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A continuous culture system containing a variant of Anacystis nidulans in logarithmic growth phase was inoculated with a lysate of what appears to be an AS-1 variant. Isolation of this variant phage had been unsuccessful until cultivation in the chemostat. The cyanophage isolated from the continuous culture system has growth and immunological characteristics very similar to AS-1 but shows varying nutritional and infection properties. The host blue-green bacterium isolated from this original continuous culture has exhibited properties associated with lysogeny. A study structured around use of a continuous culture system has been adapted to elucidate what appear to be departures from the usual virus-host relationship.

INTERACTIONS OF VARIANTS OF THE HOST BLUE-GREEN BACTERIUM *ANACYSTIS NIDULANS* WITH A VARIANT OF THE CYANOPHAGE AS-1

by

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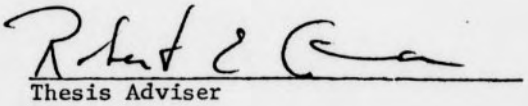

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INTRODUCTION

Since the discovery of the first virus infecting members of the class Cyanophyta (Safferman and Morris, 1964), a number of cyanophages have been isolated which clearly indicate that cyanophages are widely distributed in bodies of fresh and brackish water. As of yet, most of the phages which have been isolated have filamentous hosts (Safferman and Morris, 1963; Singh and Singh, 1967; Safferman et al. 1969b; Daft et al. 1970; Adolph and Haselkorn, 1971). Only a few cyanophages have been discovered which infect unicellular hosts (Safferman and Morris, 1969; Safferman et al. 1972; Adolph and Haselkorn, 1973; Sherman et al. 1976).

The Cyanophyceae, more commonly known as the blue-green algae, occupy a unique biological position. Due to their photoautotrophic nature and possession of the photopigments chlorophyll a and β-carotene, distinctive characteristics of plant photosynthesis, blue-greens are regarded by botanists as a class of algae (Stanier et al. 1971). However, the bacterial nature in both cellular and organismal respects of the blue-green algae has long been recognized by cell biologists (Pringsheim, 1949; Echlin and Morris, 1965; Carr and Craig, 1970). This has led to the increasing usage of the more descriptive term cyanobacteria, or, blue-green bacteria (Buchanan and Gibbons, 1974).

Bacterial Nature of the Blue-Green Bacteria

Although features common to both blue-green bacteria and heterotrophic bacteria have been observed since the nineteenth century, it has

been the presence of a typical "green plant" oxygen-evolving photosynthetic system which, in the past, has permitted the cyanobacteria to be grouped as primitive examples of green plants and algae (Carr and Craig, 1970). Examples of photosynthetic bacteria can be observed, and certain species, Rhodomicrobium vannielli, contain a photosynthetic apparatus located as a peripheral lamellae very similar to that found in blue-green bacteria (Allen, 1968a, b; Susor and Krogman, 1964).

Many basic prokaryotic features are shared by both bacteria and blue-green bacteria. These include the absence of a nuclear envelope and nucleolus, lack of membrane-enclosed organelles and mitotic apparatus, and the possession of 70S ribosomes.

The protein synthesizing system is the most closely studied aspect which reveals the fundamental prokaryotic features common to bacteria and blue-green bacteria. Both of these prokaryotes synthesize 70S ribosomes composed of 30S and 50S subunits and use formylmethionyl-t-RNA in peptide initiation (Gray and Herson, 1976). Both bacteria and blue-green bacteria contain within their 70S ribosomes 23S and 16S RNA as opposed to the 28S and 18S ribosomal RNA found associated with the 80S eukaryotic ribosome. No intermediate ribosomal RNA has been found between the two classes (Loening, 1968).

Ribosomal RNA synthesis and maturation in the unicellular blue-green bacterium Anacystis nidulans appears to follow the pattern established for bacteria (Doolittle, 1972; Doolittle, 1973). While eukaryotic 28S and 18S ribosomal RNA's are derived as a cleavage product of a single large RNA precursor containing both sequences, the prokaryotic 23S and 16S ribosomal RNA of both bacteria and blue-green bacteria originate from separate precursors (Doolittle, 1972).

Further evidence of the prokaryotic nature and close relationship between bacteria and blue-green bacteria and homology between the two protein synthesizing systems is the ability to form functional 70S hybrid ribosomes (Gray and Herson, 1976). When isolated 20S and 50S subunits from Escherichia coli and Anacystis nidulans ribosomes were allowed to reassociate, the resultant Anacystis nidulans 30S-Escherichia coli 50S and Escherichia coli 30S-Anacystis nidulans 50S hybrids were able to function in synthesizing protein in a standard in vitro poly-uridine-phenylalanine incorporating system (Gray and Herson, 1976).

Another feature common to bacteria and blue-green bacteria and found most interesting to molecular biologists is the susceptibility of both to viral infections. Since cyanophages are similar to bacteriophages both morphologically and in infection cycle, it has been suggested that they be considered as a subgroup of the bacteriophages (Padan and Shilo, 1973).

Although not necessarily an indication of biological relatedness, cyanophages show similar variation in structural complexity which has already been observed in bacteriophages (Safferman et al. 1972). While not as thoroughly studied, the attachment of the virus to the host cell and injection of nucleic acid into the cell appear to be the same for cyanophages as for bacteriophages (Safferman et al. 1972).

Biochemical aspects of cyanophage infection are more reminiscent of infection of bacteria than that found in eukaryotic plants. After viral infection of bacteria and blue-green bacteria, there is an early increase in DNAase levels which is not found in the virus infection of plants (Udvardy et al. 1976). Cell-free extracts from Anacystis nidulans

contain enzymatic activity capable of repairing *in vitro* transforming bacteriophage T4 DNA which has been damaged by X-rays or U.V. light (Shestakov et al. 1975).

Virus-Host Relationship

The typical virus-host relationship shown by the cyanophages and blue-green bacteria seems to be restricted to two classes based on host cell morphology (Sherman et al. 1976a). The cyanophages which infect filamentous blue-green bacteria cause specific ultrastructural changes in the host cell within a few hours of infection. These include an invagination of the photosynthetic lamellae leaving a virogenic stroma in which the virus replicates. The viral replication cycle, which is usually complete in 14 hours, is relatively rapid, compared to the unicellular blue-green bacterial cyanophages.

As is found in bacteriophage development, the cyanophages which infect single-celled blue-green bacteria do not significantly alter the host ultrastructure during early stages of infection. Replication takes place within the nucleoplasm and is much slower than that found in filamentous hosts. With the exception of AS-1 and AS-1M, which replicate within 16 and 12 hours respectively, replication in other unicellular cyanophages is usually not complete for 48 hours (Brown, 1972, Padan and Shilo, 1973; Carr and Whitton, 1973).

The cyanophage-blue-green bacterial interaction shows a high degree of host specificity, a rapid selection of host cell resistant mutants, and a significant dependence upon environmental as well as host factors; all of which indicates the complexity of this relationship. Most blue-green bacteria prefer an alkaline range between pH 7-11, and both host

cells and free phage survival as well as the virus-host interaction depend on temperature optima. Maintenance of viral activity of the LPP-1 cyanophage with its filamentous host requires a high magnesium ion concentration (Schneider et al. 1964) while the unicellular phages, AS-1 and SM-1, do not (Safferman et al. 1969; Safferman et al. 1972).

The viral requirements met by host cell metabolic activities show varying degrees of dependence. The LPP and N-1 cyanophages do not have an absolute requirement for host cell photosynthesis which is inhibited by the phage at an early stage of infection (Padan and Shilo, 1973). This is in contrast to SM-1 and AS-1 which are highly dependent upon photophosphorylation, which continues until just before lysis (MacKenzie and Haselkorn, 1972; Allen and Hutchison, 1976). Host cell DNA is degraded by a virus-induced DNAase, and most of the acid-soluble products serve as viral DNA precursors (Sherman and Haselkorn, 1970b; Sherman and Pauw, 1976). Blue-green bacterial proteins as well are the major precursors of phage protein. LPP multiplication does not require host cell growth in contrast to most bacteriophages which can only develop in growing cells. Bacteriophages, most likely, require exogenous substances due to the heterotrophic nature of their hosts.

Specific Interactions of AS-1 With its Host

The first cyanophages to be isolated were those having filamentous hosts. But the fact that these blue-green bacteria are filamentous complicates the elucidation of the infection and growth cycle of their cyanophages. Being in the strictest sense multicellular hosts, the established techniques used in the study of phage infection are not as

suited for use in these interactions as they are with unicellular blue-green bacteria and their viruses (Padan and Shilo, 1973).

Several cyanophages infecting unicellular hosts have been isolated (Safferman et al. 1969; Safferman et al. 1972; Adolph and Haselkorn, 1973; Sherman and Connelly, 1976). Since Anacystis nidulans has been more widely used in physiological and biochemical studies and possesses a system for genetic transformation (Bazin, 1968; Orkwiszewski, 1974), the cyanophage AS-1 has been more extensively studied.

AS-1 is infectious for the blue-green bacteria Anacystis nidulans and Synechococcus cedrorum, and contains double-stranded DNA. It is polyhedral in structure and has a contractile tail. The infection cycle in this system is 16 hours in length with an 8.5 hour latent period (Safferman et al. 1972). AS-1 is the largest cyanophage yet observed.

1. Host Ultrastructural Changes During AS-1 Infection

The normal uninfected Anacystis nidulans cell contains three concentric layers of photosynthetic membranes at the periphery of the cell surrounding fibrous nuclear material and ribosomes. Electron microscopic studies of the infection process have shown no morphological differences between normal and infected cells through the sixth hour after infection (Pearson et al. 1975). The first recognizable changes in ultrastructure can be observed by the ninth hour after infection. At this stage, the nuclear material appears more condensed and abundant. By 11 to 15 hours viral presence is detectable. An aggregation of electron-dense material, probably future viral capsids, is found in the nuclear area of the host cell. At 16 to 18 hours viral heads are obvious as dark condensates in the host cytoplasm, which now appears to lack fibrous nuclear material (Pearson et al. 1975).

AS-1 develops in the nucleoplasm without causing invagination of the photosynthetic membrane typically found at the beginning of LPP-1 infection. During the sixteenth to eighteenth hours following AS-1 infection, there is a disruption or disintegration of the lamellae in one area of the cell with the appearance of a mesosome-like structure at this site (Pearson et al. 1975). This is the only gross cytological change found in the host cell until lysis. The average burst size is 50.

Since the lytic process leaves large cell ghosts or cellular fragments, it has been suggested that lysis results from the rupture of an "osmotically labile spheroplast" which is produced by the action of phage wall-degrading enzymes or lysozymes (Pearson et al. 1975).

2. Energy Requirements During AS-1 Infection

Studies on the effect of environmental parameters on AS-1 development have shown this cyanophage to be highly dependent upon host cell photophosphorylation (Allen and Hutchison, 1976). Continuous incubation in the dark reduces the standard burst size to 2% that found under conditions of continuous illumination. Cells initially incubated in the dark and subsequently illuminated have a latent period decreased by 1 to 2 hours and an eclipse period shortened by one hour, suggesting that adsorption can take place in the dark. Once illuminated, the burst size produced under these conditions is normal or near normal in size.

Incubation of infected cells in the presence of varying concentrations of DCMU [3-(3,4-dichlorophenyl)-1,1-dimethyl urea], a photosystem II inhibitor which stops carbon dioxide fixation, reduces the burst size accordingly (Allen and Hutchison, 1976). These results are in contrast to what is found with LPP-1 and N-1 infection in which neither darkness nor DCMU completely inhibits virus production (Allen and Hutchison, 1976).

Unlike LPP-1, which requires light only during the eclipse period, AS-1 replication requires illumination until late in the developmental cycle in order to produce a maximum yield. This prolonged light requirement for AS-1 correlates with the fact that there is no alteration of host photosynthetic membranes nor cessation of photosynthesis until the final stages of infection (Pearson et al. 1975).

Despite the obligate photoautotrophic nature of the AS-1 system, dark metabolism can support the formation of a virus-induced deoxyribonuclease if synthesis is triggered in the light (Udvardy et al. 1976). The activity of this DNAase is inhibited by chloramphenicol indicating that it is the result of new protein synthesis (Udvardy et al. 1976).

