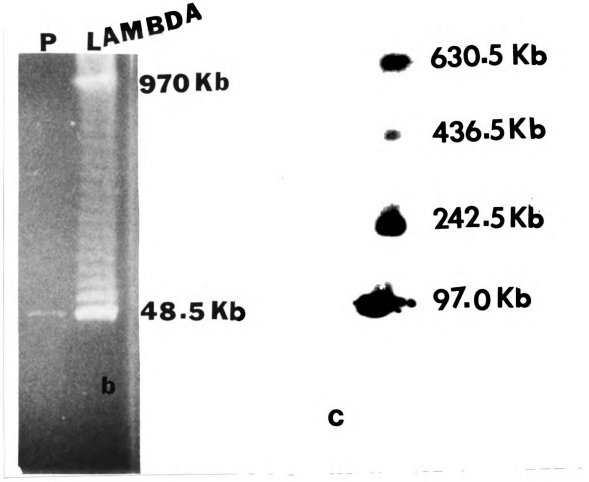

BI RN RI
BN BI
LAMBDA
RN RI
970 Kb
582 Kb
48·5 Kb

Figure 8b) Pulsed field gel electrophoresis of total *R.albus phage* DNA. Electrophoresis was carried out at 180 volts, 22 h, 14 C, pulses, 40-80 sec

c) Autoradiograph showing hybridization of phage probe to restriction (smaI) fragments from infected *R. albus*.


P
LAMBDA
970 Kb
48.5 Kb
b
630.5 Kb
436.5 Kb
242.5 Kb
97.0 Kb
c

with molecular weights, approximately 97.0, 242.5, 436.5 and 630.5 kb of the host DNA fragments (fig. 8c). There was no hybridization to DNA fragments from infected cultures of *B. fibrisolvens* D1 nor to DNA fragments from the uninfected culture of *R. albus*. The absence of hybridization to DNA from the uninfected cultures of *R. albus* probably suggests that all *R. albus* were not stably lysogenic or most probably had been cured from their prophage early in the experiment.

Effect of phage on sugar fermentation

The effect of the temperate phage on fermentation of xylan, glucose-cellobiose and cellulose in cultures of rumen bacteria was investigated. Results of the fermentations of xylan and glucose- cellobiose by the phage infected mixed ruminal culture are shown (Tables 4, 5). A mixed culture of ruminal bacteria consisting of *B. fibrisolvens*, strains D1 and 49; *R. albus*, strains 7 and 8 and wild type; *R. flavefaciens*, strain FD1; and *F. succinogenes*, strain S85 was incubated with the phage for 16 h in a medium containing a soluble xylan (0.5%), as a sole substrate, and their fermentation products were determined as described in the methods. There was a significant decrease in concentrations of lactate, formate($P < .05$) in culture infected with the phage. However, an increase in concentration of butyrate (P

<.05) was observed. There was no change in concentrations of succinate, propionate and acetate (P >.05) between control and treated culture. At 36 h of incubation, the concentration difference between the control and treated cultures were consistent. In medium containing glucose-cellobiose as the main substrate, there were no significant changes in the concentrations of succinate, and butyrate (P > .05) between control and treated culture. A significant increase in concentrations of formate, acetate and propionate (P <.05) was seen in treated culture both at 16 and 36 h incubation. Concentration of lactate decreased significantly in treated culture at 16 and 36 h incubation (P <.05).

Table 4. Effect of *R. albus* phage on fermentation[a] of xylan by mixed ruminal bacterial species[d]

	Incubation (16 h)		Incubation (36h)		
	Control	Phage	Control	Phage	SE
Succinate	0.555	0.343	0.537	0.360	0.14
Lactate	2.980[b]	1.695[c]	2.933[b]	1.743[c]	0.08
Formate	0.715[b]	0.378[c]	0.798[b]	0.295[c]	0.02
Acetate	6.725	7.990	6.870	7.847	0.24
Propionate	2.545	3.883	2.313	4.115	0.44
Butyrate	0.628[b]	0.698[c]	0.435[b]	0.890[c]	0.03

[a]each value is the mean mM concentration of duplicate samples.
[bc]means in the same row within one incubation period with different letters in their superscript differ (P<.05).
[d]represents *B. fibrisolvens*, strains D1 and 49; *R. albus*, strains 7, 8 and wild type; *R. flavefaciens*, strain FD1; *F. succinogenes*, strain S85.

Table 5. Effect of *R.albus* phage on fermentation[a] of Glucose-Cellobiose by mixed ruminal bacterial species[d]

	Incubation (16 h)		Incubation(36h)		
	control	phage	control	phage	SE
Succinate	0.718	0.770	0.683	0.805	0.08
Lactate	3.340[b]	2.365[c]	5.150[b]	0.555[c]	0.09
Formate	1.375[b]	1.690[c]	1.475[b]	1.590[c]	0.12
Acetate	12.430[b]	14.128[c]	11.863[b]	14.695[c]	0.23
Propionate	9.050[b]	11.668[c]	8.298[b]	12.420[c]	0.08
Butyrate	0.690	0.595	0.640	0.645	0.12

[a]each value is the mean mM concentration of duplicate samples.
[bc]means in the same row within one incubation period with different letters in their superscript differ (P<.05).
[d]represents *B. fibrisolvens*, strains D1 and 49; *R. albus*, strains 7, 8 and wild type; *R. flavefaciens*, strain FD1; *F. succinogenes*, S85.

DISCUSSION

The range in diversity of phage morphotypes observed in the rumen in this study was much less than what has previously been observed in other studies. While differences in sampling procedures and processing methods can not be discounted as probable causes for this difference, there seems to be no a priori reason for concluding that phage diversity is always high in the rumen. This discrepancy in phage diversity could be explained by the fact that ruminal contents of only one cow receiving an alfalfa hay diet was examined. In previous studies (Klieve, 1988) greater numbers of both cattle and sheep on different diets were sampled which could have resulted in a more diverse phage population. Under similar conditions to this study, Paynter et al. (1969) described only six distinct phage types in the rumen contents of an alfalfa hay fed cow over a one year sampling period. If most ruminal bacteria harbor phages in either a stable lysogenic or pseudolysogenic state, as the induction studies of Klieve et al. (1988) suggest, then phage diversity could be highly variable in the rumen depending on the proportions of the bacterial species present and the ruminal factors which influence their induction. Several of the phages observed in this study appear to have been reported in earlier ruminal studies. The phage with the icosahedral head, short non-

contractile tail with no collar and baseplate (Fig 1a) appears to be similar to a phage previously described by Paynter et al. (1969). Also, the cluster of particles with hexagonal heads interconnected by numerous thin strands (Fig. 1c) very closely resembles the cluster of polyhedral phage heads, with a common fibrillar attachment described by Hoogenraad et al. (1967). Typically, the non-tailed icosahedral head looked like the RNA coli phage 2J/1 described by Bradley (1964). The phage with the cuboidal head and flexible tail (Fig. 1b) somewhat resembles a lambda phage and also the phage induced from *Bacteroides ruminicola* AR by Klieve et al. (1989). The filamentous particles (Fig. 1e) does not appear to have been previously described in earlier studies.

The association of a specific phage with *T.saccharophilum* is not particularly surprising, since ruminal spirochetes are found in high numbers in the rumen of alfalfa hay fed cows. Enumeration by direct microscopic counts have shown ruminal spirochetes to be in the order of 10^8 per ml in ruminal contents (Stanton, 1979). Bryant and Burkey (1953) estimated that ruminal spirochetes constituted 2 to 5% of the culturable bacteria in the ruminal fluid of an alfalfa hay fed cow. Although, the ruminal spirochetes are not cellulolytic, they enhance cellulose digestion by their interactions

with cellulolytic species (Kudo, 1986; Stanton, 1979). *T. saccharophilum* will ferment pectin, polygalacturonic acid, soluble starch, dextrin and various monosaccharides and disaccharides (Paster, 1985). The association of a phage with *T. saccharophilum* confirms the earlier report by Hoogenraad et al. (1967) that ruminal spirochetes are heavily infected by phages. Morphologically, the phages attached to *T. saccharophilum* appears to belong to group C (e.g. short, non- contractile tail) under the classification of Bradley (1967), and appears to resemble the phages observed by Hoogenraad et al.(1967) and even much more to Staphylococcus phage 594n identified by Bradley (1963). Whether this phage is either temperate or virulent, or infects other ruminal spirochete strains remains to be determined.

The infection of *B. fibrisolvens* by a ruminal phage is of considerable interest, because *B. fibrisolvens* is one of the most predominant species in the rumen of cattle on a wide variety of diets (Bryant, 1956). Because of the high xylanolytic activity of strains, including D1 and 49, *B. fibrisolvens* is regarded as the most important species involved in the ruminal degradation of hemicellulose (Hespell, 1987). The phage-like particles which infect D1 and 49 appear to be morphologically similar. However, since a complete phage-like particle was not observed in the recovered

cells of either strain, this conclusion is still speculative. Intracellular multiplication has been examined in comparatively few cases by use of ultrathin sections. In the T-even phages, changes in the nucleoplasm have been observed prior to the formation of mature intracellular phages (Kellenbergen, 1959). With RNA phages, crystals of virions are formed prior to lysis (Schwartz, 1963). In the case of tailed mycobacteriophage B-1, the presence of empty phage heads within the host cell prior to lysis was reported (Tekeya, 1961). Klieve et al. (1989) were able to detect an unusual filamentous phage-like particle when *B. fibrisolvens* A14 was induced with mitomycin C. However, the phage-like particles we observed within the cytoplasmic space of strains D1 and 49 were clearly not a filamentous form. Whether this phage is more adventitious than the filamentous form remains to be determined, but suggest that *B. fibrisolvens* may be infected by two distinctly different phages. Our inability to develop plaques on confluent lawns of strains D1 and 49, despite the presence of phages in our infection studies with these strains is perplexing. Since the two studies were performed at different times, it is possible that the phage was not present in the ruminal fluid filtrate used for the plaque studies. The other possibility is that the phage observed in the infection studies is temperate for *B.*

fibrisolvens. The infection of *B. fibrisolvens* strains D1 and 49 by identically appearing phages suggest that these strains are very likely genetically related. Similarly, the lack of a phage infection in strain A38, suggest that strain A38 either is resistant to the phage or may not be genetically related to strains D1 and 49. In recent DNA homology studies, Hudson and Gregg (1989) concluded that, despite similar phenotypic characteristics, *B. fibrisolvens* is apparently a very genetically diverse group. Since phages are highly host specific and generally do not cross infect genetically diverse species (Ackerman, 1978), there may be an opportunity to use the ruminal phages for taxonomic classification of the indigenous bacterial population.