3. Intracellular Development

Answers to questions on the temporal expression of specific events following phage infection have been elucidated in varying degrees of detail for a variety of bacteriophage systems. Such a study has only been carried out for the filamentous blue-green bacterium Plectonema boryanum (Sherman and Haselkorn, 1970a,b,c) and recently for an AS-1 variant, AS-1M (Sherman and Pauw, 1976).

AS-1M strongly resembles AS-1 in morphology and shows a close serological relationship to this unicellular cyanophage. The two differ in growth characteristics; AS-1M adsorbs faster and has a more rapid replication cycle, 12 hours as opposed to 16 hours for AS-1 (Sherman et al. 1976). The pattern of AS-1 development as revealed by the electron microscope (Pearson et al. 1975) is very similar to that seen in AS-1M (Sherman et al. 1976). However, the timing of occurrences within the systems differs. To a large degree, this can probably be attributed to the differences found between the lengths of the two replication cycles.

In the absence of intracellular growth data on AS-1, meaningful comparison is difficult. However, hopefully the events which occur in AS-1M infection can be a useful extension of that to be found in AS-1.

AS-1M-directed protein synthesis has been divided into three classes (Sherman and Pauw, 1976). The early proteins are synthesized only during the first four hours of infection. A total of seven proteins have been identified as early; these include phage-directed DNAases which degrade the host DNA and a "factor" necessary for the initiation of the middle proteins. The middle proteins compose a large class which is synthesized around three hours after infection and continues until lysis. It is during this period that phage DNA is synthesized. Viral structural proteins comprise the late class. Synthesis of these begins around four hours after infection and continues until lysis (Sherman and Pauw, 1976).

Lysogeny

Lysogeny is ubiquitous among heterotrophic bacterial systems (Hayes, 1968). Recently this sophisticated type of prokaryotic host-parasite relationship has been discovered to occur in blue-green bacteria (Cannon et al. 1971; Cannon and Shane, 1972; Padan et al. 1972). Mitomycin-C induction of cyanophage LPP-1D, which is immunologically similar and identical in host range to LPP-1, has been shown to occur in a suspected lysogenic strain of Plectonema boryanum. The lysogeny of Plectonema boryanum by cyanophages LPP-1D and LPP-2 has been established by treating the host blue-green bacterium with chloramphenicol (Cannon and Shane, 1972). Since active host protein synthesis is vital for the production of viral progeny, interference with this condition leads to the establishment of lysogeny (Cannon and Shane, 1972).

Another phage, LPP-2-SPI, which is also temperate for the filamentous blue-green bacterium Plectonema boryanum has been isolated (Padan et al. 1972). Induction attempts using U.V. light, X-rays or Mitomycin-C proved to be unsuccessful, but induction was accomplished by growth at 37°C as opposed to 26°C. Recently, various temperature sensitive mutants of LPP-2-SPI have been isolated. These have permitted analysis of the protein synthesis which occurs during infection at the nonpermissive temperature (Rimon and Oppenhein, 1976).

Factors such as temperature, U.V. light, and unbalanced or stressful growth conditions, which are known to influcence the formation of lysogenic cultures, could have an influence on the natural blue-green bacterial population balance. Induction of the lytic cycle of a thermosensitive, lysogenic strain could occur if exposed to an elevated temperature (Padan and Shilo, 1973).

Observations Leading to This Study

Repeated attempts to isolate an unknown virulent cyanophage infectious for an Anacystis nidulans variant by standard procedures had proven to be unsuccessful. What appeared to be routine lysis in flasks containing host strains inoculated with sewage samples failed to yield the expected plaque-forming units when chloroformed and plated according to a standard plaque assay (Safferman and Morris, 1964). Lysis within the flasks appeared to be complete; repeated inoculation of lysed cultures with samples of the host gave consistent lysis. The lysing phenomenon was accomplished within 48 hours, thereby virtually eliminating host destruction by heterotrophic bacteria or protozoa. Cultivation at different temperatures and co-cultivation with other cyanophages were unsuccessful in isolation.

A sample of lysate was inoculated into an established continuous culture system containing the Anacystis nidulans variant. Under continuous culture conditions, which provided host cells at a stage of logarithmic growth, active phage particles were easily isolated before any apparent sign of lysis. The cyanophage isolated from this initial chemostat system appeared to be an AS-1 variant referred to for convenience as AS-2.

Host colonies from the original chemostat were isolated, grown, and maintained in the laboratory. For approximately three to four weeks the chemostat host blue-green bacterium exhibited growth characteristics identical to the parent bacterial strain and to wild type Anacystis nidulans. Following this period of time, the culture (referred to as A.n.-Ch for Anacystis nidulans-Chemostat) "lysed" giving a yellow-green appearance characteristic of partial lysis of Anacystis nidulans. Upon prolonged incubation at routine temperature and illumination conditions the host flasks exhibited cycles of regrowth and lysis. Plating of the filtrate from "lysed" flasks produced very small plaques. Flask cultures of A.n.-Ch have been maintained in the laboratory for over a year with no additional media supplementation.

The original problem of isolating a new cyanophage had led to additional questions. The problem of identifying what appeared to be an AS-1 variant remained. In addition, there was now the question of whether or not A.n.-Ch is lysogenic.

MATERIALS AND METHODS

Cultivation of Host Blue-Green Bacteria

Anacystis nidulans IU 625 and Synechococcus cedrorum IU 1191 were generously provided by Dr. Robert Safferman of the Environmental Protection Agency, Cincinnati, Ohio. All cultures were grown at 25-30°C in modified Chu No. 10 Medium (Cannon et al. 1971) unless otherwise specified. Illumination was provided by cool-white fluorescent lamps at an average intensity of 150 foot-candles. Cell density was measured on a Klett-Summerson Photoelectric Colorimeter, filter No. 42.

Origin of Host Variants

The first host discovered has an unknown origin. It appears to have arisen as the result of Anacystis nidulans contamination of a stock culture flask containing Chloroglea fritschii. At what time the contamination occurred is unknown, but, having a faster growth rate than Chloroglea, A. nidulans would be able to overgrow such a culture. Identity of the Anacystis variant is based on growth and pigment characteristics as well as on susceptibility to AS-1 cyanophage infection. There are no known cyanophages which infect any species of Chloroglea. In this study, this variant is subsequently referred to as A.n. var.-1 for Anacystis nidulans variant-1.

A.n. var-1 was used as the host for the original continuous culture system, which is discussed in a later section. Samples from this system were spread on Chu agar plants to isolate blue-green bacterial colonies.

An isolated colony was transferred to a ten milliliter flask containing Chu broth and allowed to grow as described above. This culture was maintained by frequent transfer to fresh medium. This host variant is referred to as A.n.-Ch for Anacystis nidulans-Chemostat. Single colonies of both variants were routinely isolated and cultivated to assure pure cultures.

Growth Curve

Ten ml samples of twenty day old A. nidulans (wild type) and variants A.n. var-1 and A.n.-Ch were inoculated into 500 ml side-arm flasks (Bellco) containing 250 ml of Chu broth. Growth was measured colorimetrically using a Klett-Summerson Photoelectric Colorimeter. One-tenth ml samples from each flask were spread on Chu agar plates daily. Results are expressed as cells per ml.

Cyanophage Detection Procedure

Ten ml of viral sample and one ml of chloroform were shaken vigorously for one minute in sterile screw-cap tubes. Chloroform was added to disrupt the cell walls of any bacteria or protozoa present. The suspension was allowed to settle for at least twenty minutes after which 1 ml of the aqueous phase was assayed for cyanophage using the soft agar layer technique of Safferman and Morris (1964). Results are described as plaque-forming units per ml (PFU/ml). Only plates containing 30-300 plaques were counted.

An attempt to detect cyanophage activity in virally-uninoculated stock blue-green bacterial cultures was carried out using cultures of A. nidulans (wild type), A.n. var-1, and A.n.-Ch, which ranged in age

from 0 to 6 months. A 1 ml sample from each host of the various ages, 0,2,3 and 6 months, was plated using the soft agar layer technique (Safferman and Morris, 1964). Any cultures indicating viral activity were filtered using a Nalgene Membrane Filter Unit equipped with a 0.45 μm filter. The filtrate was assayed on exponentially growing A. nidulans, A.n. var-1, A.n.-Ch, and Synechococcus cedrorum.

Continuous Culture

Continuous culture technology has been used to study the interactions between the blue-green bacterium A. nidulans, or variant, and a variant of the cyanophage AS-1. Continuous culture of cells in a chemostat provides a means of studying large populations under closely defined and controlled environmental conditions.

The distinctive characteristic of the chemostat is that the population is not directly controlled. Rather, the medium is provided in such a way that all essential growth factors are present in excess except for one "growth limiting" factor or nutrient. This limiting factor is present only at a concentration sufficient to support limited growth (Norris and Ribbons, 1970). In heterotrophic bacterial systems the limiting factor is normally a nutrient. For photosynthetic microorganisms, the light intensity usually becomes limiting since the mean effective light intensity on the cell mass is directly related to the dilution rate (Prokop et al. 1967).

All continuous culture devices must meet the following requirements: (1) the culture is enclosed in a manner which protects it from contamination; (2) the flow of fresh sterile medium to the growth vessel is maintained at a constant rate which can be varied; (3) the volume of the

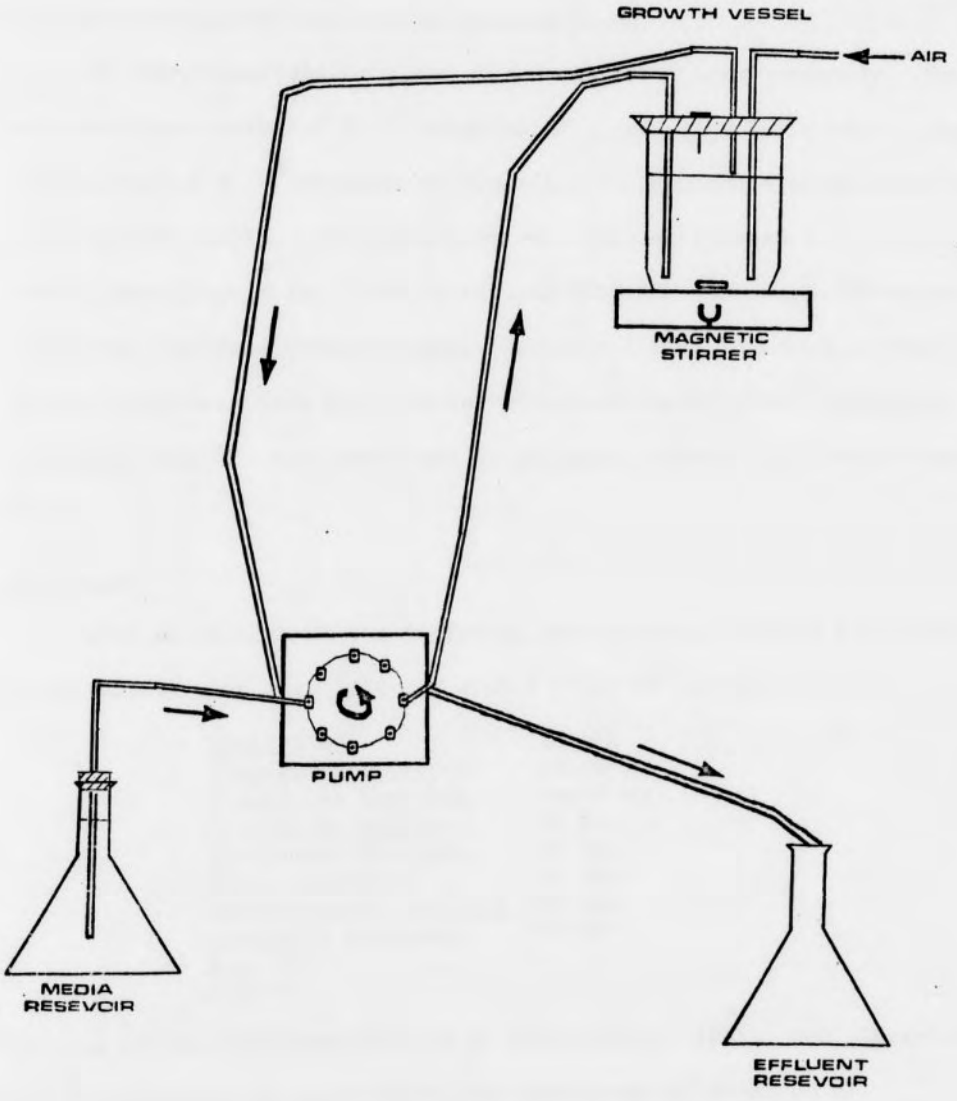
culture remains constant; (4) the culture is sufficiently agitated to provide homogeneous dispersion of media and biomass; (5) sufficient oxygen is provided if it is needed; (6) some type of temperature regulation is provided if necessary (Norris and Ribbons, 1970).