A close association between a ruminal bacteriophage and a major cellulolytic ruminal anaerobe, *R. albus*, was established. Of all the bacterial isolates and laboratory bacterial strains examined by double agar layer method, only the wild strain of *R. albus* produced plaques on its confluent lawn when plated with filtrate ruminal fluid. A ruminal phage resembling lambda was isolated. The lambda- like phage found associated with *R. albus* has the same morphology with *E. coli* lambda phage with respect to the head and tail, which is flexible. The diameter of head is the same as lambda head, 0.05 um, from which projects a tubular tail, 0.12 um long. This phage

multiplies in *R. albus* in the same manner *E. coli* phage multiplies in *E. coli*. The phage was characterized as being temperate to *R. albus* because of its ability to convert turbid plaques on the already clear plaques formed. Much of the turbidity may be attributed to surviving lysogenes or possibly due to lysogenization of host cells. The factors responsible for forming turbid plaques by the *R. albus* phage were not investigated in our study, but three genes called cI, cII, cIII, were the first genes to be identified in lambda (Kaiser, 1957). Secondary growth, as may occur particularly in long incubations of infected lawn, is usually indicative of lysogenization of host cells by temperate phages (Braksdale, 1974). Their plaques often had a target like aspect, consisting of a central colony of surviving bacteria surrounded by a clear margin, unlike clear plaques of virulent phage. A culture infected with a temperate phage does not necessarily always lyse but could continue to grow and divide normally in its original host cell. The culture fluid, however, always contains a low concentration of the phage. The reason why lysogenic cultures always contains a low level of phage is because once every several generations or so a daughter cell suddenly lyses and liberates several phages while the remaining cells carry on dividing. Because of the stable, hereditary nature of lysogeny and of the

immunity which a temperate phage confers, a recognition of the phage depended on the chance isolation of a sensitive indicator strain on which the phage will form plaques. To such effect, after screening several ruminal anaerobes, *B. fibrisolvens*, strain D1 and 49, were found to be sensitive to the phage and were used as indicators throughout the study. Because of their sensitivity, plaques always formed on their confluent lawns. Such occurrence of plaques have not been possible with *R. albus* due to immunity to super infection by the same phage. Moreover, liquid culture of *R. albus* incubated with the phage became lysogenized after a large number of phage were produced (fig. 5a). This phenomenon is in close agreement to other phages, for example, P2, which show the Boyd's effect, which is phage lysogenize host cells more efficiently when the bacterium is multiply infected or multiplies several fold in most cells before becoming a stable prophage (Braksdale, 1974). When growing cells are infected with a temperate phage, the proportion of infected cells entering the lysogenic pathway is about the same as the lytic pathway regardless of the growth medium or the cell division time (Echolas,1975). Growth of host bacteria above a threshold value is required for phage replication, which would lead to an increase in the number of phage. At some concentration of phage, the numbers would be sufficient

to decrease the number of bacteria or the metabolic activity. These interactions may even be complicated by either mutations that lead to resistant bacteria. The multiplicity of infection is also known to greatly influence the ratio of the lysogenic to the lytic response, with high multiplicities of infection favoring the lysogenic response and low multiplicities favoring the lytic response (Kovirilsky, 1973; Yen, 1980). Since the lysogeny state is only as stable as the conditions that favor it, the established prophage is continuously poised to reverse the prior decision in the light of changing circumstances and initiate a lytic cycle. Very likely many factors enter into either the lytic or the lysogenic process. Physical factors that influence establishment of immunity were studied in *E. coli* P1, and was found to lysogenize more efficiently at 20 C than at 37 C (Rosner, 1972). The effect of temperature in lysogenization of *R. albus* by its phage was not investigated in this study. However, it appears that establishment of lysogeny seem to depend on the multiplicity of infection. Studies involving growth of *R. albus* infected with the phage, indicated that a high number of phage produced prior to lysogenization of the host (Fig. 5a). Mechanisms by which *R. albus* perpetuate its temperate phage in a lysogenic or pseudolysogenic state have not been investigated in this study but is

fairly well established in lambda phage. After penetrating into the cell, the normally linear lambda genome circularizes by joining its cohesive ends , and a few " early genes" are transcribed, including repressor genes. The lysogenic decision also depends on host function, i.e, genes ihf (integrase host factor) and hfl and non-specific factors such as anti-metabolites and starvation of the host. The circularized lambda genome integrates linearly into the bacterial chromosome. Integration is mediated by phage host enzymes, integrase and/or ihf and occurs via a cross over at a preferential site. All phages do not have the same strategy as lambda. In contrast to phage lambda, P22, the DNA of Mu phage does not circularize and is integrated at random, via transposition, into the bacterial genome. Other phages including P1, certain mutants of lambda, and phages of *Streptomyces, Azomonas, Holobacterium* do not integrate at all, but behave as plasmids. Although, mechanisms that lead to a lysogenic decision whether or not to establish a prophage state in *R. albus* is not known, hybridization studies have indicated that the temperate phage could be integrated as part of the host genome. Such phenomenon was not seen with *B. fibrisolvens*, its indicator cell. The site at which the phage integrates in the host chromosome has not been determined. Nevertheless, the phage probe hybridized

with five restriction DNA fragments of the host suggest that the provirus was cut into 5 different sizes of DNA fragments by sma I. *R. albus* has also been shown by induction experiments to be lysogenic and, in some cases induction occurred spontaneously. For most of these studies, lysis of the culture was brought about following UV irradiation, and temperate phage was identified by formation of plaques on the appropriate indicator strain. Determination of phage induction by the criterion of plaque formation following exposure to inducing agents is known to be poor (Lawrence, 1976) and variable even on a prophage cured strain (Terzaghi, 1978). To avoid the possibility of a misleading result, the lysate from the induced cultures were examined and confirmed visually for the presence of phages by electron microscope rather than relying on plaque formation. Since phage was only detected in the irradiated cultures but not in the unirradiated culture, we believe that the phage was indeed temperate. It should also be recalled that every bacterium in a culture population is not lysogenic; there may be a few bacteria which do not harbor prophage. Many bacteria are also known to carry phages which cannot replicate, but may be visualized in lysate of induced bacteria, they possess variable morphology, including complete phages with full or empty heads, headless tails or tailless heads, and polyheads and polysheaths

(Ackermann, 1978). Results of the induction of the temperate phage from *R. albus* exposed to 45 or 60 sec are consistent with induction studies by Eisensark (1967), in which curing of the prophage from the host was achieved with sublethal UV irradiation. *R. albus* culture exposed to 120 sec UV irradiation did not produce lysate which could form plaques on the indicator cells. This may be due to the lethal effect of over exposure of UV irradiation. From all the results, including induction, lysogenic culture of *R. albus* must therefore be a source of lytic phage. However, in the present study, no evidence could be found that the phage investigated came directly from induced lysogenic *R. albus* in the rumen or from other ruminal species. As the temperate phage also showed some relationship by hybridization techniques with infected *R.albus*, would suggest that the phage may have been derived from lysogenic *R. albus*. However, since hybridization study did not extend to determine other ruminal species for possible lysogenes, the hypothesis that the temperate phage may have come from *R. albus* needs further study with other related species. Demonstration of the transition of a phage from lysogenic to lytic in the rumen is difficult to obtain. Only lysate obtained from induced culture provided sufficient evidence towards the lysogenic nature of *R.albus* as a source of lytic phage against *B. fibrisolvens*, strains D1

and 49. These experiments conclusively showed at least with *R. albus*, prophage can give rise to virulent phage.

Studies on the effect of temperature on replication have yielded interesting results. High titer of phage in *B. fibrisolvens*, strain D1 was produced after the phage and bacteria were incubated at 39 C. No phage or very little was produced when the culture was incubated beyond 50 C. These results indicate that indigenous phages in the rumen environments are capable of replicating at temperatures near those of the rumen.

Our preliminary studies involving effect of the isolated *R. albus* phage on fermentation of various sugars by ruminal species did not indicate consistent changes in one fermentation by-product or the other. The phage was shown to have a wide host range in its infectivity and could be expected to reduce concentration of products from fermentation of the sugars by way of lysing some of the bacteria in culture. As this did not occur, there must be a different phenomenon by which the phage exerted its effect on those bacterial species in culture. One of the possible phenomena may be that the phage could have lysogenized some of the bacteria instead of lysing them. Those bacteria that were lysogenized could increase in number and enhance fermentation products. The increase in formate concentration in the phage treated culture of *B. fibrisolvens* may be explained by lysogenization of *B.*

fibrisolvens albeit this bacteria was found to be permissive to the phage. *B. fibrisolvens* is a major formate producer compared to the other bacterial species used in the mixed culture. More research is necessary in this area to elucidate the role of the ruminal phage in fermentation of various sugars and plant polymers.

The occurrence of two types of bacteriophages in the supernatant lysate from D1 culture infected with the temperate *R. albus* phage is not clear. Nor do we have a conclusive evidence of its origin. Two possible explanations that could be thought of may be: The first is that the filamentous- like phage particle might have initially existed in the rumen fluid which was used to infect *R. albus* and perhaps passed through as a contaminant during incubation with D1 cells. As a result of the passage strain D1 may have been sensitive also to the filamentous like- phage and produced more of those filamentous like particles. The second possibility may be that some D1 cells may spontaneously have been induced to produce filamentous forms after incubation with the *R. albus* phage lysate. This possibility is much more likely since *B. fibrisolvens* AR 14 have recently been induced by mitomycin C to produce filamentous like phage (Klieve, 1989). However, no conclusive evidence was reported whether these particles were bacteriophages or bacteriocins. Although, the origin of the filamentous

like form is uncertain, their true forms and existence is unequivocal because the same forms have also been described by Bradley (1966).