The chemostat apparatus used throughout this study is diagramed in Figure 1. The volume is 800 ml. All cultures were grown at 25-30°C in Chu broth at a flow rate of 10 ml per hour. Fermentation Design fermentors, which had been converted into chemostats (Cannon et al. 1976), were used. Illumination was provided by cool-white fluorescent lights at an average intensity of 150 ft-c. Aeration was provided by an aquarium pump and agitation by a magnetic stirrer. The system was vented by a 21 gauge hypodermic needle which was also used for inoculation. The viral titer was measured by the plaque assay as previously described. Blue-green bacterial density was measured by a Klett-Summerson Colorimeter.

Four separate chemostat systems were used. The individual specifications of each are provided below.

(1) A. nidulans variant-1 was inoculated into the chemostat and allowed to equilibrate at 2×10^6 cells/ml. Fifteen ml of sample from a presumed lysate of A.n. var-1, which had been inoculated with a sewage sample, provided the viral inoculum. The lysis was presumptive since no plaques were detected in repeated platings although the flask had cleared as if lysis had occurred. A.n.-Ch in logarithmic phase growth was used as the plating host. Duplicate plates were inoculated and an average is expressed as PFU/ml. The cyanophage which is elaborated by this continuous culture is referred to as AS-2.

FIGURE 1. DIAGRAM OF CHEMOSTAT



(2) A.n.-Ch was inoculated into the growth vessel and grown as previously specified until a cell density of 2.4 X 10^6 cells/ml was attained. The system was inoculated with AS-2 at a multiplicity of infection of 0.01. A. nidulans, A.n. var-1, and A.n.-Ch in the logarithmic phase of growth were used as plating hosts.

(3) Two chemostats were set up and monitored simultaneously. One was inoculated with 1.9 X 10^5 cells/ml of A. nidulans (wild type), the other with 1.8 X 10^6 cells/ml of A.n.-Ch. No cyanophage inoculation was made. Daily samples were taken from each and centrifuged in an International Centrifuge Model V for 20 min. at 1200 RPM (100 X g). The supernatant was filtered using a Nalgene Membrane Filter Unit with a pore size of 0.45 µm. Each filtrate was plated on exponentially growing A. nidulans, A.n.-Ch, A.n. var-1 and S. cedrorum. Viable cell counts were done.

Host Range

Fifty ml of each of the following exponentially growing blue-green bacterial cultures were infected with 1 ml of 10^3 PFU/ml of AS-2:

Lyngbya sp.	IU 488
Plectonema boryanum	sensitive
Plectonema boryanum	resistant
Plectonema boryanum	IU 581
Plectonema boryanum	IU 595
Nostoc muscorum	IU 486
Synechococcus cedrorum	IU 1191
Anacystis nidulans	IU 625
A.n. var-1	
A.n.-Ch	

Clearing of the flask was used as an indication of lysis, and, therefore, such a culture is included within the host range of AS-2.

Divalent Ion Requirement

To determine whether or not the AS-2 cyanophage requires the divalent cations magnesium and calcium, and to try to determine further differences or similarities between AS-1 and AS-2, two types of approaches were taken. Dialysis was used to indicate requirement of the ions for viral adsorption or stability. Medium supplementation was undertaken in an attempt to find enhanced or diminished plaque formation. In each case, results are compared with those obtained with AS-1 as well as with experimental controls.

(1) Dialysis. Ten ml of filtered AS-1 and AS-2 lysate were each placed in a section of dialysis tubing (Fisher Scientific Co.). Each was dialyzed against four 4 liter volumes of glass distilled water at 5°C for a period of 48 hours with constant agitation. The samples and controls were then serially diluted in distilled water and plated on A. nidulans using the soft agar layer technique (Safferman and Morris, 1964).

(2) Medium Supplementation. Chu medium was prepared with the following deviations in concentrations of $Ca(NO_3)_2 \cdot 4H_2O$ and $MgSO_4 \cdot 7H_2O$:

	normal	+	-
Mg^{2+} :	2.5 gm/l	5 gm/l	1.25 gm/l
Ca^{2+} :	23.2 gm/l	46.4 gm/l	11.6 gm/l

Controls were plated on Chu-10% agar plates. The titer and plaque diameter were determined for each of the varying concentrations.

Viral Neutralization

An antiserum against AS-1 cyanophage, which had been prepared according to Smedberg (1975), was diluted 1:10 in sterile distilled

water. A 0.5 ml aliquot of 10^4 PFU/ml of each of AS-1 and AS-2 was placed in sterile 13 ml test tubes. A 0.5 ml aliquot of antiserum was added to each tube and allowed to react for 30 minutes at 25°C. The virus-antiserum mixtures were then serially diluted and plated on A. nidulans using the soft agar layer technique.

Mitomycin-C Induction

Induction of A.n.-Ch cultures at two stages of growth was undertaken. One culture was older than one month and had already shown the "lysis" customary with this variant. The other culture was younger and identical in appearance and growth features to A. nidulans (wild type). Mitomycin-C (Sigma) was added to each of these cultures to a final concentration of 10 gm/ml and incubated at 25°C for 15 minutes. Each of the cultures and controls was then centrifuged at 5000 X g for 10 minutes in a Sorvall RC2-B centrifuge rotor No. 5534. The pellets were resuspended in 30 ml of Chu broth and returned to the lights. For 8 hours, 3 ml samples were removed hourly, placed in sterile screw cap tubes and centrifuged at 4000 RPM for 10 minutes in a desk top International Clinical Centrifuge to pellet blue-green bacterial cells. After serial dilution, the supernatant was assayed for PFU/ml. Both controls and treated samples were plated on logarithmically growing S. cedrorum.

RESULTS

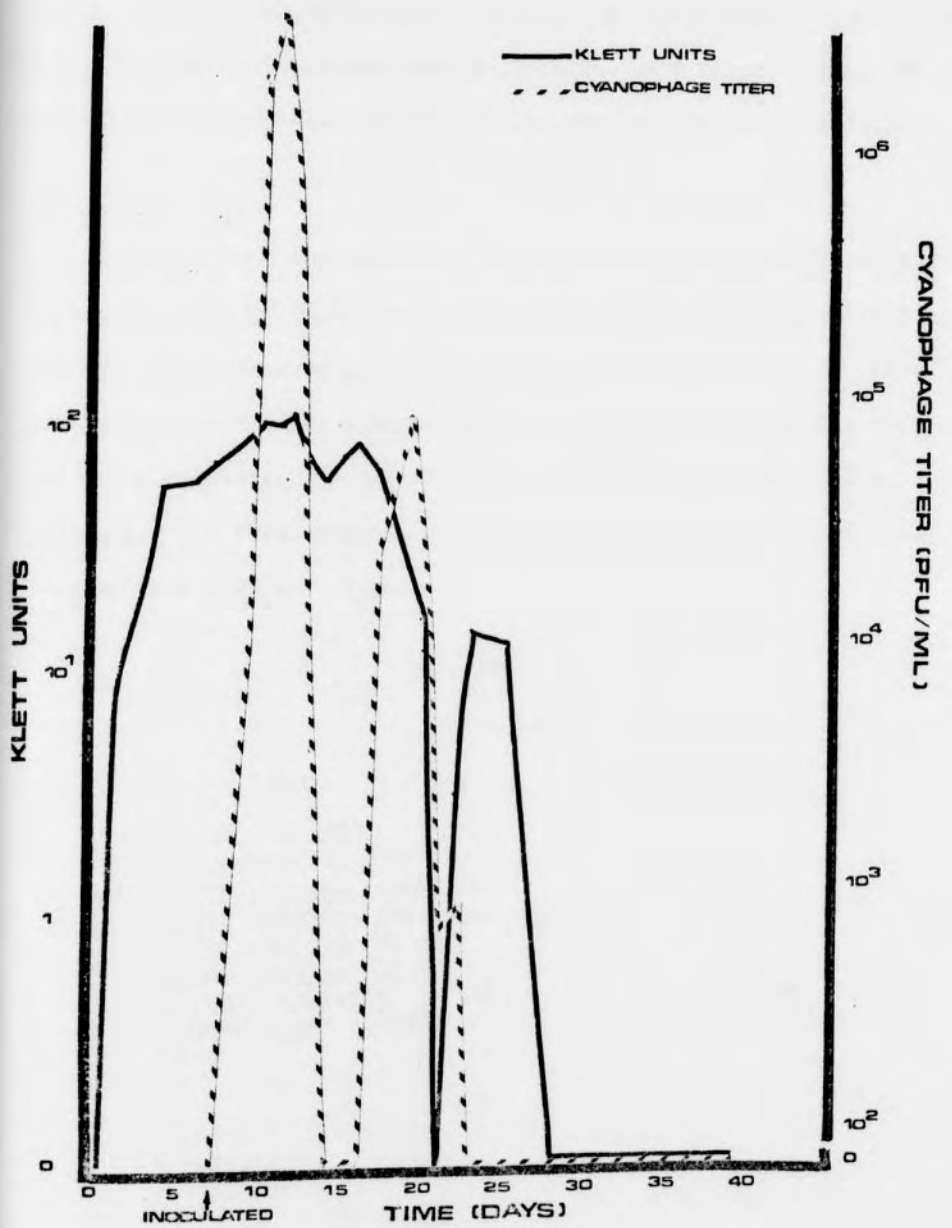
Chemostat 1

The cyanophage isolated from this first chemostat system, referred to as AS-2, had previously eluded isolation by various enrichment and concentration methods. When a sample containing presumptive viral activity was inoculated into an exponentially growing chemostat culture, plaque forming units were routinely observed upon plating.

Detection of plaques was possible within 24 hours after inoculation. The plaques were clear with a complete margin and a diameter of 1.5 to 2.0 mm. The pattern of phage growth in the chemostat appears to be cyclical (Figure 2). The first period of phage growth and decline, days 8 through 13, included a span of 6 days and reached its highest titer of 1.9×10^6 on day #10. During this same cycle, the host blue-green bacterium, A.n. var-1, also reached a peak with a Klett reading of 123 on day #12. During this first cycle, there was no visible evidence of phage activity such as would be indicated by a gradual clearing of the growth vessel.

The second phage cycle was also 6 days in length; days 17 through 22. During this period the host blue-green bacterial concentration and phage titer were lower than that found during the first phase. Both phage and host presence decreased to zero--the cyanophage after 16 days and the host within 25 days. No further viral activity was evident after this time nor was there any host detectable colorimetrically.

FIGURE 2
GROWTH OF A.N. VAR-1 AND AS-2 IN CHEMOSTAT 1



Viral Neutralization

Cyanophages AS-1 and the chemostat 1 isolate, AS-2, were reacted with AS-1 antiserum. Neutralization of both was complete. In several cases, in which the initial viral sample of either cyanophage was too high to allow total neutralization, plaques were found. These PFU were cloudy and smaller than those found on the unneutralized controls.

Host Range

Lysis by AS-2 was confined to the unicellular blue-green bacteria Anacystis nidulans (wild type) and Synechococcus cedrorum as well as the Anacystis variants A.n. var-1 and A.n.-Ch. See Table 1. AS-2 produced a lower titer when plated on A. nidulans than on the variants and had a consistently smaller plaque diameter when plated on A. nidulans. All three strains provided plaques in a variety of sizes ranging from 0.75 to 2.5 mm.

TABLE 1

Host Range

Host	Susceptibility
Lyngbya sp. IU 488	-
Nostoc muscorum IU 486	-
Plectonema boryanum sensitive	-
Plectonema boryanum resistant	-
Plectonema boryanum IU 581	-
Plectonema boryanum IU 595	-
Synechococcus cedrorum IU 1191	+ (cloudy)
Anacystis nidulans IU 625	+
A.n. var-1	+
A.n.-Ch	+

+ = lysis
- = no lysis

Divalent Ion Requirement

Dialysis. As can be seen from Table 2, there is some loss in the infection efficiency of both cyanophages after dialysis. In the case of AS-1, the loss is approximately 50% with the exception of dialysis #2 where the efficiency is only 16% that found in the control.