Host range studies of *R. albus* phage action have demonstrated a wide spectrum of interactions, ranging from highly specific to less specific. Important cellulolytic and amylolytic rumen bacteria were permissively infected with differing sensitivity; a possible explanation for this might be a restriction-modification system acting in strains. Such systems have not been studied in the rumen bacteria. The only bacteria known to have such a system is *F. succinogenes* and DNAse from supernatant culture of *F. succinogenes* degraded Lambda phage and supercoiled DNA (Forsberg, C. W., Pers. comm. as cited in P.N. Hobson, 1988). The broad host specificity of the phage on the rumen bacteria may be a reflection of the action in the rumen which may be to keep the proportion of specific bacterial species in check when their numbers exceed the ecological balance.

Conclusion

The occurrence of bacteriophages in important rumen bacterial species which are functionally significant in the ruminal degradation and fermentation processes was demonstrated. A ruminal bacteriophage that infects a

ruminal spirochete, *Treponema saccharophilum* was identified. *T. saccharophilum* is pectinolytic and is intimately associated with cellulolytic species and enhances cellulose degradation. This is the first demonstration of a phage associated with an identified rumen spirochete in pure culture. Of significance is the isolation of a temperate phage from ruminal contents which appear to lysogenize a wild isolate strain of *Ruminococcus albus*, a major cellulolytic ruminal bacteria. The phage is morphologically characterized by an icosahedral head (50 x 60 nm) and has a flexible , non contractile tail (120 nm). In contrast to the host, *R. albus*, the phage was found to be obligately lytic to *Butyrivibrio fibrisolvens*, strain D1 and 49, both of which are involved in hemicellulose degradation in the rumen. Studies conducted to determine the host range and specificity of the temperate phage confirmed that the phage infects a wide range of predominant rumen bacterial species involved in many key degradative and metabolic interactions. The phage infected predominant cellulolytic species (*Ruminococcus flavefaciens*, strain FD1, *Fibrobacter succionogenes*, strain S85, *Ruminococcus albus*, strains 7 and 8) starch degrading species (*Ruminobacter amylophilus*, strain, H18) and a major propionate producing species (*Selenomonas ruminantium*, strain HD4).

Implications of R. albus phage

The detection and isolation of ruminal bacteriophages which infect predominant indigenous rumen bacterial species raises the question as to their role in influencing rumen bacterial diversity and fermentation efficiency. The degradation of fibrous feeds in the rumen involves the concerted interactions of a number of bacterial species. Changes in the proportions and types of these bacteria, due to either temperate or lytic phage infection, could affect the efficiency of degradation and fermentation of these feeds. Considerable attention is being directed toward improvement of digestibility of dietary feedstuffs by physical and chemical treatments. Unfortunately, there has been little success. Lignin digestibility by microorganisms still remains one of the biggest problems in the rumen. To circumvent these problems, a new alternative strategy based on recombinant DNA techniques could be developed. This may be done by modifying rumen bacteria to produce high activity enzymes for cellulose and lignin degradation in the rumen. Such modification of bacteria takes into consideration the need for an appropriate bacteria to be modified, a specific genetic material for insertion into the bacteria by means of a cloning vector. Sophisticated vector systems have been exploited in the past, including high number plasmids (Keisen, 1972) and bacteriophages

(Harris, 1983). Although these gene cloning tools are available, obstacles have prevented the rapid full scale exploitation of the desired genes. Certain plasmids have been detected in some species of ruminal microorganisms (Teather, 1982). However, their use as possible vectors have not been elucidated. An alternate method which will circumvent the need for efficient replication of a recombinant gene will be a lysogenic-phage derived cloning vector containing a selectable marker, a suitable cloning site, and a defect in the normal phage chromosomal integration mechanism. Generally, lysogenic phages integrate into the host genome by their specific recombination between a unique phage DNA sequence and a non-homologous host DNA sequence (Campbell, 1962). The obvious potential of ruminant phages is then in their ability to transfer genetic material by transduction and transfection. Transduction provides a mechanism for distribution of genetic information during naturally occurring phage- host interactions. High frequency transducing ability is associated with temperate phages, and Lac+ has been shown to be transduced at high frequency in *S. lactis* L2 (McKay, 1973). Using the recent genetic engineering, stabilization of lactose and proteinase genes in the chromosomes of *Streptococcus lactis*, C2 by transduction was a most significant accomplishment for dairy starter cultures (McKay, 1973).

Zinder and Lederberg's (1952) original observation showed that many different types of characteristics could be transduced; nutritional, fermentative, antigenic, and resistance to antibiotics. It was suggested that this carriage was due to the accidental incorporation of genetic units of the host. Phages do offer some advantages over other vectors because of their high transfer frequency and the stability of their prophages within the bacterial genome. If specialized transducing ruminal phages could be identified, desirable fermentation traits could be introduced into specific ruminal bacteria for improving the efficiency of the ruminal fermentation. *R. albus* phage was isolated for its ability to infect and lysogenize *R. albus*, a major cellulolytic bacteria. In light of this it can be employed as a recombinant DNA vector in lieu of plasmids. The phage has a broad host range and can be propagated in several species of *B. fibrisolvens*. Before its use, however, a physical map which will identify sites needs to be constructed and several deletion mutants need to be isolated to determine the regions of the phage containing non-essential DNA. Such phage deletion mutants can be used as cloning vectors whereby foreign DNA can be recombined into the phage in vitro and then introduced into cell protoplasts by transfection. Since the phage DNA can integrate, the new genes can be stably maintained

and expressed. The temperate phage can provide the necessary components for recombinant DNA experiments in organisms lacking plasmids carrying selectable markers. The ability to use genetic engineering principles to construct microorganisms that can use growth substrates, such as lignified cellulose, could be extremely important to the development of better production strains and to using new raw materials as growth substances. However, for construction of microorganisms to take place, a gene product that degrades lignin must be obtained. Recently, a lignin degrading gene was described in white rot fungus, *Phanerochaete chrysosporium*, by Thompson et al. (Center for Microbial Ecology research findings, pp. 164, Fall, 1992). While this organism could offer a potential source for transferring a lignin gene to *R. albus* , a major challenge may be the establishment of the genetically modified bacteria in the rumen. The probability of the modified bacteria to survive in the rumen without being out competed by other ruminal species may be low. Even if the modified bacteria is fully competitive with the resident bacteria, its establishment would be difficult. However, a general strategy could be employed in which the constructed strain could be made capable of promoting its own transfer with high degree of efficiency so that the desired gene can be introduced into the resident bacterial population using a very small

inoculum.

Another approach where the application of gene cloning and genetic manipulation to rumen bacteria is of fundamental interest is in the alleviation of energy waste by reducing the amount of methane production in the inefficient process of rumen fermentation. Methane in the rumen is produced by the methanogens by carbon dioxide reduction with hydrogen originating from the ferredoxin mediated reoxidation of NADH produced during glycolysis. Specific inhibitors of methanogenesis, such as higher unsaturated fatty acids and chloroform have caused the desired fermentation shifts in experiments in vivo and vitro (Meyer, 1981). However, they have not been useful because of undesirable side effects or ineffectiveness in improving animal performances. The ionophores, monensin and lasolacid, have showed promise in feeding trials and now is heavily used as feed supplements for beef cattle. Monensin increases the production of propionate and decreases the production of methane in the rumen (Meyer, 1981). Nevertheless, a considerable amount of methane is still being produced. In circumventing this problem, a gene product utilizing hydrogen more competitively could be obtained and cloned into a suitable phage vector system for transferring the desired gene into a ruminal bacteria. A non ruminal species, *Clostridium aceticum*, is known to utilize hydrogen for the reduction of carbon

dioxide to acetate (Braun et al. 1981) and therefore provides a potential source for gene isolation. Such procedures involving gene manipulation of rumen bacteria provide a more logical approach than others involving introduction of acetogenic bacteria, as previously thought in some studies, directly into the rumen which is not native to the environment.

Research will be conducted in the future for a better understanding of the bacteriophages in the rumen ecosystem. Future objectives are:

1. Host range specificity of the temperate bacteriophage will be extended with other rumen bacterial species.
2. In vitro studies will be conducted to determine if the presence of a bacteriophage in pure mono and mixed bacterial cultures of ruminal bacteria affect the efficiency of degradation of various substrates or alter the fermentation end products.
3. Molecular characterization and restriction mapping of the phage will be carried out to determine appropriate sites for cloning desired genes (traits) into the phage.

REFERENCES

1. Ackermann, H.W. and W.A. Smiroff. 1978. Recherches sur la lysogenie chez *Bacillus thuringiensis* et B. Cereus. Can. J. Microbiol. 24:818

2. Adams, J.C., J.A. Gazaway, Jr., M.D. Brailsford, P.A. Hartman and N.L. Jacobson. 1966. Isolation of bacteriophages from the bovine rumen. Experientia 22:717-718.

3. Adams, M.H. 1959. Bacteriophages, Interscience publishers, INC. N.Y.59.

4. Ben-Gurion, R. 1967. On the induction of a recombination-deficient mutant of *Escherichia coli* K-12. Genet. Res. 9:309-330.

5. Bradley, D.E. 1967. Ultrastructure of bacteriophages and bacteriocins. Bacteriol. Reviews 31:230-315.

6. Bradley, D.E. 1964. The structure of some bacteriophage associated with male strains of *Escherichia coli*. J. Gen. Microbiol. 35:471-482.

7. Bradley, D.E. 1966. The structure of infective process of *Pseudomonas aeruginosa* bacteriophage containing ribonucleic acid. J. Gen. Microbiol. 45:83-96.

8. Bradley, D.E. 1963. The structure of some *Staphylococcus* and *Pseudomonas* bacteriophage. J. ultrastruct. Res. 8:552-565.

9. Brailsford, M.D. and P.A. Hartman. 1968. Characterization of *Streptococcus durans* bacteriophages. Can. J. Microbiol. 14:397-402.

10. Braksdale, L. and S.B. Arden. 1974. Persisting bacteriophage infections, Lysogeny and phage conversions. Annu. Rev. Microbiol. 28: 265-299.

11. Braun, M., M.F. Gottschalk. 1981. *Clostridium aceticum* (Wieringa), a microorganism producing acetic acid from molecular hydrogen and carbon dioxide. Arch Microbiol. 128:288-293

12. Bryant, M.P. 1972. Commentary on the Hungate technique for culture of anaerobic bacteria. Amer. J. Clin. Nutr. 25: 1324-1328.