In three of the four dialysis replicates, AS-2 infectivity was reduced to a fraction of that found normally. Again, dialysis #2 is in contrast to the other results. In no case was infectivity reduced to zero.

TABLE 2

Results of Dialysis

	AS-1 Titer	% Efficiency	AS-2 Titer	% Efficiency
Control:	1.5×10^5		3.5×10^5	
Dialysis #1	7.2×10^4	48%	8.2×10^3	.23%
Control:	6.0×10^4		2.0×10^5	
Dialysis #2	9.4×10^3	15.7%	8.4×10^4	42%
#3	4.0×10^4	67%	1.4×10^4	7%
#4	2.8×10^4	47%	1.0×10^3	.5%

Medium Supplementation. The results obtained by varying the concentrations of $MgSO_4 \cdot 7H_2O$ and $Ca(NO_3)_2 \cdot 4H_2O$, which provide the divalent cations Mg^{2+} and Ca^{2+} found in Chu No. 10 Medium, are found in Figure 3. These are presented as a comparison of the plating ability of AS-1 and AS-2 on the varying media for the hosts A. nidulans, A.n. var-1, A.n.-Ch and S. cedrorum.

Chemostat 2

Cyanophage AS-2 was inoculated into the second chemostat system in an attempt to detect differences between its growth in A.n.-Ch as

FIGURE 3

COMPARISON OF THE EFFECT OF DIVALENT CATION CONCENTRATION BETWEEN AS-1 AND AS-2

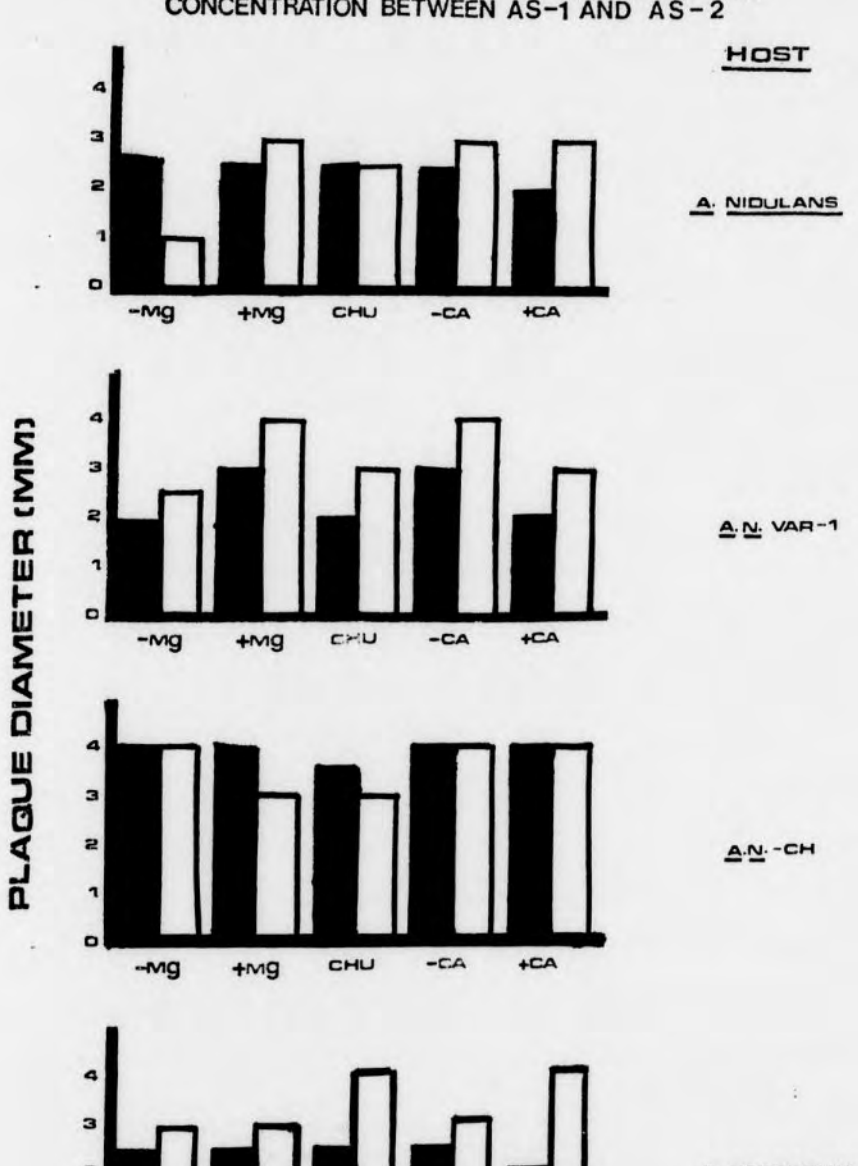
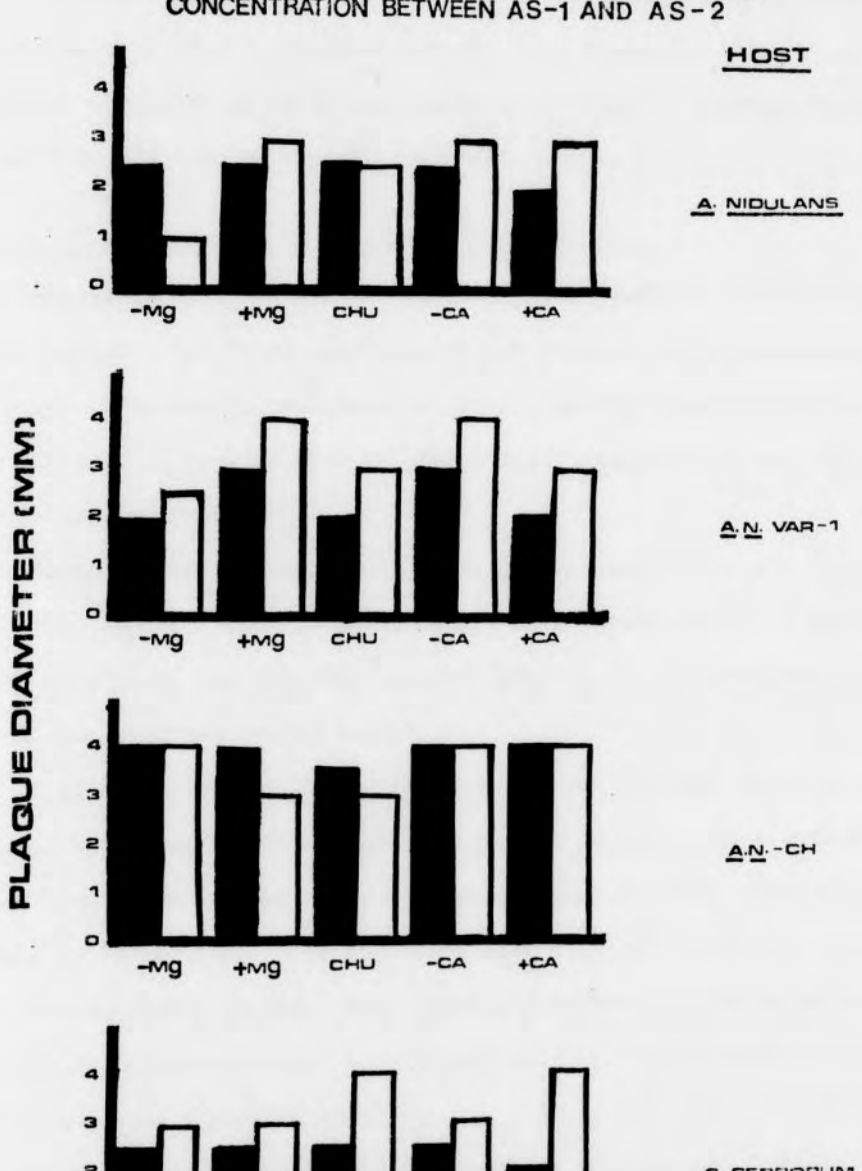


FIGURE 3

COMPARISON OF THE EFFECT OF DIVALENT CATION CONCENTRATION BETWEEN AS-1 AND AS-2



compared to A.n. var-1 (See Results, Chemostat 1). The growth pattern which developed is the more usual cyclical virus-host relationship characteristic of a continuous culture. See Figure 4. During routine assay of chemostat 2, pinpoint plaques were found in abundance upon plating in addition to the expected AS-2 plaques.

Growth Characteristics of Host Blue-Green Bacteria

Anacystis nidulans and its variants were routinely transferred to fresh medium. All three were usually subcultured at the same time and aliquots of each were transferred. This permitted visual detection of any differences between them in growth rate, pigmentation and cell death due to medium exhaustion.

Depending on the medium volume in the culture flask, all three of the hosts appeared to grow identically for a period of two to four weeks. After this time, the A.n.-Ch culture lightened to a yellow-green; results resembling clearing due to lysis.

A growth curve for A. nidulans and its two variants is found in Figures 5, 6, and 7. The visual differences found in the growth of the three blue-green bacteria can be seen in Figure 8. This composite plot of the colorimetric data of the three show virtually identical growth in the initial growth period. The visually detectable differences between A.n.-Ch on the one hand and A. nidulans and A.n. var-1 on the other were also found spectrophotometrically.

As the three cultures aged, A. nidulans and A.n. var-1 cells declined and sedimented at the bottom of the flask. The supernatant became yellow-brown in color. A.n.-Ch cultures showed cycles of clearing and regrowth. Little or no sediment was noticed during the periods of clearing. The