13. Bryant, M.P. and L.A.Burkey. 1953. Cultural methods and some characterstics of some of the more numerous groups of bacteria in the bovine rumen. J. Dairy Sci. 36:205-217.

14. Bryant, M.P. 1956. The characterstics of strains of *Selenomonas* isolated from bovine rumen contents. J. Bacteriol. 72:162-167.

15. Bryant, M.P., and I.M. Robinson. 1962. Some nutritional characteristics of predominant culturable bacteria. J. Bacteriol. 84:605-614.

16. Campbell, A. 1962. Episomes. Adv. Genet. 11 110-145.

17. Cluzel, P.J., M. Veaux, M. Rousseau and J.P. Accolas. 1987. Evidence for temperate bacteriophages in two strains of *Lactobacillus bulgaris*. J. Dairy Res. 54:397-405.

18. Echolas, H., L. Green., R. Kurda. and G. Edlin. 1975. Regulation of phage lambda development with the growth rate of host cells. A homeostatic mechanism. Virology 66:344

19. Eisensark, A. 1967. Bacteriophage techniques in methods in virology. vol.1. Maramarosch, K. and Koprowski, H., Eds., Academic press, New York. 449

20. Freifelder, D. 1983. Molecular Biology. A comprehensive introduction to Procaryotes and Eukaryotes. Jones and Bartlett Publishers, Inc.

21. Gratia, A. 1936. Des relations numerques entre bacteries lysogens et particules des bacteriophages. Ann. Inst. Pasteur 57:652

22. Harris, J.E., K.F. Chater., C.J. Bruton and J.M. Pinet. 1983. The restriction mapping of c gene detections in *Streptomyces* bacteriophage c31 and their use in cloning vector development. Gene 22: 167-174.

23. Hertman, I., and Luria, S.E. 1967. Transduction studies on the role of a rec+ gene in the ultraviolet induction of prophage lambda. J. Mol. Biol. 23:117-133.

24. Hespell, R.B., R. Wolf and R.J. Bothast. 1987. Fermentation of xylans by *Butyrivibrio fibrisolvens* and other ruminal bacteria. Appl.Environ. Microbiol. 53: 2849-2853.

25. Hobson, P.N. 1988. The Rumen Microbiol Ecosystem. ed. Elsevier Science publishers.

26. Hoogenraad, N.J., F.J.R. Hird, I. Holmes and N.F. Millis. 1967. Bacteriophages in rumen contents of sheep. J. Gen. Virol. 1:575-576.

27. Horne, R.W. 1965. Negative staining methods. P.328. In. D.H. Hay (ed.). Techniques for electron microscopy. Blackwell scientific publications, ltd., Oxford.

28. Hudson, J.F and Gregg. 1989. Genetic diversity among strains of bacteria from the rumen. Current Microbial. 19: 313-318.

29. Hungate, R.E. 1970. A roll tube method for cultivation of strict anaerobes., p.117-132. In J.R. Norris and D.W. Robbins (ed.). Methods in microbiology, vol. 3. Academic press, inc., New york.

30. Hungate, R.E. 1966. The rumen and its microbes. Academic Press, New York.

31. Hungate, R.E. 1950. The anaerobic mesophilic cellulolytic bacteria. Bacterial Revs.14:1-49.

32. Iverson, W.G. and N.F. Millis. 1976a. Lysogeny in *Streptococcus bovis*. Can. J. Microbiol. 22:853-857.

33. Iverson, W.G. and N.F. Millis. 1976b Characterization of *Streptococcus bovis* bacteriophages. Can. J. Microbiol. 22:847-852.

34. Iverson, W.G. and N.F. Millis. 1977. Succession of *Streptococcus bovis* strains with differing bacteriophages sensitivities in the rumens of two fistulated sheep. Appl. Environ. Microbiol. 33:810-813.

35. Kaisen, T., D.A. Hopwood., H.M. Wright and C.J. Thomson. 1972. PIJ107, a multicopy broad host range Streptomyces plasmid: functional analysis and development of DNA cloning vectors. Mol. Gen. Genet. 185: 223-238.

36. Kaiser, A.D. 1957. Mutations in a temperate bacteriophage affecting its ability to lysogenize *E. coli*. Virology 3:42

37. Kellenberger, E.J.Sichand, and A. Ryter. 1959. Electron microscopic studies of phage multiplication lV. The establishment of the DNA pool of vegetative phage and the maturation of phage particles. Virology 8:478-498.

38. Klieve, A.V. And T. Bauchop. 1988. Morphological diversity of ruminal bacteriophages from sheep and cattle. Appl. Environ. Microbiol. 54:1637-1641.

39. Klieve, A.V., J.F. Hudman and T. Bauchop. 1989. Inducible bacteriophages from ruminal bacteria. Appl. Environ. Microbiol. 55:1630-1634.

40. Klieve, A.V. (personal communications)

41. Klomparens, K.L., S.L. Flegler. and G.R. Hooper. 1986. Procedures for transmission and scanning electron microscopy for biological and medical science. In Ladd Research Industries (2nd ed.). Burlington, Vermont, P.130.

42. Kovirilsky, P.1973. Lysogenization by bacteriophage lambda. I.Multiple infection and the lysogenic response. Mol. Gen. Genet. 122:183

43. Kudo, K.J.Cheng and J.W. Costerton. 1986 Interaction between Treponema bryantii and cellulolytic bacteria in the in vitro degradation of straw cellulose. Can. J. Microbiol. 33:244-248.

44. Lawrence,R.C., Thomas,T.D. and Terzaghi, B.E. 1976. Reviews of the progress of dairy science: cheese starters. J. Dairy science 43:141-163.

45. Lockington, R.A., G.T. Attwood. and J.D.Brooker. 1988. Isolation and characterization of a temperate bacteriophage from the ruminal anaerobe,*Selenomonas ruminantium*. Appl.Environ. Microbiol. 54:1575-1580.

46. Maniatis, T., E.F. Fritch and J. Sambrook. 1989. Molecular cloning: a Laboratory manual. Cold Spring Harbor Laboratory. Cold Spring Harbor, N.Y.

47. Matteuzi, D.and T. Sozzi. 1971. *Bifidobacterium* bacteriophages from calf rumen. Z. Allg. Mikrobiol. 11:57- 58.

48. McClelland, M., R. Jones., Y. Patel., and M. Nelson. 1987. Restriction endonucleases for pulsed field mapping of bacterial genomes. Nucleic acids Res. 15:5985-6005.

49. Mckay,L.L, Cords, B.R. and Baldwin, K.A. 1973. Transduction of lactose metabolism in *streptococcus lactis* C2. J. of Bacteriology 115:810-815.

50. Meyer, J.W. 1981. Fermentation in the rumen and human large intestine. science 213. 25:1463-1468

51. Orpin, C.G. and E.A. Munn. 1974. The occurrence of bacteriophages in the rumen and their influence on rumen bacterial populations. Experientia 30: 1018- 1020.

52. Paster, B.J. and E. Canale-Panola. 1985. *Treponema saccharophilum* sp. nov., a large pectinolytic spirochete from the bovine rumen. Appl. Environ. Microbiol. 50:212-219.

53. Paynter, M.J.B., D.L. Ewert and W. Chalupa. 1969. Some morphological types of bacteriophages in bovine rumen contents. Appl. Microbiol.18:942- 943.

54. Playne, M.J. 1985. Determination of ethanol, volatile fatty acids, lactic and succinic acids in fermentation liquids by gas chromatography. J. Sci. Food. Agri.36:638-644.

55. Ritchie, A.E., I.M. Robinson and M.J. Allison. 1970. Rumen bacteriophages: Survey of morphological types. In P. Favard (ed.). Microscopie electronique, 1970, vol. 3. Societe Francaise de Microscopie Electronique, Paris.

56. Rosner, J.L. 1972. Formation, Induction and Curing of bacteriophage P1 lysogens. Virology 48:679-689.

57. Salanitro, J.P. and P.A. Muirhead. 1975. Quantitative method for the gas chromatographic analysis of short chain monocarboxylic and dicarboxylic acids in fermentation media. Appl. Microbiol. 29:374-381.

58. SAS, 1987. SAS user's guide: Statistics. SAS Institute, INc, Cary, NC

59. Schwartz, F.M.and N.D. zinder. 1963. Crystalline aggregates in bacterial cells infected with RNA bacteriophage f2. Virology 21 276-278.

60. Siuta. 1974. Studies toward synthesis. J. Org. Chem. 39: 3739.

61. Stanton, T.B. and E. Canale-Parola. 1979. Enumeration and selective isolation of rumen spirochetes. Appl. environ. Microbiol. 38:965-973.

62. Tamada, H., R. Harasawa and T. Shinjo. 1985. Isolation of a bacteriophage in *Fusobacterium necrophorum*. Japan J. Vet. Sci. 43:483-486.

63. Tarakanov, B.V. 1974. Lysogeny among Streptococcus bovis cultures isolated from the rumen of cattle and sheep. Microbiologiya 34: 319-320.

64. Tarakanov, B.V. 1976. Biological characterstics of *Streptococcus bovis* bacteriophages isolated from lysogenic cultures and sheep rumen. Microbiologiya 45:606-610.

65. Teather, R.M. 1982. Isolation of plasmid DNA from *Butyrivibrio fibrisolvens*. Appl. Environ. Microbiol. 43:298-302.

66. Tekeya, K.M., Koike,R.M, and T. Toda. 1961. Light and electron microscope studies of mycobacterium mycobacteriophage infections. I. Further studies on the ultra thin sections. J. Biophys. Biochem. Cytol.11:441-442.

67. Terzaghi, E. and Terzaghi, B.E. 1978. effect of Lactose concentration on the efficiency of plating of bacteriophage on *Streptococcus cremoris*. Appl. Environ. Microbiol. 35:471-478.

68. Yen, K.M. and G.N. Gussin. 1980. Kinetics of bacteriophage lambda repressor synthesis directed by the PRE promotor: influence of temperature, multiplicity of infection, and mutation of PRM or the cro gene. Mol. Gen. Genet. 179: 409

69. Zinder, N.D., and J. Lederberg. 1952. Genetic exchange in *Salmonella*. J. Bact. 64: 679