FIGURE 4

GROWTH OF A.N-CH AND AS-2 IN CHEMOSTAT 2

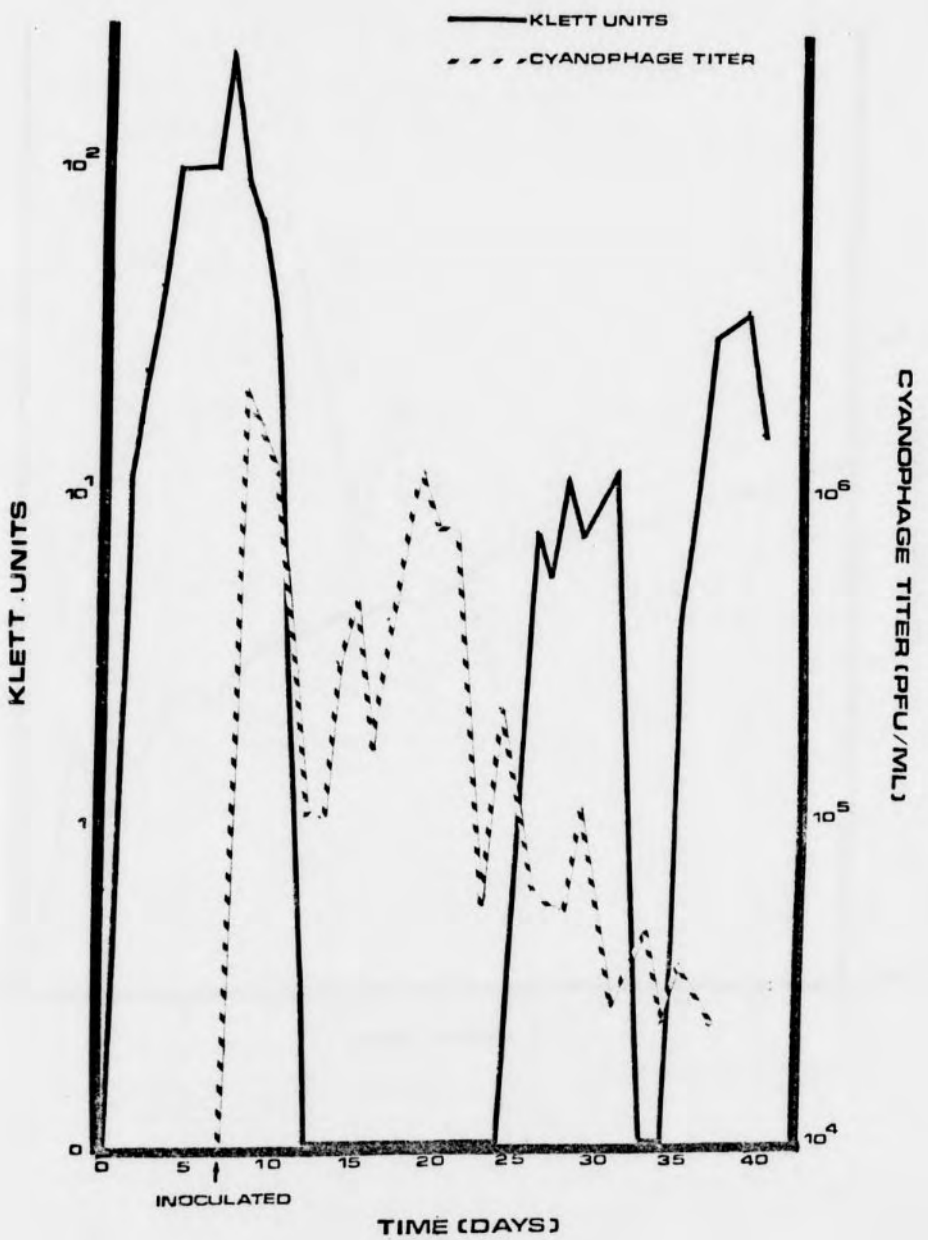


FIGURE 5 .
GROWTH CURVE: ANACYSTIS NIDULANS

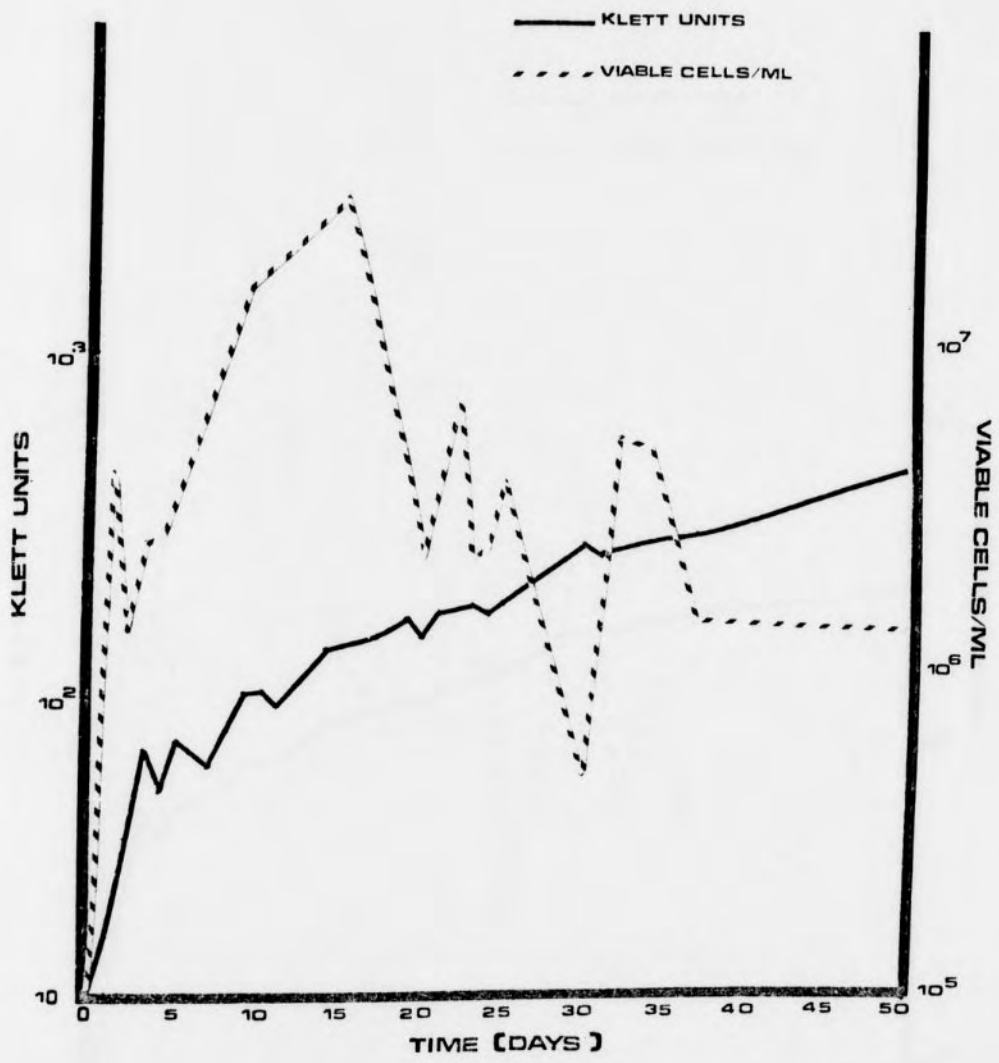


FIGURE 6
GROWTH CURVE: ANACYSTIS NIDULANS VARIANT-1

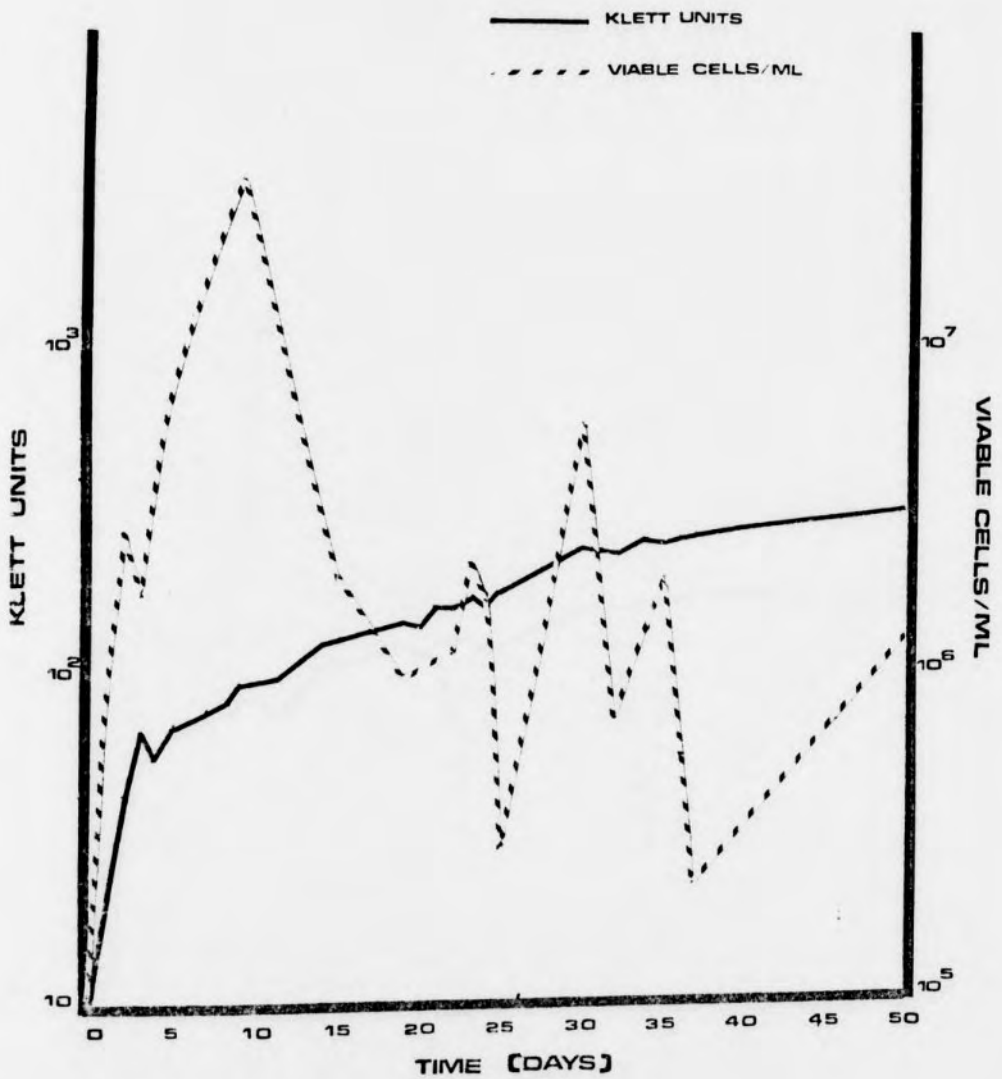


FIGURE 7
GROWTH CURVE: ANACYSTIS NIDULANS - CHEMOSTAT

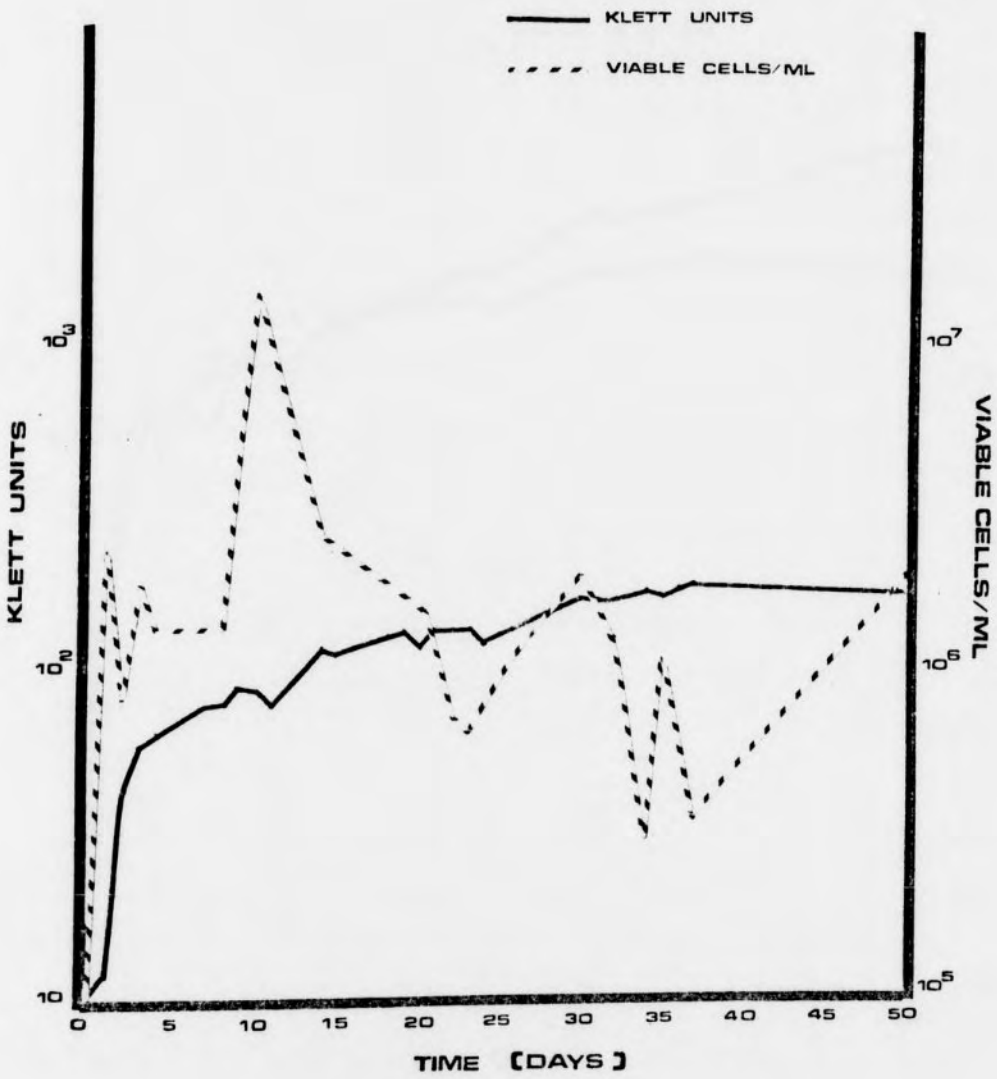
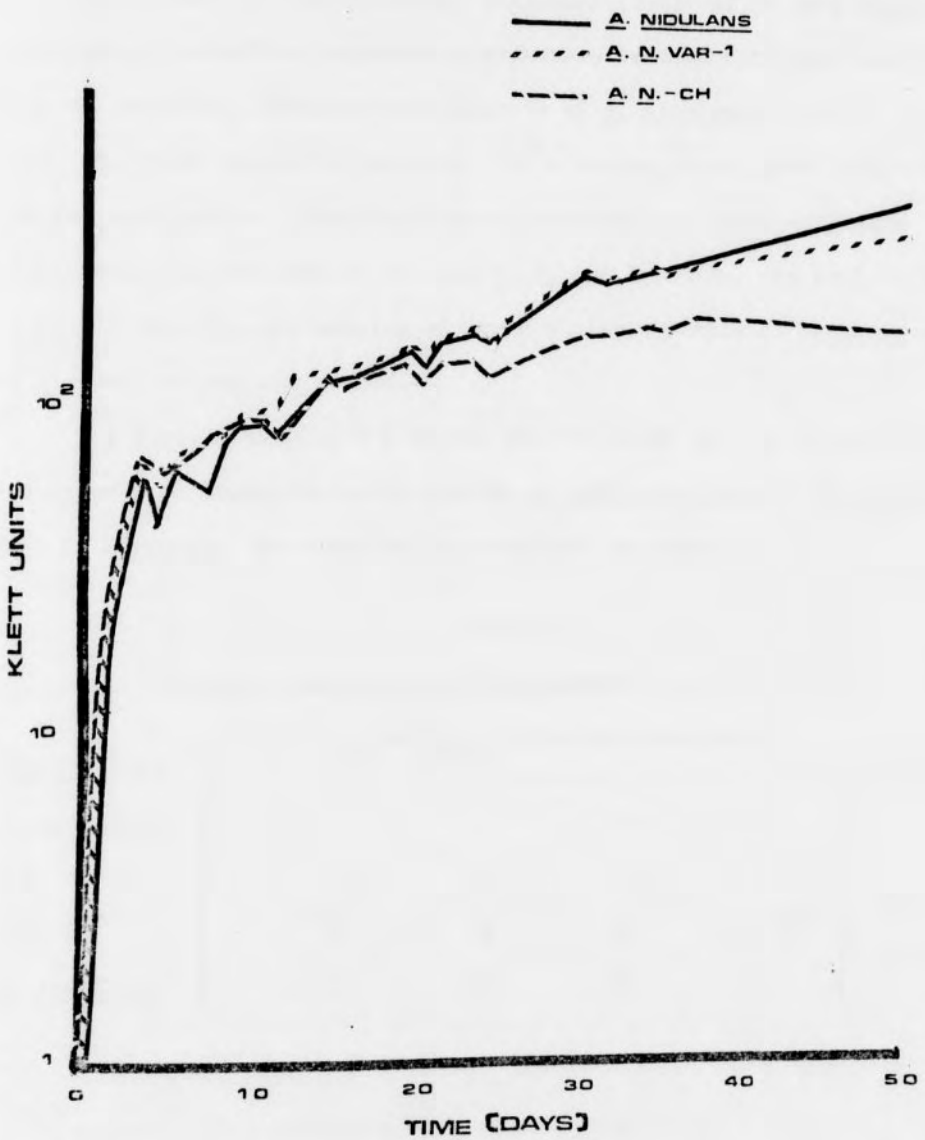


FIGURE 8
COLORIMETRIC COMPARISON OF GROWTH BETWEEN
ANACYSTIS NIDULANS AND ITS VARIANTS



growth part of the cycle was characterized by an increase in pigmentation found in normally growing cultures of A. nidulans.

Cyanophage Detection in Uninoculated Host Cultures

On account of the "clearing" phenomenon observed in aged A.n.-Ch cultures, a possible lysogenic relationship between this host and AS-2 seemed possible. Samples from cultures of A. nidulans, A.n. var-1 and A.n.-Ch, which varied in age from 0 to 6 months, were plated directly on Chu agar plates. Possible plaque formation was found on plates containing A.n.-Ch samples of ages 2, 3, and 6 months. No such activity resulted from A.n.-Ch samples of age 0 months nor from A. nidulans and A.n. var-1 of any age tested.

The A.n.-Ch samples 0-6 months were filtered and the filtrate plated separately on logarithmically growing A. nidulans, A.n. var-1, A.n.-Ch and S. cedrorum. The results are presented in Table 3.

TABLE 3

Cyanophage Detection in Uninoculated A.n.-Ch Cultures

Plating Host	Age of A.n.-Ch Culture (months) 0	2	3	6	
A. nidulans	0	0	0	50	PFU/ml
A.n. var-1	0	0	0	0	
A.n.-Ch	0	0	0	1.3×10^2	
S. cedrorum	0	0	0	0	

Chemostat 3

Presumptive viral activity had been observed in virally uninoculated cultures of A.n.-Ch (see previous section). A third continuous culture experiment was carried out to try to detect a spontaneous release of viral particles. Two chemostats were set up and monitored simultaneously. Chemostat 3-a, which was inoculated with A. nidulans (wild type), was used to compare any differences in growth which might occur in A.n.-Ch which was maintained in chemostat 3-b. No cyanophage inoculation was made.

Figure 9 shows a comparison of the growth of the two chemostats as detected colorimetrically. A.n.-Ch reached or exceeded A. nidulans growth on only a few occasions. The differences in host cell concentration were also visually observable.

Tables 4 and 5 show colorimetric and viable cell counts of the two blue-green bacteria. The presence or absence of PFU was determined by directly plating a 1.0 ml sample of cells from each chemostat. Lysis or its absence is also indicated in these tables. In the case of positive lysis, the titer of the sample was found. As can be seen from Table 4, no lysis was detected at any time from the chemostat containing A. nidulans. Chemostat 3-b, which contained A.n.-Ch, gave evidence of lysis over a 16 day period. Titering of these samples had to be repeated several times due to the difficulty in obtaining satisfactory dilutions.

The plaques were quite small ($\leq$ 0.25 mm) and were cloudy at times. A plate could contain both cloudy and clear plaques of the same size; there was no consistency noted in this phenomenon.

FIGURE 9

CHEMOSTAT-3a AND b:
COMPARISON OF COLORIMETRIC GROWTH
BETWEEN A. NIDULANS AND A.N.-CH

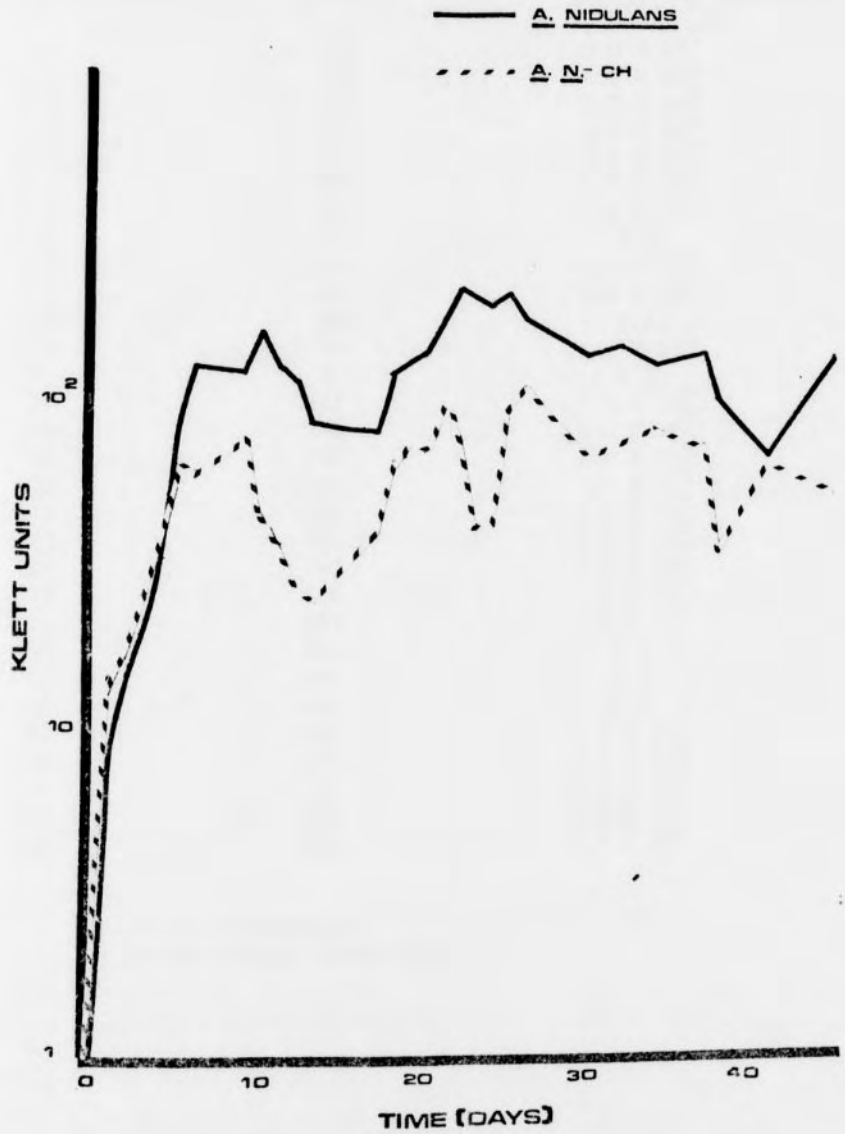


TABLE 4

Chemostat-3a:
Growth Data for *Anacystis nidulans*

Time (days)	Klett	Cell Count (cells/ml)	PFU
1	8	3.0×10^5	-
2	15	4.5×10^5	-
3	20	1.1×10^6	-
4	44	3.0×10^6	-
5	86	1.0×10^6	-
6	130	4.7×10^6	-
9	220	2.8×10^6	-
10	164	N.A.	-
11	125	4.0×10^6	-
12	113	3.0×10^6	-
13	86	N.A.	-
16	83	1.7×10^5	-
17	81	N.A.	-
18	125	5.4×10^6	-
19	136	3.0×10^6	-
20	142	3.0×10^6	-
21	183	4.9×10^6	-
22	220	8.5×10^6	-
23	208	5.0×10^7	-
24	197	1.5×10^7	-
25	216	1.4×10^7	-
26	183	1.4×10^7	-
30	143	N.A.	-
32	150	N.A.	-
34	132	N.A.	-
37	145	1.6×10^6	-
38	103	2.6×10^5	-
41	70	8.2×10^6	-
45	140	2.0×10^6	-
53	325	2.3×10^6	-

N.A. = Not Available
\- = No plaque formation

TABLE 5

Chemostat-3b:
Growth Data for *Anacystis nidulans*-Chemostat

Time (days)	Klett	Cell Count (cells/ml)	PFU	Titer
1	15	1.7×10^5	-	0
2	17	4.8×10^5	-	0
3	26	8.4×10^5	-	0
4	44	5.0×10^5	-	0
5	66	1.3×10^6	-	0
6	62	1.0×10^6	-	0
9	78	8.7×10^5	-	0
10	44	5.5×10^5	-	0
11	38	4.0×10^5	-	0
12	37	1.7×10^6	-	0
13	25	1.2×10^6	-	0
16	36	2.0×10^6	-	0
17	39	7.2×10^5	+	TNTC
18	67	7.5×10^6	+	2.7×10^4
19	75	9.0×10^6	+	3.4×10^5
20	73	3.0×10^6	+	3.0×10^4
21	100	2.6×10^6	+	2.6×10^3
22	77	1.1×10^6	+	2.5×10^3
23	42	1.4×10^5	+	2.9×10^3
24	43	7.1×10^6	+	6.0×10^3
25	97	3.2×10^6	+	N.A.
26	111	2.8×10^6	+	8.0×10^3
30	69	N.A.	+	1.6×10^4
32	75	N.A.	+	2.6×10^4
34	85	1.0×10^6	-	0
37	75	1.6×10^5	-	0
38	37	7.2×10^5	-	0
41	76	7.9×10^5	-	0
45	54	1.5×10^6	-	0
53	103	4.1×10^6	-	0

N.A. = Not Available
TNTC = Too numerous to count
\+ = Plaque formation
\- = No plaque formation

Figure 10 shows the relationship between host cell concentration and PFU titer for chemostat 3-b. The plaquing activity remained at a relatively low level and disappeared as abruptly as it had occurred.

Mitomycin-C Induction

An increase in plaque forming ability was noted in both samples of A.n.-Ch after incubation with Mitomycin-C. Neither control showed evidence of phage liberation throughout the 8 hour period. The "older" culture did show evidence of phage activity at the initial assaying but, as was found with the second culture, a rise in viral titer was not evident until approximately 6 hours after exposure to the antibiotic. See Figure 11. Precise titering was not achieved with all samples due to the difficulty encountered in plating this phage after dilution (Table 6). Obvious and clearly defined plaques were found in plating undiluted samples and those diluted 1:10. Upon higher serial dilution, the plaque formation appeared to be inhibited in some way. The plaques observed during this induction experiment appeared to be identical to those recovered from chemostat 3-b.

FIGURE 10
CHEMOSTAT - 3b :
RELATIONSHIP BETWEEN HOST CELL DENSITY
AND PFU TITER

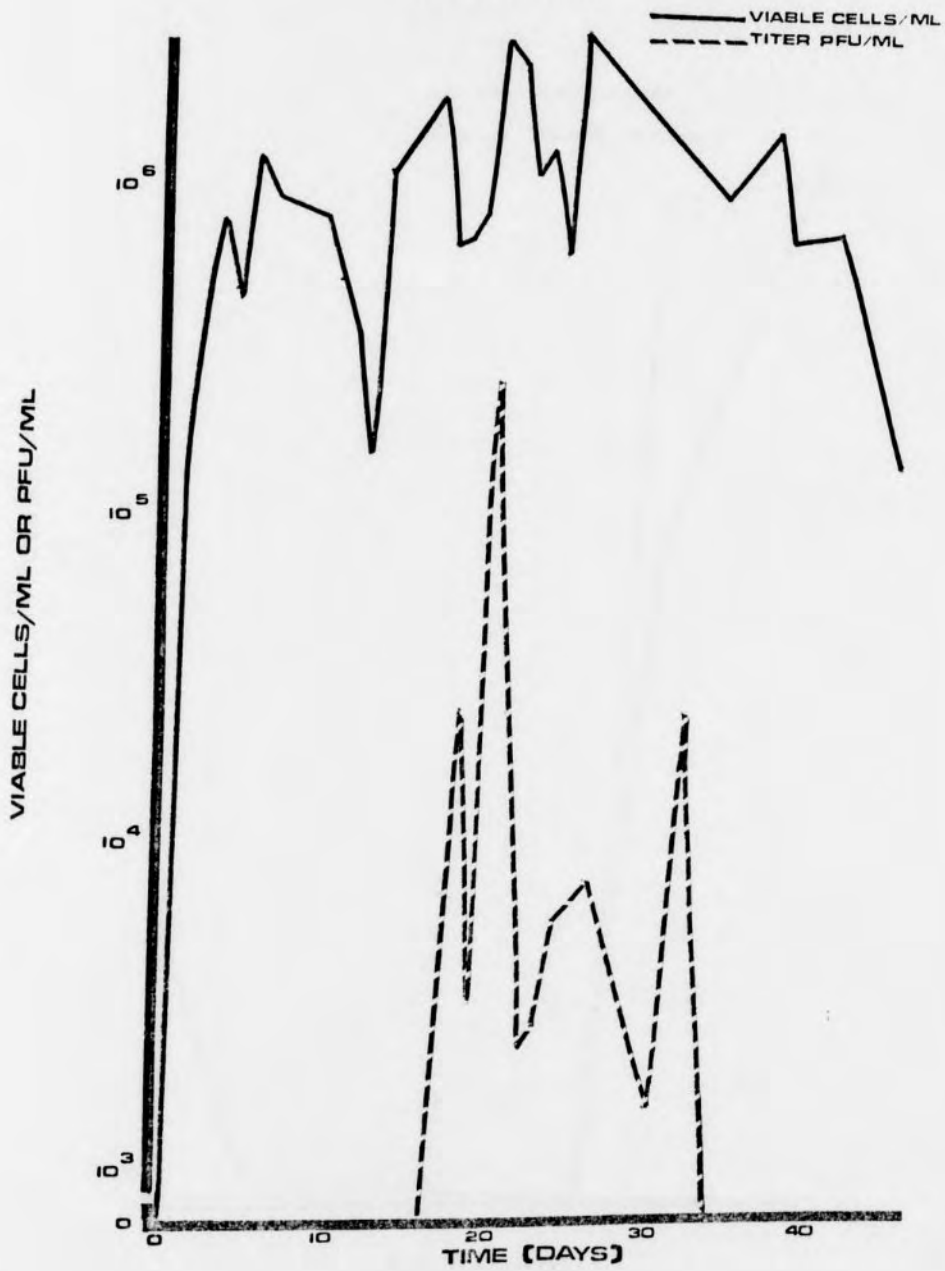


FIGURE 11
MITOMYCIN-C INDUCTION

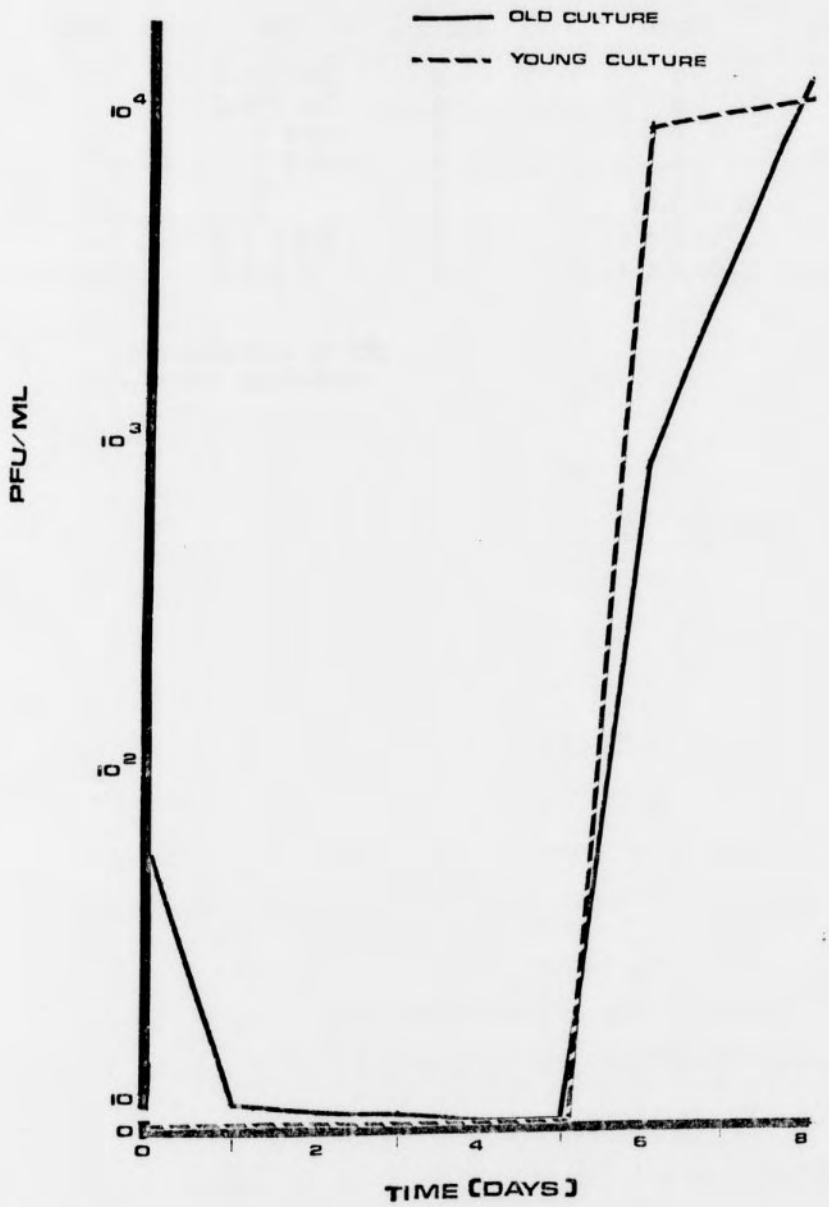


TABLE 6

Data: Mitomycin-C Induction of A.n.-Ch

A.n.-Ch Culture

Time	Old	Control	Young	Control
0	$5.3 \times 10^{1}_{1}$	0	0	0
1	1.0×10^{1}	0	0	0
2	+ N.A.	0	0	0
3	+ N.A.	0	0	0
4	0	0	0	0
5	2	0	0	0
6	$9.2 \times 10^{2}_{4}$	0	1.0×10^{4}	0
8	1.4×10^{4}	0	1.2×10^{4}	0

+ = presence of PFU
N.A. = not available

DISCUSSION

Two events, which occurred approximately simultaneously, initiated this study. One was the isolation from a continuous culture system of what appears to be an AS-1 cyanophage variant, heretofore unrecoverable. The second was the observation of the lysogenic nature, in growth, of a blue-green bacterial isolate from the same continuous culture. While research elucidating each of these phenomena was separate, the integrated study has helped to explain the origin and interrelationship of both.

Characterization of the AS-1 Variant

In the initial phase of this study, the cyanophage isolated from chemostat 1 was believed to be AS-1. But, due to the difficulty encountered in isolation and the plating differences found when compared to those of stock cultures of AS-1, this cyanophage is now thought to be an AS-1 variant, designated as AS-2.

The results of the viral neutralization experiments indicated that AS-2 is very closely related antigenically to AS-1 or is even the same phage. Both AS-1 and AS-2 have the same host range; each only plates on the unicellular blue-green bacterial strains of *A. nidulans* and *S. cedrorum*.

Differences between AS-1 and AS-2 were more evident in their nutritional requirements. AS-1 does not require the divalent cations Mg^{2+} and Ca^{2+}, which are obligatory for LPP-cyanophage infection (Schneider et al. 1964). While AS-1 does not require Mg^{2+} and Ca^{2+} for infection

(Safferman et al. 1972), dialysis did result in some loss which may be attributable to the length of dialysis and loss in stabilization of the phage particle itself. The decrease in AS-2 infectivity after dialysis is more remarkable. A complete loss in the ability to infect was never found so these cations are probably not completely essential.

A variation in infectivity was also reflected in experiments on the supplementation of medium with these ions. Figure 3 shows the results of infection as evidenced by variations in plaque diameter. There are only slight fluctuations in the PFU diameter for AS-1 regardless of the host or media used. The results from plating AS-1 on A. nidulans and S. cedrorum are identical. This is further evidence for the hypothesis that the strains susceptible to AS-1 may represent variants of a single species as has been proposed for members of the three genera comprising the LPP-1 host range (Safferman and Morris, 1967). In all cases but one, there is a decrease in AS-1 plaque size when plated on Ca^{2+}-supplemented Chu. Otherwise, plaque diameter remained fairly constant.

AS-2 showed wider variations in plaque size than AS-1. Contributing to the observation that AS-2 has a Mg^{2+} and Ca^{2+} requirement is the finding that, compared to controls, there is a decrease in plaque size when this virus is plated on Mg^{2+}-deficient medium. In two cases, plating on A. nidulans and A.n. var-1, the control plaque size is exceeded upon plating in Mg^{2+}-enriched medium. There is a decrease in plaque size when A.n.-Ch is the plating host and an increase when S. cedrorum is used.

While there are observable differences in AS-1 and AS-2 plaque size among the four hosts, AS-2 generally produced a larger plaque.

Fluctuations in size due to medium content are more noticeable with AS-2 as well as those differences which are attributable to the plating host. Perhaps the degree of divalent ion requirement found among the cyanophages, both filamentous and unicellular, which is required for infection, is due to deficiencies of these ions found between the host ranges which separate the two classes rather than to differences among the viruses themselves.

Lysogeny

The case for suspected lysogeny can be made from several positions, observable and experimental. The initial observations on the growth of A.n.-Ch, at the very least, mimiced what would be expected to occur if a blue-green bacterium were maintaining a prophage. This strain appeared to grow to a certain density after which a progressive clearing of the flask occurred. Cycles of regrowth and clearing were observed for up to a year in one flask. As a result, A.n.-Ch never reached the cell density found in A. nidulans and A.n. var-1.

Experimentally, lysogenic behavior in A.n.-Ch includes the isolation of plaques from virally uninoculated cultures and from a continuous culture system, and the induction of phage activity with Mitomycin-C. The cloudy plaques, which were frequently observed, could be due to lysogeny, poor adsorption, failure to kill the host cell, or lysis inhibition.

The finding that recovery of PFU is enhanced during exponential growth correlates well with bacterial lysogenization and induction (Hayes, 1968). Host cell physiology is one of the most influential determinants of which life cycle a prophage will exhibit. Under conditions of

maximum growth, energy and protein synthesis, a temperate phage will frequently enter the lytic cycle.

Origin and Identity of the A.n.-Ch Lysogeny

One of the more interesting questions raised by this study concerns the possible origins of this virus-host relationship. Since the blue-green bacterial strain, A.n.-Ch, which gives rise to the small, induced PFU, was isolated from chemostat 1, it seems possible that lysogenization was established by some event occurring during this period. A.n. var-1, a blue-green bacterium not yet characterized, was used as the host of this continuous culture. The origin of this variant is not certain.

If, indeed, A.n. var-1 did arise as a result of A. nidulans contamination of a Chloroglea fritschii culture, as has been discussed in the Material and Methods, there is a chance that genetic recombination could have occurred. A.n. var-1 does grow differently from both A. nidulans and A.n.-Ch, but more closely resembles A. nidulans and shows none of the lysogenic properties exhibited by A.n.-Ch.

That the inducible plaquing agent from A.n.-Ch is an AS-cyanophage would appear to be the most likely conclusion. Continuous cultivation of blue-green bacterial-cyanophage systems has given rise to variants of both the cyanophage and host (Cowlishaw and Mrsa, 1975), so it seems possible that a lysogenic relationship could be established.

There are several pieces of evidence which indicate that AS-2 is not the phage lysogenized by A.n.-Ch. The first is that A.n.-Ch is not resistant to superinfection by the cyanophages AS-1 and AS-2. Immunity of a lysogenized host to infection by the same or closely related viruses appears to be a universal phenomenon among bacteria (Hayes, 1968).

A.n.-Ch showed no decreased infectivity as a result of infection by either of these viruses.

A second finding occurred during preliminary experiments and has not been included in the Results. This was the lack of neutralization of the induced phage by AS-1 antiserum. This antiserum has been shown to neutralize both AS-1 and AS-2 cyanophages. If the chemostat 3-b isolate is an AS-derived phage, a quantitative degree of neutralization would be expected to occur. Results from these initial experiments cannot be critically analyzed at this time.

Future Investigations

The development of such a complex yet stable virus-host relationship within a laboratory environment makes the Anacystis-cyanophage system an interesting one for future study. The taxonomy of the Anacystis nidulans variants, A.n. var-1 and A.n.-Ch, needs clarification. This can be pursued through DNA studies on base composition and homology and possible life cycle and reproduction variations. Electron micrographs of both the blue-green bacterial and viral variants are needed.

Studies elucidating the exact relationship between A.n.-Ch and its possible prophage should be pursued. Is it inducible by mechanisms other than antibiotics? What is the relationship between host physiology and concentration and phage liberation in standing and continuous culture? If the plaque-forming agent proves to be a temperate phage, did it originate or was it merely induced by laboratory manipulations?

It seems possible that perhaps there is an association between the isolation of AS-2 and A.n.-Ch. Until A.n. var-1 was co-cultivated with AS-2, A.n.-Ch had not originated and AS-2 had not been isolated.

Conclusions

The behavior of the A. nidulans variant A.n.-Ch indicates that it is possibly harboring a prophage. The origin of this suspected lysogeny and the nature of the prophage have yet to be determined. At this time, it seems unlikely that it is an AS-cyanophage. It is possible that a prophage existed in A.n. var-1 but was unable to effect a lytic cycle and thus give evidence of its existence until infection with AS-2.

Cyanophage AS-2 does exhibit differences from AS-1. Whether this variant has participated, in some manner, in the establishment of the presumptive lysogeny is not known at this time.

BIBLIOGRAPHY

Adolph, K.W. and Haselkorn, R. 1971. Isolation and Characterization of a Virus Infecting the Blue-Green Alga Nostoc muscorum. Virology 46: 200-208.

Adolph, K.W. and Haselkorn, R. 1973. Isolation and Characterization of a Virus Infecting a Blue-Green Alga of the Genus Synechococcus. Virology 54: 230-236.

Allen, M.M. 1968a. Photosynthetic Membrane System in Anacystis nidulans. Jour. Bacteriol. 96: 836-841.

Allen, M.M. 1968b. Ultrastructure of the Cell Wall and Cell Division of Unicellular Blue-Green Algae. Jour. Bacteriol. 96: 842-852.

Allen, M.M. and Hutchison, F. 1976. Effect of Some Environmental Factors on Cyanophage Development in Anacystis nidulans. Arch. Microbiol. 110: 55-60.

Bazin, M.J. 1968. Sexuality in a Blue-Green Alga: Genetic Recombination in Anacystis nidulans. Nature 218: 282-283.

Bergey's Manual of Determinative Bacteriology. 1974. Buchanan, R.E. and Gibbons, N.E. (ed.). The Williams and Wilkins Co., Baltimore, Maryland.

Brown, R.M., Jr. 1972. Algal Viruses. Adv. Virus Research 17: 243-277.

Cannon, R.E., Shane, M.S. and Bush, V.N. 1971. Lysogeny of a Blue-Green Alga, Plectonema boryanum. Virology 45: 149-153.

Cannon, R.E. and Shane, M.S. 1972. The Effect of Antibiotic Stress on Protein Synthesis in the Establishment of Lysogeny of Plectonema boryanum. Virology 49: 130-133.

Cannon, R.E., Shane, M.S. and Whitaker, J.M. 1976. Interaction of Plectonema boryanum (Cyanophyceae) and the LPP-Cyanophages in Continuous Culture. Jour. Phycol. 12: 418-421.

Carr, N.G. and Craig, I.W. 1970. The Relationship Between Bacteria, Blue-Green Algae and Chloroplasts. In J.B. Harborne (ed.) Phytochemical Phylogeny. Academic Press, Inc., London.

Carr, N.G. and Whitton, B.A. (ed.) 1973. The Biology of the Blue-Green Algae. University of California Press, Los Angeles, California.

Colishaw, J. and Mrsa, M. 1975. Co-evolution of a Virus-Alga System. Appl. Microbiol. 29: 234-239.

Daft, M.J., Begg, J. and Stewart, W.D.P. 1970. A Virus of Blue-Green Algae from Freshwater Habitats in Scotland. New Phytology 69: 1029-1038.

Doolittle, W.F. 1972. Ribosomal Ribonucleic Acid Synthesis and Maturation in the Blue-Green Algae Anacystis nidulans. Jour. Bacteriol. 111: 316-324.

Doolittle, W.F. 1973. Postmaturational Cleavage of 23S Ribosomal Ribonucleic Acid and its Metabolic Control in the Blue-Green Algae Anacystis nidulans. Jour. Bacteriol. 113: 1256-1263.

Echlin, P. and Morris, I. 1965. The Relationship Between Blue-Green Algae and Bacteria. Biol. Rev. 40: 143-187.

Gray, J.E. and Herson, D.S. 1976. Functional 70S Hybrid Ribosomes from Blue-Green Algae and Bacteria. Arch. Microbiol. 109: 95-99.

Hayes, W. 1968. The Genetics of Bacteria and Their Viruses. John Wiley & Sons, Inc., New York.

Loening, U.E. 1968. Molecular Weights of Ribosomal RNA in Relation to Evolution. Jour. Mol. Biol. 38: 355-365.

MacKenzie, J.J. and Haselkorn, R. 1972. Photosynthesis and the Development of Blue-Green Algal Virus SM-1. Virology 49: 517-521.

Methods in Microbiology, Vol. II. 1970. Norris, J.R. and Ribbons, D.W. (eds.) Academic Press, New York.

Orkwiszewski, K.G. and Kaney, A.R. 1974. Genetic Transformation of the Blue-Green Bacterium Anacystis nidulans. Arch. Microbiol. 98: 31-37.

Padan, E., Shilo, M. and Oppenheim, A.B. 1972. Lysogeny of the Blue-Green Alga Plectonema boryanum by LPP-2SPI Cyanophage. Virology 47: 525-526.

Padan, E. and Shilo, M. 1973. Cyanophages--Viruses Attacking Blue-Green Algae. Bacteriol. Rev. 37: 343-370.

Pearson, N.J., Small, E.A. and Allen, M.M. 1975. Electron Microscopic Study of the Infection of Anacystis nidulans by the Cyanophage AS-1. Virology 65: 469-479.

Pringsheim, E.G. 1949. The Relationship Between Bacteria and Myxophyceae. Bacteriol. Rev. 13: 47-98.

Prokop, A., Ricica, J., Málek, I. and Thomas, J. 1967. Growth and Physiological Characteristics of a High Temperature Strain of Chorella pyrenoidosa in Continuous Culture. Nature 214: 1234-1235.

Rimon, A. and Oppenheim, A.B. 1976. Protein Synthesis Following Infection of the Blue-Green Alga Plectonema boryanum with the Temperate Virus SPI and its ts Mutants. Virology 71: 444-452.

Safferman, R.S. and Morris, M.E. 1963. Algal Virus: Isolation. Science 140: 679-680.

Safferman, R.S. and Morris, M.E. 1964. Growth Characteristics of the Blue-Green Algal Virus LPP-1. Jour. Bacteriol. 88: 771-775.

Safferman, R.S. and Morris, M.E. 1967. Observations on the Occurrence, Distribution and Seasonal Incidence of Blue-Green Algal Viruses. Appl. Microbiol. 15: 1219-1222.

Safferman, R.S., Schneider, I.R., Steere, R.L., Morris, M.E. and Diener, T.O. 1969. Phycovirus SM-1: A Virus Infecting Unicellular Blue-Green Algae. Virology 37: 386-395.

Safferman, R.S., Diener, T.O., Desjardins, P.R. and Morris, M.E. 1972. Isolation and Characterization of AS-1, a Phycovirus Infecting the Blue-Green Algae, Anacystis nidulans and Synechococcus cedrorum. Virology 47: 105-113.

Schneider, I.R., Diener, T.O. and Safferman, R.S. 1964. Blue-Green Algal Virus LPP-1: Purification and Partial Characterization. Science 144: 1127-1130.

Sherman, L.A. and Haselkorn, R. 1970a. LPP-1 Infection of the Blue-Green Alga Plectonema boryanum. I. Electron Microscopy. Jour. Virol. 6: 820-833.

Sherman, L.A. and Haselkorn, R. 1970b. LPP-1 Infection of the Blue-Green Alga Plectonema boryanum. II. Viral DNA Synthesis and Host DNA Breakdown. Jour. Virol. 6: 834-840.

Sherman, L.A. and Haselkorn, R. 1970c. LPP-1 Infection of the Blue-Green Alga Plectonema boryanum. III. Protein Synthesis. Jour. Virol. 6: 841-846.

Sherman, L.A. and Connelly, M. 1976. Isolation and Characterization of a Cyanophage Infecting the Unicellular Blue-Green Algae A. nidulans and S. cedrorum. Virology 72: 540-544.

Sherman, L.A., Connelly, M. and Sherman, D.B. 1976. Infection of Synechococcus cedrorum by the Cyanophage AS-1M. I. Ultrastructure of Infection and Phage Assembly. Virology 71: 1-16.

Sherman, L.A. and Pauw, P. 1976. Infection of Synechococcus cedrorum by the Cyanophage AS-1M. II. Protein and DNA Synthesis. Virology 71: 17-27.

Sherman, L.A. 1976. Infection of Synechococcus cedrorum by the Cyanophage AS-1M. III. Cellular Metabolism and Phage Development. Virology 71: 199-206.

Shestakov, S.V., Postnova, T.I. and Shakhnabatian, L.G. 1975. In vitro Repair of U.V. or X-Irradiated Bacteriophage T-4 by Extract from Blue-Green Alga Anacystis nidulans. Mol. Biol. Rep. 2: 89-94.

Singh, R.N. and Singh, P.K. 1967. Isolation of Cyanophages in India. Nature 216: 1020-1021.

Smedberg, C.T. 1975. Cyanophages as Indicators of Viral Presence in Sewage Wastewater. Master's Thesis, University of North Carolina at Greensboro.

Stanier, R.Y., Kunisawa, R., Mandel, M. and Cohen-Bazire, G. 1971. Purification and Properties of Unicellular Blue-Green Algae (Order Chroococcales). Bacteriol. Rev. 35: 171-205.

Susor, W.A. and Krogmann, D.W. 1964. Hill Activity in Cell-Free Preparations of a Blue-Green Alga. Bioc. Biop. A. 88: 11-19.

Udvardy, J., Sivők, B., Borbély, G. and Farkas, G.L. 1976. Formation, in the Dark, of Virus-Induced Deoxyribonuclease Activity in Anacystis nidulans, an Obligate Photoautotroph. Jour. Bacteriol. 126: 630-633.